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Duplicated FAD2 genes, fatty acid profiles and membrane integrity in cold stress response of durum wheat genotypes

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Research Article

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Abstract

Fatty acid desaturases (FADs) introduce double bonds at specific positions of fatty acyl chains and are involved in the protection of membrane integrity at low temperatures. Three genotypes of *Triticum turgidum* ssp. *durum* with differences in cold tolerance were considered. The analysis of *TdFAD2* sequences identified an intronless gene encoding a 387 amino acid protein including three histidine domains. The *in silico* mapping on the cv Svevo genome revealed the occurrence of four *FAD2* copies in tandem localized in 6A and 6B chromosomes and suggested an inversion in 6B. Three out of eight copies consisted of non-functional sequences. The exploration of the 5´regulatory region showed differences in TF binding sites among the copies suggesting certain specificity in gene expression. The evaluation of FAD2 by qRT-PCR, in plants exposed at 4 ˚C, showed transcriptional induction and variations among the genotypes. The fatty acid profiles determined by gas chromatography revealed that the major component of the fatty acids (60–80%) was linolenic acid (18:3). The more cold-tolerant genotypes increased 18:1 and 18:2 (the product of *FAD2*) content whereas the sensitive genotype did not show marked variations. Membrane integrity evaluated through electrolyte leakage was in accordance with phenotypic differences in cold tolerance. Our work provides information about the structure of *TdFAD2* locus and the *FAD2* expression and fatty acid profiles under the cold effect. Molecular and biochemical characterization of germplasm contributes to explain phenotypic variability in complex traits and thus facilitates the development of new varieties adapted to specific cultivation areas.

Key Message

FAD2 locus of durum wheat comprises four tandem copies in chromosomes 6A and 6B. Tolerance to cold in two genotypes goes along with FAD2 expression, 18:1/18:2 content, and membrane permeability.

Introduction

Temperature is one of the main environmental factors affecting the productivity of plants and their geographical distribution. At cellular level, low temperatures generate a decrease in enzymatic activity and membrane fluidity and cause protein destabilization and accumulation of reactive oxygen species (ROS) (Ruelland and Zachowski 2010). To minimize these negative effects a mechanism termed acclimation is triggered when plants are exposed to temperatures above freezing (2–6°C), which implies changes in plant growth, water balance, antioxidant production, cold-regulated (COR) gene expression and cell membrane composition (Dubey 1999). The cold signal is initially sensed at the cell membrane and mechanisms to preserve membrane fluidity are activated; a transition from crystalline to a gel-like phase occurs through variations in the unsaturation degree of the fatty acids in membrane glycerolipids (Upchurch 2008). Ion leakage is widely used as a test for estimating the extent of membrane damage under cold stress, in particular, associated with the transfer of ions K + and H+ (Uemura and Hausman 2013).

Polyunsaturated fatty acids (PUFAs) content is regulated by fatty acid desaturase (FAD) enzymes. As unsaturation of fatty acids is a key step in the biosynthesis of vegetable oils (Mikkilineni and Rocheford 2003), most of the desaturase studies focused on oilseed species such as olive (Hernández et al. 2019) and sunflower (Rousselin et al. 2002). However, in the last years, there has been an increasing interest in the regulation of these enzymes in the response to abiotic environmental stress (reviwed by He and Ding 2020) as is the case of low temperatures (Golizadeh and Kumleh 2019).

In plants, the FADs have been classified into two main groups: soluble acyl–acyl carrier protein (acyl-ACP) desaturases that introduce the first double bond into the saturated acyl chain, and acyl-lipid desaturases, which comprise membrane-bound proteins in charge of obtaining polyunsaturated fatty acids. The first group is in the chloroplast stroma and the second one localizes in the chloroplast and endoplasmic reticulum (ER) membranes (Tocher et al. 1998).

Regarding the primary structure of plant desaturases, the acyl-ACP desaturases contain two D/EEX2H motifs that are associated with iron in the active site (Shanklin and Cahoon 1998), consisting in DEKRH and EENRH residues in the case of acyl-ACP desaturases from grass (Díaz et al. 2017). On the other hand, the acyl-lipid desaturases contain three histidine domains (H-boxes) essential for catalysis, consisting of eight conserved histidine residues with structures HXXXH, HXXHH and HXXHH (Dar et al. 2017).

The desaturase enzymes in plants have been initially studied in mutant collections of *Arabidopsis thaliana* in which genes that differ in their substrate specificity and subcellular localization were described (Gibson et al. 1994). The first desaturation of stearic acid (18:0) acid is catalyzed by soluble FAB2 or SAD (stearoyl-ACP desaturase) in the chloroplast stroma. Additionally, FAD4/FAD5 (acyl lipid desaturase) acts on palmitic acid (16:0) to produce palmitoleic acid (16:1) in the chloroplast membrane. The step from oleic acid (18:1) to linoleic acid (18:2) and from 16:1 to hexadecadienoic acid (16:2) is catalyzed by FAD6 (ω-6 lipid desaturase) in the chloroplast (Zhang et al. 2009). Oleic acid (18:1) fatty acid can also be transformed to linoleic acid (18:2) by FAD2 (ω-6 lipid desaturase) in the ER. The conversion of 18:2 to linolenic acid (18:3) is carried out by FAD7/8 (ω-3 lipid desaturase) in the chloroplast and FAD3 (ω-3 lipid desaturase) in the ER, whereas the equivalent step from 16:2 to 16:3 only occurs in the chloroplast (reviewed by He et al. 2020).

The typical fatty acid profile in plant leaves and stems consists, mainly, of 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 molecules (Harwood 1996). Changes destined to preserve cellular membranes under low-temperature stress include the increase in the synthesis of unsaturated acyl chains (Makarenko et al. 2011). The changes in the unsaturated fatty acid content have been found to correlate with differences in the ability to cope with cold stress among genotypes of faba bean (Arbaoui et al. 2008) and chickpea (Javidi et al. 2022) as examples.

As the enzyme responsible for the conversion of the oleic 18:1 to linoleic 18:2 acid, FAD2 protein inserts a double bond at the delta-12/omega-6 positions relative to methyl or carboxyl terminus respectively. *FAD2* gene has an important role in determining the fatty acid composition and fluidity of plant membranes

(Pirtle et al. 2001), in plant development and in the response to stresses like cold and salinity (Dar et al. 2017).

FAD2 gene structure has evolved from a single copy state with seven or nine introns in algae to multiple copies, with introns disappearing or decreasing in land plants (Zhao et al. 2019). Increasing in *FAD2* copy number has been attributed to duplication mechanisms or polyploidy events. Enzymes coded by the extra copies have often evolved in *FAD2* isoforms as are the cases of allotetraploid cotton (Kargiotidou et al. 2008), ancient polyploid soybean (Li et al. 2007), or seed-specific expression in sunflower (Martínez-Rivas et al. 2001).

Durum wheat is the 10th most important crop worldwide and the production with a planting area of 16 M hectares represents 5% of the total wheat production (International Grains Council -IGC, 2020) (reviewed by (Beres et al. 2020). The major durum wheat-producing countries are the European Union, Canada, Syria, the United States, Argelia, and Morocco (De Vita et al. 2007). In Argentina, it is mainly cultivated in the South of Buenos Aires province where the climate is sub-humid/semi-arid, with hard winters and variable minimum temperatures in date and intensity. In this region, the freezing temperatures extend even up to the spring causing alterations during the flowering stage, with important economic consequences. Genotypes carrying spring growth habit predominate in Argentina durum germplasm (Basualdo et al. 2015). Thus, variability in the length cycle and tolerance to cold exposures become relevant traits to obtain new varieties adapted to specific climatic environments. In previous studies, we compared the performances to cold stress of a durum wheat collection (Basualdo 2013; Basualdo et al. 2015) and identified three genotypes with interesting combined data: MVTD 10–98, a winter variety, long cycle, with vernalization requirement and low damage in acclimated leaves under sub-zero temperatures; BUCK AMBAR, a spring variety, short cycle, non-vernalization requirement, and high level of damage in leaves under freezing temperature; CBW0101, a spring line, long cycle, non-vernalization requirement and levels of leaf damage closer to MVTD 10–98.

