Candidate gene expression profiling in two contrasting tomato cultivars under chilling stress

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Abstract

Tomato (*Solanum lycopersicum* Mill.) is sensitive to chilling stress during all stages of plant development. Genetic variation for chilling tolerance exists between cultivated tomato and its related wild species, but intra-specific variation has not been thoroughly investigated so far. Seedlings of 63 tomato accessions were evaluated under low temperature and two contrasting cultivars were identified for the trait: Albenga and San Marzano, the former being more chilling-tolerant. To clarify the molecular mechanisms of chilling tolerance in tomato, changes in candidate gene expressions in the two tomato genotypes were analysed, using quantitative RT-PCR. Candidate genes were chosen among those known to be induced by chilling and/or with putative roles in CBF/DREB and ROS-mediated pathways. Results show that besides a CBF regulon, whose function is conserved, ROS and C2H2-type zinc finger protein-mediated cold signalling pathways were also involved in chilling tolerance. Under the chilling stress, the up-regulation of respective transcripts was consistently higher in the chilling-tolerant genotype than in the chilling-sensitive ones.

Additional key words: C2H2-type zinc finger protein, CBF/DREB pathway, ROS-mediated pathway, *Solanum lycopersicum.*

Introduction

The chilling (0 - 12 °C) stress is one of major environmental factors limiting the productivity and geographical distribution of many important crops of tropical origin. In chilling-sensitive plants, the cellular membranes are the primary site of cold-induced injury leading to a cascade of molecular and physiological events with adverse effects on the plants (Lyons 1973, Raison and Lyons 1986).

Most cultivars of tomato are sensitive to chilling injury during all stages of plant development, including seed germination, early and late vegetative growth, and reproductive phases (Scott and Jones 1982, Wolf *et al.* 1986, Foolad and Lin 2000). In contrast, tomato interfertile wild species *Solanum habrochaites* and *Solanum chilense* grow at altitudes up to 3 300 m in the Peruvian Andes and exhibit a higher degree of chilling tolerance compared to *S. lycopersicum* (Vallejos and Pearcy 1987, Bloom *et al.* 2004, Venema *et al.* 2005). Hence, considerable genetic variation in low temperature tolerance exists between species of tomato which could be utilised to improve chilling tolerance of tomato cultivars; moreover, an intra-specific variation which has

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Abbreviations: ABA - abscisic acid; AP2/ERF - APETALA 2/ethylene response factor; Aux/IAA - auxin⁄indole-3-acetic acid; bZIP - basic region-leucine zipper; CBF/DREB - C-repeat binding factor/dehydration-responsive element binding; COR - cold-regulated; CRT/DRE - C-repeat/dehydration responsive element; Ct - cycle threshold; DHNs - dehydrins; DMRT - Duncan's multiple range test; EAR-motif - ERF-associated amphiphilic repression motif; EF-1-α - elongation factor 1-α; EL - electrolyte leakage; ERF - ethylene response factor; FAD - fatty acid desaturase; GLM - generalized linear model; ILs - introgression lines; LEA - late embryogenesis abundant; MYB - myeloblastosis; NLS - nuclear localization signal; NTC - no template control; RT-qPCR - reverse transcription-quantitative PCR; RCBD - randomized complete block design; REST© - relative expression software tool©; ROS - reactive oxygen species; SGN - sol genomics network; TFs - transcription factors; USP - universal stress protein; WT - wild type.
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not been thoroughly investigated yet could be exploited to improve such tolerance as well.

Plants respond to low temperatures by a coordinated transcriptional network (Gilmour *et al.* 2000). One of the best characterized players is represented by the *CBF/DREB1* genes with a prominent role in freezing tolerance of *Arabidopsis* (Thomashow 1999, 2001). The CBF transcription factors constitute a regulatory hub for cold acclimation, regulating the expression of the target cold regulated (*COR*) genes that contain CRT/DRE *cis*-elements in their promoters (Thomashow 2010). In *Arabidopsis*, *CBF* transcription increases within 15 min after exposure of plants to low temperature, and transcripts of the targeted *COR* genes start to accumulate within approximately 2 h (Mantyla *et al.* 1995). Overexpression of either *CBF1*/*DREB1b* or *CBF3*/*DREB1a* in *Arabidopsis* results in a strong activation of the target *COR* genes, and successively increases freezing and dehydration tolerance of non-acclimated plants (Jaglo-Ottosen *et al.* 1998, Kasuga *et al.* 1999). Moreover, the constitutive overexpression of *Arabidopsis CBF1*/*DREB1b* confers cold stress tolerance to cucumber and potato (Gupta *et al*. 2012, Movahedi *et al*. 2012). Thus, the CBF/DREB1 regulon was the first example and the most important regulatory cluster associated with cold stress in plants. CBF homologues were identified in many monocot and dicot plant species, and a recent evidence also show that the regulon is not unique to cold-tolerant plants, but it is also evolutionarily conserved in chilling-sensitive (warm-season) plants, such as tomato, rice, sunflower, and citrus (Zhang *et al.* 2004, Morsy *et al.* 2005, Fernandez *et al.* 2008, He *et al*. 2012). However, the transcriptome analysis of transgenic *Arabidopsis* plants overexpressing *CBF* revealed that only about 12 % of the *COR* genes are members of the CBF regulon (Fowler and Thomashow 2002) suggesting that other transcriptional activators/repressors also play a significant role in cold acclimation (Theocharis *et al.* 2012).

Cold stress causes the accumulation of reactive oxygen species (ROS; Asada and Takahashi 1987, Mittler 2002). ROS are able to cause oxidative injuries to DNA, lipids, and proteins (Larkindale and Knight 2002, Apel and Hirt 2004), but they are also important signals (Mittler *et al.* 2004, Torres and Dangl 2005, Cheng *et al.* 2007). Cheng *et al.* (2007) presented the evidence of a ROS-mediated regulatory module that functions as early component of the chilling stress response pathway in *japonica* rice, potentially independent of the CBF/DREB1 regulon. Moreover, genome-wide expression profiling of *Lepidium* (Zhao *et al*. 2012) and rice (Yun *et al*. 2010) showed that several regulatory clusters, including ERF, bZIP, and R2R3-MYB factors, are involved in early chilling response. The major effect on gene expression is up-regulation in the chilling-tolerant, and strong repression in the chilling-sensitive rice genotypes (Zhang *et al.* 2012).

It was also observed that some plants when exposed to chilling show anthocyanin accumulation in leaves and stems (Christie *et al.* 1994, Leyva *et al.* 1995). Recently, it was reported that, in blood oranges, cold stress induces transcriptomic modifications that increase flavonoid biosynthesis including reactions involved in anthocyanin biosynthesis (Crifò *et al.* 2011).

