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Genetic diversity and morpho-physiological assessment of drought tolerance in rapeseed (*Brassica napus* L.) cultivars

Sara Motallebinia[1], Omid Sofalian[2], Ali Asghari[2], Ali Rasoulzadeh[2] and Bahram Fathi Achachlouei[2]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran and [2]Faculty of Agricultural and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

Research Article

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Corresponding author:
Sara Motallebinia;
Email: sara.motallebi.nia@gmail.com

Abstract

Water deficit is one of the most important abiotic stresses constraining crop production in rapeseed. Understanding the mechanisms of adaptation to this stress is essential for the development and production of drought-tolerant genotypes. For this reason, this research study aims to investigate the importance of genetic diversity in identifying genotypes with a high degree of drought tolerance through assessing effectiveness of inter simple sequence repeat (ISSR) markers on 14 genotypes of rapeseed in a factorial design. Morphological and physiological characteristics were studied after the early stages of growth; in order to evaluate the genetic diversity among genotypes, 18 different ISSR markers were used. A total of 106 clear and scalable loci were amplified, of which 60 bands (56.6%) were polymorphic. The highest polymorphism information content belonged to marker number 9 with the amount of 0.365 (85.7%). Gene variation ranged from 0.081 to 0.365 and the rapeseed genotypes were divided into three groups by cluster analysis (unweighted pair group method with arithmetic mean method). The analysis of molecular variance showed that 70% of the total variation was observed within populations and 30% of this variation occurred among populations. In addition, t-test was used for comparing oil content percentage among different genotypes in control and stress levels. Adriana had the highest amount of seed oil with 36.47%, whereas Karaj 2 had the lowest amount with 27.28 and Cooper had the highest decrease in oil content percentage under stress conditions. Overall, the genotypes Likord, Hyola 401 and Sarigol 32 were identified as the most drought-tolerant.

Introduction

Globally, rapeseed (*Brassica napus* L.), also known as canola, is an economically important oilseed crop and one of the most important multipurpose edible crops (Zhu *et al.*, 2016). It is the third largest source of vegetable oil in the world after soybean and perennial oil palm (FAO, 2021). As a relatively new oilseed plant with more than 40% seed oil, rapeseed is considered as one of the most important plants, producing about 13% of the world's edible oil. Rapeseed oil contains less than 2% of erucic acid and its meal has less than 30 µg of glucosinolate (Thiyam-Holländer *et al.*, 2012). Due to its suitable agricultural characteristics, including high water consumption efficiency and relative tolerance to drought stress, rapeseed can be used as an alternative crop for grain-based crop rotations, especially in areas with arid and semi-arid climates (Hegewald *et al.*, 2018). Iran is among the hot and dry countries of the world, receiving a third of the global precipitation rate (240 mm per year), and drought is considered as one of the most important factors limiting production (Sabzevar *et al.*, 2021). Oil is the most important quality characteristic of oilseeds and the amount of oil in oilseeds is influenced by environmental conditions including drought. Therefore, understanding the effects of drought on morpho-physiological traits and identification of drought-tolerant genotypes is necessary.

Assessing genetic diversity which is a valuable component of plant breeding based on morphological characteristics is widely used, but these characteristics are affected by environmental factors and plant growth stages (Govindaraj *et al.*, 2015). To overcome this issue, molecular markers can provide effective and precise means of selection and provide detailed information about genetic variation. This information is essential for future breeding programmes aiming to better protect and use genetic resources (Shaygan *et al.*, 2021). DNA-based markers are the most widely used and have broad applications for genotyping to improve plant selection. Among these, inter simple sequence repeat (ISSR) markers are widely used in genetic diversity research due to their ease of use, high reproducibility and polymorphism. The main advantage of ISSRs is that no sequence data for primer construction are needed. And for the analytical procedures like polymerase chain reaction (PCR), only low quantities of template DNA are

required. Furthermore, ISSRs are randomly distributed throughout the genome (Godwin *et al.*, 1997). Assessing the effect of drought stress on 10 rapeseed genotypes using ISSR markers and morphological traits, Nemati stated that these markers revealed high genetic diversity for the studied rapeseed genotypes that can be used for breeding programmes (Nemati *et al.*, 2012). Moreover, there are several reports in which DNA-based markers were used. For example, in the study by Tian the genetic diversity of 127 genotypes of *B. napus*, *Brassica rapa* and *Brassica juncea* species was investigated by using genome-specific SSR primers. The results showed that 36.86% of the variance was due to a significant difference between species populations, suggesting a high genetic variation among *B. napus*, *B. rapa* and *B. juncea* genomes (Tian *et al.*, 2017). Safari and Mehrabi (2015) also studied the genetic diversity among 45 rapeseed genotypes by 15 ISSR markers.

This preliminary investigation aimed to identify rapeseed genotypes with resistance to drought and tolerance to low temperatures, suitable for cultivation in the mountainous and arid regions of northern Iran. The study investigates the genetic relationships and differentiation of 14 new genotypes, preselected from a larger screen of rapeseed utilizing molecular markers (ISSR), in addition to screening and analysing the degree of variation in morpho-physiological features and oil content between different rapeseed genotypes in response to drought stress. This is the first time that an investigation has specifically focused on drought effects in rapeseed for cultivation in northern Iran.

Materials and methods

Study location and plant materials

The present study was conducted in the greenhouse of Mohaghegh Ardabili University under controlled conditions including a 14 h photoperiodism (14 h of light and 10 h of darkness) at a temperature of 18–25°C using a factorial completely randomized design with three replications per treatment (online Supplementary Table S1). Each replicate was considered as a pot (14 litres) with six canola seedlings. A total of 14 winter rapeseed genotypes (obtained from Gene Bank of Iran) (Table 1), were evaluated for water-stress tolerance. Different levels of irrigation were evaluated, including complete irrigation (field capacity) as control and irrigation after 60 and 85% depletion of available soil moisture as two levels of stress, respectively. The moisture content was measured using a Moisture Probe Meter (MPW-160-B [12-bit resolution], Sydney, Australia). The device was calibrated before use. Leaf samples were collected on the 10th day after imposition of the stress in three levels and stored in a freezer at −70°C. FAO Irrigation and Drainage Paper No. 56 was used to determine stress levels (Allen *et al.*, 1998). The experiment was performed twice and the results obtained from either experiment were the same. All the data related to measured traits are shown in the Supplementary files.

Morpho-physiological evaluation

Ten days after applying the stress, leaf samples were collected from all the pots at three levels and stored in a refrigerator at −70°C. The following morphological traits were measured: days to flowering and plant height during flowering and grain yield. Two genotypes (Okapi 0 and Okapi 31) did not reach the flowering stage. Therefore, morphological traits were measured in 12 genotypes.