In this work, we characterized the *FAD2* of durum wheat, analyzed the role of FAD2 desaturase in the response to cold conditions and evaluated the effect of low temperatures on fatty acid content and cell membrane integrity, in three durum wheat genotypes. To address this study: i) we performed the characterization of the *FAD2* locus present in homeologous chromosomes 6 of durum genome reference as well as recognized the functional/structural elements in the predicted protein, ii) analyzed *FAD2* expression at 4°C by qRT-PCR and explored *in silico* the promoter regions, iii) determined the fatty acids composition in leaves after exposure to 4°C and iv) evaluated the electrolyte leakage in leaf samples exposed at -4 and − 13°C subzero temperatures.

Materials And Methods

Plant material

Three tetraploid wheat (*Triticum turgidum* ssp. *durum*) genotypes were included in this study: MVTD 10–98 (winter and cold-tolerant), BUCK AMBAR (spring and cold-sensitive), and CBW0101 (spring and intermediate cold-tolerant).

Fad2 Gene Isolation

Genomic DNA from leaves of the three genotypes was extracted using a CTAB protocol (Rogers and Bendich 1985). Primers for *FAD2* were designed based on CBW 0101 transcriptome (Díaz et al. 2019). The complete coding region was amplified using the following primers: Forward 5'-GTCAGTAGCATCATGGGTGC-3' and Reverse: 5'-TTGGCGGCTAGAACTTGTTG-3'. PCR reactions were performed in a volume of 25 µl containing 1X Taq DNA Polymerase Buffer, 0.2 mM each dNTP, 0.4 µM each primer, 50–100 ng DNA template, and 1 U Go Taq DNA polymerase (Promega, USA). Amplifications were carried out in a MyCycler thermal cycler (Bio-Rad, USA) with the following cycling parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, annealing at 57 C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 10 min. PCR products were cloned using pGEM-T Easy Vector Systems (Promega, USA). Clones from the three genotypes were bidirectionally sequenced.

In Silico Fad2 Mapping And Regulatory Elements Prediction

The amplified sequences were mapped on the genome of cv Svevo (https://www.interomics.eu/durum-wheat-genome). The 1500-bp upstream sequence from the start codon was analyzed to predict gene regulation. A list of transcription factor binding sites (TFBS) based on the database, are available at PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/).

Predicted Protein Sequence For Fad2 And Domain Identification

To confirm the identity of the amplified sequences, BLASTX alignments against GenBank were performed. The domains associated with the active site and the characteristic motifs were identified using Multiple Expectation Maximization for Motif Elicitation server (MEME-Suite 4.11.1, http://meme-suite.org) (Bailey et al. 2006) with the following parameters: distribution of motifs: 0 or 1 per sequence; maximum number of motifs: 6; minimum number of sites: 2; minimum motif width: 5 and maximum motif width: 50. The transmembrane helix motifs in the FAD2 sequences were predicted using the platform TransMembrane prediction using Hidden Markov Models (TMHMM 2.0, http://www.cbs.dtu.dk/services/TMHMM) and the cellular localization with subCELlular LOcalization predictor (CELLO, http://cello.life.nctu.edu.tw/).

Expression analysis of FAD2

Plants growing in a chamber were exposed, at two-leaf stage (Z1.2) (Zadoks et al. 1974), to a ramp, down-shift from 22°C (0 h, control condition) to 4°C (1°C/h) and were kept at the final temperature (cold-

stress) for 24, 72 y 144 hours. Collected leaves were frozen in liquid N_2 and kept at -80°C until use. Total RNA was isolated from leaves using the SV Total RNA Isolation System (Promega, USA) and quantified by Nanodrop DS-11 spectrophotometer (DeNovix inc., USA). The single-strand cDNAs were synthesized from 1.85 µg of total RNA using Revert-Aid M-MuLV Reverse Transcriptase (Thermo Scientific) with random primers, according to the manufacturer's instructions and an aliquot of 5 µl of 1:10 diluted cDNA was used. The expression patterns of *FAD2* were examined by quantitative PCR assay. PCR reactions were performed in a volume of 15µl containing 7.5 µl of the SYBR-Green Master Mix (Biodynamics, Argentina) and 5 pmol of each primer, Forward: 5' –CCTACGGCCCGATCTACAA-3' and Reverse: 5' –GACGACACGAGCTTCAACAA-3', using a Rotor-Gene (Qiagen). The primers were designed based on the conserved regions of the functional copies of cv Svevo. Reactions were performed on Rotor-Gene (Qiagen) with the following cycling parameters: 94°C for 2 min, 35 cycles of 94°C for 15 seg, 50°C for 20 s, 72°C for 30 s followed by one step at 72°C for 5 min. Three biological replicates were performed for each condition and genotype. Relative transcription level was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using β-actin gene (Actin) (AK362208) as reference gene with the following primers: Forward: 5' –ATGTGGATATCAGGAAGGA-3' and Reverse: 5' –CTCATACGGTCAGCAATAC-3'.

Electrical Conductivity Method

Plants at the three-leaf stage (Z1.3) (Zadoks et al. 1974) were exposed to a ramp from 22°C to 2°C (1°C/h) and were kept at the final temperature for 24 hours to get acclimation. The freezing test was conducted in a dark chamber. The ion leakage experiment was carried out according to Pra´s'il and Za´mec'nı´k, (1998) with some modifications: leaves were rinsed three times, cut into equal segments (1 cm length) and 0.25 g were put in plastic tubes. The temperature decreased 1°C/h until the two defined treatments reached − 4°C and − 13°C (C_t). The samples were kept in those conditions for 12 hours. Control samples (C_0) were held at 2°C during the same period. In the next step, 15 ml of distilled water were added to all the samples, the tubes were shacked at 150 rpm for 5 h and then measures of conductivity were registered. To achieve the maximum electrolyte leak (C_f), the samples were then transferred to -70°C and kept overnight at this temperature. The experiment included three biological replicates. Changes in membrane permeability were evaluated by Electrolyte Leakage Index (ELI) (%) = $(C_t-C_0)/(C_f-C_0)$ *100 and Relative Electrolyte Leakage (REL) = (C_t/C_f) *100.

Statistics Analysis

Differences between genotypes and treatments for each variable (relative expression, fatty acid and electrolyte leakage) were evaluated using Analysis of Variance (ANOVA), under randomized design. The mean values were compared using Fisher's Least Significant Difference test ($p < 0.05$). Statistical analysis was carried out with the Infostat software version 2019 (Di Rienzo et al., 2019).

Results

FAD2 locus characterization and in silico mapping

The *FAD2* genomic sequences from CBW 0101 and MVTD 10–98 and BUCK AMBAR were isolated. BLASTX searching in GenBank demonstrated that the sequences correspond to the intronless gene *omega 6- fatty acid desaturase* encoding a protein of 387 amino acids. These sequences were used as probes for *in silico* mapping on the durum wheat genome Svevo (https://www.interomics.eu/durum-wheat-genome-intranet) (Maccaferri et al. 2019). Four full-length tandem copies of *FAD2* gene, located in the long arm of chromosome 6, were identified in genomes A and B of the reference genome that we will refer as *TdFAD2-A1.1*, *TdFAD2-A1.2*, *TdFAD2-A1.3* and *TdFAD2-A1.4* (approx. 1.200.000 bp) and *TdFAD2-B1.1*, *TdFAD2-B1.2*, *TdFAD2-B1.3* and *TdFAD2-B1.4* (approx. 1.600.000 bp). Additional copies of *FAD2* were detected in chromosomes 1A, 5A, 2A, 7B, 7A, 3B, 5B and 4B, of cv Svevo genome, in these cases with coverage < 33%, and therefore considered incomplete copies. A scheme of the physical positions of *FAD2* copies on homoeologous 6 chromosomes and copy orientations are shown in Fig. 1. It was observed that *TdFAD2-A1.3, TdFAD2-A1.4, TdFAD2-B1.2*, *TdFAD2-B1.3* and *TdFAD2-B1.4* copies were in opposite orientation relative to *TdFAD2-A1.1*, *TdFAD2-A1.2* and *TdFAD2-B1.1*. The copies *TdFAD2-A1.3* and *TdFAD2-B1.1* presented premature stop codons at site 286 and 160/199 bp respectively and an anomalous region of 30–100 residues with discordances in amino acid sequence in comparison to described FAD2 desaturases from grasses (Díaz et al. 2017). On the other hand, the copy *TdFAD2-B1.4* included a long inside region corresponding to a Long Terminal Repeat (LTR) retrotransposon element of Ty1-*copia* superfamily, where an integrase core domain (rve), a ribonuclease type 1 (RNase_HI_RT_Ty1) and a reverse transcriptase (RVT_2) sequences could be identified (Fig. 1). Table 1 shows the identity values (%) at the nucleotide and amino acid levels obtained by the alignment of the cv Svevo copies of *TdFAD2* from 6A and 6B chromosomes. Based on the presence of a complete open reading frame, normal stop codon and no retroelement insertion; *TdFAD2-A1.1*, *TdFAD2-A1.2*, *TdFAD2-A1.4*, *TdFAD2-B1.2* and *TdFAD2-B1.3*, from cv Svevo genome, were considered functional *TdFAD2* copies.