Till now, few studies using mainly transgenic approaches or wild species *S. habrochaites* and introgression lines (ILs) have been undertaken to elucidate molecular mechanisms involved in tomato chilling tolerance (Jaglo *et al.* 2001, Zhang *et al.* 2004, Liu *et al.* 2012, Miura *et al.* 2012). The constitutive overexpression of *LeCBF1* in transgenic *Arabidopsis* plants activates CBF-targeted genes and improves freezing tolerance showing that *LeCBF1* encodes a functional homologue of the *Arabidopsis* CBF1-3 proteins. However, the constitutive overexpression of either *LeCBF1* or *AtCBF3* does not improve freezing tolerance in transgenic tomato plants. Gene expression studies identified only four genes that are induced 2.5-fold or more in the *LeCBF1* or *AtCBF3* overexpressing plants. Three of those genes, two encoding dehydrins and the third encoding a probable proteinase inhibitor, are putative members of the tomato CBF regulon as they are also induced by cold stress (Zhang *et al.* 2004). To investigate the mechanisms underlying the cold tolerance of wild tomato *S. habrochaites*, a comparative transcriptome analysis, using microarray, between cold-tolerant (LA1777 and LA3969) and cold-sensitive (LA4024) tomato genotypes were performed by Liu *et al.* (2012). The analysis identified 1613, 1523, and 1456 low temperature-responsive genes in LA1777, LA4024, and LA3969, respectively. However, to the best of our knowledge, intraspecific differences in the expression profiles of cold-tolerant and cold-sensitive cultivated tomatoes under cold stress have not been yet reported.

Therefore, to clarify the mechanisms underlying chilling tolerance in tomato, the aims of the present work were following: *1*) the identification of two tomato accessions, contrasting in their response to chilling, by means of a phenotypic characterization of a germplasm collection of wild and cultivated genotypes and a selected group of anthocyanin mutants and their corresponding background lines, and *2*) the evaluation of changes in the candidate genes expression in the two contrasting tomato accessions using RT-qPCR assays. In this study, this method was used to analyse the candidate genes expression in tomato under 1 °C, and to study genes and pathways that may play a role in chilling tolerance. The results represent the first report on candidate gene expression profiles in cultivated tomato accessions with contrasting responses to chilling stress and provide an insight into gene function and more comprehensive knowledge of the molecular mechanisms involved in the environmental responses of tomato and other crops of tropical origin.

Materials and methods

Plants, growth, and treatments: Sixty-three tomato accessions, the part of a germplasm collection held at the Department of Life Sciences, University of Modena and Reggio Emilia (UNIMORE), Italy, were used in this study for determination of chilling tolerance/sensitivity. The set of 63 accessions was composed of 32 cultivars, ecotypes, and lines of *Solanum lycopersicum* Mill., 4 genotypes of *S. habrochaites*, 2 genotypes of *S. chilense*, 1 genotype of *Solanum lycopersicoides*, 6 introgression lines (*S. lycopersicum* × *S. habrochaites*), and finally, 18 anthocyanin-pathway mutants (Table 1 Suppl.). Seeds were kindly provided by the C.M. Rick Tomato Genetics Resource Centre, Davis, CA USA (http://tgrc.ucdavis.edu) and by Dr. N. Acciarri, the CRA-ORA Research Unit for Vegetable Crops, Monsampolo del Tronto, (AP), Italy.

All accessions were germinated on moistened filter paper at 25 °C for 3 d. Seedlings were grown for 3 weeks in a growth chamber (*Binder KBW 720*, Tuttlingen, Germany) with a 16-h photoperiod under an irradiance of 180 μmol m^{-2} s^{-1} (white fluorescent tubes *Fluora 18W/77*, *Osram*, Munich, Germany), day/night temperatures of 25/19 °C, and a relative humidity of 60 %. Low temperature treatments at different temperatures (5, 3, and 1 °C) were then applied at the beginning of the photoperiod for 24 h (the irradiance decreased to 60 μmol m^{-2} s^{-1}).

For candidate genes transcript profiling, leaves from five plants of cv. Albenga (chilling-tolerant) and cv. San Marzano (chilling-sensitive) were harvested at five time points (2, 4, 8, 24, and 72 h) during the cold (1 °C) treatment and also after 11 d (264 h) of recovery at 25 °C following 72 h at 1 °C (72 + 264). The samples were immediately frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction. Two control samples were collected for each cultivar; one before the stress application, the second one 14 d later (11 d after the chilling stress termination) as control for plants collected at the recovery.

Cell membrane stability assessment: The chilling tolerance of the germplasm collection was determined using the electrolyte leakage test according to Rizza *et al.* (1994). Briefly, freshly cut leaf discs (a 0.5 cm diameter) were placed in vials containing 25 cm^3 of deionized water and stirred at 25 °C for 2 h 30 min. The conductivities of the solution and of deionized water were determined (C1 and Cw, respectively). Then, the samples were autoclaved (120 °C for 15 min), and conductivity was read again (C2). The conductivity was measured with a digital conductance meter (*GLP 31*, *Crison Instruments*, Barcelona, Spain). Electrolyte leakage, EL [%], was calculated as (C1 - Cw)/(C2 - Cw) × 100. The chilling tolerance was expressed as temperature at which 50 % leakage occurred (EL_{50}). The accessions with EL > 50 % were considered sensitive, those with EL < 50 % tolerant to chilling.

In each experiment, the treatments were arranged in a randomized complete block design (RCBD) with three replications. The analysis of variance was performed for each stress temperature (experiment) separately according to *GLM* with *SYSTAT 12* (*SPSS*, Chicago, IL, USA). Mean comparisons were performed using the Duncan's multiple range test (DMRT, $P \leq 0.05$).

RNA isolation, quality control, and cDNA synthesis: Total RNA was isolated from 50 mg of leaves according to the manufacturer's instructions using the *NucleoSpin* RNA plant kit (*Macherey-Nagel*, Düren, Germany). RNA quantity and quality were determined using a *Nanodrop ND-1000* spectrophotometer (*Thermo Fisher Scientific*, Waltham, MA, USA) and an *Agilent 2100* bioanalyzer (*Agilent Technologies*, Palo Alto, CA, USA). The extracted RNA samples were also visualized on a standard non-denaturing 1.2 % (m/v) agarose gel containing 0.5 × TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM Na_2EDTA, pH 8.3). Total RNA (0.5 μg) was reverse transcribed using an oligo (dT) primer by the *SuperScript*™ *II* reverse transcriptase (*Invitrogen Life Technologies*, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA samples were diluted to a concentration of 0.5 ng mm^{-3} (the total RNA equivalent) with nuclease-free water.

