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Co-expression gene network (WGCNA) analysis of cold-tolerant *Solanum commersonii* reveals new insights in response to low temperatures

Salvatore Esposito[1†], Riccardo Aversano[2†], James Bradeen[3], Vincenzo D'Amelia[4], Clizia Villano[2],

Domenico Carputo[2*]

† These authors contributed equally to the manuscript

[1] CREA Research Centre for Cereal and Industrial Crops (CREA-CI), S.S. 673, km 25.200 - 71122 Foggia, Italy

[2] Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy

[3] Department of Plant Pathology and The Stakman-Borlaug Center for Sustainable Plant Health, University of Minnesota, St. Paul, MN, USA

[*] Corresponding author email: carputo@unina.it, tel. (0039) 081 2539225, fax (0039) 081 7753579

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ABSTRACT

Among abiotic stressors, cold is one of the most harmful for the cultivated potato, a frost-sensitive crop. RNAseq profiling of two different clones of *S. commersonii* contrasting in their capacity to withstand low temperatures revealed a higher number of differentially expressed genes (DEGs) under non-acclimated (NAC) conditions in tolerant clone cmm1T compared to the susceptible cmm6-6 (1,002 and 8,055 DEGs, respectively). By contrast, the number of DEGs was much more comparable when both genotypes were acclimated (AC). Indeed, a total of 5,650 and 8,936 DEGs were detected in the tolerant genotype compared to the susceptible. Gene Ontology classification under NAC conditions showed a significant role for transcription regulation, lignin catabolic genes and regulation of plant type secondary cell wall in the cold-tolerant genotypes, suggesting an important role in conferring tolerance response. By contrast, response to stress and response to stimuli were enriched GO categories in both clones under AC conditions. Unsigned weighted correlation networks analysis (WGCNA) allowed identification of co-expressed hub genes with possible main regulatory functions and/or major impacts on the phenotype. Among those identified, we clarified the role of *CBF4*. This gene showed contrasting expression profiles in the two clones under NAC conditions, being induced in cold-tolerant cmm1T but suppressed in susceptible cmm6-6. By contrast, under AC conditions, *CBF4* was up-regulated in both clones. Our study provides a global understanding of mechanisms involved following exposure to NAC and AC conditions in *S. commersonii*. The mechanisms described here will inform future investigations for detailed validation in studies regarding cold tolerance in plants.

Abbreviation: AC, acclimated conditions; DEG, differentially expressed genes; NAC, non-acclimated conditions; WGCNA, unsigned weighted correlation networks analysis.

INTRODUCTION

The cultivated potato, *Solanum tuberosum* L., suffers from several abiotic constraints that affect its growth and productivity (Minhas 2012; Obidiegwu et al. 2015). Among these, cold stress, including chilling (0 - 10 C) and freezing (< 0 C), is among the more harmful suboptimal

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conditions for the cultivated potato, a frost-sensitive crop. Enhanced tolerance to cold stress provides a promising avenue for reducing production losses and increasing the cultivation area, especially important in light of a rapidly growing world population. To date, little progress has been made toward the release of tolerant varieties. This is due to various reasons, including the polyploid genetics of the tetraploid (2n=4x=48) cultivated potato, the narrow genetic base for cold tolerance within the *S. tuberosum* gene pool, and the relatively poor comprehension of cold stress mechanisms that would provide efficient breeding targets. Fortunately, *S. tuberosum* is related to a large number of tuber-bearing *Solanum* species. Many of these wild relatives have genes controlling both abiotic and biotic stress tolerance; since these genes are often lacking in cultivated *S. tuberosum,* wild potatoes represent a reservoir of allelic variability to improve noteworthy traits. So far as it is known, *S. commersonii* is one of the most tolerant to low temperatures among wild potato species. It also possesses the best capacity to cold acclimate (i.e., increase its cold tolerance after exposure to low, non-freezing temperatures) (Palta and Simon, 1993). Stone et al. (1993) provided evidence that cold tolerance and cold acclimation are independent and under polygenic control. Importantly, the genome sequence of *S. commersonii* is available (Aversano et al. 2015), making this species an attractive model to study molecular dynamics and mechanisms underlying cold tolerance and acclimation capacity. Several reports have been published documenting that low-temperature tolerance is a complex process, involving changes in the expression of numerous cold-responsive (COR) genes, mainly regulated by the *CBF* cold response pathway (Chinnusamy et al. 2010; Esposito et al. 2019a). Following microarray analyses, Carvallo et al. (2011) reported distinct *S. commersonii* and *S. tuberosum CBF* regulons (genes regulated by *CBFs*). The authors found that the overexpression of *AtCBF3* activates 160 cold-related genes in *S. commersonii* and only 54 in *S. tuberosum*. This finding suggests that differences in cold regulatory programs may explain the distinctness of these two species freezing tolerance. However, the conclusions drawn by the authors are biased by nucleotide mismatch between *S. commersonii* transcripts and *S. tuberosum* EST probes (Carvallo et al. 2011). Further, microarray experiments are limited in the range of detection and present difficulties in comparing expression levels across different experiments. Next-generation sequencing (NGS) technologies represent an improvement, allowing sequencing of entire transcriptomes (RNAseq) as demonstrated for tomato (*S. lycopersicum*), wheat (*Triticum aestivum L.*), rice *(Oryza sativa*), and many other crop species (Da Maia et al. 2017; Iquebal et al. 2019; Thomas et al. 2019; De Palma et al. 2019). Despite the substantial power of RNAseq to reveal transcriptome perturbations, the approach still has some limitations, especially in identifying co-expressed hub genes that may have main regulatory functions and/or major