Table 1
Percentage of nucleotide/amino acid sequence identity between FAD2 copies of cv Svevo. In bold are the highest identity values for each group of comparisons.

Genome A/Genome B	1B	2B	3B	4B[a]
1A	87,3/65,15	91,06/89,77	**96,82/99,48**	90,41
2A	86,53/62,15	**96,82/95**,1	91,9/88,49	88,20
3A	**92,96/67,41**	86,91/76,2	88,39/79,08	86,16
4A	84,98/60,6	88,09/82,61	89,69/84,53	**96,15**

[a]Calculated excluding retroelement sequence.

Analysis of regulatory elements in the TdFAD2 promoter from cv Svevo

The 1500 bp region upstream the start codon from the functional copies of cv Svevo was scanned aiming to identify the transcription factor binding sites (TFBS). Twenty-six different TFBS were predicted and twelve of them corresponded to families associated with the response to abiotic stress (Fig. 2). The most abundant sites were for NF-YB, bZIP, bHLH and Myb. However, the TF families were differentially represented across the *TdFAD2* copies. As examples, binding sites for NF-YB (nuclear factor Y subunit B or HAP3/CBF-A) had high frequency at *TdFAD2*-B1.3, *TdFAD2*-A1.2, *TdFAD2*-A1.4 promoters, comparatively low presence in *TdFAD2*-B1.2 and absence in *TdFAD2*-A1.1. Similarly, binding sites for ZF-HD (zinc finger-homeodomain) TF family were almost only found in *TdFAD2-A1.1* promoter, C2H2 only present in *TdFAD2-A1.4*, and sites for MyB (v-Myb myeloblastosis viral oncogene homolog) TF absent in the analyzed 1500 bp of *TdFAD2-B1.2* promoter (Fig. 2).

Characterization Of Predicted Fad2 Protein Sequences From Cv Svevo

The analysis in MEME-Suite of the five FAD2 functional copies from cv Svevo identified three motifs corresponding to the desaturase characteristic histidine boxes: HECGH (motif 1), HRRHH (motif 2) and HXXHH (motif 3) (Fig. 3). The predictor CELLO located the proteins across the membrane of the endoplasmic reticulum with high reliability (3.266–4.021) and the server TMHMM identified three transmembrane helices in all five FAD2 protein sequences.

Isolation of TdFAD2 genomic sequences and expression analysis in leaves of three durum wheat genotypes under cold conditions

The FAD2 sequences amplified from genomic DNA of MVTD 10–98 and CBW 0101 (1183 bp) and BUCK AMBAR (1195 bp) corresponded to *TdFAD2-1.3* copy. Alignment was performed excluding BUCK AMBAR sequence as it was considered a non-functional copy; thus MVTD 10–98 and CBW 0101 sequences corresponding to *TdFAD2-B1.3* copy were compared. Two polymorphic sites were found, one in the UTR 5' region (T/C) and one synonym at position 77 from the start codon (C/T). The *TdFAD2-B1.3* sequences from MVTD 10–98 and CBW 0101 were deposited in GenBank as MW175493.1 and MW175492.1 respectively.

Basal expression (22°C) of *FAD2* was observed in the three genotypes. Leaves of genotypes BUCK AMBAR and CBW0101 significantly increased the expression of *FAD2* when exposed at 4°C for 24 h while the genotype MVTD 10–98 showed a significant decrease. At 72 h and compared to 24 h, BUCK AMBAR and CBW0101 showed a significant decrease while MVTD 10–98 presented a significant increment in *FAD2* expression. Finally, at 144 h all the genotypes increased the expression relative to 0 and 72 h (Fig. 4).

Fatty Acid Analysis In Leaves Of Three Durum Wheat Genotypes

The fatty acid profiles of plants growing at 22°C (control condition) and at 4°C for 8, 24, 48, 72, and 144 h, were obtained by gas chromatography, for the three genotypes: BUCK AMBAR, CBW 0101 and MVTD 10–98. The major component of the fatty acid fraction was linolenic acid (18:3) representing 60–80% of the total. On the other hand, the least represented were palmitoleic (16:1), stearic (18:0) and oleic (18:1) acids, with mean values of around 1–3% of the total. The palmitic (16:0) and linoleic (18:2) acids showed low-intermediate levels in both cases representing around 12% of the total (Fig. 5). The effect of cold treatment on each fatty acid profile is described below.

16-carbon: A similar trend of the three genotypes (though non-significant in BUCK AMBAR) regarding 16:0 content in response to cold consisted in an increase at 8 h in comparison to control, a decrease after 24–48 h and a recovery at 72 and 144 h (Fig. 6a). In the case of 16:1, magnitudes of changes were slight across cold treatments and comprised a decrease after 8–48 h, a next increase at 72 h and a reduction of 16:1 content at the 144 h last treatment. These changes in 16:1 resulted only significantly in MVTD 10–98 genotype ($p < 0.01$) (Fig. 6b).

18-carbon: Very similar 18:0 profiles were observed in the response to low-temperature exposure in all three genotypes (Fig. 7a): a peak at 8 h, a return to 18:0 basal levels at 24 h that maintained up to 72 h (except in CBW 0101 in which it maintained up to 48 h) and a new increment at the final treatment of 144 h. Regards 18:1, CBW 0101 and MVTD 10–98 genotypes steadily increased this fatty acid from 8 h to 144 h in comparison to the control, whereas in BUCK AMBAR the 18:1 content increased, although not significantly, from 8 to 72 h but returned to basal level at 144 h. (Fig. 7b). Concerning 18:2, it was observed that in comparison to the control CBW 0101 increased significantly ($p < 0.01$) at 48 h and maintained until 144 h, MVTD 10–98 also showed a similar increase at 48 and 144 h whereas in BUCK AMBAR the cold treatments did not cause marked variations in 18:2 content (Fig. 7c). Finally, in 18:3, a decrease was observed in all genotypes in plants subjected to cold for 8 h compared to the controls, then the mean 18:3 contents increased from 24 to 48 h to values close to the control, and a reduction occurred in CBW 0101 and MVTD 10–98 genotypes around 144 h (Fig. 7d).

Relative Electrolyte Leakage

The cell membrane damage caused by exposition to cold was evaluated through REL and ELI measures. When the leaves were exposed at -4°C for 12 h the genotype BUCK AMBAR increased significantly the REL ($p < 0.0001$) from 12.94 (control) to 27.7%, whereas the genotype CBW 0101 varied from 11.90 to 13.57% and MVTD 10–98 from 6.97 to 7.9% (Fig. 8). When the leaves were exposed at -13 ˚C for 12h, the three evaluated genotypes showed an increase in the REL with similar values of around 90% (Fig. 8). At -4°C for 12 h, the corresponding ELI values presented statistically significant differences ($p < 0.0001$) between MVTD 10–98 (1.33%), CBW 0101 (2.61%) and BUCK AMBAR (15.74%) (Fig. 9a). Regarding cold treatment at -13°C, all three genotypes presented similar ELI mean values of around 90% (Fig. 9b).

Discussion

In crops of temperate areas, cold stress is one of the most important factors responsible for the decrease in productivity due to alterations at physiological, metabolic, and molecular levels. In this sense, numerous studies have focused on the role of desaturases in the response to low temperatures because they can reduce the impact on the fluidity of the cell membrane by the unsaturation of the fatty acids that compose it (Reviewed by He and Ding 2020).