Table 1. Name, origin and growth types of oilseed rape genotypes

Number	Genotype	Origin	Type of genotype
1	Karaj 1	Iran	Pure line
2	Karaj 2	Iran	Pure line
3	Tassillo	Germany	Hybrid cultivar
4	Zarfam	Iran	Cultivar
5	Talaye	Germany	Cultivar
6	Cooper	Germany	Hybrid cultivar
7	Adriana	Germany	Hybrid cultivar
8	Karun	Germany	Hybrid cultivar
9	Sarigol 32	Germany	Cultivar
10	Likord	Germany	Cultivar
11	Hyola 401	Australia	Hybrid cultivar
12	SLMO 46	Germany	Cultivar
13	Okapi 0	France	Cultivar
14	Okapi 31	France	Cultivar

Photochemical activity of photosystem II characterized by F_v/F_m and F_v/F_0 values were measured by a chlorophyll fluorometer (Opti-Sciences OS-30p+ Fluorometer, Hudson, NH, USA; Tsimilli-Michael and Strasser 2008). Soil plant analysis development (SPAD) values were monitored by a SPAD-502 meter (Konica Minolta Inc., Osaka, Japan).

The following physiological parameters were measured as well: photosynthetic pigments by the method of Lichtenthaler (1987), relative water content (RWC) of leaves by the method of Ritchie *et al.* (1990), anthocyanins by the method of Wagner (1979), total carbohydrates by the method of Irigoyen *et al.* (1992), proline using the method of Bates *et al.* (1973) and the total protein from the youngest leaves using the Bradford (1976) method.

Before the analysis of variance, a data normality test was performed using Kolmogorov and Smirnov methods for all traits. Statistical analysis was performed using SPSS 19 and SAS software and the mean comparison was calculated with the Duncan test at 5% probability level.

Oil extraction

B. napus seed samples were collected and stored in paper envelopes in a refrigerator. The seeds were powdered using a mill and then used for oil extraction. Oil content samples were extracted according to the method of Azadmard-Damirchi *et al.* (2011). And to determine the oil percentage, the weight of the oil obtained from 100 g of rapeseed samples by the Soxhlet method (Uquiche *et al.*, 2008) was used.

Molecular evaluation

DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method with slight changes (Saghai-Maroof *et al.*, 1984). The quality and concentration of the DNA were confirmed by electrophoresis on 1% agarose gel and spectrophotometry. Twenty-three primers (Metabion,

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Table 2. Sequence and annealing temperature of ISSR primers

Number	Primer sequence	Temperature (°C)	Number	Primer sequence	Temperature (°C)
1	GAG AGA GAG AGA GAG	42	13*	AGA GAG AGA GAG AGA GT	43
2*	ACA CAC ACA CAC ACA CCA	45	14*	CTC TCT CTC TCT CTC TA	42
3*	AGA GAG AGA GAG AGA GAA	45	15	CAC ACA CAC ACA CAC AT	43
4*	AGA GAG AGA GAG AGA GCC	45	16*	TCT CTC TCT CTC TCT CC	42
5*	AGA GAG AGA GAG AGA GC	42	17*	ACA CAC ACA CAC ACA CT	43
6*	AGA GAG AGA GAG AGA GG	42	18*	AGA GAG AGA GAG AGA GCT	43
7*	GAG AGA GAG AGA GAG AA	42	19*	AGA GAG AGA GAG AGA GCC	43
8*	AGA GAG AGA GAG AGA CT	42	20*	GAG AGA GAG AGA GAG ACC	43
9*	AGA GAG AGA GAG AGA CTA	43	21*	CAC ACA CAC ACA CAC AAC	44
10	GAG AGA GAG AGA GAG ATT	43	22	CTC CTC CTC CTC CTC CTC	46
11*	TGT GTG TGT GTG TGT GGG	46	23*	GGA GAG GAG AGG AGA	42
12	TCC TCC TCC TCC TCC	42			

Seoul, Korea) (Table 2) were selected for ISSR analysis. Eighteen primers marked with an asterisk (*) created the polymorphic bands. PCR amplification was carried out on the Techne PCR instrument and the reaction programme comprised an initial 5 min at 94°C; 40 cycles of 60 s at 94°C, 60 s annealing at the appropriate temperature for each primer and a 60 s extension at 72°C; finishing with a final extension of 5 min at 72°C and storage at 4°C. Agarose gel electrophoresis (1.5%) and subsequent staining with ethidium bromide were used for the identification of PCR products. Each DNA fragment was scored as a discrete variable, using 1 to indicate the presence and 0 for the absence of bands in each sample, and the analysis of molecular data was performed using NTSYSpc2, Popgen 1.32 and GenAlex 6.3 software.

To determine the efficiency of ISSR markers and to estimate the genetic divergence of the selected rapeseed genotypes, the performance of the markers was analysed using five parameters: PIC: polymorphism information contents, MRP: mean resolving power, RP: resolving power, MI: marker index and EMR: effective multiplex ratio.

The PIC value for each locus (band) was measured as follows:

$$\mathrm{PIC}_i = 1 - \sum_j f_{ij}^2$$

where f_{ij} is the frequency of the jth pattern of the ith band as presence (1) or absence (0), in case of dominant markers. After this, the PIC of every ISSR marker was measured as:

$$\mathrm{PIC} = \frac{1}{n}\sum_{i=1}^{n} \mathrm{PIC}_i$$

where n is the total number of polymorphic bands for that marker (Chesnokov and Artemyeva, 2015).

MRP is a parameter which is used to characterize the capacity of the marker integration to analyse the differences among a number of genotypes. For the calculation of RP, information of a band (BI) is measured as:

$$\mathrm{BI}_i = 1 - (2 \times |0.5 - p|)$$

where BI_i is the informative of the amplicon and p is the percentage of the 14 species having ith amplicon. The RP of each primer was measured as:

$$\mathrm{RP} = \sum_{i=1}^{n} \mathrm{BI}_i$$

$$\mathrm{MRP} = \frac{1}{n}\mathrm{RP}$$

where n is the number of polymorphic loci.

The EMR is defined as the number of polymorphic loci multiplied by the proportion of polymorphic loci:

$$\mathrm{EMR} = np(np/n)$$

where np and n are the number of polymorphic loci and the total number of loci, respectively.

