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Abstract:

The genetic control of tocopherol, phytosterol, percentage of seed protein, oil and fatty acids content in a population of recombinant inbred lines (RILs) of sunflower under various conditions are studied through QTL analysis using genetic-linkage map based on SSR markers and introducing some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive family genes and *Arabidopsis SEC14* homologue genes. Three important candidate genes (*HPPD*, *VTE2* and *VTE4*), which encode enzymes involved in tocopherol biosynthesis, are mapped to linkage group 8(LG8) and LG14. One of the most important candidate genes coding for sterol methyltransferase II (SMT2) enzyme is anchored to LG17 by CAPS marker. Four SNPs are identified for *PAT2*, *Arabidopsis* Sec14 homologue gene, between two parents (PAC2 and RHA266). *PAT2* is assigned to LG2 by CAPS marker. Squalene epoxidase (*SQE1*) is also assigned to LG15 by InDel marker. Through other candidate genes, *POD*, *CAT* and *GST* encoding enzymatic antioxidants are assigned to LG17, LG8 and LG1, respectively. The major QTL for total tocopherol content on linkage group 8 accounted for 59.5% of the phenotypic variation (*6.TTC.8*), which is overlapped with the QTL of total phytosterol content (*7.TPC.8*). Under late-sowing condition, a specific QTL of palmitic acid content on linkage group 6 (*PAC-LS.6*) is located between ORS1233 and SSL66_1 markers. Common chromosomic regions are observed for percentage of seed oil and stearic acid content on linkage group 10 (*PSO-PI.10* and *SAC-WI.10*) and 15 (*PSO-PI.15* and *SAC-LS.15*). Overlapping occurs for QTLs of oleic and linoleic acids content on linkage groups 10, 11 and 16. Seven QTLs associated with palmitic, stearic, oleic and linoleic acids content are identified on linkage group 14. These common QTLs are linked to *HPPD* homologue, HuCL04260C001. QTLs controlling various traits such as days from sowing to flowering, plant height, yield and leaf-related traits are also identified under well-, partial-irrigated and late-sowing conditions in a population of recombinant inbred lines (RILs). The results do emphasis the importance of the role of linkage group 2, 10 and 13 for studied traits. Genomic regions on the linkage group 9 and 12 are important for QTLs of leaf-related traits in sunflower. We finally identified AFLP markers and some candidate genes linked to seed-quality traits under well-irrigated and water-stressed conditions in gamma-induced mutants of sunflower. Two mutant lines, M8-826-2-1 and M8-39-2-1, with significant increased level of oleic acid can be used in breeding programs because of their high oxidative stability and heart-healthy properties. The significant increased level of tocopherol in mutant lines, M8-862-1N1 and M8-641-2-1, is justified by observed polymorphism for tocopherol pathway-related gene; *MCT*. The most important marker for total tocopherol content is E33M50_16 which explains 33.9% of phenotypic variance. One of the most important candidate genes involving fatty acid biosynthesis, *FAD2* (*FAD2-1*), is linked to oleic and linoleic acids content and explained more than 52% of phenotypic variance.

Résumé:

Le tocophérol, le phytostérol, le pourcentage de protéines des graines, l'huile et les teneurs en acides gras ont été mesurés dans une population de lignées recombinantes (RILS) de tournesol, cultivées sous conditions de sécheresse, irrigation et semis tardif. Une analyse génétique de QTL a été réalisée à partir de ces mesures, en utilisant une carte génétique basée sur des marques SSR et avec des gènes candidats (1) impliqués dans la voie métabolique de tocophérol et phytostérol, (2) des gènes codant des antioxydants enzymatiques, (3) des gènes liés à la sécheresse et (4) des gènes homologues à *SEC14* chez *Arabidopsis*. Trois gènes candidats importants (*VTE4*, *VTE2* et *HPPD*), qui codent pour des enzymes impliquées dans la biosynthèse du tocophérol, ont été cartographiés sur les groupes de liaison LG8 et LG14. Quatre SNPs sont identifiés pour *PAT2*, le gène homologue chez *Arabidopsis SEC14*, entre les deux parents (PAC2 et RHA266) et un SNP, identifié par alignement de séquences est converti en marqueur CAPS pour permettre l'analyse génotypique des RIL. Les gènes homologues à *SFH3*, *HPPD*, *CAT* et *CYP51G1* ont été cartographiés grâce à la mise au point de marqueurs dominants, tandis que des marqueurs co-dominants ont permis la cartographie des gènes homologues à *SEC14-1*, *VTE4*, *DROU1*, *POD*, *SEC14-2* et *AQUA*. Les gènes *POD*, *CAT* et *GST*, codant pour des antioxydants enzymatiques, ont également été cartographiés sur les groupes de liaison 17, 8 et 1, respectivement. Le QTL majeur pour la teneur en tocophérol a été identifié sur le groupe de liaison 8, qui explique 59,5% de la variation phénotypique (*6.TTC.8*). Il colocalsie également avec le QTL identifié pour la teneur en phytostérol (*7.TPC.8*). Sous condition de semis tardif, un QTL spécifique de la teneur en acide palmitique a été identifié sur le groupe de liaison 6 (*PAC-LS.6*). Il est situé entre les marqueurs ORS1233 et SSL66_1. Les QTLs pour le pourcentage d'huile de graines et la teneur en acide stéarique colocalisent sur les groupes de liaison 10 (*PSO-PI.10* et *SAC-WI.10*) et 15 (*PSO-PI.15* et *SAC-LS.15*). Sept QTLs associés à teneur en acides palmitique, stéarique, oléique et linoléique sont identifiés sur le groupe de liaison 14. Ils sont liés à l'homologue du gène *HPPD*. Par ailleurs, les caractères agronomiques tels que les jours du semis à la floraison, la hauteur des plantes, le rendement et la morphologie foliaire ont été étudiés. Des analyses association génétique ont permis d'identifier des QTLs intérêts sur les groupes de liaison 2, 10 et 13 pour les caractères étudiés, d'autres QTLs identifies sur les groupes de liaison 9 et 12 mettent en avant l'importance de ces régions génomiques pour les caractères de morphologie foliaire. Nous avons finalement identifié des marqueurs AFLP et quelques gènes candidats liés aux caractères impliqués dans la qualité des graines sous conditions irriguée et stress hydrique chez une population de mutants (M8). Deux lignées mutantes, M8-826-2-1 et M8-39-2-1, produisent un niveau significativement élevé d'acide oléique peuvent être utilisées dans les programmes de sélection en raison de la haute stabilité à l'oxydation et des propriétés cardiovasculaire apportés par l'acide oléique qu'elles produisent. L'augmentation du niveau de tocophérol dans les lignées mutantes, M8-862-1N1 et M8-641-2-1, est justifiée par le polymorphisme observé pour le gène, *MCT*, impliqué dans la voie métabolique du tocophérol. Le marqueur le plus important pour le contenu en tocophérol total est E33M50_16 qui explique 33,9% de la variation phénotypique. Un des gènes candidats les plus importants concernant la biosynthèse des acides gras, *FAD2 (FAD2-1)*, est lié à la teneur en acides oléique et linoléique. Il explique plus de 52% de la variation phénotypique.

Abbreviations:

RILs: recombinant inbred lines

DSF: days from sowing to flowering

PH: plant height

LN: leaf number

DLN: dried leaf number

LAF: leaf area at flowering

LAD: leaf area duration

HD: head diameter

HW: head weight

HN: head number

TGW: 1000 grain weight

GYP: grain yield per plant

BIO: biomass

PSP: percentage of seed protein

PSO: percentage of seed oil

PAC: palmitic acid content

SAC: stearic acid content

OAC: oleic acid content

LAC: linoleic acid content

TTC: total tocopherol content

TPC: total phytosterol content

CG: candidate genes

HRM: high-resolution melting

CAPS: cleaved amplified polymorphic sequence

GG: genetic gain

CIM: composite interval mapping

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Chapter 1

General introduction

1.1 Sunflower

Sunflower (*Helianthus annuus* L.) is a member of the family Compositae (Asteraceae). The basic chromosome number is n = 17. The genus includes diploid, tetraploid and hexaploid species. The closest relatives appear to be *Tithonia*, *Viguiera* and *Phoebanthus* (Heiser et al. 1969). The common sunflower (*H. annuus*) is the most important species grown commercially, although other species are also cultivated, e.g. *H. tuberosus*, which is grown for production of edible tubers, and several other species grown as ornamentals.

Sunflower (*Helianthus annuus* L.) is one of the few crop species that originated in North America. It was probably a "camp follower" of several of the western native American tribes who domesticated the crop (possibly 1000 BC) and then carried it eastward and southward of North America. Sunflower was probably first introduced to Europe through Spain, and spread through Europe as a curiosity until it reached Russia where it was readily adapted. The Spanish name for sunflower, ‘‘girasol,’’ and the French name ‘‘tournesol’’ literally mean ‘‘turn with the sun,’’ a trait exhibited by sunflower until anthesis, after which the capitula (heads) face east.

Heiser et al. (1969) proposed a species classification of the genus Helianthus including 14 annual and 36 perennial species from North America (in three sections and seven series) and 17 species from South America. More recent classifications (Jan and Seiler 2007) have introduced some modifications. The new classification brings the number of species to 51, with 14 annual and 37 perennial species.

Sunflower seed oil is the fourth most important vegetable oil in world trade at present. Conventional sunflower produces a healthful oil with great consumer acceptance because of its high content of monounsaturated and polyunsaturated fatty acids as well as high vitamin E content. In recent years, new sunflower oil types for specific applications, mainly in the food industry, have been developed through conventional breeding approaches. Unlike other oilseed crops such as soybean and canola, commercial sunflower has not been subject to transgenic breeding so far. However, sunflower breeders have been very successful in attaining a wide diversity of breeding objectives, from developing novel seed oil quality types to incorporating genetic resistance to most of the pests and diseases that threaten the crop.

1.2 Seed and germination

A sunflower seed is an ‘Achene’. When dehulled, the edible remainder is called the ‘sunflower kernel’. The ‘seed coat’ consists of the ovary wall and testa fused together. The sunflower’s germination differs from the broad bean and pea because, once the radicle has emerged, it is the hypocotyl which elongates. This has the effect of either dragging the cotyledons from the fruit wall or carrying the entire fruit above soil. Hypocotyl elongates and draws cotyledons from the fruit wall. Often the fruit wall is carried out of the soil with the cotyledons. It falls off when the cotyledons expand. All steps of sunflower seed germination are illustrated in Fig 1.1.

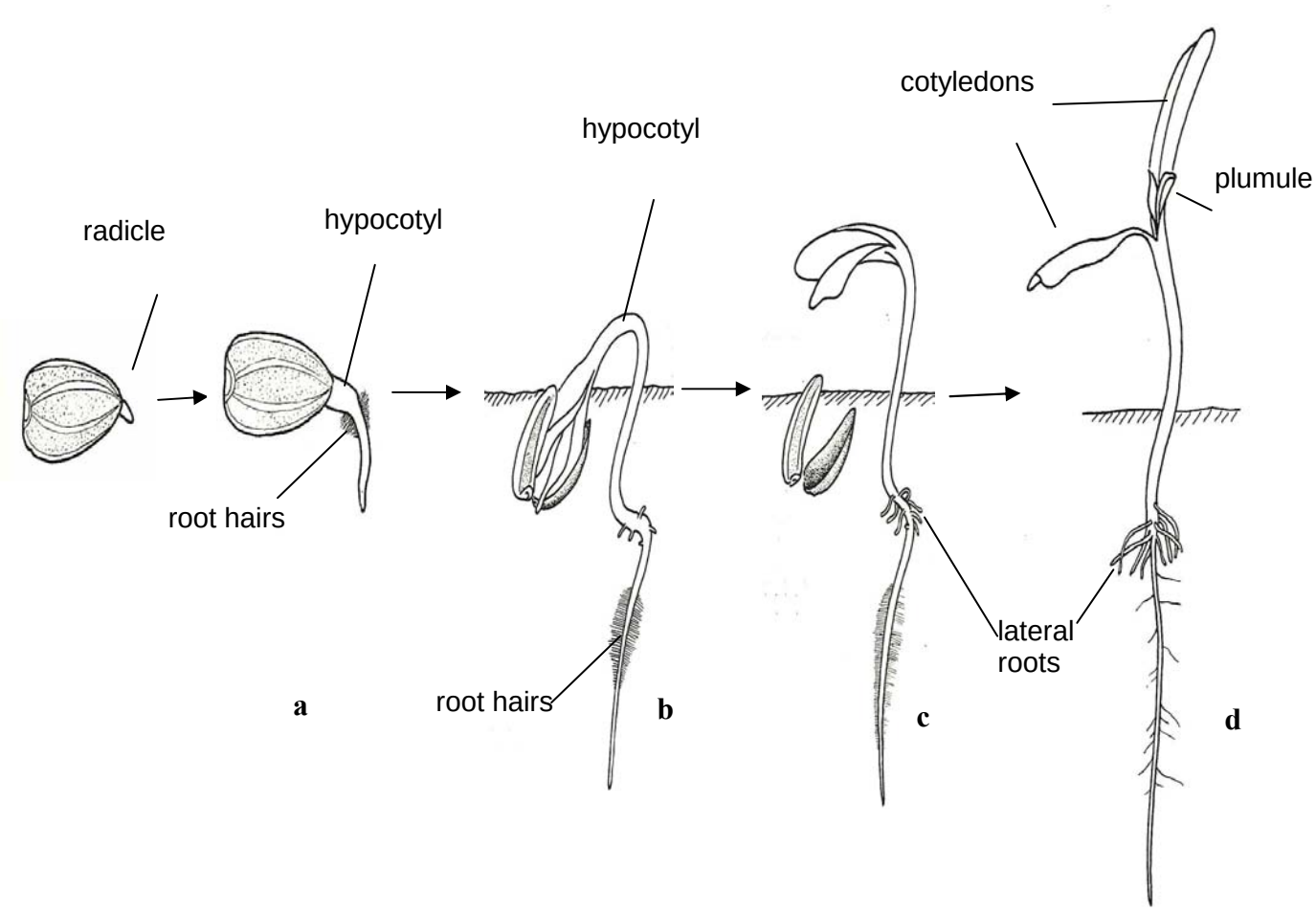


Fig. 1.1 Sunflower seed germination a) radicle emerges b) hypocotyl elongates and draws cotyledons from the fruit wall c) cotyledons are brought above the soil d) cotyledons open out, exposing the plumule
(*www.biology-resources.com/documents/plants-seeds-4.doc*)

1.3 Sunflower seed compositions

1.3.1 Protein

Sunflower is an important oil seed crop that can also be a valuable source of protein. Sunflower meal is a potential source of protein for human consumption owing to its high nutritional value and lack of antinutritional factors (Sosulski 1979). The structure and functionality of two major protein fractions in sunflower seed are described in detail by Gonzàlez-Pérez and Vereijken (2007).

.3.2 Fatty acids

Sunflower seed oil is composed of unsaturated fatty acids (90%); oleic and linoleic acids and the rest (10%) containing saturated fatty acids; palmitic and stearic acids (Dorrel and Vick, 1997; Pérez-Vich et al., 2002a).

Biosynthesis of fatty acids is now well understood in sunflower seeds. Palmitic acid can be generated by fatty acid synthetase I (FASI) (Cantisán et al., 2000). Stearic acid is formed from palmitic acid by fatty acid synthetase II (FACII), which lengthens palmitic acid (16:0) by two carbon atoms to produce stearic acid (18:0) (Pleite et al., 2006). Later, stearic acid can be either desaturated by Δ9-desaturase (stearoyl-ACP desaturase) which catalyses the first desaturation of stearic acid (18:0) to oleic acid (18:1) (Heppard et al., 1996; Lacombe et al., 2001; Vega et al., 2004) or hydrolyzed by acyl-ACP thioesterase. Finally, linoleic acid is formed from oleic acid by Δ12-desaturase (oleoyl-PC desaturase), which catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2) (Garcés and Mancha, 1991).

1.3.3 Tocopherol and phytosterol

Sunflower contains minor components such as tocopherol and phytosterol with interesting properties for human health. Tocopherols are fat-soluble compounds with vitamin E activity. In sunflower seed oil, total tocopherol content represents the sum of α, β, γ, and δ tocopherol and total phytostertol content is the sum of campesterol, stigmasterol, β-sitosterol, Δ7-campesterol, Δ5-avenasterol, Δ7-stigmasterol and Δ7-avenasterol (Ayerdi Gotor et al. 2007). Forms of tocopherol determined by the number of methyl groups on the chromanol ring (Fig 1.2). Oilseed crops reveal large variation in the levels of the different tocopherols and total

tocopherol content (Marwede et al. 2005). In sunflower seed oil, 96% of total tocopherol is α tocopherol whereas in soybean 59% of total tocopherol is γ tocopherol (Kamal-Eldin and Andersson 1997). The function of tocopherols in animal systems is generally related to the level of α tocopherol activity. Tocopherols have the ability to quench free radicals in cell membranes, protecting polyunsaturated fatty acids from damage. An imbalance in the production of free radicals and the natural protective system of antioxidants may lead to oxidized products, capable of harming tissues (Food and Nutrition Board and Institute of Medicine, 2000). Tissue damage from free radicals is considered to be related to chronic diseases such as cardiovascular disease, neurological disorders, cancer, cataracts, in flammatory diseases, and age-related macular degeneration (Bramley et al., 2000). In plants, it is suggested that the protection of photosynthetic apparatus and polyunsaturated fatty acids from oxidative damage caused by reactive oxygen species (ROS) are the main photosynthetic and non photosynthetic function of tocopherol, respectively (Trebst et al. 2002; Velasco et al. 2004; Cela et al. 2009; Semchuk et al. 2009).

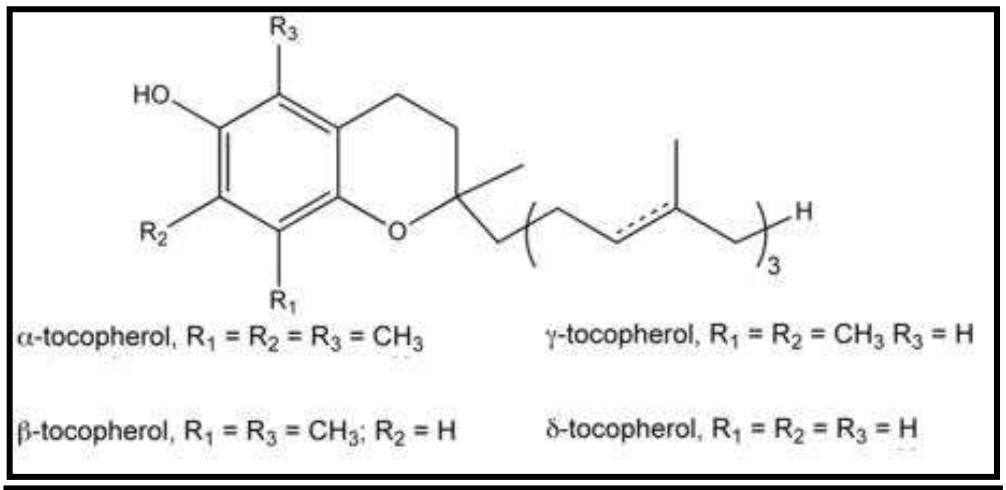


Fig. 1.2 Forms of tocopherol determined by the number of methyl groups on the chromanol ring
(http://en.wikipedia.org/wiki/Tocopherol)

1.3.3.1 Tocopherol pathway

Tocopherol biosynthetic pathway can be divided in to four following steps:

1.3.3.1.1 Synthesis of homogentisic acid (HGA)

Homogentisic acid (HGA) contributes to the chromanol head of tocopherol. HGA is the common precursor to tocopherols, can originate either via the conversion of chorismate to prephenate and then to p-hydroxyphenylpyruvate (HPP) via a bifunctional prephenate dehydrogenase in bacteria (Fig 1.3) or via the synthesis and conversion of the intermediates arogenate, tyrosine, by the shikimate pathway, and HPP in plants. HPP is then converted to HGA by p-hydroxyphenylpyruvate dioxygenase (HPPD) (Fig 1.3) (Norris et al 1998; Valentin et al. 2006).

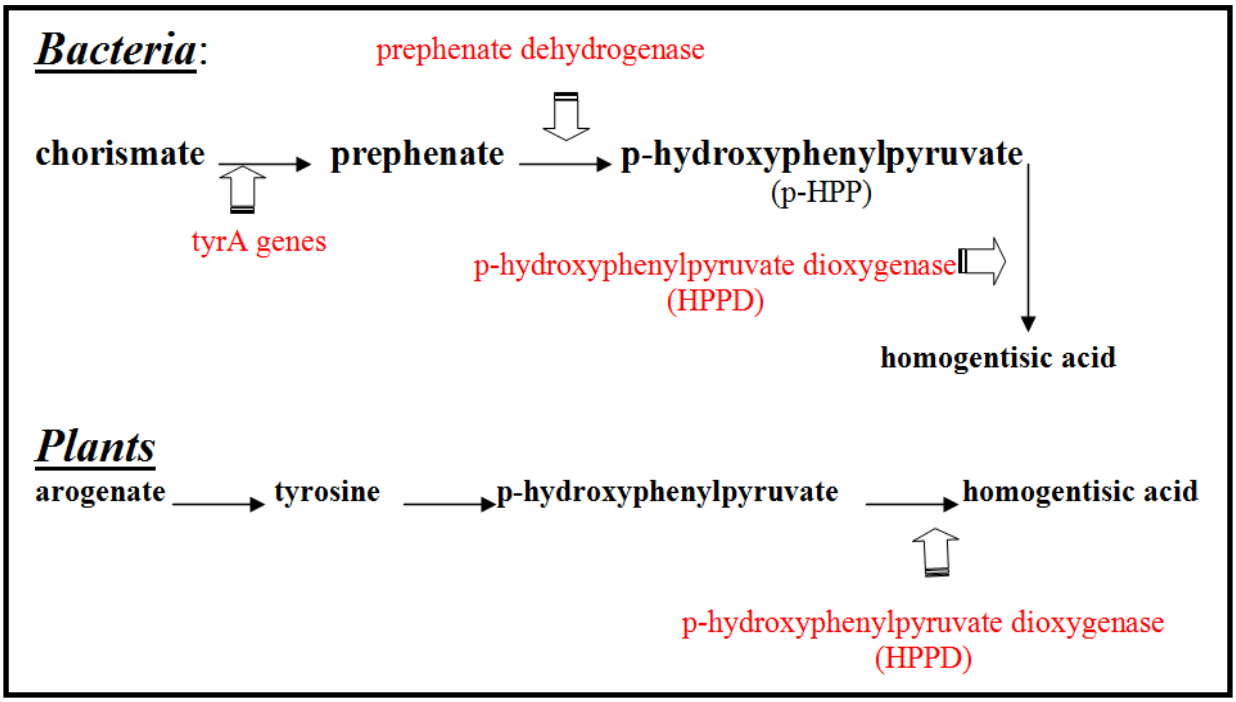


Fig. 1.3 Synthesis of homogentisic acid (HGA) in bacteria and plants

1.3.3.1.2 Synthesis of phytylpyrophosphate (PDP)

Tocopherols are a member of the class of compounds referred to as the isoprenoids. Other isoprenoids include carotenoids, gibberellins, terpenes, chlorophyll and abscisic acid. A central intermediate in the production of isoprenoids is isopentenyl diphosphate (IPP). 2 pathways, Cytoplasmic and plastid-based pathways (Fig. 1.4) to generate IPP have been reported. Phytyl pyrophosphate (PDP) is an intermediate in the side chain of tocopherol. In

plastid-based pathway or MEP pathway (Fig. 1.4); 4-phosphocytidyl-2C-methyl-D-erythritol is converted into 4-diphosphocytidyl-2C-methyl-D-erythritol-2 phosphate by the action of the translation product of *chB*. 4-diphosphocytidyl-2C-methyl-D-erythritol-2 phosphate is converted into 2-C-methyl-D-erythritol, 2, 4-cyclophosphate by the translation product of *ygbB*. The latter compound is converted by the translation product of *gcpE* into (E)-1-(4-hydroxy-3-methylbut-2-enyl) diphosphate. Subsequently, the translation product of *LytB*, in turn catalyzes the conversion of (E)-1-(4-hydroxy-3-methylbut-2-enyl) diphosphate to isopentenyl diphosphate. Geranylgeranyl diphosphate (GGDP) is synthdized from isopentenyl diphosphate by geranylgeranyl diphosphate synthase1. PDP can be generated either from GGDP directly by geranylgeranyl diphosphate reductase (GGDR) or phytol and ATP by a phytol kinase (VTE5) present in chloroplast stroma (Fig. 1.5) (Valentin et al. 2006).

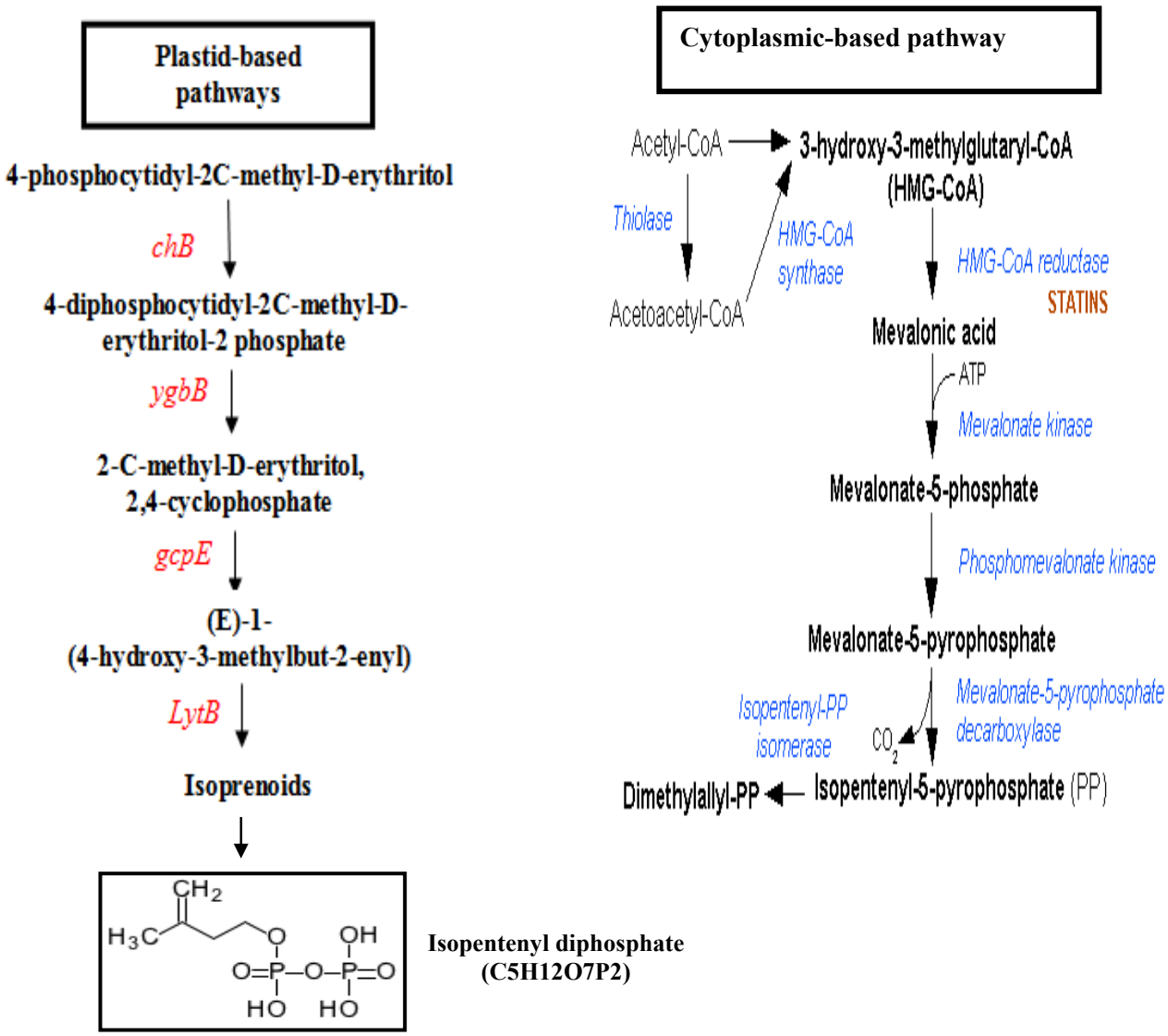


Fig. 1.4 Isopentenyl diphosphate (IPP) pathways

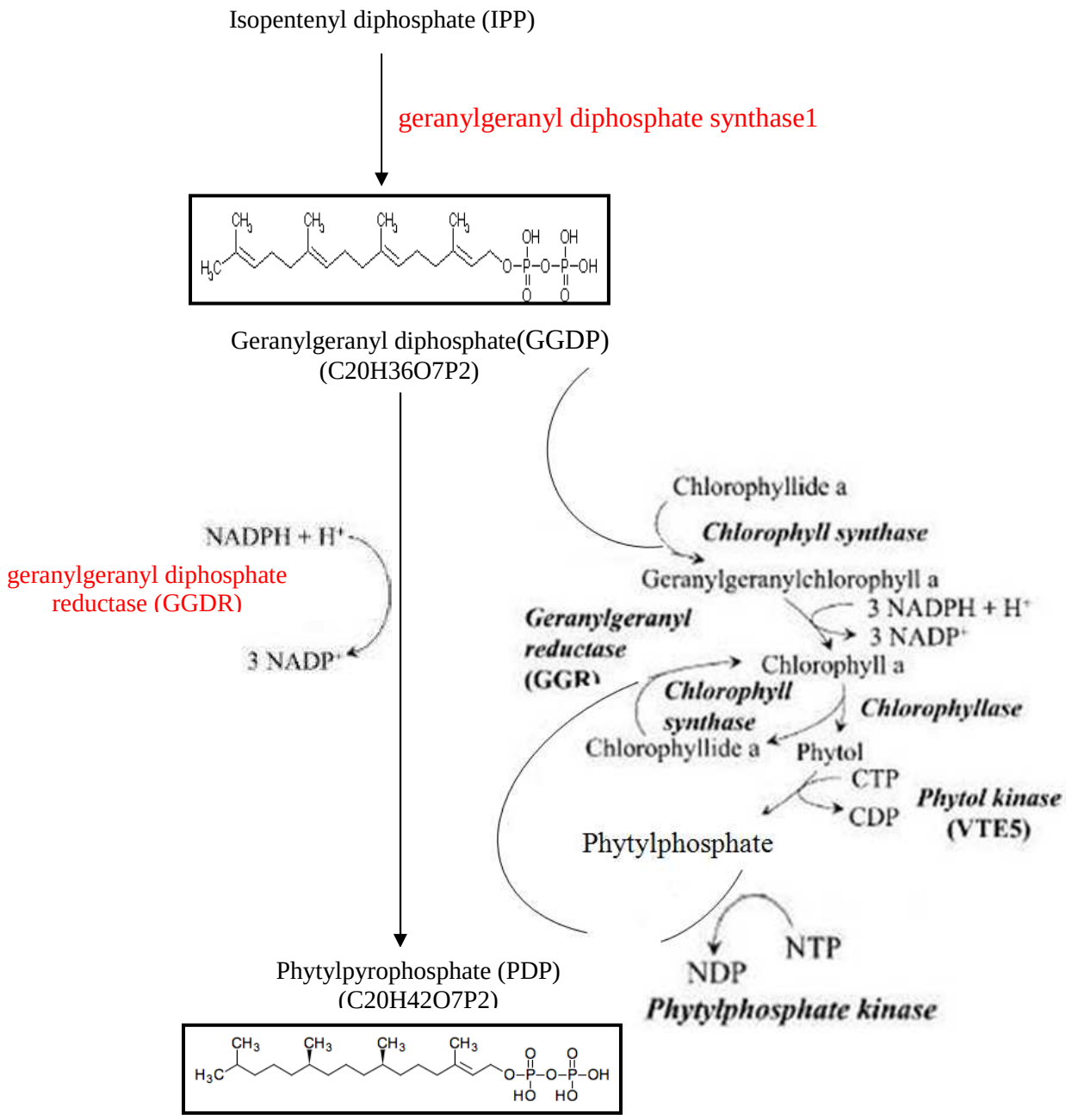


Fig. 1.5 Synthesis of Phytyl pyrophosphate (PDP)

1.3.3.1.3 Joining of HGA and PDP

2-Methyl-6-phytyl-1,4-benzoquinol (MPBQ) is formed after the condensation of HGA and PDP by homogenitisate phytyltransferase (VTE2). MPBQ can be either cyclized by tocopherol cyclase (VTE1) to form δ tocopherol or methylated by VTE3 to form 2, 3-Dimethyl-5-phytyl-1, 4- benzoquinol (DMPBQ) (Fig. 1.6) (Porfirova et al. 2002; Shintani et

al. 2002; Collakova and DellaPenna 2003; Semchuk et al. 2009). DMPBQ can be cyclized by VTE1 to form γ tocopherol (Sattler et al. 2003).

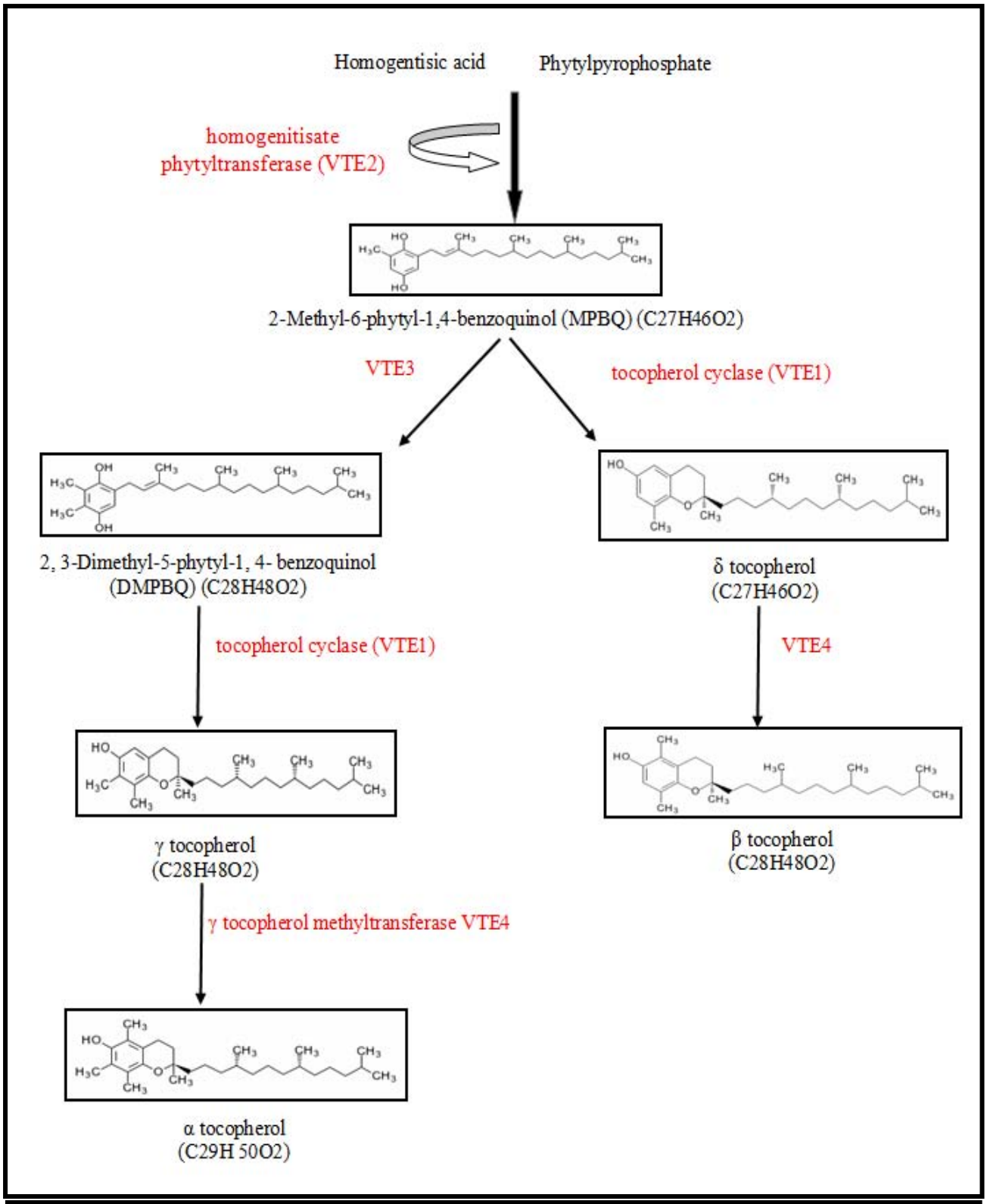


Fig. 1.6 Condensation of homogentisic acid (HGA) and phytylpyrophosphate (PDP)

1.3.3.1.4 Methylation of an aromatic ring

α-tocopherol can be generated by methylation of γ-tocopherol via γ-tocopherol methyl-transferase (VTE4) (D'Harlingue and Camara 1985). β-tocopherol is formed from δ-tocopherol by methylation of the 5 position by VTE4 (Fig. 1.6). Also β-tocopherol can be directly converted to α-tocopherol by tMT2 via the methylation of the 3 position (Norris et al. 2004).

1.4 Molecular map of sunflower

Sunflower, as one of the most important dicot crops, has been studied for construction of molecular genetic map. The first map of sunflower was reported by Gentzbittel et al. (1995) and Berry et al. (1995), using RFLPs markers. Gentzbittel et al. (1999) presented an updated version of the above-mentioned map, using more RFLPs markers. Two other maps were also published, using RFLPs (Jan et al. 1998) and RFLPs and AFLPs (Gedil et al. 2001). Molecular genetic-linkage map based on 459 SSRs, has been constructed by Tang et al. (2002), which is the first reference map of sunflower based on single- or low-copy public SSR markers. Genetic map of our department (Poormohammad Kiani et al. 2007) contains 304 AFLP and 191 SSR markers with the total length of 1824.6 cM and a mean density of one locus every 3.68 cM.

1.5 Objectives

The objectives of this research are:

- To map some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive family genes and *Arabidopsis Sec14* homologue genes,
- To identify chromosomal regions associated with quantitative variation of tocopherol, phytosterol, protein, oil and fatty acid compositions under several environments and conditions and to validate the extent to which these candidate genes affect quantitative phenotypic variability for the studied traits in sunflower grains.
- to map QTLs for leaf-related traits such as dried leaf number (DLN), leaf number (LN), leaf area at flowering (LAF) and leaf area duration (LAD), yield-related traits such as head diameter (HD), head weight (HW), 1000 grain weight (TGW), grain yield per plant (GYP), biomass (BIO) and other traits such as days from sowing to flowering (DSF) and plant

height (PH) in recombinant inbred lines by using saturated SSR and AFLP linkage map of our department (Poormohammad Kiani et al. 2007a), in the field with well-, partial-irrigated and late-sowing conditions

- To assess the genetic variability and to identify AFLP markers and polymorphic candidate genes associated with seed-quality traits under well-irrigated and water-stressed conditions in gamma-induced mutants of sunflower.

Chapter 2

Materials and methods

2.1 Plant materials

2.1.1 Population of recombinant inbred lines (RILs)

The recombinant inbred lines (RILs) used in this research have been developed through single seed descent from F2 plants, derived from a cross between PAC2 and RHA266 (Flores Berrios et al. 2000). Both parental lines are sunflower public inbred lines. RHA266 has been obtained from a cross between wild *H. annuus* and Peredovik by USDA and PAC2 is an INRA-France inbred line from a cross between *H. petiolaris* and 'HA61' (Gentzbittel et al. 1995).

2.1.2 Population of mutants (M8)

The sunflower restorer inbred line 'AS613' has been produced in our laboratory from a cross between two genotypes ('ENSAT-125' and 'ENSAT-704') through a single-seed descent (SSD) programme (Sarrafi et al. 2000). The seeds of 'AS613' were exposed to gamma rays at the Atomic Energy Center (Cadarache, France) with a dose of 75 Grays. Mutants population have been developed through modified SSD method (Sarrafi et al. 2000). Regarding to morpho-physiological studies, among a population of about 2000 gamma-induced mutants of sunflower, 23 M8 mutants were selected for quantitative analysis.

2.2 Experimental conditions

2.2.1 Experimental conditions for RILs

Three independent experiments were undertaken at Teheran University campus of Karaj. Experimental design was a randomized complete block with three replications. Seeds of 89 RILs and their two parents were sown in the field under well-, partial-irrigated and late-sowing conditions. Each genotype per replication consisted of one row, 4m long, 50cm between rows and 25cm between plants in rows. The distance between the replications of well-irrigated and partial-irrigated treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (partial-

irrigated), irrigation was controlled and adjusted by the observation of wilting threshold of the leaves. Partial water deficit was started 45 day after sowing at the near flower bud formation stage and continued up to maturity. The sowing dates were: normal sowing on May and late sowing on July.

2.2.2 Experimental conditions for mutants

Two independent experiments were undertaken in randomized complete block design with three replications. Seeds of mutants and original line (AS613) were sown in the field under well-irrigated and water-stressed conditions. Each genotype per replication consisted of one row, 4m long, 50 cm between rows and 25 cm between plants in rows. The distance between replications of well-irrigated and water-stressed treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (water-stressed), water deficit was started 45 days after sowing at the stage near flower bud formation and continued up to maturity.

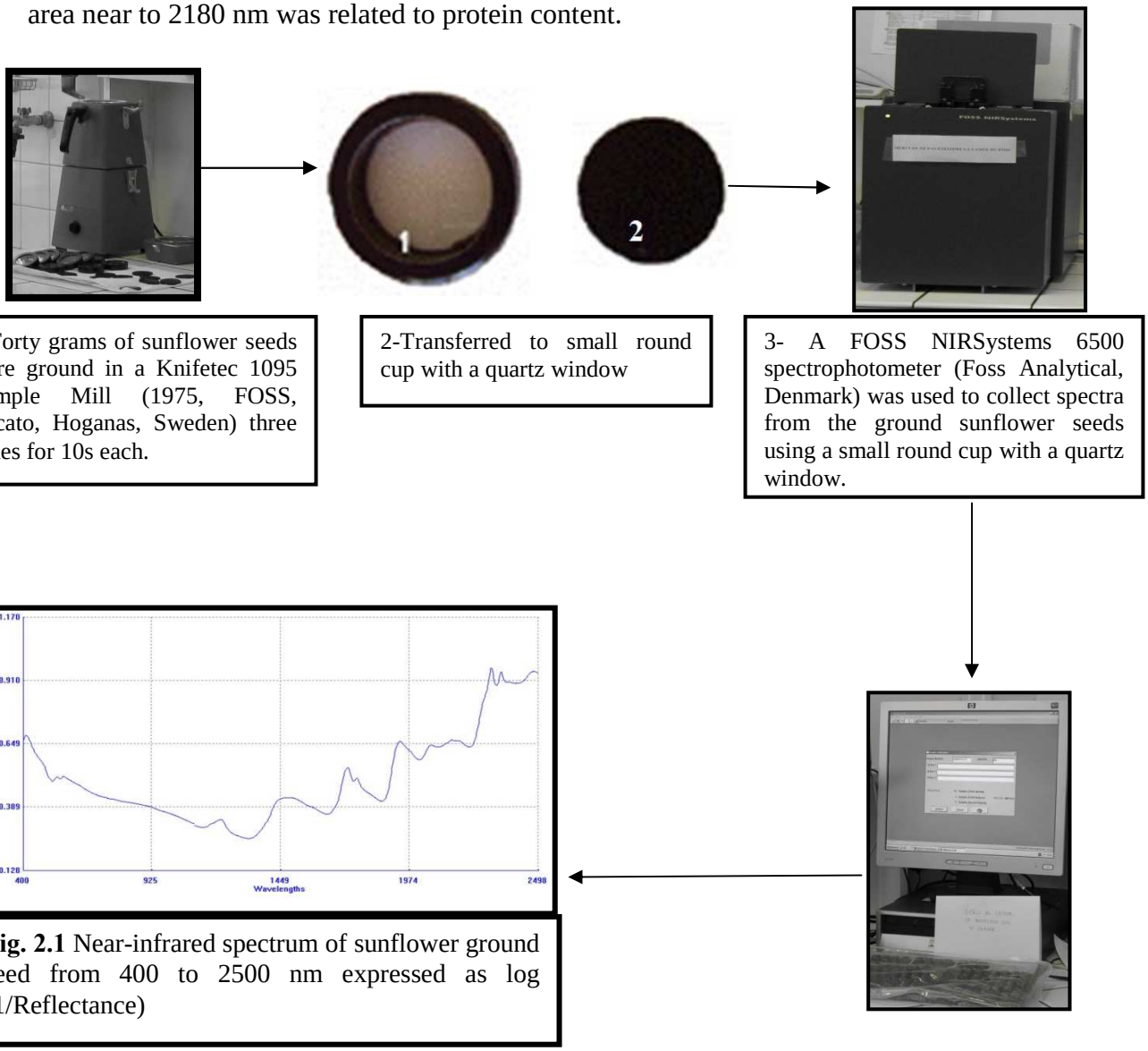
2.3 Trait measurements

2.3.1 Morpho-physiological traits (Fig. 2.2)

DSF (days from sowing to flowering) and PH (plant height) were measured for RILs and their parental lines at the plot scale (on the line, when 50% of the plants were at the flowering stage). Leaf-related traits such as LN (leaf number), DLN (dried leaf number), were recorded at anthesis, and leaf length (L) and width (W) of all green leaves were measured at flowering stage, and total leaf area at flowering (LAF) was calculated with the formula: LAF= 0.7L×W (Alza and Fernandez-Martinez 1997). Green leaf area of the plants was determined weekly from flowering to harvest in order to evaluate green leaf area with respect to time. An integral of weekly leaf area was considered as being an estimate of leaf area duration (LAD, m^2 days). At harvest, yield components such as HD (head diameter), HW (head weight), HGW (100 grain weight), GYP (grain yield per plant) and BIO (biomass) were measured. HN (head number) was also measured when 50% of the plants were at the anthesis. Three plants per genotype per condition per replication were randomly chosen for evaluation of the mentioned traits.

2.3.2 Seed-quality traits (Fig. 2.2)

Percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC), linoleic acid content (LAC), total tocopherol content (TTC) and total phytosterol content (TPC) were measured by the FOSS NIRSystems 6500. Forty grams of sunflower seeds per genotype per condition per replication were ground in a Knifetec 1095 Sample Mill (1975, FOSS, Tecato, Hoganas, Sweden) three times for 10 s each. A FOSS NIRSystems 6500 spectrophotometer (Foss Analytical, Denmark) was used to collect spectra from the ground sunflower seeds using a small round cup with a quartz window. The reflectance (R) of each sample was measured as log of 1/R from 400 to 2500 nm at 2 nm intervals. Eight hundred sixty and 660 spectra database were used for tocopherol and phytosterol prediction, respectively. The absorption maximum around 1700-1800 and 2300-2400 nm were due to oil and fatty acid content. The area near to 2180 nm was related to protein content.



1-Forty grams of sunflower seeds were ground in a Knifetec 1095 Sample Mill (1975, FOSS, Tecato, Hoganas, Sweden) three times for 10s each.

2-Transferred to small round cup with a quartz window

3- A FOSS NIRSystems 6500 spectrophotometer (Foss Analytical, Denmark) was used to collect spectra from the ground sunflower seeds using a small round cup with a quartz window.

Fig. 2.1 Near-infrared spectrum of sunflower ground seed from 400 to 2500 nm expressed as log (1/Reflectance)

2.3.2 .1 Solvent extraction of lipids

The extraction of the total oil content was performed by hexane (n-hexane, Prolabo/Subra, Toulouse, France) extraction using an accelerated solvent extractor apparatus (ASE 200, Dionex, France) with an isopropanol/hexane mixture (5:95 v/v) during 20 min. Then, the solvent was removed from the extracts under low pressure evaporation (Rotavapor, Bioblock Scientific HS 40 HUBER, Heildorph, Germany). Lipid extracts were weighed and tocopherol content was analyzed.

2.3.2.2 Tocopherol determination

Total tocopherol for some samples was achieved using a high-performance liquid chromatography (HPLC) (SpectraPhysics, Thermo Separation Products, USA) with a normal-phase LiChrosorb Si60 column, 250cm x 4mm x 5µm (CIL, Cluzeau, France) (ISO 9936, 1997). The mobile phase was a mixture of hexane/isopropanol (99.7:0.3 v/v) at 1mL/min flow rate. One gram of oil sample was diluted in 25 mL of hexane and 20µl was injected into the HPLC. Detection was performed with fluorescence detector (excitation wavelength = 298 nm and emission wavelength=344nm: Waters 2475 multi λ). Total tocopherol content was calculated as the sum of α, β, γ, and δ-tocopherol contents and expressed in mg kg^{-1} oil.

2.3.2.3 NIRS calibration

Prediction equations were calculated with a modified partial least-squares regression (MPLS) model after 4 outlier elimination passes (WINISI 1.02 - Infrasoft International LLC). With the MPLS regression method, factors are extracted in decreasing order of reliance measured by covariance with the response variable. To prevent overfitting in calibration, the number of factors is optimized by cross-validation in calibration set. Previous mathematical treatment was applied on each spectrum: a standard normal variate and detrend (SNV/detrend) scatter correction, a first-derivative transformation, and a smoothing on four data points. The equation with the highest coefficient of determination (R^2) and the lowest standard error (SE) of the calibration was used to predict the tocopherol and the phytosterol values of the validation set.

The performance of this NIRS model, for the estimation of tocopherols and phytosterols, was determined by the following parameters: the standard error of calibration (SEC), the coefficient of determination in calibration (RSQ), the standard error of cross-validation (SECV), the coefficient of determination of cross-validation (1–VR) and the standard error

of prediction (SEP). Near infrared reflectance (NIR) spectroscopy, has been successfully used as an alternative technique to classical methods in due to determine multiple parameters such as proteins, oil content, fatty acid compositions (Pérez-Vich et al., 1998; Velasco & Becker, 1998; Biskupek-Korell & Moschner, 2007; Ebrahimi et al., 2008; Ebrahimi et al., 2009). In previous study in our laboratory,a relatively good correlation between NIRS results and HPLC and GC method for total tocopherol (R^2 = 0.64) (Ayerdi Gotor et al., 2007) and total phytosterol content (R^2 = 0.61) (Ayerdi Gotor et al., 2008; Calmon et al. 2009) were observed. We have obtained a very good correlation between the HPLC analysis and the NIRS predictions (R^2 = 0.76).

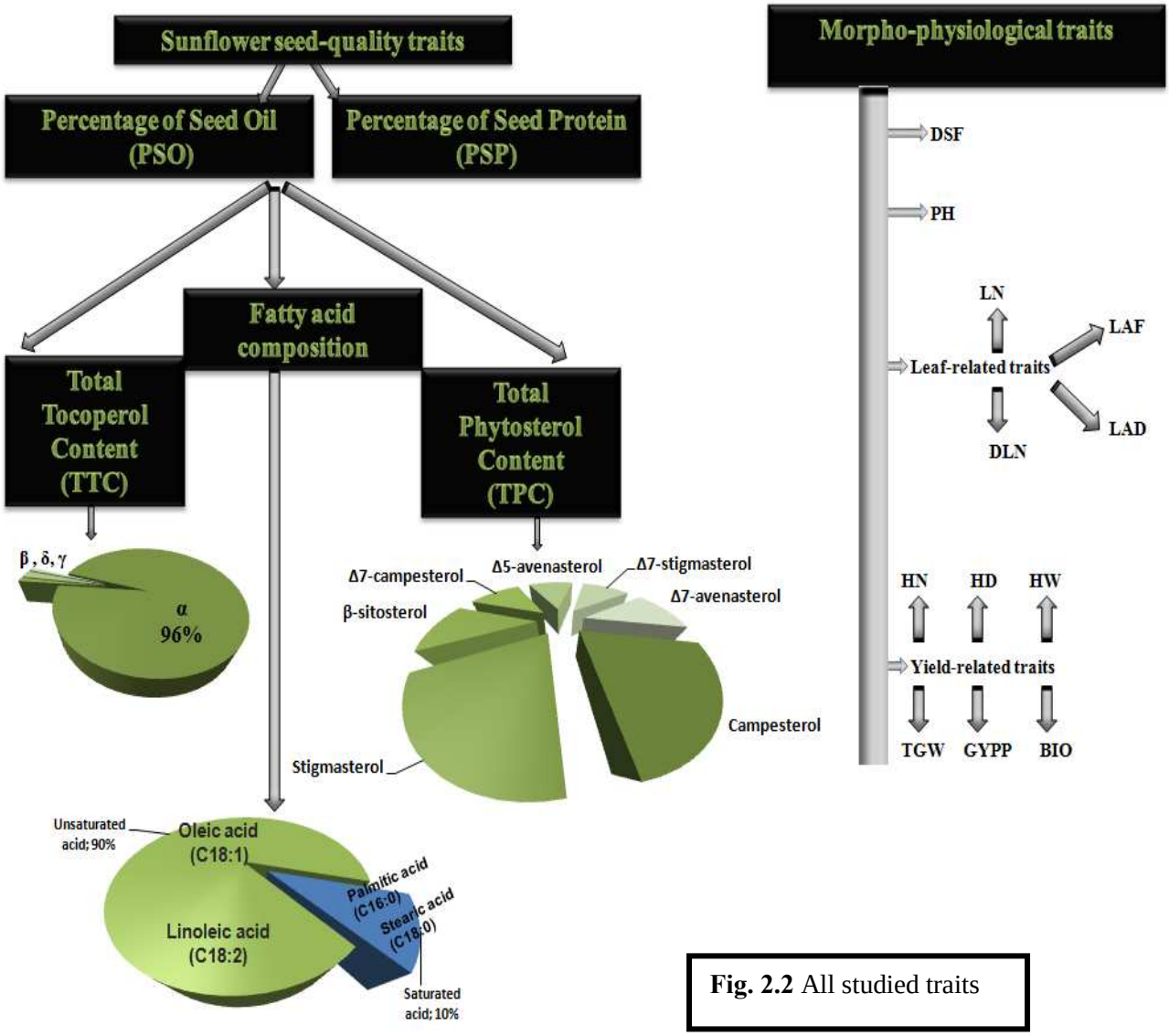


Fig. 2.2 All studied traits

2.4 Molecular analysis

2.4.1 DNA Extraction and PCR program

The genomic DNA of PAC2, RHA266, RILs, original and mutant lines were isolated and DNA quantification was performed by picogreen (protocole is included in appendix). The PCR program was: 4 min at 94 °C followed by 35 cycles; 30 s at 94 °C, 30 s at (55 °C or 58 °C), 1 min at 72 °C and at last, 5 min at 72 °C.

2.4.2 AFLP genotyping

Different *MseI/EcoRI* primer combinations were used for AFLP genotyping. The AFLP procedure was conducted as described by Rachid Al-Chaarani et al. (2004).

2.4.3 Candidate Genes (CGs)

Some important tocopherol pathway-related genes, phosphoglyceride transfer-related genes, enzymatic antioxidant-related genes, drought-responsive genes and fatty acid biosynthesis-related genes were studied. Respective sequence data for candidate genes coding for these proteins were obtained from The *Arabidopsis* Information Resource (*www.arabidopsis.org*). In order to seek the *helianthus* homolog sequences to the *Arabidopsis* genes, we used the Compositae EST assembly clusters, available at the *Helianthus*-devoted bioinformatics portal Heliagene (*www.heliagene.org*). The *Helianthus* EST clusters presenting the reciprocal blast with the highest score and lowest E value with regarding to the original *Arabidopsis* genes were chosen for our studies. All primers were designed by MATLAB. Between 2 to 4 various primer combinations per each candidate gene were tested on agarose gel. After sequencing; SNP-PHAGE, through the website at *http://www.heliagene.org/*, was applied for analyzing sequence traces from both parents to identify SNPs. Genotyping was done by SNP-based CAPS marker and high resolution melting (HRM) as well as directly on agarose gel.