The molecular characterization of *TdFAD2* gene was carried out to detect alleles or isoforms associated with cold tolerance. The sequence of *FAD2* is well conserved in plants and encompasses 1164 nucleotides that encode a protein of 387 amino acids (Tao et al. 2006). In durum wheat, the *TdFAD2* genes are alike in length and intronless. *In silico* mapping of *FAD2* on cv Svevo genome (Maccaferri et al. 2019), revealed multiple copies of the gene consisting in four tandem copies in the long arm of chromosomes 6A and 6B. Duplication events of *FAD2* gene seem to have occurred early in the origin of Triticum/Aegilops species considering that three or four copies have been identified in 6A, 6B and 6D homeologous of *T. aestivum* (Hajiahmadi et al. 2020) and likely even earlier, during *Poaceae* radiation taking into account the existence of three *FAD2* copies in chromosome 7 of the rice genome (Zhiguo et al. 2019). Tandemly duplicated copies of *FAD2* genes have been also described in different plant families, with *Gossypium raimondii* (Liu et al. 2015) and *Glycine max* (Chi et al. 2011) as examples. An increase in copies of individual genes or chromosomal segments is an important source of new evolutionary characteristics apart from the duplication of the entire genome (polyploidization) (Kanazawa et al. 2009).

A segment of around 5 kb was found inserted in the *TdFAD2-B1.4* copy, assigned to a LTR retroelement class. The proportion of transposable elements reaches up to 86% and 85% of the sequence of A and B subgenomes of common wheat (Wicker et al. 2018); in the *TdFAD2-B1.4* case the retroelement seems to completely disrupt the desaturase protein sequence suggesting gene inactivation. This retroelement sequence might have been misdescribed as a long intronic region in a previous analysis of FAD genes in 6B chromosome of hexaploid wheat (Hajiahmadi et al. 2020).

Identity values at nucleotide level among the *FAD2* copies of cv Svevo 6A and 6B chromosomes ranged from 85–97%. This is in accordance with the average 95% identity value for homoeologous gene comparisons in allopolyploids wheat (Krasileva et al. 2013). An examination of identities revealed that the highest values correspond to the pairs *TdFAD2-A1.1* - *TdFAD2-B1.3* (97%), *TdFAD2-A1.2* - *TdFAD2-B1.2* (97%), *TdFAD2-A1.3* - *TdFAD2-B1.1* (93%) and *TdFAD2-A1.4*- *TdFAD2-B1.4* (96%) (retroelement excluded) suggesting non-collinear relationships between 6A and 6B chromosomes in the region surrounding copies 1–3. These observations could reflect the occurrence of an inversion covering at least 1 Mb with one of the breakpoints between copies 3B and 4B. The inclusion in the analysis of chromosome 6D of common wheat (https://wheat.pw.usda.gov/GG3/) allows us to speculate that this inversion occurred on chromosome 6B since 6A and 6D retain the same directionality in the copies. A chromosomal rearrangement belonging to a pericentric inversion type has been identified in chromosome 6B of several accessions of common wheat using C-banding patterns (Badaeva et al. 2007); they also observed that the highest number of chromosomal rearrangements was recorded in the B genome. Differences in directionality among tandem *FAD2* copies along each chromosome also evidence structural changes in

the segment that could be favored by ectopic recombination events between transposable elements. Figure 10 resumes a proposal for describing the structural relationships within the *FAD2* region of cv Svevo. It is well known that the complexity of the wheat genome is not only given by its multiple subgenomes but also by the occurrence of diverse and numerous chromosomal rearrangements, mostly inversions and translocations (Ma et al. 2013, 2014; Zhou et al. 2020), gene duplication and inclusively homoeologous exchanges (Zhang et al. 2020).

After exploring cv Svevo sequences covering promoter regions of *FAD2* genes, we focused on binding sites for 12 TFs families related to stress responses. Several of them such as NF-YB, ZF-HD, C2H2 and MyB were differentially represented among the copies of *FAD2* desaturase genes suggesting they could separately evolve towards determined expression patterns. NF-YB is one of the subunits of the NF-Y complex involved in plant response to abiotic stress, flowering time, and development (Zhang et al. 2023). ZF-HD (Liu et al. 2021) and C2H2 (Han et al. 2020) are zinc finger proteins with important roles in regulating gene expression under conditions such as salt, drought, and low temperature. MyB comprises a large, functionally diverse TF family, participants in a variety of plant-specific processes like development, morphogenesis, hormone, and stress response (Ambawat et al. 2013). Most of these mentioned TF families were recently found to be differentially expressed in plants of durum (Díaz et al. 2019) and common (Jiang et al. 2022) wheats, in response to low temperatures. Moreover, the multiple copies of *FAD2* varying in their binding sites for TFs could be differentially sensitive to internal or external signals and acquire distinct functionalities with time. In this regard, there have been described some *FAD2* isoforms with determined expression patterns such as the seed-specific *FAD2* in cotton (Kargiotidou et al. 2008) and sunflower (Martínez-Rivas et al. 2001) in comparison to constitutively expressed isoforms. In the same sense, *FAD2* isoforms can be differentially responsive to changes in temperature as occurred in *Medicago truncatula* where *FAD2.2* and *FAD2.3* resulted respectively induced and down-regulated relative to control after 24 hours at 4 C (Zhang et al. 2018).

Regarding *FAD2* expression analysis, it has first to be noted that the utilized primers were designed on conserved sequences of the functional *FAD2* genes localized in both 6A and 6B chromosomes of cv Svevo. Therefore, the registered level of transcripts in analyzed genotypes would correspond to a sum value originating from several or even all the functional copies. Although with some differences in their profiles, the three evaluated durum wheat genotypes showed constitutive expression of *FAD2* in leaves and induction after six days at 4°C, in comparison to control conditions. Similar increases in *FAD2* transcription as part of the response to low temperature have been observed in cotton (Kargiotidou et al. 2008) and rice (Shi et al. 2012) as examples of direct transcriptional control. This mechanism contrasts with that observed in some *FAD2* forms such as from soybean (Li et al. 2007) that did not modify their expression profile under cold stress, suggesting that in this case a translational/post-translational regulation of the enzyme would be rather responsible for the changes in fatty acid composition after cold exposure. When comparing FAD2 expression profiles among the three durum wheat genotypes it was observed that CBW 0101 and MVTD 10–98 reached a peak after the extended cold period of six days (144 h) whereas BUCK AMBAR exhibit its maximum value very soon after exposure (24 h). Thus, the susceptible genotype had a comparatively earlier and higher *FAD2* expression increment although it was

unable to maintain it along the experiment time course. Differences in patterns of FAD2 expression have been similarly observed between bread wheat cultivars varying in cold tolerance grade (Golizadeh and Kumleh 2019).

The fatty acid analysis indicated that 18:3 linolenic acid is the major component (80%) in leaves of durum wheat growing at 22°C, followed at a great distance by both saturated and unsaturated chains such as 16:0 and 18:2. This profile agrees with that reported by (Nejadsadeghi et al. 2014) using leaves of durum plants but differed from that obtained from durum grain samples in which 18:2 linoleic was the predominant fatty acid (Narducci et al. 2019), suggesting tissue/organ specific fatty acid patterns.

Synthesis *de novo* of plant fatty acid occurs in plastid. The 16:0-ACP chain may be elongated to 18:0 and then desaturated to 18:1 so that 16:0-ACP and 18:1-ACP are the primary products of plastid fatty acid synthesis (He and Ding 2020). The 16:0 and 18:0 contents increased after a few hours of cold exposure in the three durum genotype plants, though the response was stronger for 18:0. Thus, the precursor 16:0 and its first elongation product 18:0 raised their synthesis rates under cold stress, in agreement with (Zhang et al. 2010). The fact that 16:1 content remained rather stable or even decreased suggests that 16:0 elongation towards 18:0 was the favored pathway in comparison to 16:0 desaturation (Shanklin and Cahoon 1998). The 18:1 content also increased after cold exposure but with differences among genotypes and at more advanced stages of the treatment (> 48 h), reasonably associated with the preceding increase of 18:0 (8 h). Whereas CBW 0101 and MVTD 10–98 steadily incremented 18:1 content above control values, BUCK AMBAR showed slight variations, providing evidence about differential desaturation capacity among genotypes. This trend is confirmed when the 18:2 profile is examined, since after two days of cold exposure, CBW 0101 and MVTD 10–98 also showed a higher accumulation of linoleic acid in comparison to the controls, suggesting an 18:1/18:2 correlated pattern. Even though 18:2 content increased under low temperatures in two genotypes, a subsequent increment in 18:3 was not observed in any case and on the contrary, an early sharp reduction of 18:3 occurred. A significant reduction of unsaturated fatty acids content at the beginning of low-temperature treatment (i.e 2–4 h) has been similarly observed in olive (Hernández et al. 2019). The 18:3 profile suggests that along six days of cold treatment, the 18:2 unsaturation process occurs at similar or lower rates than that observed under normal growing conditions. A slight effect of cold temperatures on 18:3 content has been previously suggested (Román et al. 2012).