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impacts on phenotypes. This is largely due to the huge amount of data that RNAseq studies produce, presenting an analytical, organizational, and interpretive challenge. Weighted gene correlation network analysis (WGCNA) is emerging as an efficient and effective tool to elucidate biological networks and predict individual genes functional role. The approach has been used to study flower development in *Arabidopsis* (Xie et al. 2015), endosperm development in wheat (Pfeifer et al. 2014), soybean domestication (Lu et al. 2016) and blood transcriptome of ewes following hemp seed supplementation in their diet (Iannaccone et al. 2019). It also helped researchers to reveal stress-related genes in *O. sativa* (Sircar et al. 2015; Zhu et al. 2019) and *Medicago truncatula* (Burks et al. 2016). In the current study, we resorted to RNA-seq and WGCNA to analyze gene expression patterns of two clones of *S. commersonii* contrasting in their cold response, namely cmm1T and cmm6-6. Cmm1T displays a high freezing tolerance (LT50=-6.4 C), whereas cmm6-6 is cold-sensitive (LT50=-2.8 C) (Carputo et al. 2013) Conversely, both cmm1T and cmm6-6 exhibit a common ability to cold acclimate as, after two weeks of acclimatization at 4 C, their killing temperatures drop down to -8.9 and -6.7 C, respectively (Carputo et al. 2013). The findings described in this work contribute to a better understanding of the molecular and physiological mechanisms involved in cold response in *S. commersonii*. Our study provides a list of target genes that might be used in future biotechnological approaches for efficient exploitation of the wide genetic background of wild potatoes to improve the current breeding strategies of cultivated potato.

MATERIALS AND METHODS

Plant materials and determination of DNA diversity

In our experiment, we used two different genotypes of *S. commersonii*: cmm1T (a clone of PI243503) and cmm6-6 (a clone of PI590886). The former is freezing tolerant and able to cold acclimate. The latter is freezing susceptible but retains capacity to cold acclimate (Carputo et al. 2013). Both clones were provided as seed by the Inter-Regional Potato Introduction Project (IR-1), Sturgeon Bay, WI (USA). Plants of each clone were maintained *in vitro* on Murashige and Skoog (MS) medium (Sigma-Aldrich, http://www.sigmaaldrich.com) with 1% (w/v) sucrose and 0.8% (w/v) agar, at 24 C with irradiance of 200 μ mol m-2^{s-1}, under a 16/8 h (light/dark) photoperiod. To investigate the genetic diversity underlying the phenotypic differences

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between cmm1T and cmm6-6, a genotype by sequencing (GBS) approach was implemented. 1µg of genomic DNA of each clone extracted through TRIZOL was digested with *ApeKI* and incubated at 37C for 16-20 hours. Fragmented DNA was then purified with Agencourt AMPureXP beads (Beckman Coulter, Indiana, USA) and ligated to barcoded adapters. Samples were pooled, and the libraries were created using the TruSeq indexes kit (Illumina, San Diego, CA, USA). The resulting libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and Bioanalyzer DNA assay (Agilent Technologies, Santa Clara, CA). Libraries were finally processed with Illumina cBot for cluster generation on the flow cell, following the manufacturer's instructions and sequenced with V4 chemistry paired-end 50bp mode on the HiSeq2500 instrument (Illumina, San Diego, CA). Once produced, Illumina reads were mapped to the reference genome through BWA-MEM-0.7.10. Duplicated reads were removed using MarkDuplicate implemented in Picard (http://broadinstitute.github.io/picard) and a variant calling was run using Freebayes-0.9.18 with default parameters (Garrison et al. 2012).