The marker index (MI) is the product of the information polymorphism value and the effective multiplex ratio:

$$\mathrm{MI} = \mathrm{PIC} \times \mathrm{EMR}$$

Results

Analysis of morphological traits

The results of variance analysis (online Supplementary Table S2) showed that the effects of drought stress, genotype and their interaction were significant in all the studied traits at 1% probability level. Hayola 401 had the least days to flowering in all control and stress levels, while Talaye and SLMO 46 had the highest plant height and grain yield under stress conditions, respectively. General heritability of traits varied from 0.8146% for days to flowering to 81.09% for grain yield. The effectiveness of phenotypic selection is affected by the genotype × stress (E) interaction (Sallam *et al.*, 2019).

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Analysis of physiological traits

The variance analysis (online Supplementary Table S3) showed that the effect of water stress affected all the traits significantly at 1% probability level except for chlorophyll b. The studied genotypes showed significant differences in chlorophyll content, RWC, chlorophyll a, total chlorophyll, carotenoid, carbohydrate and proline. The interaction effect of genotype × stress was also significantly different in chlorophyll content, maximum fluorescence, relative content of water, chlorophyll b, total chlorophyll, carotenoid and carbohydrate. General heritability also varied from 0.185 in the fluorescence trait up to 65.52 in the chlorophyll content trait. The results related to mean comparison of the morphological and physiological traits showed that all traits except for plant height, seed yield, maximum fluorescence, RWC and chlorophyll b increased under stress conditions. Cluster analysis was performed by the Ward method using Euclidean distance, based on the arithmetic averages of morphological and physiological traits. The dendrograms obtained from cluster analysis divided the genotypes into two groups at the control and the second level of stress. Also, at the first stress level, the genotypes were divided into three groups (Fig. 1). At the control level, the first group had higher levels of fluorescence compared to the other group. And the second had high levels of chlorophyll, photosynthetic pigments and proline. The genotypes with a high genetic distance are suitable for hybridization and access to high heterozygous hybrids.

Discriminant analysis (online Supplementary Table S4) showed that genotypes in the three drawn dendrograms were correctly grouped based on the studied traits (control level, stress levels 1 and 2).

ISSR molecular analysis

In this study, in order to detect and evaluate the diversity between rapeseed genotypes, ISSR molecular primers were used. After screening 23 primers, 18 primers produced clear patterns of multiple bands. A total of 106 clear and scalable loci were amplified, of which 60 bands (56.6%) were polymorphic (Table 3). An example of ISSR primer banding pattern (no. 17) is presented in online Supplementary Fig. S3. The polymorphism metrics (EMR, MRP, PIC, RP and MI) of the 16 ISSR primers are summarized in Table 3. Primer no. 9 revealed the highest PIC (0.365) and MI (1.881). The EMR ranged from 5.14 for primer no. 9 to 0.2 for primer no. 4 (mean: 2.09). Three primers (17, 9 and 5) with high RP values (4.46, 3.92 and 3.78) were introduced as the most efficient markers. Shannon's information index was 0.325 in our study. The primer no. 9 revealed pronounced

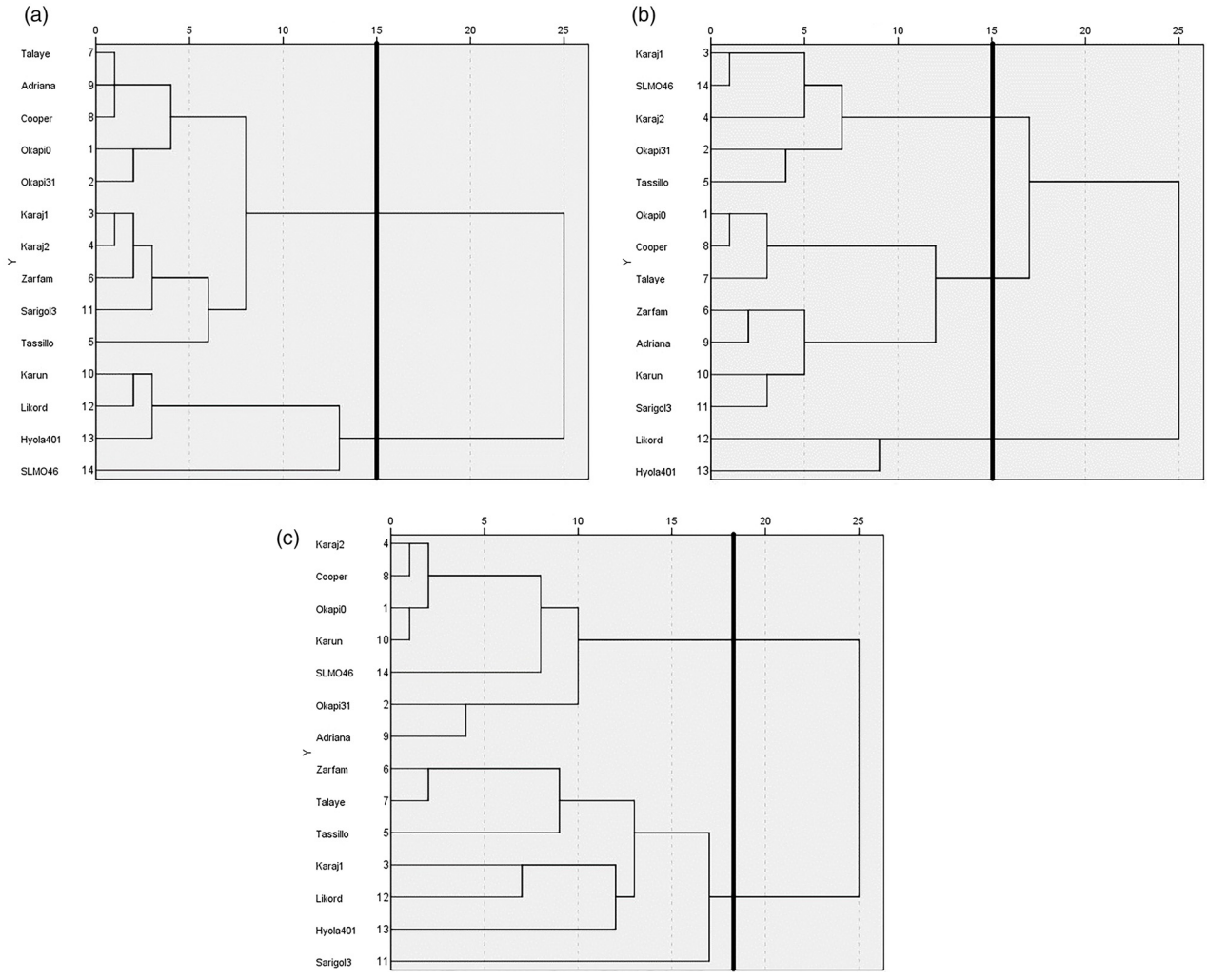

Figure 1. Grouping of canola genotypes based on the measured traits under control, first and second stress levels using the Ward method.