2.4.4 High-Resolution Melting (HRM)

HRM was performed in capillaries of a Light Cycler® 480 Real-Time (Roche Applied Science) with the cycling program consisting of 5 min of initial denaturation at 95 °C and 60 cycles of 15 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C. Melting curves were generated by ramping from 70 to 95 °C at 0.02 °C/s. The gene scanning module in the LightCycler® 480

software was used to normalize raw melting curve data by setting pre-melt and post-melt signals of all RILs and parental lines to uniform values and then to modify the normalized curves across a temperature axis as well as to plot the melting curve differences between them.

2.4.5 Cleaved Amplified Polymorphic Sequence (CAPS) marker

SNPs between PAC2 and RHA266 for sterol C-methyltransferase (*SMT2*), delta24-sterol reductase (*DWF1*) and patellin2 (*PAT2*), *Arabidopsis* Sec14 homologue gene, were identified through multiple sequence alignments using SNP-PHAGE. Then they were converted to CAPS marker to allow genotyping of RILs via *www.biophp.org/minitools/restriction_digest/demo.php* and *http://helix.wustl.edu/dcaps/dcaps.html* (alll sequences are included in appendix). The following protocol was used for DNA digestion: sterile and deionized water 6.8µl, RE 10X buffer 1 µl, PCR-product 2 µl and restriction endonuclease (10 u/µl) 0.2 µl. Incubation at 37 °C for 2 hours was performed (Promega-usage information). All samples were incubated at 65 °C for 10 min to deactivate restriction enzyme.

2.5 Statistical analysis

2.5.1 Statistical analysis for RILs

Data were analyzed using SAS PROC GLM (SAS Institute Inc. 1996) and SPSS. Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. The mean of RILs and that of their parents were compared for both traits. Genetic gain (GGB) when the best RIL is compared with the best parent and genetic gain (GG10%) when the mean of the top 10% selected RILs is compared with the mean of the parents, were determined for the studied traits. Additive and environmental variances as well as narrow-sense heritability were calculated according to Kearsey and Pooni (1996), using least-square estimates of genetic parameters.

2.5.2 Statistical analysis for mutants

The data were analyzed using SPSS. Correlations among traits in each of condition were determined. The association between AFLP markers and candidate genes with the quantitative traits was estimated through stepwise multiple regression analysis, where each quantitative trait was considered as an dependent variable while AFLP markers and candidate genes were treated as an independent variable. To select independent variables for the regression

equation, *F*-values with 0.045 and 0.099 probability were used to enter and remove, respectively.

2.6 QTL and map construction

Some of the studied candidate genes were introduced in our map (Poormohammad Kiani et al. 2007) based on SSR markers using CarthaGene 0.999 (Schiex and Gaspin 1997). Chi-square-tests were performed for segregation distortion of each locus. Loci were assembled into groups using likelihood odds (LOD) ratios, with a LOD threshold of 4.0 and a maximum recombination frequency threshold of 0.35. Multiple locus order estimates were performed for each linkage group. The likelihoods of different locus orders were compared and the locus-order estimate with the highest likelihood was selected for each linkage group. The Kosambi mapping function was used to calculate map distances (cM) from recombination frequencies. Mapchart 2.1 was used for graphical presentation of linkage groups and map position of the studied candidate genes. The chromosomal locations of QTLs for the studied traits were resolved by composite interval mapping (CIM), using Win QTL Cartographer, version 2.5 (Wang et al. 2005) with the mean values of three replications for each RIL in each condition. The genome was scanned at 2-cM intervals; with a window size of 15 cM. Up to 15 background markers were used as cofactors in the CIM analysis with the programme module Srmapqtl (model 6). Additive effects of the detected QTLs were estimated with the Zmapqtl program. The percentage of phenotypic variance (R^2) explained by each QTL was estimated by Win QTL Cartographer.

Chapter 3

Genetic dissection of tocopherol and phytosterol in recombinant inbred lines of sunflower through QTL analysis and the candidate gene approach

Submitted

Abstract

Sunflower contains tocopherol, non-enzymatic antioxidant known as lipid-soluble vitamin E, and phytosterol with interesting properties, which can result in decreased risk for chronic diseases in human and with several beneficial effects in plants. The genetic control of tocopherol and phytosterol content in a population of recombinant inbred lines (RILs) of sunflower under several environments and conditions is studied through quantitative trait loci analysis (QTL) using genetic-linkage map based on SSR markers and introducing some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive family genes and *Arabidopsis Sec14* homologue genes. Three important candidate genes (*HPPD*, *VTE2* and *VTE4*), which encode enzymes involved in tocopherol biosynthesis, are mapped to linkage group 8(LG8) and LG14. One of the most important candidate genes coding for sterol methyltransferase II (SMT2) enzyme is anchored to LG17 by CAPS marker. Four SNPs are identified for *PAT2*, *Arabidopsis* Sec14 homologue gene, between two parents. *PAT2* is assigned to LG2 by CAPS marker. Squalene epoxidase (*SQE1*) is also assigned to LG15 by InDel marker. Through other candidate genes, *POD*, *CAT* and *GST* encoding enzymatic antioxidants are assigned to LG17, LG8 and LG1, respectively. One to 6 QTLs are identified, depending on the trait and environments. The major QTL for total phytosterol content on linkage group 8 accounted for 55.1% of the phenotypic variation, which is overlapped with the QTL of total tocopherol content. *GST*, *POD*, *SMT2* and *SEC14-2* genes showed co-localization with QTL for phytosterol content. Two candidate genes, *HPPD* and *SFH3,* exhibited co-localization with QTL for tocopherol content (*7.TTC.14, 6.TTC.14, 7.TTC.16, 5.TTC.16*). The candidate genes associated with tocopherol and phytosterol, especially *HPPD* and *SMT2,* could be precisely used for alternation of the tocopherol and phytosterol content of sunflower seeds by development of functional markers.

Key words: Tocopherol, Phytosterol, QTL, candidate gene, Sunflower

Abbreviation: **RIL**, recombinant inbred line; **SSR**, simple sequence repeats; **CG**, candidate gene; **SNP**, single-nucleotide polymorphism; **CAPS**, cleaved amplified polymorphic sequence; **InDel**, insertion/deletion; **HRM**, high-resolution melting; **NIRS**, near-infrared reflectance spectrometry; **HPLC**, high-performance liquid chromatography; **TTC**, total tocopherol content; **TPC**, total phytosterol content; **CIM**, composite interval mapping; **QTL**, quantitative trait locus.

3.1. Introduction

Sunflower contains minor components such as tocopherol and phytosterol with interesting properties for human health. Phytosterols are products of the isoprenoid biosynthetic pathway naturally present in plants and occurring exclusively in the cytoplasm. Phytosterols are present in different plant parts and mostly in seeds. Their level depends on species and sunflower seeds contain a quite high concentration (Mouloungui et al., 2006). The role of phytosterols in plant growth and developmental processes like cell division, polarity and morphogenesis (Lindsey et al. 2003; Schaller 2004), embryogenesis (Clouse, 2000), membrane fluidity and permeability (Schaller 2003), as anti-inflammatory (Bouic, 2001) and as anti-oxidation activities (Van Rensburget et al., 2000) has been also well known. In sunflower seed oil, total phytostertol content represents the sum of campesterol, stigmasterol, β-sitosterol, Δ7-campesterol, Δ5-avenasterol, Δ7-stigmasterol and Δ7-avenasterol (Ayerdi Gotor et al. 2007). Tocopherol (α, β, γ, and δ-tocopherol) belongs to the Vitamin E class of lipid soluble antioxidants that are essential for human nutrition. The function of tocopherol in human and animal systems is generally related to the level of α-tocopherol activity. Alpha-tocopherol has a maximum vitamin E activity (Kamal-Eldin and Appelqvist 1996). Oilseed crops reveal large variation in the levels of the different tocopherols and total tocopherol content (Marwede et al. 2005). Among oil seed crops sunflower grains mainly contain α tocopherol, which accounts for more than 95% of the total tocopherols, whereas in soybean 59% of total tocopherol is γ tocopherol (Kamal-Eldin and Andersson 1997). In plants, it is suggested that the protection of photosynthetic apparatus and polyunsaturated fatty acids from oxidative damage caused by reactive oxygen species (ROS) are the main photosynthetic and non photosynthetic function of tocopherol, respectively (Trebst et al. 2002; Velasco et al. 2004; Cela et al. 2009; Semchuk et al. 2009). Epidemiological evidence indicates that tocopherol and phytosterol supplementation can result in decreased risk for chronic diseases such as cardiovascular disease, cancer, neurological disorders, cataracts, and age-related macular degeneration (Bramley et al. 2000) and reduction of cholesterol levels in blood (Ostlund 2002). The amount of α tocopherol in sunflower seed can be controlled by 3 loci; *Tph1 (m), Tph2 (g)* and *d* (Demurin 1993; Hass et al. 2006; Tang et al. 2006). The epistasy between *Tph1* and *d* loci are reported by Tang et al. (2006) .The level of β tocopherol is increased by *d* locus in mutant inbred lines (*m m*). The level of γ tocopherol is increased by *g* locus in mutant inbred lines (*g g*) as a result of knockout of γ tocopherol methyl transferase (Hass et al. 2006). The

d locus is mapped to linkage group 4 and the *Tph2* locus is placed in linkage group 8 between ORS312 and ORS599 makers (Hass et al. 2006). The co-segregation of *Tph1* with SSR markers ORS1093, ORS222 and ORS598 is observed (Vera-Ruiz et al. 2006). 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (*MPBQ*/ *MSBQ-MT*) paralogs from sunflower (*MT1* and *MT2*) are isolated and sequenced (Tang et al. 2006). InDel markers are developed for *MT1* and *MT2* and the *MT1* Locus is assigned to linkage group 1 (Tang et al. 2002; Tang et al. 2003).

Sunflower has been considered for construction of molecular map. The first map of sunflower was reported by Gentzbittel et al. (1995) and Berry et al. (1995), using RFLPs markers. Gentzbittel et al. (1999) presented an updated version of the above-mentioned map, using more RFLPs markers. Two other maps were also published, using RFLPs (Jan et al. 1998) and RFLPs and AFLPs (Gedil et al. 2001). Genetic-linkage map based on 459 SSR, has been also constructed (Tang et al. 2002), which is the first reference map of sunflower based on single- or low-copy public SSR markers. Genetic map of our department (Poormohammad Kiani et al. 2007) contains 304 AFLP and 191 SSR markers with the total length of 1824.6 cM and a mean density of one locus every 3.7 cM. Identification of chromosomal regions with effects on tocopherol and phytosterol would increase our understanding of the genetic control of these traits. As far as we know QTLs controlling total tocopherol content in sunflower have not been reported in the literature. One QTL for total sterols was identified on linkage group one that explained 14.3 % of the total variability (Alignan et al. 2008). QTLs associated with tocopherol in maize and winter oilseed rape are detected (Wong et al. 2003; Marwede et al. 2005). One to five QTLs are identified for α, γ and total tocopherol content and α/β tocopherol ratio in winter oilseed rape (Marwede et al. 2005). Thirty-one QTLs associated with tocopherol content and its compositions were identified by composite interval mapping (CIM) in RILs of maize (Chander et al. 2008). These QTLs are mapped on sixteen linkage groups except linkage group4. Eight QTLs were identified for total tocopherol. On LG5, the QTL of total tocopherol was linked to *HPPD* gene and explained 7.13% of phenotypic variance (Chander et al. 2008). Three QTLs controlling total phytosterol content are identified on LG8, 13, 18 in a population consisted of 148 DH lines of rapeseed and explained 60% of phenotypic variance (Amar et al. 2008). The objectives of this research are to map some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive family genes and *Arabidopsis Sec14* homologue genes, to identify chromosomal regions associated with quantitative variation of tocopherol and phytosterol content under several environments

and conditions and to validate the extent to which these candidate genes affect quantitative phenotypic variability for tocopherol or phytosterol content in sunflower grains.

3.2. Materials and methods

3. 2.1 Plant materials and experimental conditions

The recombinant inbred lines (RILs) used in this research have been developed through single seed descent from F2 plants, derived from a cross between PAC2 and RHA266 (Flores Berrios et al. 2000). Both parental lines are sunflower public inbred lines. RHA266 has been obtained from a cross between wild *H. annuus* and Peredovik by USDA and PAC2 is an INRA-France inbred line from a cross between *H. petiolaris* and 'HA61' (Gentzbittel et al. 1995).

3.2.1.1 First year - France-Toulouse

3.2.1.1a Greenhouse experiment- Seventy-two RILs and their parents (PAC2 and RHA266) were grown in a greenhouse in plastic pots containing a mixture of 40% soil, 40% compost, and 20% sand. Temperature was controlled at 25/18 ± 2°C (day/night), relative humidity was 65/85 ± 5%, and light was provided to obtain a 16h light period. The experiment consisted of a split-plot design with three blocks and one plant per genotype per water treatment per block. The main plot was water treatment (well-watered and water-stressed) and subplot was genotype (RILs and their parents). The RILs and their parents were randomized within each treatment–block combination. To simulate water-deficit conditions similar to the field, a progressive water stress was imposed at the stage near flower bud formation (R1; Schneiter and Miller 1981) by progressively decreasing the irrigation to 30% of field capacity during 12 days. Water-stressed plants were then irrigated at 30% of field capacity until harvest. Well-watered plants received sufficient water to maintain soil water content close to field capacity. Both well-watered and water-stressed plants were weighed to maintain the desired soil water content. Midday leaf water potential of the youngest fully expanded leaf was about –1.8 MPa in water-stressed plants, corresponding to a severe water stress (Maury et al. 1996). According to Tezara et al. (2002), field water capacity of about 60% is considered as mild stress and 33% is considered as severe stress in sunflower.

3.2.1.1b Field experiment- Ninety-nine RILs and their parents (PAC2 and RHA266) were grown in the field under both well-irrigated and non-irrigated (rainfall) treatments. In each water treatment, 3 replications were designed and each replication consisted of 2 rows, each 4.6 m long, with 50 cm between rows and 25 cm between plants in a row. Thirty-two plants were obtained per plot. Plants in the well- irrigated treatment were watered two times, at two critical stages: before flowering and during grain filling (determined according to the sunflower irrigation programme in the Toulouse region, France). Plants in the water-stress (rainfall) treatment were not irrigated at all. Four plants per genotype per water treatment per replication were randomly chosen for our studies.

3.2.1.2 Second year- Iran-Karaj

Three independent experiments were undertaken at Teheran University campus of Karaj. Experimental design was a randomized complete block with three replications. Seeds of 89 RILs and their two parents (PAC2 and RHA266) were sown in the field under well-, partial-irrigated and late-sowing conditions. Each genotype per replication consisted of one row, 4m long, 50cm between rows and 25cm between plants in rows. The distance between the replications of well-irrigated and partial-irrigated treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (partial-irrigated), plots were irrigated once every two weeks. Partial water deficit was started 45 day after sowing at the near flower bud formation stage and continued up to maturity. The sowing dates were: normal sowing on May and late sowing on July.

3.2.2 Trait measurements

3. 2.2.1 Tocopherol measurement

Pre-measurements for total tocopherol content (TTC) were carried out by both FOSS NIRSystems 6500 and reference method (HPLC, ISO 9936, 1997) for core collection. Forty grams of sunflower seeds were ground in a Knifetec 1095 Sample Mill (1975, Foss Tecator, Höganäs, Sweden) three times for 10 s each. A FOSS NIR Systems 6500 spectrophotometer (Foss Analytical, Denmark) was used to collect spectra from the ground sunflower seeds using a small round cup with a quartz window. The reflectance (R) of each sample was measured as log of 1/R from 400 to 2500 nm at 2nm intervals. Total oil content was extracted and TTC was thus determined using the following protocol:

Solvent extraction of lipids

The extraction of the total oil content was performed by hexane (n-hexane, Prolabo/Subra, Toulouse, France) extraction using an accelerated solvent extractor apparatus (ASE 200, Dionex, France) with an isopropanol/hexane mixture (5:95 v/v) during 20 min. Then, the solvent was removed from the extracts under low-pressure evaporation (Rotavapor, Bioblock Scientific HS 40 HUBER, Heildorph, Germany). Lipid extracts were weighed and tocopherol content was analyzed.

Tocopherol determination

Total tocopherol was achieved using a high-performance liquid chromatography (HPLC) (SpectraPhysics, Thermo Separation Products, USA) with a normal-phase LiChrosorb Si60 column, 250cm x 4mm x 5µm (CIL, Cluzeau, France) (ISO 9936, 1997). The mobile phase was a mixture of hexane/isopropanol (99.7:0.3 v/v) at 1mL/min flow rate. One gram of oil sample was diluted in 25 mL of hexane and 20µl was injected into the HPLC. Detection was performed with fluorescence detector (excitation wavelength = 298 nm and emission wavelength=344nm: Waters 2475 multi λ). Total tocopherol content was calculated as the sum of α, β, γ, and δ-tocopherol contents and expressed in mg kg^{-1} oil.

3.2.2.2 Phytosterol measurement

Pre-measurements for total phytosterol content (TPC) were also carried out by both FOSS NIRSystems 6500 and reference method (GC) for core collection. Total phytosterol content for core collection was measured by gas chromatography after saponification with KOH 0.5M during 15 min and a purification on an aluminium oxide basic colum. One µl of trimethylsylil (TMS) solutions were injected into fused silica capillary (ZB-5) column (Phenomenex, Paris, France) in a Fisons gas chromatography (GC 8000 series MMFC 800 Multi-function controller, Italy) fitted with a flame ionization detector. Sterols were identified by their retention time relative to betulin (Internal standard –Sigma-France). Total phytosterol was calculated as campesterol, stigmasterol, β-sitosterol, Δ7-campesterol, Δ5-avenasterol, Δ7-stigmasterol and Δ7-avenasterol content and expressed in mg 100 g^{-1} oil. A modified partial least-squares regression (MPLS) model, after 4 outlier elimination passes (WINISI 1.02 – Infrasoft International LLC) was used. The performance of our NIRS model, for the estimation of tocopherols and phytosterols was determined by the following

parameters: the standard error of calibration (SEC), the coefficient of determination in calibration (RSQ), the standard error of cross-validation (SECV), the coefficient of determination of cross-validation (1–VR) and the standard error of prediction (SEP). We have obtained a high significant correlation between the HPLC analysis and the NIRS predictions for TTC ($R^2 = 0.76$) and good relatively correlation between the GC analysis and the NIRS predictions for TPC. In previous studies in our department, a relatively good correlation between NIRS results and GC method for total phytosterol content ($R^2 = 0.61$) (Ayerdi Gotor et al. 2008; Calmon et al. 2009) was also observed. Then, TTC and TPC were measured for all 1827 samples.

3. 2.3 Molecular and statistical analysis

3.2.3.1 DNA Extraction and PCR program

A set of 123 RILs and their parents (PAC2, RHA266) were used for DNA extraction and DNA quantification was performed by picogreen. The PCR program was: 4 min at 94 °C followed by 35 cycles; 30 s at 94 °C, 30 s at (55 °C or 58 °C), 1 min at 72 °C and at last, 5 min at 72 °C.

3.2.3.2 Candidate Genes (CGs)

Some important tocopherol and phytosterol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive genes and *Arabidopsis Sec14* homologue genes were selected to introduce in our map (Poormohammad Kiani et al. 2007). Reactions catalyzed by proteins of the tocopherol and phytosterol biosynthetic pathway are illustrated in Fig. 3.1 and 3.2, respectively. Respective sequence data for candidate genes (CGs) coding for these proteins were obtained from The *Arabidopsis* Information Resource (*www.arabidopsis.org*). In order to seek the *helianthus* homolog sequences to the *Arabidopsis* genes, we used the Compositae EST assembly clusters, available at the *Helianthus*-devoted bioinformatics portal Heliagene (*www.heliagene.org*). The *Helianthus* EST clusters presenting the reciprocal blast with the highest score and lowest E value with regarding to the original *Arabidopsis* genes were chosen for our studies. All primers were designed by MATLAB. Between 2 to 4 various primer combinations per each candidate gene were tested on agarose gel**.** After sequencing; SNP-PHAGE, through the website at *http://www.heliagene.org/*, was applied for analyzing sequence traces from both parents to identify SNPs. Genotyping was

done by SNP-based CAPS markers, InDel marker and high resolution melting (HRM) as well as directly on agarose gel. Primers used for PCR, HRM, InDel and CAPS makers are summarized in Table 3.1.

3.2.3.3 High-Resolution Melting (HRM)

HRM was performed in capillaries of a Light Cycler® 480 Real-Time (Roche Applied Science) with the cycling program consisting of 5 min of initial denaturation at 95 °C and 60 cycles of 15 s at 95 °C, 15 s at 58 °C and 20 s at 72 °C. Melting curves were generated by ramping from 70 to 95 °C at 0.02 °C/s. The gene scanning module in the LightCycler® 480 software was used to normalize raw melting curve data by setting pre-melt and post-melt signals of all RILs and parental lines to uniform values and then to modify the normalized curves across a temperature axis as well as to plot the melting curve differences between them. All RILs and their parents were clustered into groups, based on their melting curves.

3.2.3.4 Cleaved Amplified Polymorphic Sequence (CAPS) marker

SNPs between PAC2 and RHA266 for sterol methyltransferase II (*SMT2*), delta24-sterol reductase (*DWF1*) and patellin2 (*PAT2*), *Arabidopsis* Sec14 homologue gene, were identified through multiple sequence alignments using SNP-PHAGE. Then they were converted to CAPS marker to allow genotyping of RILs via *www.biophp.org/minitools/restriction_digest/demo.php* and *http://helix.wustl.edu/dcaps/dcaps.html*. The following protocol was used for DNA digestion: sterile and deionized water 6.8µl, RE 10X buffer 1 µl, PCR-product 2 µl and restriction endonuclease (10 u/µl) 0.2 µl. Incubation at 37 °C for 2 hours was performed (Promega-usage information). All samples were incubated at 65 °C for 10 min to deactivate restriction enzyme.

3.2.4 Statistical analysis and map construction

Data were analyzed using SAS PROC GLM (SAS Institute Inc. 1996) and SPSS. Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. The mean of RILs and that of their parents were compared for both traits. Genetic gain (GGB) when the best RIL is compared with the best parent and genetic gain (GG10%) when the mean of the top 10% selected RILs is compared with the mean of the parents, were determined for the studied traits. Some of the studied candidate genes were introduced in our map (Poormohammad Kiani et al. 2007) based on SSR markers using CarthaGene 0.999

(Schiex and Gaspin 1997). Chi-square-tests were performed for segregation distortion of each locus. Loci were assembled into groups using likelihood odds (LOD) ratios, with a LOD threshold of 4.0 and a maximum recombination frequency threshold of 0.35. Multiple locus order estimates were performed for each linkage group. The likelihoods of different locus orders were compared and the locus-order estimate with the highest likelihood was selected for each linkage group. The Kosambi mapping function was used to calculate map distances (cM) from recombination frequencies. Mapchart 2.1 was used for graphical presentation of linkage groups and map position of the studied candidate genes. The chromosomal locations of QTLs for the studied traits were resolved by composite interval mapping (CIM), using Win QTL Cartographer, version 2.5 (Wang et al. 2005) with the mean values of three replications for each RIL in each conditions. The genome was scanned at 2-cM intervals; with a window size of 15 cM. Up to 15 background markers were used as cofactors in the CIM analysis with the programme module Srmapqtl (model 6). Additive effects of the detected QTLs were estimated with the Zmapqtl program. The percentage of phenotypic variance (R^2) explained by each QTL was estimated by Win QTL Cartographer.

3.3. Results

3. 3.1 Phenotypic variation

Results of analysis of variance for the two studied traits in France and Iran are presented in Table 3.2 and 3.3, respectively. Significant genotypic effect is observed for aforementioned traits in all experiments. The effect of water treatment was significant for TPC in greenhouse conditions, and TTC was significantly affected in the field (Table 3.2). The RIL x Water treatment interaction was highly significant for TTC and TPC in greenhouse conditions (Table 3.2). Genetic gain and phenotypic performance of RILs and their parents for above-mentioned traits in all environments and conditions are calculated. The difference between parents is significant for total tocopherol content (TTC) and total phytosterol content (TPC) in all experiments. The differences between the mean of RILs (MRILs) and the mean of their parents (MP) for TTC and TPC are not significant. The mean of RILs for TTC and TPC is higher under late-sowing and partial-irrigated conditions compared to the well-irrigated RILs (Table 3.4). The comparison between the best parent (BP) and the best RIL (BRIL), presented as genetic gain (GGB), showed a significant difference for both traits in all conditions. A large genetic variation is observed for the two studied traits resulting in significant differences between the 10% selected RILs

(10%SRILs) and the mean of the parents for all conditions. Frequency distribution of TTC and TPC for RILs under several environments and conditions is presented in Fig. 3.3.

3.3.2 Linkage map

The latest sunflower map of our department (Poormohammad Kiani et al. 2007) based on SSR markers was used to introduce some important candidate genes which can be directly or indirectly associated with TTC and TPC. The distribution of SSR markers and candidate genes (CGs) among the 17 linkage groups is presented in Table 3.5. Dominant markers are developed for *GST*, *CAT*, *HPPD*, *SFH3*, 2,3 oxidosqualene cyclase and sterol 14-demethylase (*CYP51G1*) (Fig. 3.4). Glutation s-transferase (*GST*), enzymatic antioxidant, is assigned to linkage group 1 (LG1). The best map position corresponding to *GST* can be considered after ORS803 marker (Fig. 3.4). P-hydroxyphenylpyruvate dioxygenase (*HPPD*) involving in the conversion of p-hydroxyphenylpyruvate to homogentisic acid (Norris et al. 1998; Valentin et al. 2006) is placed in linkage group 14 (LG14) between ORS1152_1 and ORS391 makers (Fig. 3.4). 2,3 oxidosqualene cyclase and sterol 14-demethylase (*CYP51G1*) are not assigned to our map. Co-dominant markers are developed for *SEC14-1*, *SEC14-2* and *POD* genes (Fig. 3.4). Peroxidase, (*POD*) is mapped at the end of linkage group 17 (LG17), at the interval of 13.1 cM of ORS727 (Fig. 3.4). *SEC14-2*, *SFH3* are linked to LG15 and LG16, respectively. We identified twelve SNPs for γ-tocopherol methyl-transferase (*VTE4*), one of the most important candidate genes involving tocopherol biosynthetic pathway, between both parents (PAC2 and RHA266). Dominant and co-dominant are also developed for *VTE4* (Fig. 3.4). Five candidate genes (CGs); catalase (*CAT*), γ-tocopherol methyl-transferase (*VTE4*), homogenitisate phytyltransferase (*VTE2*), *PSI P700* and drought-responsive family gene (*DROU1*) are anchored to linkage group 8 (LG8). Mapping of *VTE2* and *PSI P700* genes is done by using HRM. SNP-based CAPS markers are also developed for *PAT2*, *SMT2* and *DWF1* (Fig. 3.4). Four SNPs are identified for *PAT2*, *Arabidopsis* Sec14 homologue gene, between two parents and identified SNP at the 477th position is converted to CAPS marker (Fig. 3.4). Patellin2 (*PAT2*) is localized to linkage group 2 (LG2). We also identified 8 and 2 SNPs for sterol methyltransferase II (*SMT2*) and delta24-sterol reductase (*DWF1*), respectively (Fig. 3.4). Identified SNPs at the 402^{ed} and 296th position by sequence alignment analysis were converted to CAPS marker to allow genotyping of RILs for *SMT2* and *DWF1*, respectively (Fig. 3.4). In phytosterol pathway, 24- methylene–lophenol is converted to 24- ethylidene lophenol by *SMT2*. Campesterol is formed from 24- methylene cholesterol by *DWF1*. Sitosterol is also

generated from Isofucosterol by *DWF1*. *SMT2* is mapped to linkage group 17 (LG17) whereas *DWF1* is not assigned to our molecular map. Squalene epoxidase (*SQE1*) is also assigned to LG15 by InDel marker (Fig. 3.4). Nine primer combinations have been tested for *VTE5* gene but no SNP has been observed between both parents. No polymorphism is also observed between two parents for *VTE3* gene (Fig. 3.4).

3.3.3 QTL analysis

The map position, genetic effect and percentage of variation explained by QTLs associated with total tocopherol content (TTC) and total phytosterol content (TPC) under various environments and conditions are presented in Table 6. The QTLs are designated by 1, 2, 3, 4, 5, 6, 7 for well-watered green house, water-stressed green house, well-irrigated field–France, non-irrigated (rainfall) field–France, well-irrigated field–Iran, partial-irrigated field–Iran and late-sowing field–Iran conditions, respectively, followed by an abbreviation for the trait, the corresponding linkage group, and the number of QTLs in linkage group. One to six QTLs are identified depending on trait and growth conditions (Table 3.6). Both parental lines contributed to the expression of the different target traits and positive and negative additive effects are presented (Table 3.6). Co-localized QTLs were detected for both traits on linkage groups 1, 8, 10, 11, 16 and 17 (Fig. 3.4). The major QTL of TTC on linkage group 8 (6.*TTC.8*) accounted for 59.51% of the phenotypic variation, which is overlapped with the QTL of TPC (7.*TPC.8*). This overlapped QTL is linked to candidate gene, HuCL02051C001. Common QTL for TTC (5.*TTC.15, 6.TTC.15* and 7.*TTC.15*) is detected in linkage group 15. The most important QTL for TPC is `7.*TPC.8*`, which is positioned on linkage group 10 at 53.6 cM. Individual effect of this QTL on the expression of phenotypic variation (R^2) is 55.1% (Table 3.6). The favorable alleles for this QTL come from RHA266 (Table 3.6). *GST*, *POD*, *SEC14-2* and *SMT2* genes showed co-localization with QTL for phytosterol content. Two candidate genes, *HPPD* and *SFH3*, exhibited co-localization with QTL for tocopherol content (Fig. 3.4).

3.4. Discussion

Significant differences between the parents under all conditions for mentioned traits indicate genetic differences between them. This suggests that the parental lines carry different genes, which are inherited in some RILs via transgressive segregation. Non significant differences between the means of the RILs (MRILs) and the mean of their parents (MP) reveal that the RILs used in this research are representative of possible

genotypic combinations of the cross ‘PAC2’ x ‘RHA266’. Genetic gain (GGB) when the best RIL is compared with the best parent and GG10% Sel. considered as the differences between the mean of the top 10% selected RILs and the mean of the parents, are significant for total tocopherols content (TTC) and total phytosterols content (TPC), revealing transgressive segregation for the mentioned traits. Transgressive segregation was also reported for other traits in the same population (Poormohammad Kiani et al. 2007; Ebrahimi et al. 2008). Transgressive segregation would be the result of the accumulation of favorable alleles coming from different parental lines. The positive and negative signs of additive effect at the different loci (Table 3.6) indicate the contribution of both parental lines and confirm the transgressive segregation observed at the phenotypic level.

In this study QTLs associated with TTC and TPC in sunflower using the gene-based linkage map under several environments and conditions are identified by composite interval mapping (CIM) method. The influences of genetic background can be eliminated by CIM method (Wu et al. 2007). In this method, the power of QTL detection will be increased by considering a set of QTLs outside the interval being tested as cofactors and finally the bias in the estimated position and effect(s) of QTL will be reduced (Zeng 1994). As far as we know QTLs for these two traits are not reported in the literature. The QTLs detected in our research reveal that several putative genomic regions are involved in the expression of the mentioned traits under various conditions. QTLs associated with TTC and TPC in our research are overlapped with QTLs for some other traits identified in previous studies. The overlapped QTLs for TTC and TPC under late-sowing condition (Exp.7) on linkage group1 (*7.TTC.1, 7.TPC.1*) in our study are in the same region where *Tph1* gene associated with increased β-tocopherol content is mapped (Vera-Ruiz et al. 2006). This chromosomic region was reported by Poormohammad Kiani et al. (2009) for days from sowing to flowering. A common QTL for TTC is identified on linkage group 16 (*5.TTC.16, 7.TTC.16*). These QTLs, controlled by the RHA266 alleles, appear to be important in both well-irrigated conditions. This region of linkage group 16 is reported for oil content by Ebrahimi et al. (2008) and leaf number by Poormohammad Kiani et al. (2009). Another common QTL for TTC was observed on linkage group 16 under well-irrigated conditions in the green house and field. These QTLs (*1.TTC.16, 3.TTC.16.1*) are associated to an SSR marker; ORS333. Common QTLs for TPC are identified on linkage group 9 (*5.TPC.9, 6.TPC.9*). This chromosomic region was reported by Ebrahimi et al. (2008) for palmitic acid content and by Ebrahimi et al. (2009) for percentage of grain protein. A stable QTL for TPC (*4.TPC.10, 5.TPC.10*) is found on linkage group 10 under non-(rainfall) and well-irrigated conditions in

the field between two SSR markers; HA928 and HA3847. Another stable QTL for TPC is found on linkage group 10 (*5.TPC.10.2, 7.TPC.10*). These QTLs, controlled by the RHA266 alleles, appear to be important in both well-irrigated conditions. This region on linkage group 10 was detected for leaf area at flowering stage by Poormohammad Kiani et al. (2009). The major QTL of TTC on linkage group 8 (*6.TTC.8*) accounted for 59.51% of the phenotypic variation, which is overlapped with the QTL of TPC (*7.TPC.8*) where two other QTLs for TPC were detected under well-irrigated conditions in the green house and field (*1.TPC.8*, *3.TPC.8*). Left marker on the LOD peak is a drought-related candidate gene (Fig. 4). In a previous study the positive and significant correlation between high tocopherol content and drought resistance has been recognized (Munné-Bosch 2005). A common QTL for TTC is identified on linkage group 14 (*6.TTC.14, 7.TTC.14*). This chromosomic region on linkage group 14, between ORS1152_1 marker and candidate gene (HuCL04260C001), appears to have important role for increasing of TTC under partial-irrigated and late-sowing conditions. One of the most important candidate genes (HuCL04260C001), that modulates the expression of p-hydroxyphenylpyruvate dioxygenase (HPPD), is assigned to LG14 between ORS1152_1 and ORS391 markers. A major QTL near *HPPD* gene is reported by Gilliland et al. (2006) for α-tocopherol in *Arabidopsis* which explained 40 % of phenotypic variance. In maize, on LG5, the QTL of total tocopherol was linked to *HPPD* gene and explained 7.13% of phenotypic variance (Chander et al. 2008).With a seed-specific over-expression of *HPPD*, a 24-28% increase in seed total tocopherol content has been observed in *Arabidopsis* (Shintani and DellaPenna 1998).Trebst et al. (2002) reported the induction of *HPPD* under senescence and stress condition. TTC is increased under partial-irrigated conditions. We can mention some reasons for it; under drought stress, tocopherol biosynthesis may be stimulated by recognized abscisic acid-specific motif in the promoter region of *HPPD* gene (Munné-Bosch 2005). Under water stress condition, phytol can be released as a result of the degradation of chlorophyll a by chlorophyllase. Phytolphosphate can be formed from phytol by *VTE5* (Valentin et al. 2006). The latter compound is converted by phytylphosphate kinase into phytyl pyrophosphate (PDP) (Valentin et al. 2006). PDP is as a precursor for tocopherol biosynthesis (Norris et al. 2004). Genes involved in chlorophyll degradation and tocopherol pathway can be activated by stress-induced jasmonic acid accumulation (Munné-Bosch and Falk 2004). It has been demonstrated that tocopherol synthesis and stress hormones such as jasmonic acid, abscisic acid and salicylic acid might be coupled in plant responses to stress (Munné-Bosch 2005). An increase of tocopherol synthesis under moderate stress and a decrease of tocopherol synthesis under

severe stress have been reported (Munné-Bosch 2005). *PSI P700* gene is anchored to LG8. The interdependence between PS II/PSI activity and amount of tocopherol has also been recognized and photosystem activity decreases when the tocopherol level is low (Trebst et al. 2002). Common regulatory mechanisms of photoprotection and photosynthesis refer to ATCTA element in both promoter of tocopherol pathway-related genes and photosynthesis-related genes (Welsch et al. 2003). In the present study some enzymatic antioxidant-related genes such as peroxidase (*POD*), glutation s-transferase (*GST*) and catalase (*CAT*) are assigned to LG17, LG8 and LG1, respectively. A stable QTL for TPC (*3.TPC.1, 4.TPC.1*) is identified on linkage group 1 under non-(rainfall) and well-irrigated conditions in the field (Fig. 4). *GST* gene showed co-localization with these QTLs. The correlation between enzymatic and non enzymatic antioxidant was studied in a previous research (Semchuk et al. 2009). Through candidate genes, some *Arabidopsis Sec14* homologue genes are anchored to our new map. It has been reported that SEC14 domains exist in proteins from plants, yeast and mammals (Saito et al. 2007). Wide range of lipids, phosphatidylglycerol and tocopherols were known as ligands for SEC14 domain-containing proteins (Saito et al. 2007). One of the most important candidate genes coding for sterol methyltransferase II (SMT2) enzyme is anchored to LG17 by CAPS marker. Co-localization between *SMT2* gene and stable QTL for phytosterol (*1.TPC.17, 2.TPC.17*) is observed under well-watered and water-stressed conditions in green house (Fig. 3.4). γ-tocopherol methyl-transferase (*VTE4*), one of the most important candidate genes involving tocopherol biosynthetic pathway, is assigned to linkage group 8. Endrigkeit et al. (2009) reported that in rape seed population, *VTE4* was anchored to the end of chromosome A02, where also two QTLs for alpha tocopherol content had been identified. The increase of tocopherol content is observed by over-expressed *VTE4* in Arabidopsis seed (Shintani and DellaPenna 1998). The amount of tocopherol was also increased by delay in planting time in chickpea (Gül et al. 2008). As conclusion, 15 candidate genes were introduced in the latest linkage map of our department (Poormohammad Kiani et al. 2007) based on SSR markers. Regarding to all recognized tocopherol and phytosterol pathway–related genes, we have used respective sequences of *A. thaliana* to find homologues in *H. annuus* via heliagene web site. This strategy led us to find sequences of some *H. annuus* candidate genes, which play directly or indirectly a role in tocopherol and phytosterol biosynthesis. *GST*, *POD*, *SMT2* and *SEC14-2* genes showed co-localization with QTL for phytosterol content. Two candidate genes, *HPPD* and *SFH3*, exhibited co-localization with QTL for tocopherol content. The candidate genes associated with tocopherol and phytosterol, especially *HPPD* and *SMT2*, could be precisely used for

alternation of the tocopherol and phytosterol content of sunflower seeds by development of functional markers. Detection of QTLs influencing various traits could enhance the efficiency of marker-assisted selection and increase genetic progress. The relatively low number of RILs used in current research may have a negative influence on the accuracy of the calculated QTL effects, the ability to detect QTLs with small effects and R^2 maybe overestimation (Beavis et al. 1994; Bachlava et al. 2008). This was, to some degree, compensated by the higher precision of the phenotyping and the use of our map including candidate genes. A combination of biotechnological and genomic-based approaches will provide many facilities for more precise understanding of the function of tocopherol and phytosterol under various environments and conditions.

Table 3.1: Primers used for PCR, HRM, CAPS and InDel makers

	Target gene	Accession		Sequence of primer (5' to3')	
		AGI-Arabidobsis	Homologue with Heliagene Cluster	Forward +M13F (**F**+CACGACGTTGTAAAACGAC)	Reverse+ M13R (**R**+ACAGGAAACAGCTATGAC)
Tocopherol pathway-related genes	*VTE4*	AT1G64970	HuCL02246C001	TGAATCTGACGGTTTAGAAC ATCCGTATGATTGAACAAGC	AAACTCCGTTCAGAAAGCG ATGTGCTCTCCACTCTCCATTG
	HPPD	AT1G06570	HuCL04260C001	GCTCTGAGAAGTTCCCTTTC	ATGTGTTGTCGGATGAGCAG
	VTE1	AT4G32770	HuCL01457C001	AAACCTTGATGCCATAGGG	TCCTCTGTTAGGCTCAAAC
	VTE2	AT2G18950	HuCL02840C003	TGCCACAAGAGCAAATCGCTTC	TTTGGGCACTCTTCATAAG
	VTE3	AT3G63410	HuCL02127C001	TGTCTCGTTTCTTTGCGGAC	TAGTAGATTCCAGCCAACGC
	VTE5	AT5G04490	HuCL15929C001	ACAGAATCTAAGCAGAAAGC	CACCACTGTTGCCATCATTG
	GGPS	AT4G36810	HuCL07599C001	AGTTTGTTACACCATCTTCC	AAGTGGTCAACTCCAACATCCG
	MCT	AT2G02500	HuCL00002C009	CAAAGTCTTCACCACAAATG	ACCTCATCCCATCTTCTTCC
	HMBPP	AT5G60600	HuCL00358C002	TGTGCTTGGTATGCCATTC	CCCTTTGGGAATGTTATGTGG
	TAT	AT5G53970	HuCL00730C001	GCAGATGAAGTGTATGGTC	TATTTGTGGGTAAAGTGCC
Enzymatic antioxidant-related gene	*POD*	AT1G14540	HuCL03143C001	TCGTCGGGATAGTCTTTAC	CGATAGGTAGAGGACTGTTG
	CAT	AT1G20620	HuCL00001C054	AAACTACCCTGAGTGGAAG	AATGAATCGTTCTTGCCTG
	GST	AT1G02930	HuCL00790C003	AAAGAGCACAAGAGTCCTG	ACTTATTTGAGTGGGCAAC
	PAT2	AT1G22530	HuCL00156C004	CTTGGAACAACTGAAGAGC	TGAGTTTACTGCTGTTCCG
	PAT3	AT1G72160	HuCL09971C001	TGCTCTTAGTTCTTTGAGTC	TTACCCTGAGTTTGTTTCG
	PAT6	AT3G51670	HuCL07229C001	GTGTGTTACAATGCTTATGG	TGACTGTGAACTCAGAAGC
	SEC14-1	AT1G75170	HuCL10527C001	TATGTCCATCTTTCGGCGTC	ATGGTGTCTTTAGCGGTTC
	SEC14-2	AT3G24840	HuCL09897C001	ATGATAACCGTGTGGATAGC	ATGCTAAACTGGAGGAAAGC
	SEC14-3	AT4G39170	HuCL01370C001	TTGAGCAATGTCTGGACCTC	CGGTATTCCAAAGCAATCG
	SFH3	AT2G21540	HuCL00667C001	CAAGGAAGGATTTCACCGTG	AAGGCGGTTGATGCTTTACG

The candidate genes are: tocopherol methyl-transferase (***VTE4***), p-hydroxyphenylpyruvate dioxygenase (***HPPD***), tocopherol cyclase (***VTE1***), homogenitisate phytyltransferase (***VTE2***), MPBQ/MSBQ methyltransferase (***VTE3***), Phytol Kinase (***VTE5***), geranylgeranyl pyrophosphate synthetase (***GGPS***), 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (***MCT***), 4-hydroxy-3-methylbut-2-enyl diphosphate (***HMBPP***), tyrosine aminotransferase (***TAT***), peroxidase (***POD***), glutation s-transferase (***GST***), catalase (***CAT***), pattelin 2 (***PATL2***), patellin3 (***PATL3***), patellin-6 (***PATL6***), phosphoglyceride transfer (***SEC14-1***, ***SEC14-2***, ***SEC14-3***) and phosphatidylinositol transporter (***SFH3***).

Table 3.1 (continued)

	Target gene	Accession		Sequence of primer (5' to3')	
		AGI-Arabidobsis	Homologue with Heliagene Cluster	Forward +M13 (**F**+CACGACGTTGTAAAACGAC)	Reverse+ M13 R (**R**+ACAGGAAACAGCTATGAC)
Phytosterol pathway-related genes	*SQE1*	AT1G58440	HuCL04368C001	AGGCTACAGTCATTCCGC	TTAAGTTAAGAGATTTTGGTCATTGTG
	OC	AT1G16600	HuCL20280C001	CCGTCCTCCATCTGTGTTTC	TCCTGAGCAACTGTATGATGC
	CYP51G1	AT1G11680	HuCL03272C001	CGAGTCAGCAGGAGGTGTATC	GCAAACAGGGCGGCAATG
	SMT2	AT1G20330	HuCL02933C001	CCTTCTACAACCTCGTAACCG	ATCCTTCTCTTTCACCACCTC
	STE1	AT3G02580	HuCL00001C314	GCACGGATAAGCGATGTCG	ACCGAACATCCAGTCCATC
	DWF1	AT3G19820	HuCL01845C001	TGTCGGAGTCTACTACACACC	GATAACCTATTCGGGCTGGTC
	DROU1	AT5G26990	HuCL02051C001	TTGTTGAGGAGGGAACTAAG	GTCATCACCAAGAATCGTCG
	DROU2	AT5G48870	HuCL07710C001	GCTCAATGGAAATAACATAGCC	TCCTGTTGCTAAGGCGAAAC
	PSIP700	ATCG00350.1	HuCX944063	CAGCCAAAGGAAGATGATG	TAGCCATACCAGTGATTTG
	AQUA	AT1G52180.1	HuCL00086C003	GCCTACATTGCTGAGTTCATC	TTAGTATTCATTGGTGAGGG

The candidate genes are: squalene epoxidase **(*SQE1*)**, 2,3 oxidosqualene cyclase **(*OC*)**, sterol 14-demethylase **(*CYP51G1*)**, sterol methyltransferase II **(*SMT2*)**, lathosterol oxidase **(*STE1*),** delta 24-sterol reductase **(*DWF1*)**, drought-responsive family gene **(*DROU1*)**, supersensitive to drought **(*DROU2*)**, PSI P700, aquaporin **(*AQUA*)** and delta 24-sterol reductase **(*DWF1*)**.

Table 3.2: Analysis of variance (mean squares) for total tocopherol and total phytosterol content in a population of sunflower recombinant inbred lines (RILs) for first year in France

	df		Total tocopherol content		Total phytosterol content	
	Field	Green house	Field	Green house	Field	Green house
Water treatment	1	1	34341.67*	450.76^{NS}	66.03^{NS}	67957.78**
Block	2	2	9540.61^{NS}	4666.68^{NS}	310.11^{NS}	377.57^{NS}
Block x Water treatment	2	2	833.25^{NS}	2679.61^{NS}	204.63^{NS}	616.85^{NS}
Genotype (RILs)	98	71	**49199.44****	**23966.04****	**3352.11****	**2446.31****
RILs x Water treatment	98	71	4452.04^{NS}	9976.89**	170.09^{NS}	858.40**
Error	392	284	5557.17	4518.52	166.42	314.87

*, **: significant at 0.05 and 0.01 probability level, respectively. ^{NS}: non-significant.

Table 3.3: Analysis of variance (mean squares) for total tocopherol and total phytosterol content in a population of sunflower recombinant inbred lines (RILs) for second year in Iran

Source of variance	df	Total tocopherol content			Total phytosterol content		
		WI	PI	LS	WI	PI	LS
RILs	88	**18102.06****	**19124.36****	**24528.86****	**1526.24****	**1282.06****	**737.13****
Blocks	2	13388.78^{NS}	102794.09**	24902.52**	418.50^{NS}	138.28^{NS}	117.37^{NS}
Error	176	4510.62	5644.55	4313.31	309.49	410.18	225.15

**, ^{NS}: significant at 0.01 probability level and non-significant, respectively.
WI: well-irrigated, PI: partial-irrigated and LS: late-sowing

Table 3.4: Genetic variability and genetic gain for total tocopherol and total phytosterol content in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated, partial-irrigated and late-sowing conditions

	Total tocopherol content (mg/kg of oil)			Total phytosterol content (mg/100 g of oil)		
	WI	PI	LS	WI	PI	LS
PAC-2 (P1)	165.43	194.86	249.02	294.29	308.54	324.17
RHA-266 (P2)	338.87	353.47	413.96	318.51	345.57	347.98
/P1-P2/	173.44**	158.61**	164.94**	24.22*	37.03*	23.81*
MP: (P1+P2)/2	252.15	274.17	331.49	306.40	327.05	336.08
Mean of RILs (MRILs)	259.6	287.13	340.15	317.1	327.6	350.26
/MRILs -MP/	7.44NS	12.96NS	8.65NS	10.69NS	0.55NS	14 .18 NS
GGB: (BRIL-BP)	155.17**	196**	235.22**	73.33**	43.09**	44**
GG10%: (10% SRILs - MP)	185.23**	198.08**	249.09**	60.27**	45.95**	31.96*

PAC2' (P1) and 'RHA266' (P2): parental lines; MP: mean of two parental lines; MRILs: mean of recombinant inbred lines; BRIL: the best RIL; BP: the best parent; 10%SRILs: the mean of the top 10% selected RILs; GGB: genetic gain when the best RIL is compared with the best parent; GG10%: genetic gain when the mean of the top 10% selected RILs is compared with the mean of the parents. *, **: significant at 0.05 and 0.01 probability level, respectively. NS: non-significant. WI: well-irrigated, PI: partial-irrigated and LS: late-sowing

Table 3.5: The distribution of SSR markers and candidate genes among the 17 linkage groups in sunflower recombinant inbred lines

Linkage group	Candidate gene	SSR marker					Total marker Number	Length (cM)	Average distance (cM)
	Heliagene -Cluster	SSL	SSU	ORS	HA	IUB			
LG1	HuCL0079C003	0	0	5	1	0	7	68.6	9.8
LG2	HuCL00156C004 HuCL10527C001	0	0	10	2	0	14	102.1	7.29
LG3		1	2	3	2	0	8	53	6.63
LG4		0	0	4	3	0	7	32	4.57
LG5		1	0	6	2	0	9	58.5	6.50
LG6		1	0	3	1	0	5	53.6	10.72
LG7		0	0	3	2	0	5	33.9	6.78
LG8	HuCL02840C003 HuCX944063 HuCL02051C001 HuCL02246C001 HuCL00001C054	1	0	6	3	0	15	182.9	12.19
LG9		3	0	7	4	0	14	77.3	5.52
LG10		4	0	6	5	0	15	79.7	5.31
LG11	HuCL00358C002	1	0	3	1	0	6	66.2	11.03
LG12		1	3	5	4	0	13	72.2	5.55
LG13		0	1	3	3	0	7	71.6	10.23
LG14	HuCL04260C001	4	2	4	3	0	14	89.8	6.41
LG15	HuCL09897C001 HuCL04368C001	0	2	6	4	0	14	113.6	8.11
LG16	HuCL00667C001	3	0	16	5	1	26	153.6	5.91
LG17	HuCL03143C001 HuCL02933C001	1	0	7	1	0	11	96.9	8.80
Total	13	21	10	97	46	1	190	1405.3	7.65

'SSL' and 'SSU' SSR are GIE CARTISOL, France markers, 'ORS' SSR markers from the SSR database; 'IUB' and 'HA' are SSR markers developed by INTA. All SSR markers are public and can be provided upon request.

Table 3.6: QTLs detected for total tocopherol and total phytosterol content under various environments and conditions.

Trait	QTL	LG	Position cM	LOD	Additive effects	R^2
1- First year-France-Toulouse						
1-1 Green house						
1-1-1 Well-watered						
TTC	*1.TTC.8*	8	123.8	4.9	-32.4	9.8
	1.TTC.9	9	6.1	5.0	-70.4	44.0
	1.TTC.16	**16**	**8.4**	**4.0**	**26.9**	**8.0**
TPC	*1.TPC.2*	2	18.0	6.5	20.2	41.1
	1.TPC.8	**8**	**30.9**	**5.1**	**21.0**	**20.0**
	1.TPC.16	16	116.8	4.6	-20.7	20.0
	1.TPC.17	**17**	**79.61**	**4.5**	**11.1**	**10.5**
1-1-2 Water-stressed						
TTC	***2.TTC.11***	**11**	**10.1**	**3.5**	**37.0**	**11.4**
	2.TTC.16	16	15.21	5.0	26.0	7.0
	2.TTC.17	17	30.71	3.5	-25.7	5.2
TPC	***2.TPC.17***	**17**	**71.61**	**4.0**	**14.1**	**16.7**
1-2Field						
1-2-1 Well-irrigated						
TTC	***3.TTC.16.1***	**16**	**4.41**	**3.2**	**29.0**	**7.0**
	3.TTC.16.2	16	36.41	3.2	34.0	9.0
TPC	***3.TPC.1***	**1**	**18.1**	**6.0**	**-8.0**	**6.8**
	3.TPC.8	**8**	**38.6**	**6.0**	**-7.0**	**5.6**
	3.TPC.10	10	76.6	5.0	-10.6	10.0
1-2-2 Non-irrigated (rainfall)						
TTC	*4.TPC.17*	17	42.5	5	12.1	10.0
TPC	***4.TPC.1***	**1**	**18.1**	**7.0**	**-5.7**	**4.5**
	4.TPC.10	**10**	**8.0**	**4.6**	**-18.6**	**27.0**
	4.TPC.13	13	54.5	3.3	-4.4	2.5

Table 3.6. (Continued)

Trait	QTL	LG	Position cM	LOD	Additive effects	R^2
2-Second year-Iran-Karaj						
2-1 Well-irrigated						
TTC	*5.TTC.2*	2	49.7	5.9	84.7	44.3
	5.TTC.10	10	61.7	5.4	39.6	16.0
	5.TTC.15	**15**	**75.3**	**6.2**	**-52.6**	**12.0**
	5.TTC.16	**16**	**64.6**	**4.5**	**-36.5**	**13.9**
TPC	***5.TPC.9***	**9**	**54.2**	**3.6**	**11.4**	**13.5**
	5.TPC.10.1	**10**	**20.1**	**3.7**	**13.9**	**17.0**
	5.TPC.10.2	**10**	**47.6**	**6.7**	**-10.7**	**11.6**
	5.TPC.11	**11**	**12.0**	**3.2**	**-9.4**	**8.9**
	5.TPC.13	13	67.6	4.59	21.1	39.2
2-2 Partial-irrigated						
TTC	***6.TTC.8***	**8**	**49.6**	**3.5**	**24.5**	**59.5**
	6.TTC.14	**14**	**29.6**	**3.3**	**-24.3**	**2.5**
	6.TTC.15	**15**	**77.3**	**3.3**	**-19.9**	**7.9**
	6.TTC.16	16	136.4	3.6	-27.6	8.3
TPC	***6.TPC.9.1***	**9**	**49.4**	**7.2**	**14.3**	**33.3**
	6.TPC.9.2	9	72.8	3.7	-9.7	12.2
2-3 Late-sowing						
TTC	***7.TTC.1***	**1**	**10.0**	**4.0**	**108.6**	**51.0**
	7.TTC.10	**10**	**13.7**	**3.5**	**-30.0**	**13.0**
	7.TTC.11	11	8.0	4.3	106.5	14.5
	7.TTC.14	**14**	**31.6**	**4.6**	**-25.1**	**4.6**
	7.TTC.15	**15**	**70.7**	**4.0**	**-36.2**	**9.2**
	7.TTC.16	**16**	**64.6**	**4.5**	**-27.9**	**7.0**
TPC	***7.TPC.1***	**1**	**8.0**	**5.2**	**-7.6**	**12.4**
	7.TPC.8	**8**	**53.6**	**7.9**	**-18.1**	**55.1**
	7.TPC.10	**10**	**42.0**	**3.0**	**-4.8**	**6.4**
	7.TPC.15	15	104.1	6.4	-6.3	8.5

QTLs are named as follows: a number indicating the experiments (**1**, well-watered - green house – France-Toulouse; **2**, water-stressed - green house – France-Toulouse; **3**, well-irrigated- field – France-Toulouse; **4,** non-irrigated (rainfall) - field – France-Toulouse; **5**, well-irrigated- field– Iran-Karaj; **6**, partial-irrigated - field – Iran-Karaj; **7**, late-sowing - field – Iran-Karaj), followed by an abbreviation for the trait, the corresponding linkage group, and the number of QTLs in linkage group. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it.