Cold stress treatment

The cold stress experiment was performed in 2019 and described by Esposito et al. (2020). Briefly, four-weeks old *in vitro* plantlets were transplanted into 14-cm pots filled with sterile soil and grown for two weeks in a growth chamber under cool white fluorescent lamps (350–400 µmol $m^{-2} s^{-1}$) at 24C (16 dark/8 light long) prior submitting them to cold stress under either non-acclimated (NAC) or acclimated (AC) conditions. In particular, in NAC experiments, three plants of each clone were challenged for 30 min at -2C, while three control plants were kept at 24C. In AC experiments, six plants/clone were acclimated at 4C for two weeks. Then, three were transferred to -2C for 30min, while the others, used as controls, were kept at 4C, as also reported by Iovene et al. (2004). In both experiments, an environmentally controlled cold room was used. At the end of each stress, young leaf samples were collected from all replicates and control plants and individually stored at -80C.

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RNA extraction and sequencing

For each sample, total RNA was isolated from leaf tissues using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA), and its integrity was verified using a bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Three µg of total RNA of each sample was sent to UMN Genomic Center (University of Minnesota, USA) for library preparation. Twenty-four cDNA libraries (three biological replicates from leaves from control and stress conditions for both experiments) were subsequently sequenced using the Illumina HiSeq 2500 sequencing platform, providing 125bp paired-end reads and a total of 30M reads/samples. Sequence quality assessment was carried out using the FASTQC tool version 0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Trimming was performed using Trimmomatic-0.3330 (Bolger et al. 2014).

RNAseq data analysis and validation

All sequence data sets were loaded into the Artificial Intelligence RNAseq (AIR) online tool (Vara et al. 2019), and a new RNA-seq experiment was chosen. Gene expression levels were calculated using geometric normalization and the per-condition dispersion method by quantifying the Illumina reads according to the FPKM (fragments per kilobase per million mapped fragments). These values were then used to perform a principal component analysis (PCA) and to validate biological replicates (Supplemental Figure S1). Fold-changes (FC) were reported as the log (base 2) of normalized read count abundance for the cold-stressed samples divided by the read count abundance of the control samples. Gene ontology (GO) terms were examined with AgriGO (http://bioinfo.cau.edu.cn/agriGO/) with the following parameters: hypergeometric statistical test method, multi-test adjustment Hochberg FDR, significance level: <0.05, and 3 minimum number of mapping entries. Significant values were sorted by enrichment score (Query_item/Query_total)/(Background_item/Background_total) and GO redundancy was removed with the REVIGO tool (http://revigo.irb.hr). MAPMAN software was also used to understand further the biological role of differentially expressed genes (DEGs). Since a physical map for *S. commersonii* is not available yet, we used the Mercator online tool to

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associate *S. commersonii* proteins to MapMan bins. Then, the list of DEGs was mapped to MapMan bins for data visualization and pathway analysis. Finally, to validate the reliability of the expression profiles observed in the RNA-seq data, 10 genes were used for quantitative real-time PCR (qPCR) analyses using the iTaq SYBR Green supermix (Bio-Rad, Munich, Germany). Gene-specific primers, designed by Primer3, are listed in Table S1. The elongation factor gene *EF* was used as an internal control (Nicot et al. 2005). The same RNA samples employed subjected to RNA-seq were used as template for this validation. A 2µl aliquot of 1/10 cDNA, 0.3µM of each specific primer, and the FAST SYBR Green master mix (Applied Biosystems, Foster City, CA) comprised a final qRT-PCR volume of 20µl. The qRT-PCRs were performed using an ABI 7900HT Real-Time PCR System (Applied Biosystems), and the relative expression value was calculated through the ΔΔCt method (Livak and Schmittgen, 2001).

Unsigned weighted correlation networks analysis (WGCNA)

WGCNA was used to perform the hierarchical clustering and to identify co-expressed genes ("hub genes"), which may have main regulatory functions and/or significant impacts on genes conditioning phenotypes. In detail, four different adjacency matrices (cmm1T and cmm6-6, each under NAC and AC conditions) were created using all expression values (FPKM). The pickSoftThreshold () function was used to choose the proper soft-thresholding power (Langfelder and Horvath, 2008; 2011). In particular, for each analysis, the lowest power for which the scale-free topology fit index reaches 0.90 was used (Supplemental Figure S2). The weighted adjacency matrices were then transformed into a topological overlap matrix (TOM), which allows the calculation of dissimilarity values used to minimize spurious associations effects. The result was used as input for the linkage hierarchical clustering, and the modules (clusters of highly interconnected genes) were identified in the resulting dendrogram through a dynamic hybrid tree cutting algorithm (DynamicTreeCut algorithm). Finally, we estimated the relationships between each module and the temperature causing 50% lethal injury (LT50) in both clones by calculating the Pearson's correlation using the module eigengene values.