When 18:2 content and *FAD2* expression data were combined, it became visible that though BUCK AMBAR was the most sensitive genotype with the fastest and highest *FAD2* induction, this increase in gene transcription was not reflected in 18:2 changes. Thus, the observed changes in the regulation of *FAD2* in BUCK AMBAR under low-temperature conditions result in only maintaining constitutive levels of 18:2. The lack of correlation between the effect of cold on desaturase gene expression and its corresponding fatty acid level might be due to a later, out of timescale, response of BUCK AMBAR in respect of 18:2 synthesis although post-transcriptional mechanisms for enzyme regulation has also been suggested (Hernández et al. 2019). The *FAD2* induction in the case of MVTD 10–98 genotype looks delayed (peak at six days) in comparison to the other two genotypes. However, considering that hundreds

of wheat genes involved in Ca2 + and hormones signal, transcription and starch-sucrose metabolism have proved to modify their expression after a cold period as short as 4 h (Jiang et al. 2022), the possibility of an earlier, non-detected *FAD2* induction in MVTD 10–98 cannot be discarded, considering our initial sample was collected at 24 h. Here, it has to be noted that 18:2 content is the resulting level of linoleic acid produced through FAD2 and FAD6 enzymes with activity in endoplasmic reticulum and plastid respectively (He et al. 2020).

To cope with cold environments, plants increase osmolytes that favor water retention and modify the ratio of unsaturated fatty acids to saturated to preserve membrane fluidity (Golizadeh and Kumleh 2019). The integrity of the cellular membranes can be monitored during exposure to cold through the ion leakage (EL) measure, which uses the electrical conductivity of surrounding water as an index of membrane damage (Wilner 1960). The indexes ELI and REL are both based on electrical conductivity measures in samples after cold treatment relative to a maximum leakage, though differ in that ELI calculation includes the values obtained from control samples (2°C in this study). The two indexes demonstrated that genotypes MVTD 10–98 followed by CBW 0101 were able to maintain greater cell membrane integrity at freezing temperatures of − 4°C than BUCK AMBAR, indicating a better response to deal with stress. Data obtained from − 13°C treatment did not render useful information regarding genotypes as resulted in similar ELI and REL values.

Conclusions

Combined data reveal that durum plants at two-three leaf stage mainly modify 16:0, 18:0, 18:1 and 18:2 contents in response to low, above zero, temperatures as would occur in the period prior to a frost in the field. In addition, the plants increase the transcription of FAD2 in charge of 18:2 synthesis. Among these variables, 18:1/18:2 profiles and FAD2 gene expression differed depending of genotypes and showed that tolerant winter MVTD 10–98 presented the largest increases in unsaturated fatty acid content, sensitive spring BUCK AMBAR the lowest ones and mild tolerant spring CBW101 was intermediate or close to those observed in the winter genotype. The same conclusions were obtained from conductivity measures that indicated that MVTD 10–98 and CBW 0101 were able to sustain better preservation of the cell membrane integrity in accordance with their more pronounced changes in unsaturated fatty acid contents. Both, adjustment of unsaturation grade and cell membrane preservation, could partially explain the superior cold tolerance of a genotype.

The sequence information previously generated from the transcriptome analysis of the CBW 0101 line, allowed us to know the localization of *FAD2* locus in durum wheat genome. *FAD2* mapping revealed four gene copies in chromosomes 6A and 6B, and omega-6 fatty acid desaturase (*TdFAD2*) sequences of durum wheat became first available. Physical distance among copies, gene denominations according to wheat nomenclature rules and positions of functional protein motifs are provided. Duplication and inversion rearrangements in the segment evolved to produce a structurally complex *FAD2* locus containing non-functional and functional copies with variability in their regulatory regions. On this basis,

any attempt to alter FAD2 activity in durum wheat should necessarily entail expression analysis that will ensure FAD2 copy specificity.

Durum wheat crop comprises a predominantly spring germplasm. The line CBW 0101 originated from winter x spring crosses and possesses the spring allele in the determinant of growth habit VRN-A1 locus (Basualdo et al. 2015). Here, CBW 0101 showed intermediate phenotypes relative to MVTD 10–98 and BUCK AMBAR or closer to MVTD 10–98. Characterization of germplasm regarding responses to environmental stresses represents useful information for breeding, in order to obtain new varieties adapted to specific cultivation areas. In addition, molecular and biochemical descriptions provide the genetic bases underpinning the phenotypic variability in a complex trait.

Declarations

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Selva Cuppari. Data analysis was performed by all the authors as well as the writing of the first draft of the manuscript.

Data Availability

The genomic DNA sequences of *TdFAD2* desaturase of the genotypes CBW 0101 and MVTD-1098 are deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the accessions MW175492.1 and MW175493.1 respectively.

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Figures

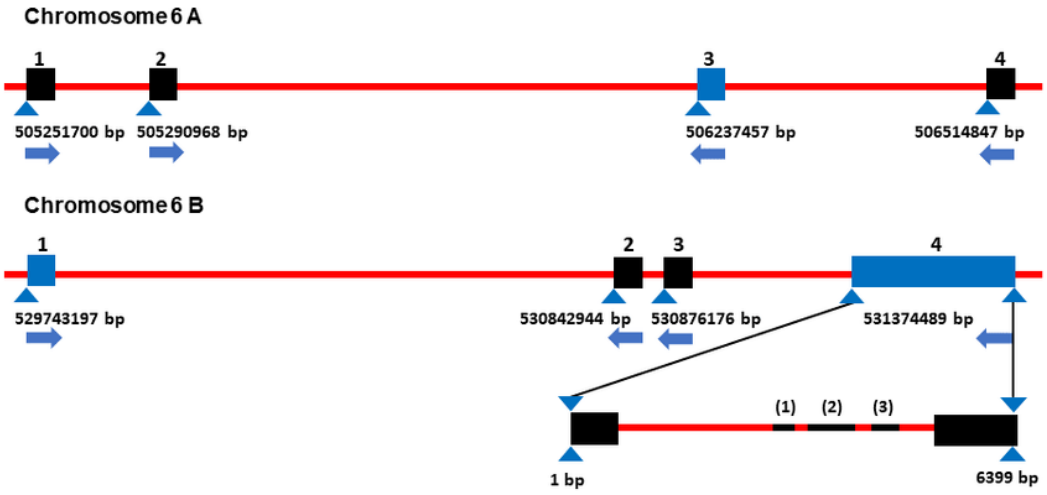


Figure 1

Physical mapping of *TdFad2* copies on 6A and 6B chromosomes (red lines) of cv Svevo. In boxes were represented the gene copies (1200 bp), the functional (black boxes) and the nonfunctional ones (blue boxes). At the bottom is shown a close-up of *TdFad2-B1.4* structure carrying a retroelement with rve (1), RVT_2 (2), and RNase_HI_RT_Ty1 (3) regions. The numbers indicated the start points of the coding sequence in each copy and the blue arrows indicate the orientation of the sense strand.

