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A B S T R A C T

This study was conducted to identify physiological traits associated with cold tolerance in sunflower and to identify the genomic regions involved in their variation. A population of 98 recombinant inbred lines (RILs) and their two parents were sown in the field as usual sowing date (control) and one or two months earlier (long-term low temperature treatments). A trait commonly used to underlying cold tolerance related to the degree of membrane damage, as well as traits associated with growth capacity (chlorophyll content, potential photochemical efficiency of photosystem II and plant dry weight) and finally those reflecting acclimation mechanism to stress conditions (osmotic potential at full turgor, and specific leaf area) have been investigated at early development stages. Significant differences were observed among the three sowing dates for all traits. Chlorophyll content and specific leaf area are genetically associated with cold tolerance. Genetic gains were observed for chlorophyll content and osmotic potential traits in some of early sowing dates, which suggest that they could be used for cold tolerance in breeding programs. QTL analyses show that several putative genomic regions are involved in the variation of the physiological traits studied under low temperature. Major QTLs for cold tolerance associated with SSR markers such as *ORS331_2* for the cell membrane stability should be checked in several environments to see if they can be used in marker-assisted selection programs.

1. Introduction

Sunflower is one of the most important oil crops worldwide. This summer crop is mainly cultivated under rather high temperature. Water deficit stress which can take place during critical periods of flowering and grain filling induces yield decline [1]. Two main strategies have been studied to maximize the sunflower production under drought stress conditions. The first way is to improve the drought tolerance of sunflower cultivars [2,3]. Early sowing is the second way to avoid the critical water stress period [4]. The effect of early sowing and winter planting has been studied in sunflower in several Mediterranean countries [5,6]. Authors have shown that early sowings improve the water availability [7] and increase the yield of the crop [8,6]. In temperate regions, early sowing compared with conventional sowing is associated with long-term low temperature exposure during first stages of development in sunflower.

Low temperature exposure has consequences for most biological processes. It initiates a number of physiological changes which lead the plant to be more cold tolerant [9]. Among the numerous metabolic changes, photosynthesis is the main physiological process studied under low temperature condition in many species including *Arabidopsis* [10], alfalfa [11], rice [12], maize [13], wheat [14] and barley [15]. Photosynthetic modifications are notably characterized by changes in photochemical efficiency [16,17] in response to photooxidation [18], photoprotection [13] and photoinhibition [14,19]. Accumulation of metabolites and low molecular weight solutes such as carbohydrates into the cytoplasm constitute another major metabolic change observed in plants after exposure to low temperature [20]. These solutes contribute to decrease the osmotic potential which leads to decrease the cytoplasmic freezing point and prevent dehydration in cells [21,22]. However, low temperature exposure can induced cell structural alterations as observed in several warm-season crop species [23] due to membrane lipid degradation [24].

The genetic dissection of the quantitative traits controlling the adaptive response of crops to abiotic stress is a prerequisite to allow cost-effective applications of genomics-based approaches to breeding programs [25]. Sunflower QTL mapping was conducted for agronomical traits [26–28], and for photosynthesis and water status traits under water stress condition [29,30]. QTLs for cold

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tolerance during the first stages of development has been identified in rice [31,12], winter wheat [32], maize [33,17] and sorghum [34]. As far as we know, no study in the literature refers to genetic analysis of physiological traits associated with cold tolerance in sunflower.

The objectives of this research are to study a set of physiological traits associated with cold tolerance, to understand which are affected in the first development stage in sunflower subjected to early sowing associated with long-term low temperature exposure and to analyze the genetic basis of low temperature tolerance in sunflower. Identifying physiological traits associated with molecular markers involved in low temperature tolerance would be useful in breeding programs.

2. Materials and methods

2.1. Plant material and experimental conditions

A population of 98 RILs of sunflower (*Helianthus annuus*) and their parents RHA266 and PAC2 were sown in the field to investigate cold tolerance response under early sowing condition. Recombinant inbred lines were sown at three dates: usual sowing date as control (S3 in April) and one or two months earlier (S2 in March and S1 in February). For the last 10 years the mean temperature registered during April was 12.7 °C ranging from 7.9 °C to 17.5 °C, the mean temperature during March was 10.4 °C ranging from 5.6 °C to 15.2 °C and during February 7.5 °C ranging from 3.7 °C to 11.3 °C. Air temperature was recorded daily at 2 m above the soil surface close to the experimental site.

For each sowing date three replications per genotype were performed. The replications were two rows of 3 m long with 50 cm between rows and 25 cm between plants in rows. Each replication per genotype consisted of an experimental unit of 24 plants. Plants were exposed to periods of low temperature depending on the sowing dates (Fig. 1). Date of emergence was determined for each experimental unit when 50% of emergence was reached [35]. Sampling was realized at 800 °Cd in each sowing date. Cumulative degree days (°Cd) was calculated as the sum of the average daily temperature. Each sample consisted of the aerial part of a single plant per experimental unit. Samples have been placed 24 h at 4 °C before physiological traits measurement.

2.2. Trait measurements

Long-term low temperature exposure effects were determined using two photosynthetic, three non-photosynthetic traits and the plant dry weight measurement. The photosynthetic traits are chlorophyll fluorescence and chlorophyll content. Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany) for the younger fully expanded leaves after keeping 1 h under dark condition. The minimum fluorescence (Fo) and the maximum fluorescence (Fm) following a saturating light pulse (8000 μmol m^{-2} s^{-1}) were measured. The variable fluorescence (Fv = Fm − Fo) and the ratio of variable maximum fluorescence (Fv/Fm) were calculated. The Fv/Fm ratio following a saturating light pulse represents a measure of the potential photochemical efficiency of photosystem II electron transport [36]. Chlorophyll content (CC) was determined with a portable SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan). Measurements were performed for three samples through the middle section of the younger fully expanded leaves.

Non-photosynthetic traits studied are osmotic potential, relative electrolyte leakage and specific leaf area. Osmotic potential at full turgor (OP_{FT}) was measured on expressed sap of frozen and thawed leaves using 10 mL aliquots placed in an osmometer (Wescor Model 5520, Logan, Utah, USA) calibrated with manufacturer solutions. The relative electrolyte leakage (REL) was performed according to the protocol of Campos et al. [24] using leaf discs (2 cm diameter) from young fully expanded leaves which were rinsed three times with demineralised water then placed in tube with 10 mL of demineralised water. Electrolyte leakage (EL) was measured with conductimeter (WTW LF 95, sonde TetraCon 96, Germany) after 24 h of floating at room temperature. Then, tubes were autoclaved for 15 min (121 °C) to kill the leaf tissue and release the total electrolytes (TE). Results were expressed as relative electrolyte content (REL) calculated as (EL/TE) × 100 (%). The specific leaf area (SLA) was determined with discs (2 cm diameter) cut on the third fully expanded leaves and dried (48 h, 80 °C). SLA was calculated as leaf area/leaf dry weight (m^2 kg^{-1}). The total aerial part of three plants per genotype per sowing date per replication was dried at 80 °C for 48 h and the plant dry weight (PDW) was determined.

2.3. Statistical analysis

Means comparison between the three sowing dates for each studied trait was tested with the Kruskal–Wallis test [37]. The normality of the traits distribution was tested with the Shapiro–Wilk test. For each sowing date, a mixed model with genotypes (RILs and parents) and replication effects, was used for analysis of the experimental data. Statistical analyses were performed with SPSS for Windows (15. 0). Genetic gain for each trait was calculated as the differences between the mean of the top 10% selected RILs and the mean of the parents.