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RESULTS

Plant material genotyping

To sample the global genetic diversity of cmm1t and cmm6-6, we assayed the DNA sequence variation on a genome-wide scale via GBS. Overall, we identified 16,491 heterozygous variants (GT= 0/1) (SNPs and Indels) in cmm1T and 17,186 in cmm6-6. Most of the heterozygous variants (13,413) were in common, whereas 2,708 were private in cmm1T and 3,279 in cmm6-6 (Supplemental Figure S3A). By contrast, 6,841 in the former and 7,863 in the latter were homozygous for the altered allele (GT= 1/1). Among them, 3,891 were in common, 2,456 were private in cmm1T, and 3,602 were in cmm6-6 (Supplemental Figure S3B). 864 common variants presented a different genotype, being homozygous in cmm1T but heterozygous in cmm6-6 (494), and *vice-versa* (370). Most of the variants were mainly due to transition events, mainly C > T and G > A (Supplemental Figure S3C). Fifty-eight percent of the identified variants were localized in intergenic regions, followed by downstream (13.8%) and upstream (11.9%) gene regions (Supplemental Figure S3D). Using *S. tuberosum* as reference genome (*S. commersonii* chromosomes are not available yet), the average number of total variants in each chromosome was calculated. Variants were widely distributed on all chromosomes, from a minimum of 5,051 SNPs on Chr 00 to a maximum of 28,420 on Chr 1 (data not shown).

Major differences in transcriptome reprogramming occur in cmm1T and cmm6-6 under NAC conditions

RNAseq analysis produced more than one billion reads, with an average of 33M reads from each sample. A total of 411,579,508 in cmm1T and 414,682,788 in cmm6-6 cleaned reads were obtained in NAC conditions (Supplemental Table S2). Overall, 19,322 and 20,203 transcripts originated from cmm1T and cmm6-6, respectively (Figure 1). Among DEGs, 1,002 and 8,055 were identified in cmm1T and cmm6-6, including 797 (80%) and 4,537 (56%) up-regulated genes, and 205 (20%) and 3,517 (44%) down-regulated, respectively (Figure 2A; Supplemental Table S3). Out of all, 761 DEGs were common between the two clones, whereas 241 were unique to cmm1T, and 7,294 to cmm6-6 (Figure 2B). Enrichment analysis of all DEGs was performed to increase the likelihood of correctly assigning their roles to significant common pathways. Around 180 and 1,160 unique enriched GO terms were associated with significantly up-regulated or down-regulated genes in cmm1T and cmm6-6, respectively (Supplemental

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Table S4 and Supplemental Table S5). The "lipid biosynthetic metabolism" term applied to DEGs from cmm6-6 but not those from cmm1T, whereas "lignin catabolic and biosynthetic processes" and "regulation of plant type secondary cell wall biogenesis" terms were mostly enriched in the cold-tolerant cmm1T rather than in susceptible cmm6-6. In addition, in both cmm1T and cmm6-6, the "RNA regulation" bin included many DEGs. Among transcription factors belonging to the *ap2/ERF* family, we observed that *CBF3* and *CBF4* were up-regulated in cmm1T (FC = 4.2 and 2.7, respectively) but down-regulated in cmm6-6 (FC = -2.2 and -3.5). We looked for possible variants within the promoter region to explain the contrasting expression response of the two clones. The GBS data analysis did not provide evidence of polymorphisms within the *CBF3* and *CBF4* promoters (data not shown), although we cannot exclude that our approach did not cover these regions. To further confirm the RNAseq results, we performed qRT-PCR expression analyses on ten genes (*CBF1*, *CBF2*, *CBF3*, *CBF4*, *HOS1*, *ZAT12, SIZ1, CIPK17, CIPK23,* and *COR47*) chosen among the main regulators involved in the cold stress pathway (Table 1). The FC detected by qRT-PCR were consistent with the expression means inferred from sequencing reads analysis (Table 1A). For example, *CBF3*, *CBF4,* and *ZAT12* were induced in cmm1T but repressed in cmm6-6. By contrast, *HOS1*, known to mediate ubiquitination and subsequent proteasomal degradation of the activator *ICE1,* was down-regulated in cmm1T but induced in cmm6-6. *SIZ1* was repressed in both clones. No significant changes were found for *CBF1*, *CBF2*, *CIPK17, CIPK23* and *COR47.*