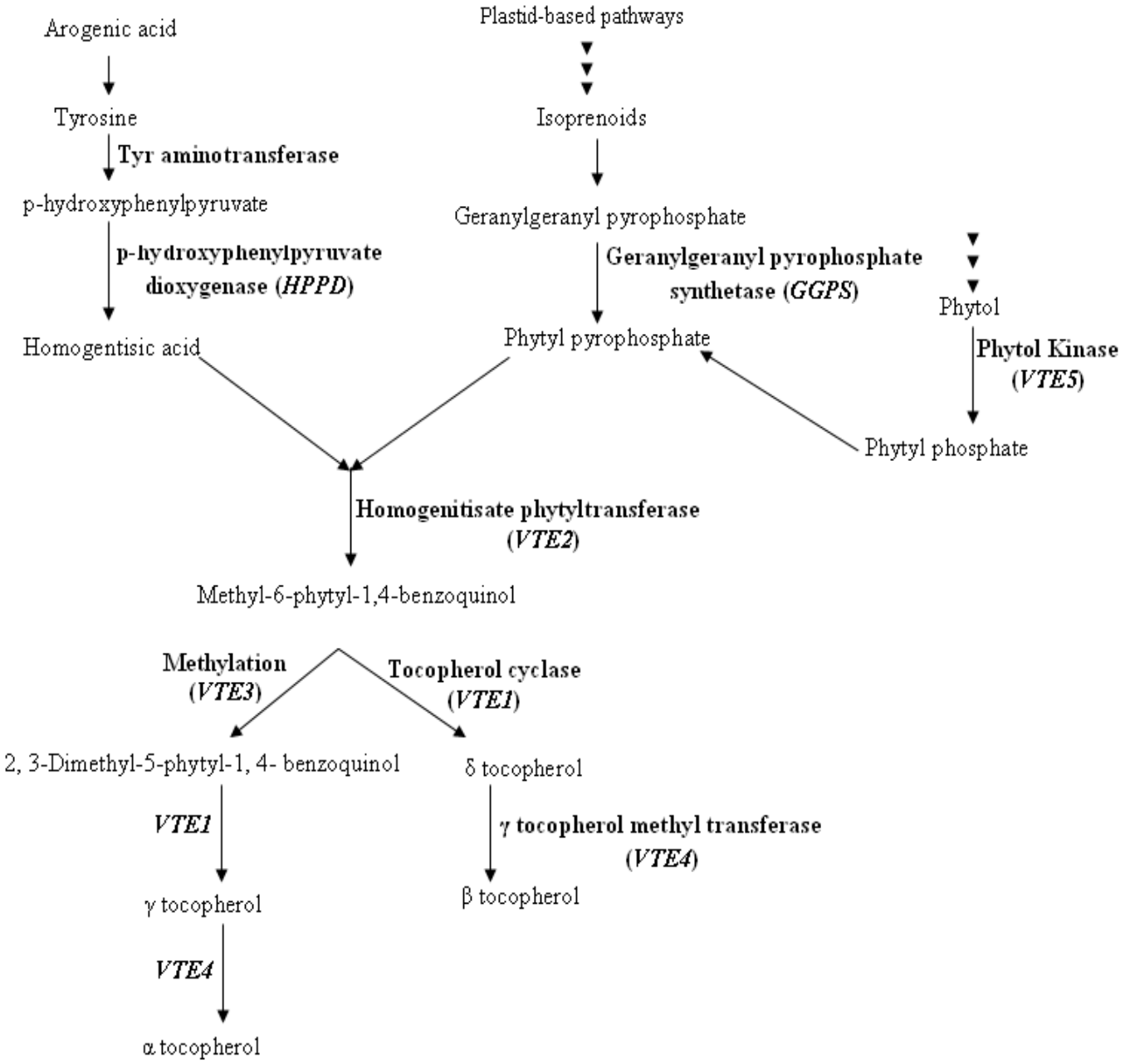


Fig. 3.1. Tocopherol pathway contains four parts: A-synthesis of homogentisic acid (HGA), the common precursor to tocopherol, which contributes to the chromanol head of tocopherol (Norris et al. 2004). B-synthesis of phytyl pyrophosphate (PDP), an intermediate in the side chain of tocopherol. C- joining HGA and PDP: 2-Methyl-6-phytyl-1,4-benzoquinol (MPBQ) is formed after the condensation of HGA and PDP by homogenitisate phytyltransferase (VTE2). MPBQ can be either cyclized by tocopherol cyclase (VTE1) to form δ tocopherol or methylated by VTE3 to form 2, 3-Dimethyl-5-phytyl-1, 4- benzoquinol (DMPBQ) (Porfirova et al. 2002; Shintani et al. 2002; Collakova et al. 2003). DMPBQ can be cyclized by VTE1 to form γ tocopherol (Sattler et al. 2003). D- methylation of an aromatic ring; α-tocopherol can be generated by methylation of γ-tocopherol via γ-tocopherol methyl-transferase (VTE4) (D'Harlingue and Camara 1985). β-tocopherol is formed from δ-tocopherol by methylation of the 5 position by VTE4 (Norris et al. 2004). The studied candidate genes are highlighted in bold.

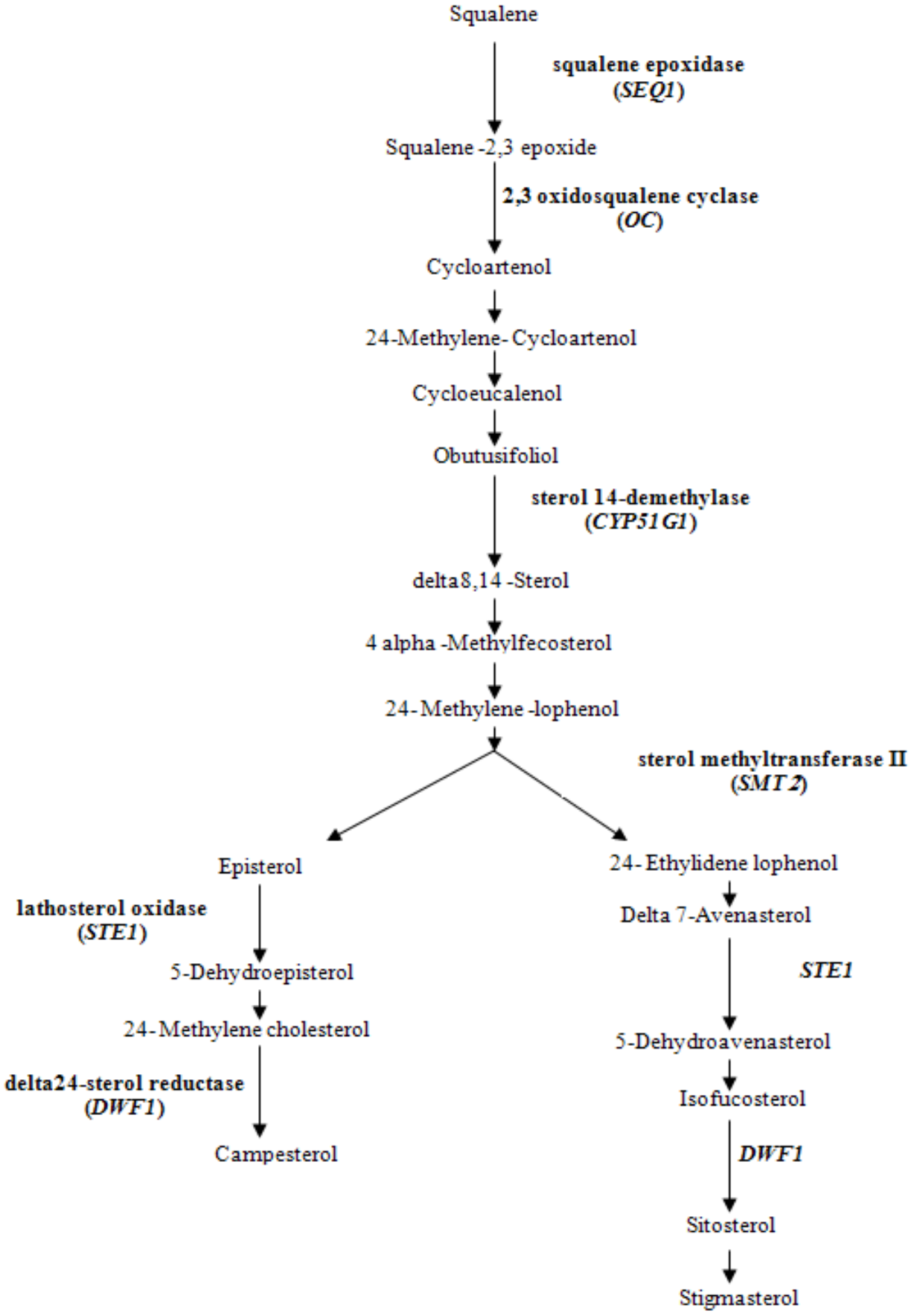


Fig 3.2. Simplified phytosterol biosynthetic pathway. The studied candidate genes are highlighted in bold.

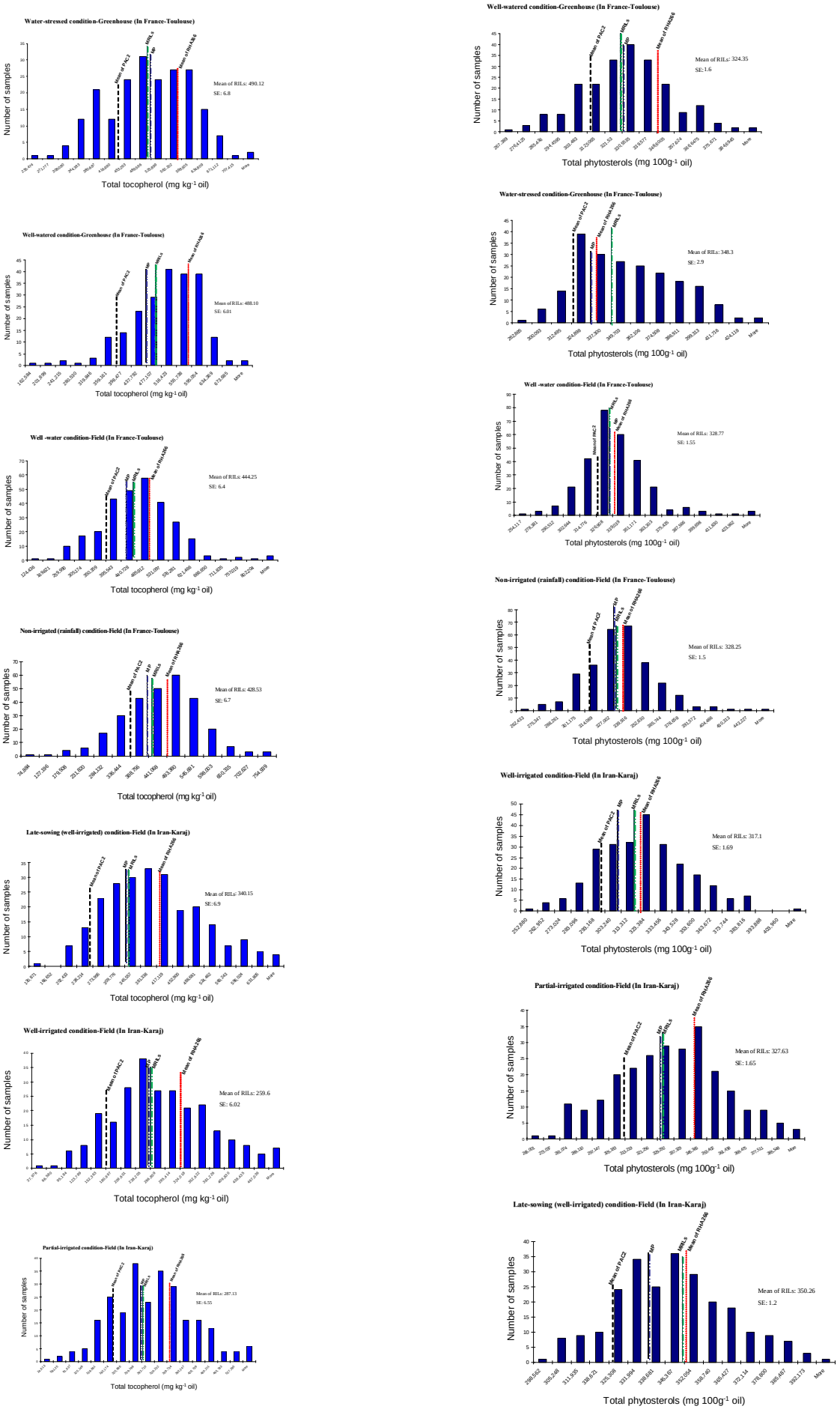
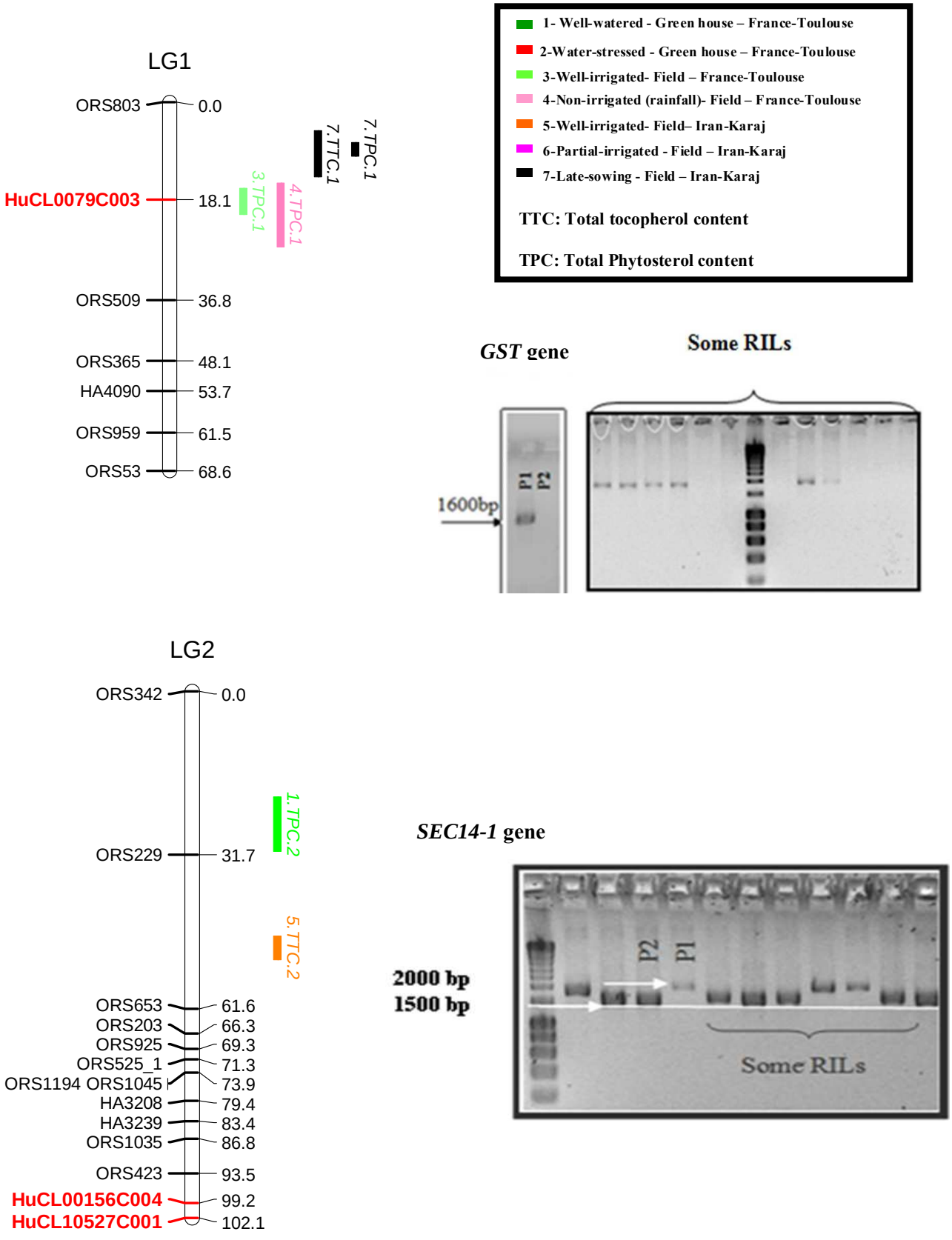
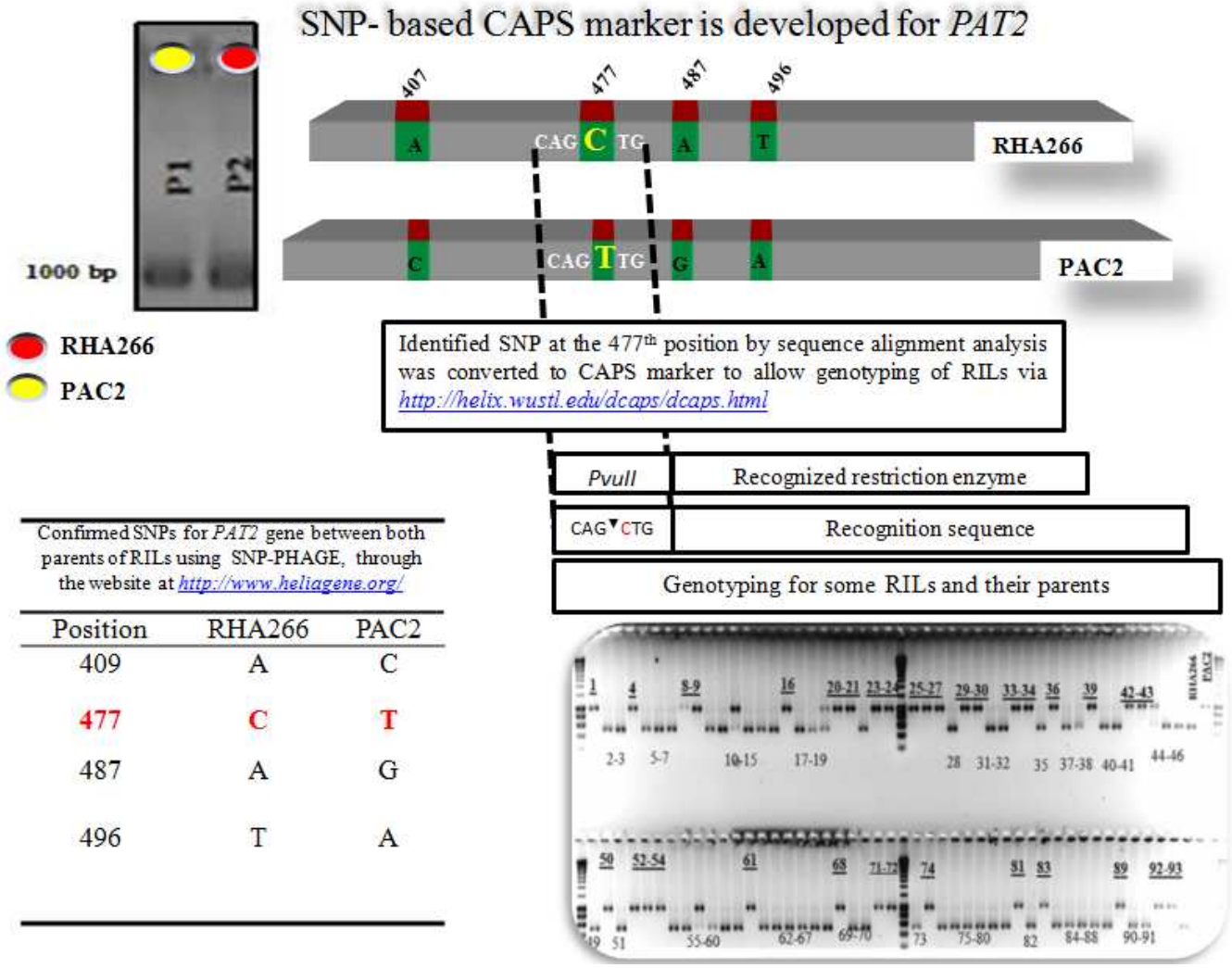


Fig 3.3. Frequency distribution of total tocopherol content (mg kg^{-1} oil) and total phytosterol content (mg $100g^{-1}$ oil) for RILs under several environments and conditions. In dotted lines are signalled the values of mean of three repetitions of the parental lines, mean of parents (MP) and mean of RILs (MRILs).

Fig. 3.4. Molecular genetic linkage map of sunflower based on 175 SSRs and 15 candidate genes (CGs) using 123 recombinant inbred lines from the cross of PAC2 X RHA266. The positions of QTLs are shown on the right side of the linkage groups. Bars represent intervals associated with the QTLs. The polymorphic banding pattern was used for genotyping all RILs. Gelelectrophoretic separation of candidate gene-PCR products from both parents, PAC2 (P1), RHA266 (P2) and some RILs are presented with the corresponding linkage group.





Confirmed SNPs for *PAT2* gene between both parents of RILs using SNP-PHAGE, through the website at http://www.heliagene.org/

Position	RHA266	PAC2
409	A	C
477	C	T
487	A	G
496	T	A

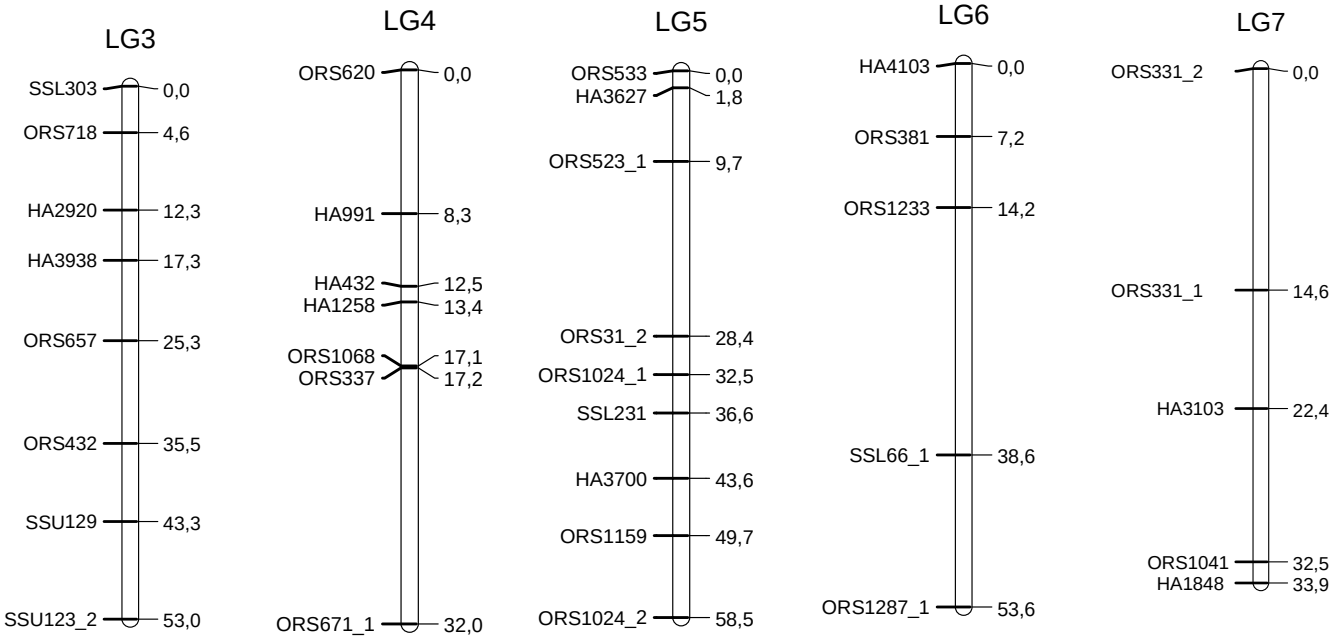
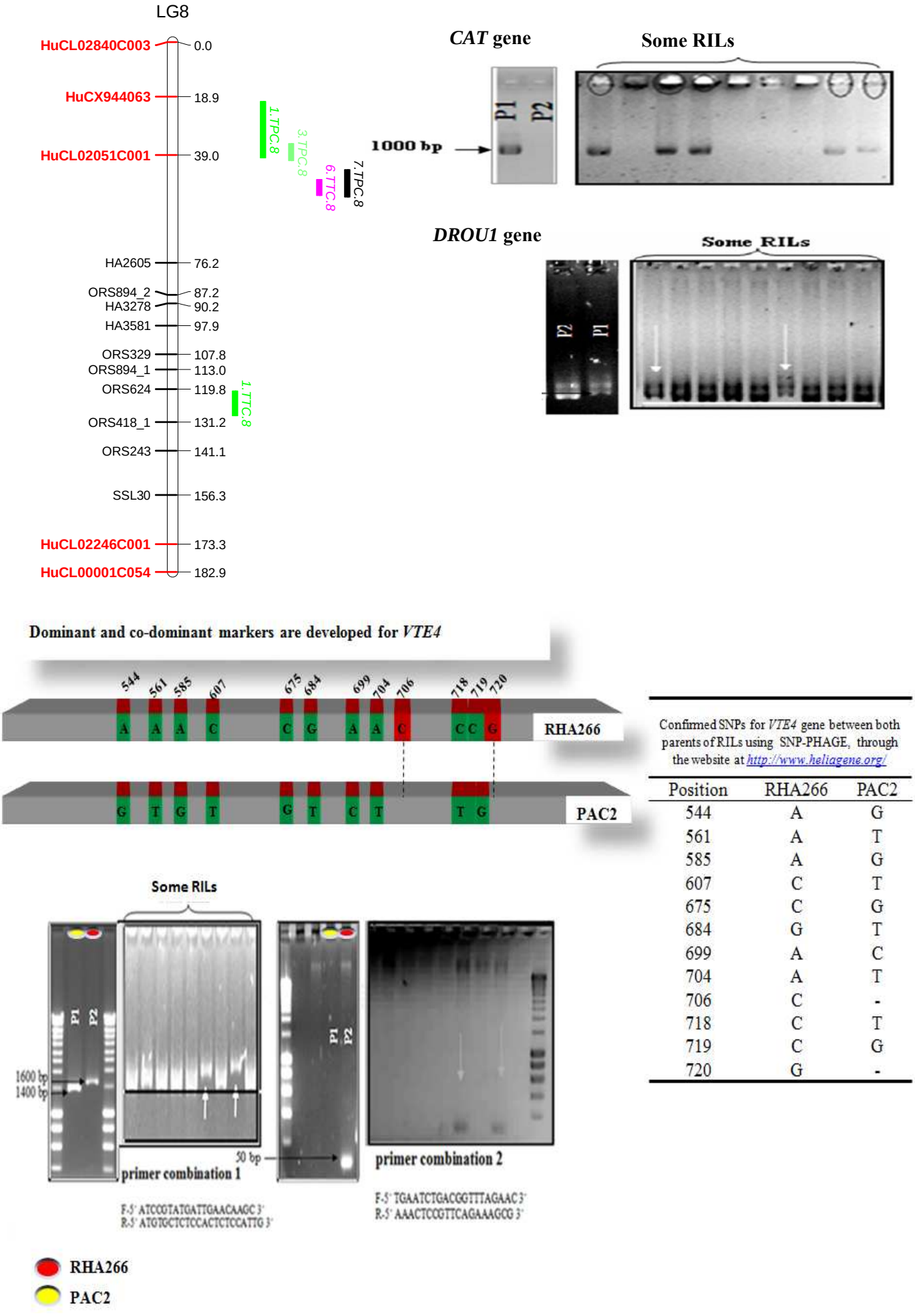


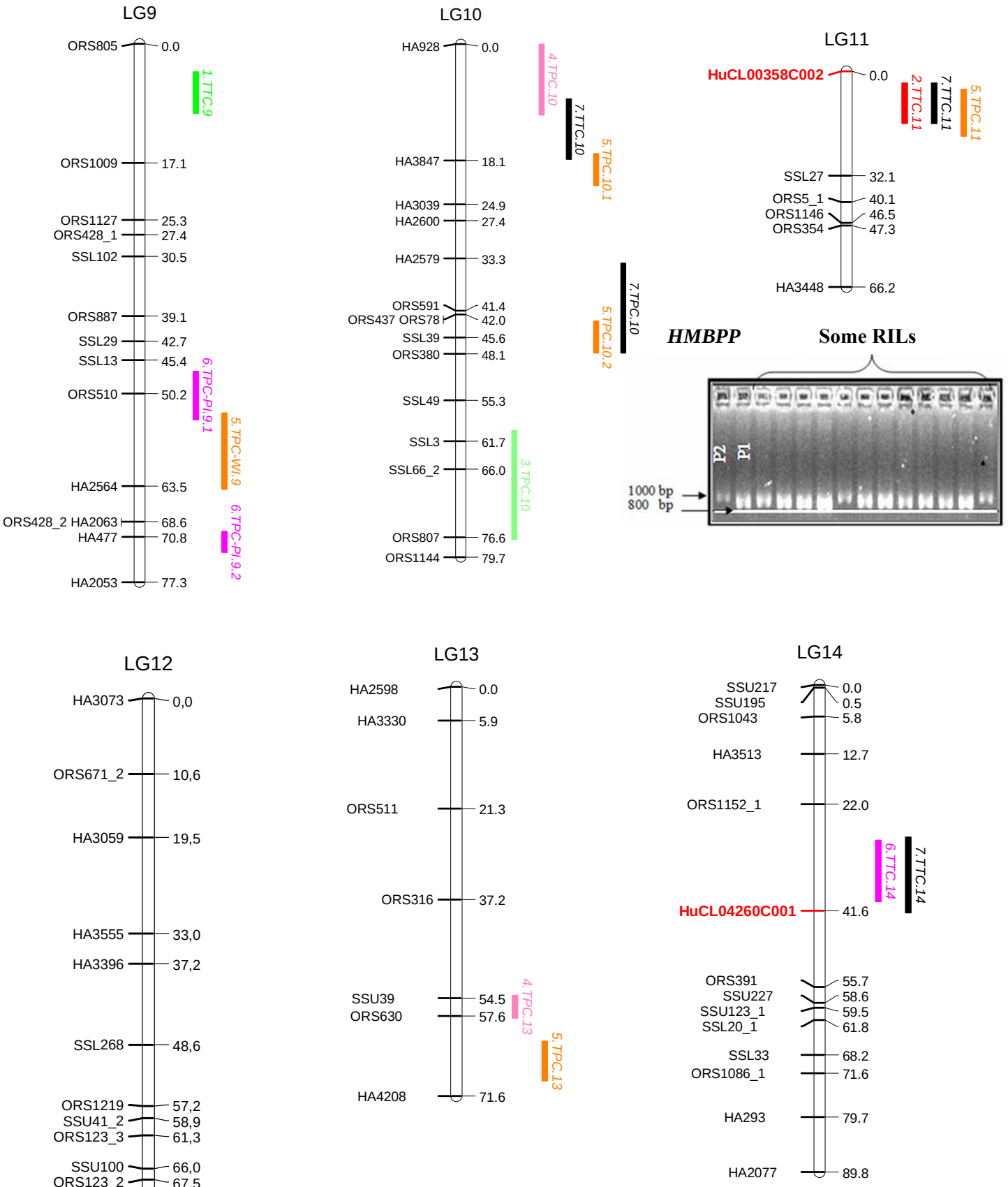
Fig. 3.4. (Continued)



Confirmed SNPs for *VTE4* gene between both parents of RILs using SNP-PHAGE, through the website at http://www.heliagene.org/

Position	RHA266	PAC2
544	A	G
561	A	T
585	A	G
607	C	T
675	C	G
684	G	T
699	A	C
704	A	T
706	C	-
718	C	T
719	C	G
720	G	-

Fig. 3.4. (Continued)



Dominant marker observed for ***HPPD* gene**

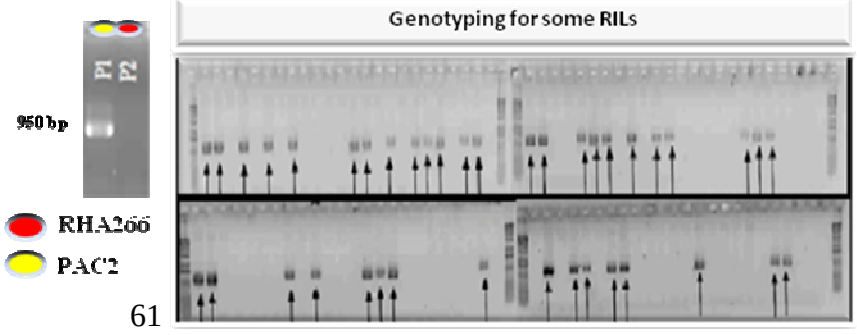


Fig. 3.4. (Continued)

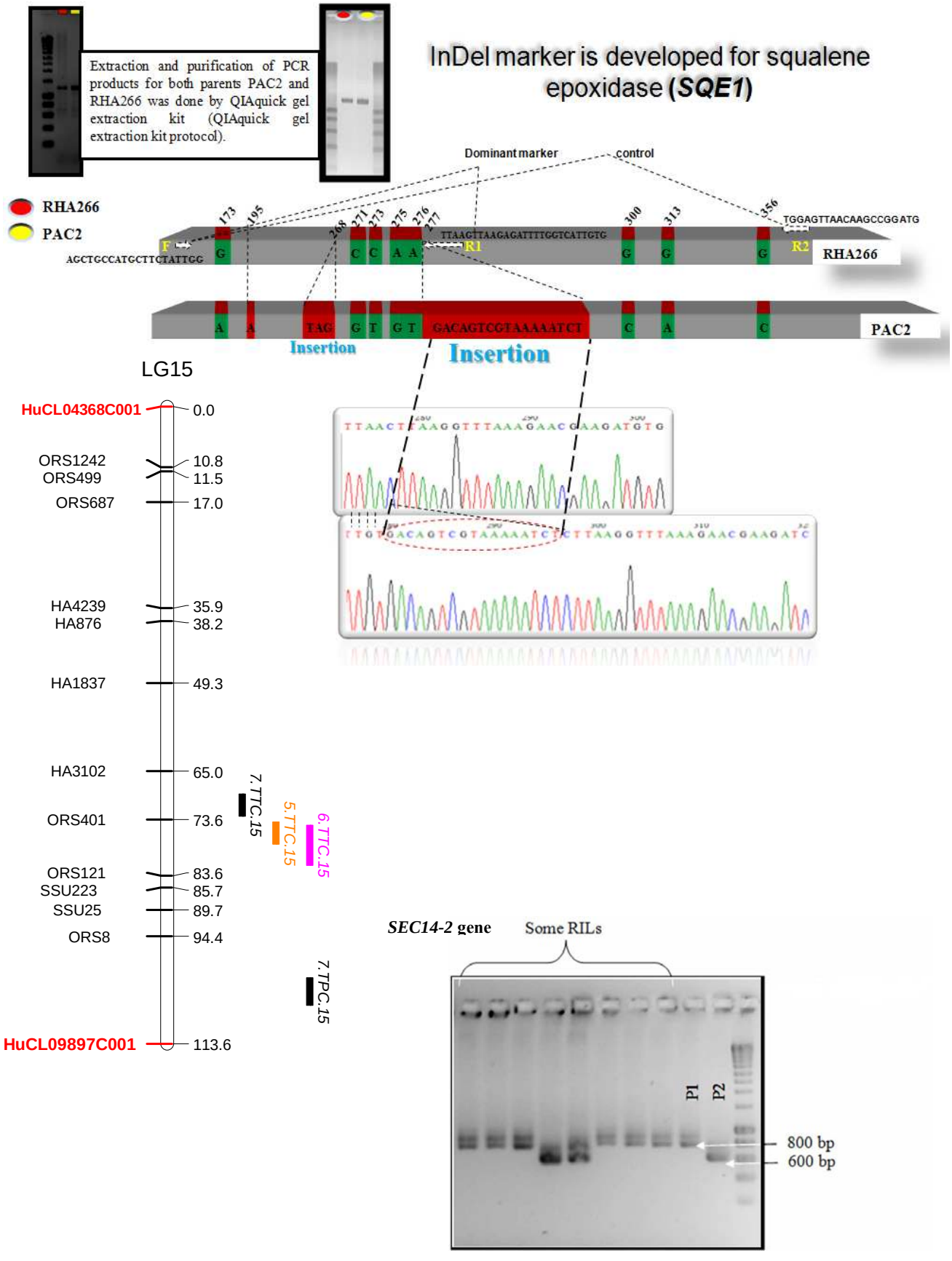


Fig. 3.4. (Continued)

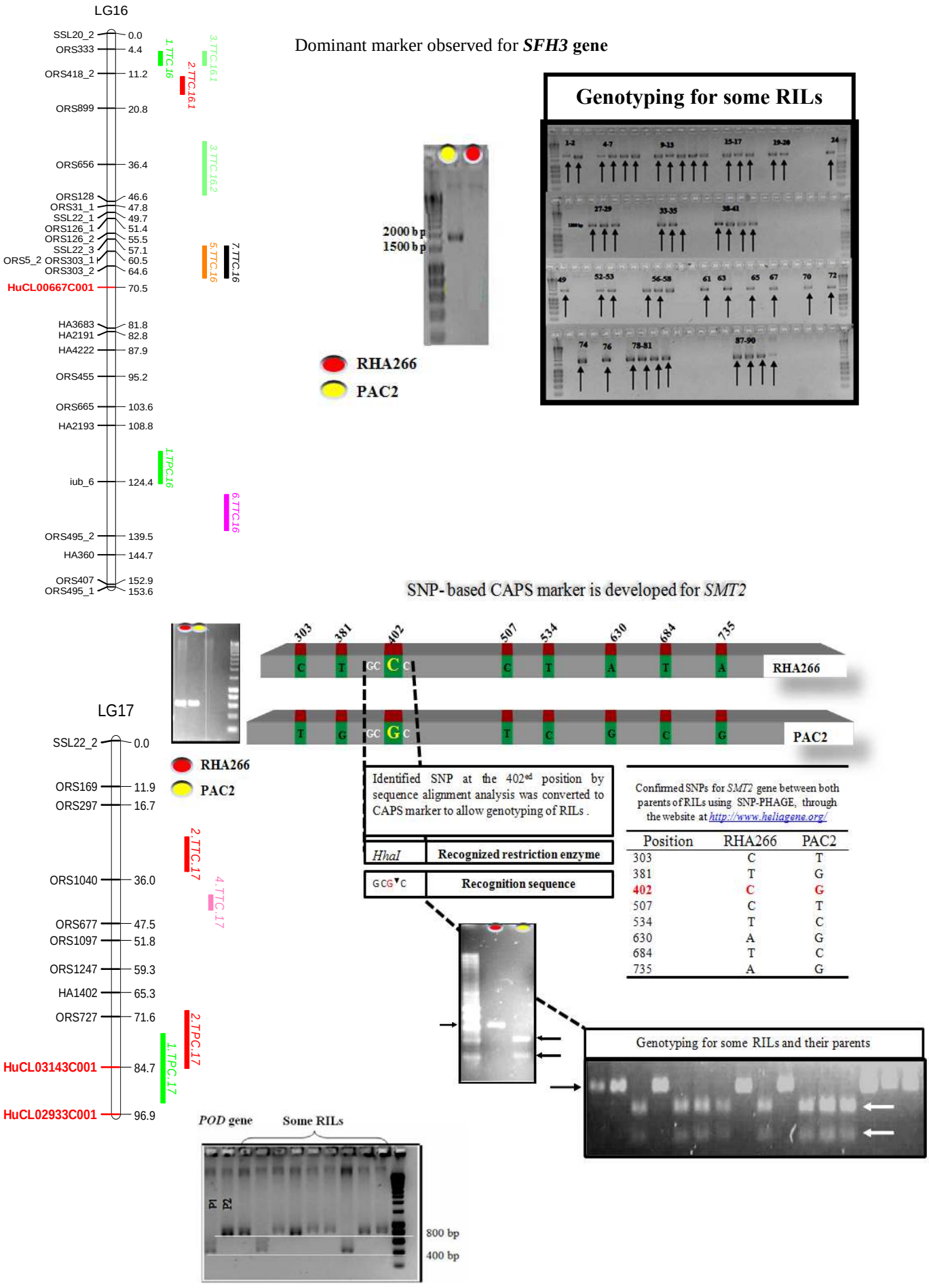


Fig. 3.4. (Continued)

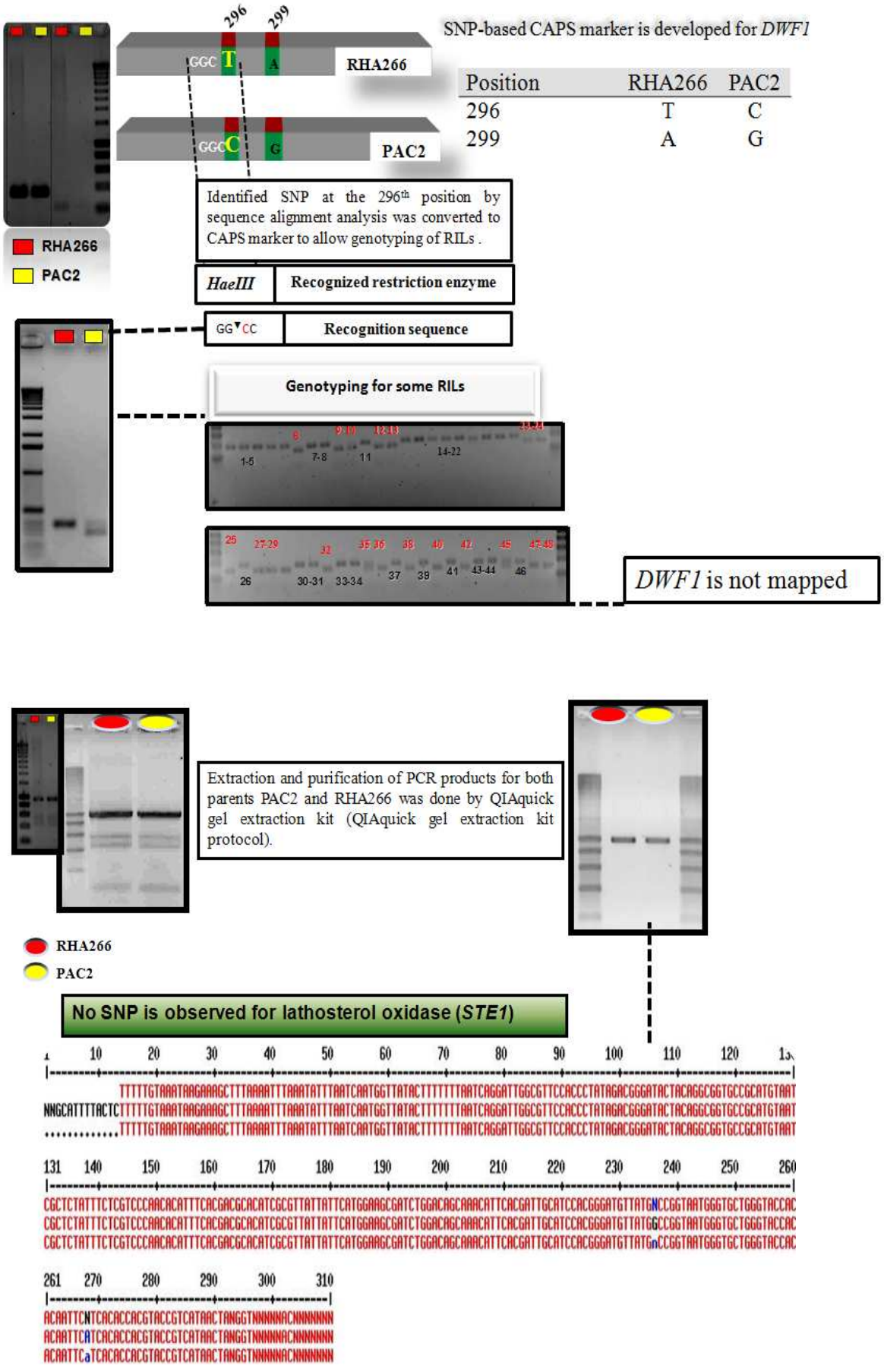
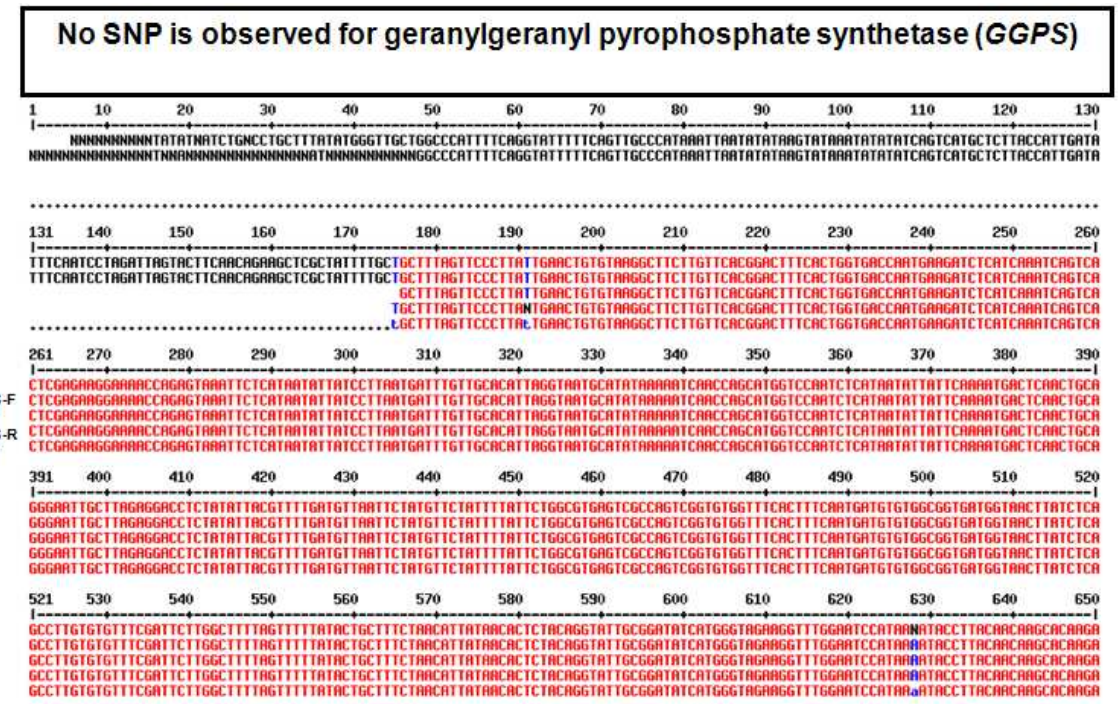


Fig. 3.4. (Continued)



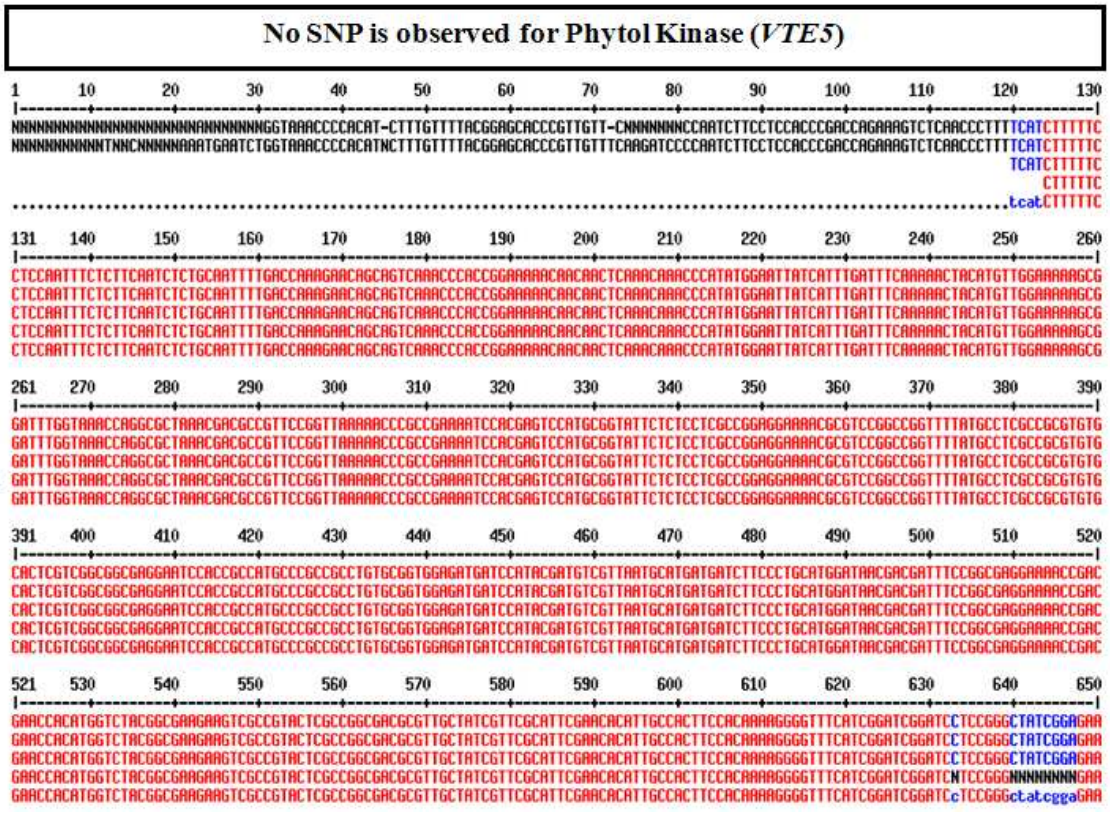


Fig. 3.4. (Continued)

Chapter 4

Genetic control of protein, oil and fatty acids content under partial drought stress and late sowing conditions in sunflower

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Under press

Abstract

The purpose of the present study was to map QTLs associated with percentage of seed protein, oil and fatty acids content under different conditions in a population of recombinant inbred lines (RILs) of sunflower. Three independent field experiments were conducted with well-, partial-irrigated and late-sowing conditions in randomized complete block design with three replications. High significant variation among genotypes is observed for the studied traits in all conditions. Several specific and non specific QTLs for the aforementioned traits were detected. Under late-sowing condition, a specific QTL of palmitic acid content on linkage group 6 (*PAC-LS.6*) is located between ORS1233 and SSL66_1 markers. Common chromosomic regions are observed for percentage of seed oil and stearic acid content on linkage group 10 (*PSO-PI.10* and *SAC-WI.10*) and 15 (*PSO-PI.15* and *SAC-LS.15*). Overlapping occurs for QTLs of oleic and linoleic acids content on linkage groups 10, 11 and 16. Seven QTLs associated with palmitic, stearic, oleic and linoleic acids content are identified on linkage group 14. These common QTLs are linked to *HPPD* homologue, HuCL04260C001. Coincidence of the position for some detected QTLs and candidate genes involved in enzymatic and nonenzymatic antioxidants would be useful for the function of the respective genes in fatty acid stability.

Key words: Sunflower, QTL, SSR, Oil content, Protein content, Fatty acids

Abbreviation: **QTL**, quantitative trait locus; **CIM**, composite interval mapping; **RIL** recombinant inbred line; **NIRS**, near-infrared reflectance spectrometry; **SSR**, simple sequence repeats; **PSP**, percentage of seed protein; **PSO**, percentage of seed oil; **PAC**, palmitic acid content; **SAC**, stearic acid content; **OAC**, oleic acid content; **LAC**, linoleic acid content

4.1 Introduction

Sunflower (*Helianthus annuus* L.) is cultivated as a source of vegetable oil and protein. Oil, fatty acid composition and protein contents are the main factors determining seed nutritional properties. Sunflower seed oil is composed of unsaturated fatty acids (90%); oleic and linoleic acids and the rest (10%) containing saturated fatty acids; palmitic and stearic acids (Dorrel and Vick, 1997; Pérez-Vich et al., 2002a). The role of unsaturated fatty acids on the quality of vegetable oil, the protection of membrane under low temperature and membrane fluidity is

more important than the effect of saturated fatty acids because of their lower melting point (Neidleman, 1987; Thompson, 1993; Heppard et al., 1996).

The classical method, gas chromatography (GC), is widely used to determine the fatty acid composition of the oil in sunflower seeds (Pérez-Vich et al., 1998). This technique is reliable but also expensive, long and use hazardous chemicals (Pérez-Vich et al., 1998). In plant breeding programs, a more rapid, accurate, low-cost, simple and nondestructive method is needed for screening of fatty acid composition. Near-infrared reflectance spectrometry (NIRS) is a more rapid, simple and nondestructive method that is nowadays used by breeders and food industry to determine multiple parameters such as proteins, oil content and fatty acid compositions (Pérez-Vich et al., 1998; Velasco and Becker, 1998; Biskupek-Korell and Moschner, 2007).

Sunflower has been considered for construction of molecular map. Various molecular markers, such as RFLP (Gentzbittel et al. 1995, Berry et al. 1995) and AFLP (Gedil et al. 2001) have been used whereas simple sequence repeats (SSR), as a highly reproducible molecular marker, is one of the most efficient marker for molecular linkage map (Tang et al., 2003). Genetic-linkage map based on 459 SSR, has been also constructed (Tang et al. 2002), which is the first reference map of sunflower based on single- or low-copy public SSR markers. The value of SSRs is that they usually detect single loci and are specific to a given place in the genome. SSRs are also highly variable and scored as co-dominant markers.

The genetic studies on sunflower mutant lines; CAS-12 and CAS-5, with high palmitic acid content, revealed that high palmitic acid can be controlled by three partially recessive alleles (*p1*, *p2* and *p3*) at 3 loci (Pérez-Vich et al., 2002a). CAS-14, CAS-3, CAS-4 and CAS-8 lines, containing respectively 35%, 28%, 15% and 14% stearic acid, have been released as high and medium stearic acid sunflower mutants (Garcés and Mancha, 1991; Cantisán et al., 2000). The level of stearic acid is controlled by *ES1* and *ES2* genes in CAS-3 mutant whereas it is increased by the *ES3* gene in CAS14 mutant (Garcés and Mancha, 1991). *ES3* gene is also mapped to Linkage group 8 (Garcés and Mancha, 1991). Three genes, designated *Ol1*, *Ol2*, and *Ol3,* are reported which are associated with high oleic acid content in sunflower seed (Fernández-Martínez, 1989). Among three microsomal oleate desaturase, *FAD2-1* is strictly correlated with high oleic acid content in sunflower seed oil (Martínez-Rivas et al., 2001). *Eco*RI and *Hin*dIII fragments, which are polymorphic in association with low and high oleic acid content genotypes, are also identified (Lacombe et al., 2001).