2.4. QTL analysis

The sunflower reference map recently constructed in our department by Poormohammad Kiani et al. [30] was used for detection of QTLs. The mapping population was developed through single seed descent from F2 plants derived from a cross between 'PAC2' and 'RHA266' [38]. RHA266 was 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" [39]. RHA266 compared to PAC2 have a higher values for yield, leaf area at flowering, and lower values for plant height and total dry matter [39–41]. Under water stress conditions the two parental lines differed significantly for leaf area at flowering, leaf area duration, plant height, total dry matter, head weight and seed quality traits [42,41]. This map consisted of 495 markers (304 AFLP and 191 SSR), placed in 17 linkage groups with a mean density of one locus for 3.7 cM. Each linkage group presumably corresponds

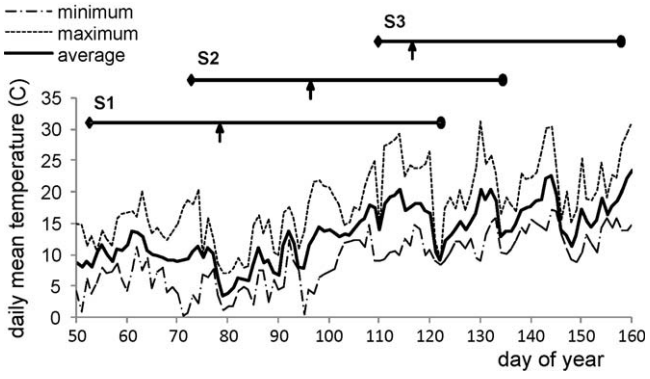

Fig. 1. Daily mean temperature during the growing season. The three sowing dates are indicated S1, S2 and S3. The dates of emergence are indicated by vertical arrows and the sampling date for each sowing date is marked by the black circle.

Table 1
Analysis of variance for physiological traits in a population of sunflower recombinant inbred lines (RILs) and their two parents grown in three sowing dates: one control sowing date (S3) and two early sowing dates associated with low temperature (S1 and S2).

Sowing date		Fv/Fm	CC	SLA	REL	OP_{FT}	PDW
S3	Mean	0.830 a	31.7 a	26.6 a	21.6 a	−0.64 a	3.84 a
	Range	0.711/0.862	21.2/45.2	17.9/36.9	13.6/37.5	−0.92/−0.44	0.74/18.21
	MS_G	0.001***	41.085***	15.182^{NS}	17.519^{NS}	0.011^{NS}	28.348***
S2	Mean	0.812 b	29.4 b	24.9 b	23.6 b	−0.74 b	2.38 b
	Range	0.634/0.855	18.5/41.0	16.2/33.7	15.8/34.1	−1.10/−0.56	0.34/10.80
	MS_G	0.001***	26.195***	12.919***	13.865**	0.006**	9.476***
S1	Mean	0.792 c	27.9 c	25.6 b	25.7 c	−0.76 c	0.67 c
	Range	0.594/0.858	19.5/35.0	18.0/34.5	16.4/38.1	−0.99/−0.58	0.05/2.42
	MS_G	0.002***	15.180***	13.221***	28.862***	0.006***	0.150^{NS}

Fv/Fm: potential photochemical efficiency of photosystem II; CC: chlorophyll content (SPAD values); SLA: specific leaf area (m^2/kg); REL: relative electrolyte leakage (%); OP_{FT}: osmotic potential at full turgor (MPa); PDW: plant dry weight (g); MSG: genotype mean square; NS: non-significant. Values with a common letter in the same column are not significantly different at $p = 0.05$ (Kruskal–Wallis test)
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level.

to one of the 17 chromosomes in the haploid sunflower genome ($x = 17$) [30]. The QTL location was estimated with the composite interval mapping method of QTL Cartographer version 2.5 software [43,44] using mean values of the three replicates for each RIL in each sowing date. The control marker number and the window size were 15 and 15 cM, respectively. The LOD score criterion for QTL significance was estimated by mean of a permutation test with 1000 permutations [45]. Mapchart 2.1 was used for graphical presentation of linkage groups and map positions of the SSR and AFLP markers.

3. Results

Contrasted thermal conditions are observed for the three sowing dates (Fig. 1). The mean temperatures from sowing to sampling are lower in the two early sowing than in control with 11.7 °C for S1, 12.9 °C for S2 and 16.4 °C for S3. The mean temperature from sowing to emergence is lower in S1 (10.3 °C) and S2 (8.4 °C) compared with S3 (17.8 °C), whereas temperatures recorded from emergence to sampling period are lower in S1 (12.4 °C) compared with S2 (15.6 °C) and S3 (16.2 °C).

The means comparison between the three sowing dates for the studied traits shows that sowing date has significant effect on all physiological traits (Table 1). A normal distribution was observed for the specific leaf area SLA and the osmotic potential at full turgor (OP_{FT}) as presented in Fig. 2. According to the Shapiro–Wilk test the distribution of some other traits deviate from normality. As normalizing data through transformation may misrepresent differences among individuals by pulling skewed tails toward the centre of the distribution [45], all phenotypic analyses were performed on untransformed data. Significant differences are observed between S1, S2 and S3 for all the studied traits except for SLA, in which means of S1 and S2 are not significantly different. The mean values of the potential photochemical efficiency of photosystem II (Fv/Fm), chlorophyll content (CC), SLA, (OP_{FT}) and the plant dry weight (PDW) are lower in early sowing dates (S1 and S2) compared with the control S3 (Table 1). On the contrary, the mean value of REL is higher in S1 and S2 than in S3 (Table 2).

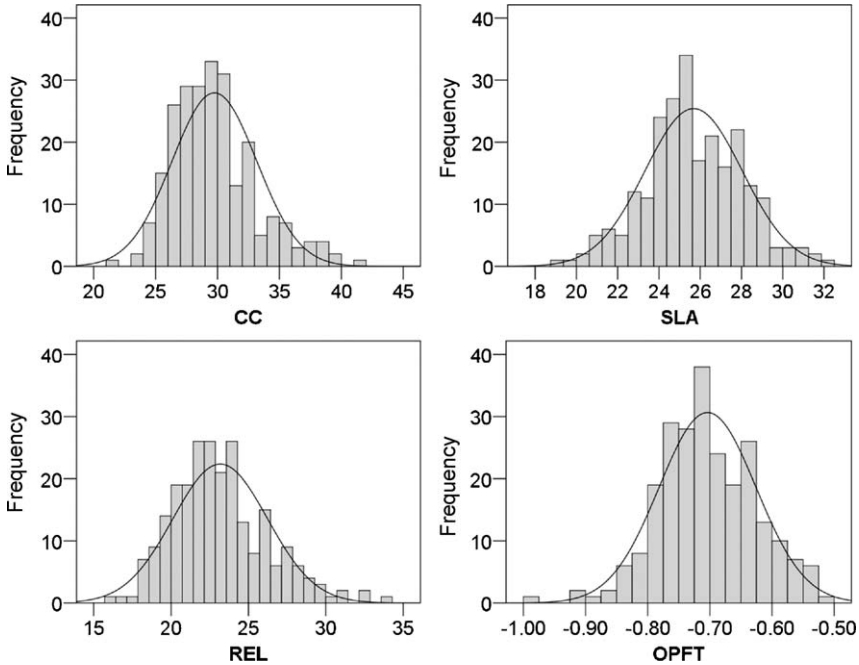

Fig. 2. Distribution for physiological traits: chlorophyll content—CC (SPAD values), the specific leaf area—SLA (m^2/kg), the relative electrolyte leakage—REL (%) and the osmotic potential at full turgor—OPFT (MPa) in a population of sunflower recombinant inbred lines (RILs) and their two parents grown in three sowing dates.