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Table 3. ISSR primers and their indices

Primer	Primer sequence (5′–3′)	Total number of scored bands	Total number of polymorphic bands	Total number of monomorphic bands	Percentage of polymorphic bands	PIC	MRP	RP	MI	EMR
ISSR 2	ACA CAC ACA CAC ACA CCA	3	1	2	0.3333	0.1531	0.714	0.714	0.05103	0.3333
ISSR 3	AGA GAG AGA GAG AGA GAA	5	3	2	0.6	0.1704	0.7126	2.138	0.30672	1.8
ISSR 4	AGA GAG AGA GAG AGA GCC	5	1	4	0.2	0.0816	0.285	0.285	0.01632	0.2
ISSR 5	AGA GAG AGA GAG AGA GC	6	5	1	0.8333	0.2908	0.7566	3.783	1.21166	4.166
ISSR 6	AGA GAG AGA GAG AGA GG	4	1	3	0.25	0.1224	0.571	0.571	0.0306	0.25
ISSR 7	GAG AGA GAG AGA GAG AA	5	3	2	0.6	0.2469	0.4046	1.214	0.44442	1.8
ISSR 8	AGA GAG AGA GAG AGA CT	4	1	3	0.25	0.125	0.5	0.5	0.03125	0.25
ISSR 9	AGA GAG AGA GAG AGA CTA	7	6	1	0.85714	0.3659	0.65383	3.923	1.8817	5.1428
ISSR 11	TGT GTG TGT GTG TGT GGG	9	6	3	0.6666	0.2268	0.3326	1.996	0.9072	4
ISSR 13	AGA GAG AGA GAG AGA GT	4	3	1	0.75	0.3018	0.692	2.076	0.67905	2.25
ISSR 14	CTC TCT CTC TCT CTC TA	6	4	2	0.6666	0.2636	0.6605	2.642	0.70293	2.66
ISSR 16	TCT CTC TCT CTC TCT CC	6	2	4	0.3333	0.1241	0.6785	1.357	0.08273	0.666
ISSR 17	ACA CAC ACA CAC ACA CT	10	7	3	0.7	0.2817	0.637	4.46	1.38033	4.9
ISSR 18	AGA GAG AGA GAG AGA GCT	7	4	3	0.5714	0.2332	0.46375	1.855	0.5330	2.2857
ISSR 19	AGA GAG AGA GAG AGA GCC	8	5	3	0.625	0.2832	0.6282	3.141	0.885	3.125
ISSR 20	GAG AGA GAG AGA GAG ACC	8	4	4	0.5	0.2156	0.6245	2.498	0.4312	2
ISSR 21	CAC ACA CAC ACA CAC AAC	3	1	2	0.3333	0.0868	0.846	0.846	0.02893	0.3333
ISSR 23	GGA GAG GAG AGG AGA	6	3	3	0.5	0.165	0.7853	2.356	0.2475	1.5
Total		106	60	46	–	3.7379	10.94598	36.355	9.85157	37.6621
Percentage		–	56.60	43.40	–	–	–	–	–	–
Average		5.88	–	–	0.5316	0.2076	0.608	2.0197	0.54731	2.0923

PIC, polymorphism information contents; MRP, mean resolving power; RP, resolving power; MI, marker index; EMR, effective multiplex ratio.

Table 4. Genetic diversity parameters based on ISSR markers

Primer number	Primer sequence 5′–3′	Observed number of alleles	Effective number of alleles	Nei's gene diversity (H_e)	Shannon's information index (I)
ISSR 2	ACA CAC ACA CAC ACA CCA	1.33	1.28	0.153	0.217
ISSR 3	AGA GAG AGA GAG AGA GAA	1.6	1.25	0.170	0.270
ISSR 4	AGA GAG AGA GAG AGA GCC	1.20	1.13	0.018	0.119
ISSR 5	AGA GAG AGA GAG AGA GC	1.83	1.48	0.290	0.437
ISSR 6	AGA GAG AGA GAG AGA GG	1.25	1.24	0.122	0.170
ISSR 7	GAG AGA GAG AGA GAG AA	1.60	1.45	0.246	0.357
ISSR 8	AGA GAG AGA GAG AGA CT	1.25	1.25	0.125	0.137
ISSR 9	AGA GAG AGA GAG AGA CTA	1.85	1.66	0.365	0.526
ISSR 11	TGT GTG TGT GTG TGT GGG	1.66	1.36	0.226	0.345
ISSR 13	AGA GAG AGA GAG AGA GT	1.75	1.52	0.301	0.442
ISSR 14	CTC TCT CTC TCT CTC TA	1.66	1.47	0.263	0.386
ISSR 16	TCT CTC TCT CTC TCT CC	1.33	1.22	0.124	0.183
ISSR 17	ACA CAC ACA CAC ACA CT	1.70	1.48	0.281	0.413
ISSR 18	AGA GAG AGA GAG AGA GCT	1.57	1.41	0.233	0.339
ISSR 19	AGA GAG AGA GAG AGA GCC	1.62	1.52	0.283	0.403
ISSR 20	GAG AGA GAG AGA GAG ACC	1.50	1.38	0.215	0.310
ISSR 21	CAC ACA CAC ACA CAC AAC	1.33	1.11	0.086	0.143
ISSR 23	GGA GAG GAG AGG AGA	1.50	1.25	0.165	0.253
	(Mean)	1.56	1.38	0.221	0.325

discrimination of 85.7% polymorphism. On the other hand, primer no. 4 recorded the lowest polymorphism of 20%. Moreover, the observed and effective numbers of alleles were 1.56 and 1.38, respectively (Table 4). Considering sub-populations as groups, the average genetic variation (H_s) was 0.1344 and the total genetic variation (H_t) was 0.1982. The average degree of gene differentiation (G_{st}) among rapeseed genotypes was 0.3218 in all locations. This result suggests that a larger share of the total variation is related to the diversity within the genotypes. The genetic distance between the genotypes based on the genetic distance of Nei (Nei, 1987) ranged from 0.109 between Karun and Adriana up to 0.400 between Sarigol 32 and Tassillo.

Three different genetic distance coefficients (Dice, simple matching and Jacard) and three different clustering methods (unweighted pair group method with arithmetic mean [UPGMA], single linkage and complete linkage) were used in this analysis. Based on the highest cophenetic correlation coefficient, Jacard's genetic distance coefficient and UPGMA method were selected. Based on the results of the Mantel test, the cophenetic correlation coefficient ($r = 0.81$) was obtained, which indicates a high correlation between similarity matrices and final cluster diagrams. In this analysis, 14 rapeseed genotypes were divided into three groups (Fig. 2).