The genetic control of stearic and oleic acids in sunflower seed oil is also investigated through QTL analysis and cosegregation between stearoyl-ACP desaturase locus (SAD17A)

and *ES1* gene and between oleoyl-PC desaturase locus (OLD7) and *OL* gene are reported (Pérez-Vich et al., 2002b). In this study two QTLs controlling stearic and oleic acids content are also mapped to LG1 (SAD17A) and LG14 (OLD7). Several QTLs for oil and fatty acid content are identified by Ebrahimi et al. (2008). Six QTLs are detected for oil content in a population of F3 families of sunflower and the most important QTL is located on linkage group 13 (*pog-13-1*) which explained 47% of phenotypic variance (R^2) (Mokrani et al., 2002). As far as other species are concerned, overlapping chromosomic regions and nine epistatic locus pairs are identified for oil and protein content in rapeseed (Zhao et al., 2006). In *Brassica juncea*, 6 and 5 QTLs are detected for oil and protein content, respectively (Mahmmod et al., 2006). Protein, oil and fatty acids content are influenced by environmental factors. Water stress significantly decreases oil content in sunflower (Muriel and Downes, 1974; Nel et al., 2002) whereas protein content at maturity is increased in sunflower (Ebrahimi et al., 2009), wheat (Ozturk and Aydin, 2004) and peanuts (Dwivedi et al., 1996). An increase of oleic acid content in sunflower (Baldini et al., 2002) and peanuts (Dwivedi et al., 1996) is also observed under water deficit. The ratio of oleic to linoleic acid can be strongly affected by temperature regimes in sunflower (Trémoliéres et al., 1982), whereas it can be hardly affected in safflower (Browse and Slack, 1983) and rapeseed (Trémoliéres et al., 1982). The effect of sowing time (temperature regimes) on fatty acid content depends on species and genotypes. In sunflower, the ratio of oleic to linoleic acid is increased under water stress (Talha and Osman, 1974) whereas under early-sowing condition, it is decreased (Flagella et al., 2002). Activation of enzymatic and nonenzymatic antioxidant-related genes can result in the protection of fatty acids against oxidative stress and finally increasing their stability (Munné-Bosch and Alegre, 2002; Collakova and DellaPenna, 2003; Kanwischer et al., 2005; Marwede et al., 2005; Semchuk et al., 2009).

In this research for the first time we used genetic-linkage map based only on SSR markers and some important candidate genes for enzymatic, non-enzymatic antioxidant, drought-responsive family and phosphoglyceride transfer in due to genetic study of protein, oil and fatty acids content in a population of recombinant inbred lines (RILs) of sunflower under well-, partial-irrigated and late-sowing conditions. Objectives of this investigation are to identify chromosomal regions associated with quantitative variation of protein, oil and fatty acid compositions under various conditions and validate the extent to which these candidate genes affect quantitative phenotypic variability for the studied traits in sunflower grains.

4.2 Materials and methods

4. 2.1 Plant materials and experimental conditions

The recombinant inbred lines (RILs) used in this research were developed through single seed descent from F2 plants, derived from a cross between PAC2 and RHA266 (Flores Berrios et al., 2000). Three independent experiments were undertaken at Teheran University campus of Karaj. Experimental design was randomized complete block with three replications. Seeds of 89 RILs and their two parents were sown in the field under well-, partial-irrigated and late-sowing conditions. Each genotype per replication consisted of one row, 4m long, 50 cm between rows and 25 cm between plants in rows. The distance between replications of well-irrigated and partial-irrigated treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (partial-irrigated), irrigation was controlled and adjusted by the observation of the wilting threshold of the leaves. Partial water deficit was started 45 day after sowing at the stage near flower bud formation and continued up to maturity. The sowing dates were: normal sowing on May and late sowing on July.

4.2.2 Trait measurements

Percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC) and linoleic acid content (LAC) were measured in RILs and their parental lines in each replication for all conditions by the FOSS NIRSystems 6500. Forty grams of sunflower seeds per genotype per condition per replication were ground in a Knifetec 1095 Sample Mill (1975, FOSS, Tecato, Hoganas, Sweden) three times for 10s each. No sample material adhered to the walls of the mill because the sample was mixed at each interval.A FOSS NIRSystems 6500 spectrophotometer (Foss Analytical, Denmark) was used to collect spectra from the ground sunflower seeds using a small round cup with a quartz window. The reflectance (R) of each sample was measured as log of 1/R from 400 to 2500 nm at 2 nm intervals. The absorption maximum around 1700-1800 and 2300-2400 nm were due to oil and fatty acid content. The area near to 2180 nm was related to protein content. In order to validity of NIRS results, some samples used for NIRS were also tested by gas chromatography (GC) and high correlation was observed.

4.2.3 Statistical analysis and map construction

Data were analyzed using SAS PROC GLM (SAS Institute Inc., 1996) and SPSS. Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. The mean of RILs and that of their parents were compared for all the traits. Genetic gain when the best RIL is compared with the best parent (GGB) and when the mean of the top 10% selected RILs is compared with the mean of the parents (GG10%), were calculated for the traits. Simple correlation coefficients (Pearson) among the studied traits were also determined. Some important tocopherol pathway-related genes, enzymatic antioxidant-related genes, drought-responsive genes and phosphoglyceride transfer-related genes were studied. Respective sequence data for candidate genes were obtained from The *Arabidopsis* Information Resource (*www.arabidopsis.org*). In order to seek the *helianthus* homolog sequences to the *Arabidopsis* genes, we used the compositae EST assembly clusters, available at the *Helianthus*-devoted bioinformatics portal Heliagene (*www.heliagene.org*). The *Helianthus* EST clusters presenting the reciprocal blast with the highest score and lowest E value with regarding to the original *Arabidopsis* genes were chosen for our studies. Genotyping was done by SNP-based CAPS marker and high resolution melting (HRM) as well as directly on agarose gel. The chromosomal locations of QTLs were resolved by composite interval mapping (CIM), using Win QTL Cartographer, version 2.5 (Wang et al., 2005) with the mean values of three replications for each RIL in each conditions. The genome was scanned at 2-cM intervals; with a window size of 15 cM. Up to 15 background markers were used as cofactors in the CIM analysis with the program module Srmapqtl (model 6). Additive effects of the detected QTLs were estimated with the Zmapqtl program (Basten et al., 2002). The percentage of phenotypic variance (R^2) explained by each QTL was estimated by Win QTL Cartographer.

4.3 Results

4.3.1 Phenotypic variation

Results of analysis of variance for PSP, PSO, PAC, SAC, OAC and LAC are presented in Table 4.1. A normal distribution was observed for studied traits under all conditions and it is shown in Fig. 4.1 for well-irrigated condition. Significant genotypic effect is observed for

aforementioned traits in well-, partial-irrigated and late-sowing conditions. Genetic gain and phenotypic performance of RILs and their parents for above-mentioned traits in all conditions are presented in Table 4.2. The differences between the mean of RILs (MRILs) and the mean of their parents (MP) for all studied traits are nonsignificant. The comparison between the best parent (BP) and the best RIL (BRIL), considered as genetic gain (GGB), showed a significant difference for all traits in all conditions. A large genetic variability is observed for all studied traits resulting in significant differences between the 10% selected RILs (10%SRILs) and the mean of the parents for all conditions. Phenotypic correlations among different traits in different conditions are presented in Table 4.3. A significant and positive correlation is observed between PSP and SAC, between PSO and LAC, between PAC and SAC and between PAC and LAC in all conditions. A significant and negative correlation is also observed between PSP and PSO, between PSP and PAC, between PSO and SAC and between PAC and OAC under all conditions. There is a high significant and negative correlation between OAC and LAC. Under well- and partial-irrigated conditions, the correlation between PSP and OAC and between PSO and PAC is significant and positive whereas a significant and negative correlation is observed between PSP and LAC and between PSO and OAC.

4.3.2 QTL analysis

The map position and characteristics of QTLs associated with the studied traits in the field under well-, partial-irrigated and late-sowing conditions are presented in Table 4.4. QTLs are designated as the abbreviation of the trait followed by *'WI'*, *'PI'* and *'LS'* for well-irrigated, partial-irrigated and late-sowing conditions. The corresponding linkage group and the number of QTLs in the group are also indicated for each QTL. Two to six QTLs are identified depending on the trait and conditions. Both parental lines contribute to the expression of the different target traits and positive or negative additive effects are presented (Table 4.4). Co-localized QTLs are detected for all traits on various linkage groups (Fig. 4.2). Detected QTLs for PSP explain from 5.4 % to 28.8% of the phenotypic variance (R^2). The most important QTL for PSP (*PSP-WI.13*) is identified on linkage group 13. Favorable alleles for this QTL come from RHA266. The percentage of phenotypic variance (R^2) explained by QTLs of PSO ranged from 5.92 % to 38.18 %. The most important QTL for PSO on linkage group 16 (*PSO-PI.16*) under partial-irrigated condition controls 38.18 % of phenotypic variance (R^2). On linkage group 16, we also identified 2 other QTLs for this trait under well-irrigated and late-sowing conditions. They are co-located with several QTLs controlling fatty acids content

(Fig. 4.2). The percentage of phenotypic variance (R^2) explained by QTLs of PAC ranged from 4.65 % to 38%. Most of the favorable alleles for these QTLs come from PAC2. Under late-sowing condition, identified QTLs for SAC explain from 7.31 % to 54.38% of the phenotypic variance. Several QTLs are detected for OAC under all conditions. The percentage of phenotypic variance (R^2) explained by these QTLs ranged from 5.31 % to 67.35 %. The most important QTL for OAC (*OAC-WI.10*) is located on linkage group 10 where several QTLs controlling fatty acids are found. Under late-sowing condition, five QTLs for LAC are identified which explained from 4.36 % to 53.72% of the phenotypic variance.

4.4 Discussion

Significant differences between the parents are observed only for percentage of seed oil (PSO) in well- irrigated condition and percentage of seed protein (PSP) under partial-irrigated conditions, indicating gene expression differences between them for these two traits under the two conditions (Table 4.2). Non significant differences between the mean of the RILs (MRILs) and the mean of their parents (MP) reveal that the RILs used in this research are representative of all possible genetic combinations between the two parents 'PAC2' and 'RHA266'. Genetic gain (GGB) when the best RIL is compared with the best parent and GG10% Sel, considered as the differences between the mean of the top 10% selected RILs and the mean of the parents, are significant for all the studied traits, revealing transgressive segregation for all the studied traits. Transgressive segregation is also reported for water status traits (Poormohammad Kiani et al., 2007) and yield-related traits (Poormohammad Kiani et al., 2009) in the same population. Transgressive segregation would be the result of the accumulation of favorable alleles coming from different parental lines. The positive and negative signs of additive effect at the different loci (Table 4) indicate the contribution of both parental lines and confirm the transgressive segregation observed at the phenotypic level. The mean of late-sown RILs for LAC is increased (64.03 %) compared to the well-irrigated RILs (50.34 %). Highly significant correlations are observed among most of the studied traits. High negative correlation between OAC and LAC in all conditions is similar to the results of Lagravère et al. (2004) and Ebrahimi et al. (2008) in sunflower. A significant and positive correlation between PSP and OAC and between PSO and PAC is observed under well- and partial-irrigated conditions whereas correlation between them is not significant under late-sowing condition. Non significant correlation between PSP and LAC and between PSO and OAC is observed under late-sowing condition whereas correlation between them is significant

and negative under well- and partial-irrigated conditions. Correlation between OAC and PAC is negative in all conditions, which is similar to the results of Ebrahimi et al. (2008) in sunflower and Möllers and Schierholt (2002) in rapeseed.

The QTLs detected in current research reveal that several putative genomic regions are involved in the expression of the mentioned traits under all conditions. A specific QTL for PSP is identified on linkage group 8 (*PSP-LS.8*). This QTL, controlled by RHA266 alleles, appears to be important in late-sowing condition. Overlapping occurs for QTLs of PSP and PSO on linkage group 9 (*PSP-PI.9* and *PSO-PI.9*) and 11 (*PSP-PI.11* and *PSO-PI.11*). Significant and negative correlation between PSP and PSO (Table 4.3) is justified by opposite additive effects of their overlapped QTLs (Table 4.4). Negative correlation between PSP and PSO (Table 4.3) and their overlapped QTLs with opposite additive effects (Table 4.4) are also reported in the previous studies (Lee et al., 1996; Zhao, 2002; Mohamood et al., 2006; Ebrahimi et al., 2008). This phenomenon poses potential challenges to breeders for simultaneous improvement of both traits. However, independent segregation of QTLs for PSP and PSO provides opportunity for simultaneous improvement of these two traits in sunflower. Under partial-irrigated condition, a specific QTL of PSP on linkage group 8 (*PSP-PI.8*) is linked to candidate gene, HuCL02840C003 (Fig. 4.2). This candidate gene, homogenitisate phytyltransferase (VTE2), involves in tocopherol pathway (Kanwischer et al. 2005). The most important QTL of PSO (*PSO-PI.16*) is mapped to linkage group 16 between ORS492_2 and ORS899 markers. This chromosomic region is important for oil content as it is also reported by Tang et al. (2006) and Ebrahimi et al. (2008) for seed oil content. A common QTL for PSO is identified on linkage group 2 (*PSO-WI.2, PSO-LS.2*). These QTLs, controlled by the PAC2 alleles, appear to be important in both well-irrigated conditions. This region on linkage group 2, linked to ORS525_1 marker, is also reported for oil content under greenhouse condition (Ebrahimi et al., 2008). Under late-sowing condition, a specific QTL of PSO on linkage group 8 (*PSO-LS.8*) is assigned to candidate gene, HuCX944063 which involves in photosystem I. The oil content is positively associated with leaf area which determines the photosynthetic capacity of sunflower (Hervé et al., 2001). Overlapped chromosomic regions for PSO and SAC are identified on linkage group 10 (*PSO-PI.10* and *SAC-WI.10*) and 15 (*PSO-PI.15* and *SAC-LS.15*). A significant and negative association between PSO and SAC (Table 4.3) is strengthened by opposite additive effects of their overlapped QTLs (Table 4.4). There is an important overlapped region for PAC, LAC and PSO on linkage group 17 (*PAC-LS.17*, *LAC-LS.17* and *PSO-LS.17*). This chromosomic region is located between ORS297 and ORS1040 markers. A specific QTL for linoleic acid content (LAC) which was also linked to

ORS297 marker was already detected in this region (Ebrahimi et al., 2008). A common QTL of PAC on linkage group 16 (*PAC-WI.16* and *PAC-PI.16*) is linked to the SSR marker, ORS418_2. Several QTLs of PSO, OAC and LAC are also identified in this region. Seven QTLs associated with PAC, SAC, OAC and LAC are identified on linkage group 14. These overlapped QTLs are linked to candidate gene, HuCL04260C001 which modulates the expression of p-hydroxyphenylpyruvate dioxygenase (HPPD). This candidate gene is located between ORS1152_1 and ORS391 markers. Homogentisic acid (HGA), the common precursor to tocopherols (Valentin et al., 2006), can originate either via the conversion of chorismate to prephenate and then to p-hydroxyphenylpyruvate (HPP) via prephenate dehydrogenase in bacteria or via the synthesis and conversion of the intermediates arogenate, tyrosine, by the shikimate pathway, and HPP in plants. HPP is then converted to HGA by p-hydroxyphenylpyruvate dioxygenase (HPPD) (Norris et al., 1998). The interdependence between the amount of tocopherol and lipid peroxidation has also been recognized (Munné-Bosch, 2005). In plants, the protection of photosynthetic apparatus and polyunsaturated fatty acids from oxidative damage caused by reactive oxygen species (ROS) are the main function of tocopherol (Trebst et al., 2002; Velasco et al., 2004; Cela et al., 2009; Semchuk et al., 2009). Under late-sowing condition, a specific QTL of PAC on linkage group 6 (*PAC-LS.6*) is located between ORS1233 and SSL66_1 markers (Fig. 2). Overlapping occurs for QTLs of PAC and SAC on linkage groups 2, 8, and 14. This can be explained by correlation between PAC and SAC as well as by a specific gene for fatty acid synthetase II (FACII), which lengthens palmitic acid (16:0) by two carbon atoms to produce stearic acid (18:0) (Cantisán et al., 2000; Pleite et al., 2006). In previous studies, several overlapped QTLs of PAC and SAC are reported (Burke et al., 2005; Ebrahimi et al., 2008). Overlapping also occurs for QTLs of SAC and OAC on linkage groups 1, 2, and 14. This can be explained by the existence of specific gene for Δ9-desaturase (stearoyl-ACP desaturase), which catalyses the first desaturation of stearic acid (18:0) to oleic acid (18:1) (Heppard et al., 1996; Cantisán et al., 2000). We detected overlapped QTLs for SAC and OAC under late-sowing condition on linkage group1 (*SAC-LS.1* and *OAC-LS.1*). This chromosomic region is reported for days from sowing to flowering (Poormohammad Kiani et al., 2009). A significant negative correlation between days to flowering and seed-oil content in areas with short growing season was reported by Leon et al. (2003). They also detected two overlapped QTLs for seed oil content and days to flowering. Common QTLs of SAC on linkage group 10 (*SAC-WI.10 and SAC-LS.10*) and linkage group 14 (*SAC-WI.14, SAC-PI.14 and SAC-LS.14*) are identified. A specific QTL of SAC is detected on linkage group 5 which is linked to HA3627 marker.

Common QTLs of OAC are observed on linkage group 2 (O*AC-PI.2 and OAC-LS.2*) which overlap with QTLs controlling PAC and SAC. The high negative correlation (Table 3) between OAC and LAC in all conditions is justified by opposite additive effects of their linked QTLs (Tables 4.4; Figs. 4.1). Another overlapping for QTLs of OAC and LAC is observed on linkage groups 10, 11 and 16. This can be explained by correlation between OA and LA as well as by a specific gene for Δ12-desaturase (oleoyl-PC desaturase), which catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2) (Garcés and Mancha, 1991). Regarding to identified QTLs for SAC, OAC and LAC on linkage group 14 between ORS1152_1 and ORS391 markers, we can consider this overlapping as a chromosomic region that controls two pathways, FatA (stearoyl-ACP desaturase) and FatB (acyl-ACP thioesterase), in sunflower (Pleite et al., 2006).

As conclusion, we have detected several specific and non specific QTLs under well-, partial-irrigated and late-sowing conditions for PSP, PSO, PAC, SAC, OAC and LAC. Detection of QTLs influencing various traits could increase the efficiency of marker-assisted selection and increase genetic progress. The relatively low number of RILs used in current research may have a negative influence on the accuracy of the calculated QTL effects and the ability to detect QTLs with small effects and R^2 overestimation (Bachlava et al., 2008; Beavis, 1994). This was, to some degree, compensated by the higher precision of the phenotyping and the use of our map including candidate genes. The absence of significant difference between the mean of RILs and the mean of parents (Table 4.3) shows also that RILs used in our study can present all possible genetic combination from two parents for the studied traits. Coincidence of the position for some detected QTLs and candidate genes would be useful for the function of the respective genes in fatty acid pathway and its stability.

Table 4.1: Analysis of variance (mean squares) for percentage of seed protein, percentage of seed oil, palmitic, stearic, oleic and linoleic acids content in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated (WI), partial-irrigated (PI) and late-sowing (LS) conditions

Source of variance	df	condition	Protein	oil	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid
RILs	88	WI	31.68**	46.70**	0.86**	1.53**	198.77**	195.86**
		PI	22.10**	41.12**	0.97**	0.95**	177.21**	171.27**
		LS	11.23**	27.36**	0.49**	0.89**	83.82**	82.62**
Blocks	2	WI	1.58 NS	13.82 NS	1.22**	3.69**	71.88 NS	26.10 NS
		PI	4.32 NS	47.57*	2.24**	8.47**	94.70*	57.91 NS
		LS	1.15 NS	50.07**	2.57**	6.64**	71.01**	26.91 NS
Error	176	WI	3.96	11.89	0.14	0.47	23.76	22.91
		PI	5.50	13.49	0.15	0.54	21.25	20.86
		LS	2.83	10.05	0.09	0.32	11.40	11.07

*, **: significant at 0.05 and 0.01 probability level, respectively. NS: non-significant.

Table 4.2: Genetic variability and genetic gain for percentage of seed protein, percentage of seed oil, palmitic, stearic, oleic and linoleic acids content (in percentage of oil) in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated (WI), partial-irrigated (PI) and late-sowing (LS) conditions

		Protein (%)	Oil (%)	Palmitic acid	Stearic acid	Oleic acid	Llinoleic acid
PAC-2 (P1)	WI	21.18	40.59	4.80	3.73	41.95	48.81
	PI	21.51	38.13	4.76	3.92	43.22	46.93
	LS	25.37	39.23	5.51	5.25	24.20	64.96
RHA-266 (P2)	WI	21.69	45.22	5.58	3.95	38.84	52.50
	PI	24.64	38.81	5.32	5.00	41.37	49.23
	LS	25.27	40.45	6.15	4.96	25.40	64.58
/P1-P2/	WI	0.51^{NS}	4.62^{**}	0.77^{NS}	0.21^{NS}	3.11^{NS}	3.68^{NS}
	PI	3.12^{*}	0.68^{NS}	0.56^{NS}	1.07^{NS}	1.84^{NS}	2.29^{NS}
	LS	0.09^{NS}	1.21^{NS}	0.63^{NS}	0.29^{NS}	1.19^{NS}	0.37^{NS}
MP :(P1+P2)/2	WI	21.433	42.904	5.187	3.839	40.397	50.657
	PI	23.08	38.47	5.04	4.46	42.30	48.08
	LS	25.32	39.84	5.83	5.10	24.80	64.77
MRILs	WI	22.26	40.30	5.21	4.31	40.19	50.34
	PI	23.10	39.51	5.17	4.45	41.48	49.22
	LS	25.22	37.29	5.96	5.39	25.11	64.03
/MRIL -MP/	WI	0.83^{NS}	2.60^{NS}	0.01^{NS}	0.47^{NS}	0.21^{NS}	0.31^{NS}
	PI	0.01^{NS}	1.03^{NS}	0.13^{NS}	0.01^{NS}	0.81^{NS}	1.13^{NS}
	LS	0.10^{NS}	2.55^{NS}	0.12^{NS}	0.28^{NS}	0.31^{NS}	0.73^{NS}
GGB: (BRIL-BP)	WI	11.3^{**}	5.11^{**}	1.11^{*}	1.31^{**}	19.44^{**}	17.65^{*}
	PI	8.22^{**}	11.01^{**}	1.49^{**}	1.9^{**}	17.4^{**}	19.08^{**}
	LS	7.85^{**}	5.9^{**}	1.08^{**}	2.14^{*}	16.52^{**}	15.95^{**}
GG10%: (10% SRILs - MP)	WI	7.06^{**}	4.78^{**}	1.187^{**}	2.18^{**}	15.15^{**}	15.09^{**}
	PI	5.69^{**}	8.52^{**}	1.33^{**}	1.52^{**}	13.85^{**}	14.99^{**}
	LS	4.39^{**}	3.74^{**}	0.94^{*}	1.59^{*}	10.74^{**}	9.11^{*}

PAC2' (P1) and 'RHA266' (P2): parental lines; MP: mean of two parental lines; MRILs: mean of recombinant inbred lines; BRIL: the best RIL; BP: the best parent; 10%SRILs: the mean of the top 10% selected RILs; GGB: genetic gain when the best RIL is compared with the best parent; GG10%: genetic gain when the mean of the top 10% selected RILs is compared with the mean of the parents. *, **: significant at 0.05 and 0.01 probability level, respectively. ^{NS}: non-significant.

Table 4.3: Simple correlation coefficients (Pearson) among percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic, stearic, oleic and linoleic acids content (PAC, SAC, OAC and LAC, respectively) in sunflower (RILs) under well-irrigated (WI), partial-irrigated (PI) and Late-sowing (LS) conditions

	PSP			PSO			PAC			SAC			OAC		
	WI	PI	LS	WI	PI	LS	WI	PI	LS	WI	PI	LS	WI	PI	LS
PSO	-0.65**	-0.63**	-0.35**												
PAC	-0.41**	-0.24**	-0.23**	0.27**	0.27**	0.10ns									
SAC	0.64**	0.56**	0.45**	-0.46**	-0.30**	-0.25**	0.15**	0.31**	0.42**						
OAC	0.44**	0.33**	0.08 ns	-0.28**	-0.21**	-0.10ns	-0.84**	-0.84**	-0.72**	-0.07 ns	-0.07 ns	-0.15*			
LAC	-0.48**	-0.36**	-0.07 ns	0.33**	0.25**	0.18**	0.81**	0.81**	0.67**	-0.15*	-0.02ns	0.06 ns	-0.99**	-0.99**	-0.98**

*, **: significant at 0.05 and 0.01 probability level, respectively. NS: non-significant.

Table 4.4: QTLs detected for percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC) and linoleic acid content (LAC) under well-, partial-irrigated and late-sowing conditions

Trait	QTL	LG	Position cM	LOD	Additive effects	R^2
Well-irrigated						
PSP	*PSP-WI.10.1*	10	2.01	4.67	-1.67	14.00
	PSP-WI.10.2	10	35.31	4.32	-1.71	12.00
	PSP-WI.13	13	25.31	3.09	-3.22	28.80
PSO	*PSO-WI.2*	2	71.31	3.29	1.17	6.23
	PSO-WI.11	11	47.31	3.94	1.38	9.25
	PSO-WI.13	13	13.91	4.00	3.51	28.31
	PSO-WI.15	15	0.61	5.86	1.82	15.06
	PSO-WI.16	16	19.21	4.88	1.88	15.63
Partial-irrigated						
PSP	*PSP-PI.8.1*	8	0.01	5.36	-0.93	8.00
	PSP-PI.9	9	44.71	6.86	0.94	8.90
	PSP-PI.11	11	2.01	5.02	-1.41	17.20
PSO	*PSO-PI.9*	9	47.41	6.15	-1.77	12.07
	PSO-PI.10	10	45.61	5.91	1.92	14.23
	PSO-PI.11	11	24.01	6.11	2.31	20.00
	PSO-PI.15	15	20.21	5.85	2.26	12.58
	PSO-PI.16	16	15.21	6.30	3.58	38.18
Late-sowing						
PSP	*PSP-LS.8*	8	156.31	7.50	-0.75	10.50
	PSP-LS.11	11	40.11	3.91	-0.58	5.40
PSO	*PSO-LS.2*	2	71.31	5.58	1.58	18.91
	PSO-LS.8	8	26.91	3.20	1.10	5.92
	PSO-LS.11	11	42.11	3.23	1.22	9.78
	PSO-LS.16	16	144.71	4.88	1.31	11.89
	PSO-LS.17	17	32.71	6.14	-1.48	11.95

Table 4.4: continued

Trait	QTL	LG	Position cM	LOD	Additive effects	R^2
Well-irrigated						
PAC	*PAC-WI.10*	10	0.01	5.56	0.24	10.64
	PAC-WI.14	14	41.61	3.67	-0.13	4.65
	PAC-WI.16	16	11.21	5.70	0.17	6.40
SAC	*SAC-WI.3*	3	0.01	3.40	-0.31	14.70
	SAC-WI.7	7	12.01	3.10	-0.31	11.16
	SAC-WI.10	10	45.61	4.07	-0.34	17.42
	SAC-WI.14	14	38.01	3.00	-0.33	14.07
Partial-irrigated						
PAC	*PAC-PI.2*	2	2.01	4.04	0.47	36.04
	PAC-PI.16	16	11.21	3.47	0.17	5.80
SAC	*SAC-PI.2*	2	8.01	4.60	0.70	65.17
	SAC-PI.8	8	53.61	6.63	-0.70	12.00
	SAC-PI.14	14	26.01	3.087	-0.69	15.00
Late-sowing						
PAC	*PAC-LS.2*	2	6.01	4.97	0.27	20.11
	PAC-LS.6	6	28.21	5.29	0.16	7.18
	PAC-LS.8	8	55.61	6.24	0.45	38.00
	PAC-LS.10	10	8.01	5.00	0.24	10.00
	PAC-LS.11	11	10.01	6.50	0.28	11.04
	PAC-LS.17	17	26.71	6.20	-0.21	11.80
SAC	*SAC-LS.1*	1	6.01	4.67	0.57	54.38
	SAC-LS.5	5	3.81	3.07	0.23	10.75
	SAC-LS.10	10	52.11	4.38	0.20	8.75
	SAC-LS.14	14	26.01	3.87	-0.21	7.31
	SAC-LS.15	15	10.21	4.85	-0.24	10.52

Table 4.4: continued

Trait	QTL	LG	Position cM	LOD	Additive effects	R^2
Well-irrigated						
OAC	*OAC-WI.4*	4	17.11	4.00	-2.78	7.20
	OAC-WI.10	10	6.01	12.98	-9.69	67.35
	OAC-WI.11	11	4.01	3.81	-3.13	8.74
	OAC-WI.14	14	34.01	4.54	3.51	10.11
	OAC-WI.16	16	11.21	3.55	-2.77	6.06
LAC	*LAC-WI.3*	3	25.31	5.18	-2.42	5.65
	LAC-WI.4	4	17.11	4.80	1.89	3.70
	LAC-WI.10	10	6.01	11.09	9.48	68.00
	LAC-WI.13	13	56.51	3.15	-2.05	4.50
	LAC-WI.16	16	139.51	6.68	-2.83	7.90
Partial-irrigated						
OAC	*OAC-PI.2*	2	14.01	3.3	-3.57	10.93
	OAC-PI.4	4	17.11	6.44	-3.70	11.20
	OAC-PI.10	10	6.01	11.55	-5.90	36.45
	OAC-PI.11	11	14.01	4.44	-3.68	8.98
	OAC-PI.14	14	34.01	3.79	7.98	30.83
LAC	*LAC-PI.2*	2	45.71	3.29	7.35	30.38
	LAC-PI.4	4	17.21	6.10	3.73	12.47
	LAC-PI.10	10	6.01	11.04	5.42	31.01
	LAC-PI.11	11	0.01	6.07	2.16	6.17
	LAC-PI.13	13	61.61	3.69	-2.32	5.88
	LAC-PI.16	16	8.41	4.49	2.52	5.64
Late-sowing						
OAC	*OAC-LS.1*	1	2.01	3.13	1.61	5.31
	OAC-LS.2	2	18.01	3.96	-6.561	58.20
	OAC-LS.10	10	10.01	3.03	-3.031	14.87
	OAC-LS.16	16	11.21	3.70	-2.351	10.76
LAC	*LAC-LS.4*	4	17.21	5.12	1.35	4.36
	LAC-LS.10	10	0.01	3.32	2.27	10.29
	LAC-LS.11	11	10.01	4.31	5.19	18.52
	LAC-LS.14	14	53.61	3.87	-2.34	12.19
	LAC-LS.17	17	24.71	7.84	-6.31	53.72

The QTLs are designated as the abbreviation of the trait followed by `*WI* `, `*PI* ` and `*LS* ` for well-irrigated, partial-irrigated and late-sowing. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it. Fatty acids are measured as percentage of oil.

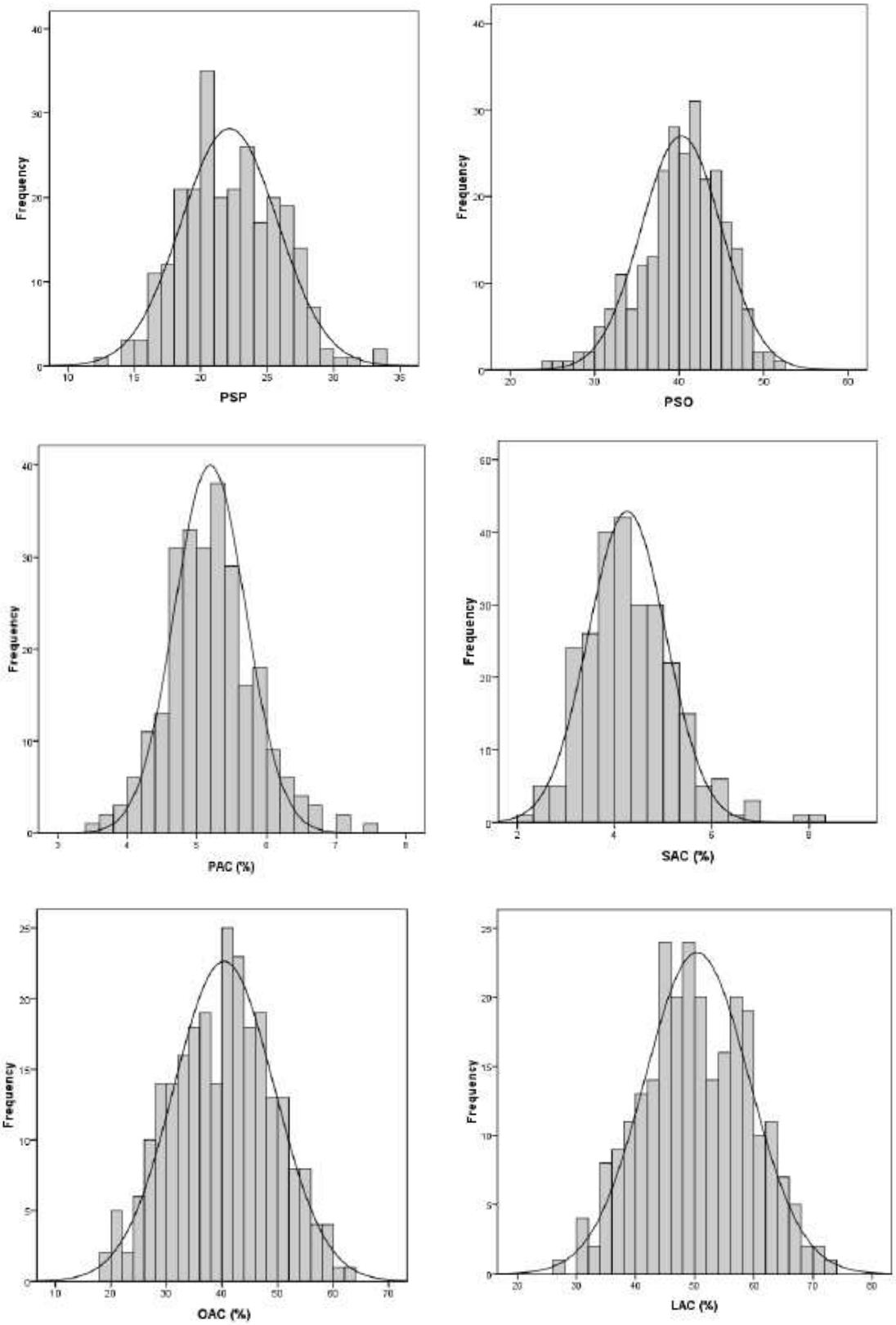
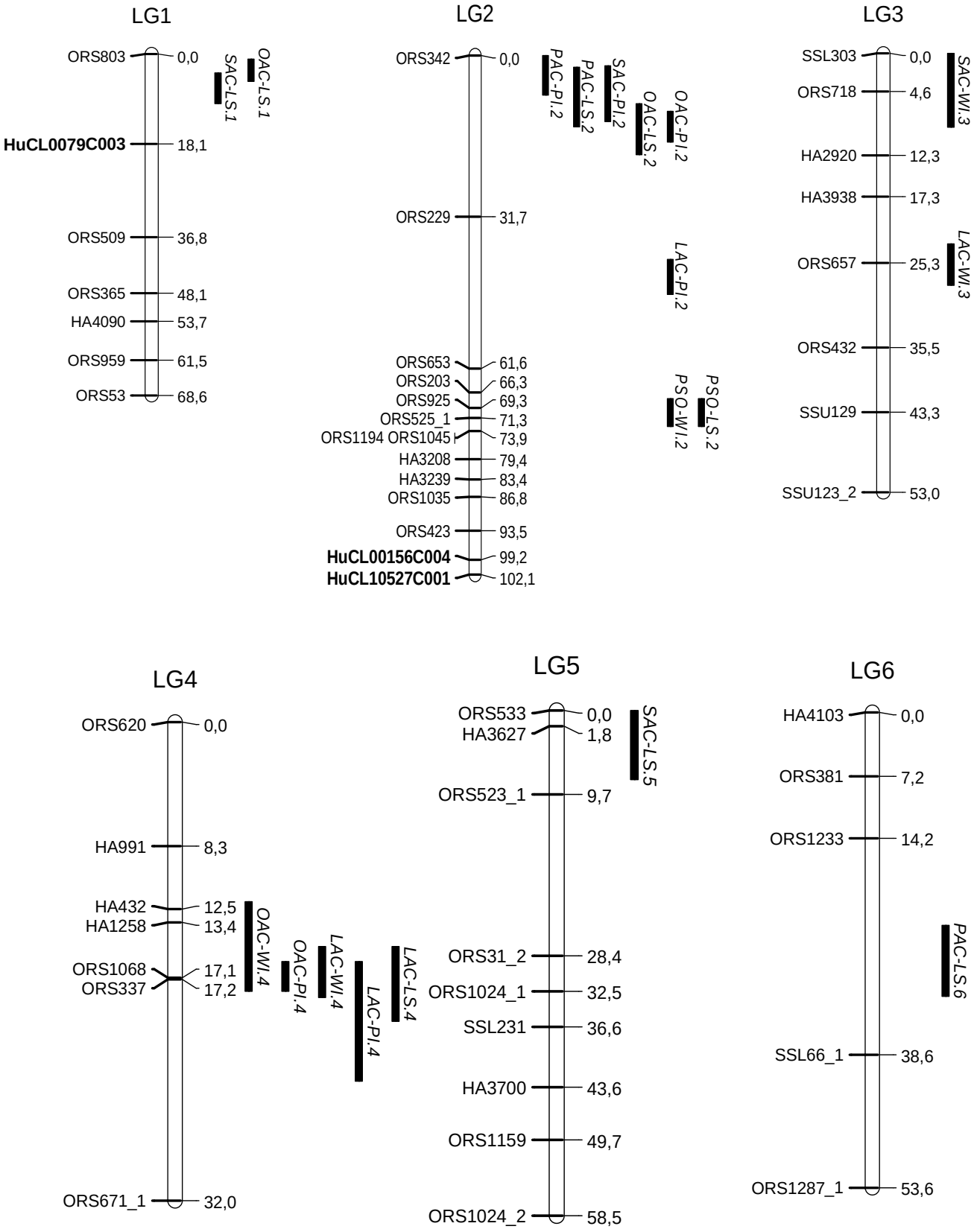


Fig. 4.1. Distribution for percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC) and linoleic acid content (LAC) in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated condition.

Fig. 4.2. Molecular linkage groups of sunflower map presenting QTLs for percentage of seed protein (PSP), percentage of seed oil (PSO), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC) and linoleic acid content (LAC).The positions of QTLs are shown on the right side of the linkage groups. Bars represent intervals associated with the QTLs.



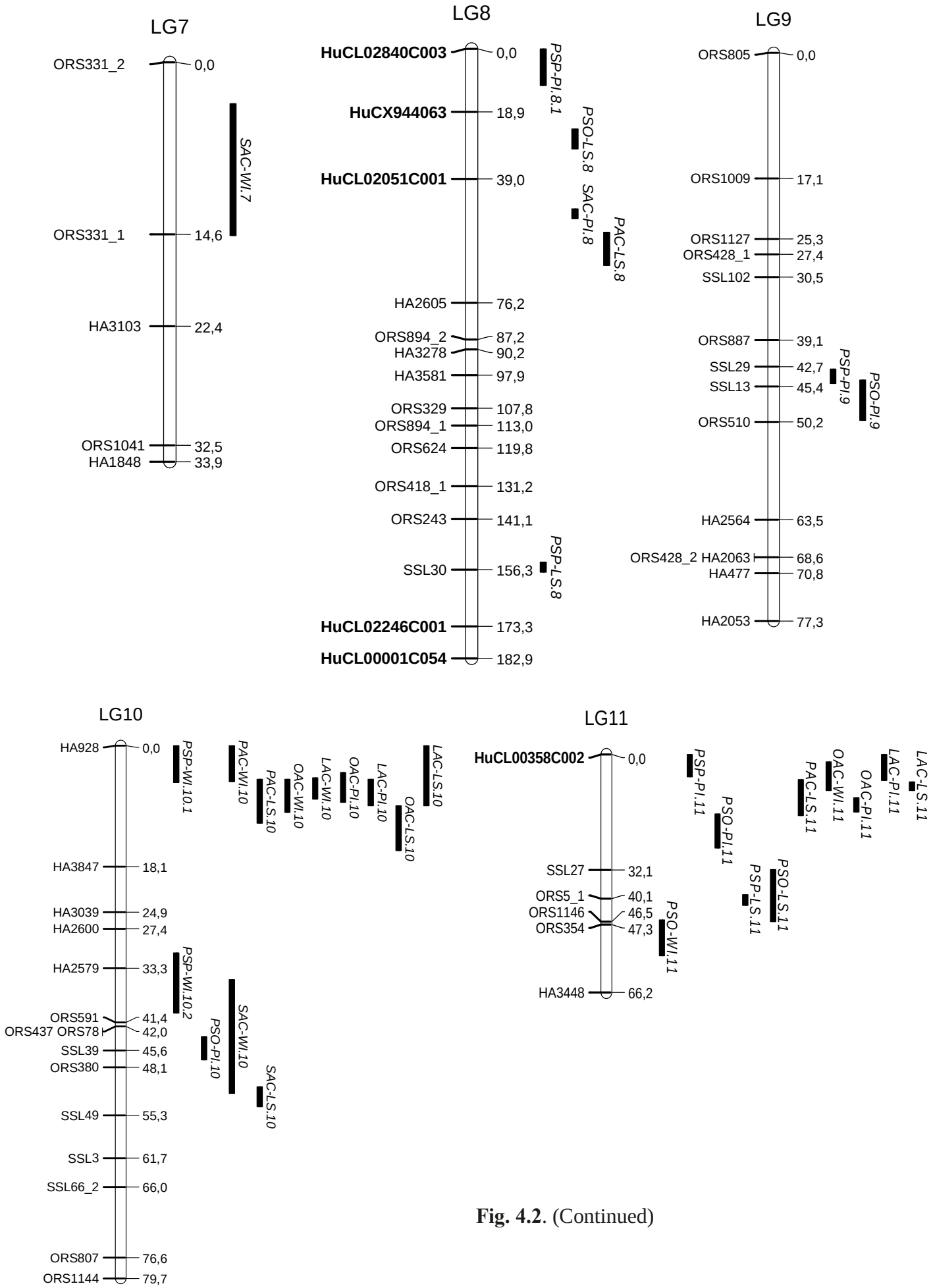


Fig. 4.2. (Continued)

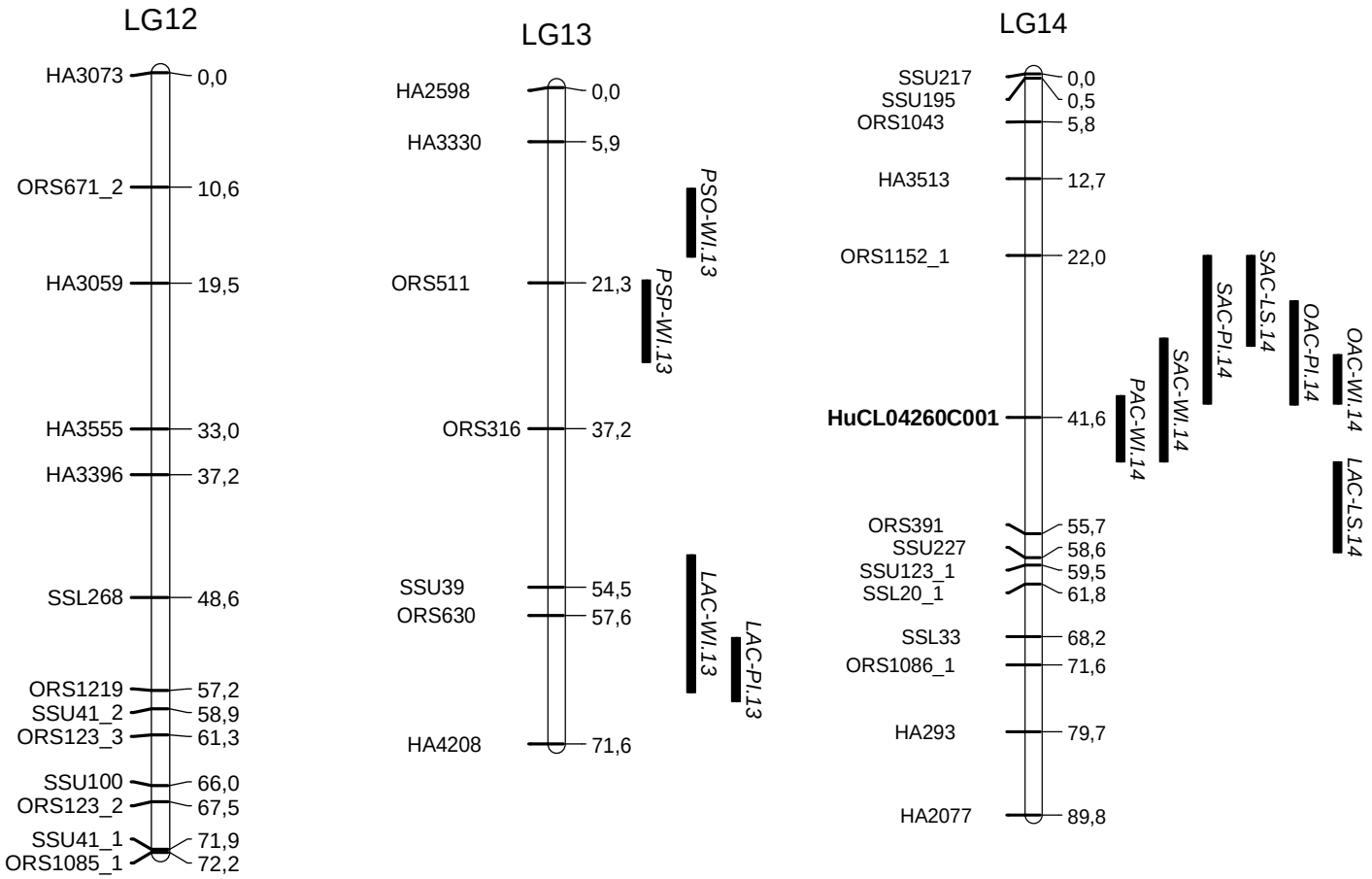


Fig. 4.2. (Continued)

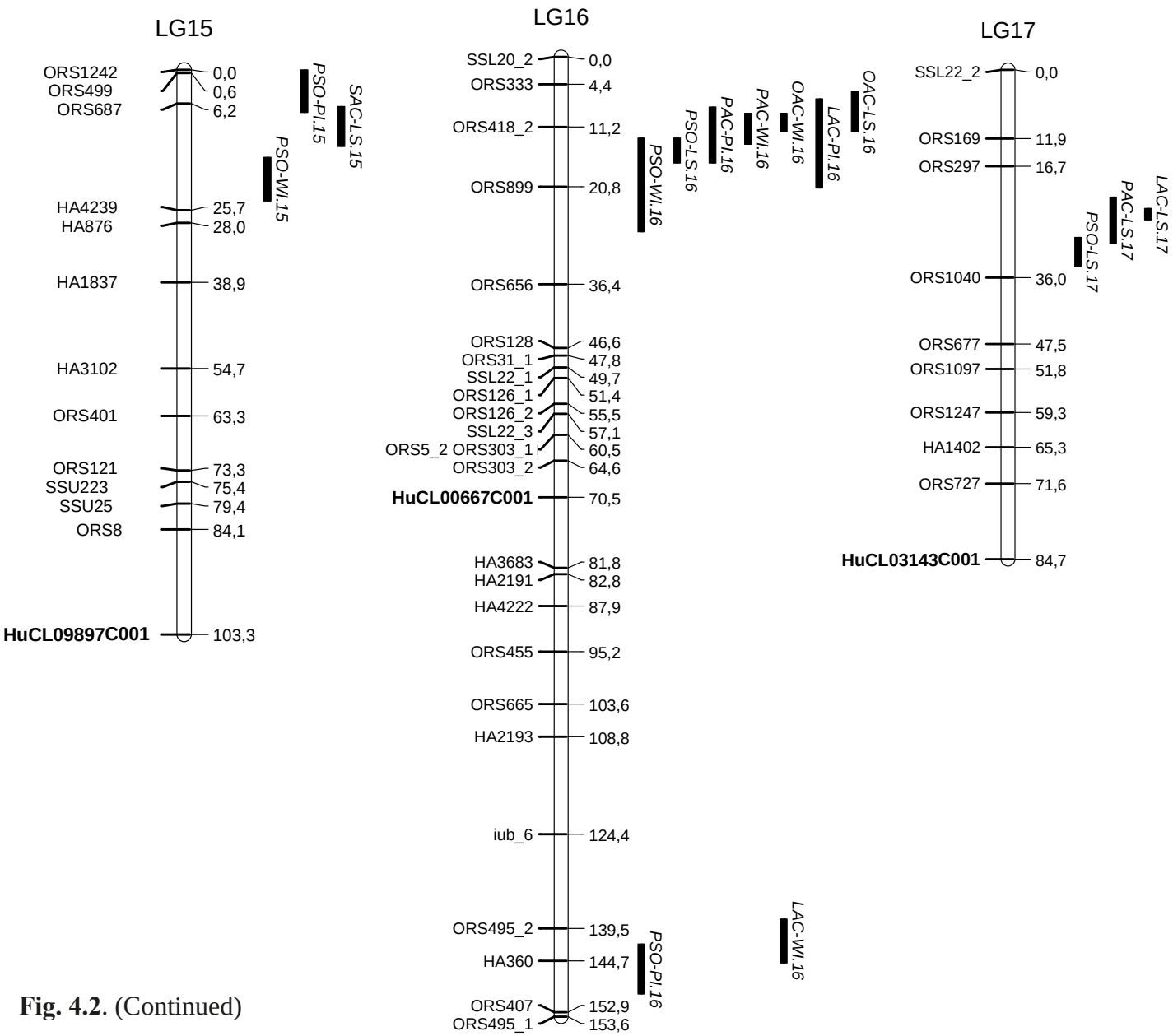


Fig. 4.2. (Continued)

The candidate genes are: HuCL00790C003; glutation s-transferase (GST), HuCL00156C004; pattelin 2 (PATL2), HuCL10527C001; phosphoglyceride transfer (SEC14-like protein), HuCL02840C003; homogenitisate phytyltransferase (VTE2), HuCX944063; PSI P700, HuCL02051C001; drought-responsive family protein, HuCL00001C054; catalase (CAT), HuCL02246C001; tocopherol methyl-transferase (VTE4), HuCL00358C002; 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), HuCL04260C001; p-hydroxyphenylpyruvate dioxygenase (HPPD), HuCL09897C001; cytosolic factor (SEC 14- like protein), HuCL00667C001; phosphoglyceride transfer and HuCL03143C001; peroxidase (POD).

Chapter 5

QTL analysis of agronomic traits in recombinant inbred lines of sunflower under partial irrigation

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Abstract:

The objective of the present research is to map QTLs associated with agronomic traits such as days from sowing to flowering, plant height, yield and leaf-related traits in a population of recombinant inbred lines (RILs) of sunflower. Two field experiments were conducted with well- and partial-irrigated conditions in randomized complete block design with three replications. A map with 304 AFLP and 191 SSR markers with a mean density of one marker per 3.7 cM was used to identify QTLs related to the studied traits. The difference among RILs is significant for all studied traits in both conditions. Three to seven QTLs are found for each studied trait in both conditions. The percentage of phenotypic variance (R^2) explained by QTLs ranged from 4% to 49%. Three to six QTLs are found for each yield-related trait in both conditions. The most important QTL for grain yield per plant on linkage group 13 (*GYP-P-13-1*) under partial-irrigated condition controls 49% of phenotypic variance (R^2). The most important QTL for 1000-grain weight (*TGW-P-11-1*) is identified on linkage group 11. Favorable alleles for this QTL come from RHA266. The major QTL for days from sowing to flowering (*DSF-P-14-1*) is observed on linkage group 14 and explained 38% of the phenotypic variance. The positive alleles for this QTL come form RHA266. The major QTL for HD (*HD-P-13-1*) is also identified on linkage group 13 and explained 37% of the phenotypic variance. Both parents (PAC2 and RHA266) contributed at QTLs controlling leaf-related traits in both conditions. Common QTL for leaf area at flowering (*LAF-P-12-1, LAF-W-12-1*) is detected in linkage group 12. The results do emphasize the importance of the role of linkage group 2, 10 and 13 for studied traits. Genomic regions on the linkage group 9 and 12 are specific for QTLs of leaf-related traits in sunflower.

Key Words: sunflower, recombinant inbred lines, QTL, partial-irrigation, agronomic traits

Abbreviations:

QTL Quantitative trait locus

CIM Composite interval mapping

5.1 Introduction

Sunflower (*Helianthus annuus* L., 2n =34) has been considered for construction of molecular genetic map. A saturated and reference genetic map based on 304 AFLP and 191 SSR markers with a mean density of one marker per 3.7 cM was recently constructed (Poormohammad Kiani et al. 2007a). Drought stress can have major impacts on plant growth and development. Drought can result in lower yield and possible crop failure. Upon partial water stress, photosynthetic activity decreases mainly due to stomatal closure, which constitutes the stomatal limitation of photosynthesis (Hugo et al. 2004). Low heritability of yield and large genotype x environment interaction are main obstacles in achieving progress in enhancing yield through a direct selection (Tuberosa et al. 2002). Many agricultural important traits such as yield, quality and disease resistance are controlled by many genes and are known as quantitative traits. The regions within genomes that contain genes associated with particular quantitative trait are recognized as quantitative trait loci (QTLs). QTLs controlling important traits such as *in vitro* regeneration parameters (Flores Berrios et al. 2000a–c), resistance to *Sclerotinia sclerotiorum* and *Phoma macdonaldii* (Bert et al. 2004, and Micic et al. 2005**),** physiological parameters (Hervé et al. 2001), resistance to downy mildew and black stem (Rachid Al-Chaarani et al. 2002), agronomic traits (Rachid Al-Chaarani et al. 2004), germination and seedling development (Rachid Al-Chaarani et al. 2005), yield components and percentage of oil in grain (Mestries et al. 1998, Mokrani et al. 2002, Leon et al. 2003), water status traits and osmotic adjustment under well-watered and water-stressed conditions (Poormohammad Kiani et al. 2007a) and seed-quality traits (Ebrahimi et al. 2008) are identified in sunflower.

Gimenez and Fereres (1986) and Prieto Losada (1992) showed that duration of leaf area is related to rainfed sunflower yield. The yield differences were also associated with variation in total biomass (Alza and Fernandez-Martinez 1997).

According to Flagella et al. (2002), flower bud formation and flowering are critical growth stages for water stress in sunflower. Some researchers have imposed water stress at flower bud formation stage in order to evaluate the effect of water stress on physiological traits such as plant water status and photosynthesis in sunflower (Pankovic et al. 1999, Maury et al. 2000, Tezara et al. 2002). Gimenez and Fereres (1986) and Prieto Losada (1992) showed that leaf area duration is related to rainfed sunflower yield. The yield differences were also associated with variation in total biomass (Alza and Fernandez-Martinez 1997).

The goals of this research are to identify QTLs in recombinant inbred lines (RILs) of sunflower for leaf area at flowering (LAF), leaf area duration (LAD), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), days from sowing to flowering (DSF) and plant height (PH) by using a saturated SSR and AFLP linkage map (Poormohammad Kiani et al. 2007a), in the field condition with well- and partial-irrigated treatments.

5.2 Materials and methods

5.2.1 Plant materials and experimental conditions

The recombinant inbred lines (RILs) used in this research were developed through single seed descent from F2 plants, derived from a cross between PAC2 and RHA266 (Flores Berrios et al. 2000a). Both parental lines are sunflower public inbred lines. RHA266 has been obtained from a cross between wild *H. annuus* and Peredovik by USDA and PAC2 is an INRA-France inbred line from a cross between *H. petiolaris* and 'HA61'(Gentzbittel et al. 1995). RHA266 is more resistant to downy mildew with higher values for yield,1000-grain weight and oil percentage compared with PAC2 (Gentzbittel et al. 1995, Rachid Al-Chaarani et al. 2004). Two independent experiments were undertaken at Teheran University campus of Karaj. Experimental design was randomized complete block with three replications. Seeds of 87 RILs and their two parents were sown in the field under well- and partial-irrigated conditions. Each genotype per replication consisted of one row, 4m long, 50 cm between rows and 25 cm between plants in rows. The distance between replications of well-irrigated and partial-irrigated treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (partial-irrigated), irrigation was controlled and adjusted by the observation of the wilting threshold of the leaves. Partial water deficit was started 45 day after sowing at the stage near flower bud formation and continued up to maturity.