Table 2
Genetic gain for physiological traits in a population of sunflower recombinant inbred lines (RILs) and their two parents grown in three sowing dates: one control sowing date (S3) and two early sowing dates associated with low temperature (S1 and S2).

Sowing date		Fv/Fm	CC	SLA	REL	OP_{FT}	PDW
S3	PAC2 (P1)	0.839	30.9	24.4	18.5	−0.65	2.15
	RHA266 (P2)	0.818	28.0	26.5	21.3	−0.68	2.15
	P1–P2	0.021^{NS}	2.88^{NS}	-2.0^{NS}	-2.8^{NS}	0.03^{NS}	0.00^{NS}
	$\bar{X}_P$	0.828	29.4	25.5	19.9	−0.66	2.15
	$\bar{X}_{RIL}$	0.830	31.8	26.6	21.6	−0.64	3.87
	$\bar{X}_{10\%\ best\ RIL}$	0.852	38.9	30.8	17.8	−0.75	11.28
	$\bar{X}_{RIL} - \bar{X}_P$	0.002^{NS}	2.4^{NS}	1.2^{NS}	1.7^{NS}	0.02^{NS}	1.72^{NS}
	$GG10\% = \bar{X}_{10\%\ best\ RIL} - \bar{X}_P$	0.023^{NS}	$\mathbf{9.4}^{***}$	5.3^{NS}	-2.1^{NS}	-0.09^{NS}	$\mathbf{9.13}^{***}$
S2	PAC2 (P1)	0.801	29.3	24.7	21.3	−0.71	0.91
	RHA266 (P2)	0.752	27.5	23.6	26.0	−0.72	1.43
	P1–P2	0.050^{**}	1.78^{NS}	1.0^{NS}	-4.7^{NS}	0.00^{NS}	-0.52^{NS}
	$\bar{X}_P$	0.777	28.4	24.1	23.7	−0.71	1.17
	$\bar{X}_{RIL}$	0.813	29.4	24.9	23.6	−0.74	2.40
	$\bar{X}_{10\%\ best\ RIL}$	2.006	34.5	28.3	20.4	−0.85	6.70
	$\bar{X}_{RIL} - \bar{X}_P$	0.036^{*}	1.0^{NS}	0.8^{NS}	0.0^{NS}	-0.03^{NS}	1.23^{NS}
	$GG10\% = \bar{X}_{10\%\ best\ RIL} - \bar{X}_P$	$\mathbf{1.229}^{***}$	$\mathbf{6.1}^{***}$	4.1^{NS}	-3.3^{NS}	$\mathbf{-0.14}^{***}$	$\mathbf{5.53}^{***}$
S1	PAC2 (P1)	0.838	29.8	24.8	23.3	−0.75	0.89
	RHA266 (P2)	0.793	28.9	26.6	25.7	−0.72	0.90
	P1–P2	0.045^{NS}	0.91^{NS}	-1.9^{NS}	-2.4^{NS}	-0.03^{NS}	-0.01^{NS}
	$\bar{X}_P$	0.815	29.3	25.7	24.5	−0.74	0.89
	$\bar{X}_{RIL}$	0.791	27.9	25.6	25.7	−0.76	0.67
	$\bar{X}_{10\%\ best\ RIL}$	0.831	31.7	29.3	20.7	−0.85	1.10
	$\bar{X}_{RIL} - \bar{X}_P$	-0.024^{NS}	-1.4^{NS}	-0.2^{NS}	1.2^{NS}	-0.02^{NS}	-0.22^{NS}
	$GG10\% = \bar{X}_{10\%\ best\ RIL} - \bar{X}_P$	0.016^{NS}	2.4^{NS}	3.6^{NS}	-3.8^{NS}	$\mathbf{-0.12}^{***}$	0.21^{NS}

Fv/Fm: potential photochemical efficiency of photosystem II; CC: chlorophyll content (SPAD values); SLA: specific leaf area (m^2/kg); REL: relative electrolyte leakage (%); OP_{FT}: osmotic potential at full turgor (MPa); PDW: plant dry weight (g). The significant differences are presented as bold-face. 'PAC2' (P1) and 'RHA266' (P2): parental lines; $\bar{X}_P$: mean of two parental lines; $\bar{X}_{RIL}$: mean of recombinant inbred lines (RILs); $\bar{X}_{10\%\ best\ RIL}$: the mean of the top 10% selected RILs; GG10%: genetic gain when the mean of the top 10% selected RILs is compared with the mean of the parents. NS: non-significant.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level.

Analysis of variance of the 98 recombinant inbred lines and their parents ('PAC2' and 'RHA266') is summarized in Table 1. Photosynthetic traits (Fv/Fm and CC) present significant differences between genotypes for each sowing date. Non-photosynthetic traits SLA, REL and $OP_{FT,}$ show significant variability only for S1 and S2. Concerning the plant dry weight (PDW), significant genotypic variability is observed in S2 and S3. The difference between the two parents is significant only for Fv/Fm in S2 (Table 2), in spite of the existence of genetic variability in RILs for all traits (Table 1). Differences for the mean value of all traits between RILs (X_{RIL}) and their parents (X_P) are not significant, except for Fv/Fm in S2 (Table 2). Genetic gain, as the difference between the mean of 10% selected RILs ($X_{10\%\ best\ RIL}$) and the mean of parents (X_P), is significant for OP_{FT} in S1 and S2, for Fv/Fm in S2 and for CC and PDW in S3.

The map position and the characteristics of QTLs associated with the studied traits for the three sowing dates are presented in Table 3. For an easier overview of overlapping QTLs between the traits and the sowing dates, an image of all QTL regions is presented in Fig. 3. Three to nine QTLs are identified depending to the traits and sowing dates. QTLs explain from 4.6% to 23% of the phenotypic variance of the traits (R^2). Additive effects present positive or negative values showing that both parental lines contribute to the expression of the different traits. The largest amount of phenotypic variance explains by a QTL detected for Fv/Fm is 22% (*Fv/Fm-S3-16.2*). This QTL is co-located with two overlapping QTLs detected for CC in S2 and S3 conditions. The most important QTL for CC is identified on linkage group 9 (*ORS805*) and explains 19% of the total phenotypic variance. The major QTL for SLA (*SLA-S3-16.1*) is located on the linkage group 16 and explain 22% of the phenotypic variance. The most important QTL detected for REL on the linkage group 7 is associated with the SSR marker *ORS331_2* and explain 23% of the phenotypic variance. This QTL is identified in the two early sowing dates (S1 and S2). The major QTL for PDW (*PDW-S3-5.1*) explains 20% of phenotypic variance in S3 and is overlapped with QTL detected for PDW in S2. Among the identified QTLs, one is stable for SLA across the three sowing dates on linkage group 12 (Table 3). Two QTLs are common between S1 and S2 for REL on linkage group 7 and for OP_{FT} on linkage group 10. Eight QTLs are common between S2 and S3 for Fv/Fm (linkage group 9), for CC (linkage groups 2, 12 and 16) and for PDW (linkage groups 5, 15 and 17).