Selection of candidate genes: Eighteen candidate genes were selected among those known to be induced by chilling and/or with putative roles in CBF/DREB and ROS-mediated pathways in tomato and in other plants (Kodama *et al.* 1994, Zhang *et al.* 2004, Cheng *et al.* 2007, Yu *et al.* 2009, Sharma *et al.* 2010, Zhang *et al.* 2011, Pan *et al.* 2012). Additional gene coding for cold inducible histidine kinase 1 was retrieved from *Tomato Gene Index* data mining (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=tomato).

Sequences of the candidate genes chosen were evaluated for homology to tomato genes using *BLASTN* and *TBLASTX* against the *Sol* genomics network (*SGN*) database (http://solgenomics.net/), setting the E value cut off at $1e^{-20}$, and a *SGN* tomato gene model was identified. The gene annotations were attributed to gene models querying the *UniProt/TrEMBL* database using *BLASTX*. *UniProt/TrEMBL* (http://www.uniprot.org/) gene ontology was taken when available; if not, the data from the *SGN* databases were used.

The list of candidates comprises both transcription factor and non-transcription factor genes (Table 1). The nineteen RT-qPCR primer pairs used were designed and evaluated using the *Oligo Explorer* software, *v. 1.1.0* (T. Kuulasma, University of Kuopio, Finland) and the *Oligo Analyzer* software (www.idtdna.com). Four serial two-fold dilutions of cDNA (0, 1:2, 1:4, and 1:8), were made to determine the gene specific PCR amplification efficiency for each primer pair in RT-qPCR experiments.

The amplification efficiency was calculated from the slope of the standard curve based on the cycle threshold (Ct) values for all dilution points in a series. The *REST*© (relative expression software tool, http://b2b.qiagen.com/products/rest2009software.aspx#Tabs=t0) (Pfaffl *et al.* 2002) was used to calculate the gene specific PCR amplification efficiency of the primer pairs.

RT-qPCR assays were performed on a *7300* real-time PCR system (*Applied Biosystems*, Foster City, CA, USA) in 25 mm^3 of reaction mixture containing 12.5 mm^3 of *SYBR Green PCR Master Mix* with *ROX* (*Applied Biosystems*), 2.5 mm^3 of each primer (in a 0.2 - 0.5 µM range), 5 mm^3 of cDNA (0.5 ng mm^{-3}), and 2.5 mm^3 of water. PCRs with no template controls were also performed for each primer pair (NTC). PCR conditions were following: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. To identify nonspecific PCR products, a final dissociation stage was also added: 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Following amplification, the *7300 Sequence Detection System* software was used to set both the baseline and the threshold for each reaction. The assays were performed in triplicate. Relative gene expression was calculated using the relative expression software tool (*REST*©; Pfaffl *et al.* 2002) with a normalization against the expression level of the gene encoding an elongation factor 1-α (*EF-1-α,* a *SGN* gene model *Solyc06g005060*), which was selected from the literature as the best housekeeping gene in chilling conditions; primer sequences for the housekeeping gene were forward 5'-GGAACTTGAGAAGGAGCCTAAG-3', and reverse 5'-CAACACCAACAGCAACAGTCT-3' (Løvdal and Lillo 2009).

Statistics: The statistical analysis was performed with the *REST© 2009* software which uses a mathematical model that compares treated and control samples using the pair wise fixed reallocation randomization test (Pfaffl *et al.* 2002). The data presented here were calculated using 10 000 randomizations. A relative fold change of ± 2 in gene expression was set as the cut-off to reject the null hypothesis that there was no difference between treated and untreated samples.

Results

Chilling-damage evaluation and two contrasting genotypes selection: A damage to cell membranes after the chilling treatments (5, 3, and 1 °C for 24 h) was measured by EL [%], based on the principle that damage to cell membranes results in an enhanced leakage of solutes into the apoplastic water. EL was about 10 % in all the untreated genotypes (data not shown). There was no difference in EL among the plants after they were treated at the temperature of 5 °C (data not shown), whereas at 3 °C, EL values ranged between 13.3 % (Rotonda Ligure) and 61.2 % (LA3957) (Fig. 1 Suppl.). Statistically significant differences ($P \leq 0.05$) were detected among groups of the genotypes suggesting that a useful variation in chilling tolerance exists within the collected germplasm (Fig. 1 Suppl.). However, the majority of accessions showed EL values between 15 and 35 % suggesting that the imposed temperature was not sufficient to determine clearly differences in chilling

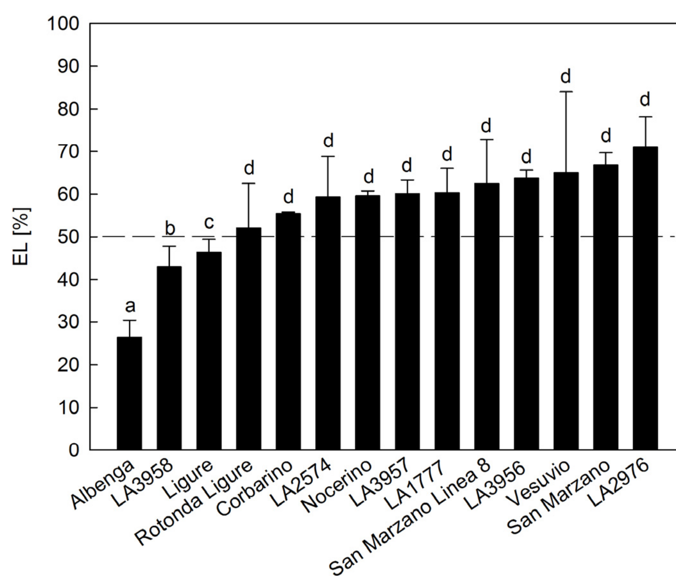

Fig. 1. Chilling tolerance at 1 °C for 24 h as electrolyte leakage (EL [%]) measured in fourteen selected accessions. Values of electrolyte leakage above 50 % are interpreted as indication of strong membrane injury (the dashed line). Mean ± SE, $n = 3$. Different letters indicate statistically significant differences (DMRT, $P \leq 0.05$).

Table 1. Candidate genes selected for RT-qPCR analysis.