5.2.2 Investigation of agronomic traits

Various traits such as days from sowing to flowering (DSF) and plant height (PH) were measured for RILs and their parental lines at the plot scale (on each line, when 50% of the plants were at flowering stage). Moreover, leaf length (L) and width (W) of all green leaves were measured at flowering stage, and total leaf area at flowering (LAF) was calculated with the formula: LAF= 0.7L×W (Alza and Fernandez-Martinez 1997). Green leaf area of the

plants was determined weekly from flowering to harvest in order to evaluate green leaf area with respect to time. An integral of weekly leaf area was considered as being an estimate of leaf area duration (LAD, m^2 days). At harvest, yield components such as head diameter (HD), 1000 grain weight (TGW) and grain yield per plant (GYP) were measured. Three plants per genotype per condition per replication were randomly selected for evaluation of the mentioned traits. All traits were measured for RILs and their parental lines in each replication for both experiments.

5.2.3 QTL and statistical analysis

Various traits data were analyzed using SAS PROC GLM (SAS Institute Inc. 1996) and SPSS. Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. Genetic correlations between the traits were also determined.

Sunflower map recently constructed by Poormohammad Kiani et al. (2007a) was used for identification of QTLs. The chromosomal locations of QTLs for the studied traits were resolved by composite interval mapping (CIM), using Win QTL Cartographer, version 2.5 using mean values of three replications for each RIL in each conditions (Wang et al. 2005). The control marker number and the window size were 15 and 15 cM, respectively. The percentage of phenotypic variance (R^2) explained by each QTL, the percentage of phenotypic variance (TR^2) explained by the QTLs given all the covariants, were estimated also by Win QTL Cartographer .

5.3 Results

Results of analysis of variance for the agronomic traits are presented in Table 5.1. Results showed significant genotypic effect among RILs for all the studied traits in both well- and partial-irrigated conditions. Correlations among the studied traits are shown in Table 5.2. Positive and significant correlations are observed between LAF and LAD as well as between yield components in both well- and partial-irrigated conditions. The effect of blocks on GYP was significant under both conditions where as it was significant for TGW and DSF under well-irrigated condition. Correlations among the studied traits are shown in Table 2. Positive and significant correlations are observed between LAF and LAD as well as between yield components in both well- and partial-irrigated conditions. Head diameter (HD) is positively correlated with TGW, GYP, LAF and LAD in both conditions (Table 5.2). High significant

and positive correlation between TGW and GYP is observed in both well- and partial-irrigated conditions (Table 5.2). Under well-irrigated condition, the correlation between DSP and LAF is significant. Grain yield per plant (GYP) is also positively correlated with LAF and LAD in both conditions (Table 5.2).

The characteristic of Linkage map has been explained in detail in previous study (Poormohammad Kiani et al. 2007a). This map contains 304 AFLP and 191 SSR markers which are placed in 17 groups (Fig. 5.1). The total map length is 1824.6 cM with mean density of one marker per 3.7 cM, which has a density close to sunflower reference map (3.1) presented by Tang et al. (2002). The map position and characteristics of QTLs associated with studied traits in the field condition under well- and partial-irrigated conditions are presented in Tables 3 and 4, respectively. The bold QTLs are stable across both well- and partial-irrigated conditions for each trait. The QTLs are designated as the abbreviation of the trait followed by '*W*' or '*P*' for well-irrigated or partial-irrigated. The corresponding linkage group and the number of QTLs in the group were also indicated for each QTL. QTLs explained from 4% to 30% and 4% to 49% of the total phenotypic variance of the studied traits (R^2) in well- and partial-irrigated conditions respectively. A large phenotypic variation was also explained when considering cofactors TR^2 (Tables 5.3 and 5.4). Both parental lines contributed to the expression of the different target traits. The percentage of phenotypic variance explained by QTLs of DSF ranged from 5% to 38 %. The most important QTL for DSF (*DSF-P-14-1*) is found on linkage group 14 and explained 38% of the phenotypic variance. The positive alleles for this QTL come form RHA266 (Table 5.4). A total of eleven QTLs are found for PH in well- and partial-irrigated conditions and the phenotypic variance varies from 4% to 27%. The most important QTL for PH is *PH-W-16-1*, which is located on linkage group 16 at 75.61 cM. Individual effect of this QTL on the expression of R^2 is 27%. Two common QTLs are identified for PH in both conditions on linkage groups 10 and 15 (*PH-W-10-1*and *PH-P-10-1; PH-W-15-1 and PH-P-15-1*). Three co-localized QTLs for leaf-related traits, (LAF and LAD), are detected on linkage groups 2, 5 and 12. Both parents (PAC2 and RHA266), contributed in controlling the QTLs of the two mentioned traits in both conditions. Three to six QTLs are found for each yield-related trait in well- and partial-irrigated conditions. QTLs explained from 4% to 30% and 4% to 49% of the total phenotypic variance of yield-related traits (R^2) in well- and partial-irrigated conditions respectively. The major QTL for HD (*HD-P-13-1*) is found on linkage group 13 and explained 37% of the phenotypic variance. The positive alleles for this QTL come form PAC2 (Table 5.4). A total of nine QTLs are identified for grain yield

per plant under both conditions. The most important is *GYP-P-13-1*, in partial-irrigated condition, which is positioned on linkage group 13 at 48.21 cM. The LOD score is 7.7 and individual effect of this QTL on the expression of R^2 is 49%, whereas the TR^2 is 91% (Table 5.4). The major QTL for 1000-grain weight (*TGW-P-11-1*) is identified on linkage group 11. For yield-related traits, the favorable alleles of identified QTLs come from both parents.

5.4 Discussion

Detection of genomic regions associated with leaf-related traits and yield components under well- and partial-irrigated conditions will be useful for marker-based approaches to improve aforementioned traits in sunflower. In our experiments, variation was observed for all studied traits in both well- and partial-irrigated conditions. Genetic variation for some agronomic traits, are also reported by Rachid-Al-Chaarani et al. (2004). The QTLs detected in our research revealed that several putative genomic regions are involved in the expression of the studied agronomic traits under well- and partial-irrigated conditions. The percentage of phenotypic variance explained by the QTLs (R^2) ranged from 4% to 49%. The positive and negative signs of additive effect at the different loci indicate the contribution of both parental lines and confirm the transgressive segregation observed at the phenotypic level (Table 5.3 and 5.4). Transgressive segregation would be the result of the accumulation of favourable alleles coming from different parental lines. Transgressive segregation was also reported for other traits in the same population (Poormohammad Kiani et al. 2007a; Ebrahimi et al. 2008).

In several cases, one QTL was identified to be associated with more than one trait. Under well-irrigated condition, co-localization occurs for QTLs of HD and TGW on linkage group 2 (*HD-W-2-1 and TGW-W-2-1*). Significant and positive correlation between HD and TGW (Table 5.2), is justified by the effects of their co-localized QTLs (Table 5.3). Co-localized QTLs for HD and LAD are identified on linkage group 5 (*HD-W-5-1 and LAD-W-5-1*). Under partial-irrigated condition, co-localized QTLs are also identified on linkage group 2 for TGW and GYP (*TGW-P-2-1 and GYP-P-2-1*), linkage group 4 for LAD and GYP (*LAD-P-4-1 and GYP-P-4-1*), Linkage group 10 for PH and LAD (*PH-P-10-1 and LAD-P-10-1*) and linkage group 13 for HD and GYP (*HD-P-13-1 and GYP-P-13-1*) (Table 5.4). Identification of co-localized QTLs, influencing several traits shows that genes controlling the traits are in the same genomic region. The correlation among different traits as well as their co-localization observed is relevant to effort for manipulating multiple traits simultaneously (Poormohammad Kiani 2007b). The locations of QTLs identified in the present research for different traits when compared with those controlling some agronomic traits reported by Rachid-Al-Chaarani

et al. (2004), revealed 3 co-localized QTLs. The co-localized QTLs are observed on linkage group 5 (*TGW-P-5-1* and 1000 grain weight), linkage group 7 (*PH-P-7-1* and stem diameter*; LAF-W-7*-1 and sowing-to-flowering date).

Several QTLs for LAF and LAD are detected on different linkage groups (Fig 5.1). The effect of leaf area duration (LAD) on grain yield per plant is more important than leaf area at flowering (LAF) (Poormohammad Kiani 2007b). This indicates that maintaining green leaf area longer after anthesis is important for a high yield production under both water treatments. It has been reported that maintaining green leaf area and consequently a longer duration of photosynthetic activity has contributed to increased yield in most of major crops (Russel 1991, Evans 1993). Genetic differences in photosynthetic duration have been also associated with a longer grain filling duration and higher yield in maize (Richards 2000).

The QTL detected for grain yield per plant (*GYP-W-3-1*) co-localized with a QTL controlling stearic acid content (*4.SA.3.1*) identified by Ebrahimi et al.(2008). Some QTLs detected in our research co-localized also with QTLs controlling germination and seedling development identified by AL-chaarani et al. (2005). The co-localized QTLs are identified on linkage group 1 (*DSF-W-1-2* and shoot dry weight), linkage group 5 (*TGW-P-5-1* and percentage or normal seedlings), linkage group 6 (*LAF-W-6-1* and shoot length) and linkage group 16 *(PH-W-16-1* and root length).

Some QTLs detected in our research co-localized with QTLs controlling plant water status and osmotic adjustment in RILs of sunflower under two water treatments in greenhouse condition reported by Poormohammad Kiani et al. (2007a). The co-localized QTLs are also identified on linkage group 7 (*LAF-W-7-1* with leaf water potential and relative water content) on linkage group10 (*LAF-W-10-1* with relative water content and on linkage group11 (*LAD-W-11-1* with osmotic potential).

Eleven QTLs are in common across two well and partial-irrigated conditions for the studied traits. Stable QTLs, contain genes controlling the trait in both conditions. Our results do highlight the importance of the role of linkage groups 9 and 12 for controlling leaf-related traits. The most important are, on linkage group 9 (*LAF-P-9-1 and LAD-W-9-1*) and 12 (*LAD-W-12-1, LAF-W-12-1* and *LAF-P-12-1*). Detailed characterization of these genomic regions through the development and evaluation of near-isogenic lines will lead to an improved understanding and might set the stage for the positional cloning of genes-related to them. The relatively low number of RILs used in current research may have a negative influence on the accuracy of the calculated QTL effects, the ability to detect QTLs with small effects and R^2

may be overestimation (Beavis et al. 1994, Bachlava et al. 2008). This was, to some degree, compensated by the higher precision of the phenotyping and the use of our saturated map.

Table 5.1: Analysis of variance (mean squares) for days from sowing to flowering (DSF), plant height (PH), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), leaf area at flowering (LAF) and leaf area duration (LAD) in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated (WI) and partial-irrigated (PI) conditions.

	df	Conditions	DSF	PH	HD	TGW	GYP	LAF	LAD
RILs	86	WI	34.56**	1212.70**	54.41**	1377.1**	1917.25**	13.59**	65.79**
		PI	28.81**	885.48**	43.45**	1067.2**	1340.72 **	8.30**	40.19**
Blocks	2	WI	30.27**	640.60NS	1.70 NS	428**	704.02*	2.50 NS	26.07NS
		PI	20.25 NS	226.59NS	9.94NS	194NS	1142.34 **	5.30 NS	12.22NS
Error	172	WI	6.32	218.01	3.76	77	159.92	4.40	21.58
		PI	8. 60	145.10	5. 50	86	188.01	3.70	17.97

*, **: significant at 0.05 and 0.01 probability level, respectively. NS: non-significant.

Table 5.2: Simple correlation coefficients (Pearson) days from sowing to flowering (DSF), plant height (PH), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), leaf area at flowering (LAF) and leaf area duration (LAD) in a population of sunflower recombinant inbred lines (RILs) grown under well-irrigated (WI) and partial-irrigated (PI) conditions.

	DSF		PH		HD		TGW		GYP		LAF	
	WI	PI	WI	PI	WI	PI	WI	PI	WI	PI	WI	PI
PH	0.33**	0.29**										
HD	-0.09NS	-0.21**	0.33**	0.21**								
TGW	-0.25**	-0.24**	0.25**	0.18**	0.78**	0.80**						
GYP	-0.14*	-0.16**	0.31**	0.29**	0.83**	0.83**	0.79**	0.77**				
LAF	0.15*	0.12 NS	0.45**	0.36**	0.19**	0.25**	0.19**	0.24**	0.23**	0.24**		
LAD	0.10 NS	0.12 NS	0.46**	0.40**	0.22**	0.30**	0.24**	0.30**	0.30**	0.34**	0.65**	0.56**

*, **: significant at 0.05 and 0.01 probability level, respectively. NS: non-significant.

Table 5.3: QTLs detected for days from sowing to flowering (DSF), plant height (PH), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), leaf area at flowering (LAF) and leaf area duration (LAD) under well -irrigated condition.

Trait	QTL	Linkage group	Marker On the left of LOD peak	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2b}
DSF	*DSF-W-1-1*	1	ORS959	68.21	3.7	-1.06	0.07	0.45
	DSF-W-1-2	1	E32M61_10	43.01	3.1	1.26	0.08	0.53
	DSF-W-2-1	2	E40M62_17	65.81	5.5	1.75	0.17	0.54
PH	*PH-W-7-1*	7	E38M50_2	45.21	6.6	-7.72	0.13	0.63
	PH-W-8-1	8	ORS329	86.91	4.3	-5.72	0.06	0.64
	PH-W-10-1	10	E40M62_15	91.41	5	8.38	0.11	0.67
	PH-W-10-2	10	HA3039	172.71	3.7	-7.39	0.07	0.60
	PH-W-13-1	13	ORS511	54.71	10.1	10.60	0.16	0.60
	PH-W-15-1	15	ORS401	34.81	4.9	6.46	0.06	0.67
	PH-W-16-1	16	E40M50_1	75.61	6.7	-25.33	0.27	0.84
HD	*HD-W-2-1*	2	E35M60_4	100.61	4.1	-0.88	0.04	0.63
	HD-W-5-1	5	SSL231	25.61	5.8	1.41	0.10	0.60
	HD-W-8-1	8	ORS894_1	86.21	3.8	-1.13	0.04	0.64
	HD-W-10-1	10	ORS591	101.21	3.7	-1.43	0.05	0.63
	HD-W-15-1	15	HA1837	46.41	3	-1.08	0.04	0.64
TGW	*TGW-W-2-1*	2	E35M60_4	100.61	11	-1.03	0.18	0.85
	TGW-W-8-1	8	E33M48_5	30.61	6.5	-0.67	0.07	0.74
	TGW-W-10-1	10	E35M48_8	80.41	6	-1.02	0.10	0.73
GYP	*GYP-W-3-1*	3	ORS657	54.31	5.6	6.18	0.04	0.46
	GYP-W-5-1	5	ORS533	84.91	7.7	6.12	0.04	0.48
	GYP-W-6-1	6	ORS381	30.61	6	3.56	0.04	0.46
	GYP-W-8-1	8	ORS243	57.31	5.9	-6.90	0.04	0.49
	GYP-W-10-1	10	HA928	24.21	8.8	-16.62	0.30	0.53
	GYP-W-13-1	13	ORS630	51.91	6.4	12.76	0.14	0.58
LAF	*LAF-W-1-1*	1	ORS509	57.91	4	-1.84	0.11	0.45
	LAF-W-2-1	2	ORS229	80.11	5	-0.39	0.04	0.52
	LAF-W-5-1	5	ORS1024_1	27.31	6.5	1.60	0.04	0.41
	LAF-W-6-1	6	SSL66_1	62.21	3.9	-0.55	0.04	0.39
	LAF-W-7-1	7	E35M60_22	60.81	4.8	-0.80	0.11	0.41
	LAF-W-10-1	10	E37M49_5	69.61	3.8	-0.66	0.07	0.33
	LAF-W-12-1	12	E40M59_8	68.90	4.5	0.8	0.11	0.61
LAD	*LAD-W-2-1*	2	E33M48_4	94.51	4.1	-0.33	0.04	0.58
	LAD-W-5-1	5	SSL231	25.61	6.7	0.64	0.05	0.57
	LAD-W-8-1	8	E36M59_13	43.31	6.7	-1.04	0.10	0.60
	LAD-W-9-1	9	E36M59_17	73.51	6.4	0.91	0.10	0.58
	LAD-W-11-1	11	E38M50_24	72.51	3.9	-0.70	0.06	0.57
	LAD-W-12-1	12	E35M61_2	5.01	4.3	0.92	0.09	0.59

The QTLs are designated as the abbreviation of the trait followed by `*W* ` for well-irrigated. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it.
a Percentage of individual phenotypic variance explained. Value determined by Win QTL Cart., version 2.5
b Percentage of phenotypic variance explained by the QTLs given all the covariants

Table 5.4: QTLs detected for days from sowing to flowering (DSF), plant height (PH), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), leaf area at flowering (LAF) and leaf area duration (LAD) under partial -irrigated condition.

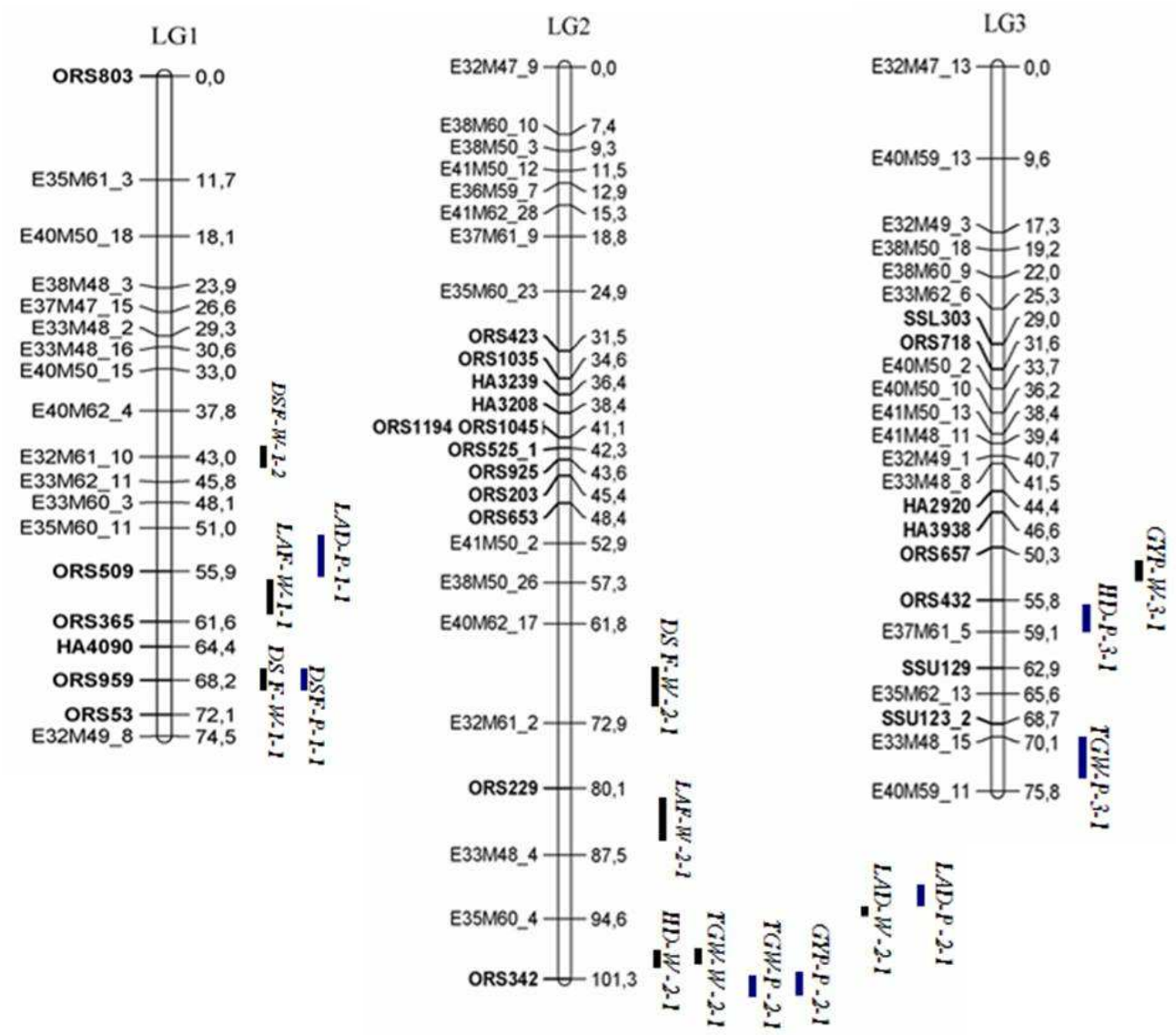
Trait	QTL	Linkage group	Marker On the left of LOD peak	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2b}
DSF	***DSF-P-1-1***	**1**	**ORS959**	**68.21**	**4.5**	**-1.34**	**0.05**	**0.53**
	DSF-P-7-1	7	ORS1041	20.71	4	-1.17	0.06	0.50
	DSF-P-14-1	14	E37M47_24	101.01	8.1	-2.60	0.38	0.67
	DSF-P-17-1	17	ORS31_3	2.01	4.1	1.13	0.08	0.59
PH	*PH-P-4-1*	4	E41M62_24	77.41	4	-6.60	0.10	0.53
	PH-P-7-1	7	E40M62_1	56.31	3	-4.33	0.04	0.42
	PH-P-10-1	**10**	**HA3039**	**172.71**	**4.3**	**-4.82**	**0.04**	**0.41**
	PH-P-15-1	**15**	**ORS401**	**40.81**	**4.6**	**4.60**	**0.05**	**0.43**
HD	*HD-P-3-1*	3	ORS432	57.81	3.6	1.14	0.06	0.50
	HD-P-8-1	**8**	**ORS894_1**	**86.81**	**5.2**	**-1.05**	**0.04**	**0.55**
	HD-P-10-1	**10**	**ORS591**	**101.21**	**5.1**	**-1.59**	**0.08**	**0.52**
	HD-P-13-1	13	ORS630	48.21	5	3.29	0.37	0.79
TGW	***TGW-P-2-1***	**2**	**E35M60_4**	**100.61**	**5.3**	**-1.03**	**0.07**	**0.74**
	TGW-P-3-1	3	E33M48_15	70.11	5.8	1.00	0.10	0.71
	TGW-P-5-1	5	E41M62_30	19.51	3.9	0.72	0.06	0.71
	TGW-P-6-1	6	E33M60_1	6.91	4.4	-0.93	0.08	0.72
	TGW-P-10-1	**10**	**E35M48_8**	**82.41**	**3.6**	**-0.99**	**0.09**	**0.74**
	TGW-P-11-1	11	ORS733	8.01	6.4	-1.62	0.22	0.93
GYP	*GYP-P-2-1*	2	E35M60_4	98.61	8.4	-6.31	0.04	0.54
	GYP-P-4-1	4	E41M59_3	80.61	8	-4.07	0.04	0.54
	GYP-P-13-1	**13**	**ORS630**	**48.21**	**7.7**	**18.00**	**0.49**	**0.91**
LAF	*LAF-P-5-1*	5	ORS523_1	85.31	5	0.70	0.13	0.59
	LAF-P-9-1	9	HA477	42.81	5.4	0.50	0.10	0.61
	LAF-P-11-1	11	E35M60_21	19.61	3.4	-0.58	0.08	0.54
	LAF-P-12-1	**12**	**E40M59_8**	**68.91**	**5.1**	**1.01**	**0.16**	**0.63**
LAD	*LAD-P-1-1*	1	E35M60_11	53.01	3.1	0.51	0.04	0.57
	LAD-P-2-1	**2**	**E33M48_4**	**93.51**	**3.5**	**-0.70**	**0.04**	**0.56**
	LAD-P-4-1	4	E41M59_3	82.61	4.6	-0.62	0.06	0.55
	LAD-P-5-1	**5**	**SSL231**	**27.01**	**4**	**0.30**	**0.04**	**0.54**
	LAD-P-10-1	10	HA3039	170.71	6.2	-1.20	0.15	0.55

The QTLs are designated as the abbreviation of the trait followed by `*P* ` for partial-irrigated. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it. The bold QTLs are in common, presented as stable QTLs, across both well- and partial-irrigated condition for each trait.

a Percentage of individual phenotypic variance explained. Value determined by Win QTL Cart., version 2.5

b Percentage of phenotypic variance explained by the QTLs given all the covariants

Fig. 5.1 Molecular linkage groups of sunflower map presenting QTLs for days from sowing to flowering (DSF), plant height (PH), head diameter (HD), 1000 grain weight (TGW), grain yield per plant (GYP), leaf area at flowering (LAF) and leaf area duration (LAD). The positions of QTLs are shown on the right side of the linkage groups. Bars represent intervals associated with the QTLs.



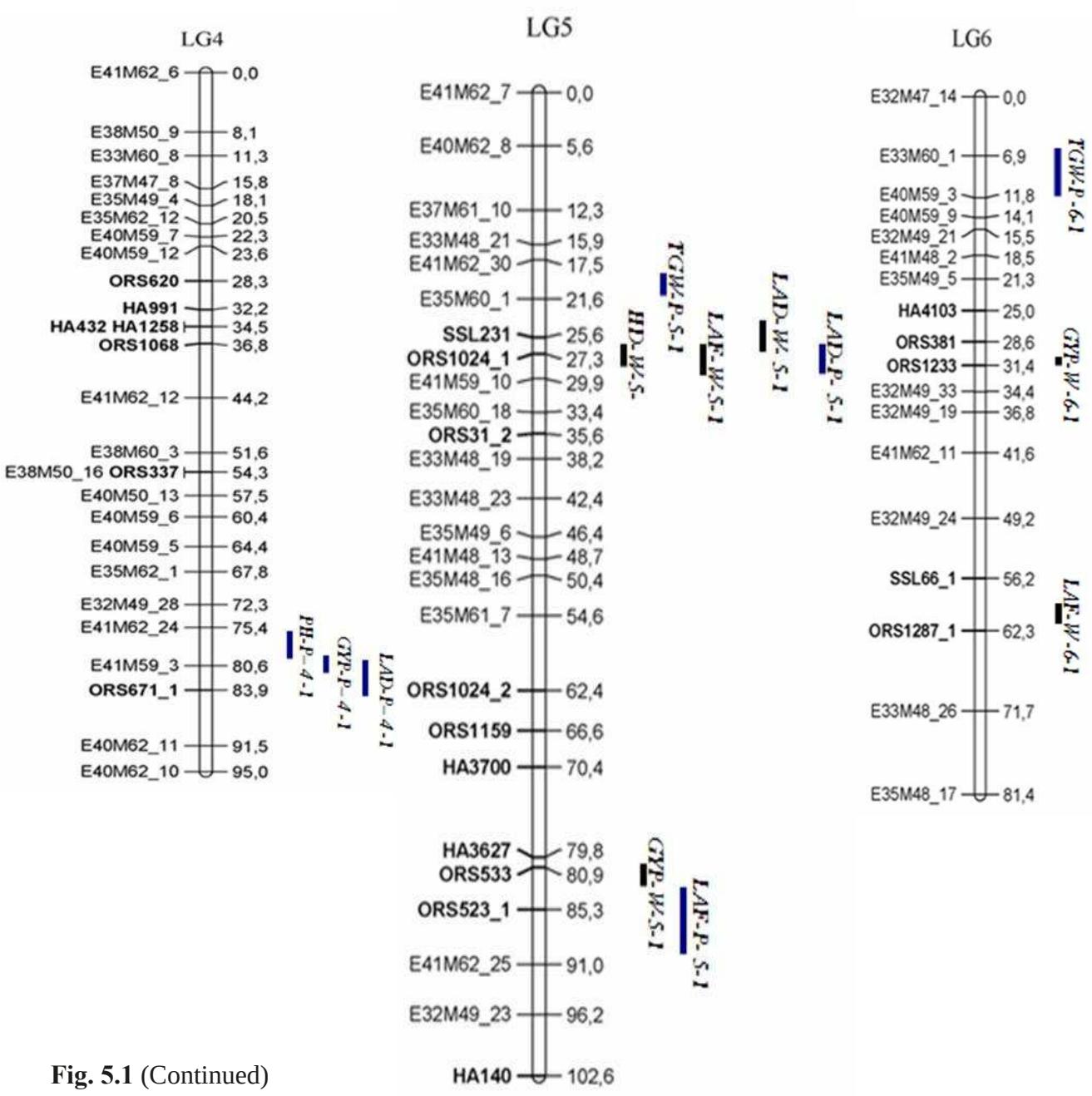


Fig. 5.1 (Continued)

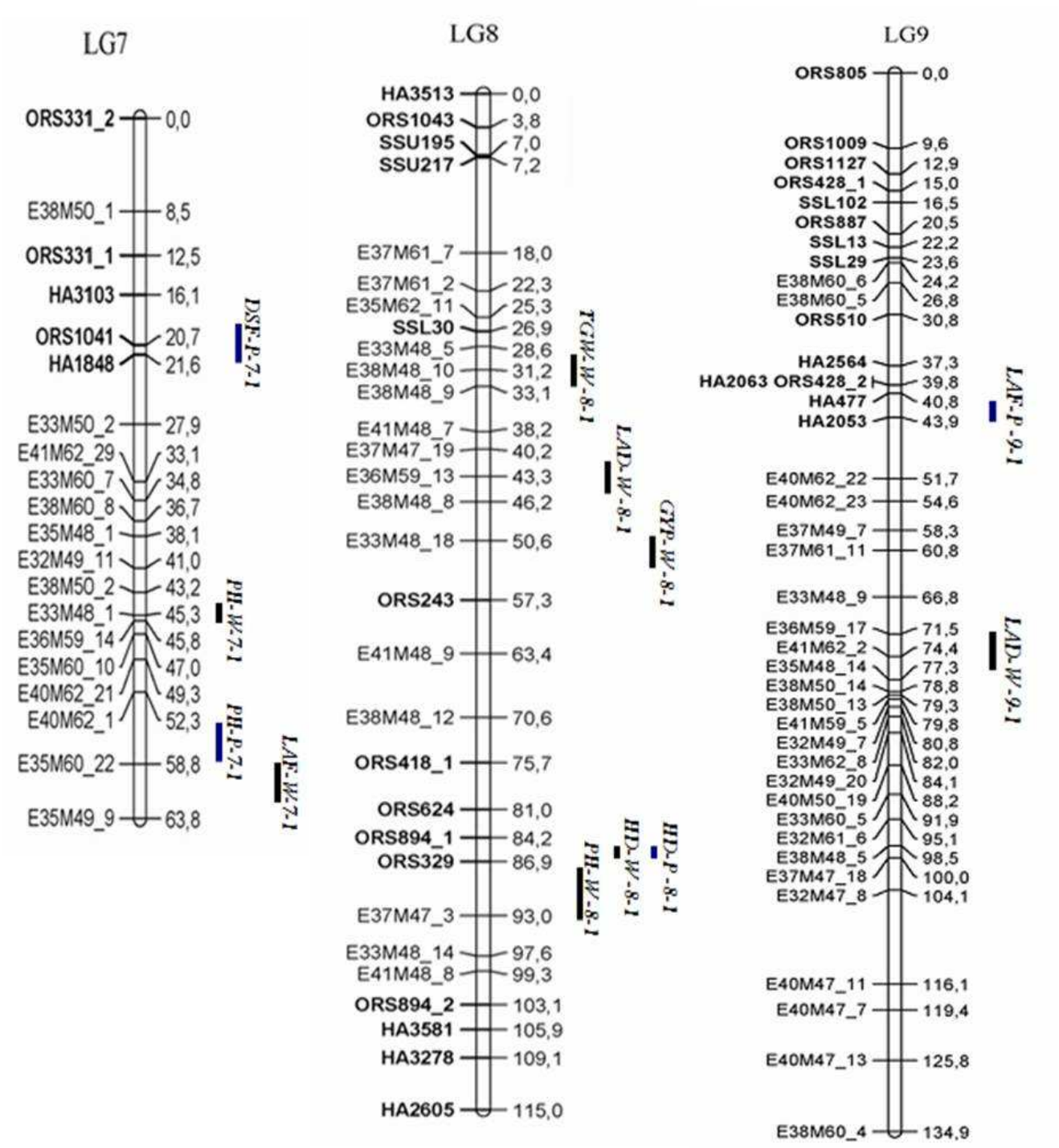


Fig. 5.1 (Continued)

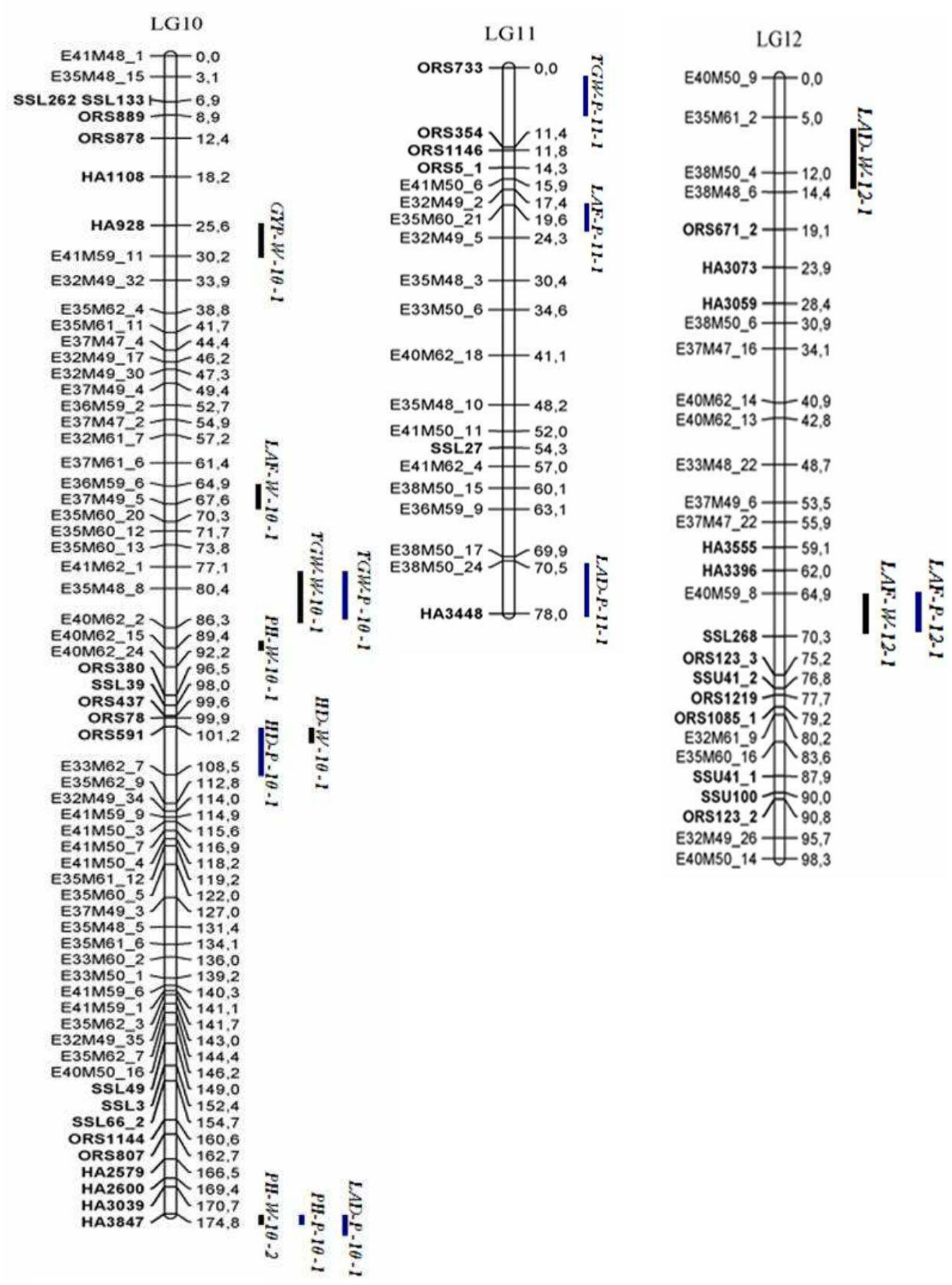


Fig. 5.1 (Continued)

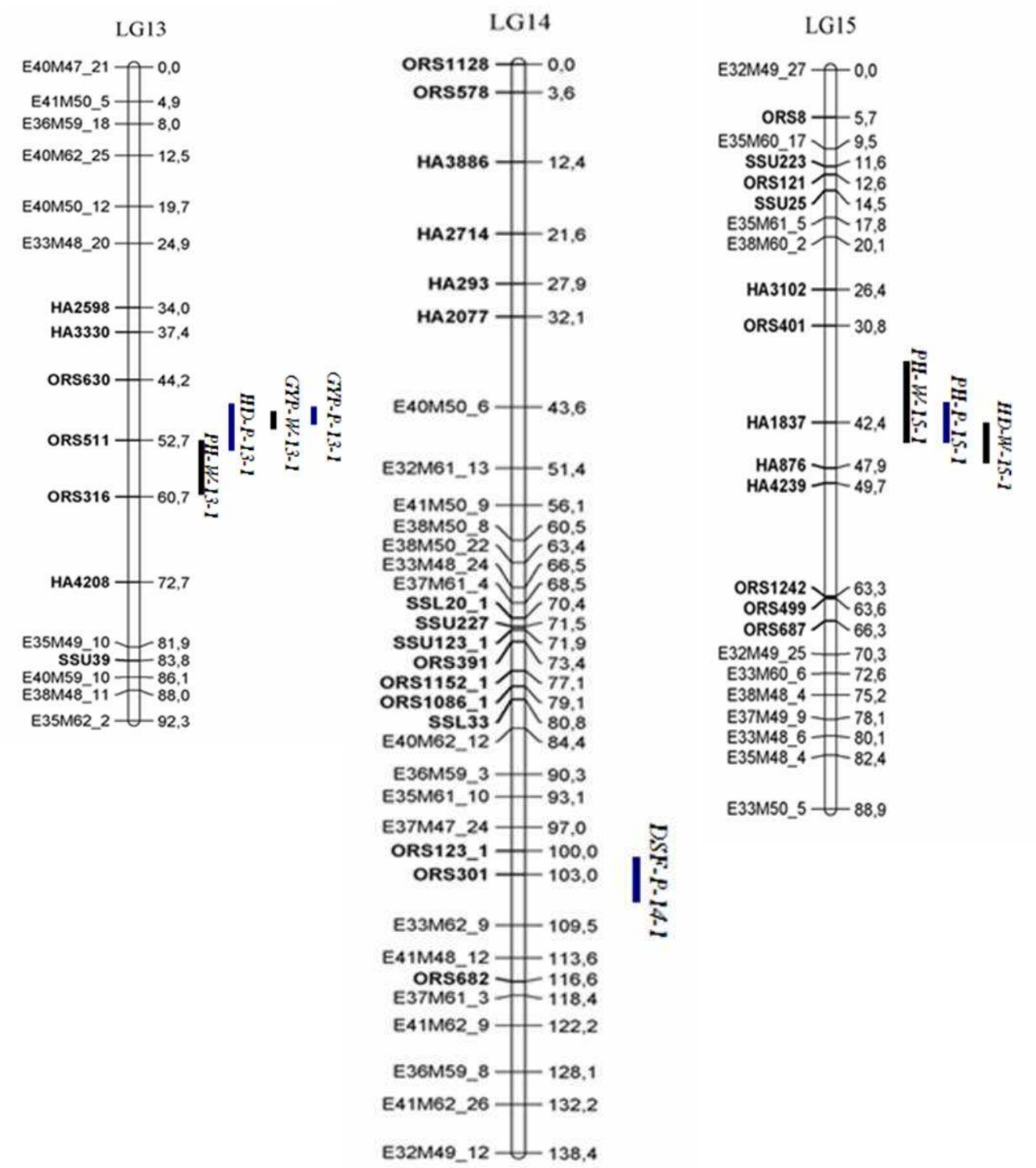


Fig. 5.1 (Continued)

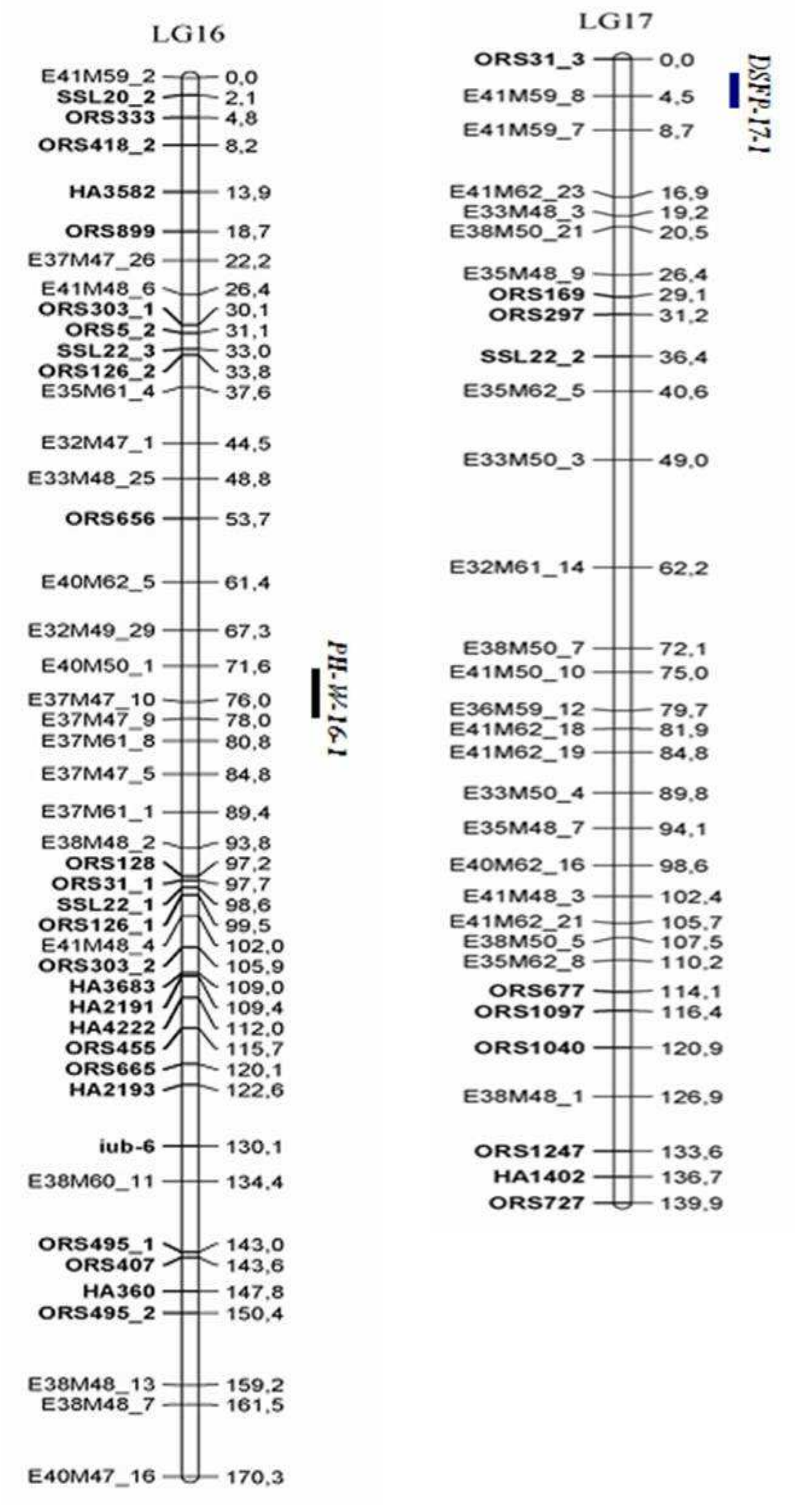


Fig. 5.1 (Continued)

Chapter 6

QTL mapping of morpho-physiological traits in recombinant inbred lines of sunflower under different sowing dates

Abstract

The objectives of the current research are to map QTLs controlling different traits such as days from sowing to flowering, plant height, yield and leaf-related traits under different sowing dates (temperature regimes) in a population of recombinant inbred lines (RILs). Two field experiments consisted of a randomized complete block design with three replications under normal- and late-sowing conditions. Several QTLs for the studied traits were identified on different linkage groups. Three to 8 QTLs were found, depending on trait and growth conditions, and the percentage of phenotypic variance explained by the QTLs ranged from 4% to 38%. Common chromosomic region on linkage group 8 was linked to an SSR marker ORS243 for grain yield per plant and head weight. Co-location of QTLs for mentioned traits with QTLs associated with seed-quality, plant water status and osmotic adjustment, agronomic, germination and seedling development identified in previous studies was observed.

6.1 Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important sources of vegetable oil in the world (Flores Berrios et al. 2000). Sowing date (temperature regimes), drought and salinity constitute some of the most serious limitations to crop growth and productivity (Poormohammad Kiani et al. 2007a).

It has been demonstrated that the sowing date may have a bigger effect on yield, and quality of sunflower seed (Jasso de Rodriguez et al. 2002). Studies have been carried out on sowing dates in sunflower revealed that growth, yield and oil is reduced when normal sowing dates are delayed (Robinson 1970, Unger 1980, Beard and Geng 1982, Miller et al. 1984, Thompson and Heenan 1994, Jasso de Rodriguez et al. 2002, Vega and Hall 2002, Gupta and Rathore 1994). Planting time is a crucial factor for obtaining desirable yield (Qasim et al. 2008). Both sowing date and genotype x sowing date interaction effect on yield mostly involved the variation of attributes and processes expressed postanthesis. Biomass differences between planting dates were the dominant determinant of the sowing effect on yield (Vega and Hall 2002). Genotype and sowing date (temperature regime) can modify the oleic/linoleic ratio (Harris et al., 1978, Champolivier and Merrien 1996, Lagravère et al., 2000).

Yield in sunflower, as in all other crops, depends on many characters, especially yield components which are controlled by several genes, their effects being modified with

environment (Fick and Miller 1997). Heritability for yield is relatively low compared to other agronomic traits (Fick 1978). Polygenic inheritance patterns are reported for sowing to flowering date in sunflower (Machacek 1979). Identification of QTLs for leaf, yield-related and various traits using a molecular marker map, not only allows genetic dissection of mentioned traits, but also expedites transfer of QTLs through a process known as marker-assisted selection (Foolad et al. 2oo3). The availability of locus-specific molecular markers for yield-related and leaf- related traits would be of great benefit, providing the potential for more rapid screening of beneficial combinations of alleles in breeding programmes. Sunflower has been considered for construction of molecular genetic maps and several genetic maps are available (Gentzbittel et al. 1995, Berry et al. 1995, Jan et al. 1998, Gentzbittel et al. 1999, Gedil et al. 2001). Molecular genetic-linkage map based on 459 SSR, has been constructed by Tang et al. (2002), which is the first reference map of sunflower based on single- or low-copy public SSR markers. Genetic map of our department (Poormohammad Kiani et al. 2007a) contains 304 AFLP and 191 SSR markers with the total length of 1824.6 cM and a mean density of one locus every 3.7 cM. QTLs controlling important traits such as physiological parameters (Hervé et al. 2001), agronomic traits (Rachid Al-Chaarani et al. 2004), grain weight per plant (GWP), 1,000-grain weight (TGW), percentage of oil in grain (POG) and STF date (Mestries et al. 1998, Mokrani et al. 2002, Leon et al. 2003), water status traits and osmotic adjustment under well-watered and water-stressed conditions (Poormohammad Kiani et al. 2007a) and seed-quality traits (Ebrahimi et al. 2008) are detected in sunflower.

The objective of current research is to map QTLs for leaf-related traits such as dried leaf number (DLN), leaf number (LN), leaf area at flowering (LAF) and leaf area duration (LAD), yield-related traits such as head number (HN), head diameter (HD), head weight (HW), 1000 grain weight (TGW), grain yield per plant (GYP), biomass (BIO) and other traits such as days from sowing to flowering (DSF) and plant height (PH) in recombinant inbred lines in normal- and late-sowing dates by using recently saturated SSR and AFLP linkage map Poormohammad Kiani et al. (2007a). The formulation of breeding strategies to improve yield at late-sowing dates would be facilitated by identification of QTLs involved in processes underlying the observed yield reduction.

6.2 Materials and methods

6.2.1 Plant materials and experimental conditions

The recombinant inbred lines (RILs) used in this research were developed through single seed descent from F2 plants, derived from a cross between PAC2 and RHA266 (Flores Berrios et al. 2000). Two independent experiments were undertaken at Teheran University campus of Karaj. Experimental design was randomized complete block with three replications. Seeds of 87 RILs and their two parents were sown on May and July, as a normal- and late-sowing, respectively. Each genotype per replication consisted of one row, 4m long, 50 cm between rows and 25 cm between plants in rows.

6.2.2 Traits measurement

DSF (days from sowing to flowering) and PH (plant height) were measured for RILs and their parental lines at the plot scale (on the line, when 50% of the plants were at the flowering stage). Leaf-related traits such as LN (leaf number), DLN (dried leaf number), were recorded at anthesis, and leaf length (L) and width (W) of all green leaves were measured at flowering stage, and total leaf area at flowering (LAF) was calculated with the formula: LAF= 0.7L×W (Alza and Fernandez-Martinez 1997). Green leaf area of the plants was determined weekly from flowering to harvest in order to evaluate green leaf area with respect to time. An integral of weekly leaf area was considered as being an estimate of leaf area duration (LAD, m^2 days). At harvest, yield components such as HD (head diameter), HW (head weight), HGW (100 grain weight), GYP (grain yield per plant) and BIO (biomass) were measured. By the way, HN (head number) was measured when 50% of the plants were at the anthesis. Three plants per genotype per condition per replication were randomly chosen for evaluation of the mentioned traits. All traits measured for RILs and their parental lines in each replication for both experiments.

6.2.3 QTL and statistical analysis

Various traits data were analyzed using SAS PROC GLM (SAS Institute Inc. 1996) and SPSS. Statistical analysis was carried out in order to determine the main effect of RILs for the studied traits. Genetic correlations between the traits were also determined.

Sunflower map recently constructed by in our department Poormohammad Kiani et al. (2007a) was used for identification of QTLs. The chromosomal locations of QTLs for the

studied traits were resolved by composite interval mapping (CIM), using Win QTL Cartographer, version 2.5 (Wang et al. 2005) using mean values of three replications for each RIL in each conditions. The control marker number and the window size were 15 and 15 cM, respectively. The percentage of phenotypic variance (R^2) explained by each QTL, the percentage of phenotypic variance (TR^2) explained by the QTLs given all the covariants, were estimated by Win QTL Cartographer (Wang et al., 2005).

6.3 Results

6.3.1 Analysis of variance and phenotypic variation

Results of analysis of variance for the agronomic traits are presented in Table 6.1. Results showed significant genotypic effect among RILs for all the studied traits in both well- and partial-irrigated conditions. Phenotypic correlations among different traits in two conditions are presented in Table 6.2. Positive and significant correlations were observed between grain yield per plant and other components of yield such as HD, HW, TGW and BIO in both conditions. Positive and significant correlations were observed among leaf-related traits whereas LAF and LAD were not correlated with DLN in both normal and late-sowing conditions (Table 2).

6.3.2 Linkage map and QTL mapping

The characteristic of Linkage map has been explained in detail in previous study (Poormohammad Kiani et al. 2007a). This map contains 304 AFLP and 191 SSR markers which are placed in 17 groups (Fig. 6.1). The total map length is 1824.6 cM with mean density of one marker per 3.7 cM, which has a density close to sunflower reference map (3.1) presented by Tang et al. (2002). The map position and characteristics of the QTLs associated with various traits such as DSF, PH and MBN, leaf-related traits and yield-related traits are summarized in Tables 6.3, 6.4, and 6.5 in normal- and late-sowing conditions. The bold QTLs are stable across both well- and partial-irrigated conditions for each trait. The QTLs are designated as the abbreviation of the trait followed by '*N*' or '*L*' for normal-sowing or late-sowing. The percentage of phenotypic variance explained by QTLs (R^2) ranged from 4% to 38%, but a large phenotypic variation was also explained when considering cofactors 'TR^2'. For an easier overview of overlapping QTLs between traits and growth conditions, an image of all QTL regions is presented in Fig. 6.1. Both parental lines contributed to the expression of

the different target traits and positive or negative additive effects are explained. Overlapping QTLs were detected for different traits on different linkage groups (Fig. 6.1).

6.3.2.1 QTLs for DSF and PH

The most important QTL for DSF (*DSF-L-14-1*) was found on linkage group 14 and explained 18% of the phenotypic variance. The positive alleles for this QTL come form RHA266 (Table 6.3). Two common QTLs were identified for PH in both conditions on linkage groups 10 and 13 (*PH-N-10-1*and *PH-L-10-2; PH-N-13-1 and PH-L-13-1*).

6.3.2.2 QTLs controlling leaf-related traits

Several QTLs were found for leaf-related traits, among which 6 were common across both conditions (Table 6.4). The largest amount of phenotypic variance explained by a QTL of DLN was 20% (*DLN-L-4-1*). Among 9 detected QTLs for LN under normal- and late-sowing conditions, two QTLs were in common (*LN-N-4-1* and *LN-L-4-1*; *LN-N-10-1*and *LN-L-10-1*). The most important QTL for For LN was *LN-L-10-1*, which is located on linkage group 10 at 24.21 cM. The LOD score was 3.9 and individual effect of this QTL on the expression of R^2 was 13%. Six QTLs controlling leaf-related traits were identified on the different regions of linkage group 9. Among 10 QTLs for LAF, the positive alleles for 8 QTLs come from RHA266 and for 2 QTLs they come from PAC2 in normal- and late-sowing conditions. Six and 4 QTLs were found for LAD in normal- and late-sowing condition, respectively (Table 6.4). PAC2 and RHA266 contributed at QTLs controlling leaf-related traits in both conditions.

6.3.2.2 QTLs controlling yield-related traits

The percentage of phenotypic variance explained by QTLs controlling yield-related traits ranged from 4% to 38% (Table 6.5). A total of 8 QTLs were identified for HN under both conditions the phenotypic variance explained from 4% to 27%. The most important was *HN-L-10-1*, in late sowing condition, which is situated on linkage group 10 at 32.21 cM. The LOD score was 9.2 and individual effect of this QTL on the expression of R^2 was 27%, whereas the TR^2 was 67%. PAC2 alleles increased head number. The largest amount of phenotypic variance explained by a QTL of HW was 38%, whereas the TR^2 was 92% (*HW-N-13-1*), which is linked to SSR marker ORS630. Seven QTLs were identified for TGW and the percentage of phenotypic variance explained by these QTLs ranged from 4% to 18%. Six and 3 QTLs were detected for BIO in late- and normal-sowing conditions, respectively. For GYP,

the positive alleles for 3 QTLs come from RHA266 and for 7 QTLs they come from PAC2. The most important was *GYP-N-10-1* with 30% R^2 and LOD 8.8.

6.4 Discussion

6.4.1 Phenotypic variation among genotypes

Detection of genomic regions associated with leaf-related traits and yield components under normal- and late-sowing conditions will be useful for marker-based approaches to improve aforementioned traits in sunflower. In our experiments, variation was observed for all studied traits in both conditions. Genetic variation for some agronomic traits, are also reported by Rachid Al-Chaarani et al. (2004). The QTLs detected in our research revealed that several putative genomic regions are involved in the expression of the studied agronomic traits under both conditions. The percentage of phenotypic variance explained by the QTLs (R^2) ranged from 4% to 38%. The positive and negative signs of additive effect at the different loci indicate the contribution of both parental lines and confirm the transgressive segregation observed at the phenotypic level (Table 6.3, 6.4 and 6.5). Transgressive segregation would be the result of the accumulation of favourable alleles coming from different parental lines. Transgressive segregation was also reported for other traits in the same population (Poormohammad Kiani et al. 2007a; Ebrahimi et al. 2008).