Overlapping QTLs between traits are observed mainly in control sowing date. In S3, QTL for plant dry weight (PDW) is co-located with the QTL detected for REL on linkage group 4 and with the QTL detected for Fv/Fm on the linkage group 15. Two QTLs detected for Fv/Fm are co-located with QTLs identified for CC in linkage groups 15 and 16. Overlapping QTLs for CC, OP_{FT} and SLA are also detected in the linkage group 9. In the early sowing S1, QTLs for SLA and REL are co-located in linkage group 4.

4. Discussion

Physiological changes induced by low temperature affected all the traits studied. Non-significant differences were found for all traits studied between the means of the RILs and the mean of the parents (Table 2). This indicates that the RILs are representative of possible genotypic combinations of the two parents for the studied traits, as it was also previously reported by Poormohammad Kiani et al. [30], for the water status and the osmotic adjustment of sunflower under two water treatments. Genetic gain was significant for the chlorophyll content (CC) and plant dry weight (PDW) in S2 and S3 and for the osmotic potential at full turgor (OP_{FT}) in S1 and S2 (Table 2). This might be due to positive transgressive segregation resulting from the accumulation of favorable alleles in some RILs. Transgressive segregation has

Table 3
Map position and effect of QTLs for potential photochemical efficiency of photosystem II (Fv/Fm), chlorophyll content (CC), specific leaf area (SLA), relative electrolyte leakage (REL), osmotic potential at full turgor (OP_{FT}) and plant dry weight (PDW) detected in RILs under three sowing conditions: one control sowing date (S3) and two early sowing dates associated with low temperature (S1 and S2). The threshold level of the LOD score for each trait was estimated by means of a permutation test with 1000 permutations.

Sowing date	Trait	QTL	Linkage group	Nearest marker	Position cM[a]	LOD score	R^{2b}	Additive effect
S3	Fv/Fm	*Fv/Fm-S3-9.1*	9	*HA477*	42.8	3.9	0.12	−0.007
		Fv/Fm-S3-10.1	10	*E35M61_11*	41.7	3.5	0.07	−0.005
		Fv/Fm-S3-14.1	14	*ORS301*	103.0	3.6	0.11	0.007
		Fv/Fm-S3-15.1	15	*E35M48_4*	86.4	3.6	0.11	−0.007
		Fv/Fm-S3-16.1	16	*E37M47_10*	76.0	5.0	0.17	−0.009
		Fv/Fm-S3-16.2	16	*E37M61_1*	89.4	6.6	0.22	−0.010
	CC	*CC-S3-2.1*	2	*E41M50_12*	11.5	4.1	0.07	1.386
		CC-S3-6.1	6	*E41M48_2*	18.5	7.4	0.16	−2.038
		CC-S3-9.1	9	*ORS805*	2.0	7.3	0.19	−1.721
		CC-S3-12.1	12	*ORS671_2*	21.1	3.2	0.07	1.072
		CC-S3-16.1	16	*ORS418_2*	8.2	3.5	0.07	1.095
		CC-S3-16.2	16	*E37M47_5*	86.8	5.1	0.12	−1.523
	SLA	*SLA-S3-2.1*	2	*E35M60_4*	98.6	3.6	0.08	−0.726
		SLA-S3-7.1	7	*E33M50_2*	27.9	3.7	0.07	0.673
		SLA-S3-9.1	9	*ORS1009*	9.6	6.0	0.16	0.996
		SLA-S3-12.1	12	*E40M50_9*	2.0	3.9	0.09	−0.755
		SLA-S3-13.1	13	*HA3330*	37.4	3.4	0.08	−0.715
		SLA-S3-14.1	14	*E41M62_9*	126.2	3.7	0.09	−0.714
		SLA-S3-16.1	16	*ORS303_2*	105.9	7.7	0.22	−1.168
	REL	*REL-S3-4.1*	4	*HA991*	34.2	4.8	0.10	−1.209
		REL-S3-4.2	4	*E40M59_6*	60.4	7.1	0.12	1.268
		REL-S3-9.1	9	*E33M60_5*	91.9	4.2	0.07	−0.857
		REL-S3-10.1	10	*E37M49_5*	67.6	8.0	0.14	1.559
		REL-S3-10.2	10	*E35M62_9*	112.8	11.9	0.23	−2.254
		REL-S3-13.1	13	*E40M47_21*	0.0	5.9	0.11	0.902
	OP_{FT}	*OPFT-S3-5.1*	5	*E41M62_7*	0.0	4.3	0.10	0.022
		OPFT-S3-7.1	7	*ORS331_2*	0.0	4.8	0.15	−0.027
		OPFT-S3-9.1	9	*ORS805*	0.0	3.1	0.10	0.020
	PDW	*PDW-S3-2.1*	2	*E38M50_26*	61.3	3.7	0.08	−0.997
		PDW-S3-4.1	4	*E40M59_12*	25.6	4.5	0.11	−1.306
		PDW-S3-4.2	4	*E40M59_6*	64.4	5.0	0.09	1.203
		PDW-S3-5.1	5	*HA3700*	70.4	8.9	0.20	−1.751
		PDW-S3-13.1	13	*HA4208*	72.7	3.8	0.09	1.026
		PDW-S3-14.1	14	*ORS1128*	0.0	7.1	0.16	1.417
		PDW-S3-15.1	15	*E35M48_4*	84.4	4.9	0.13	−1.530
		PDW-S3-17.1	17	*E41M48_3*	102.4	4.5	0.10	−1.214
		PDW-S3-17.2	17	*E35M62_8*	110.2	3.5	0.07	−1.063
S2	Fv/Fm	*Fv/Fm-S2-7.1*	7	*ORS331_1*	12.5	6.2	0.13	−0.008
		Fv/Fm-S2-7.2	7	*HA1848*	25.6	3.4	0.10	−0.007
		Fv/Fm-S2-8.1	8	*SSU217*	7.2	8.1	0.16	0.010
		Fv/Fm-S2-9.1	9	*HA2053*	43.9	3.1	0.06	−0.005
		Fv/Fm-S2-15.1	15	*E37M49_9*	78.1	3.7	0.07	−0.006
	CC	*CC-S2-1.1*	1	*E40M50_18*	20.1	3.5	0.07	0.954
		CC-S2-2.1	2	*E41M50_12*	11.5	4.0	0.06	0.894
		CC-S2-4.1	4	*E35M62_1*	71.8	7.4	0.14	1.414
		CC-S2-10.1	10	*E35M61_6*	134.1	3.6	0.06	0.970
		CC-S2-12.1	12	*ORS671_2*	23.1	4.4	0.08	0.889
		CC-S2-14.1	14	*E32M61_13*	51.4	3.7	0.08	0.886
		CC-S2-15.1	15	*ORS687*	66.3	3.4	0.06	0.773
		CC-S2-16.1	16	*E37M47_5*	86.8	3.8	0.07	−0.887
	SLA	*SLA-S2-5.1*	5	*E41M62_30*	17.5	4.7	0.08	−0.637
		SLA-S2-8.1	8	*SSL30*	26.9	7.7	0.14	−0.853
		SLA-S2-8.2	8	*E37M47_19*	40.2	3.5	0.09	−0.689
		SLA-S2-10.1	10	*E35M62_4*	38.8	3.8	0.07	0.669
		SLA-S2-11.1	11	*E36M59_9*	65.1	4.6	0.09	0.714
		SLA-S2-12.1	12	*E40M50_9*	0.0	3.4	0.06	−0.576
		SLA-S2-14.1	14	*ORS1128*	0.0	3.3	0.07	0.595
		SLA-S2-15.1	15	*SSU223*	11.6	3.4	0.05	0.544
	REL	*REL-S2-5.1*	5	*E35M60_1*	25.6	4.4	0.09	−0.752
		REL-S2-5.2	5	*E35M49_6*	48.4	6.0	0.21	1.168
		REL-S2-6.1	6	*E41M48_2*	18.5	7.5	0.15	−1.212
		REL-S2-7.1	7	*ORS331_2*	2.0	9.5	0.23	−1.192
		REL-S2-11.1	11	*ORS354*	11.4	4.1	0.08	0.688
		REL-S2-13.1	13	*E33M48_20*	32.9	7.6	0.18	1.071
		REL-S2-13.1	13	*ORS316*	60.7	5.2	0.11	−0.840
	OP_{FT}	*OPFT-S2-10.1*	10	*E32M61_7*	59.2	4.9	0.11	−0.031
		OPFT-S2-10.2	10	*HA2579*	166.5	3.3	0.08	0.022