Moreover, principal coordinate analysis indicated that the first three principal coordinates explained 64.67% of the molecular variations among the genotypes (online Supplementary Table S5).

Oil content

By applying stress, oil content decreased in all genotypes. There was no noticeable difference between the two levels of water stress (moderate and severe). Therefore, the t-test was used for comparison. There was a significant difference (0.01) between the amount of oil in the two levels of control and water stress (online Supplementary Table S6). Among the genotypes, Adriana with 36.47% had the highest amount and Karaj 2 with 27.82% had the lowest oil content in the control level. Among the genotypes, Karaj 1, Karaj 2, Sarigol 32 and Hayola 401 had the lowest decrease in oil content, which is a sign of better stability under stress conditions. Cooper, on the other hand, had the highest decrease in oil content.

Results of canonical correlation analysis

Canonical correlation analysis was used to find out the potential relationship between the measured traits and genetic parameters in the studied population. Canonical correlations of physiological traits and molecular markers and the proportion of variability explained by each pair of canonical variates were calculated at the control level and two stress levels separately.

At the control level and the first level of stress (online Supplementary Table S7), the first canonical correlations (0.977 and 0.9629, respectively) between the first canonical variates (V1U1) were significant ($P < 0.01$). But, at the second level of stress, both the first (0.9662) and second (0.9446) canonical correlations between the first two canonical variates (V1U1 and V2U2) were found significant, so these functions were used to interpret the results of canonical correlation analysis at different treatment levels (online Supplementary Table S8). According to Tabachnick and Fidell (2001), correlations between the canonical variates and main variables that are greater than 0.300 are

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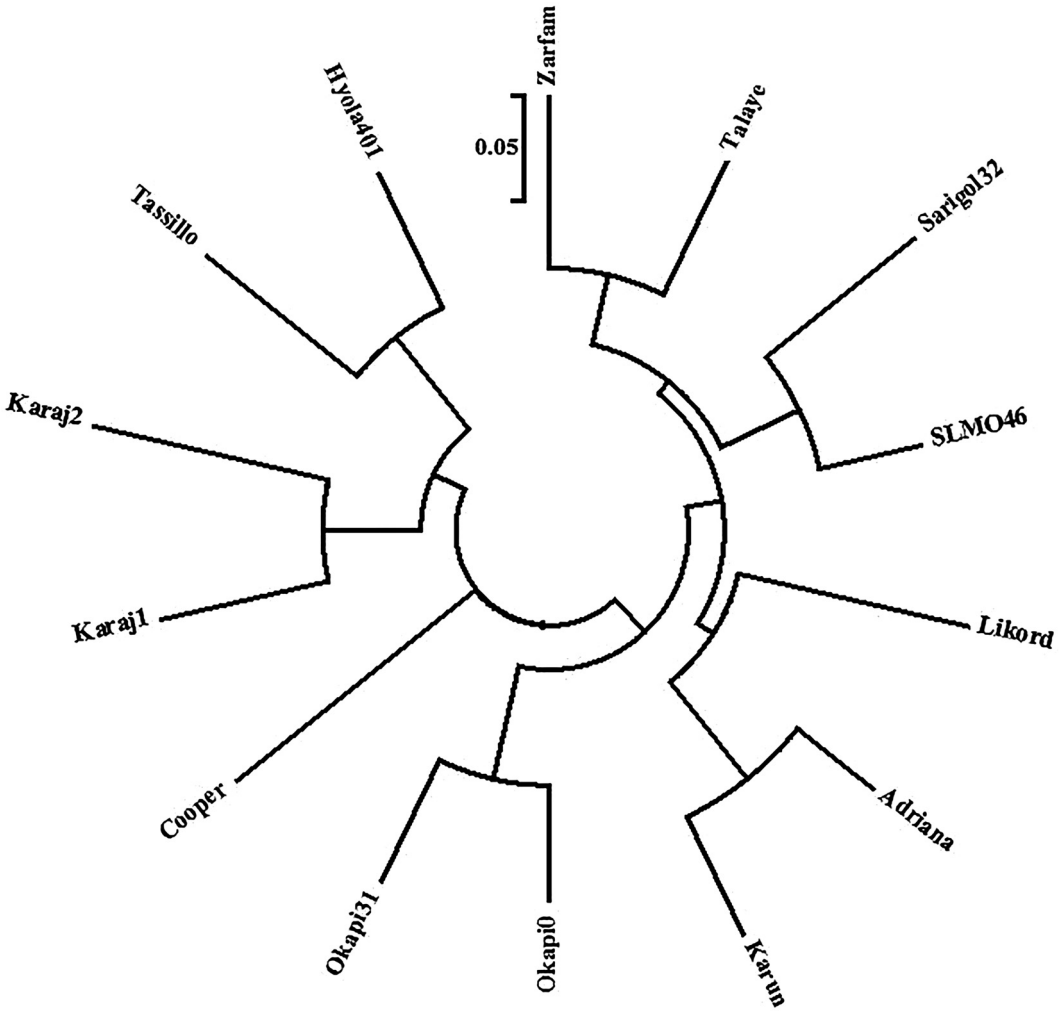

Figure 2. Grouping of canola genotypes based on ISSR data using Jacard similarity coefficient and UPGMA method.

interpreted regardless of their positive or negative sign (±). At the control level, it was found that the first canonical variate of physiological traits (V1) positively correlated with chlorophyll content (SPAD), chlorophyll a, chlorophyll b, total chlorophyll, proline and anthocyanin, whereas high-negative correlations were observed between that of physiological traits (V1) and maximum fluorescence, carotenoid and the percentage of protein (online Supplementary Table S8). The first canonical variate of molecular markers (U1) had high-positive correlations with loci m3l7, m5l15, m5l19, m6l23, m8l31, m11l42, m17l65, m17l66, m17l67, m17l69, m17l72, m18l76, m18l78, m19l87, m20l91, m21l99, m23l104 and m23l106. High-negative correlations were obtained between that of molecular markers (U1) and loci m9l38, m11l43, m13l49, m13l50, m13l51 and m20l94. The degree to which the canonical variates of physiological traits can justify the variation in their primary variables is equal to sum of the squared powers of the canonical structures divided by the number of variables within that group (online Supplementary Table S8). However, the redundancy index is defined as a percentage of variance captured by canonical variates of the opposite group (online Supplementary Table S8). Each value presented in online Supplementary Table S9 represents the variability of each variable in one group (the variable in physiological traits) which is explained by the first canonical variate of another group (by the first canonical variate of molecular traits). All data points representing relevant variables on the *X* and *Y* axes and lying within a circle (the blue circle) with a radius of 0.3 were ignored (online Supplementary Fig. S5–S7). It means that correlations between the original variables and their canonical variates are less than 0.30. At the first stress level (60% depletion of available soil moisture), the results indicated that the physiological traits including chlorophyll content (SPAD), chlorophyll a, chlorophyll b, total chlorophyll, proline and carotenoid had high-positive correlations with their first canonical variate (V1), whereas negative correlations were observed between quantum yield potential, initial fluorescence, maximum fluorescence, total carbohydrates, the percentage of protein and their first canonical variate (V1). The first canonical variate of molecular markers (U1) presented high-positive correlations with loci m4l10, m5l15, m8l31, m9l39, m11l40, m11l41, m11l45, m17l65, m17l66, m17l69, m17l72, m18l76, m18l78, m23l104 and m23l106 (online Supplementary Table S8). At the second stress level (85% depletion of available soil moisture), it was observed that the first canonical variate of molecular markers (U1) had high-positive correlations with loci m4l10, m5l15, m5l17, m8l31, m9l35, m11l40, m11l41, m17l67, m18l76 and m18l78. On the other hand, there were significant negative correlations between loci m7l27, m9l34, m9l38, m19l84, m19l85, m19l86, m19l87, m19l89, m20l94, m20l96,