6.4.2 QTLs controlling DSF and PH

QTLs controlling DSF and PH in normal- and late-sowing conditions in the current research which are overlapped with QTLs for some traits identified in previous studies are summarized in Table 6.6. In our research most of the QTLs for DSF and PH were co-located with the seed-quality (Tang et al. 2006, Ebrahimi et al. 2008), plant water status and osmotic adjustment (Poormohammad Kiani et al. 2007a), agronomic (Rachid Al-Chaarani et al. 2004, Poormohammad Kiani 2007b) and germination and seedling development (Rachid Al-Chaarani et al. 2005) QTLs. The QTL for DSF (*DSF-N-1-1*; associated with marker ORS959) overlapped with oil and oleic acid content. Therefore, change in days from sowing to flowering, due to sowing date, will affect the levels of oil content in sunflower. Rachid Al-Chaarani et al. (2004) observed a non-significant correlation between sowing to flowering time and oil content. In contrast, Leon et al. (2003) reported a significant negative correlation between days to flowering and seed-oil concentration in areas with short growing seasons

(Fargo), and they also detected two QTLs that were associated with both seed oil concentration and days to flowering. Duration of growth from sowing to physiological maturity was reduced by 15 days when sowing time was delayed (Thompson and Heenan 1994). In several cases, one QTL was identified to be associated with more than one trait (Fig 6.1). Overlapped QTLs were located on: linkage group 13 (linked to ORS511) for PH, HD and GYP (*PH-L-13-1*, *PH-N-13-1*, *HD-L-13-1* and *GYP-L-13-1*), linkage group 14 (linked to ORS301) for DSF and BIO (*DSF-L-14-1* and *BIO-L-14-1*). Genetic correlations between the studied traits confirm also these relations (Table 6.2). Significant and positive correlation between DSF and BIO and between PH and GYP (Table 2) is justified by the effects of their overlapped QTLs (Table 6.3 and 6.5).

6.4.3 QTLs controlling leaf-related traits

QTLs associated with leaf-related traits in normal- and late-sowing conditions in our research which are overlapped with QTLs for some traits identified in previous studied are presented in Table 7. Some QTLs detected for LAF in our research overlapped with QTLs controlling plant water status and osmotic adjustment in RILs of sunflower under two water treatments in greenhouse condition reported by Poormohammad Kiani et al. (2007a). The overlapping QTLs are located on linkage group 7 (*LAF-N-7-1* with leaf water potential and relative water content; linked to E35M60_22) and 10 (*LAF-N10-1* with relative water content; linked to E37M49_5) (Table 6.7). Overlapped QTLs are identified on: linkage group5 (linked to marker SSL231) for leaf area duration (LAD) and oleic acid content (*LAD-N-5-1 and 1.OA.5.1*), linkage group6 (linked to marker E32M49_19) for LAD and oil content (*LAD-L-6-1 and 2.OC.6.1*) and linkage group12 (linked to marker E35M61_2) for LAD and stearic acid content (*LAD-N-12-1 and 2.SA.12.1*) were detected (Table 6.7). Canopy stay green (defined as green leaf area duration) proved to be associated with adaptation to late-sowing dates. This indirect selection index appears to be a more reliable aspect for use in breeding for adaptation to late sowings than some other genotype characteristics associated to yield (Vega and Hall 2002). Variations in BIO and harvest index were strongly linked to the amount of intercepted radiation during grain filling which, in turn, was associated to LAD (Vega and Hall 2002). The effect of leaf area duration (LAD) on grain yield per plant is more important than leaf area at flowering (LAF) (Poormohammad Kiani 2007b). This indicates that maintaining green leaf area longer after anthesis is important for a high yield production under both water treatments. It has been reported that maintaining green leaf area and consequently a longer

duration of photosynthetic activity has contributed to increased yield in most of major crops (Russel 1991, Evans 1993). Genetic differences in photosynthetic duration have been also associated with a longer grain filling duration and higher yield in maize (Richards 2000). Several QTLs associated with leaf-related traits were identified on the different regions of linkage group 9; indicating genomic regions on the linkage group 9 were important for marker-based breeding programme for leaf-related traits in sunflower. In both normal- and late-sowing conditions, the co-location of QTLs for LAF and BIO (*LAF-L-2-1*, *LAF-N-2-1* and *BIO-L-2-1*; associated with marker ORS1024_1), LN, HN and DLN (*LN-L-4-1*, *LN-N-4-1*, *HN-L-4-1* and *DLN-L-4-1*; associated with marker ORS671_1), LN and HN (*LN-N-4-1* and *HN-L-4-1*; associated with marker ORS671_1), LAD, HD and GYP (*LAD-N-5-1*, *HD-N-5-1* and *GYP-L-5-1*; associated with marker SSL231), LN and GYP (*LN-N-10-1*, *LN-L-10-1* and *GYP-N-10-1*; associated with marker HA1108), LAF, GYP and HW (*LAF-L-10-1*, *GYP-L-10-1* and *HW-L-10-1*; associated with marker SSL39) and LAD and BIO (*LAD-L-11-1*, *LAD-N-11-1* and *BIO-L-11-1*; associated with marker E38M50_24) was determined. Identification of overlapped QTLs, influencing several traits shows that genes controlling the traits are in the same genomic region. The correlation among different traits as well as their co-localization observed is relevant to effort for manipulating multiple traits simultaneously.

6.4.4 QTLs controlling yield-related traits

It can be concluded that grain yield and its components were affected considerably by sowing date. Progress in increasing yield and its stability through a direct selection has been hampered by the low heritability of yield. The observed lower yields associated with late sowings have been variously hypothesized as due to warmer temperatures during the early growth period, which promotes excessive early stem growth (Beard and Geng, 1982) and reduce time to flowering (Andrade, 1995), and to cooler temperatures and reduced incident radiation postanthesis, which affects the dynamics of grain filling (Andrade, 1995; Bange et al., 1997). In both condition normal- and late-sowing condition, stable QTLs for head weight (*HW-L-2-1*, *HW-N-2-1*), head diameter (*HD-L-2-1*, *HD-N-2-1*) and 1000 grain weight (*HGW-L-2-1*, *HGW-N-2-1*) were identified on linkage group 2. It can be explained by positive correlation among yield components. We also detected another co-located region for HW and GYP in linkage group 8 and 6 that were linked to marker ORS243 and ORS381 in both conditions, respectively (Fig. 6.1). Total biomass produced will depend on incident radiation, canopy fractional interception, and the efficiency with which intercepted radiation is

converted into biomass (radiation use efficiency, RUE). Biomass accumulation was mostly influenced by the amount of intercepted radiation rather than by radiation use efficiency. On linkage group 7, 8, 9, 11 and 14, five regions were detected where Biomass, stearic acid content, oil content, head diameter, 1000 grain weight and leaf number (linked to E33M50_2), Biomass, linoleic acid content, oleic acid content and stearic acid content (linked to SSU217), Biomass and osmotic potential (linked to E38M50_24) and Biomass and sowing to flowering (linked to ORS301) were co-located (Table 6.8).

Nineteen QTLs were in common across two normal- and late-sowing conditions for the studied traits. The effect of sowing date on number of seed was significant (Uslu 1998). Dry matter production at flowering and harvest index were lowered under late sowing condition (Thompson and Heenan 1994). In our research, SSR marker ORS243 was associated with QTLs controlling HW in both normal- and late-sowing condition (*HW-L-8-1*, *HW-N-8-1*) and GYP (*GYP-N-8-1*). It is possible that overlapping regions are loci with pleiotropic effects. The current research enabled us to investigate the genetic basis of trait association by looking for co-location of corresponding QTLs for leaf-related traits, yield-related traits and other traits on the genetic map under different sowing date (temperature regimes). Detailed characterization of these genomic regions through the development and evaluation of near-isogenic lines will lead to an improved understanding and might set the stage for the positional cloning of genes-related to them. The relatively low number of RILs used in current research may have a negative influence on the accuracy of the calculated QTL effects, the ability to detect QTLs with small effects and R^2 may be overestimation (Beavis et al. 1994, Bachlava et al. 2008). This was, to some degree, compensated by the higher precision of the phenotyping and the use of our saturated map.

Table 6.1: Analysis of variance (mean squares) for leaf-, yield-related traits, DSF and PH in sunflower (RILs) in a population of sunflower recombinant inbred lines (RILs) grown under normal-sowing (N) and late-sowing (L) conditions,

		Leaf-related traits										Yield-related traits						DSF		PH	
	dF	LN		DLN		LAF		LAD		HN		HW		HD		GYP		BIO		TGW				
Condition		N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L
RILs	86	4658.76**	4227.68**	29.60**	8.75**	13.59**	7.44**	65.79**	35.03**	391.72**	345.38**	7785.17**	4644.34**	54.41**	41.48**	1917.25**	1261.08**	26343.59**	20603.95*	1377.10**	907.00**	34.56**	18.22*	1212.70**	1571.43**
Blocks	2	1026.00	159.60	6.01	30.26**	2.50	5.06*	26.07	26.93	264.78	55.20	466.33	1915.29**	1.70	27.25**	704.02*	1236.91**	16957.97*	5334.97*	428.00**	31.00	30.27**	15.22*	640.6	1571.43**
Error	172	708.17	909.46	11.78	4.85	4.40	2.60	21.58	12.68	139.18	30.38	304.76	205.41	3.76	2.82	159.92	113.30	6265.76	2075.65	77.00	52.00	6.32	3.87	218.01	203.46

* Significant at 0.05 probability level, ** Significant at 0.01 probability level.

Table 6.2: Simple correlation coefficients (Pearson) among leaf-related traits, yield-related traits, DSF and PH in sunflower (RILs) under normal-sowing (N) and late-sowing (L) conditions.

	DSF		DLN		LN		PH		HN		BIO		HD		HW		TGW		GYP		LAF	
	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L	N	L
DLN	0.44**	0.24**																				
LN	0.31**	0.15*	0.29**	0.21**																		
PH	0.33**	0.10	0.24**	0.20**	0.11	-0.17**																
HN	0.26**	0.23**	0.08	-0.05	0.75**	0.85**	0.09	-0.27**														
BIO	0.16**	0.07	0.05	0.05	0.38**	0.19**	0.47**	0.27**	0.31**	0.20**												
HD	-0.09	-0.29**	-0.11	-0.09	-0.54**	-0.55**	0.33**	0.38**	-0.45**	-0.69**	0.24**	0.23**										
HW	-0.13*	-0.22**	-0.09	0.02	-0.54**	-0.57**	0.34**	0.43**	-0.45**	-0.72**	0.27**	0.22**	0.89**	0.92**								
TGW	-0.25**	-0.14**	-0.11	0.04	-0.51**	-0.55**	0.25**	0.46**	-0.45**	-0.70**	0.25**	0.33**	0.78**	0.76**	0.85**	0.86**						
GYP	-0.14*	-0.26**	-0.01	0.01	-0.48**	-0.49**	0.31**	0.39**	-0.43**	-0.63**	0.29**	0.22**	0.83**	0.83**	0.91**	0.90**	0.79**	0.75**				
LAF	0.15*	0.02	0.09	0.06	0.49**	0.31**	0.45**	0.35**	0.40**	0.08	0.78**	0.65**	0.19**	0.30**	0.23**	0.33**	0.19**	0.34**	0.23**	0.29**		
LAD	0.10	0.06	0.11	0.06	0.45**	0.31**	0.46**	0.37**	0.38**	0.10	0.63**	0.67**	0.25**	0.30**	0.30**	0.33**	0.24**	0.41**	0.30**	0.29**	0.65**	0.67**

* Significant at 0.05 probability level, ** Significant at 0.01 probability level.

Table 6.3: Map position and effect of QTLs detected in RILs for DSF and PH

Trait	QTL	Linkage group	Marker On the left of LOD peak	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2b}
Normal-sowing								
DSF	*DSF-N-1-1*	1	ORS959	68.21	3.7	-1.06	0.07	0.45
	DSF-N-1-2	1	E32M61_10	43.01	3.1	1.26	0.08	0.53
	DSF-N-2-1	2	E40M62_17	65.81	5.5	1.75	0.17	0.54
PH	*PH-N-7-1*	7	E38M50_2	45.21	6.6	-7.72	0.13	0.63
	PH-N-8-1	8	ORS329	86.91	4.3	-5.72	0.06	0.64
	PH-N-10-1	10	E40M62_15	91.41	5	8.38	0.11	0.67
	PH-N-10-2	10	HA3039	172.71	3.7	-7.39	0.07	0.60
	PH-N-13-1	13	ORS511	54.71	10.1	10.60	0.16	0.60
	PH-N-15-1	15	ORS401	34.81	4.9	6.46	0.06	0.67
	PH-N-16-1	16	E40M50_1	75.61	3.6	-25.33	0.27	0.84
Late-sowing								
DSF	*DSF-L-2-1*	2	E35M60_23	26.91	3.8	0.67	0.05	0.60
	DSFL-2-2	**2**	**E40M62_17**	**63.81**	**3.3**	**0.98**	**0.04**	**0.58**
	DSF-L-9-1	9	E40M47_13	127.81	3.8	0.80	0.08	0.56
	DSF-L-10-1	10	E35M61_6	134.11	5.5	1.12	0.09	0.59
	DSF-L-10-2	10	E32M61_7	57.21	3.5	-0.72	0.04	0.52
	DSF-L-14-1	14	ORS301	105.01	6.8	-1.27	0.18	0.50
	DSF-L-16-1	16	E37M47_5	86.81	3.1	0.99	0.06	0.55
	DSF-L-17-1	17	E35M62_8	110.21	5.3	1.00	0.07	0.59
PH	*PH-L-7-1*	7	E35M60_22	62.81	5.4	-6.51	0.09	0.64
	PH-L-10-1	10	E32M49_17	46.21	5.6	-9.14	0.11	0.65
	PH-L-10-2	**10**	**E40M62_15**	**91.41**	**4.5**	**9.38**	**0.10**	**0.70**
	PH-L-13-1	**13**	**ORS511**	**54.71**	**6.5**	**9.27**	**0.15**	**0.67**
	PH-L-13-2	13	HA3330	39.41	4.4	6.08	0.06	0.58
	PH-L-14-1	14	HA3886	20.41	3.1	4.66	0.04	0.69

The QTLs are designated as the abbreviation of the trait followed by '*N*' or '*L*' for normal-sowing or late-sowing. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it. The bold QTLs are in common, presented as stable QTLs, across both well- and partial-irrigated condition for each trait.

a Percentage of individual phenotypic variance explained. Value determined by Win QTL Cart., version 2.5

b Percentage of phenotypic variance explained by the QTLs given all the covariants

Table 6.4: Map position and effect of QTLs detected in RILs for leaf-related traits

Trait	QTL	Linkage group	Marker On the left of LOD peak [a]	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2b}
Normal-sowing								
DLN	*DLN-N-3-1*	3	E32M47_13	2.01	3	1.14	0.08	0.58
	DLN-N-8-1	8	E37M61_7	18.01	4.4	-1.00	0.05	0.50
	DLN-N-11-1	11	SSL27	56.31	7.2	1.01	0.07	0.43
	DLN-N-11-2	11	E41M62_4	59.01	7.3	1.05	0.09	0.45
LN	*LN-N-4-1*	4	ORS671_1	85.91	3.3	6.34	0.05	0.62
	LN-N-9-1	9	ORS887	20.51	4.1	-14.89	0.09	0.64
	LN-N-9-2	9	E37M47_18	100.01	4.7	14.19	0.09	0.64
	LN-N-10-1	10	HA1108	21.31	5	18.23	0.11	0.62
	LN-N-10-2	10	E32M61_7	59.21	3.2	-22.61	0.09	0.73
LAF	*LAF-N-1-1*	1	ORS509	57.91	4	-1.84	0.11	0.45
	LAF-N-2-1	2	ORS229	80.11	5	-0.39	0.04	0.52
	LAF-N-5-1	5	ORS1024_1	27.31	6.5	1.60	0.04	0.41
	LAF-N-6-1	6	SSL66_1	62.21	3.9	-0.55	0.04	0.39
	LAF-N-7-1	7	E35M60_22	60.81	4.8	-0.80	0.11	0.41
	LAF-N-10-1	10	E37M49_5	69.61	3.8	-0.66	0.07	0.33
	LAF-N-12-1	12	E40M59_8	68.90	4.5	0.8	0.11	0.61
LAD	*LAD-N-2-1*	2	E33M48_4	94.51	4.1	-0.33	0.04	0.58
	LAD-N-5-1	5	SSL231	25.61	6.7	0.64	0.05	0.57
	LAD-N-8-1	8	E36M59_13	43.31	6.7	-1.04	0.10	0.60
	LAD-N-9-1	9	E36M59_17	73.51	6.4	0.91	0.09	0.58
	LAD-N-11-1	11	E38M50_24	72.51	3.9	-0.70	0.06	0.57
	LAD-N-12-1	12	E35M61_2	5.01	4.3	0.92	0.09	0.59
Late-sowing								
DLN	***DLN-L-3-1***	**3**	**E32M47_13**	**2.01**	**3**	**0.97**	**0.08**	**0.56**
	DLN-L-4-1	4	ORS671_1	85.91	7.6	-1.02	0.20	0.64
	DLN-L-9-1	9	E38M60_5	28.81	7.8	0.66	0.11	0.58
	DLN-L-10-1	10	E33M60_2	138.01	6.3	-0.94	0.04	0.54
	DLN-L-17-1	17	E35M48_7	96.11	10.6	-0.79	0.17	0.58
LN	***LN-L-4-1***	**4**	**ORS671_1**	**85.91**	**3**	**9.64**	**0.05**	**0.51**
	LN-L-8-1	8	E38M48_12	74.61	3	-8.17	0.04	0.50
	LN-L-9-1	9	E38M48_5	98.51	3.1	11.01	0.06	0.51
	LN-L-10-1	**10**	**HA1108**	**24.21**	**3.9**	**20.34**	**0.13**	**0.51**
LAF	***LAF-L-2-1***	**2**	**ORS229**	**80.11**	**6.7**	**-0.35**	**0.04**	**0.57**
	LAF-L-9-1	9	ORS1127	14.91	6.7	-0.59	0.06	0.59
	LAF-L-10-1	10	SSL39	99.91	7.6	-0.55	0.08	0.56
LAD	***LAD-L-2-1***	**2**	**E33M48_4**	**93.51**	**5.6**	**-0.45**	**0.05**	**0.62**
	LAD-L-6-1	6	E32M49_19	40.81	5	-0.55	0.09	0.59
	LAD-L-11-1	**11**	**E38M50_24**	**74.51**	**4.3**	**-0.33**	**0.04**	**0.57**
	LAD-L-15-1	15	SSU25	16.51	9.1	0.61	0.04	0.59

The QTLs are designated as the abbreviation of the trait followed by '*N*' or '*L*' for normal-sowing or late-sowing. The positive additive effect shows that PAC2 alleles increase the trait and negative additive effect shows that RHA266 alleles increase it. The bold QTLs are in common, presented as stable QTLs, across both well- and partial-irrigated condition for each trait.

a Percentage of individual phenotypic variance explained. Value determined by Win QTL Cart., version 2.5

b Percentage of phenotypic variance explained by the QTLs given all the covariants

Table 6.5: Map position and effect of QTLs detected in RILs for yield-related traits

Trait	QTL	Linkage group	Marker On the left of LOD peak	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2b}
Normal-sowing								
HN	*HN-N-4-1*	4	E38M50_16	56.31	5.9	-5.47	0.11	0.65
	HN-N-13-1	13	E33M48_20	28.91	6	-3.15	0.06	0.64
	HN-N-14-1	14	E36M59_3	92.31	4.9	-3.86	0.09	0.67
HD	*HD-N-2-1*	2	E35M60_4	100.61	4.1	-0.88	0.04	0.63
	HD-N-5-1	5	SSL231	25.61	5.8	1.41	0.10	0.60
	HD-N-8-1	8	ORS894_1	86.21	3.8	-1.13	0.04	0.64
	HD-N-10-1	10	ORS591	101.21	3.7	-1.43	0.05	0.63
	HD-N-15-1	15	HA1837	46.41	3	-1.08	0.04	0.64
HW	*HW-N-2-1*	2	E35M60_4	98.61	8.5	-18.02	0.09	0.76
	HW-N-4-1	4	E41M59_3	80.61	9.8	-18.60	0.11	0.73
	HW-N-8-1	8	ORS243	57.55	5	-11.23	0.05	0.45
	HW-N-13-1	13	ORS630	48.21	10.7	22.12	0.38	0.92
TGW	*TGW-N-2-1*	2	E35M60_4	100.61	11	-1.03	0.18	0.85
	TGW-N-8-1	8	E33M48_5	30.61	6.5	-0.67	0.07	0.74
	TGW-N-10-1	10	E35M48_8	80.41	6	-1.02	0.10	0.73
BIO	*BIO-N-7-1*	7	E33M50_2	29.91	4.1	-6.68	0.04	0.38
	BIO-N-12-1	12	SSL268	70.31	5	18.43	0.06	0.59
	BIO-N-15-1	15	SSU25	14.51	10.8	6	0.05	0.53
GYP	*GYP-N-3-1*	3	ORS657	54.31	5.6	6.18	0.04	0.46
	GYP-N-5-1	5	ORS533	84.91	7.7	6.12	0.04	0.48
	GYP-N-6-1	6	ORS381	30.61	6	3.56	0.04	0.46
	GYP-N-8-1	8	ORS243	57.31	5.9	-6.90	0.04	0.49
	GYP-N-10-1	10	HA1108	24.21	8.8	-16.62	0.30	0.53
	GYP-N-13-1	13	ORS630	51.91	6.4	12.76	0.14	0.58
Late -sowing								
HN	*HN-L-4-1*	4	ORS671_1	87.91	4.4	4.29	0.09	0.60
	HN-L-4-2	**4**	**E38M50_16**	**56.31**	**3.7**	**-2.71**	**0.04**	**0.60**
	HN-L-10-1	10	E41M59_11	32.21	9.2	7.22	0.27	0.67
	HN-L-13-1	13	E40M50_12	23.71	4	-3.89	0.08	0.59
	HN-L-14-1	**14**	**E36M59_3**	**90.31**	**3.6**	**-2.10**	**0.05**	**0.57**
HD	***HD-L-2-1***	**2**	**E35M60_4**	**96.61**	**5.7**	**-1.17**	**0.10**	**0.58**
	HD-L-4-1	4	E41M62_6	8.01	3	1.12	0.07	0.51
	HD-L-10-1	**10**	**ORS591**	**102.01**	**3.8**	**-1.07**	**0.05**	**0.52**
	HD-L-13-1	13	ORS511	54.71	3.1	1.40	0.07	0.58
HW	*HW-L-2-1*	2	ORS525_1	42.31	5.4	-12.57	0.06	0.45
	HW-L-2-2	**2**	**E35M60_4**	**96.61**	**4.8**	**-13.63**	**0.07**	**0.53**
	HW-L-4-1	4	E41M62_24	77.41	3.9	-12.57	0.05	0.47
	HW-L-8-1	**8**	**ORS243**	**57.31**	**5.7**	**-10.23**	**0.05**	**0.43**
	HW-L-10-1	10	SSL39	98.01	6.6	-21.91	0.10	0.51
	HW-L-13-1	**13**	**ORS630**	**52.21**	**3.7**	**12.32**	**0.06**	**0.45**
TGW	***TGW-L-2-1***	**2**	**E35M60_4**	**98.61**	**9.4**	**-1.08**	**0.04**	**0.76**
	TGW-L-4-1	4	E41M62_24	79.41	10.7	-0.67	0.10	0.71
	TGW-L-5-1	5	HA3700	74.41	5.9	1.00	0.11	0.73
	TGW-L-10-1	10	E41M59_11	32.21	7.4	-0.91	0.07	0.73
BIO	*BIO-L-1-1*	1	ORS509	57.91	4.1	-14.58	0.08	0.42
	BIO-L-2-1	2	ORS229	80.11	5.2	-16.18	0.10	0.51
	BIO-L-8-1	8	SSU217	13.21	4.8	-11.04	0.04	0.41
	BIO-L-11-1	11	E38M50_24	76.51	8.3	17.16	0.10	0.37
	BIO-L-14-1	14	ORS301	107.01	5.1	-12.79	0.06	0.43
	BIO-L-16-1	16	ORS656	55.71	4.5	-13.23	0.04	0.41
GYP	*GYP-L-5-1*	5	SSL231	25.61	9.5	5.33	0.05	0.41
	GYP-L-6-1	**6**	**ORS381**	**30.61**	**6.8**	**4.83**	**0.04**	**0.40**
	GYP-L-10-1	10	SSL39	98.01	7.6	-8.74	0.07	0.44
	GYP-L-13-1	13	ORS511	54.71	5.5	9.15	0.11	0.50

Table 6.6: QTLs controlling DSF and PH in normal- and late-sowing conditions in the present study, which are overlapped with QTLs for some traits identified in previous studies

Days from sowing to flowering and plant height (in our present study)		Previuose study	Overlapped QTLs	marker linked to Overlapped QTL
Normal -sowing condition	**Late -sowing condition**			
Seed-quality traits (Tang et al. 2006 [A], Ebrahimi et al. 2008[B])				
Days from sowing to flowering		Oil content [B] Oleic acid content [B] Oil content [A]	*DSF-N-1-1;* *1.OC.1.1* *1.OA.1.1*	ORS959
Plant height		Linoleic acid content [B]	*PH-N-7-1;* *4.LA.7.1*	E38M50_2
Plant height		Palmitic acid content [B]	*PH-N-8-1;* *2.PA.8.2*	ORS329
Plant height		Stearic acid content [B]	*PH-N-15-1;* *3.SA.15.1*	ORS401
	Plant height	Stearic acid content [B]	*PH-L-14-1;* *1.SA.14.1*	HA3886
	Days from sowing to flowering	Palmitic acid content [B]	*DSF-L-16-1;* *2.PA.16.1*	E37M47_5
Plant water status and osmotic adjustment traits (Poormohammad Kiani et al. 2007a) [C]				
	Plant height	Leaf water potential [C] Relative water content [C]	*PH-L-7-1;* *LWP.WS.7.1* *RWC.WS.7.1*	E35M60_2
Germination and seedling development traits (Rachid Al-Chaarani et al. 2005) [D]				
Days from sowing to flowering		Shoot dry weight [D]	*DSF-N-1-2;* *sdw-3-1*	E32M61_10
	Days from sowing to flowering	Shoot length [D]	*DSF-L-1-2;* *sl-15*	E35M62_8
Plant height		Root length [D]	*PH-N-16-1;* *rl-8*	E40M50_1
Agronomic traits (Rachid Al-Chaarani et al. 2004 [E], Poormohammad Kiani 2007b[F])				
Days from sowing to flowering		Sowing-to-flowering date [F]	*DSF-N-1-1;* *DSFI.1.1*	ORS959
Plant height		Total dry matter [F] Head weight [F] Head weight [F]	*PH-N-10-2;* *HWD.10.1* *HWN.10.2*	HA3039
	Plant height	Sowing-to-flowering date [E] Leaf number [F]	*PH-L-7-1;* *stf-4-1* *LNN.7.1*	E35M62_22
	Days from sowing to flowering	Leaf area [F]	*DSF-L-9-1;* *LAFW.9.1*	E40M47_13
	Days from sowing to flowering	Total dry matter [F]	*DSF-L-10-1;* *BIOI.10.1*	E35M61_6
	Days from sowing to flowering	Sowing-to-flowering date [F]	*DSF-L-16-1;* *DSFD.16.1*	E37M47_5

Table 6.7: QTLs associated with leaf-related traits in normal and late-sowing conditions in our research, which are overlapped with QTLs for some traits identified in previous studies

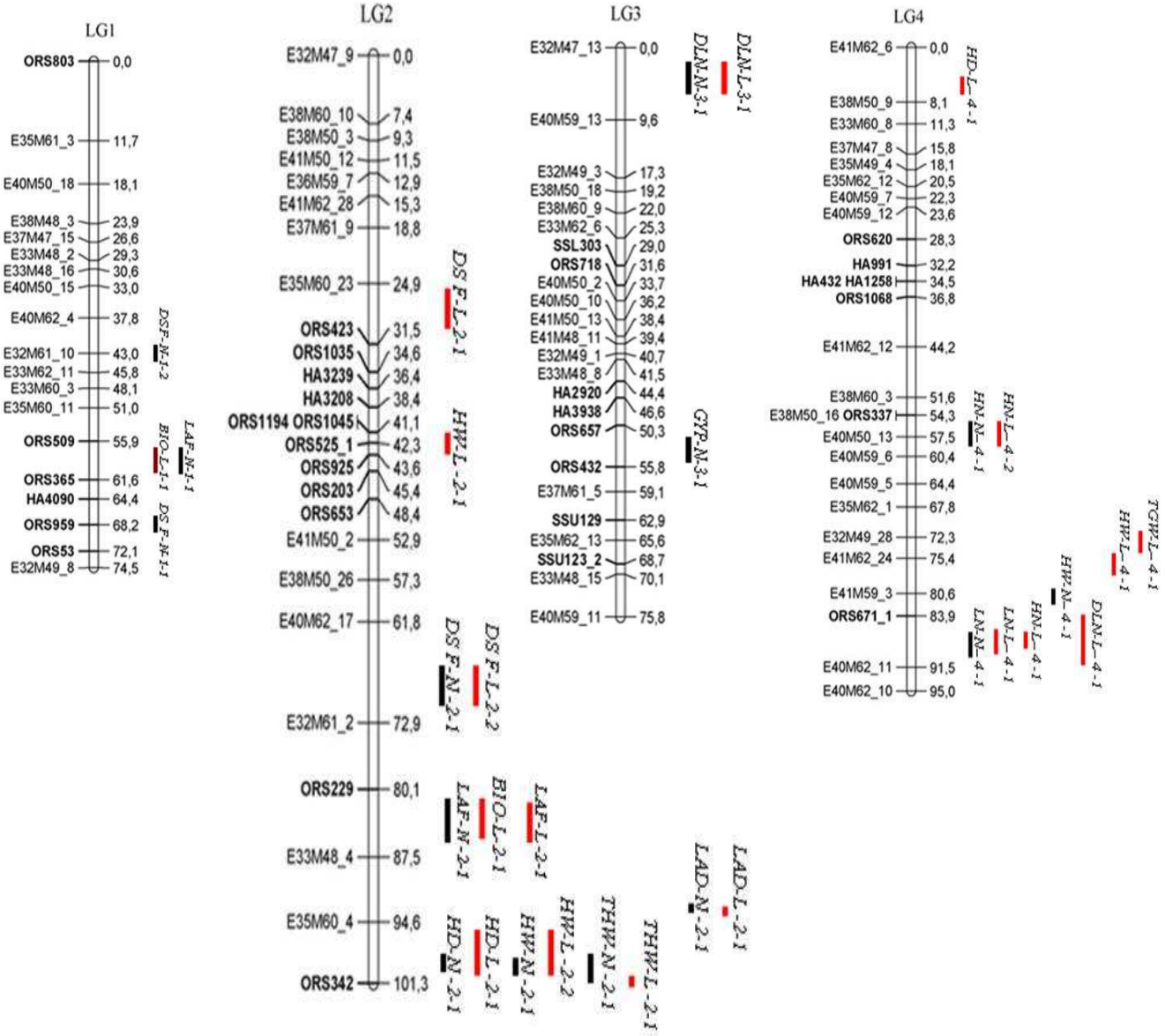
leaf-related traits (in our present study)		Previuose study	Overlapped QTLs	marker linked to Overlapped QTL
Normal -sowing condition	**Late -sowing condition**			
Seed-quality traits (Ebrahimi et al. 2008) [B]				
Leaf area duration		Oleic acid content [B]	*LAD-N-5-1; 1.OA.5.1*	SSL231
Leaf number at flowering		Palmitic acid content [B]	*LN-N-9-1; 3.PA.9.1*	ORS887
Leaf area duration		Stearic acid content [B]	*LAD-N-12-1; 2.SA.12.1*	E35M61_2
	Leaf area duration	Oil content [B]	*LAD-L-6-1; 2.OC.6.1*	E32M49_19
	Dried leaf number at flowering	Palmitic acid content [B]	*DLN-L-10-1; 1.PA.10.2*	E33M60_2
	Leaf number at flowering	Oleic acid content [B]	*LN-L-9-1; 1.OA.9.1*	E38M48_5
	Dried leaf number at flowering	Linoleic acid content [B]	*DLN-L-17-1; 3.LA.17.2*	E35M48_7
Plant water status and osmotic adjustment traits (Poormohammad Kiani et al. 2007a) [C]				
Leaf area at flowering		Leaf water potential [C] Relative water content [C]	*LAF-N-7-1; LWP.WS.7.1* *RWC.WS.7.1*	E35M60_22
Leaf area at flowering		Relative water content [C]	*LAF-N-10-1; RWC.WW.10.1*	E37M49_5
Leaf area duration	Leaf area duration	Osmotic potential [C]	*LAD-N-11-1, LADL-11-1; OP.WS.11.1*	E38M50_24
Germination and seedling development traits (Rachid Al-Chaarani et al. 2005) [D]				
Leaf area duration		Shoot length [D]	*LAF-N-6-1; sl-5*	SSL66_1
Agronomic traits (Rachid Al-Chaarani et al. 2004 [E], Poormohammad Kiani 2007b [F])				
Leaf area duration		Leaf number [F]	*LAD-N-5-1; LNN.5.1*	SSL231
Leaf number at flowering		Leaf number [F]	*LAF-N-5-1; LND.5.1*	ORS1024_1
Leaf number at flowering		Sowing-to-flowering date[E]	*LAF-N-7-1; stf-4-1*	E35M60_22
Leaf number at flowering		Duration of photosynthetic [F]	*LN-N-9-1; LADD.9.1*	E37M47_18
Leaf area duration		Total dry matter [F]	*LAD-N-12-1; BIOW.12.1*	E35M61_2
Dried leaf number at flowering	Dried leaf number at flowering	Total dry matter [F]	*DLN-L-3-1, DLN-N-3-1; BIOI.3.1*	E32M47_13

Table 6.8: QTLs controlling yield-related traits in normal- and late-sowing conditions in the present study, which are overlapped with QTLs for some traits identified in previous studies

Yield-related traits in our present study		Previuose study	Overlapped QTLs	marker linked to Overlapped QTL
Normal-sowing condition	**Late -sowing condition**			
Seed-quality traits (Ebrahimi et al. 2008 [B] and Pérez-Vich et al., 2006[G])				
Head diameter 1000-grain weight Head weight	Head diameter 1000-grain weight Head weight	Palmitic acid content [B]	*HD-N-2-1,* *TGW-N-2-1,* *HW-N-2-1,* *HD-L-2-1,* *TGW-L-2-1,* *HW-N-2-2;* *2.PA.2.1*	E35M60_4
Grain yield per plant		Oil content [B] Stearic acid content [B]	*GYP-N-3-1;* *2.OC.3.1* *4.SA.3.1*	ORS657
Head diameter	Grain yield per plant	Oleic acid content [B]	*HD-N-5-1,* *GYP-L-5-1;* *1.OA.5.1*	SSL231
Biomass		Oil content [B] Stearic acid content [B]	*BIO-N-7-1;* *1.OC.7.2* *2.SA.7.1*	E33M50_2
1000-grain weight		Oil content [B Stearic acid content [B]	*TGW-N-10-1;* *4.OC.10.1* *3.SA.10.1*	E35M48_8
Head number		Linoleic acid content [B]	*HN-N-13-1;* *2.LA.13.2*	E33M48_20
	Biomass	Linoleic acid content [B] Oleic acid content [B] Stearic acid content [B]	*BIO-L-8-1;* *1.LA.8.1* *1.OA.8.1* *2.SA.8.1*	SSU217
	Head weight	Oil content [B]	*HW-L-2-1;* *1.OC.2.1*	ORS525_1
Grain yield per plant Head weight	Head weight	*Es3* gene (associated with stearic acid level) [G] Palmitic acid content [B]	*HW-N-8-1,* *HW-L-8-1,* *GYP-L-8-1;* *4. PA.8.2*	ORS243
Plant water status and osmotic adjustment traits (Poormohammad Kiani et al. 2007a) [C]				
	Head diameter	Turgor potential [C]	*HD-L-4-1;* *TP.WW.4.1*	E41M62_6
	Biomass	Osmotic potential [C]	*BIO-L-11-1;* *OP.WS.11.1*	E38M50_24
Germination and seedling development traits (Rachid Al-Chaarani et al. 2005) [D]				
Head number		Shoot fresh weight [D] Shoot dry weight [D]	*HN-N-13-1;* *sfw-12* *sdw-12*	E33M48_20
	Head number	Shoot fresh weight [D] Root fresh weight [D] Shoot dry weight [D]	*HN-L-13-1;* *sfw-12* *rfw-12* *sdw-12*	E40M50_12
	Head number 1000-grain weight	Shoot length [D]	*HN-L-10-1,* *TGW-L-10-1;*	E41M59_11

			sl-9	
Agronomic traits (Rachid Al-Chaarani et al. 2004 [E], Poormohammad Kiani 2007b[F])				
Head diameter 1000-grain weight Head weight	Head diameter 1000-grain weight Head weight	Head weight [F] Grain yield [F]	*HD-N-2-1,* *TGWN-2-1,* *HW-N-2-1,* *HD-L-2-1,* *TGW-L-2-1,* *HW-N-2-2;* *HWN.2.1* *GYPN.2.1*	E35M60_4
Head weight		Grain yield [F]	*HW-N-4-1;* *GYPI.4.2*	E41M59_3
Grain yield per plant		Leaf area [F]	*GYP-N-5-1;* *LAFN.5.1*	ORS533
Biomass		Head diameter [E] 1000 grain weight [E] Leaf number [F]	*BIO-N-7-1;* *hd-4-1* *tgw-4-1* *LNI.7.2*	E33M50_2
Head diameter	Head diameter	Leaf area [30]	*HD-N-10-1;* *HD-L-10-1* *LAFD.10.1*	ORS591
Head number	Head number	Plant height [E]	*HN-N-14-1,* *HN-L-14-1* *ph-11-1;*	E36M59_3
	Head number 1000-grain weight	Sowing to flowering [E]	*HN-L-10-1,* *TGW-L-10-1;* *stf-9-1*	E41M59_11
	Head number	Head weight [F]	*HN-L-4-1;* *HWW.5.1*	ORS671_1
	Head weight 100-grain weight	Grain yield [F]	*HW-L-4-1,* *TGW-L-4-1;* *GYPD.4.1*	E41M62_24
	Grain yield per plant	Leaf number [F]	*GYP-L-5-1;* *LNN.5.1*	SSL231
	Biomass	Sowing to flowering [F]	*BIO-L-14-1;* *DSFD.14.1*	ORS301

Fig. 6.1 Molecular linkage groups of sunflower map presenting QTLs for leaf-related traits, yield-related traits, DSF and PH The positions of QTLs are shown on the right side of the linkage groups. Bars represent intervals associated with the QTLs.



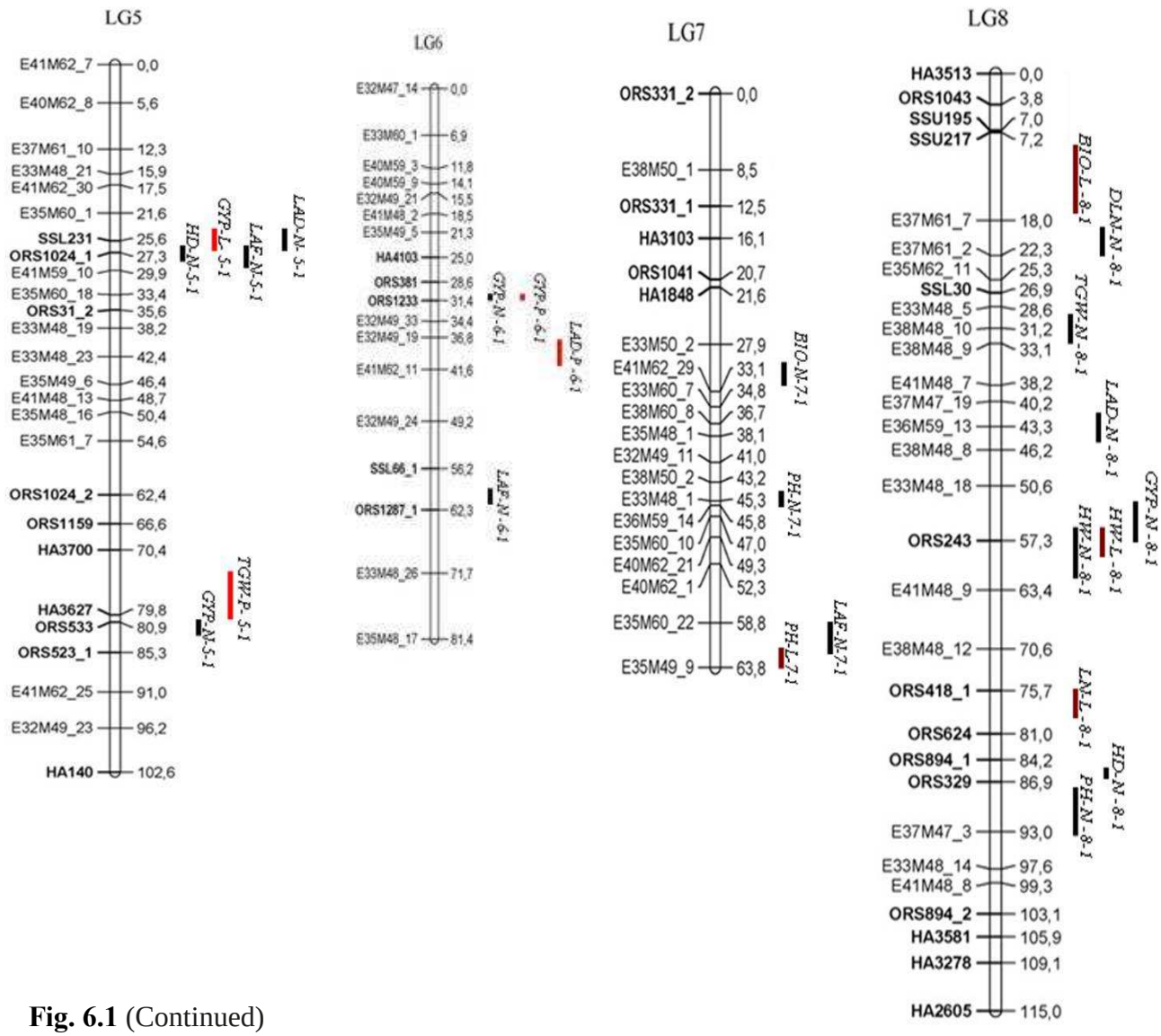


Fig. 6.1 (Continued)

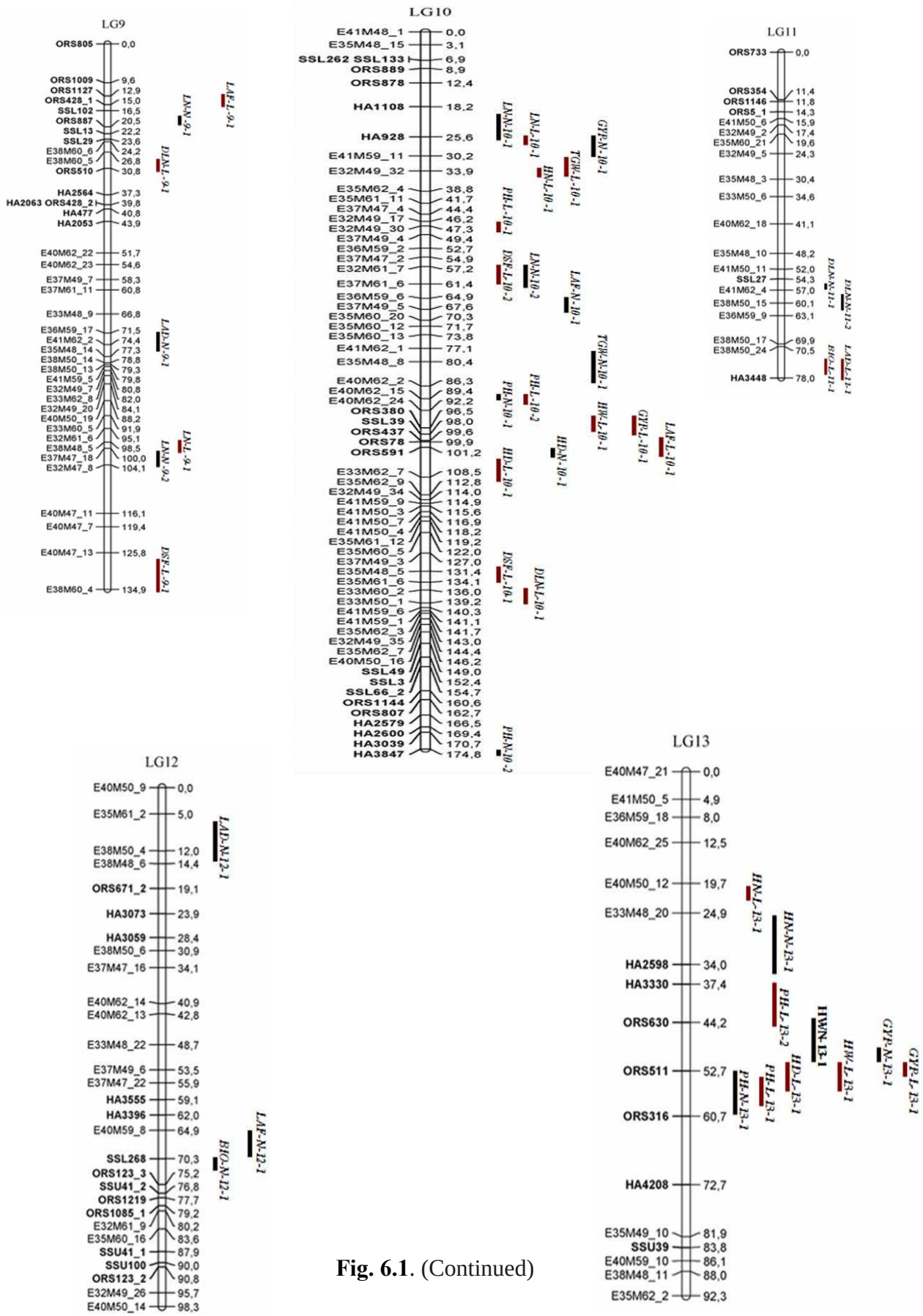


Fig. 6.1. (Continued)

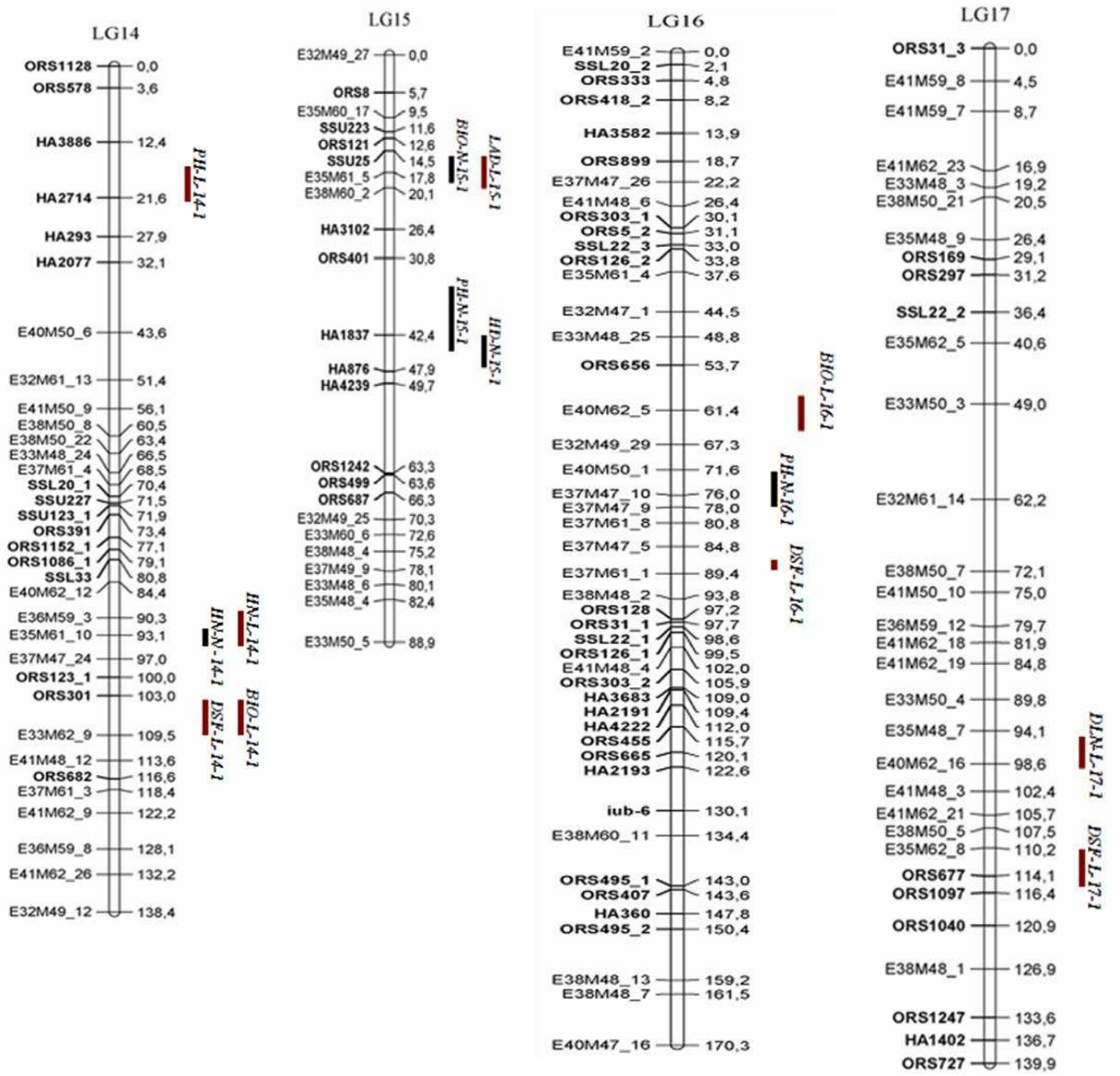


Fig. 6.1 (Continued)

Chapter 7

Genetic variability of seed-quality traits in sunflower mutants under water-stressed condition

Submitted

Abstract

Sunflower is one of the major annual world crops grown for edible oil and its meal is a potential source of protein for human consumption. It contains tocopherol can result in decreased risk for chronic diseases in human. The objectives of the current research are to assess the genetic variability and to identify AFLP markers and polymorphic candidate genes associated with seed-quality traits under well-irrigated and water-stressed conditions in gamma-induced mutants of sunflower. Two mutant lines, M8-826-2-1 and M8-39-2-1, with significant increased level of oleic acid can be used in breeding programs because of their high oxidative stability and heart-healthy properties. The significant increased level of tocopherol in mutant lines, M8-862-1N1 and M8-641-2-1, is justified by observed polymorphism for tocopherol pathway-related gene; *MCT*. The most important marker for total tocopherol content is E33M50_16 which explains 33.9% of phenotypic variance. One of the most important candidate genes involving fatty acid biosynthesis, *FAD2* (*FAD2-1*), is linked to oleic and linoleic acids content and explained more than 53% of phenotypic variance. Common markers associated with different seed-quality traits in well-irrigated and water-stressed conditions could be used for marker-assisted selection (MAS) in both conditions. Other markers, which are specific for one condition whereas linked to different traits or specific for a trait, could be useful for a given water treatment.

Key words: Seed-quality traits, Gamma-induced mutants, AFLP markers, Candidate genes, Sunflower

7.1Introduction

Sunflower seed oil contains saturated and unsaturated fatty acids, as the lipid part of the oil, as well as tocopherol conferring antioxidant properties to the non-lipid part of oil. Fatty acids with 18 carbons are either saturated (C18:0; stearic acid) or unsaturated (C18:1; oleic acid and C18:2; linoleic acid) (Dorrell and Vick 1997; Pérez-Vich et al. 2002). Among C18 fatty acids, oleic acid is more important because of higher oxidative stability, more resistance to heating and heart-healthy properties (Smith et al. 2007).

Tocopherol belongs to the Vitamin E class of lipid soluble antioxidants that are essential for human nutrition. The function of tocopherol in human and animal systems is generally

related to the level of α-tocopherol activity. Alpha-tocopherol has a maximum vitamin E activity (Kamal-Eldin and Appelqvist 1996). Among oil seed crops sunflower grains mainly contain α tocopherol, which accounts for more than 95% of the total tocopherols (Marwede et al. 2005). In sunflower seed oil, total tocopherol content represents the sum of α, β, γ, and δ tocopherol (Ayerdi Gotor et al. 2007).

In sunflower, gamma-irradiation has been used for inducing genetic variability for different characters such as osmotic-related traits (Poormohammad kiyani 2007), resistance to *Phoma macdonaldii* (Abu AL Fadi et al. 2004), germination traits (Alejo-James et al. 2004), morphological traits (Nabipour et al. 2004) and organogenesis (AL-Chaarani et al. 2004). Mutagenesis has been successfully used for developing variation in the fatty acid profile of sunflower and some mutants with altered fatty acid content have been developed.

High palmitic acid mutants; 275HP, CAS-5 and CAS-12 (Fernandez-Martinez et al. 1997), and the high stearic acid line, CAS-3, as well as two lines with midstearic acid content, CAS-4 and CAS-8, were obtained (Osorio et al. 1995). Developing midstearic acid sunflower lines (CAS-19, *es1es1Es2Es2*, and CAS-20, *Es1Es1es2es2*,) from a high stearic acid mutant is also reported (Pérez-Vich et al. 2004). The genetic studies of CAS-3, CAS-4 and CAS-8 revealed that total stearic acid increased as a result of reduced conversion rate of stearic to oleic acid while conversion rate of palmitic to stearic was not changed (Cantisán et al. 2000). Enzymatic actives for stearoyl-ACP desaturase and acyl-ACP thioesterase in above-mentioned high stearic acid mutant showed that stearoyl-ACP desaturase activity was reduced whereas acyl-ACP thioesterase activity was increased (Cantisán et al. 2000). Prevenets, high oleic acid mutants, have been so far developed by chemical mutagenesis (dimethyl-sulfate) (Soldatov 1976). In previous studies *Eco*RI and *Hin*dIII fragments, that are polymorphic in association with normal and high oleic acid mutants, were identified by Lacombe et al. (2001). In genotypes with high oleic acid content, in addition to 5.7 kb an extra 7.9 kb *Eco*RI fragment (*Eco*RI-Δ12HOS) was observed in comparison with genotypes with normal oleic acid content. A novel *Hin*dIII fragment of more than 15 kb (*Hin*dIII - Δ12HOS) instead of 8 kb was also reported (Lacombe et al. 2001). Co-segregation of *FAD2-1* with *Ol* in high-oleic sunflower mutant was also reported (Schuppert et al. 2006). Three loci; *Tph1 (m), Tph2 (g)* and *d*, can control the level of α tocopherol in sunflower seed (Hass et al. 2006; Tang et al. 2006; Vera-Ruiz et al. 2006). The amount of β tocopherol is increased by *d* locus in mutant inbred lines (*m m*) where as the level of γ tocopherol is enhanced by *g* locus in mutant inbred lines (*g g*) as a result of knockout of γ tocopherol methyl transferase (Hass et al. 2006). 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-

1,4-benzoquinone methyltransferase (*MPBQ*/*MSBQ-MT*) paralogs from sunflower (*MT1* and *MT2*) are isolated and sequenced (Tang et al. 2006). INDEL markers are developed for *MT1* and *MT2* and the *MT1* Locus is mapped to linkage group 1 (Tang et al. 2006). In this research the genetic variation of seed-quality traits such as total tocopherol, protein, oil and fatty acids contents, as well as polymorphism for AFLP markers and some candidate genes (CGs) in gamma-induced mutants of sunflower under well-irrigated and water-stressed conditions are studied.