Table 3 (*Continued*)

Sowing date	Trait	QTL	Linkage group	Nearest marker	Position cM[a]	LOD score	R^{2b}	Additive effect
		OPFT-S2-14.1	14	*HA2714*	21.6	3.5	0.10	0.023
		OPFT-S2-15.1	15	*E38M60_2*	20.1	3.8	0.07	0.018
		OPFT-S2-16.1	16	*E37M47_26*	22.2	4.8	0.11	−0.022
		OPFT-S2-16.2	16	*E38M48_7*	161.5	5.1	0.10	0.021
	PDW	*PDW-S2-1.1*	1	*HA4090*	66.4	3.6	0.09	0.529
		PDW-S2-5.1	5	*HA3700*	70.4	3.3	0.07	−0.499
		PDW-S2-10.1	10	*SSL49*	151.0	3.8	0.09	−0.603
		PDW-S2-10.2	10	*ORS807*	162.7	4.9	0.11	−0.647
		PDW-S2-15.1	15	*E35M48_4*	86.4	3.8	0.10	−0.692
		PDW-S2-17.1	17	*ORS297*	31.2	3.4	0.06	0.520
		PDW-S2-17.2	17	*E41M48_3*	102.4	5.9	0.13	−0.702
		PDW-S2-17.3	17	*E35M62_8*	110.2	3.0	0.07	−0.519
S1	Fv/Fm	*Fv/Fm-S1-10.1*	10	*E35M61_6*	131.0	6.0	0.13	−0.017
		Fv/Fm-S1-14.1	14	*ORS391*	73.4	4.0	0.09	0.009
		Fv/Fm-S1-17.1	17	*E41M48_3*	102.4	4.4	0.10	−0.010
	CC	*CC-S1-5.1*	5	*E41M62_30*	19.5	4.9	0.12	−0.987
		CC-S1-14.1	14	*HA3886*	14.4	3.2	0.11	−0.789
		CC-S1-17.1	17	*ORS1040*	120.4	6.8	0.16	1.151
	SLA	*SLA-S1-4.1*	4	*E37M47_8*	17.8	5.2	0.13	0.934
		SLA-S1-12.1	12	*E40M50_9*	0.0	3.7	0.07	−0.598
		SLA-S1-13.1	13	*ORS511*	52.7	6.5	0.15	0.978
		SLA-S1-14.1	14	*E41M48_12*	115.6	3.9	0.08	0.743
	REL	*REL-S1-2.1*	2	*E32M47_9*	2.0	3.9	0.11	1.176
		REL-S1-4.1	4	*E35M49_4*	18.1	6.0	0.12	−1.914
		REL-S1-5.1	5	*ORS533*	80.9	4.3	0.11	−1.198
		REL-S1-7.1	7	*ORS331_2*	0.0	7.3	0.17	−1.549
		REL-S1-17.1	17	*E38M48_1*	128.9	3.5	0.11	−1.105
	OP_{FT}	*OPFT-S1-1.1*	1	*E33M48_2*	29.3	5.4	0.12	−0.018
		OPFT-S1-5.1	5	*E37M61_10*	12.3	6.3	0.12	−0.021
		OPFT-S1-5.2	5	*E41M59_10*	31.9	3.1	0.10	0.016
		OPFT-S1-10.1	10	*ORS1144*	162.7	4.8	0.11	−0.017
		OPFT-S1-11.1	11	*ORS1146*	11.8	6.1	0.12	0.018
		OPFT-S1-13.1	13	*ORS316*	72.7	4.6	0.10	−0.016
		OPFT-S1-15.1	15	*E35M48_4*	82.4	3.9	0.07	−0.014
		OPFT-S1-16.1	16	*E38M60_11*	138.4	6.0	0.17	−0.021
		OPFT-S1-17.1	17	*E35M62_5*	40.6	3.4	0.07	0.014
	PDW	*PDW-S1-9.1*	9	*ORS428_2*	39.8	5.6	0.14	0.112
		PDW-S1-14.1	14	*ORS1128*	0.0	3.6	0.09	0.081
		PDW-S1-16.1	16	*HA3582*	13.9	4.8	0.13	−0.103
		PDW-S1-16.2	16	*E32M47_1*	44.5	4.8	0.17	0.148

[a] From the north of linkage group.
[b] Percentage of individual phenotypic variance explained.

already been observed for drought adaptive traits on the same RIL population [42,46]. QTLs identified in the present study showed that several putative genomic regions were involved in the expression of the physiological traits under the three sowing dates (Table 3). The positive and negative signs of additive effect at the different loci indicated the genetic contribution of both parental lines. This confirms the transgressive segregation observed at phenotypic level. Most of QTLs were detected only in one specific environmental condition and constitute adaptative QTLs [25]. The analysis of genes expression showed that 108 cDNA clones were found to be differentially expressed in response to low temperature in sunflower and about 90% of these genes were down regulated [47]. It has been established that the expression of hundreds of genes is altered in response to low temperature [48].

REL was higher after long-term low temperature exposure compared to control (S3) (Table 1) which shows that low temperature leads to a decrease in cell membrane stability. Higher cell membrane stability (which is associated with low values of REL) in response to cold stress after long-term low temperature exposure is considered as a cold acclimation process [10]. This phenomenon was reported in *Arabidopsis* and in rose clover [10]. In contrast, our results show that the cell membrane stability in RILs studied was lower after a long-term low temperature exposure indicating cold damage. This confirms results obtained by Hewezi et al. [47]. The authors have evaluated the frost tolerance of sunflower at −3.8 °C, −4.8 °C and −5.8 °C by measurements of electrolyte leakage. They found that after exposure to low temperature, no cold acclimation but cold damage was observed in the sunflower genotypes studied. In our study, the most important QTL for REL found on linkage group 7 linked to the SSR marker (*ORS331_2*), was common between S1 and S2 (Table 3). These genomic regions should be more investigated to see if they present QTLs related to cold tolerance which are stable in many environments and also in other genetic backgrounds, then they should be used for marker-assisted selection programs.