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m20l97 and the first canonical variate of molecular markers (U1). Also, high correlations were observed between the first canonical variate of physiological traits (V1) and chlorophyll content (SPAD), initial fluorescence (F_0), maximum fluorescence (F_m), RWC, total carbohydrates and the percentage of protein. However, RWC and total carbohydrate traits had negative correlations with the first function of physiological traits (V1). The results demonstrate that the percentage of protein has significantly increased at the second stress level compared to the other treatment levels.

Discussion

In the present study, three measured morphological traits were reduced under stress conditions. Decreased plant height and grain yield under stress conditions indicate photosynthetic dysfunction due to dehydration, reduced production and distribution of photosynthetic materials. These results are consistent with reports by other researchers (Sinaki *et al.*, 2007; Nasri *et al.*, 2008; Faraji *et al.*, 2009).

In comparison to the control, flowering time in Tassillo, Likord and Talaye genotypes was hindered by drought stress levels. The flowering time of rapeseed is influenced by a number of genetic factors, including vernalization (Shah *et al.*, 2018) and photoperiod genes (Raman *et al.*, 2019). Studies have also shown that drought stress can alter the expression level of miRNAs like miR156s, which regulate the expression of flowering time genes in rapeseed. It was found that miR156 genes were differentially expressed under drought stress, possibly in response to altered sugar levels under photosynthetic limitations (Schiessl *et al.*, 2020).

A significant increase of total carbohydrate content was also observed for all genotypes under drought stress that is consistent with findings reported by Schiessl *et al.* (2020). Karaj 1, Karaj 2, Zarfam, Adriana and Karun had early flowering time phenotypes in response to drought stress, which is regarded as a drought escape strategy to alleviate adverse effects of drought stress on crop productivity (Song *et al.*, 2013; Khanna-Chopra and Singh, 2015). Therefore, these genotypes can be suitable for areas that experience late season drought (Song *et al.*, 2013; Khanna-Chopra and Singh, 2015). Several factors can influence the early flowering time in rapeseed during drought stress including genes such as FLC (Cockram *et al.*, 2007) and VRN-1 (Diallo *et al.*, 2012), plant hormones like salicylic acid, brassinosteroids, indoleacetic acid and cytokinin (Diezel *et al.*, 2011; Wasternack *et al.*, 2013) and epigenetic factors (Zhang *et al.*, 2011; Pu *et al.*, 2013). No significant difference in days to flowering was observed in the Hyola 401, Sarigol 32 and Cooper suggesting that the flowering time was not influenced by drought stress.

Among the measured physiological traits, the highest RWC was obtained in the control and was significantly higher from the stress levels. It is likely that abscisic acid is involved in signalling from the root under drought stress conditions and causes the pores to close and results in a decrease in photosynthesis (Ritchie *et al.*, 1990). The amount of chlorophyll a and total chlorophyll increased with stress. The application of stress was associated with a decrease in the initial fluorescence and maximum fluorescence, which indicates the decay of the reaction centres of the photosystem II under drought stress conditions.

Carotenoid increased with stress so that the highest amount was observed at the second level of stress. Carotenoids can protect the light-harnessing system of the photosynthetic apparatus from the radical oxygen molecules and indirectly reduce the production of oxygen species. Carotenoids also consume nicotinamide adenine dinucleotide phosphate hydrogen and protect chlorophyll against photo-oxidation through a mechanism called the xanthophyll cycle (Koyro, 2006). Total carbohydrates, anthocyanins, proline and protein levels increased under drought stress conditions, which are consistent with previous studies (Khan *et al.*, 2007; Ebrahimi *et al.*, 2010; Nasibi *et al.*, 2011; Karimi and Vafaeezadeh, 2012; Schiessl *et al.*, 2020).

All the traits except for anthocyanin, F_0, F_m, F_v/F_m had high heritability. For this reason, the response to selection using marker-assisted selection (MAS) for these traits could be highly effective, as a higher heritability suggests that the traits are more likely to be passed on to the next generation. MAS has several advantages over conventional phenotypic selection, including the ability to select plants based on their genotype which can considerably shorten the time for new crop varieties to be brought to the market (Collard and Mackill, 2008; Hasan *et al.*, 2021). In the context of stress conditions, such as drought, MAS has been used to identify and validate quantitative trait loci (QTLs) associated with yield traits (Hashem *et al.*, 2023).

Techniques to identify the level of genetic diversity in rapeseed are very important (Havlickova *et al.*, 2014) and ISSR markers are one of the most widely used techniques for this purpose. ISSR primers studied in the investigation were mostly dinucleotide repeats with either one or two nucleotide residues anchored at the 3′ terminus. Primer no. 17 with repeats $(AC)_8$ amplified the most frequent number of bands, whereas the second most frequent one belonged to primer nos. 11, 19 and 20 with repeats $(TG)_8$, $(AG)_8$ and $(GA)_8$, respectively. These results are consistent with those previously reported in the literature (Shi *et al.*, 2013), where dinucleotides with repeats AT, AG and AC accounted for 23.5% of repeat sequences founded in *B. napus* genome. According to online Supplementary Table S9 and Table 3, primer nos. 5, 9, 17, 18 and 19 were determined to be informative, as the first canonical variate of physiological traits (V1) explained the majority of the variation in banding pattern amplified by the primers. Numerous studies have indicated that microsatellites are commonly found in genic regions, including cDNA clones (Piquemal *et al.*, 2005), expressed sequence tags (Batley *et al.*, 2007), unique transcript sequences (Wang *et al.*, 2012), BAC-end sequences (Ling *et al.*, 2007) and genome survey sequences (Cheng *et al.*, 2009). Given that ISSR markers consist of microsatellite core sequences with a few selective nucleotides serving as anchors in the adjacent regions (Amiteye, 2021), these markers have the capability to amplify partial sequences of microsatellites found in coding or untranslated regions and potentially have associations with specific traits (Parida *et al.*, 2010).