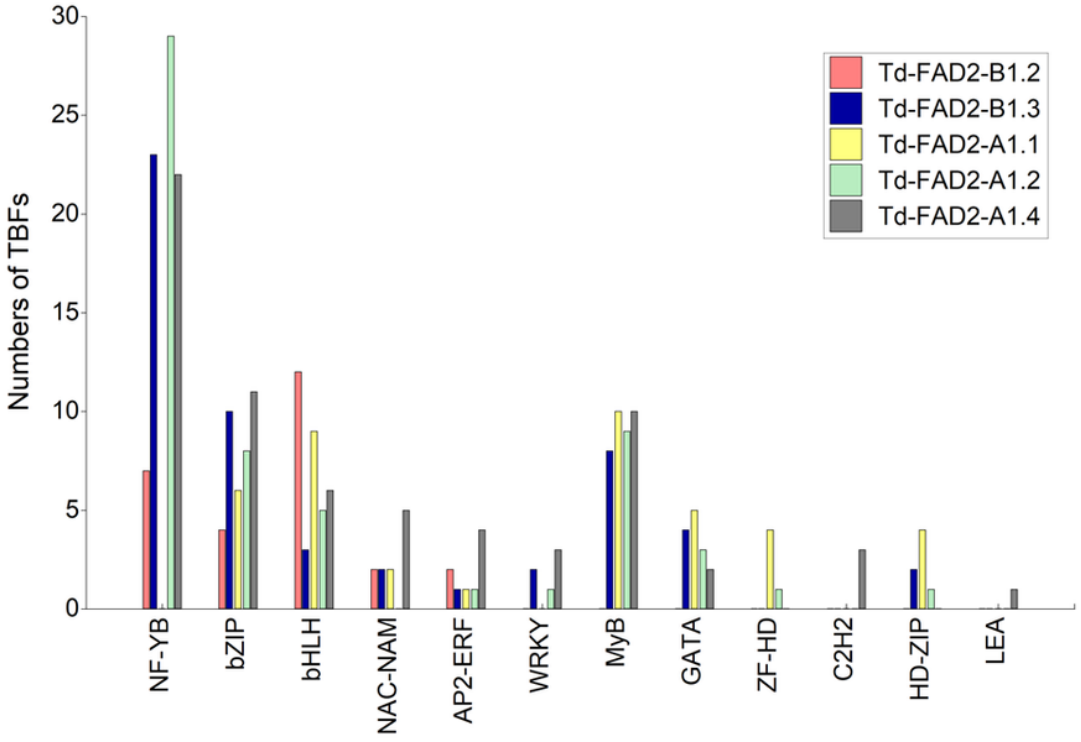


Figure 2

Abundance of abiotic stress TFBS identified by the PlantPAN tool along the 5' promoter region (1500 bp) of five genes of *FAD2* in *T. turgidum* ssp. *durum* cv Svevo.

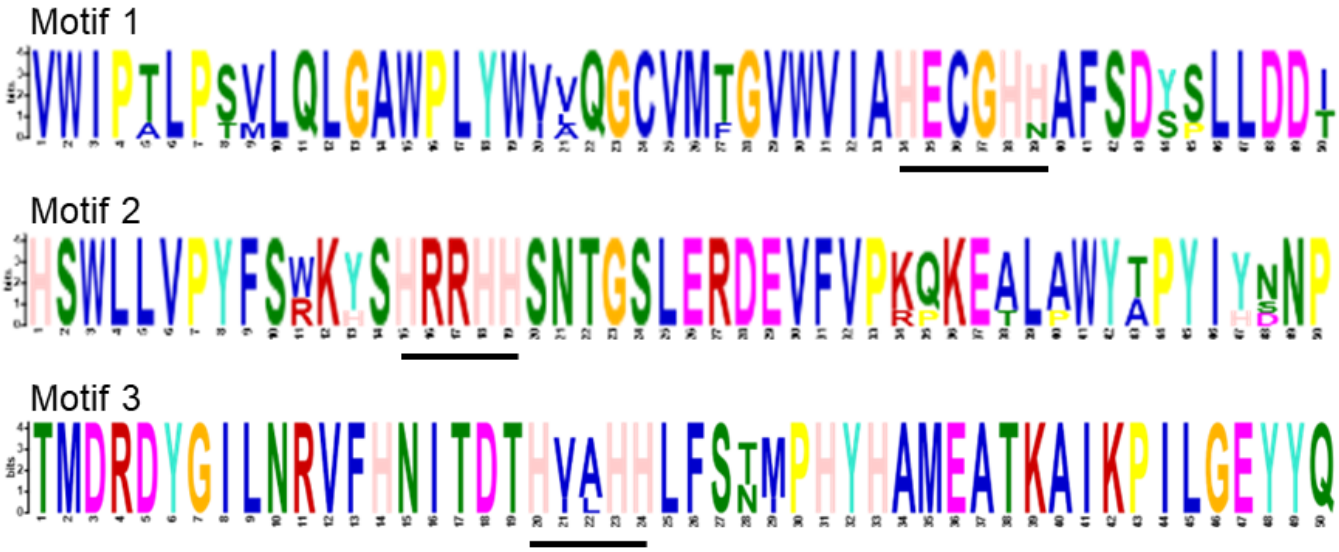


Figure 3

Logo representation of amino acid motifs of five *TdFAD2* functional copies, containing histidine boxes: HECGH in motif 1, HRRHH in motif 2 and HXXHH in motif 3.

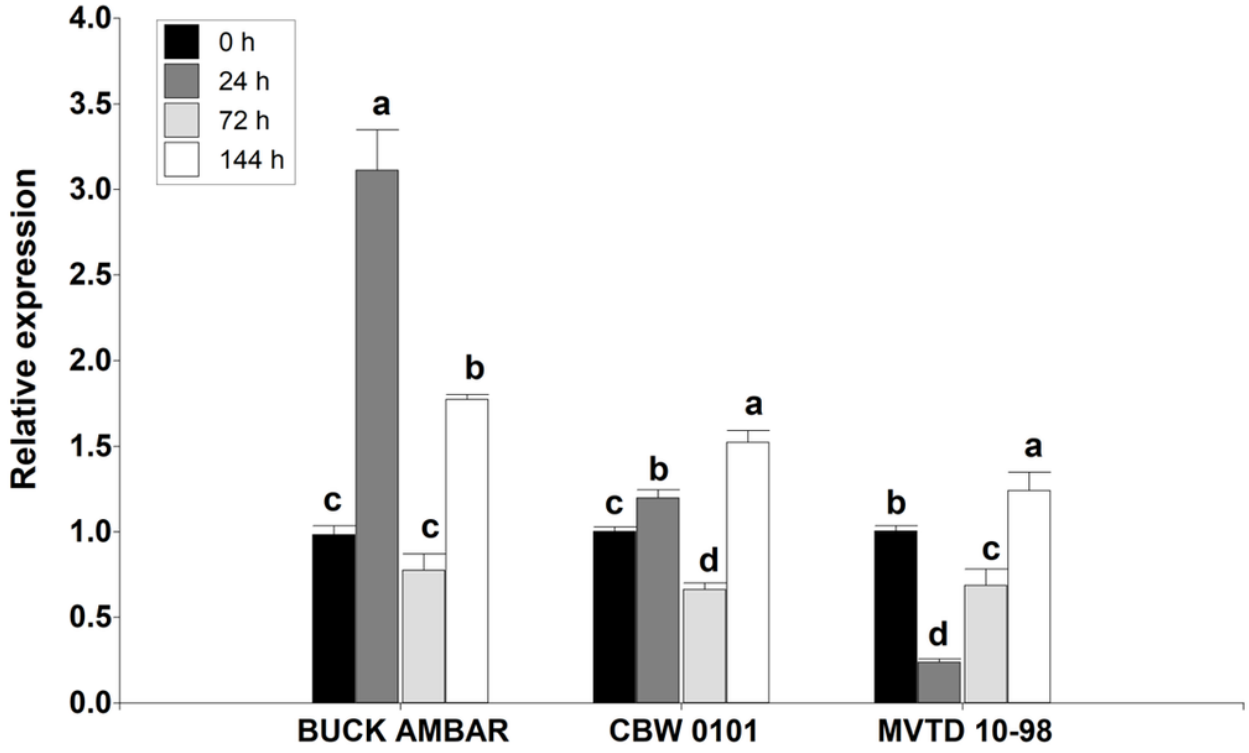


Figure 4

Relative expression levels of *FAD2* in leaves of three durum wheat genotypes at control conditions (plants growing at 22°C = 0 hours at 4°C), or after 24, 72, and 144 hours at 4°C. Different letters indicate significant differences between treatments ($p < 0.05$, Fisher test). The error bars represent the standard deviation (± SD) for three replicates.