The long-term low temperature exposure induced a reduction of growth capacity as indicated by the reduction of photosynthetic activity (CC and Fv/Fm) and a reduction of dry matter accumulation (Table 1). Common genomic regions were involved between S2 and S3 conditions for photosynthetic traits and dry matter accumulation on the linkage groups 2, 9, 12, and 15–17 (Fig. 3). Two regions in linkage groups 10 and 16 were detected where QTLs for photosynthetic traits (Fv/Fm and CC) were co-located. These two genomic regions should contain genes with pleiotropic effect which control at the same time efficiency of photosystem II and chlorophyll content. A strong reduction of the plant dry matter was

observed in S1 compared to S2 and S3 (Table 1). Low temperatures did not occur during the same period of plant development for S1 and S2 (Fig. 1): plants in S1 were maintained at low temperature after emergence stage whereas in S2, the temperature after emergence was close to control condition (daily mean temperature of 15.6 °C and 16.2 °C for S2 and S3, respectively). Photosynthetic apparatus has been activated for S2 and S3 under the same temperature conditions whereas in S1 temperatures were colder

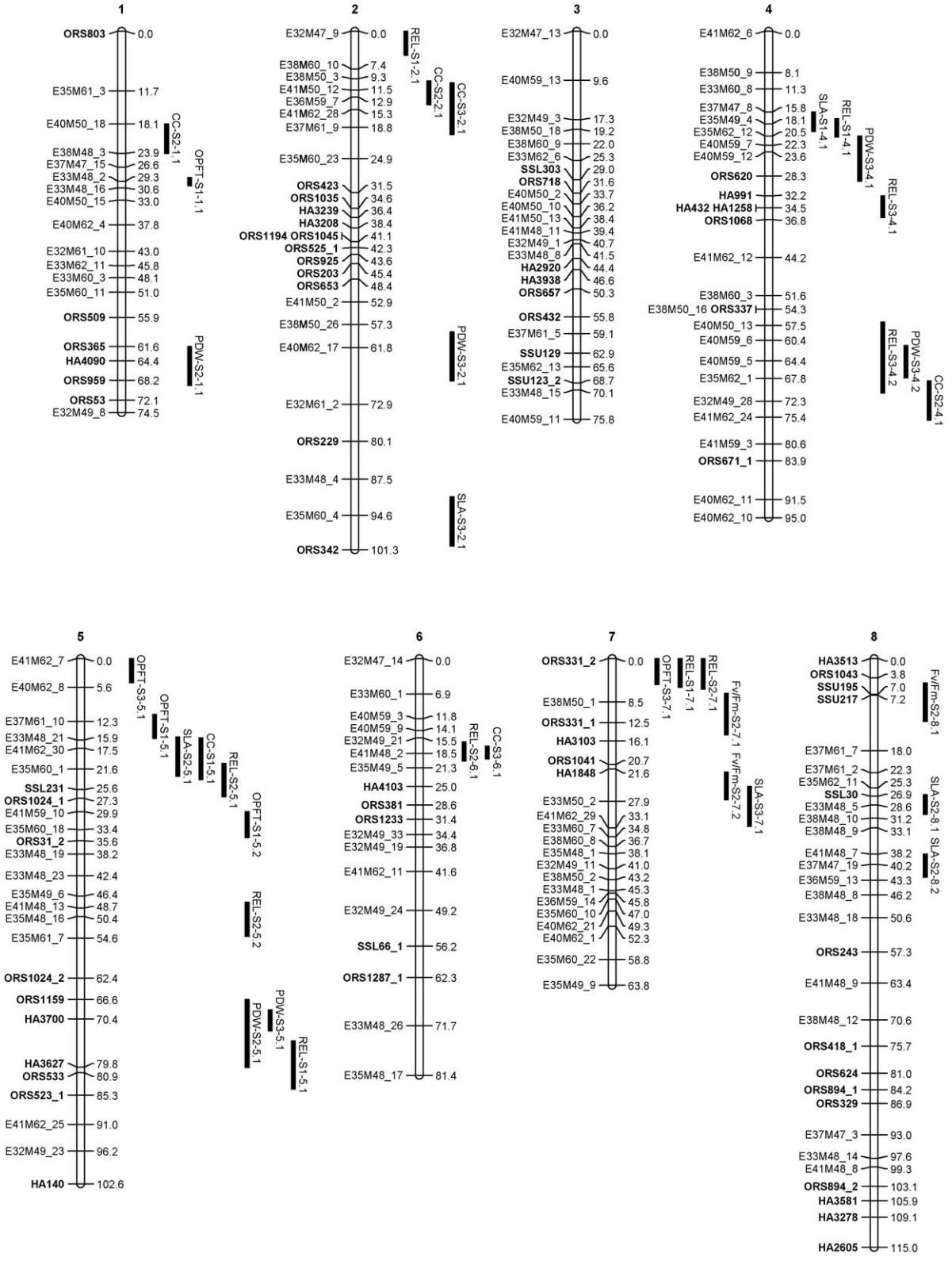

Fig. 3. Sunflower genetic linkage map showing the position of QTL associated with physiological traits in three sowing dates: one control sowing date (S3) and two early sowing dates associated with low temperature (S1 and S2). The physiological traits investigated are the potential photochemical efficiency of photosystem II (Fv/Fm), the chlorophyll content (CC), the specific leaf area (SLA), the relative electrolyte leakage (REL), the osmotic potential at full turgor (OPFT) and the plant dry weight (PDW). The positions of the QTLs are represented on the right side of the linkage groups. Bars represent intervals associated with the QTLs.