Overall, the results from this study identified rapeseed genotypes suitable for cultivation in northern Iran and provided new insight into the heritability of traits.

Conclusion

The present study showed that Likord, Sarigol 32 and Hayola 401 provide better performance stability under drought stress conditions and would be suitable for incorporating into breeding programmes. The utility of ISSR–PCR marker primer nos. 5, 9, 17, 18 and 19 for discriminating genotypes was demonstrated. The markers identified in this study could be useful for QTL mapping to identify the chromosomal regions for drought tolerance to breed rapeseed genotypes suitable for the dry and mountainous regions in northern Iran.

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References

Allen RG, Pereira LS, Raes D and Smith M (1998) Crop evapotranspiration guidelines for computing crop water requirements. FAO Irrigation and drainage paper 56. Food and Agriculture Organization, Rome.

Amiteye S (2021) Basic concepts and methodologies of DNA marker systems in plant molecular breeding. *Heliyon* **7**, e08093. doi: 10.1016/j.heliyon.2021.e08093

Azadmard-Damirchi S, Alirezalu K and Fathi Achachlouei B (2011) Microwave pretreatment of seeds to extract high quality vegetable oil. *World Academy of Science, Engineering and Technology* **57**, 72–75.

Bates L, Waldren R and Teare I (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205–207.

Batley J, Hopkins CJ and Cogan NOI (2007) Identification and characterization of simple sequence repeat markers from *Brassica napus* expressed sequences. *Molecular Ecology Notes* **7**, 886–889.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Analytical Biochemistry* **72**, 248–254.

Cheng X, Xu J and Xia S (2009) Development and genetic mapping of microsatellite markers from genome survey sequences in *Brassica napus*. *Theoretical Applied Genetics* **118**, 1121–1131.

Chesnokov YV and Artemyeva AM (2015) Evaluation of the measure of polymorphism information of genetic diversity. *Agricultural Biology* **50**, 571–578.

Cockram J, Jones H, Leigh FJ, O'Sullivan D, Powell W, Laurie DA and Greenland AJ (2007) Control of flowering time in temperate cereals: genes, domestication, and sustainable productivity. *Journal of Experimental Botany* **58**, 1231–1244.

Collard BC and Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B. Biological Sciences* **363**, 557–572.

Diallo AO, Ali-Benali MA, Badawi M, Houde M and Sarhan F (2012) Expression of vernalization responsive genes in wheat is associated with histone H3 trimethylation. *Molecular Genetics and Genomics* **287**, 575–590.

Diezel C, Allmann S and Baldwin IT (2011) Mechanisms of optimal defense patterns in *Nicotiana attenuata*: flowering attenuates herbivory-elicited ethylene and jasmonate signaling. *Journal of Integrative Plant Biology* **53**, 971–983.

Ebrahimi S, Nikkhah A and Sadeghi A (2010) Changes in nutritive value and digestion kinetics of canola seed due to microwave irradiation. *Asian-Australasian Journal of Animal Science* **23**, 347.

FAO (2021) FAOSTAT. Rome, Italy. Available at www.fao.orgfaostaten%23dataQI.pdf

Faraji A, Latifi N, Soltani A and Shirani Rad AH (2009) Seed yield and water use efficiency of canola (*Brassica napus* L.) as affected by high temperature stress and supplemental irrigation. *Agricultural Water Management* **96**, 132–140.

Godwin ID, Aitken EAB and Smith LW (1997) Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis* **18**, 1524–1528.

Govindaraj M, Vetriventhan M and Srinivasan M (2015) Importance of genetic diversity assessment in crop plants and its recent advances: an overview of its analytical perspectives. *Genetics Research International* **2015**, 1–14.

Hasan N, Choudhary S, Naaz N, Sharma N and Laskar RA (2021) Recent advancements in molecular marker-assisted selection and applications in plant breeding programmes. *Journal of Genetic Engineering and Biotechnology* **19**, 128.

Hashem M, Sandhu KS, Ismail SM, Börner A and Sallam A (2023) Validation and marker-assisted selection of DArT-genomic regions associated with wheat yield-related traits under normal and drought conditions. *Frontiers in Genetics* **14**, 1195566.

Havlickova L, Jozova E, Rychla A, Klima M, Kucera V and Curn V (2014) Genetic diversity assessment in winter oilseed rape (*Brassica napus* L.) collection using AFLP, ISSR and SSR markers. *Czech Journal of Genetics and Plant Breeding* **50**, 216–225.

Hegewald H, Wensch-Dorendorf M, Sieling K and Christen O (2018) Impacts of break crops and crop rotations on oilseed rape productivity: a review. *European Journal of Agronomy* **101**, 63–77.

Irigoyen J, Einerich D and Sánchez-Díaz M (1992) Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum* **84**, 55–60.

Karimi B and Vafaeezadeh M (2012) SBA-15-functionalized sulfonic acid confined acidic ionic liquid: a powerful and water-tolerant catalyst for solvent-free esterifications. *Chemical Communications* **48**, 3327–3329.

Khan MN, Siddiqui MH, Mohammad F, Khan M and Naeem M (2007) Salinity induced changes in growth, enzyme activities, photosynthesis, proline accumulation and yield in linseed genotypes. *World Journal of Agricultural Sciences* **3**, 685–695.

Khanna-Chopra R and Singh K (2015) Drought resistance in crops: physiological and genetic basis of traits for crop productivity. In Tripathi BN and Müller M (eds), *Stress Responses in Plants: Mechanisms of Toxicity and Tolerance*. New York, NY: Springer, pp. 267–292.

Koyro HW (2006) Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte *Plantago coronopus* (L.). *Environmental and Experimental Botany* **56**, 136–146.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology* **148**, 350–382.

Ling AE, Kaur J and Burgess B (2007) Characterization of simple sequence repeat markers derived in silico from *Brassica rapa* bacterial artificial chromosome sequences and their application in *Brassica napus*. *Molecular Ecology Notes* **7**, 273–277.

Nasibi F, Manuchehri Kalantari KH and Yaghoobi MM (2011) Comparison the effects of sodium nitroprusside and arginine pretreatment on some physiological responses of tomato plant (*Lycopersicun esculentum*) under water stress. *Iranian Journal of Biology* **24**, 833–847 (in Persian).