7.2 Materials and methods

7.2.1Plant materials and experimental conditions

The sunflower restorer inbred line 'AS613' has been produced in our laboratory from a cross between two genotypes ('ENSAT-125' and 'ENSAT-704') through a single-seed descent (SSD) programme (Sarrafi et al. 2000). The seeds of 'AS613' were exposed to gamma rays at the Atomic Energy Center (Cadarache, France) with a dose of 75 Grays. Mutants population have been developed through modified SSD method (Sarrafi et al. 2000). Regarding to morpho-physiological studies, among a population of about 2000 gamma-induced mutants of sunflower, 23 M8 mutants were selected for quantitative analysis. Two independent experiments were undertaken in randomized complete block design with three replications at Tehran University-Iran-2007. Seeds of mutants and original line (AS613) were sown in the field under well-irrigated and water-stressed conditions. Each genotype per replication consisted of one row, 4m long, 50 cm between rows and 25 cm between plants in rows. The distance between replications of well-irrigated and water-stressed treatments was 7m. The so-called 'well-irrigated' condition plots were irrigated once every week, whereas for the second condition (water-stressed), water deficit was started 45 days after sowing at the stage near flower bud formation and continued up to maturity.

7.2.2 Trait measurements

Morpho-physiological traits

Various traits such as days from sowing to flowering (DSF) and plant height (PH) were measured for mutants and original line (AS613) at the plot scale (on each line, when 50% of the plants were at flowering stage). Moreover, leaf length (L) and width (W) of all green leaves were measured at flowering stage, and total leaf area at flowering (LAF) was

calculated with the formula: LAF= 0.7L×W (Alza and Fernandez-Martinez 1997). Green leaf area of the plants was determined weekly from flowering to harvest in order to evaluate green leaf area with respect to time. An integral of weekly leaf area was considered as being an estimate of leaf area duration (LAD, m^2 days). At harvest, yield components such as head diameter (HD), head weight (HW), 1000 grain weight (TGW), grain yield per plant (GYP) and biomass (Bio) were measured. Three plants per genotype per condition per replication were randomly selected for evaluation of the mentioned traits. All traits were measured for mutants and original line in each replication for both experiments.

Seed-quality traits

Near infrared reflectance (NIR) spectroscopy, has been successfully used as an alternative technique to classical methods in due to determine multiple parameters of seed quality traits in sunflower, such as proteins, oil content, fatty acid compositions (Pérez-Vich et al. 1998; Velasco and Becker, 1998; Biskupek-Korell and Moschner, 2007; Ebrahimi et al. 2008; Ebrahimi et al. 2009). Seed protein content (SPC), seed oil content (SOC), palmitic acid content (PAC), stearic acid content (SAC), oleic acid content (OAC) and linoleic acid content (LAC) were measured in mutants and original line (AS613) in each replication for both conditions by the FOSS NIRSystems 6500. Twenty grams of sunflower seeds per genotype per condition per replication were ground in a Knifetec 1095 Sample Mill (1975, Foss Tecator, Höganäs, Sweden) three times for 10 s each. A FOSS NIR Systems 6500 spectrophotometer (Foss Analytical, Denmark) was used to collect spectra from the ground sunflower seeds using a small round cup with a quartz window. The reflectance (R) of each sample was measured as log of 1/R from 400 to 2500 nm at 2nm intervals.

Pre-measurements for total tocopherol content (TTC) were carried out by both FOSS NIRSystems 6500 and reference method (HPLC, ISO 9936, 1997) for core collection (forty four samples). Total oil content was extracted and TTC was thus determined using the following protocol:

Solvent extraction of lipids

The extraction of the total oil content was performed by hexane (n-hexane, Prolabo/Subra, Toulouse, France) extraction using an accelerated solvent extractor apparatus (ASE 200, Dionex, France) with an isopropanol/hexane mixture (5:95 v/v) during 20 min. Then, the solvent was removed from the extracts under low-pressure evaporation (Rotavapor,

Bioblock Scientific HS 40 HUBER, Heildorph, Germany). Lipid extracts were weighed and tocopherol content was analyzed.

Tocopherol determination

Total tocopherol was achieved using a high-performance liquid chromatography (HPLC) (SpectraPhysics, Thermo Separation Products, USA) with a normal-phase LiChrosorb Si60 column, 250cm x 4mm x 5µm (CIL, Cluzeau, France) (ISO 9936, 1997). The mobile phase was a mixture of hexane/isopropanol (99.7:0.3 v/v) at 1mL/min flow rate. One gram of oil sample was diluted in 25 mL of hexane and 20µl was injected into the HPLC. Detection was performed with fluorescence detector (excitation wavelength = 298 nm and emission wavelength=344nm: Waters 2475 multi λ). Total tocopherol content was calculated as the sum of α, β, γ, and δ-tocopherol contents and expressed in mg kg^{-1} oil. A modified partial least-squares regression (MPLS) model, after 4 outlier elimination passes (WINISI 1.02 - Infrasoft International LLC) was used. The performance of our NIRS model, for the estimation of tocopherols was determined by the following parameters: the standard error of calibration (SEC), the coefficient of determination in calibration (RSQ), the standard error of cross-validation (SECV), the coefficient of determination of cross-validation (1–VR) and the standard error of prediction (SEP). We have obtained a high significant correlation between the HPLC analysis and the NIRS predictions for TTC (R^2 = 0.76) indicating the NIRS method can be used to determine total tocopherol content. Then, TTC was measured in mutants and original line (AS613) in each replication for both conditions by the FOSS NIRSystems 6500. In previous studies in our department, a relatively good correlation between NIRS results and HPLC and GC method for total tocopherol (R^2 = 0.64) (Ayerdi Gotor et al. 2007) and total phytosterol content (R^2 = 0.61) (Ayerdi Gotor et al. 2008; Calmon et al. 2009) were also observed.

7.2.3 Molecular analysis

The genomic DNA of original and mutant lines were isolated according to the method of extraction and purification presented by Porebski et al. (1997) and DNA quantification was performed by picogreen. The AFLP procedure is previously described by Darvishzadeh et al. (2008). Polymorphism of some important candidate genes; tocopherol pathway-related, phosphoglyceride transfer-related, enzymatic antioxidant-related, drought-responsive and fatty acid biosynthesis-related genes were studied. Reactions catalyzed by proteins of the

tocopherol and fatty acid pathways are illustrated in Figure 1 and Figure 2, respectively. Respective sequence data for candidate genes coding for these proteins were obtained from The *Arabidopsis* Information Resource, TAIR, (*www.arabidopsis.org*). In order to seek the *helianthus* homolog sequences to the *Arabidopsis* genes, we used the Compositae EST assembly clusters, available at the *Helianthus*-devoted bioinformatics portal Heliagene (*www.heliagene.org*). The *Helianthus* EST clusters presenting the reciprocal blast with the highest score and lowest E value with regarding to the original *Arabidopsis* genes were chosen for our studies. All Primers were designed by MATLAB. Four various primer combinations per each candidate gene were tested on agarose gel**.** Primers used for PCR are summarized in Table 7.1. The PCR program was: 4 min at 94 °C followed by 35 cycles; 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and at last, 5 min at 72 °C.

7.2.3 Statistical analysis

The data were analyzed using SPSS. The association between AFLP markers and candidate genes with the quantitative traits was estimated through stepwise multiple regression analysis, where each quantitative trait was considered as a dependent variable while AFLP markers and candidate genes were treated as an independent variable. To select independent variables for the regression equation, *F*-values with 0.045 and 0.099 probabilities were used to enter and remove, respectively. Multiple regression analysis has been used to identify molecular markers associated with morphological and yield traits in some crops (Virk et al. 1996; Vijayan et al. 2006).

7.3 Results

7.3.1Phenotypic variation

Results of analysis of variance show significant genotypic effect (Mutants) for morpho-physiological (Table 7.2) and seed-quality (Table 7.3) traits under well-irrigated and water-stressed conditions. Characteristics of sunflower M8 mutant lines in both conditions for morpho-physiological and seed-quality traits are also summarized in Table 3 and 4, respectively. Regarding the range of mutant lines, variation for all studied traits was observed and some mutants presented significant higher values compared with the original line.

7.3.2 Molecular analysis

Seventeen AFLP primer combinations and their polymorphic markers used for genotyping mutants and their original line (AS613). The number of polymorphic markers varied from 8 to 27 for different primer combinations (Darvishzadeh et al. 2008). Polymorphisms are also observed for the studied candidate genes for original line (AS 613) and some mutants (Fig. 3). The results of marker identification for different traits under well-irrigated and water-stressed conditions are summarized in Table 7.4. Results revealed that the number of AFLP marker and candidate gene (CG) associated with seed-quality traits ranged from 4 to 6 depending on trait and conditions. The percentage of phenotypic variance (R^2) explained by each marker or candidate gene associated with the traits ranged from 4.4% to 53.3%. The most important marker for TTC is E33M50_16 and explained 33.9% of phenotypic variance. One of the most important candidate genes involving tocopherol pathway (Fig. 1), homogenitisate phytyltransferase (*VTE2*), is linked to TTC (Table 7.4). The E31M50_12 marker correlated with SPC is identified in well-irrigated condition and explained 35% of phenotypic variance. The largest amount of phenotypic variance (R^2) explained by E37M50_10 for SOC is 35.3 %. Under water-stressed condition, association between *SEC14* and SOC is observed. Among 11 identified markers for PAC in both conditions, E37M50_20 is most important with R^2=28.6%. Under well-irrigated condition, the correlation between *POD* and PAC is also observed. Two common markers; E33M59_6 and E31M50_5, are detected for SAC. One of the most important candidate genes involving fatty acid biosynthesis (Fig. 7.2), *FAD2* (*FAD2-1*), is linked to OAC and LAC and explains more than 53% of phenotypic variance (Table 7.4).

7.4 Discussion

The large genetic variability observed among mutant lines for the studied traits revealed that the efficiency of gamma-irradiation for inducing genetic variation in sunflower for seed-quality traits. Some mutants have advantages over the original line 'AS613' for different traits. Mutant line, M8-862-1N1, presents significant increased level of tocopherol (403.78 mg kg $^{-1}$oil compared with 314.3 mg kg $^{-1}$oil in original line AS-613; Table 7.3). Mutant lines, M8-826-2-1 and M8-39-2-1, with significant increased level of oleic acid (70.3 mg 100mg $^{-1}$oil in M8-826-2-1 mutant compared with 29.2 mg 100mg^{-1}oil in original line AS-613; Table 7.3) are developed by gamma rays with a dose of 75 Grays in our research. These mutants can be used in breeding programs because of high oxidative stability and heart-healthy properties.

Molecular genetic studies have been carried out in the aforementioned population through AFLP markers and candidate genes (CGs). The results of marker identification show that some AFLP markers and candidate genes are associated with several traits and some others are specific for only one trait (Table 7.4). Among all studied candidate genes involving tocopherol biosynthetic pathway, polymorphisms were observed for *VTE4*, *VTE2* and *MCT* genes among some mutants and original line (Fig. 1). Endrigkeit et al. (2009) reported that in rape seed genotypes, *VTE4* was anchored to the end of chromosome A02, where also two QTLs for α-tocopherol content had been identified. Primer combination corresponding to *MCT* gene (PC 4; Table 7.1) led to PCR fragment of about 1.2 kb in M8-862-1N1 and M8-641-2-1 Lines and 1.6 kb in AS613 (Fig. 7.1). High level of tocopherol in mutant lines, M8-862-1N1 and M8-641-2-1, is justified by observed polymorphism for *MCT* gene (Fig. 7.1). The polymorphisms for some Phosphoglyceride transfer-related genes are also observed among mutants and original line (Fig. 7.3).

The level of TTC, SOC, PAC and LAC are significantly increased in M8-186-1 line (Table 7.3) and polymorphism for phosphatidylinositol transporter (*SEC14*) is also observed compared with AS613 (Fig. 7.3). The level of SOC is significantly increased in M8-52-1-1 line **(**40.2 g 100g $^{-1}$ dry matter in M8-52-1-1 mutant compared with 32.6 g 100g $^{-1}$ dry matter in original line AS-613) and polymorphism for stearoyl ACP desaturase, phosphatidylinositol transporter (*SEC14*), *CAT*, *POD* and *PGT* genes is also observed compared with AS613 (Fig. 7.2 and 7.3). It has been reported that SEC14 domains exist in proteins from plants, yeast and mammals (Saito et al. 2007). Wide range of lipids, phosphatidylglycerol and tocopherols were known as ligands for SEC14 domain-containing proteins (Saito et al. 2007).

Some enzymatic antioxidant-related genes such as peroxidase (*POD*) and catalase (*CAT*) present polymorphisms in some mutant lines compared with the original line; AS613 (Fig. 7.3). The association between *POD* gene, enzymatic antioxidant, and PAC is observed (Table 7.4). The interdependence between antioxidant and lipid peroxidation has also been recognized (Semchuk et al. 2009). In plants, the protection of photosynthetic apparatus and polyunsaturated fatty acids from oxidative damage caused by reactive oxygen species (ROS) are the main function of antioxidant (Trebst et al. 2002; Velasco et al. 2004; Cela et al. 2009; Semchuk et al. 2009). Primer combination corresponding to *Dehydrin* gene (PC 13; Table 7.1) led to specific PCR fragment of about 1 kb in M8-417-1, M8-826-2-1, M8-39-2-1 and M8-186-1 Lines (Fig. 7.3). *Dehydrin* is a gene of the D-11 subgroup of late-embryogenesis-abundant (LEA) proteins (Dure et al. 1989; Close et al. 1993), associated with drought

tolerance in sunflower (Ouvard et al. 1996; Cellier et al. 1998). In sunflower three *FAD2* genes have been isolated whereas from both cotton and soybean two and from *Arabidopsis* only a single *FAD2* genes have been identified (Heppard et al. 1996; Liu et al. 1997; Martinez-Rivas et al. 2001). In the present study, sequences of *FAD2* used for primer design were obtained from GenBank, closet homologue in *Helianthus* is indicated as reference. Primer combinations corresponding to *FAD2_1* gene (PC 18; Table 7.1) led to high oleic acid specific amplification fragments of about 1.5 kb in M8-826-2-1 and M8-39-2-1 Lines. In contrast, primer combinations corresponding to *FAD2_2* and *FAD2_3* genes just led to nonspecific bands in mutants and original line (Fig. 7.2). These results suggest that the high oleic mutation in gamma-induced sunflower population interferes with the mutation in *FAD2_1* gene. *FAD2-1* is seed specific and strongly expressed in developing seeds (Martinez-Rivas et al. 2001). Co-segregation of *FAD2-1* with *Ol* gene has been shown and it has been also assigned to linkage group 14 in sunflower (Schuppert et al. 2006). The high association between *FAD2-1* gene and E33M60_8 with OAC and LAC can be explained by correlation between OA and LA as well as by a specific gene for Δ12-desaturase (oleoyl-PC desaturase), which catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2) (Garcés and Mancha, 1991). High oleic acid mutants can be developed either by the upstream desaturation of stearic acid into oleic acid by Δ9 desaturase or by the downstream desaturation of oleic acid by Δ12 desaturase. High significant and negative correlation between OA and LA (Ebrahimi et al. 2008) is justified by opposite coefficient of their common markers (Table 7.4). This phenomenon poses potential challenges to breeders for simultaneous improvement of both traits. However, independent markers for OA and LA identified in our research (Table 7.4) provide opportunity for simultaneous improvement of these two traits in sunflower.

Two stable markers, E40M59_5 and E37M50_7, for TTC are identified in both conditions (Table 7.4). The changes in TTC during plant responses to drought stress can be characterized by two phases. In the first phase, increased TTC contribute to avoid oxidative damage by quenching reactive oxygen species (ROS). The second phase occurs when the stress is severe. TTC decreases during the second phase and consequently, lipid peroxidation increases and cell death happens if tocopherol deficiency cannot be compensated by other mechanisms of protection (Munné-Bosch 2005). An increase of tocopherol synthesis under moderate stress and a decrease of tocopherol synthesis under severe stress have been reported (Munné-Bosch 2005). Under water-stressed condition, common marker; E37M50_20, for PAC and SAC is detected (Table 7.4). This can be explained by a specific

gene for fatty acid synthetase II (FACII), which lengthens palmitic acid (16:0) by two carbon atoms to produce stearic acid (18:0) (Pleite et al. 2006). Common markers associated with different seed-quality traits in well-irrigated and water-stressed conditions could be used for marker-assisted selection (MAS) in both conditions. Other markers, which are specific for one condition whereas linked to different traits or specific for a trait, could be useful for a given water treatment.

Table 7.1: Primers used for PCR

Target gene	Accession		Primer pair combination	Sequence of primer (5' to3')	
	AGI-Arabidobsis	Homologue with Heliagene Cluster		Forward	Reverse
Tocopherol pathway-related genes					
VTE4	AT1G64970.1	HuCL02246C001	1 2	ATCCGTATGATTGAACAAGC GTTTGGTCAATGGAGAGTG	ATGTGCTCTCCACTCTCCATTG ATCCTTCAATCATTAGTGGC
VTE2	AT2G18950.1	HuCL02840C003	3	TGCCACAAGAGCAAATCGCTTC	TTTGGGCACTCTTCATAAG
MCT	AT2G02500.1	HuCL00002C009	4	CAAAGTCTTCACCACAAATG	ACCTCATCCCATCTTCTTCC
Phosphoglyceride transfer-related genes					
PGT	AT1G75170.1	HuCL10527C001	5	TATGTCCATCTTTCGGCGTC	ATGGTGTCTTTAGCGGTTC
Cytosolic	AT3G24840.1	HuCL09897C001	6	ATGATAACCGTGTGGATAGC	ATGCTAAACTGGAGGAAAGC
SEC14	AT2G21540.2	HuCL00667C001	7	CAAGGAAGGATTTCACCGTG	AAGGCGGTTGATGCTTTACG
Enzymatic antioxidant-related genes					
POD	AT1G14540.1	HuCL03143C001	8	GACTTGGAAGAAGAGATTCAC	ATTGTCAGCATACTCGGTC
CAT	AT1G20620.1	HuCL00001C054	9	AAACTACCCTGAGTGGAAG	AATGAATCGTTCTTGCCTG
GST	AT1G02930.1	HuCL00790C003	10	AAAGAGCACAAGAGTCCTG	ACTTATTTGAGTGGGCAAC
Drought-responsive genes					
Drou	AT5G26990.1	HuCL02051C001	11	TTGTTGAGGAGGGAACTAAG	GTCATCACCAAGAATCGTCG
SPL2	AT5G43270.1	HuCL10252C001	12	ATTTGATGGGAAGAAGCGG	CATTGTGGTCAGAAAGCCTC
Dehydrin	AT3G50980.1	HuCL00053C009	13	AAGTTCTCCAAACCGACGAG	ACAACCACAGTGAAACCAC

The candidate genes are: tocopherol methyl-transferase (*VTE4*), homogenitisate phytyltransferase (*VTE2*), 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (*MCT*), phosphoglyceride transfer (*PGT*), Cytosolic, phosphatidylinositol transporter (*SEC14*), peroxidase (*POD*), glutation s-transferase (*GST*), catalase (*CAT*), drought-responsive (*Drou, SPL2*), Dehydrin,

Table 7.1(Continued)

	Target gene	Accession		Primer pair combination	Sequence of primer (5' to3')	
		AGI-Arabidobsis	Homologue with Heliagene Cluster		Forward	Reverse
Fatty acid biosynthesis -related genes						
FAD2[*1]	*FAD2-3*	–	AY802998.1	14	GCCTTATTCTACATTCTGCTC	ATCCCATAGTCTCGGTCTAC
			HuCL07925C001	15	GCCTTATTCTACATTCTGCTC	AATCGCCTTTGTTGCTTCC
	FAD2-2	–	AY802993.1	16	GGTCTGTCATCCGTTCATTC	GCGAATCGGTCATAATACC
			HuCL00141C001	17	GGTCTGTCATCCGTTCATTC	AGTCCCGTCAAACTGATAG
	FAD2-1	–	DQ075691.1	18	GAGAAGAGGGAGGTGTGAAG	GCCATAGCAACACGATAAAG
			HuCL00406C001	19[*2]	GAGAAGAGGGAGGTGTGAAG	ACAAAGCCCACAGTGTCGTC
	stearoyl ACP desaturase	AT2G43710.1	HuCL00103C001	20	GACGTTTCAATCAGACCTGT	GCATTGTTTGGTAAGTAGGC
				21	GCCTACTTACCAAACAATGC	TATTTTTGTGTAGGCGGTTT
	FatB	AT1G08510.1	HuCL03123C002	22	TTACACATTCGGCTTATCG	TGGTTGATAAAGGTTCTCGGG
				23	TTACACATTCGGCTTATCG	GCACATTTCTGGTGTTGAACCG
				24	ACTGAGGTGAATGGGAGTAG	GCACATTTCTGGTGTTGAACCG
	FatA	AT3G25110.1	HuCL04107C001	25	AATAAGACGGCGACTGTTG	TCTCAATTTCAACCACATCA

FAD2 (*FAD2-1, FAD2-2, FAD2-3*), stearoyl ACP desaturase and acylACP thioesterase (*FatA, FatB*).

[*1]: Sequences of *FAD2* used for primer design were obtained from GenBank, closet homologue in *Helianthus* is indicated as reference.

[*2]: Specific primer for high OAC (Berville et. Al. 2009)

Table 7.2: Characteristics of sunflower M8 mutant lines for morpho-physiological traits in well-irrigated (WI) and Water-stressed (WS) conditions

Trait	Well-irrigated condition			Water-stressed condition			Effect	
	Original line (AS613)	Mutants		Original line (AS613)	Mutants		M[a]	
		Mean	Range		Mean	Range	WI	WS
Sowing to flowering date	52	54.91	52-58	51	53.21	51-57	**	**
Plant height (cm)	150.5	170.04	144.3-218	147.5	165.04	144.3-209	**	**
Leaf number	32	43.29	32-59	32	41.29	32-59	**	**
Leaf area at flowering stage (m^2)	0.715	0.716	0.417-1.353	0.715	0.716	0.407-1.303	**	**
Leaf area duration (m^2 days)	14.293	14.329	8.339-27.069	12.985	13.019	7.328-25.122	**	**
Head weight (g)	114.78	115	42.5-169.1	77.62	90.82	40.32-138.54	**	*
Head diameter (cm)	21	21.13	13.5-24.5	19.25	20.07	11.5-25	**	**
1000 grain weight (g)	71.8	71.6	36.9-102.1	53.9	67.2	32.8-96.7	**	**
Grain yield per plant (g)	66.69	58.43	18.15-94.29	55.64	47.92	16.43-96.7	**	*
Biomass (g)	118.65	173.09	74.55-297	99.85	154.93	70.7-259.6	**	*

[a] M: mutants effect

*, **: significant at 0.05 and 0.01 probability level, respectively.

Table 7.3: Characteristics of sunflower M8 mutant lines for seed-quality traits in well-irrigated (WI) and Water-stressed (WS) conditions

Trait	Original line (AS613)		Mutants				Some mutant lines										Effect	
			mean		Range		M8-826-2-1		M8-133-2		M8-186-1		M8-143-2		M8-862-1N1		M [a]	
	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS
TTC (mg kg $^{-1}$ oil)	314.3	277.5	312.1	274.9	228.9-403.7	134.9-395.4	292.9	303.1	284	261.5	387.4*	365.1*	342.1	345.7	**403.7***	**395.5***	**	**
SPC (g 100 g $^{-1}$ dry matter)	26.8	27.2	27.26	27.9	22.2-31.0	23.2-31.1	30.0	31.2	**31.0***	**30.0***	22.2	27.7	30.5	30.0	24.4	23.3	**	**
SOC (g 100 g $^{-1}$ dry matter)	32.6	32.0	34.8	32.4	27.2 – 42.5	27.0– 39.7	36.3	35.9	27.8	29.8	**42.5***	33.8	37.3*	35.7*	40.7*	39.2*	**	**
PAC (mg 100 mg $^{-1}$ oil)	5.9	6.5	5.9	6.0	4.6-7.1	4.8-6.8	4.7*	4.8*	5.5	6.1	**7.0****	**6.8**	6.4	6.1	6.5	6.9	**	**
SAC (mg 100 mg $^{-1}$ oil)	5.8	7.0	6.0	6.8	5.1-7.4	5.1-7.3	5.8	6.3	6.7	7.3	5.3	6.1	**7.4***	**6.8**	5.2	5.2	**	**
OAC (mg 100 mg $^{-1}$ oil)	29.2	28.1	29.1	29.7	13.2-65.3	14.3-68.8	**70.3***	**68.8***	29.1	22.9	13.2*	14.3*	35.2	36.6	18.9	16.3	**	**
LAC (mg 100 mg $^{-1}$ oil)	59.5	60.6	60.2	59.6	26.0-75.9	23.0-74.6	26.0*	23.0*	59.3	65.7	**75.9****	**74.5****	53.9	52.6	70.6	72.9	**	**

Note: TTC, total tocopherol content; SPC, seed protein content; SOC, seed oil content; PAC, palmitic acid content; SAC, stearic acid content; OAC, oleic acid content; LAC, linoleic acid content.

*, **: significant difference with original line (AS613) at 0.05 and 0.01 probability level, respectively.

[a] M: mutants effect

Table 7.4: AFLP markers and candidate genes correlated with seed-quality traits in a population of sunflower mutants and original line (AS613) under well-irrigated and water-stressed conditions

Traits	Well-irrigated condition					Water-stressed condition				
	Marker	R^2 %	T-Value	P-Value	Standardized coefficient	Marker	R^2 %	T-Value	P-Value	Standardized Coefficient
TTC	**E37M50_9**	**32.7**	**6.8**	**<0.0001**	**-0.6**	**E40M59_5**	**30.7**	**6.7**	**<0.0001**	**-0.7**
	E33M50_16	33.9	10.1	<0.0001	0.7	**E37M50_7**	**15.7**	**5.1**	**<0.0001**	**-0.6**
	E40M59_5	**10.5**	**5.5**	**<0.0001**	**-0.6**	E33M59_7	11.2	3.5	0.003	0.4
	E40M50_7	7.0	5.1	<0.0001	0.4	E33M59_2	10.7	3.5	0.002	0.4
	E37M50_7	**5.0**	**3.0**	**0.007**	**-0.3**	***VTE2***	**7.0**	**2.6**	**0.01**	**0.3**
SPC	E31M50_12	35.0	8.4	<0.0001	-0.6	E37M50_12	23.0	7.7	<0.0001	0.7
	E37M50_10	**19.0**	**5.8**	**<0.0001**	**-0.5**	**E40M59_7**	**19.2**	**4.8**	**<0.0001**	**0.4**
	E31M48_1	11.2	5.2	<0.0001	-0.4	**E33M61_6**	**13.8**	**6.2**	**<0.0001**	**-0.6**
	E37M62_7	12.1	5.2	<0.0001	0.4	E33M49_7	16.0	7.2	<0.0001	0.8
	E40M59_7	**6.1**	**3.8**	**<0.0001**	**0.3**	E33M47_2	11.3	3.5	0.003	-0.3
SOC	**E37M50_10**	**35.3**	**7.1**	**<0.0001**	**0.5**	E33M59_4	21.3	6.4	<0.0001	-0.5
	E31M50_6	**22**	**6.3**	**<0.0001**	**-0.4**	E33M47_6	32.0	7.7	<0.0001	-0.5
	E38M62_7	**12.9**	**7.3**	**<0.0001**	**0.6**	**E37M50_6**	**14.3**	**7.4**	**<0.0001**	**-0.6**
	E38M62_8	10.5	4.4	<0.0001	-0.2	E33M47_1	10.4	4.9	<0.0001	0.4
						E37M50_10	**9.1**	**4.1**	**0.001**	**0.3**
						SEC14	**4.9**	**3.2**	**0.005**	**-0.2**
PAC	**E33M60_8**	**26.8**	**6.3**	**<0.0001**	**-0.6**	**E37M50_20**	**28.6**	**10.1**	**<0.0001**	**-0.9**
	FAD2-1	**25.1**	**6.8**	**<0.0001**	**0.7**	E33M61_5	22.9	7.9	<0.0001	-0.6
	E40M59_6	13.6	3.6	0.002	0.3	E40M50_8	17.0	6.0	<0.0001	-0.5
	E33M50_13	6.9	3.5	0.003	-0.3	E37M50_3	8.5	6.1	<0.0001	0.5
	E33M59_11	6.0	3.2	0.005	-0.3	**E33M50_8**	**8.7**	**3.9**	**0.001**	**0.3**
	POD	6.7	2.7	0.01	-0.2					
SAC	E33M50_13	28.9	9.6	<0.0001	-0.8	E33M61_3	37.4	5.5	<0.0001	0.4
	E33M59_6	**27.3**	**8.8**	**<0.0001**	**0.8**	**E33M59_6**	**21.7**	**6.0**	**<0.0001**	**0.5**
	E31M50_5	**14.1**	**5.2**	**<0.0001**	**-0.4**	**E31M50_5**	**14.1**	**6.0**	**<0.0001**	**0.5**
	Drou	12.1	5.3	<0.0001	0.4	**E37M50_20**	**10.2**	**4.0**	**0.001**	**0.3**
	E33M61_6	**7.5**	**3.7**	**0.002**	**-0.3**	**E33M60_4**	**6.3**	**3.3**	**0.004**	**0.2**

TTC, total tocopherol content; SPC, seed protein content; SOC, seed oil content; PAC, palmitic acid content; SAC, stearic acid content. The candidate genes are: homogenitisate phytyltransferase (*VTE2*), phosphatidylinositol transporter (*SEC14*), peroxidase (*POD*), drought-responsive (*Drou*). Common markers are also shown as bold-face.

Table 7.4: Continued

Traits	Well-irrigated condition					Water-stressed condition				
	Marker	R^2 %	T-Value	P-Value	Standardized coefficient	Marker	R^2 %	T-Value	P-Value	Standardized Coefficient
OAC	***FAD2-1***	**44.3**	**11.7**	<0.0001	-0.9	***FAD2-1***	**52.8**	**7.5**	**<0.0001**	**-0.7**
	E33M60_8	**19.4**	**3.6**	**0.002**	**0.3**	**E33M60_8**	**16.4**	**3.9**	**0.001**	**0.3**
	E33M59_10	8.0	5.4	<0.0001	-0.5	E33M61_1	7.5	2.8	0.012	-0.3
	E33M59_8	9.0	4.3	<0.0001	0.3	**E37M50_9**	**6.2**	**2.6**	**0.017**	**0.2**
	E33M59_11	**4.4**	**3**	**0.009**	**0.2**					
LAC	***FAD2-1***	**43.7**	9.5	<0.0001	0.7	***FAD2-1***	**53.3**	**9.7**	**<0.0001**	**0.8**
	E33M60_8	**19.3**	**6.1**	**<0.0001**	**-0.5**	**E33M60_8**	**18.2**	**5.7**	**<0.0001**	**-0.5**
	E40M59_6	**8.6**	**3.3**	**0.004**	**0.2**	E38M62_6	6.8	4.4	<0.0001	-0.4
	E33M49_13	6.6	4.2	0.001	0.3	E33M47_10	7.1	3.9	0.001	0.3

OAC, oleic acid content; LAC, linoleic acid content and common markers are shown as bold-face.

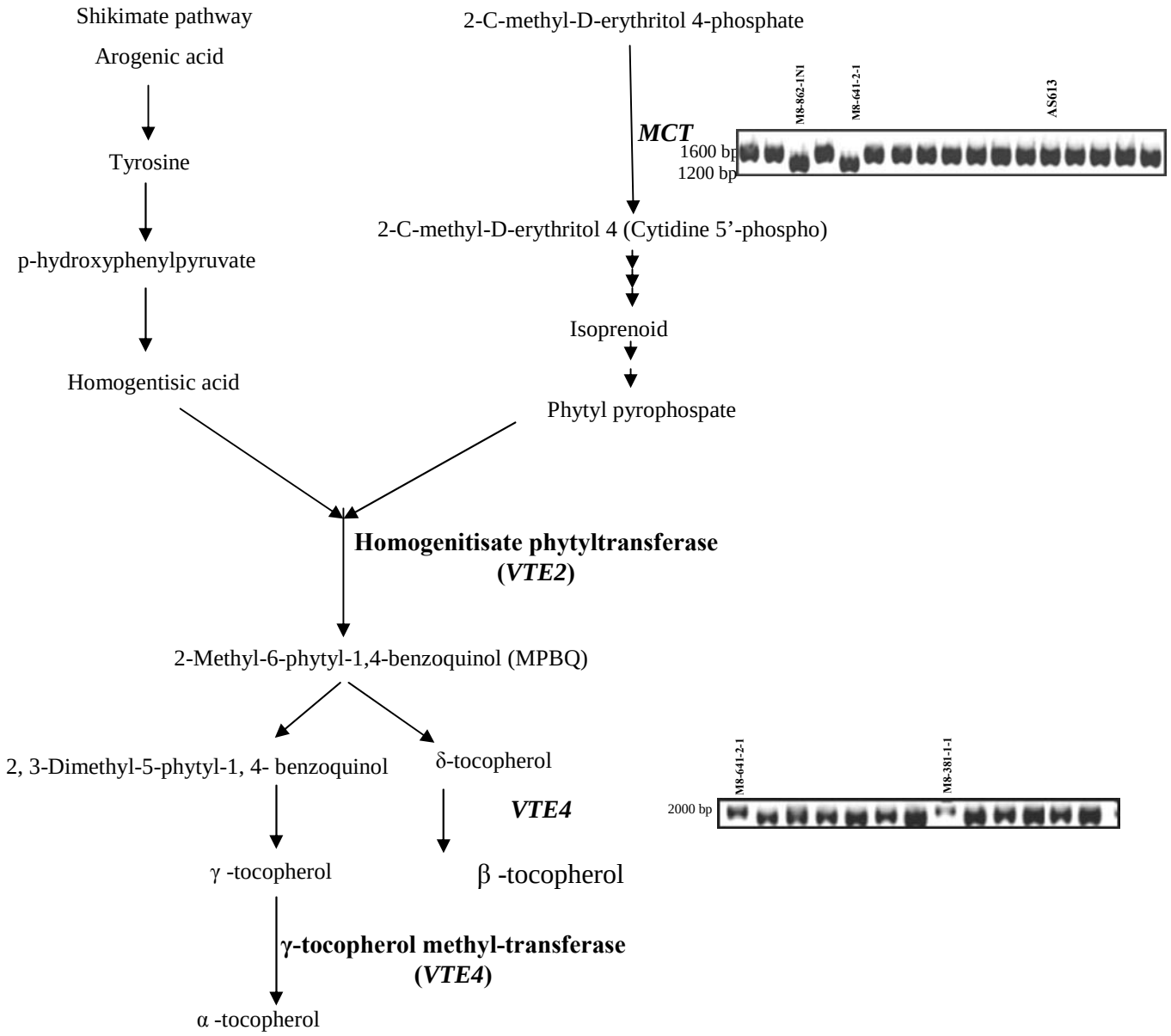


Fig. 7.1 Tocopherol biosynthetic pathway; '2-C-methyl-D-erythritol 4-phosphate' is converted into '2-C-methyl-D-erythritol 4 (Cytidine 5'-phospho)' by 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (MCT).'2-Methyl-6-phytyl-1,4-benzoquinol' (MPBQ) is formed after the condensation of homogentisic acid (HGA) and phytyl pyrophosphate (PDP) by homogenitisate phytyltransferase (VTE2). α-tocopherol can be generated by methylation of γ-tocopherol via γ-tocopherol methyl-transferase (VTE4) (D'Harlingue and Camara 1985). β-tocopherol is formed from δ-tocopherol by methylation of the 5 position by VTE4 (Norris et al. 2004).The studied candidate genes are also highlighted in bold. Gelelectrophoretic separation of candidate gene-PCR products from original line (AS613) and some mutants are presented with the corresponding metabolic pathway.

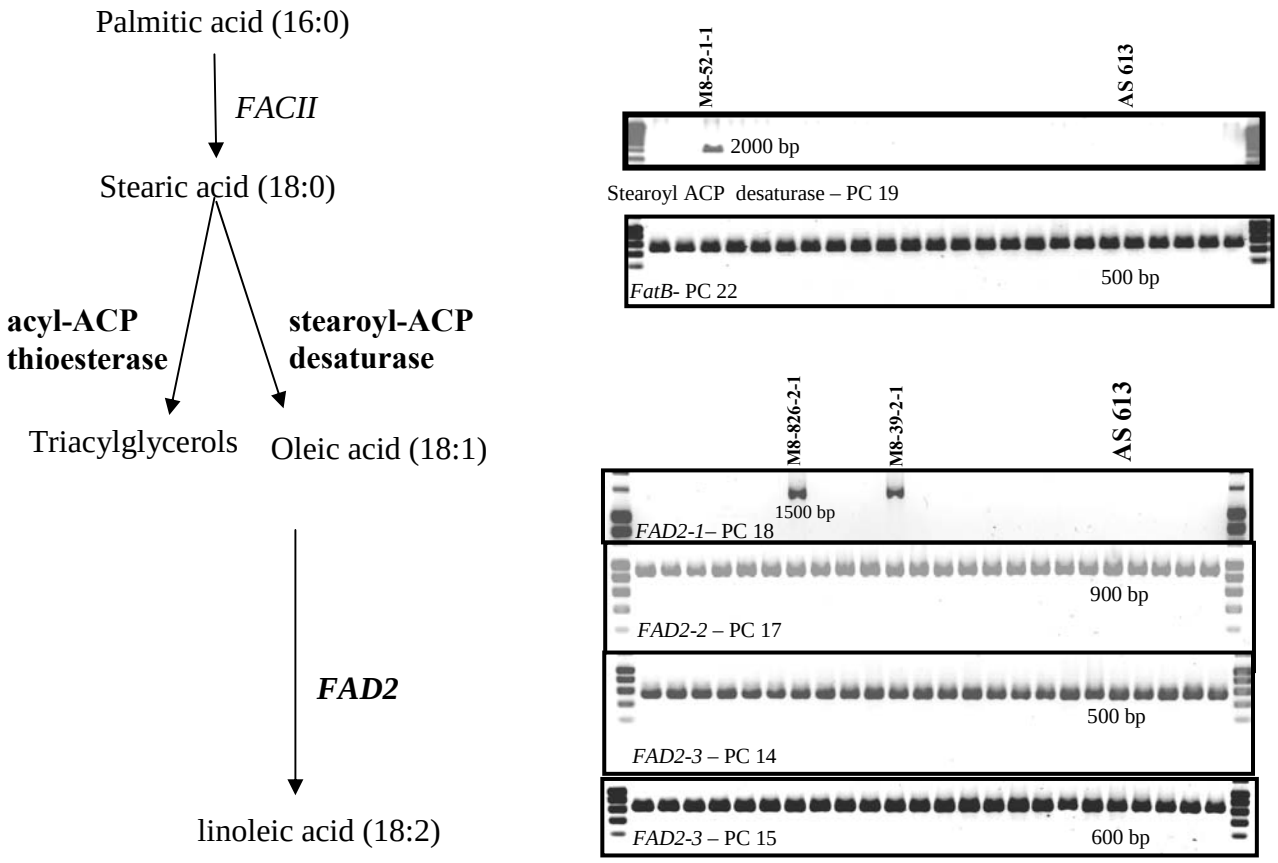


Fig. 7.2 Simplified fatty acid biosynthetic pathway, Stearic acid is formed from palmitic acid by FACII, which lengthens palmitic acid (16:0) by two carbon atoms to produce stearic acid (18:0) (Pleite et al., 2006). Stearic acid can be either desaturated by Δ9-desaturase (stearoyl-ACP desaturase) which catalyses the first desaturation of stearic acid (18:0) to oleic acid (18:1) or hydrolyzed by acyl-ACP thioesterase (Heppard et al., 1996; Lacombe et al., 2001; Vega et al., 2004).Finally, linoleic acid is formed from oleic acid by Δ12-desaturase (oleoyl-PC desaturase; FAD2), which catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2) (Garcés and Mancha, 1991).The studied candidate genes are also highlighted in bold. Gelelectrophoretic separation of candidate gene-PCR products from original line and mutants are presented with the corresponding metabolic pathway. PC: primer combination

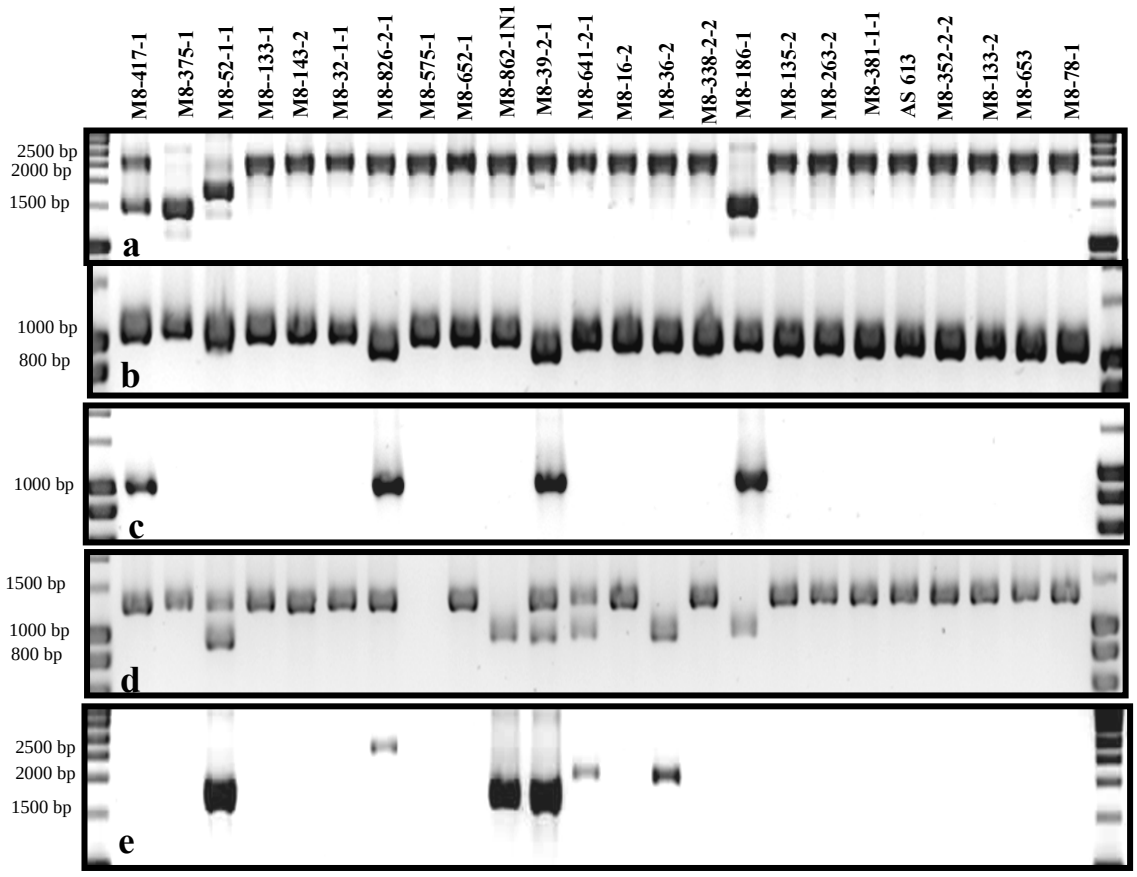


Fig. 7.3 Polymorphisms for candidate gene-PCR products among original line (AS613) and mutants. a) phosphatidylinositol transporter (*SEC14*)-PC7, b) catalase (CAT)-PC9, c) dehydrin-PC13, d) peroxidase (*POD*)-PC8 and e) phosphoglyceride transfer (*PGT*)-PC5
PC: Primer combination

CONCLUSION ET PERSPECTIVES

L’objectif principal de ce travail était d’identifier les régions génomiques contrôlant des caractères morphophysiologiques du tournesol ainsi que ceux associés à la qualité de l’huile (teneur en tocophérol, phytostérol, acides palmitique, stéarique, oléique et linoléique) et à la composition de la graine (pourcentage de protéines et d’huile), ainsi que leur stabilité au travers de différents environnements (conditions de sécheresse, irrigation et semis tardif). Une population de lignées recombinantes issues du croisement entre ‘PAC2’ et ‘RHA266’ a été étudiée sous différents traitements. En effet, l’amélioration de la tolérance à la sécheresse nécessite l'identification des QTL contrôlant ces caractères de qualité en conditions sèches et le transfert des allèles d’intérêt aux cultivars productifs. Dans ce cadre, le développement des outils de marquage moléculaire permet de rechercher des relations entre la présence de marqueurs et les caractères de qualité. Les cartes génétiques facilitent ainsi l'identification des zones génomiques contrôlant les caractères de qualité. Nous avons cartographié des gènes candidats ; (1) impliqués dans la voie métabolique de tocophérol et phytostérol, (2) des gènes codant des antioxydants enzymatiques, (3) des gènes liés à la sécheresse et (4) des gènes homologues à *Sec14* chez *Arabidopsis* dans une carte génétique du tournesol construite dans notre laboratoire basée sur des SSRs (Poormohammad kiyani et al. 2007). Un autre objectif était d’identifier des marqueurs AFLP et quelques gènes candidats liés aux caractères impliqués dans la qualité de l’huile et la qualité des graines en conditions de stress hydrique et irriguée dans une population de mutants (M8).

Développement de la carte génétique

En utilisant une population de lignées recombinantes issues du croisement entre ‘PAC2’ et ‘RHA266’, une carte génétique du tournesol a été construite dans notre laboratoire (Poormohammad kiyani et al. 2007). Nous avons amélioré cette carte et établi une nouvelle carte génétique basée sur 190 SSRs et gènes candidats. Cette nouvelle carte a une longueur de 1405.3 cM et une densité d’un marqueur pour 7.6 cM. Les groupes de liaisons ont été nommés en comparant les positions des SSRs communs entre notre carte et la carte génétique référence du tournesol construit par Tang et al. (2002). L'incorporation des gènes candidats dans notre carte a permis

d'augmenter la précision de détection de QTL pour les différents caractères analysés dans cette étude. Les gènes homologues à *SFH3*, *HPPD*, *CAT* et *CYP51G1* ont été cartographiés grâce à la mise au point de marqueurs dominants, tandis que des marqueurs co-dominants ont permis la cartographie des gènes homologues à *SEC14-1*, *VTE4*, *DROU1*, *POD*, *SEC14-2* et *AQUA.* Trois gènes candidats importants (*VTE4*, *VTE2* et *HPPD*), qui codent pour des enzymes impliquées dans la biosynthèse du tocophérol, ont été cartographiés sur les groupes de liaison LG8 et LG14. Nous avons identifié douze SNPs pour *VTE4* entre les deux parents (PAC2 et RHA266). Quatre SNPs sont identifiés pour *PAT2*, le gène homologue chez *Arabidopsis* de *SEC14*, entre les deux parents et un SNP, identifié par alignement de séquences est converti en marqueur CAPS pour permettre l'analyse génotypique des RIL. Les gènes *POD*, *CAT* et *GST*, codant pour des antioxydants enzymatiques, ont également été cartographiés sur les groupes de liaison 17, 8 et 1, respectivement. *SMT2* produit le phytosterol 24-Ethylidene lophenol à partir de 24-Methylene–lophenol. Huit SNPs sont identifiés pour *SMT2* entre les deux parents et le SNP identifié par alignement de séquences est converti en marqueur CAPS pour le génotypage des RIL. La nouvelle carte génétique ainsi produite fournit un outil de base important pour l'analyse des caractères quantitatifs et qualitatifs chez *Helianthus annuus.*

Variabilité génétique des caractères associés à la qualité de l'huile, à la qualité des graines et des caractères morpho-physiologiques chez le tournesol

Les résultats montrent qu'il existe une variabilité génotypique pour les paramètres associés à la qualité de l'huile (teneur en tocophérol, phytostérol, acides palmitique, stéarique, oléique et linoléique), à la qualité des grains (le pourcentage de protéines et huiles) ainsi que les paramètres morpho-physiologiques en condition de sécheresse et de semis tardif au sein de la population de lignées recombinantes issues du croisement entre 'PAC2' et 'RHA266'. Une différence significative a été notée entre les deux parents PAC2 et RHA266 pour les teneurs en tocophérol et phytostérol en condition de sécheresse et semi tardif, et pour le pourcentage de protéines sous contrainte hydrique. La lignée parentale RHA266 a montré certains avantages significatifs concernant les

teneurs en tocophérol et phytostérol en condition de sécheresse et de semis tardif par rapport au parent PAC2.

Le génotype RHA266 présente des valeurs plus élevées pour le pourcentage d'huiles, teneur en acide palmitique et linoléique par comparaison avec le parent PAC2.

Un gain génétique significatif a été observé pour tous les caractères étudiés sous contrainte hydrique, semis tardif et témoin. Ce phénomène pourrait être dû à l'accumulation des allèles favorables venant des deux lignées parentales chez les lignées recombinantes sélectionnées. Il a été observé une forte corrélation négative entre teneur en acide oléique et linoléique. De tels résultats ont également été observés par Lagravère et al. (2004) et Ebrahimi et al. (2008). La contrainte hydrique et le semis tardif augmente la teneur en tocophérol et phytostérol, ce qui confirme les resultats rapportés par Munné-Bosch (2005). D'une manière générale, nous remarquons dans notre étude que le pourcentage de protéines des graines augmente en réponse au déficit hydrique chez les RILs confirmant ainsi les résultats de Ozturk et Aydin (2004) obtenus chez le blé. Une forte ségrégation transgressive a été observée pour les paramètres associés à la qualité de l'huile (teneur en tocophérol, phytostérol, palmitique, stéarique, oléique et linoléique), à la qualité des grains (le pourcentage de protéines et huiles) et morpho-physiologique en condition de sécheresse et semis tardif. La ségrégation transgressive serait le résultat de l'accumulation d'allèles positifs venant des deux lignées parentales.

La population de mutants présente également une variabilité génétique significative pour tous les caractères étudiés. Le gain génétique significatif pour tous les caractères étudiés chez les mutants montre l'efficacité de l'irradiation aux rayons gamma pour induire une variation génétique au niveau des caractères de qualité de l'huile (teneur en tocophérol, phytostérol, acides palmitique, stéarique, oléique et linoléique), de qualité des grains (le pourcentage de protéines et huiles) et de caractères morpho-physiologiques chez le tournesol. Les lignées mutantes, M8-862-1N1 et M8-641-2-1, ont montré les meilleures valeurs pour le teneur en tocophérol. Deux lignées mutantes, M8-826-2-1 et M8-39-2-1, produisent un niveau significativement élevé d'acide oléique peuvent être utilisées dans les programmes de sélection en raison de la haute stabilité à l'oxydation et des propriétés cardiovasculaires apportées par l'acide oléique.

Dissection génétique de la qualité de l'huile, des graines et de caractères morpho-physiologiques par analyse QTL et colocalisation de gènes candidats chez le tournesol

Nous avons identifié plusieurs QTL communs à nos différentes études. Nous avons également identifié plusieurs QTL co-localisés avec des QTL déjà identifiés pour d'autres caractères dans la littérature. Ces QTL, utiles au développement de programmes de sélection pour les caractères de qualité et quantité chez le tournesol, sont présentés ci-après. Les co-localisations de QTLs aident à mieux comprendre les bases génétiques des caractères étudiés et éventuellement à développer plus rapidement des génotypes d'intérêt. Un QTL majeur pour la teneur en tocophérol a été identifié sur le groupe de liaison 8, qui explique 59,5% de la variation phénotypique (6.*TTC.8*). Il est co-localisé également avec le QTL identifié pour la teneur en phytostérol (7.*TPC.8*). Sous condition de semis tardif, un QTL spécifique de la teneur en acide palmitique a été identifié sur le groupe de liaison 6 (*PAC-LS.6*). Il est situé entre les marqueurs ORS1233 et SSL66_1. Nous avons détecté deux régions chromosomiques sur les groupes de liaison 10 et 15, où les QTL contrôlant le pourcentage d'huiles ont été co-localisés avec des QTL de teneur en acide stéarique (*PSO-PI.10*, *SAC-WI.10* et *PSO-PI.15*, *SAC-LS.15*). Sept QTL associés à teneur en acides palmitique, stéarique, oléique et linoléique sont identifiés sur le groupe de liaison 14. Ils sont co-localisés à l'homologue du gène *HPPD*. Les quatre QTL contrôlant la teneur en tocophérol (7.*TTC.1*), en phytostérol (7.*TPC.1*), en acide stéarique (*SAC-LS.1*) et en acide oléique (*OAC-LS.1*) sont situés dans la même position, sur le groupe de liaison 1 où Le gène *GST*, a été cartographié. Le gène, *Tph1*, associé à la teneur en β-tocophérol a été également cartographié dans la même région chromosomique (Vera -Ruiz et al. 2006). Cette région chromosomique a été rapportée par Poormohammad Kiani et al. (2009) pour la précocité de floraison. L'activité antioxydante des tocophérols protège les acides gras en éliminant les radicaux libres, il a d'ailleurs été montré chez le tournesol une corrélation positive entre l'acide gras 18:2 et tocophérol (Kamal-Eldin et Andersson, 1997).