Gene annotation	*SGN* gene model	Primer sequence	Conc.
CBF/DREB1 regulon			
Q8S9N5_SOLLC	Solyc03g026280	F 5'-CTGCCTATCCCTGCTTCCTC-3'	5 µM
CBF1		R 5'-AAGATCGCCTCCTCATCCAC-3'	
Q675A0_SOLLC	Solyc03g026270	F 5'-AGTCGGAGGAAGAAGAATCAGTG-3'	2 µM
CBF3		R 5'-TCCCATTTCAGTACATTGAGGTG-3'	
E5F3A5_SOLPE	Solyc04g082200	F 5'-GGCAATTTCATCTGAGTTGTCTG-3'	4 µM
Dehydrin		R 5'-TTGATCTTCTGTCCATCCTCTCC-3'	
C6F3B6_SOLCO	Solyc05g053080	F 5'-ATTGAAGAGAAACTCCACATCGG-3'	4 µM
DHN10		R 5'-ACAAATCCTTCCTTATGCTCATCC-3'	
B1N681_SOLPI	Solyc07g007750	F 5'-GCTTGTTATGGCTACTGAAATGGG-3'	4 µM
Defensin protein		R 5'-GAGGCACAATTCTTATCGCTCAC-3'	
ROS-mediated regulon			
Q9SPF7_SOLLC	Solyc01g079480	F 5'-CTGATGCTAATGGACTTGCTGTC-3'	3 µM
BZIP DNA-binding protein		R 5'-CAGGTTGTGCTCAAAACTGAAAC-3'	
A7ULI0_DATME	Solyc09g090130	F 5'-CGATGTGGAAAAAGTTGCAG-3'	4 µM
Myb-related transcription factor		R 5'-TTATCTGTTCGTCCCGGTAATC-3'	
Q308A9_SOLTU	Solyc06g050980	F 5'-CGACAGAAATACTGCGATGAATC-3'	4µM
Ferritin		R 5'-TTCCTTGAAAAACTTGGCTAGACC-3'	
Q571X7_SOLLC	Solyc04g076850	F 5'-GATCTCAGTCTCCCGAAAGAGG-3'	5 µM
Aux/IAA protein		R 5'-GAGAATCCATCCATAGCGTCTG-3'	
VATL_SOLLC	Solyc10g054560	F 5'-TTCTGCTGGAATGGCTATTGG-3'	4 µM
V-type proton ATPase 16 kDa proteolipid subunit		R 5'-GCTCGGGAAGACAAGATAATGC-3'	
Chilling induced genes			
A6ZIC0_SOLLC	Solyc04g077980	F 5'-TGTCACAAGTGTTTTCCGACTG-3'	4 µM
C2H2-type zinc finger protein		R 5'-TTAGCGTTAGCATTTCCGTTACC-3'	
G8GTS3_CITLI	Solyc01g109170	F 5'-GTTTGTTCAGGGGAGATTTCG-3'	4 µM
WCOR413-like protein		R 5'-AATGCTGCTGGTGCTTCAAG-3'	
B0YQX0_GOSAR	Solyc09g011660	F 5'-CCACATCAAGACTCATTCCTCTG-3'	4 µM
SGN Universal stress protein 1		R 5'-AACACCTCAGCCTCCCTAAACTC-3'	
E1AWE4_9ROSI	Solyc02g065380	F 5'-CTCCTCCAAGAGCACATCAGAG-3'	4 µM
COR413-PM1		R 5'-GAAGAACCAGAGCCCAGATTG-3'	
Q6Q4I4_SOLLC	Solyc03g093560	F 5'-CGAAGCCTGTTGTTGTTGTAGAG-3'	4 µM
Ethylene response factor 5		R 5'-TTCTATTTGGGTCACGAATCTCC-3'	
Q8L9K1_ARATH	Solyc04g054910	F 5'-TCCCTTTCTGTTGTCGTCTGTC-3'	4 µM
SGN Ethylene-responsive transcription factor 13		R 5' -GTTCTGGATCACCTGGCTTTC-3'	
Q71BY9_CATRO	Solyc02g083680	F 5'-CCTCTCAAGAAGGATGGTTGC-3'	4 µM
Cold inducible histidine kinase 1		R 5'-CGGGGTCTTCAGAATCAATAGC-3'	
A9LNM9_SOLLC	Solyc06g007130	F 5'-GAGGAGAAGTCCAGGAAAAAAAGG-3'	4 µM
Omega-3 fatty acid desaturase		R 5'-AAGCCAACGACGGTAGAAAGATAG-3'	
Q7X7I9_SOLLC	Solyc06g051400	F 5'-CTTCTTCCGTTTCTGTTTCCG-3'	5 µM
Omega-3 fatty acid desaturase		R 5'-CACTTACCCTCAATGCCCAAC-3'	

tolerance among the accessions. With the objective to select two accessions with a clear contrasting response to the stress in our germplasm collection, a further chilling-tolerance assay was conducted. The EL analysis of fourteen genotypes with differential sensitivity at 3 °C revealed that differences in chilling tolerance between the accessions were the most apparent when the plants were exposed to 1 °C for 24 h; the EL values ranged between 26.4 and 71.0 % (Fig. 1).

Due to the consistent phenotypic variation observed in the sub-sample of the cultivated material, a definite validation of our results was performed using six fresh market tomato genotypes selected from the 14 tested accessions. Three chilling-sensitive accessions: Nocerino, San Marzano, and Vesuvio were compared to three chilling-tolerant accessions: Rotonda Ligure, Ligure, and Albenga. The EL test performed at the most discriminating conditions (1 °C for 24 h) highlighted the different phenotypic behaviour of the two groups of accessions (Fig. 2 Suppl.). Finally, the Albenga and San Marzano cultivars were selected by visual scoring for their contrasting response to chilling (1 °C for 24 h) (Fig. 3 Suppl.).

Since some authors hypothesized a role for phenolic compounds in determining phenotypic variation in response to plant exposure to a low-temperature stress (Prasad 1996, Rice-Evans *et al.* 1997, Pennycooke *et al.* 2005, Crifò *et al.* 2011), 18 anthocyanin mutants and

their relative genetic backgrounds were included in the *UNIMORE* tomato germplasm collection (Table 1 Suppl.). All the accessions were thus exposed to the same chilling stress conditions (1 °C for 24 h), but no accession showed an EL less than 50 %, as well as no clear differences were observed between anthocyanin mutants and their corresponding wild types (Fig. 4 Suppl.).