Nasri M, Khalatbari M, Zahedi H, Paknejad F and Tohidi Moghadam HR (2008) Evaluation of micro and macro elements in drought stress condition in cultivars of rapeseed (*Brassica napus* L.). *American Journal of Agricultural and Biological Sciences* **3**, 579–583.

Nei M (1987) *Molecular Evolutionary Genetics*. New York, USA: Columbia University Press.

Nemati M, Asghari A, Sofalian O, Rasoulzadeh A and Mohamaddoust Chamanabad H (2012) Effect of water stress on rapeseed cultivars using morpho-physiological traits and their relations with ISSR markers. *Journal of Plant Physiology & Breeding* **2**, 55–66.

Parida S, Pandit A, Gaikwad K, Sharma T, Srivastava P, Singh N and Mohapatra T (2010) Functionally relevant microsatellites in sugarcane unigenes. *BMC Plant Biology* **2010**, 251.

Piquemal J, Cinquin E and Couton F (2005) Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers. *Theoretical and Applied Genetics* **111**, 1514–1523.

Pu L, Liu MS, Kim SY, Chen LF, Fletcher JC and Sung ZR (2013) EMBRYONIC FLOWER1 and ULTRAPETALA1 act antagonistically on Arabidopsis development and stress response. *Plant Physiology* **162**, 812–830.

Raman H, Raman R, Qiu Y, Yadav AS, Sureshkumar S, Borg L, Rohan M, Wheeler D, Owen O, Menz L and Balasubramanian S (2019) GWAS hints at pleiotropic roles for FLOWERING LOCUS T in flowering time and yield-related traits in canola. *BMC G Raman enomics* **20**, 636.

Ritchie SW, Nguyen HT and Holaday AS (1990) Leaf water content and gas-exchange parameters of two wheat genotypes differing in drought resistance. *Crop Science* **30**, 105–111.

Sabzevar MS, Rezaei A and Khaleghi B (2021) Incremental adaptation strategies for agricultural water management under water scarcity condition in northeast Iran. *Regional Sustainability* **2**, 224–238.

Safari S and Mehrabi AA (2015) Evaluation of genetic diversity in rapeseed genotypes using ISSR markers. *Agricultural Biotechnology* **5**, 37–43.

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences* **81**, 8014–8018.

Sallam A, Alqudah AM, Dawood MF, Baenziger PS and Börner A (2019) Drought stress tolerance in wheat and barley: advances in physiology, breeding and genetics research. *International Journal of Molecular Sciences* **20**, 31–37.

Schiessl SV, Quezada-Martinez D, Orantes-Bonilla M and Snowdon RJ (2020) Transcriptomics reveal high regulatory diversity of drought tolerance strategies in a biennial oil crop. *Plant Science* **297**, 110515.

https://doi.org/10.1017/S1479262124000145 Published online by Cambridge University Press

Shah S, Weinholdt C, Jedrusik N, Molina C, Zou J, Große I, Schiessl S, Jung CH and Emrani N (2018) Whole transcriptome analysis reveals genetic factors underlying flowering time regulation in rapeseed (*Brassica napus* L.). *Plant Cell Environment* **41**, 1935–1947.

Shaygan N, Etminan A, Majidi Hervan I, Azizinezhad R and Mohammadi R (2021) The study of genetic diversity in a minicore collection of durum wheat genotypes using agro-morphological traits and molecular markers. *Cereal Research Communications* **49**, 141–147.

Shi J, Huang S, Zhan J, Yu J, Wang X, Hua W, Liu S, Liu G and Wang H (2013) Genome-wide microsatellite characterization and marker development in the sequenced brassica crop species. *DNA Research* **21**, 53–68.

Sinaki JM, Majidi Heravan E, Shirani AH, Noormohamadi G and Zarei G (2007) The effects of water deficit during growth stages of canola (*B. napus* L.). *American–Eurasian Journal of Agricultural and Environmental Sciences* **2**, 417–422.

Song YH, Ito S and Imaizumi T (2013) Flowering time regulation: photoperiod- and temperature-sensing in leaves. *Trends Plant Science* **18**, 575–583.

Tabachnick B and Fidell LS (2001) *Using Multivariate Statistics*. Needham Heights, USA: A Pearson Education Company, 966 pp.

Thiyam-Holländer U, Eskin NA and Matthäus B (2012) *Canola and Rapeseed: Production, Processing, Food Quality, and Nutrition*. Boca Raton, Florida, USA: CRC Press. 374 pp.

Tian HY, Channa SA and Hu SW (2017) Relationships between genetic distance, combining ability and heterosis in rapeseed (*Brassica napus* L.). *Euphytica* **213**, 1.

Tsimilli-Michael M and Strasser RJ (2008) In vivo assessment of stress impact on plant's vitality: applications in detecting and evaluating the beneficial role of mycorrhization on host plants. In Varma A. (ed.), *Mycorrhiza*. Berlin Heidelberg: Springer, pp. 679–703.

Uquiche E, Jerez M and Ortiz J (2008) Effect of pretreatment with microwaves on mechanical extraction yield and quality of vegetable oil from Chilean hazelnuts (*Gevuina avellana* Mol). *Innovative Food Science and Emerging Technologies* **9**, 495–500.

Wagner GJ (1979) Content and vacuole/extravacuole distribution of neutral sugars, free amino acids, and anthocyanin in protoplasts. *Plant Physiology* **64**, 88–93.

Wang F, Wang XF, Chen X, Xiao Y, Li H, Zhang SH, Xu J, Fu J, Huang L, Liu CH, Wu J and Liu K (2012) Abundance, marker development and genetic mapping of microsatellites from unigenes in *Brassica napus*. *Molecular Breeding* **30**, 731–744.

Wasternack C, Forner S, Strnad M and Hause B (2013) Jasmonates in flower and seed development. *Biochimie* **95**, 79–85.

Zhang Z, Zhang S and Zhang Y (2011) Arabidopsis floral initiator SKB1 confers high salt tolerance by regulating transcription and pre-mRNA splicing through altering histone H4R3 and small nuclear ribonucleoprotein LSM4 methylation. *The Plant Cell* **23**, 396–411.

Zhu M, Monroe JG, Suhail Y, Villiers F, Mullen J, Pater D, Hauser F, Jeon BW, Bader JS, Kwak JM, Schroeder JI, McKay JK and Assmann SM (2016) Molecular and systems approaches towards drought-tolerant canola crops. *New Phytologist* **210**, 1169–1189.

https://doi.org/10.1017/S1479262124000145 Published online by Cambridge University Press