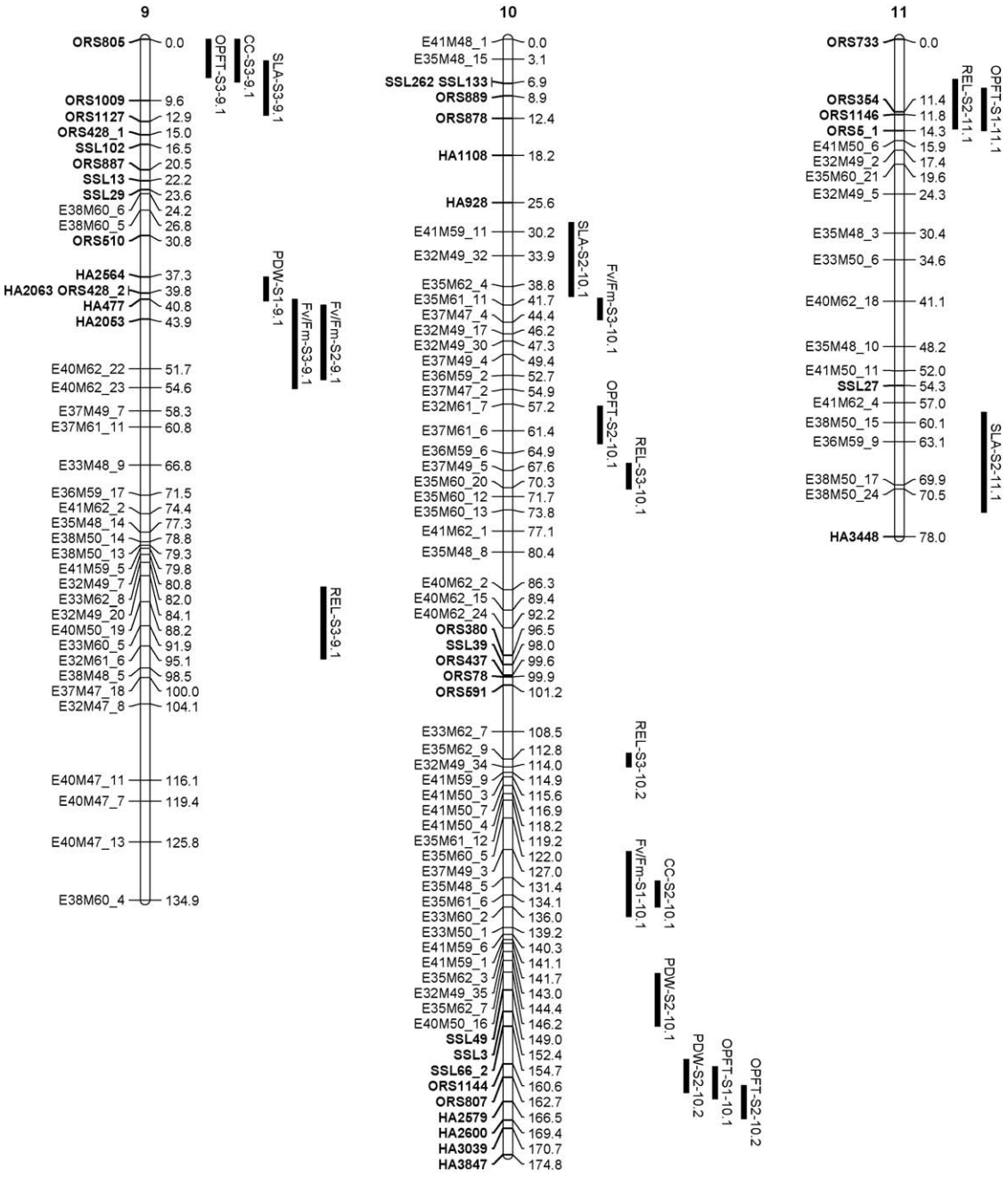

Fig. 3. (*Continued*).

on this stage. These low temperatures occurring in S1 have probably induced the detection of specific QTLs associated to low temperature adaptation of the photosynthetic apparatus. The effect of low temperature on photosynthetic efficiency has been reported in *Arabidopsis thaliana* [10], maize [49], rice [50], wheat [14] and barley [15] but as far as we know, it is not studied in sunflower. In our study, plants grown under low temperature conditions were characterized by a lower photochemical efficiency of photosystem II (Fv/Fm) compared with plants grown in control condition (S3). As shown in wheat, the decrease in the photochemical efficiency of photosystem II (Fv/Fm) can result from the photodamage of the D1 protein of PSII reaction centres and the increase of dissipating excitation energy in the PSII antennae as heat [14]. Low temperature exposure induced lower chlorophyll content compared with control (Table 1). This could reflect the photoprotective process due to the modification of the pigment composition to improve the ability to dissipate the excess light energy as heat via the xanthophyll cycle [51,52]. Photoprotective process leads to a decrease of the chlorophyll content and an increase of the zeaxanthin content as shown by Leipner et al. [53] in maize under low temperature condition. Specific QTLs detected for CC, Fv/Fm and PDW in S1 may be involved in these acclimation processes. These QTLs associated with SSRs marker as *ORS331_1* for photochemical efficiency of photosystem II (Fv/Fm), *ORS1040* for the chlorophyll content (CC) and *ORS428_2* for the plant dry weight (PDW) could be QTLs of interest for cold acclimation of the growth capacity. Co-located QTLs for CC and REL traits in S1 were detected in the linkage group 17. This genomic region may be implied in cold tolerance and growth acclimation to low temperature (Fig. 3).

Early sowing associated with low temperature induced a significant reduction of the specific leaf area (SLA). In our experiment, sunflower shows a significant genetic variability for

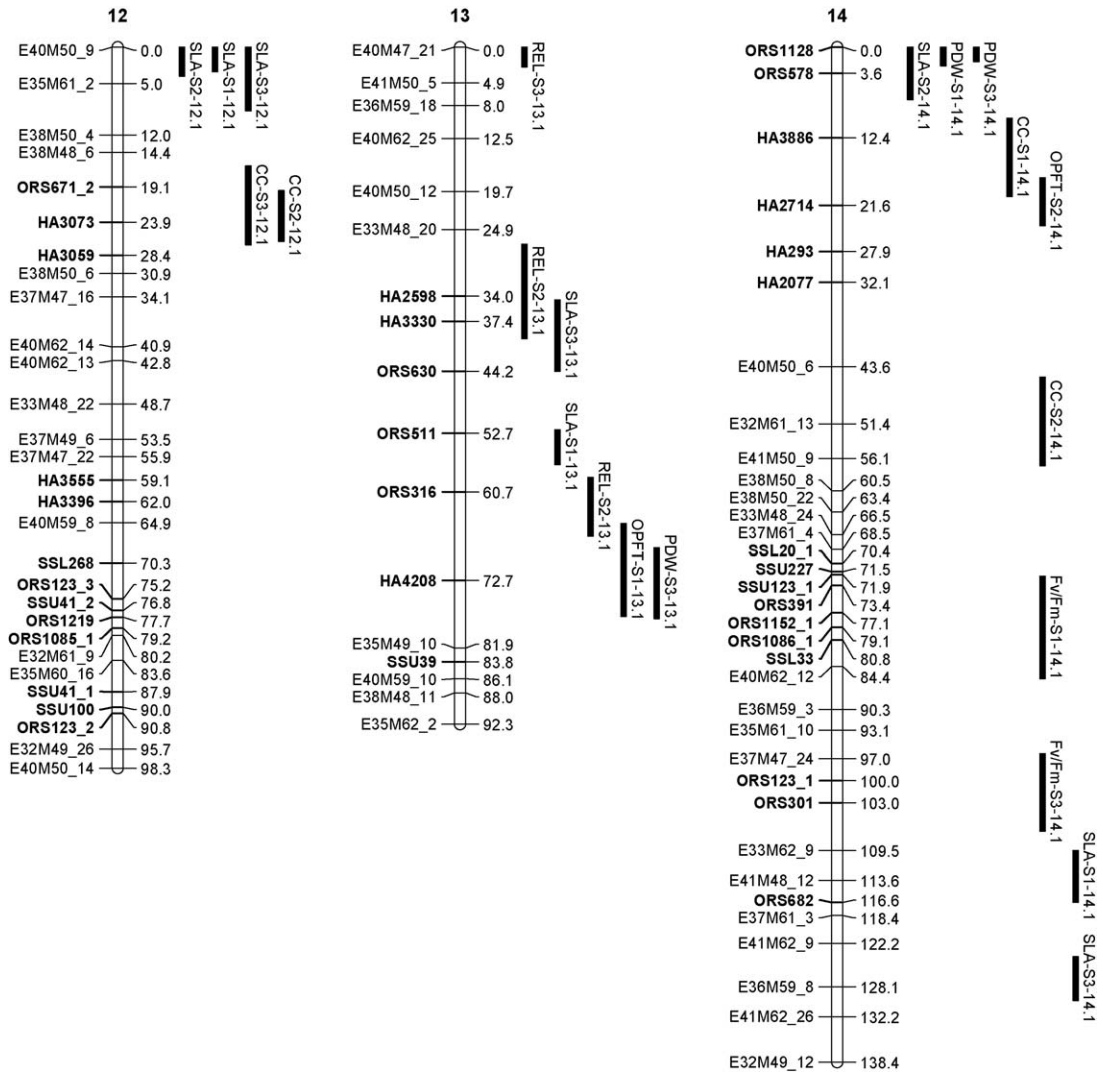

Fig. 3. (*Continued*).