Candidate gene expression patterns: Quantitative RT-PCRs were performed to examine changes in transcript abundance of the selected candidate genes in response to different exposure to the chilling stress in cvs. Albenga and San Marzano (Tables 2 to 4 and Table 2 Suppl.). Five candidate genes analysed belonged to the CBF/DREB1 regulon and were already identified by Zhang *et al.* (2004) as differentially expressed in transgenic tomato plants under chilling stress. Two of them, genes coding for CBF1 and CBF3, were transcription factors (*i.e.*, regulators), and three genes encoding dehydrin (formerly annotated as homologue of potato dehydrin Ci7 cLER1E13/TC116013), DHN10 (formerly annotated as dehydrin-like protein cLER17C11/TC116174), and a defensin protein (formerly annotated as probable proteinase inhibitor cLER1P6/TC115955) were *COR* genes (*i.e.*, effectors). *COR* genes annotations were updated after the tomato genome release (the Tomato Genome Consortium 2012). *CBF1* and *CBF3* were rapidly induced in response to the low temperature in both the cultivars, but transcripts were more strongly induced in the tolerant genotype than in the sensitive one. The amount of *CBF1* transcripts reached the maximum within few hours in San Marzano and Albenga with nearly 300- and 5 000-fold increases, respectively. In Albenga, the *CBF1* transcripts remained stable until 72 h of the cold treatment, whereas in San Marzano gradually decreased but were still strongly induced compared with the control (Table 2). On the other hand, *CBF3* was less induced in response to the low temperature as compared to *CBF1* in both the cultivars. The amount of *CBF3* transcripts reached the maximum within few hours and subsequently gradually decreased in both the cultivars (Table 2). The amount of *COR* genes transcripts was higher in Albenga than in San Marzano. In Albenga, the amount of transcripts for a dehydrin protein increased to 19-fold, and *DHN10* transcripts increased to 8-fold after 72 h of the exposure at 1 °C (Table 2). In addition, during the recovery period, the transcripts abundance of *CBFs* and the dehydrin protein decreased and returned to the control level in both the cultivars. The transcript abundance of *DHN10* during the recovery period returned to the control level only in Albenga, whereas in San Marzano, it reached a peak of expression. Finally, the gene encoding a defensin protein, a proteinase inhibitor, was slightly induced after 24 h of exposure at 1 °C but only in San Marzano (Table 2 Suppl.).

Table 2. The induction of the CBF/DREB1 regulon in cvs Albenga and San Marzano under a chilling stress (1 °C) for the indicated time points [h]. Gene expression values are represented as means and SE (expression of standard error as calculated by the software *REST*), $n = 3$. The relative gene expression was normalised to the *EF-1-α* expression and then to the respective control for each time point (the pair wise fixed reallocation randomization test at * - $P \leq 0.05$, ** - $P \leq 0.01$, and *** - $P \leq 0.001$).

Gene	Time point [h]	Albenga expression	SE range		San Marzano expression	SE range	
Solyc03g026280	2	4304.83*	2354.67	8684.60	281.85*	272.63	291.66
CBF1	4	4924.47***	2666.45	9900.90	204.99***	196.80	211.62
	8	4552.13***	2416.61	8645.32	187.69***	153.13	212.20
	24	2862.56***	1573.36	5794.85	100.53*	85.08	118.84
	72	1265.60***	680.94	2396.68	38.52**	36.03	40.95
	72 + 264	-1.81	-1.93	-1.70	-2.00	-2.21	-1.85
Solyc03g026270	2	38.28***	21.50	63.16	58.35*	29.99	127.00
CBF3	4	83.93*	58.96	113.91	21.33***	14.45	29.99
	8	50.02***	37.57	69.46	21.20*	14.17	30.17
	24	46.81*	32.46	64.66	14.03***	9.68	23.69
	72	22.05***	16.64	32.02	5.07***	2.76	9.95
	72 + 264	1.95	1.09	3.33	-5.24*	-6.45	-4.50
Solyc04g082200	2	3.99***	3.42	4.68	3.90***	2.82	6.48
Dehydrin	4	1.95	1.63	2.27	4.89***	3.48	8.19
	8	2.34***	2.12	2.55	8.10*	5.84	13.36
	24	5.85***	5.28	6.62	6.76***	4.79	11.34
	72	18.92*	17.32	20.85	15.57***	10.65	24.84
	72 + 264	1.20***	1.09	1.32	-1.09	-1.48	1.19
Solyc05g053080	2	2.60	1.75	3.51	-1.03	-1.22	1.18
DHN10	4	1.67	1.27	2.58	1.72*	1.44	2.07
	8	2.01*	1.38	2.78	1.99*	1.69	2.56
	24	5.69*	4.15	8.19	3.10***	2.50	3.87
	72	7.99*	5.54	11.22	2.72**	2.20	3.16
	72 + 264	-1.23	-1.41	-1.05	4.52*	3.99	5.01

Table 3. The induction of the ROS-mediated regulon in cvs Albenga and San Marzano under a chilling stress (1 °C) for the indicated time points. Gene expression values are represented as means and SE, $n = 3$. The relative gene expression was normalized to the *EF-1-α* expression and then to the respective control for each time point (the pair wise fixed reallocation randomization test at * - $P \leq 0.05$, ** - $P \leq 0.01$ and *** - $P \leq 0.001$).

Gene	Time point [h]	Albenga expression	SE range		San Marzano expression	SE range	
Solyc01g079480	2	4.74*	3.37	7.18	2.15***	1.68	2.82
BZIP DNA-binding	4	5.85*	4.16	8.56	2.20***	1.70	2.65
protein	8	5.62***	4.06	8.51	2.82*	2.19	3.50
	24	9.41***	6.70	13.47	3.40***	2.70	4.33
	72	8.31***	5.43	10.94	2.11*	1.72	2.82
	72 + 264	1.17	-1.10	1.38	-1.08	-1.62	1.23
Solyc09g090130	2	3.49*	2.38	4.64	4.05*	2.63	5.20
Myb-related	4	1.59	1.24	2.12	1.95*	1.25	2.93
transcription factor	8	2.59*	2.00	3.48	2.11*	1.55	3.10
	24	7.88*	5.96	10.97	2.87***	2.04	3.72
	72	4.01*	2.41	5.56	2.09**	1.63	2.98
	72 + 264	-1.09	-1.27	1.15	-2.26*	-3.21	-1.30
Solyc06g050980	2	7.97***	5.90	10.86	5.68***	3.33	13.04
Ferritin	4	5.51***	4.70	6.54	5.35***	4.12	6.77
	8	4.84***	4.09	5.69	3.52*	2.87	4.92
	24	5.37*	4.58	6.34	3.42*	2.64	4.01
	72	2.66*	2.23	3.16	-1.00	-1.26	1.21
	72 + 264	-1.43	-2.37	1.16	-5.78*	-6.29	-5.38

Five of the candidate genes analysed belong to the ROS mediated metabolic pathway as proposed by Cheng *et al*. (2007) in rice, two functionally annotated as transcription factors of the bZIP and Myb families, and three genes coding for ferritin, an Aux/IAA protein, and a V-type proton ATPase 16 kDa proteolipid subunit. A gene encoding the BZIP DNA-binding protein was rapidly induced already at 2 h only in Albenga and its induction reached the maximum after 24 h in both the cultivars, but the induction was higher in Albenga over all timings of the chilling treatment (Table 3). Since the BZIP DNA-binding protein may be one of upstream regulators of the Myb-related transcription factor and ferritin protein as reported by Cheng *et al.* (2007), the induction of those two genes was investigated. Transcripts for the Myb-related transcription factor, a putative orthologue of rice *OsMyb4*, and the ferritin protein were rapidly induced already at 2 h in both the cultivars (Table 3). *Myb* transcripts accumulated more in Albenga than in San Marzano and reached a peak after 24 h of the cold treatment in Albenga (Table 3). The expression of a gene coding for ferritin remained stable until 24 h, then decreased in both the cultivars, and returned to the control level only in San Marzano (Table 3). Finally, genes encoding the Aux/IAA protein and V-type proton ATPase 16 kDa proteolipid subunit were not modulated in our experiments (Table 2 Suppl.). In Albenga during the recovery period from the stress, the transcripts abundance of all ROS-mediated genes decreased and returned to the control level. This was also true for San Marzano except for a gene coding for ferritin that was down-regulated (Table 3).