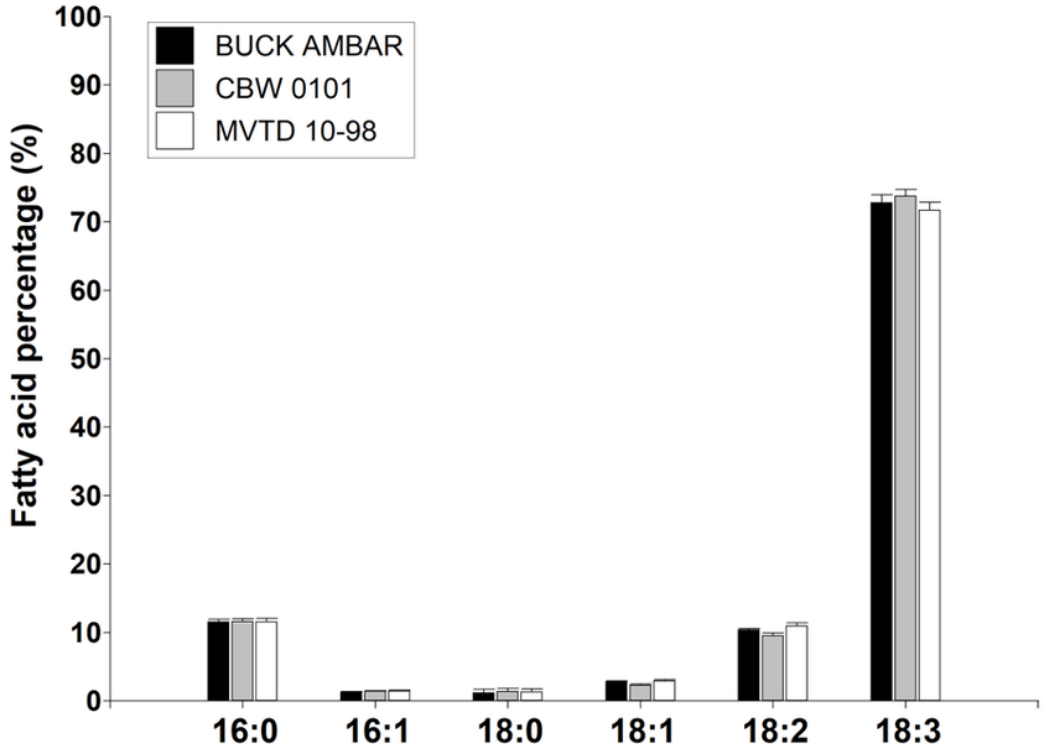


Figure 5

Mean values of fatty acid (16:0, 16:1, 18:0, 18:1, 18:2 and 18:3) of the control plants growing at 22°C, of the genotypes BUCK AMBAR, CBW 0101 y MVTD 10-98. The error bars represent the standard deviation (± SD) for three replicates per treatment.

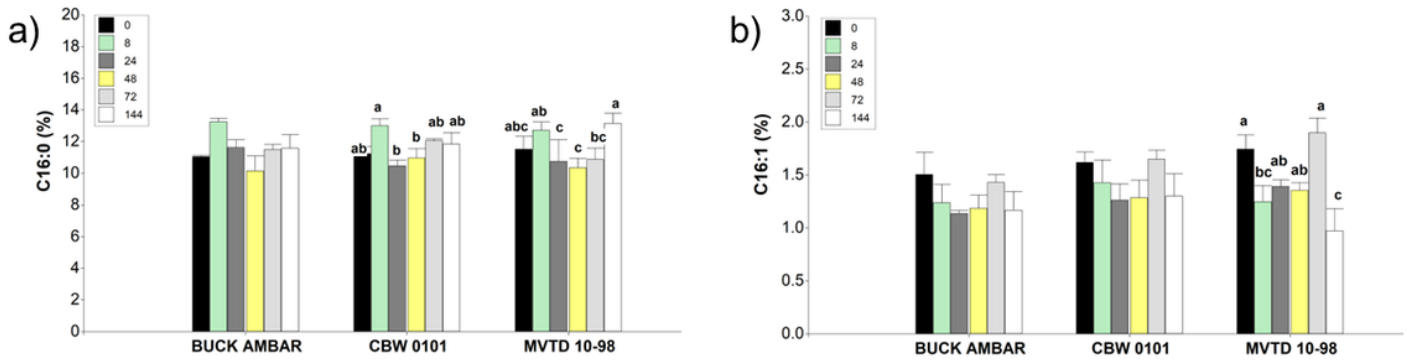


Figure 6

a) Palmitic (16:0) and b) palmitoleic (16:1) acid content at control conditions (seedlings growing at 22 C or 0 hours at 4 C) and after 8, 24, 48, 72 and 144 hours at 4 C, in three durum wheat genotypes. The vertical axis shows the percentage of total fatty acid. Different letters indicate significant differences between treatments ($p < 0.05$, Fisher test). The absence of letters indicates no significant differences between treatments. The error bars represent the standard deviation (± SD) for three replicates.

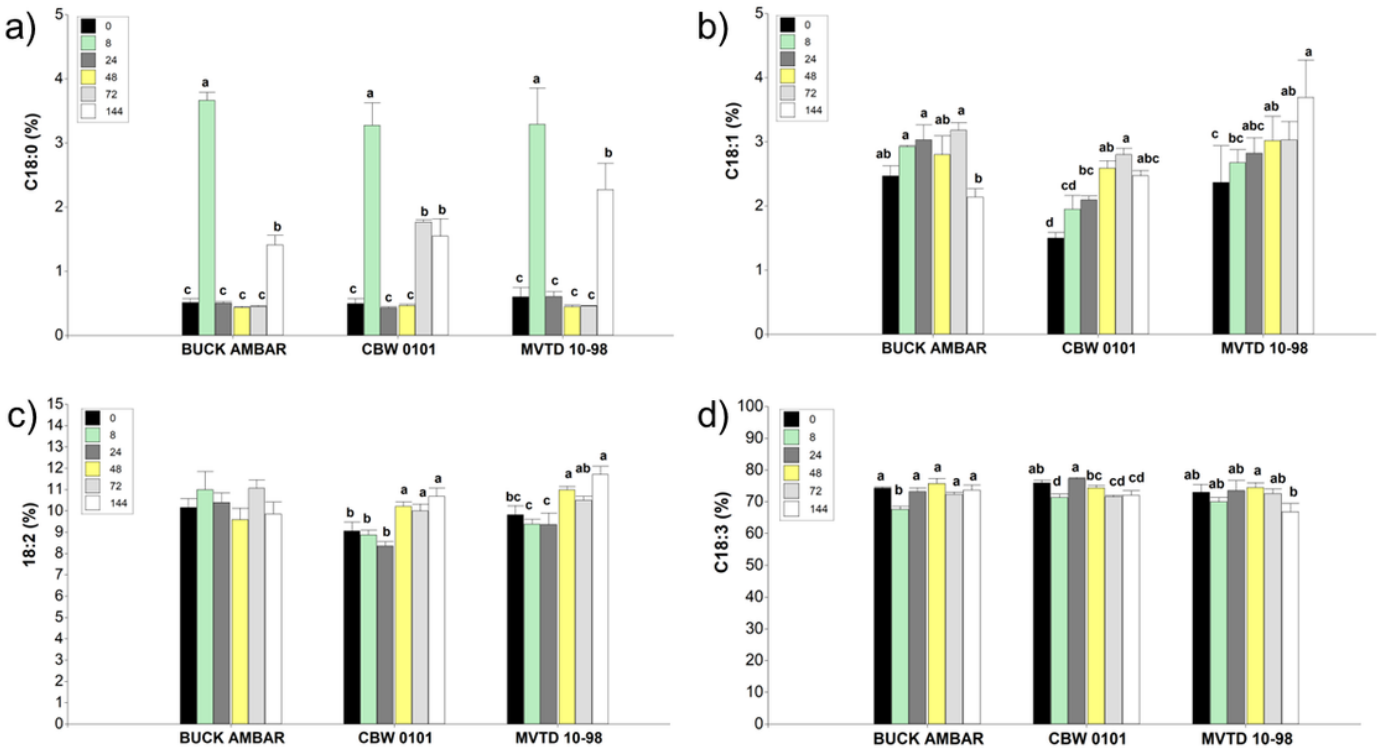


Figure 7

a) Stearic (18:0), b) oleic (18:1), c) linoleic (18:2), and d) linolenic (18:3) acid content at control conditions (seedlings growing at 22 C or 0 hours at 4 C) and after 8, 24, 48, 72 and 144 hours at 4°C, in three durum wheat genotypes. The vertical axis shows the percentage of total fatty acid. Different letters indicate significant differences between treatments ($p < 0.05$, Fisher test). The absence of letters indicates no significant differences between treatments. The error bars represent the standard deviation (± SD) for three replicates.