SLA in response to low temperature. On linkage group 12, one region was detected where QTLs for SLA were co-located in all conditions indicated that this genomic region is stable for the control of the SLA (Fig. 3). Two overlapped QTLs between SLA and REL were detected on the linkage group 4 in S1 and on the linkage group 5 in S2, which show that SLA may be a component of cold tolerance. This phenomenon has been also reported in maize [54–56] and different annual legumes [57]. Verheul et al. [56] suggested that low SLA could arise from thicker leaves due to a thicker mesophyll and wax layers and a thicker cuticle. These morphological modifications are similar to xerophytic adaptation, and are commonly exhibited by freeze-tolerant plants [58].

Low temperature implied a decrease of the osmotic potential at full turgor (OP_{FT}) (Table 1). This indicates an increase of the intracellular osmolyte concentration in sunflower genotypes in response to low temperature exposure. The osmotic potential presented a substantial genetic variability after low temperature exposure (Table 1), which shows that the osmotic potential is a useful trait to screen genotypes under low temperature treatment. This trait is a well-known indicator for cold acclimation in Citrus, Spinach and Petunia [22], white clover [59] and Puma Rey [21]. The locations of QTLs identified in the present study for osmotic potential at full turgor (OP_{FT}) when compared with those controlling water status related traits reported by Poormohammad Kiani et al. [30], showed overlapping on linkage groups 1, 5, 9, 16 and 17. On linkage group 16 we have identified the QTL, *OPF-S2-16.1* under low temperature condition which is overlapped with five QTLs identified by Poormohammad Kiani et al. [30] for relative water content, leaf water potential, turgor potential and osmotic potential at full turgor under water stress condition. This suggests that osmotic regulation observed in response to cold and drought stress were regulated by common genomic region. It was shown by Nakashima and Yamaguchi-Shinozaki [60] in *Arabidopsis* that the same genes (dehydration-responsive elements) were activated for osmotic regulation under cold and drought stress.

The whole results of our experiment do highlight that long-term low temperature exposure leads to a decrease in cell membrane stability associated with a decrease of the plant growth capacity (decrease of the plant dry weight, reduction of the chlorophyll content and the potential photochemical efficiency of photosystem II) and the osmotic potential at full turgor. We have also identified the traits associated with cold tolerance, as CC and SLA which are genetically related to REL. These traits would be more investigated to be used to screen genotypes of sunflower for cold tolerance improvement. Genomic regions presenting QTLs associated with SSR markers for REL are interesting for cold tolerance and should be more investigated for their stability across different environments.

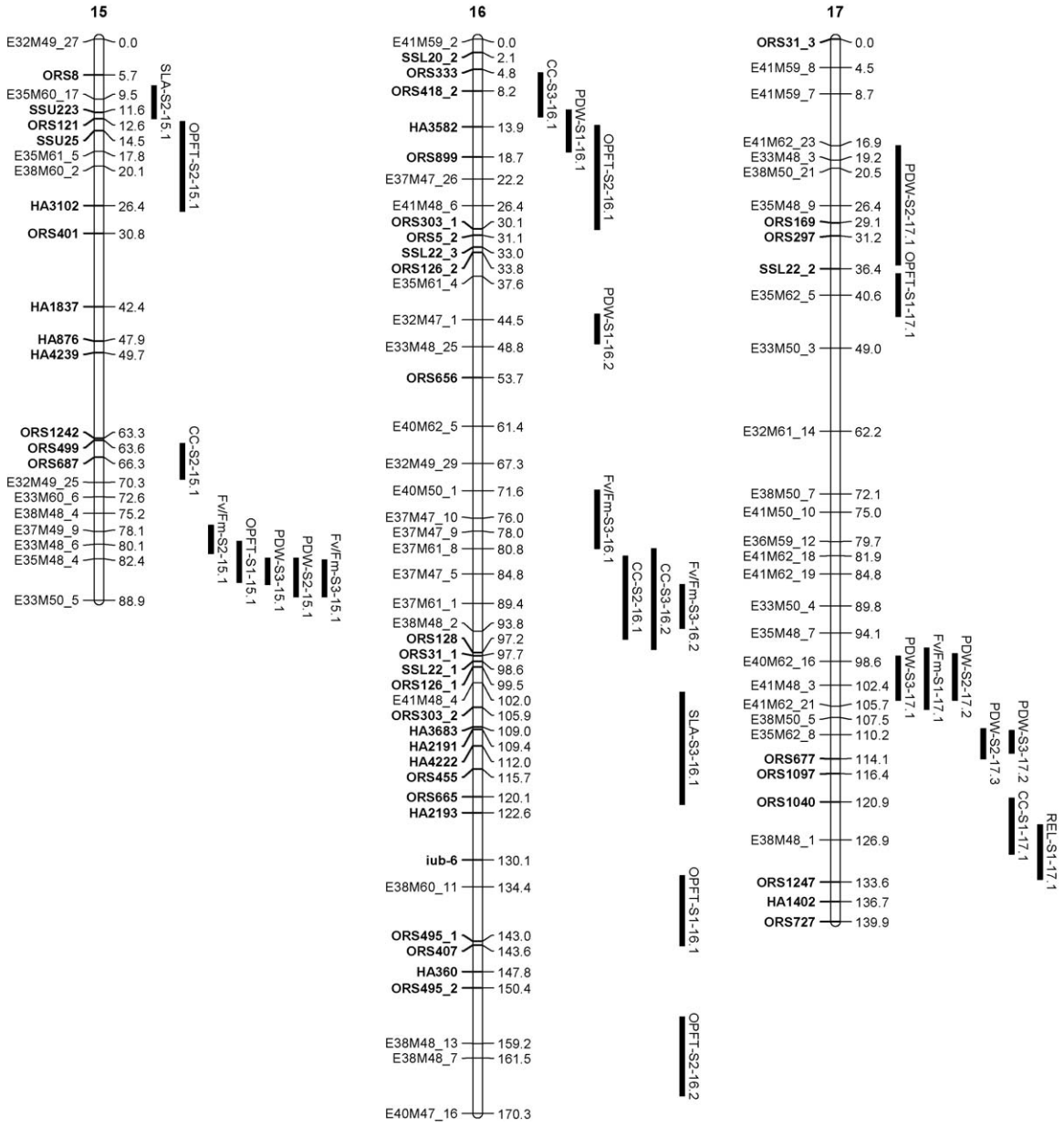

Fig. 3. (*Continued*).

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