Finally, nine candidate genes were selected from the literature and from the *Tomato Gene Index* website, as induced by chilling but not belonging to the two main chilling response pathways. Three out of nine candidate genes were functionally annotated as transcription factors of the ERF and C2H2 zinc-finger families (Tables 4 and 2 Suppl.). Genes coding for a C2H2-type zinc finger protein and ethylene response factor 5 were rapidly induced in response to chilling in both the cultivars and were more strongly induced in the tolerant genotype than in the sensitive one (Table 4). Transcripts for the C2H2-type zinc finger protein were strongly induced already at 2 h in both the cultivars and remained substantially stable until 72 h of the cold treatment (Table 4). Transcripts for ethylene response factor 5 reached very high levels already after 2 h in San Marzano and in Albenga and then decreased at 72 h in both the cultivars (Table 4). A gene encoding ethylene-responsive transcription factor 13 (named ERF7 by Sharma *et al.* 2010) was slightly induced in response to chilling already at 2 h in San Marzano, the expression remained stable until 4 h of the treatment and then returned to the control level after 8 h (Table 2 Suppl.). The C2H2-type zinc finger protein may be one of upstream regulators of genes coding for a WCOR413-like protein, COR413-PM1, and universal stress protein 1, and was reported to be involved in the complicated network controlling the expression of cold-responsive genes (Zhang *et al.* 2011). Transcripts for the WCOR413-like protein were induced in both the cultivars, but more rapidly and accumulated to a higher level in Albenga with respect to San Marzano (Table 4), whereas transcripts for universal stress protein 1 were slightly induced only in Albenga after 24 h of chilling, and then returned to the control level (Table 4). A gene

encoding COR413-PM1 was not modulated in our experiments, except after 72 h of chilling in San Marzano where it was slightly down-regulated (Table 2 Suppl.).

A gene coding for cold inducible histidine kinase 1 was rapidly induced but not at high levels and its transcript abundance decreased and returned to the control level after 8 - 24 h in both the cultivars (Table 2 Suppl.). Genes for two omega-3 fatty acid desaturases displayed a different expression pattern; a gene encoding A9LNM9 (named *LeFAD3* by Yu *et al.* 2009) was down-regulated at nearly all considered time points in both the cultivars, whereas a gene coding for Q7X719 (named *LeFAD7* by Liu *et al.* 2006) was slightly cold-induced only in Albenga and reached a peak of expression after 8 h, then its transcript abundance decreased and returned to the control level (Table 2 Suppl.).

Table 4. The induction of the chilling induced genes in cvs Albenga and San Marzano under a chilling stress (1 °C) for the indicated time points. Gene expression values are represented as means and SE, $n = 3$. The relative gene expression was normalized to the *EF-1-α* expression and then to the respective control for each time point (the pair wise fixed reallocation randomization test at * - $P \leq 0.05$, ** - $P \leq 0.01$ and *** - $P \leq 0.001$).

Gene	Time point [h]	Albenga expression	SE range		San Marzano expression	SE range	
Solyc04g077980	2	244.38***	185.59	360.74	60.21***	39.57	89.80
C2H2-type zinc finger	4	249.01**	172.73	320.64	51.17***	34.31	73.66
protein	8	198.70***	145.05	267.73	53.17***	34.31	73.78
	24	317.45***	247.42	464.10	94.18***	61.16	133.52
	72	169.41***	120.79	260.15	34.34***	22.60	49.25
	72 + 264	-1.38*	-1.51	-1.24	-4.98	-5.35	-4.61
Solyc01g109170	2	7.54***	6.38	9.32	2.05***	1.81	2.37
WCOR413-like protein	4	5.00*	4.23	5.78	3.99***	3.47	4.63
	8	4.86***	4.00	5.48	5.81***	4.67	7.16
	24	4.96*	4.15	6.44	3.70***	3.19	4.23
	72	2.78***	2.42	3.35	1.22	-1.13	1.68
	72 + 264	-1.21	-1.57	1.17	1.45	1.29	1.68
Solyc09g011660	2	2.43*	1.81	3.35	1.09	-1.07	1.25
SGN universal stress	4	1.93***	1.37	2.44	-1.29*	-1.48	-1.12
protein 1	8	1.71***	1.29	2.32	-1.45*	-1.64	-1.22
	24	3.06***	2.28	4.19	-1.24*	-1.39	-1.10
	72	2.04***	1.50	2.70	-1.58*	-1.80	-1.34
	72 + 264	1.10	1.02	1.16	1.33	-1.02	1.71
Solyc03g093560	2	30.14*	25.84	32.95	13.99***	10.50	17.53
Ethylene response	4	34.17**	29.80	36.85	11.53***	8.39	14.20
factor 5	8	29.38***	26.98	32.88	11.60***	8.46	14.38
	24	22.61*	17.31	27.37	7.01***	5.10	8.56
	72	12.64***	11.45	14.12	2.13***	1.62	2.71
	72 + 264	-1.49*	-1.64	-1.35	-13.89***	-14.71	-12.65

Discussion

A germplasm collection of cultivated fresh market and processing tomatoes (*S. lycopersicum*), a series of accessions of wild-relative species, and a selected group of anthocyanin mutants with their corresponding background lines has been established and maintained at *UNIMORE*. The EL assays indicated that the majority of the accessions had EL_{50} at approximately 1 °C suggesting that this temperature is clearly a stress temperature for tomato and it is able to discriminate sensitive and tolerant tomato accessions (Figs. 1 and 2 Suppl.). Venema *et al.* (2005) found that high-altitude genotypes of wild tomato *S. habrochaites* and *S. chilense* exhibit a relatively high degree of chilling tolerance in comparison to *S. lycopersicum.* However, in the EL assays performed in our study, the wild tomato accessions turned out to be substantially much more sensitive to chilling than expected (Fig. 1). This discrepancy could probably be explained by the fact that the EL test in other studies was done at higher temperature but upon a longer stress application (Liu *et al.* 2012). Moreover, wild species were reported to be chilling tolerant due to the ability to recover rapidly after exposure to sub-optimal temperatures which is expressed through a greater growth capacity and a decreased inhibition of photosynthesis (Elizondo and Oyanedel 2010) and should be thus examined either by the degree of shoot wilting after an episode of root chilling (Truco *et al.* 2000, Bloom *et al.* 2004, Goodstal *et al.* 2005) or by the rate of seed germination (Wolf *et al.* 1986).