Nous avons détecté trois régions chromosomiques sur les groupes de liaison 2, 8, 14 où les QTL contrôlant la teneur en acide palmitique ont été co-localisés avec des QTL

de teneur en acide stéarique. Ceci peut être expliqué par la corrélation entre l'acide palmitique et l'acide stéarique, ainsi que par un gène spécifique de l'acide gras synthétase II (FACII), qui allonge l'acide palmitique (16:0) par deux atomes de carbone pour produire l'acide stéarique (18:0) (Cantisán et al., 2000; Pleite et al., 2006). Trois co-localisations de QTL contrôlant les teneurs en acides stéarique et oléique sur les groupes de liaison 1, 2, 14 ont été identifiés. Ceci peut être expliqué par la corrélation entre l'acide stéarique et l'acide oléique, ainsi que par un gène spécifique de la Δ9-desaturase (stearoyl-ACP desaturase), responsable de la conversion de l'acide stéarique en acide oléique dans les graines chez le tournesol (Heppard et al., 1996; Cantisán et al., 2000). Trois QTL contrôlant la teneur en acide oléique sont co-localisés avec ceux contrôlant la teneur en acide linoléique sur les groupes de liaison 10, 11, 16. Ceci peut être expliqué par un gène spécifique de la Δ12-desaturase (oleoyl-PC desaturase), responsable de la conversion de l'acide oléique (18:1) en acide linoléique (18:2) dans les graines chez le tournesol (Garcés and Mancha, 1991). La corrélation négative entre le pourcentage de protéines et huiles est justifiée par des effets additifs contraires de leurs QTL co-localisés (*PSP-PI.9*, *PSO-PI.9*, *PSP-PI.11* et *PSO-PI.11*). L'identification de QTL influençant plusieurs caractères simultanément pourrait augmenter l'efficacité de la sélection assistée par marqueurs (SAM) et ainsi augmenter le progrès génétique (Upadyayula et al. 2006). Un QTL commun pour la teneur en tocophérol est identifié sur le groupe de liaison 16 (*TTC-WI.16, TTC-LS.16*). Les allèles favorables pour les deux QTL viennent du parent RHA266. Cette région apparaît importante dans les deux conditions bien irriguées. Cette région du groupe de liaison 16 a été rapportée pour la teneur en huile par Ebrahimi et al. (2008) et le nombre de feuilles par Poormohammad Kiani et al. (2009). Un autre commune QTL pour la teneur en phytostérol est identifiés sur le groupe de liaison 9 (*TPC-WI.9, TPC-PI.9.1*). Cette région chromosomique a été rapportée par Ebrahimi et al. (2009) pour le pourcentage de protéines du grain. Un QTL commun pour la teneur en tocophérol est également identifié sur le groupe de liaison 14 (6.*TTCI.14, 7.TTC.14*). Cette région chromosomique, entre le marquer SSR ‘ORS1152_1’ et le gène candidat ‘HuCL04260C001’ semble avoir un rôle important pour le contrôle de tocophérol en condition de sécheresse et semis tardif. Le gène candidat, *HPPD*, qui code pour l’

enzyme impliqué dans la biosynthèse du tocophérol, a été cartographié sur le groupe de liaison LG14 entre les deux marqueurs ‘ORS1152_1’ et ‘ORS391’ . L’induction de *HPPD* a été rapporté par Trebst et al. (2002) en condition de stress. Une augmentation de la synthèse de tocophérol en condition de stress modéré et une diminution de la synthèse de tocophérol en condition contrainte sévère ont été rapportés par Munné-Bosch (2005). *PSI P700* à été cartographié sur le groupe de liaison 8 par la technique HRM . L'interdépendance entre l’activité ‘PS II et PSI’ et le montant de tocophérol a également été démontré (Trebst et al. 2002). Un QTL majeur pour le pourcentage d’huiles (*PSO. PI.16*) a été identifié sur le groupe de liaison 16 entre les marqueurs ‘ORS492_2’ et ‘ORS899’. Cette région chromosomique est importante pour la teneur en huile chez le tournesol. Il est également rapporté par Tang et al. (2006) et Ebrahimi et al. (2008) pour la teneur en huile de graines. Une association significative et négative entre le pourcentage d’huiles et le teneur en acide stéarique (chapitre 3) est justifiée par des effets additifs contraires de leurs QTL co-localisés . Nous avons également détecté une région chromosomique sure le groupe de liaison 17, où les QTL contrôlant le pourcentage d’huiles et la teneur en acide palmitique ont été co-localisés avec un QTL de teneur en acide linoléique (*PAC-LS.17*, *LAC-LS.17* et *PSO-LS.17*). Cette région chromosomique est situé entre les marqueurs SSR ‘ORS297’ et ‘ORS1040’.

Des analyses association génétique ont permis d’identifier des QTL d’intérêt sur les groupes de liaison 2, 10 et 13 pour les caractères morphologiques et agronomiques, d’autres QTL identifiés sur les groupes de liaison 9 et 12 mettent en avant l'importance de ces régions génomiques pour les caractères de morphologie foliaire. En condition de stress partiel, plusieurs co-localisations de QTL contrôlant les caractères morphologique et agronomique ont été détectés, par exemple sur le groupe de liaison 2 pour le poids de 1000 graines et le rendement en poids de graines par plante (*TGW-P-2-1* et *GYP-P-2-1*), le groupe de liaison 4 pour la durée de surface foliaire post floraison et le rendement en poids de graines par plante (*LAD-P-4-1* et *GYP-P-4-1*), le groupe de liaison 10 pour la hauteur de la plante et la durée de surface foliaire post floraison (*PH-P-10-1* et *LAD-P-10-1*) et le groupe de liaison 13 pour le diamètre du capitule et le rendement en poids de graines par plante (*HD-P-13-1* and *GYP-P-13-1*).

Nous avons détecté une région chromosomique sur le groupe de liaison 2, où le QTL contrôlent la teneur en acide linoléique a été co-localisés avec des QTL de surface foliaire à la floraison et teneur en tocophérol (*5.TTC.2, LAC-PI.2* et *LAF-W.2.1*). Cette région est liée à un marquer SSR « ORS229». Nous avons également détecté une région chromosomique sur le groupe de liaison 5, où le QTL contrôlent la teneur en acide stéarique a été co-localisés avec des QTL de surface foliaire à la floraison et rendement en poids de graines (*GYP-WI.5.1*, *LAF-PI.5.1* et *SAC-LS.5*). Cette région est liée au marquer SSR, 'ORS533'.Co-localisasions entre les QTL contrôlant la teneur en acide palmitique et le rendement en poids de graines par plante à été observé sur le groupe de liaison 6. Cette région est liée au marquer SSR, 'ORS1233'. Nous remarquons deux co-localisations de QTL contrôlant des caractères de qualité d'huile, qualité de la graine et morphophysiologiques du tournesol sur le groupe de liaison 10. Un tel résultat pourrait s'expliquer par des effets pléiotropiques des gènes (QTL) ou par des loci étroitement liés. Les trois QTL contrôlent le pourcentage de protéines, la biomasse totale par plante et la durée de surface foliaire post floraison sont situés dans la même position sur le groupe de liaison 11. Cette région est liée à un marquer SSR « HA3446». Plusieurs QTL contrôlent la qualité de l'huile (teneur en tocophérol et acide linoléique) et les caractères morphophysiologiques (le rendement en poids de graines par plante, le pois du capitule et le diamètre du capitule) sont situés dans la même position sur le groupe de liaison 13. Cette région est liée à un marquer SSR « ORS630».

L'augmentation du niveau de tocophérol dans les lignées mutantes, M8-862-1N1 et M8-641-2-1, est justifiée par le polymorphisme observé pour le gène, *MCT*, impliqué dans la voie métabolique du tocophérol. Le marqueur le plus important pour le contenu en tocophérol total est E33M50_16 qui explique 33,9% de la variation phénotypique. Un des gènes candidats les plus important concernant la biosynthèse des acides gras, *FAD2 (FAD2-1),* est lié à la teneur en acides oléique et linoléique. Il explique plus de 52% de la variation phénotypique. Trois gènes de *FAD2* (*FAD2-1, FAD2-2 et FAD2-3)* ont été isolés chez le tournesol, alors que deux ont été identifié chez le coton et soja et un seul chez *Arabidopsis* (Heppard et al. 1996, Liu et al. 1997, Martinez-Rivas et al. 2001). Pour le gène *FAD2_1,* une combinaison d'amorces (chapitre 7) a montré une

bande spécifique d'environ 1,5 kb pour les deux lignées mutantes M8-826-2-1 et M8-39-2-1. En revanche, les combinaisons d'amorces correspondant aux gènes; *FAD2_2* et FAD2_3 ont montré des bandes non spécifiques chez les mutants et la lignée d'origine. Ces résultats suggèrent que la mutation du gène *FAD2_1* par irradiation aux rayons gamma entraine une haute teneur en acide oléique. Chez la lignée mutante M8-52-1-1, le niveau d'huile est significativement augmenté par rapport à la lignée d'origine, AS613, ainsi que le nombre de polymorphismes observé pour les gènes stearoyl ACP desaturase, *SEC14*, *CAT, POD* et *PGT*. Pour le gène *Dehydrin*, une combinaison d'amorces (chapitre 7) a montré une bande spécifique d'environ 1kb pour les quarte lignées mutantes M8-417-1, M8-826-2-1, M8-39-2-1 et M8-186-1.

Perspectives

Ce travail pourrait être développé par :

1) la réalisation d'expérimentations complémentaires dans différents environnements pédoclimatiques et dans d'autres fonds génétiques, ce qui permettrait de valider les QTL constitutifs et les QTL spécifiques aux environnements

2) l'identification de nouveaux polymorphismes moléculaires associés aux caractères de la qualité de l'huile, des graines et des caractères morpho-physiologiques.

3) la réalisation de croisements entre les RILs présentant un polymorphisme dans l'intervalle de la zone étudiée. Après recombinaison génétique, il devrait être possible d'identifier des génotypes présentant des recombinaisons dans cette zone et d'évaluer ces génotypes pour identifier les marqueurs étroitement liés au caractère étudié.

4) la cartographie fine et la réalisation de contig de BAC au voisinage des QTL, ce qui permettrait d'envisager le clonage positionnel.

5) l'analyse de l'expression d'un grand nombre de gènes à l'aide de la technique microarray qui peut aider à mieux comprendre les mécanismes impliqués dans le contrôle de la qualité de l'huile et de la qualité de la graine du tournesol. Cette technique peut être déployée pour deux objectifs :

a- Analyser l'expression de gènes en utilisant les génotypes contrastés et corréler le niveau d'expression des gènes aux différences phénotypiques relatives aux caractères de qualité de la graine.

b- Analyser l’expression des gènes sur toutes les lignées recombinantes (RILs) issues du croisement 'PAC 2' × 'RHA266' pour identifier les QTL contrôlant l’expression de gène (eQTL). Cette dernière approche permettrait de localiser les QTL impliqués dans l’expression différentielle globale des gènes et, par conséquent, d’associer la variation phénotypique avec la variation transcriptomique. L’analyse du transcripotome par puce à ADN peut fournir des informations quantitatives sur plusieurs dizaines de milliers de gènes simultanément.

6) TILLING sur les gènes candidats pour leur validation fonctionnelle.

References

Abou Al Fadil, T., Dechamp-Guillaume, G., Poormohammad Kiani, S. and Sarrafi, A. 2004. Genetic variability and heritability for resistance to black stem (*Phoma macdonaldii*) in sunflower (*Helianthus annuus* L.). J. Genet. Breed.. 58: 323-328.

Ahloowalia, B.S. and Maluszynski, M. 2001. Induced mutations- A new paradigm in plant breeding. Euphytica 118: 167-173.

Alejo-James, A., Jardinaud, M.F., Maury, P., Aliber, J., Gentzbittel, L., Sarrafi, A., Grieu, P. and Petitprez, M. 2004. Genetic variation for germination and physiological traits in sunflower mutants induced by gamma rays. J. Genet. Breed. 58: 285-294.

Alignan, M., Roche, J., Vear, F., Vincourt, P., Bouniols, A., Cerny, M., Mouloungui, Z. and Merah, O. 2008. Variability and genetic analysis of sterols content in sunflower seeds, Internat. sunflower conference Cordoba.

Alza, J.O., Fernandez-Martinez, J.M. 1997. Genetic analysis of yield and related traits in sunflower (*Helianthus annuus* L.) in dryland and irrigated environments. Euphytica 95: 243-251.

Amar, S., Ecke, W., Becker, H., and Möllers, C. 2008. QTL for phytosterol and sinapate ester content in *Brassica napus* L. collocate with the two erucic acid genes. Theor. Appl. Genet. 116: 1051-1061.

Andrade, F. H. 1995. Analysis of growth and yield of maize, sunflower and soybean grown at Balcarce, Argentina. Field Crops Res. 41: 1–12.

Ayerdi Gotor, A., Farkas, E., Berger, M., Labalette, F., Centis, S., Daydé, J., and Calmon, A. 2007. Determination of tocopherols and phytosterols in sunflower seeds by NIR spectrometry. Eur. J. Lipid Sci. Technol. 109: 525–530.

Ayerdi Gotor, A., Berger, M., labalette, F., Centis, S., Dayde J., Calmon, A. 2008. Near infrared spectrometry (NIRS) prediction of minor components in sunflower seeds. Proc. 17th International Sunflower Conference, June 8-12, 2008. Córdoba (Spain), vol.2:763-769.

Bachlava, E., Dewey, R.E., Auclair, J., Wang, S., Burton, J.W. and Cardinal, A.J. 2008. Mapping genes encoding microsomal ω-6 desaturase enzymes and their cosegregation with QTL affecting oleate content in soybean. Crop Sci. 48: 640–650.

Baldini, M., Giovanardi, R., Tahmasebi-Enferadi, S., Vannozzi, G.P. 2002. Effects of water regime on fatty acid accumulation and final fatty acid composition in the oil of standard and high oleic sunflower hybrids. Ital. J. Agron. 16: 119–126.

Bange, M.P., Hammer, G.L. and Rickert, K.G. 1997. Environmental control of potential yield of sunflower in the subtropics. Aust. J. Agric. Res. 48: 231–240.

Basten, C.J., Weir, B.S. and Zeng, Z.B. 2002. QTL Cartographer version 1.16, Programme in Statistical Genetics, Department of statistics, North Carolina State University.

Beard, B.H. and Geng, S. 1982. Interrelationships of morphological and economic characters of sunflower. Crop Sci. 22: 817–822.

Beavis, W.D. 1994. The power and deceit of QTL experiments: lessons from comparative studies. In Proceedings of the 49th Annual Corn and Sorghum Industry Research Conference, Chicago, 7–8 December 1994. Edited by D. Wilkinson. American Seed Trade Association, Washington. pp. 250–266.

Berry, S.T., Leon, A.J., Hanfrey, C.C., Challis, P., Burkolz, A., Barnes, S.R., Rufener, G.K., Lee, M. and Caligari, P.D.S. 1995. Molecular-marker analysis of *Helianthus annuus* L. 2. Construction of an RFLP map for cultivated sunflower. Theor. Appl. Genet. 91: 195–199.

Bert, P.F., Dechamp-Guillaume, G., Serre, F., Jouan, I., de Labrouhe, D.T., Nicolas, P. and Vear, F. 2004. Comparative genetic analysis of quantitative traits in sunflower (*Helianthus annuus* L.). 3. Characterisation of QTL involved in resistance to *Sclerotinia sclerotiorum* and *Phoma macdonaldii*. Theor. Appl. Genet. 109: 865–874.

Berville, A., Lacombe, S., Veillet, S., Granier, C., Leger, S. and Jouve P. 2009. Method of selecting sunflower genotypes with high oleic acid content in seed oil. United States Patent Application 20090258346.

Bhatia, C.R., Nichterlein, K. and Maluszynski, M. 1999. oilseed cultivars developed from induced mutations and mutations altering fatty acid composition. Mutation Breeding. No. 11. Joint FAO/IAEA division of nuclear techniques in food and agriculture and FAO/IAEA agriculture and biotechnology laboratory, Seibersdorf International Atomic Energy Agency Vienna .

Biskupek-Korell, B. and Moschner, C.R. 2007. Near-Infrared Spectroscopy (NIRS) for quality assurance in breeding, cultivation and marketing of high-oleic sunflowers. Helia 29: 73-80.

Bramley, P.M., Elmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A. and Wagner. K.H. 2000. Vitamin E. J. Sci. Food Agric. 80: 913–938.

Browse, J. and Slack, R. 1983. The effect of temperature and oxygen on the rates of fatty acid synthesis and oleate desaturation in safflower *(Carthamus tinetorius)* seed. Biochim. Biophys. Acta 753: 145-152.

Burke, J.M., Knapp, S.J. and Rieseberg, L.H. 2005. Genetic consequences of selection during the evolution of cultivated sunflower. Genetics 171: 1933–1940.

Cantisán, S., Martínez-Force, E. and Garcés, R. 2000. Enzymatic studies of high stearic acid sunflower seed mutants. Plant Physiology and Biochemistry 38: 377-382.

Calmon, A., Ayerdi Gotor, A., Berger, M., labalette, F., Moreau P., Gaillard, A., Dayde, J. 2009, Near infrared spectroscopy (NIRS) for improvement in sunflower breeding for fatty acids and minor components. Word congress on oils and fats & 28th ISF Congress. September 27-30, 2009. Sydney (Australia).

Cela, J., Falk, J. and Munné-Bosch, S. 2009. Ethylene signaling may be involved in the regulation of tocopherol biosynthesis in Arabidopsis thaliana. FEBS Letters. 583: 992-996.

Cellier, F., Conejero, G., Breitler, J.C. and Casse, F. 1998. Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower accumulation of dehydrin transcripts correlates with tolerance. Plant Physiol. 116: 319–328.

Close, T.J., Fenton, R.D. and Moonan, F.A. 1993. View of plant dehydrins using antibodies specific to the carboxy terminal peptide. Plant Mol. Biol. 23: 279–286.

Champolivier, L.and Merrien, A. 1996. Evolution de la teneur en huile et de sa composition en acides gras chez deux variétés de tournesol (oléique ou non) sous l'effet de températures différentes pendant la maturation des graines. OCL 3: 140-144.

Collakova, E., and DellaPenna, D. 2003. The role of homogentisate phytyltransferase and other tocopherol pathway enzymes in the regulation of tocopherol synthesis during abiotic Stress. Plant Physiol. 133: 930-940.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B.and Pang, E.C.K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and market-assisted selection for crop improvement: The basic concepts. Euphytica 142: 169-196.

Demurin, Y. 1993. Genetic variability of tocopherol composition in sunflower seeds. Helia. 16: 59–62.

D'Harlingue, A. and Camara, B. 1985. Plastid enzymes of terpenoid biosynthesis. Purification and characterization of gamma-tocopherol methyltransferase from Capsicum chromoplasts. The Journal of Biological Chemistry 260: 15200-15203.

Dorrell, D.G. and Vick, B.A. 1997. Properties and processing of oilseed sunflower. In: A. A. Schneiter (ed.), Sunflower Production and Technology. Agron. Monogr. 35: 709-746. ASA, CSSA and SSSA, Madison.

Dure III, L., Crouch, M., Harada, J., Ho, T-HD, Mundy, J., Quatrano, R., Thomas, T. and Sung, Z.R. 1989. Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol. Biol. 12: 475–486.

Dwivedi, S.L., Nigam, S.N., Rao, R.C.N., Singh, U. and Rao, K.V.S. 1996. Effect of drought on oil, fatty acids and protein contents of groundnut (*Arachis hypogaea* L.) seeds. Field Crops Res. 48: 25–133.

Ebrahimi, A., Maury, P., Berger, M., Poormohammad Kiani, S., Nabipour, A., Shariati, F., Grieu, P. and Sarrafi, A. 2008. QTL mapping of seed-quality traits in

sunflower recombinant inbred lines under different water regimes. Genome 51: 599-615.

Ebrahimi, A., Maury, P., Berger, M., Calmon, A., Grieu, P. and Sarrafi, A. 2009. QTL mapping of protein content and seed characteristics under water-stress conditions in sunflower. Genome 52: 419-430.

Endrigkeit J, Wang X, Cai D, Zhang C, Long Y, Meng J and JungC, Genetic mapping, cloning, and functional characterization of the BnaX.VTE4 gene encoding a gamma-tocopherol methyltransferase from oilseed rape. *Theor Appl Genet* 119:567-575 (2009).

Endrigkeit, J., Wang, X., Cai, D., Zhang, C., Long, Y., Meng, J. and Jung, C. 2009. Genetic mapping, cloning, and functional characterization of the BnaX.VTE4 gene encoding a gamma-tocopherol methyltransferase from oilseed rape. Theor. Appl. Genet. 119: 567-575.

Evans, L.E. 1993. Crop evolution, adaptation and yield, New York, Cambridge.

Fernández-Martínez, J., Jimenez, A., Dominguez, J., Garcia, J.M., Garces, R. and Mancha, M. 1989. Genetic analysis of the high oleic acid content in cultivated sunflower (*Helianthus annuus* L.). Euphytica 41: 39–51.

Fernández-Martínez, J.M., Osorio, J., Mancha, M. and Garcés, R. 1997. Isolation of high palmitic mutants on high oleic background. Euphytica 97:113-116.

Fick, G.N. 1978. Sunflower breeding and genetics. In: Carter JF (ed) Sunflower science and technology. ASA, CSSA and SSSA, Madison, pp 279–327.

Fick, G.N. and Miller, J.F. 1997. The genetics of sunflower. In: Shneither AA (ed) Sunflower technology and production. ASA, CSSA and SSSA, Madison, pp 441–495.

Flagella, Z., Rotunno, T., Tarantino, E., Di Caterina, R., De Caro, A. 2002. Changes in seed yield and fatty acid composition of high oleic sunflower (*Helianthus annuus* L.) hybrids in relation to the sowing date and the water regime. Eur. J. Agron. 17: 221–230.

Flores Berrios, E., Gentzbittel, L., Kayyal, H., Alibert, G. and Sarrafi, A. 2000a. AFLP mapping of QTLs for in vitro organogenesis traits using recombinant inbred

lines in sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 101: 1299–1306.

Flores Berrios, E., Sarrafi, A., Fabre, F., Alibert, G. and Gentzbittel, L., 2000b. Genotypic variation and chromosomal location of QTLs for somatic embryogenesis revealed by epidermal layers culture of recombinant inbred lines in the sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 101: 1307–1312.

Flores Berrios, E., Gentzbittel, L., Mokrani, L., Alibert, G. and Sarrafi, A. 2000c. Genetic control of early events in protoplast division and regeneration pathways in sunflower. Theor. Appl. Genet. 101: 606–612.

Foolad, M.R., Zhang, L.P. and Subbiah, P. 2003. Genetic of drought tolerance during seed germination in tomato, inheritance and QTL mapping, Genome 46: 536–545.

Garcés, R. and Mancha, M. 1991. *In vitro* oleate desaturase in developing sunflower seeds. Phytochemistry 30: 2127-2130.

Gedil, M.A., Berry, S.K., Jones, R., Leon, A., Wye, C., Peleman, J. and Knapp, S.J. 2001. An integrated RFLP-AFLP linkage map for cultivated sunflower. Genome 44: 213–221.

Gentzbittel, L., Vear, F., Zhang, Y.-X., Bervillé, A. and Nicolas, P. 1995. Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 90: 1079–1086.

Gentzbittel, L., Mestries, E., Mouzeyar, S., Mazeyrat, F., Badaoui, S., Vear, F., Tourvieille de Labrouhe, D. and Nicolas, P. 1999. A composite map of expressed sequences and phenotypic traits of the sunflower (*Helianthus annuus* L.) genome. Theor. Appl. Genet. 99: 218–234.

Gimenez, C. and Fereres, E. 1986. Genetic variability in sunflower cultivars under drought. II. Growth and water relations. Aust. J. Agric. Res. 37: 583–597.

Gonzàlez-Pérez, S. and Vereijken, J.M. 2007. Sunflower proteins: overview of their physicochemical, structural and functional properties. J. Sci. Food Agric. 87: 2173–2191.

Gül, M., Egesel, C. and Turhan, H. 2008. The effects of planting time on fatty acids and tocopherols in chickpea. European Food Research and Technology. 226: 517-522.

Gupta, S., Subrahmanyam, D. and Rathore, V.S. 1994. Influence of sowing dates on yield and oil quality in sunflower. Journal of Agronomy and Crop Science 172: 137 – 144.

Harris, H.C, William, J.R. and Mason, W.K. 1978. Influence of temperature on oil content and composition of sunflower seed. Aus. J. Agric. Res. 29: 1203-1212.

Hass, C.G., Tang, S., Leonard, S., Traber, M., Miller, J.F. and Knapp, S.J. 2006. Three non-allelic epistatically interacting methyltransferase mutations produce novel tocopherol (vitamin E) profiles in sunflower. Theor. Appl. Genet. 113: 767-782.

Heppard, E.P., Kinney, A.J., Stecca, K.L. and Miao, G.H. 1996. Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. Plant Physiol. 110: 311-319.

Hervé, D., Fabre, F., Flores Berrios, E., Leroux, N., Al Chaarani, G., Planchon, C., Sarrafi, A. and Gentzbittel, L. 2001. QTL analysis of photosynthesis and water status traits in sunflower (*Helianthus annuus* L.) under greenhouse conditions. J. Exp. Bot. 52: 1857– 1864.

Heiser, C.B., Smith, D.M., Clevenger, S.B. and Martin, W.C. 1969. The North American sunflowers (Helianthus). Mem. Torr. Bot. Club 22 : 1–218.

Jan, C.C. and Seiler, G. 2007. Sunflower. In: R.J. Singh (Ed.), Oilseed Crops, Genetic Resources, Chromosome Engineering, and Crop Improvement. CRC Press, Boca Raton, FL, USA, Vol. 4, pp. 103–165.

Jan, C.C., Vick, B.A., Miller, J.F., Kahler, A.L. and Butler, E.T. 1998. Construction of an RFLP linkage map for cultivated sunflower. Theor. Appl. Genet. 96: 15–22.

Jasso de Rodriguez, D., Phillips, B.S., Rodriguez-García, R. and Angulo-Sánchez, J.L. 2002. Grain yield and fatty acid composition of sunflower seed for cultivars developed under dry land conditions. In: J. Janick and A. Whipkey (eds.), Trends in new crops and new uses. ASHS Press, Alexandria, VA. 139–142.

Kamal-Eldin, A. and Andersson, R. 1997. A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. Journal of the American Oil Chemists' Society. 74: 375-380.

Kanwischer, M., Porfirova, S., Bergmuller, E. and Dörmann, P. 2005. Alterations in tocopherol cyclase activity in transgenic and mutant plants of arabidopsis affect tocopherol content, tocopherol composition, and oxidative stress. Plant Physiol. 137: 713-723.

Kearesey, M.J. and Pooni, H.S. 1996. The genetical analysis of quantitative traits. London, UK: Chapman and Hall.

Lacombe, S. and Berville, A. 2001. A dominant mutation for high oleic acid content in sunflower (*Helianthus annuus* L.) seed oil is genetically linked to a single oleate-desaturase RFLP locus. Mol. Breed. 8: 129–137.

Lagravère, T., Champolivier, L., Lacombe, S., Kleiber, D., Bervillé, A. and Daydé, J. 2000. Effects of temperature variations on fatty acid composition in oleic sunflower oil (*Helianthusannuus L.*) hybrids. Proc. 15th International Sunflower Conference, Toulouse, France.Tome I, A: 73-78.

Lagravère, T., Kleiber, D., Surel, O., Calmon, A., Berville, A. and Daydé, J. 2004. Comparison of fatty acid metabolism of two oleic and one conventional sunflower hybrids: a new hypothesis. J. Agron. Crop Sci. 190: 223–229.

Lee, S.H., Bailey, M.A., Mian, M.A.R., Carter, T.E., Shipe, E.R., Ashley, D.A., Parrott, W.A., Hussey, R.S. and Boerma, H.R. 1996. RFLP loci associated with soybean seed protein and oil content across populations and locations. Theor. Appl. Genet. 93: 649-657.

Leon, A.J., Andrade, F.H. and Lee, M. 2003. Genetic analysis of seed oil percentage across generations and environments in sunflower (*Helianthus annuus* L.). Crop Sci. 43: 135–140.

Liu, Q., Singh, S.P., Brubaker, C.L., Sharp, P.J., Green, A.G. and Marshall, D.R. 1997. Isolation and characterization of two different microsomal ω-6 desaturase genes in cotton (*Gossypium hirsutum* L.). In: Williams J.P., Khan M.U. and Lem N.W. (eds), Physiology, Biochemistry and Molecular Biology of Plant Lipids. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 383–385.

Machacek, C. 1979. Study of the inheritance of earliness in sunflower (*Helianthus annuus* L.). Genet Sel .15: 225–232.

Mahmood, T., Rahman, M., Stringam, G., Yeh, F. and Good, A. 2006. Identification of quantitative trait loci (QTL) for oil and protein contents and their relationships with other seed quality traits in *Brassica juncea*. Theor. Appl. Genet. 113: 1211-1220.

Martínez-Rivas, J.M., Sperling, P., Lühs, W. and Heinz, E. 2001. Spatial and temporal regulation of three different microsomal oleate desaturase genes (*FAD2*) from normal-type and high-oleic varieties of sunflower (*Helianthus annuus* L.). Molecular Breeding 8: 159-168.

Marwede, V., Gul, M.K., Becker, H.C. and Ecke, W. 2005. Mapping of QTL controlling tocopherol content in winter oilseed rape. Plant Breeding. 124: 20-26.

Maury, P., Berger, M., Mojayad, F. and Planchon, C. 2000. Leaf water characteristics and drought acclimation in sunflower genotypes. Plant Soil 223: 153–160.

Mestries, E., Gentzbittel, L., Tourvieille de Labrouhe, D., Nicolas, P. and Vear, F. 1998. Analysis of quantitative trait loci associated with resistance to *Sclerotinia sclerotiorum* in sunflower (*Helianthus annuus* L.) using molecular markers. Mol. Breed 4: 215–226.

Micic, Z., Hahn, V., Bauer, E., Schon, C.C. and Melchinger, A.E. 2005. QTL mapping of resistance to Sclerotinia midstalk-rot in RIL of sunflower population NDBLOSsel x CM625. Theor. Appl. Genet. 110: 1490–1498.

Miller, B.C., Oplinger, E.S., Rand, R., Peters, J. and Weis, G. 1984: Effect of planting date and plant population on sunflower performance. Agron. J. 76: 511–515.

Mokrani, L., Gentzbittel, L., Azanza, F., Fitamant, L., Al-Chaarani, G. and Sarrafi, A. 2002. Mapping and analysis of quantitative trait loci for grain oil and agronomic traits using AFLP and SSR in sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 106: 149–156.

Möllers, C. and Schierholt, A. 2002. Genetic variation of palmitate and oil content in a winter oilseed rape doubled haploid population segregating for oleate content. Crop Sci. 42: 379–384.

Munné-Bosch, S. and Alegre, L. 2002. Plant aging increases oxidative stress in chloroplasts. Planta 214: 608–615.

Munné-Bosch, S. and Falk, J. 2004. New insights into the function of tocopherols in plants. Planta. 218: 323-326.

Munné-Bosch, S. 2005. The role of alpha-tocopherol in plant stress tolerance. J. Plant Physiol. 162: 743-748.

Muriel, J.L. and Downes, R.W. 1974. Effects of periods of moisture stress during various phases of growth of sunflower in the greenhouse. *In* Proceedings of the 6th International Sunflower Conference, Bucharest, Romania, 22–24 July 1974. *Edited by* A.V. Vranceanu. ISA Bucharest, Romania. pp. 127–131.

Nabipour, A., Yazdi-Samadi, B. and Sarrafi, A. 2004. Genetic control of some morphological mutants in sunflower. J. Genet. Breed. 58: 157-162.

Neidleman, S.L. 1987. Effects of temperature on lipid unsaturation. Biotechnol Genet. Eng. Rev. 5: 245-268.

Nel, A.A., Loubser, H.L. and Hammes, P.S. 2002. Development and validation of relationships between sunflower seed quality and easily measurable seed characteristics for grading purposes. S. Afr. J. Plant Soil 19: 201–205.

Norris, S.R., Shen, X. and Della Penna, D. 1998. Complementation of the arabidopsis pds1 mutation with the gene encoding p-hydroxyphenylpyruvate dioxygenase, Plant Physiol. 117: 1317-1323.

Norris, S.R., Lincoln, K., Abad, M.S., Eilers, R., Hartsuyker K.K., Hirschberg, J., Karunanandaa, B., Moshiri, F., Stein, J., Valentin, H.E. and Venkatesh, T.V. 2004. Tocopherol biosynthesis related genes and uses thereof. United States Patent 7230165 .

Ostlund, R.E. 2002. Phytosterols in human nutrition, Annual Review of Nutrition 22: 533-549.

Osorio, J., Fernández-Martínez, J.M., Mancha, M. and Garcés, R.1995. Mutant sunflower with high concentration in saturated fatty acid in the oil. Crop Sci. 35: 739-742.

Ouvrard, O., Cellier, F., Ferrare, K., Tousch, D., Lamaze, T., Dupuis, J.M. and Casse-Delbart, F. 1996. Identification and expression of water stress- and abscisic acid-regulated genes in a droughttolerant sunflower genotype. Plant Mol Biol 31: 819–829(1996).

Ozturk, A. and Aydin, F. 2004. Effect of water stress at various growth stages on some quality characteristics of winter wheat. J. Agron. Crop Sci. 190: 93–99.

Pankovic, D., Sakac, Z., Kevresan, S. and Plesnicar, M. 1999. Acclimation to longterm water deficit in the leaves of two sunflower hybrids: photosynthesis, electron transport and carbon metabolism. J. Exp. Bot. 330: 127–138.

Pérez-Vich, B., Velasco, L. and Fernández-Martínez, J. 1998. Determination of seed oil content and fatty acid composition in sunflower through the analysis of intact seeds, husked seeds, meal and oil by near-infrared reflectance spectroscopy. Journal of the American Oil Chemists' Society 75: 547-555.

Pérez-Vich, B., Garcés, R. and Fernández-Martínez, J.M. 2002a. Inheritance of high palmitic acid content in the sunflower mutant CAS-12 and its relationship with high oleic content. Plant Breeding 121: 49-56.

Pérez-Vich, B., Fernández-Martínez, J.M., Grondona, M., Knapp, S.J. and Berry, S.T. 2002b. Stearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. Theor. Appl. Genet. 104: 338-349.

Pérez-Vich, B., Muñoz-Ruz, J. and Fernández-Martínez, J.M. 2004. Developing midstearic acid sunflower lines from a high stearic acid mutant, Crop Sci. 44:70-75.

Pérez-Vich, B., Leon, A.J., Grondona, M., Velasco, L., and Ferna´ndez-Martı´nez, J.M. 2006. Molecular analysis of the high stearic acid content in sunflower mutant CAS-14. Theor. Appl. Genet. 112: 867–875.

Pleite, R., Martinez-Force, E. and Garces, R. 2006. Increase of the stearic acid content in high-oleic sunflower (*Helianthus annuus*) seeds. Journal of Agricultural and Food Chemistry 54: 9383-9388.

Poormohammad Kiani, S., Talia, P., Maury, P., Grieu, P., Heinz, R., Perrault, A., Nishinakamasu, V., Hopp, E., Gentzbittel, L., Paniego, N. and Sarrafi, A.

2007a. Genetic analysis of plant water status and osmotic adjustment in recombinant inbred lines of sunflower under two water treatments. Plant Science. 172: 773-787.

Poormohammad Kiani, S. 2007. Analyse génétique des réponses physiologiques du tournesol (*Helianthus annuus* L.) soumis à la sécheresse. Ph.D. thesis. Doctorat de l'institut national polytechnique de Toulouse. pp. 148-163.

Poormohammad Kiani, S. 2007b. Analyse génétique des réponses physiologiques du tournesol (*Helianthus annuus* L.) soumis à la sécheresse. Ph.D. thesis. Doctorat de l'institut national polytechnique de Toulouse. pp. 59-84

Poormohammad Kiani, S., Maury, P., Nouri, L., Ykhlef, N., Grieu, P. and Sarrafi, A. 2009. QTL analysis of yield-related traits in sunflower under different water treatments. Plant Breed 128: 363-373.

Porfirova, S., Bergmuller, E., Tropf, S., Lemke, R. and Dormann, P. 2002. Isolation of an Arabidopsis mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis, Proceedings of the National Academy of Sciences of the United States of America. 99: 12495-12500.

Prieto Iosada, H. 1992. Response to drought conditions of sunflower genotypes differing in yield potential and length of cycle. PhD Thesis University of Cordoba (in Spanish).

Qasim, M., Qamer, M., Alam F. and Alam M. 2008. Sowing dates effect on yield and yield components of different wheat varieties. J. Agric. Res. 46: 135-140.

Rachid Al-Chaarani, G., Roustaee, L., Gentzbittel, L., Mokrani, L., Barrault, G., Dechamp-Guillaume, G. and Sarrafi, A 2002. A QTL analysis of sunflower partial resistance to downy mildew (*Plasmopara halstedii*) and black stem (*Phoma macdonaldii*) by the use of recombinant inbred lines (RILs). Theor. Appl. Genet. 104: 490–496.

Rachid Al-Chaarani, G., Gentzbittel, L., Huang, X. and Sarrafi, A. 2004. Genotypic variation and identification of QTLs for agronomic traits using AFLP and SSR in recombinant inbred lines of sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 109: 1353-1360.

Rachid Al-Chaarani, G., Gentzbittel, L., Barrault, G., Lenoble, S. and Sarrafi, A. 2004. The effect of gamma rays and genotypes on sunflower organogenesis traits. J. Genet. Breed 58:73-78.

Rachid Al-Chaarani, G., Gentzbittel, L., Wedzony, M. and Sarrafi, A. 2005. Identification of QTLs for germination and seedling development in sunflower (*Helianthus annuus* L.). Plant Sci. 169: 221–227.

Richards, R.A. 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. J. Exp. Bot. 51: 447-458.

Robinson, R.G. 1970. Sunflower date of planting and chemical composition at various growth stages. Agron. J. 62: 665–666.

Russel, W.A. 1991. Genetic improvement of maize yields. Adv. Agron. 46: 245-298.

Saito, K., Tautz, L. and Mustelin, T. 2007. The lipid-binding SEC14 domain. Biochim. Biophys. Acta.1771: 719-726.

Sarrafi, A., Kayyal, H., Rachid Al-Chaarani, G., Cantin, F., Chaline, A.S. and Durielle, A.S. 2000. Inheritance of organogenesis parameters in cotyledons of sunflower (*Helianthus annuus* L.). J Genet. Breed. 54: 227-231.

SAS Institute Inc. 1996. SAS/STAT user's guide, version 6, 4th ed., Vols. 1 and 2. SAS Institute Inc., Cary, N.C.

Sattler, S.E., Cahoon, E.B., Coughlan, S.J. and DellaPenna, D. 2003. Characterization of Tocopherol Cyclases from Higher Plants and Cyanobacteria. Evolutionary Implications for Tocopherol Synthesis and Function, Plant Physiol. 132: 2184-2195.

Schiex, T. and Gaspin, C. 1997. CARTHAGENE, construction and joining maximum likelihood genetic maps, in: Proceeding of ISMB, vol. 5, Porto Carras, Halkidiki, Greece, pp. 258–267.

Schuppert, G.F., Tang, S., Slabaugh, M.B. and Knapp, S.J. The sunflower high-oleic mutant *Ol* carries variable tandem repeats of *FAD2-1*, a seed-specific oleoyl-phosphatidyl choline desaturase. Mol. Breeding 17: 241–256.

Semchuk, N.M., Lushchak, O.V., Falk, J., Krupinska, K. and Lushchak V.I. 2009. Inactivation of genes, encoding tocopherol biosynthetic pathway enzymes,

results in oxidative stress in outdoor grown Arabidopsis thaliana. Plant Physiology and Biochemistry. 47: 384-390.

Shintani, D.K., Cheng, Z. and DellaPenna, D. 2002 The role of 2-methyl-6-phytylbenzoquinone methyltransferase in determining tocopherol composition in Synechocystis sp. PCC6803. FEBS Letters. 511: 1-5.

Smith, S.A., King, R.E. and Min, D.B. 2007. Oxidative and thermal stabilities of genetically modified high oleic sunflower oil. Food Chem 102:1208-1213.

Soldatov, K.I. 1976. Chemical mutagenesis in sunflower breeding. In: Proc 7th Intl. Sunflower Conf Krasnodar, USSR, Vlaardingen, the Netherlands: Intl. Sunflower Assoc., pp. 352–357.

Tang, S., Yu, J.K., Slabaugh, M.B., Shintani, D.K. and Knapp, S.J. 2002. Simple sequence repeat map of the sunflower genome. Theor. Appl. Genet. 105: 1124–1136.

Tang, S., Kishor, V.K. and Knapp, S.J. 2003. PCR-multiplexes for a genome-wide frame work of simple sequence repeat marker loci in cultivated sunflower. Theor. Appl. Genet. 107: 6-19.

Tang, S., Hass, C.G., and Knapp, S.J. 2006. *Ty3/gypsy*-like retrotransposon knockout of a 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase is non-lethal, uncovers a cryptic paralogous mutation, and produces novel tocopherol (vitamin E) profiles in sunflower. Theor. Appl. Genet. 113: 767-782.

Tang, S., Leon, A., Bridges, W.C. and Knapp, S.J. 2006. Quantitative trait loci for genetically correlated seed traits are tightly linked to branching and pericarp pigment loci in sunflower. Crop Sci. 46: 721–734.

Talha, M. and Osman, F. 1974. Effect of soil water stress on water economy and oil composition in sunflower (*Helianthus annuus* L.). J. Agric. Sci. Cambridge 84: 49–56.

Tezara, W., Mitchall, V., Driscoll, S.P. and Lawlor, D.W. 2002. Effects of water deficit and its interaction with CO2 supply on the biochemistry and physiology of photosynthesis in sunflower. J. Exp. Bot. 375: 1781–1791.

Thompson, G.A.J. 1993. Response of lipid metabolism to developmental change and environmental perturbation. In TS Moore Jr, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 591-619.

Thompson, J.A. and Heenan, D.P. 1994. Effect of sowing time on growth, yield, and oil characteristics of irrigated sunflower in southern New South Wales. Australian Journal of Experimental Agriculture 34: 255 – 258.

Trémoliéres, A., Dubacq, J.P. and Drapier, D.1982. Unsaturated fatty acids in maturing seeds of sunflower and rape: Regulation by temperature and light intensity. Phytochemistry 21: 41-45.

Trebst, A., Depka, B. and Holländer-Czytko H. 2002. A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Letters. 516:156-160.

Unger, P.W. 1986. Growth and development of irrigated sunflower in the Texas high plains. Agron. J. 7: 507-515.

Uslu, N. 1998. Response to inoculation and sowing date of soybean under bafra plain conditions in the northern region of turkey. Tr. J. of Agriculture and Forestry **22:** 525-531.

Valentin, H.E., Lincoln, K., Moshiri, F., Jensen, P.K.**,** Qi, Q., Venkatesh, T.V., Karunanandaa, B., Baszis, SR., Norris, S.R., Savidge, B., Gruys, K.J. and Last, R.L. 2006. The Arabidopsis vitamin E pathway gene5-1 Mutant Reveals a Critical Role for Phytol Kinase in Seed Tocopherol Biosynthesis. Plant Cell. 18: 212-224.

Velasco, L. and Becker, H.C. 1998. Estimating the fatty acid composition of the oil in intact-seed rapeseed (*Brassica napus* L.) by nearinfrared reflectance spectroscopy. Euphytica 101: 221–230.

Velasco, L., Pérez-Vich, B. and Fernández-Martínez, J.M. 2004. Development of sunflower germplasm with high delta-tocopherol content. HELIA. 27: 99-106.

Vega**,** A.J. and Hall**,** A.J. 2002. Effects of planting date, genotype, and their interactions on sunflower yield. Crop Science 42: 1191-1201.

Vega, S., del Rio, A., Bamberg, J. and Palta, J. 2004. Evidence for the up-regulation of stearoyl-ACP (Δ9) desaturase gene expression during cold acclimation. American Journal of Potato Research 81: 125-135.

Vera-Ruiz, E., Velasco, L., Leon, A., Fernández-Martínez, J. and Pérez-Vich, B. 2006. Genetic Mapping of the Tph1 Gene Controlling Beta-tocopherol Accumulation in Sunflower Seeds. Molecular Breeding. 17: 291-296.

Vijayan, K., Srivatsava, P.P., Nair, C.V., Awasthi, A.K., Tikader, A., Sreenivasa, B. and Urs, SR. 2006. Molecular characterization and identification of markers associaterd with yield traits in mulberry using ISSR markers. Plant Breed. 125: 298-301.

Virk, P.S., Ford-Lloyd, B.V., Jackson, M.T., Pooni, H.S., Clemeno, T.P. and Newbury, H.J. 1996. Predicting quantitative variation within rice germplasm using molecular markers. Heredity 76: 296-304.

Wang, S., Basten, C.J. and Zeng, Z.B. 2005. Windows QTL Cartographer V2.5. Department of Statistics, North Carolina State University, Raleigh, N.C.. Available from http://statgen.ncsu.edu/qtlcart/WQTLCart.htm.

Welsch, R., Medina, J., Giuliano, G., Beyer, P. and Von Lintig, J. 2003. Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana.* Planta. 216: 523-534.

Wong, J.C., Lambert, R.J., Tadmor, Y. and Rocheford, T.R. 2003. QTL associated with accumulation of tocopherols in maize. Crop Sci. 43: 2257-2266.

Wu, R., Casella, G. and Ma, C. 2007. Statistical genetics of quantitative traits Linkage, Maps, and QTL, Springer, New York, 2007, pp. 287-302.

Zeng, Z.B. 1994. Precision mapping of quantitative trait loci. Genetics. 136:1457-1

Zhao, J., Becker, H., Zhang, D., Zhang, Y. and Ecke, W. 2006. Conditional QTL mapping of oil content in rapeseed with respect to protein content and traits related to plant development and grain yield. Theor. Appl. Genet. 113: 33-38.

Appendix

Midi préparation d'ADN génomique de tournesol *(N.Pouilly-2008)*

Objectif : Extraire l'ADN de plantes en vue de tests PCR, de digestion par des enzymes de restriction.

Matériel : distributeur sur bouteilles et/ou combi-tips
cônes 10-100µl & 100-1000µl autoclavés
transfert pipettes stériles 7mL
Pour chaque échantillon, prévoir 3 séries de tubes 15mL type Falcon PP.

Matériel végétal :
Le matériel végétal est conservé à -80°C jusqu'à lyophilisation.

Réactifs :

	pour n=1	pour n=32	
Tampon CTAB	5mL	160mL	cf. protocole « Sol_Tampon_CTAB_V1 »
1,4-Dithiothreitol (DTT) 1M	50µl	1,6mL	Ajouter le DTT extemporanément.
Chloroforme/IAA 24/1 (v/v)	10mL	320mL	Pour un mélange préparé à l'avance, conserver dans une bouteille hermétiquement close, sous sorbonne.
Isopropanol	8mL	256mL	Placer à -20°C avant utilisation.
Ethanol 70%	2,5mL	80mL	
Chlorure de sodium NaCl 5M	1,5mL	48mL	Autoclavé.
Tampon TE 10-1 stérile	3mL	96mL	Autoclavé.
RNase 10 mg/ml	1.5µl	48µl	
Tampon TE 10-0.1 stérile	200µl	6,4mL	Autoclavé.

Noter le n° de lot pour l'ensemble des produits utilisés.

Timing :
Vérifier la disponibilité des réactifs et de l'équipement (centrifugeuse, bain-marie).
Une extraction de 32 tubes peut être réalisée (2 séries de 16 tubes en décalé)

Prévention :
- Travailler sous la sorbonne pour les étapes utilisant les réactifs suivants : tampon CTAB, DTT, chloroforme/IAA, isopropanol.
- Prévoir l'élimination des déchets (bidon de 20L)

Méthodologie

En arrivant: Mettre en route le bain-marie à 65°C.
Régler la centrifugeuse sur 10°C.

Préparation des feuilles
Récolter environ 0.5g de feuilles, de préférence jeunes et de bon aspect, dans des tubes de 15mL type Falcon PP correctement identifiés.
Après lyophilisation, ajouter 2 mL de billes de verre par tube et réaliser le broyage avec le mélangeur à peinture.

Extraction

☞ *Sorbonne + gants*

1. Pour chaque échantillon, prévoir 5 mL de tampon CTAB + 50µl DTT 1M dans une bouteille. Faire chauffer le mélange dans le bain-marie pendant une dizaine de minutes (le CTAB froid précipite et peut ainsi faire précipiter l'ADN).

2. Ajouter 5 mL de tampon CTAB/DTT dans chaque tube et bien agiter.
Placer une série de 32 tubes 2 heures à 65°C (détruit les membranes et libère l'ADN). Agiter régulièrement pour décoller la poudre et la laisser s'imbiber.

3. Compléter avec 7mL de chloroforme/IAA 24/1.
Bien homogénéiser en agitant jusqu'à formation d'une soupe (élimination des débris cellulaires, précipitation les protéines restantes).

Mélanger à nouveau en inversant les tubes une vingtaine de fois.

4. Centrifuger à 4200g à 10°C pendant 30 min. Pendant la centrifugation, préparer une nouvelle série de tubes de 15 mL identifiés contenant 5 mL d'isopropanol 100% froid.

5. Récupérer la phase aqueuse (environ 5 mL) à l'aide d'une transfert pipette stérile.
Transférer dans le tube correspondant identifié.

Mélanger avec l'isopropanol en inversant les tubes : l'ADN précipite.

Laisser décanter l'ADN durant 1 heure minimum à 4°C (dans la glace ou au réfrigérateur).

6. Centrifuger 4200g à 4°C pendant 15 min. Eliminer le surnageant et laisser sécher le tube à l'envers sur du papier absorbant pendant ¼ heure.
Attention : vérifier que les culots ne se décrochent pas !

Mettre en route le bain-marie à 37°C.

7. Dissoudre le culot dans 3 mL de TE 10-1 stérile contenant 0.5 µg/mL de RNAse (soit 0.5µl de solution stock à 10 mg/mL par mL de TE).
Placer les tubes à 37°C durant 1 heure, en agitant de temps en temps.

Remarque : la manipulation peut être stoppée à ce stade en conservant les tubes à 4°C jusqu'au lendemain.

8. Ajouter 3 mL de chloroforme-IAA 24 :1, agiter et centrifuger 4200g à 4°C pendant 15 min. Récupérer le surnageant dans un tube neuf (environ 3mL).

9. Ajouter 1.5 mL de NaCl 5M, agiter, puis 3 mL d'isopropanol 100% froid. Mélanger par inversion: l'ADN précipite. Laisser précipiter au minimum ½ heure à 4°C (dans la glace ou au réfrigérateur).

10. Centrifuger 4200g à 4°C pendant 5 min. Eliminer le surnageant. Ajouter 2.5 mL d'Ethanol 70%. Bien rincer le culot.

☞ *Fin de manipulation sous sorbonne*

11. Centrifuger 4200g à 4°C pendant 5 min. Eliminer le surnageant et laisser sécher le tube à l'envers sur du papier absorbant pendant ¼ heure. Coucher le tube sur le côté et laisser sécher quelques heures (en fonction de la taille du culot).

12. Reprendre le culot dans 200µl de TE10-0.1, en laissant une nuit à 4°C.

Vérification

13. Vérifier la qualité des ADN extraits par spectrométrie (Nanodrop), fluorimétrie et/ou électrophorèse.
Résultat attendu : un ADN de haut poids moléculaire non dégradé doit être obtenu, dans une quantité comprise entre 1 à 50 µg d'ADN.

Dosage de l'ADN au Picogreen®
sur le fluorimètre BioTEk FL600

(N.Pouilly-2008)

Objectif :

Ce mode opératoire a pour but de définir le protocole d'utilisation du fluorimètre Biotek FL600 pour le dosage d'ADN au Picogreen® sur microplaque 96. Ce protocole permet le dosage d'ADN de faible concentration, entre 50 à 300ng/µl.

Le Picogreen® utilisé vient s'intercaler dans l'hélice de l'ADN double brin. Après excitation à 485 nm, le picogreen® intercalé dans l'ADN émet une fluorescence à 530 nm dont l'intensité est proportionnelle à la quantité d'ADN présent.

Cette quantité est calculée par rapport à une courbe étalon de 8 points correspondants à 8 concentrations différentes en ADN (de 0 à 2 ng/µl).

Matériel :

- Fluorimètre Biotek FL600
- Micropipettes mono & multicanaux
- Microplaque noire demi puits fond plat (réf. xxx)

Réactifs :

- Kit Quanti-iT™ Picogreen® (Invitrogen)
- H_2O ultra pure

Prévention :

Le Picogreen® est un intercalant de l'ADN. A ce titre, il est à considérer comme un produit mutagène potentiel.

Prévoir une poubelle déchet spécifique (ex : sac plastique résistant) identifiée pour les consommables plastiques contaminé par le Picogreen®. (tube, cônes, réservoir, plaques, gants). Ce sac identifié Picogreen® sera à éliminer dans un contenant déchets chimiques en salle BET en fin de manipulation.

Méthodologie :

1. Préparation des ADN

Préalable : faire un dosage préalable par spectrophotométrie (nanodrop), afin d'ajuster si besoin le coefficient de dilution des ADN.

Le dosage s'effectue sur des ADN dilués au 1/300ème dans du TE 10/1.
Le TE 10/1 est obtenu en diluant au 1/20ème le tampon fourni dans le kit (TE 20X).

2. Préparation du Picogreen®

Décongeler un aliquot de Picogreen® **à l'abri de la lumière**.
Lorsqu'il est **totalement décongelé**, faire une dilution au 1/200ème dans du TE 0/1.

La quantité minimale de Picogreen® dilué au 1/200ème est la suivante :

(N échantillons + 8 points de gamme) X 100 µl.

soit pour une plaque 96 complète: 50µl Picogreen® stock + 9.95mL TE 10/1.

Vortexer et stocker à l'abri de la lumière avant utilisation.
Le Picogreen® dilué n'est pas stable, il se dégrade très vite, ne pas le conserver plus d'une demi-journée.

3. Préparation de la gamme étalon

- Dilution ADN λ standard à 2ng/µL

Diluer l'ADN λ stock (100µg/mL) au 1/50ème : 40µl ADN λ 100µg/mL + 1960µl TE10/1.

- Préparation de la gamme haute 0-2ng/µl

[ADN] finale	ADN λ 2ng/µl V(µl)	TE10/1 V(µl)
0	0	1000
2pg/µl	1	999
0,02ng/µl	10	990
0,1ng/µl	50	950
0,2ng/µl	100	900
0,4ng/µl	200	800
1ng/µl	500	500
2ng/µl	1000	0

Les 8 points de gammes sont stockés à 4°C, dans la boite contenant les différentes gammes d'ADN.

Si la quantité restante est inférieure à 50 µl, la gamme est à refaire.

4. Préparation de la plaque de dosage

Déposer 100µl d'ADN (gammes étalons, ADN à doser dilués au 300ème), en commençant par les 8 points de la gamme sur les puits A1 à H1.

Ajouter 100µl de Picogreen® dilué au 1/200 à la pipette multicanaux (mélanger par un aller/retour).
Stocker la plaque à température ambiante avant le dosage, en la couvrant avec du papier alu. Le dosage doit être effectué après 5 minutes d'incubation.

Lors du dosage, la plaque est agité par l'appareil pendant 2 min. avant la lecture de la fluorescence. Cela permet l'homogénéisation des réactifs et une bonne intégration du Picogreen® dans l'ADN.

5. Utilisation du fluorimètre Biotek FL600

Allumer le fluorimètre et l'ordinateur de pilotage.
Ouvrir le logiciel KC4
Lancer le protocole DNA_FLUO3.PRT sous D :/Nicolas_P

Vérifier les paramètres « Settings » comme ci-après (en précisant notamment les puits si la plaque est incomplète).
Créer et enregistrer la plaque à doser.
Compléter le plan de plaque (« Layout »), en précisant : la gamme étalon utilisée, les puits contenant les échantillons à doser et leur dilution (300 d'après ce protocole).

Paramètres protocole :

ShakingIntensity=2
ShakingTime=120
Pre-Heating=No
Incubation=No
Temperature=0
LagTime=00:00:00
PlateSize=8,12
PlateType=96 WELL PLATE
Wells= à définir
ReadBlankPlate=No
MonitorWell=No
EjectPlateBetweenMeasurement=No
Method=Fluorescence
Reading Direction=VERTICAL

Filter1=485/20
Filter2=530/25
Gain=80
OpticsPos=Bottom
Mode=Static
SamplesNb=100
DelayBeforeSampling=350
DelayBetweenSamples=10
GainAdjust_MinValue=100
GainAdjust_MaxValue=90000
SkipReferenceWell=No
CheckLampBeforeMeasurement=Yes
SetLampOffDuringMeasurement=No

6. Résultats

Après lecture de la plaque, le logiciel réalise la courbe étalon à partir des points de gamme.

Cette courbe est une droite qui doit passer par zéro et dont le coefficient de corrélation (R^2) doit être le plus proche de 1. Cette droite étalon permet le calcul des concentrations en ADN des échantillons.

Imprimer le rapport de dosage, avec la courbe étalon.
Le rapport de dosage (fichier txt) est enregistré sous le répertoire D:// et transférable par disquette.