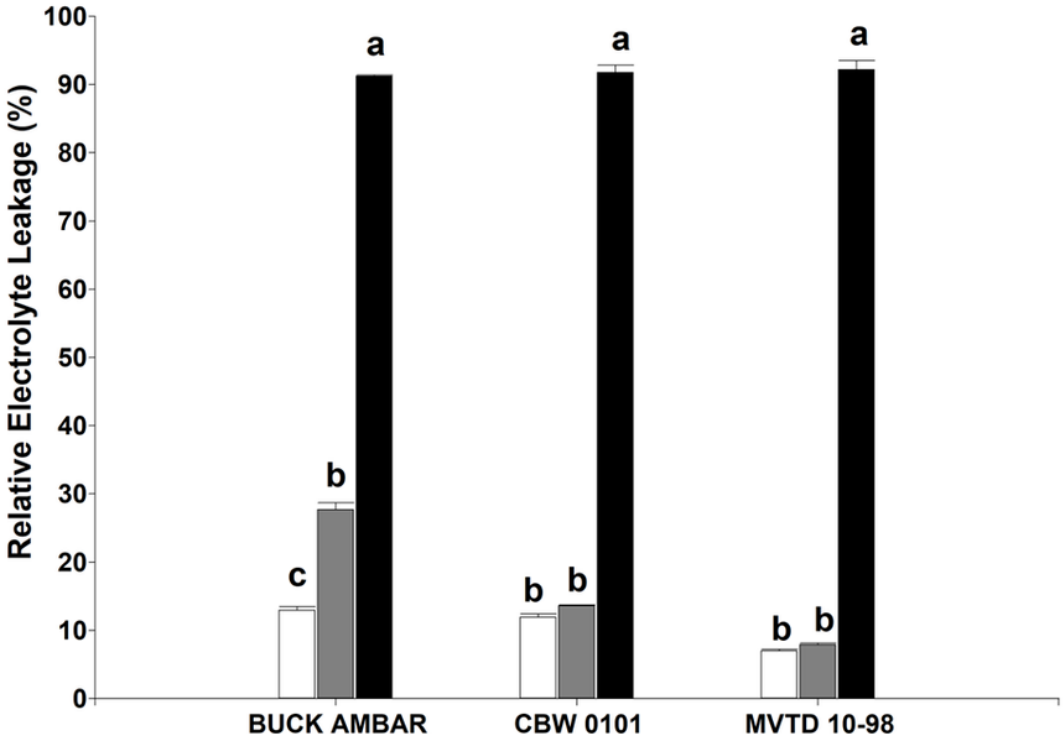


Figure 8

Relative electrolyte leakage REL (%) in leaves of three durum genotypes at 2 C (white bars), -4°C (gray bars), and -13°C (black bars) for 12 hours. Different letters indicate significant differences between treatments ($p < 0.05$, Fisher test). The error bars represent the standard deviation (± SD) for three replicates.

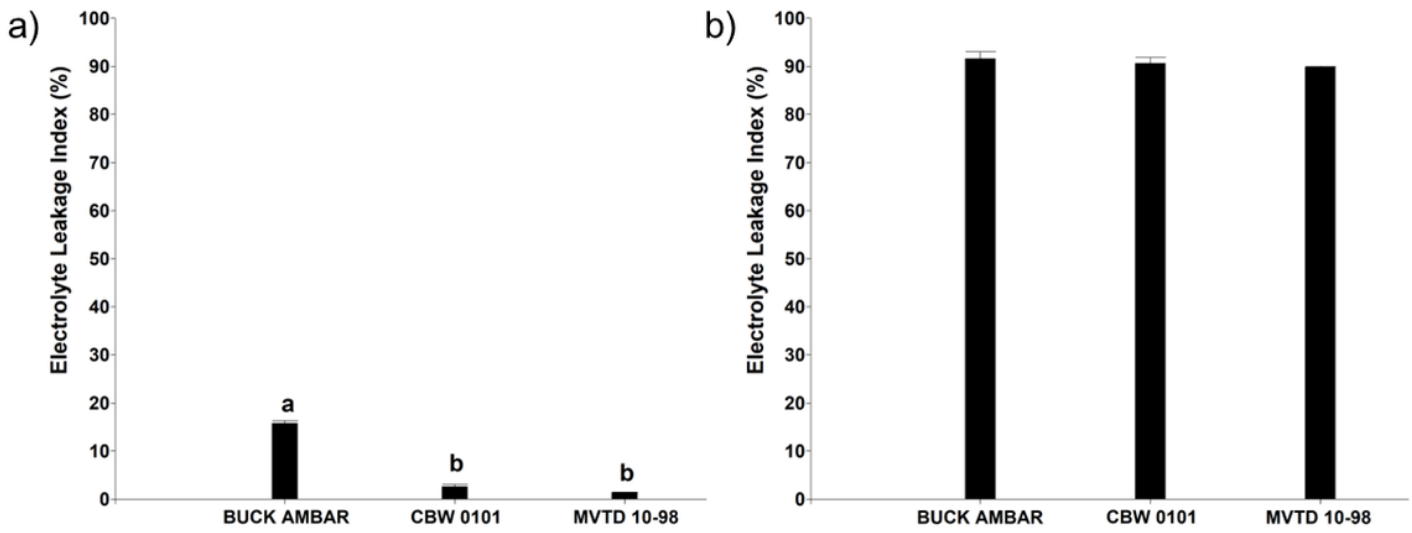


Figure 9

a) Electrolyte leakage index ELI (%) in leaves of three durum genotypes at a) -4°C for 12 h and b) -13°C for 12 h. Different letters indicate significant differences between genotypes ($p< 0.05$, Fisher test) and the absence of letters indicates no significant differences between genotypes ($p> 0.05$, Fisher test). The error bars represent the standard deviation (± SD) for three replicates.

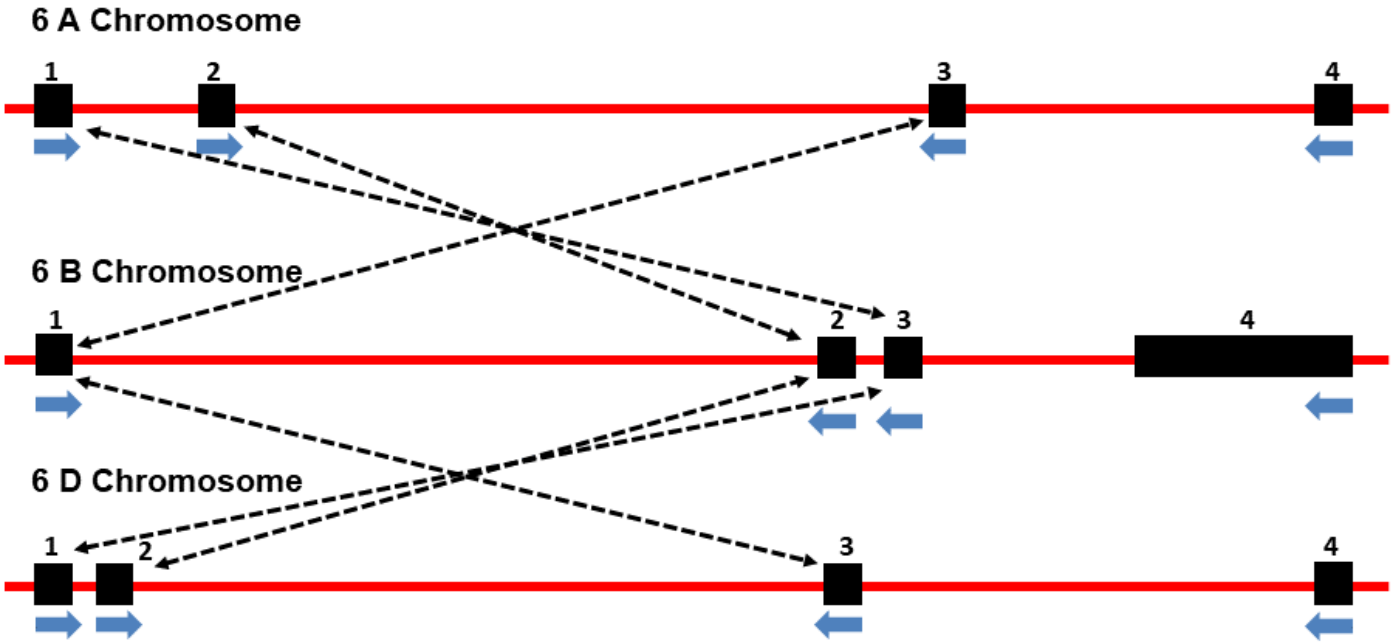


Figure 10

Chromosomal rearrange inferred from sequence similarities and orientations of the *TdFAD2* copies on 6A, 6B (cv Svevo) and 6D (cv Chinese Spring) chromosomes (red lines). In boxes were represented the gene copies. The broken arrows join the copies with the highest nucleotide identity.