The collection of anthocyanin mutants was evaluated

for chilling tolerance as anthocyanins are an important group of polyphenolic pigments (Tanaka *et al.* 2008), and some studies have investigated increases in phenolic acids relative to low temperature stress (Prasad 1996, Rice-Evans *et al.* 1997, Pennycooke *et al.* 2005). Polyphenolic compounds are accumulated perhaps as results of an acclimation mechanism to get over cold stress (Pennycooke *et al.* 2005). Unlike temperate plants, tomato cannot cold acclimate (Jaglo *et al.* 2001), and this may explain why no clear differences were observed between the anthocyanin mutants and their corresponding wild types (Fig. 4 Suppl.).

Exploiting the genetic variability present in the *UNIMORE* tomato germplasm collection, we showed that the two tomato cultivars Albenga and San Marzano were widely contrasting in their phenotypic response to chilling, with the former being more tolerant than the latter. This was further confirmed by the scored survival at the end of an 11-d recovery period after chilling at 1 °C for 72 h. In San Marzano, the initiation of new leaves was completely suppressed, whereas the Albenga plants showed regrowth (data not shown). Both the onset of growth and the initiation of new leaves after chilling could be a consequence of the fact that the treatment had milder adverse effects on the photosynthetic apparatus in Albenga than in San Marzano, as shown in wild tomato species by Venema *et al.* (1999, 2005). The difference in chilling stress tolerance in these two cultivars might be triggered by their different origin, pedigree, and breeding history. The tolerant cv. Albenga is adapted to the Liguria region (44 ° N 8 ° E, North Italy) which is characterized by a cooler climate in comparison to the Campania region (40 ° N 14 ° E, South Italy).

Since relatively few studies have been reported to date on the molecular mechanisms underlying the response of tomato to chilling (Jaglo *et al.* 2001, Zhang *et al.* 2004, Liu *et al.* 2012), the two selected cultivars can thus be considered a useful plant material. The two main response pathways were monitored: the CBF/DREB1 regulon and the ROS-mediated signalling pathway. The present work represents the first insight into candidate gene expression profiling of chilling tolerance in cultivated tomato.

The CBF/DREB1 response pathway regulon has been widely studied in *Arabidopsis* which is able to cold acclimate and survive freezing, whereas tomato is a chilling-sensitive plant that does not cold acclimate. The difference in freezing tolerance between *Arabidopsis* and tomato reflects a specific adaptation and differences in selection for the genetic systems that impart cold tolerance. Tomato has a *CBF* locus comprising three *CBF* genes arranged in tandem on chromosome 3, therefore, cold-regulated *CBF-like* genes are not limited to plants that cold acclimate (Jaglo *et al.* 2001, Zhang *et al.* 2004). In this work, the expression analysis of the *CBF1* and *CBF3* genes was performed showing that these genes were rapidly accumulated in response to the low temperature in both the cultivars (Table 2). Our results are partially in disagreement with the work of Zhang *et al.* (2004), who found that only *LeCBF1* is induced in response to low temperature (4 °C) in tomato, but in agreement with Mboup *et al.* (2012) who found also *CBF3* to be up-regulated during chilling stress in wild tomato species. Moreover, in the present study, the expression of the *CBF1* and *CBF3* genes was maintained for several days which is quite uncommon for *CBF* genes, but this finding is in agreement with the results described in grapevine and in rice (Xiao *et al.* 2008, Mao and Chen 2012). Previous reports on transgenic tomato and on Versailles core collections of *Arabidopsis* and rice show that *CBF* genes expression was not always proportional to the degree of freezing tolerance (Zhang *et al.* 2004, McKhann *et al.* 2008, Mao and Chen 2012). However, in the present study, the induction was much lower in San Marzano adapted to warmer environments as already reported in *Arabidopsis* by Lin *et al.* (2008), who found that several mutated nucleotides in the promoters of *CBF3* and *CBF1* of ecotypes from lower latitude may be responsible for its consistently low expression. While tomato appears to have a functional CBF gene set, the composition of the tomato CBF cold response regulon is considerably different from that of *Arabidopsis* (Zhang *et al.* 2004). The analysis of the transcriptome of *Arabidopsis* plants overexpressing *CBF* genes revealed that the CBF/DREB1 regulon consists of about one hundred CBF/DREB1 responsive genes (Fowler and Thomashow 2002, Cook *et al.* 2004). On the contrary, Zhang *et al.* (2004) surveyed the expression of about 8 700 tomato genes in tomato transgenic plants overexpressing either *LeCBF1* or *AtCBF3*, and identified only four induced *COR* genes. Two genes, coding for dehydrin and DHN10, showed a similar response to chilling to that reported by Zhang *et al.* (2004). A gene encoding dehydrin was up-regulated in both the cultivars consistently with previous reports that considered it to be a marker for a cold stress response in tomato (Weiss and Egea-Cortines 2009, Liu *et al.* 2012). Genes that encode dehydrins (DHNs) are expressed during late stages of embryogenesis, as well as in vegetative tissues subjected to ABA application, drought, low temperature, and high salt conditions (for review see Close 1996, 1997), suggesting their role in the protection of cells from damage caused by stress-induced dehydration. The accumulation of hydrophilic proteins predicted to form an amphipathic α-helix is one of the best documented responses of plants to cold treatment (Eriksson *et al.* 2011). It has been proposed that dehydrins possess cryoprotective (Bravo *et al.* 2003) or antifreeze (Puhakainen *et al.* 2004) activities, but their precise mechanisms remain elusive. They could prevent membrane destabilization by acting as chaperones to inhibit the aggregation and/or inactivation of proteins under stress conditions (Kovacs *et al.* 2008). The results of the present study show that the functions of CBF regulon were conserved at least in part in low temperature-sensitive plants as already reported (Zhang *et al.* 2004, Mao and Chen 2012). In addition, the early induction of the *CBFs* was observed in both the cultivars

(Table 2) and thus showed no obvious relationship between their early expression and the degree of chilling tolerance. This can be explained by the fact that besides the CBF/DREB1 cold-responsive pathway, other pathways could be involved in this process.

Chilling-induced physiological imbalance leads to an elevated content of ROS in plant cells (Alam and Jacob 2002, Neill *et al.* 2002). It was established that H_2O_2 plays a very important role in mediating signal transduction in response to both biotic and abiotic stresses. Although many ROS-inducible genes were identified in *Arabidopsis* (Desikan *et al.* 2001), a direct involvement of a ROS-mediated regulatory module in the low temperature transcriptional networks was not analysed to the same level of detail as the CBF/DREB1. Cheng *et al.* (2007) identified the components of a putative ROS-mediated regulatory module by an integrative analysis of genomic data in rice, hypothesizing that this regulon is probably independent of the CBF/DREB1 regulon. ROS were found to regulate a key component of the early-response to chilling stress, the *ROS-bZIP1* (Cheng *et al.* 2007). A gene encoding the BZIP DNA-binding protein analysed in the present work was chosen due to high homology to *Arabidopsis* bZIP transcription factors belonging to the S-group which are associated with a stress response (*e.g.*, cold, drought, anaerobiosis, wounding) and sucrose signalling (Kusano *et al.* 1995, Jakoby *et al.* 2002). Recently, a tomato gene encoding BZIP DNA-binding protein, named *LebZIP1*, was described to up-regulate oxidative stress-related genes when expressed transiently in *Nicotiana benthamiana* (Seong *et al.* 2008). In our study, *LebZIP1* was rapidly induced at 2 h only in Albenga, and, moreover, its up-regulation was higher in Albenga than in San Marzano at all timings (Table 3). The S-group of the bZIP TFs bind to the as1/ocs-like elements (TGACGT, Jakoby *et al.* 2002) that were found in the promoter of rice *OsMyb4* (Cheng *et al.* 2007), an important transcription factor up-regulated in response to several abiotic stresses (Vannini *et al.* 2006). In the present study, a putative tomato orthologue of rice *OsMyb4* was differentially expressed in the tolerant and sensitive tomato cultivars at 24 h and its up-regulation was consistently higher in Albenga (Table 3). A gene coding for ferritin was chosen among the genes putatively involved in the ROS-mediated regulatory module as "effectors" (activated after 2 h) as reported by Cheng *et al.* (2007). It has been demonstrated that ferritin could reduce the accumulation of ROS in response to an oxidant challenge (Orino *et al.* 2001). Worth noting is that ferritin was up-regulated in both the cultivars, however, at the later timings, the up-regulation was higher (at 24 h) or exclusive (at 72 h) in the tolerant cv. Albenga (Table 3), underlining the putative importance of ROS scavenging in tolerance to chilling.

The C2H2-type (also called TFIIIA-type) zinc finger protein family is one of the largest families of transcription factors in plants involved in the defence and acclimation responses of plants to different environmental stress conditions and controlling the expression of many stress-activated genes (for review see Kiełbowicz-Matuk 2012). *SlCZFP1* was cloned and characterized in tomato, its expression was strongly induced by cold, salt, and drought, but not by exogenously applied ABA, and it was suggested that *SlCZFP1* and *LeCBF1* can act in separate parallel pathways (Zhang *et al.* 2011). In the present study, the transcripts of the C2H2-type zinc finger protein were more strongly induced in the tolerant genotype (4 to 5-fold higher) than in the sensitive one during all timings (Table 4). The overexpression of this gene in *Arabidopsis* and rice resulted in COR induction and an enhanced tolerance to freezing. It was suggested that *OsWCOR413-like* and *OsUSP1* genes can function in a SlCZFP1 mediated cold signalling pathway (Zhang *et al.* 2011). A *COR413* gene encodes a transmembrane protein and plays important roles in stabilizing the plasma membrane, in perceiving extracellular signals, and in regulating plant tolerance to abiotic stresses in different plant species (Breton *et al*. 2003, Okawa *et al.* 2008). In the present study, the *WCOR413-like* transcripts were induced in both the cultivars, but the accumulation was more rapid and higher already at 2 h in the tolerant genotype (Table 4). OsUSP1, an universal stress protein, is regulated by ethylene and may play a role in the adaptation of rice to submergence (Sauter *et al.* 2002). A tomato orthologue of *OsUSP1* was shown to be differentially expressed between the tolerant and sensitive tomato cultivars and its expression appeared to be positively correlated with chilling tolerance of Albenga (Table 4). These data suggest that the higher induction of genes coding for the C2H2-type zinc finger protein, WCOR413-like protein, and universal stress protein 1 observed in Albenga could be determinant for its chilling tolerance.

A novel member of the AP2/ERF transcription factor family, *SlERF5*, that may act as regulator linking diverse signalling networks involving ABA and ethylene to mediate abiotic stress responses is induced in both tomato leaves and stems after exposure to 4 °C (Pan *et al.* 2012). In our study, a gene coding for ethylene response factor 5 was rapidly induced in both the cultivars, but its up-regulation was consistently higher in Albenga than in San Marzano at all timings (Table 4).

Low temperature is an abiotic stress known to induce modifications in membrane lipid structure (Orlova *et al.* 2003, Yu *et al.* 2009). The ω-3 fatty acid desaturases are key enzymes for the formation of trienoic fatty acids, and catalyse the desaturation of dienoic fatty acids in membrane lipids (Somerville and Browse 1991). In tomato, the expression of *LeFAD7* and *LeFAD3* was reported to be inducible by a chilling stress (4 °C) (Liu *et al.* 2006, Yu *et al.* 2009). Transgenic tomato overexpressing *LeFAD7* showed alleviated membrane damage and photoinhibition of photosystems I and II under chilling at low irradiance, and had a higher chilling tolerance in comparison to WT (Liu *et al.* 2008). In our study, a gene coding for ω-3 fatty acid desaturase

(Q7X7I9) was slightly cold-induced only in Albenga (Table 2 Suppl.).

In conclusion, on the basis of the analysis of candidate genes we may say that different regulatory mechanisms might be involved in chilling tolerance in tomato. It could be hypothesized that the activation of TFs (*e.g.*, CBFs, bZIPs, and C2H2 zinc fingers) caused differential expressions of many transcripts among the tolerant and sensitive genotypes, such as genes encoding dehydrins and universal stress protein 1, and genes associated with physiological and metabolic processes, such as scavenging systems (ferritin). This differential expressions make Albenga more cold tolerant than San Marzano, probably by reducing cell membrane damage, regulating metabolism, and maintaining hormone and ROS homeostasis. To the best of our knowledge, the present work represents the first targeted insight into candidate gene expression profiling of chilling tolerance in tomato. The results not only provide potential candidate genes for improving cold tolerance of the cultivated tomato, but also give insights into the molecular mechanisms of cold tolerance in tomato and other important crops of tropical origin.

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