Modest differences in transcriptome reprogramming occur in cmm1T and cmm6-6 under AC conditions

Cold stress under AC resulted in 408,578,084 in cmm1T and 406,660,312 in cmm6-6 cleaned reads (Supplemental Table S2). A total of 18,471 and 16,947 transcripts were recovered for cmm1T and cmm6-6, respectively (Figure 1), of which 5,650 and 8,936 were DEGs (Figure 3A; Supplemental Table S6). As was true under NAC, cmm1T yielded a lower number of DEGs compared to cmm6-6 under AC. Specifically, 2,774 (49%) *vs.* 4,090 (46%) of up-regulated DEGs and 2,876 (51%) *vs.* 4,846 (54%) of down-regulated DEGs originated from cmm1T and cmm6-6, respectively (Figure 3A). About 3,000 DEGs were common between the two clones, whereas 2,642 were unique to cmm1T, and 5,928 to cmm6-6 (Figure 3B). One thousand nine and 1,298 unique GO terms were associated with the significantly up-regulated or down-regulated DEGs in cmm1T and cmm6-6, respectively (Supplemental Table S7 and Supplemental Table S8). Similar

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terms were enriched in each clone. For example, categories such as "cellular process", "biosynthetic process", "primary metabolic process", "response to stress" and "response to stimuli" and "transport" were enriched in both clones (data not shown). Under AC, some *ap2/ERF* transcription factors were enriched in both clones. In particular, *CBF3* and *CBF4* were up-regulated in both cmm1T and cmm6-6 (FC = 2.8 and 3.3, respectively), further underlying their potential role in cold acclimation processes. For both genes, the expression values obtained via qRT-PCR were comparable to RNAseq data (Table 1). *ZAT12* was induced in both clones, whereas *HOS1* and *SIZ1* were repressed. No significant changes were found for *CBF1*, *CBF2*, *CIPK17, CIPK23,* and *COR47.* Notably, in both cmm1T and cmm6-6, 38% of DEGs were assigned to an unknown function, suggesting that many of the genes involved in cold tolerance following acclimation have yet to be fully described. Genotype-specific modules such as secondary metabolism in cmm1T and response to abiotic stress in cmm6-6 were also identified.

WGCNA identifies potential candidate genes associated with cold tolerance under NAC and AC conditions

Under NAC, WGCNA analysis identified 26 different highly co-expressed clusters of genes (modules) in both clones (Figure 4A). The largest module ('turquoise' in both cmm1T and cmm6-6) consisted of 8,851 and 5,246 genes, whereas the smallest ('darkgrey' in both cmm1T and cmm6-6) contained 45 and 128 genes, respectively (Supplemental Table S9). The 'salmon' cluster ($r = 0.64$) in cmm1T and the 'purple' ($r = 0.72$) in cmm6-6 were correlated with the stress tolerance phenotype (Figure 4A). For both modules, we focused on the genes with the highest intramodular connectivity (hub genes, MM > 0.65), as these genes may represent points of biological interest in defining our phenotypes (Figure 4B). Eighty-three genes belonging to the 'salmon' module in cmm1T (MM > 0.65) were involved in "RNA regulation", "lignin biosynthesis", "lipid metabolism", "stress-responsive genes" and "protein transport" (Supplemental Table S10). Among those involved in RNA regulation, the *AP2/ERF* gene family was highly associated with cold tolerance in cmm1T (data not shown). Genes involved in the phenylpropanoid-lignin biosynthetic pathway (i.e., *HCT, C3H, F5H, LAC4* and *PRX72*) also clustered in the 'salmon' group and were all down-regulated in cmm1T. These data are in agreement with GO enrichment analysis, where "lignin catabolic and biosynthetic processes" and "plant type secondary cell wall biogenesis" terms were particularly enriched in the cold-tolerant cmm1T rather than in the cold-sensitive cmm6-6 (data not shown). Additionally, an acyl carrier protein *(ACP)* involved in fatty acid biosynthesis was also found in the same salmon

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module and was significantly induced. The cmm6-6 'purple' included 821 genes (MM > 0.65, Figure 4B) involved in "DNA repair", "DNA synthesis", "chromatin organization", "protein activation and degradation" (Supplemental Table S10). Although WGCNA analysis clarifies the role of some genes involved in cold stress tolerance, further studies are needed to investigate DEGs for which descriptions and GO terms are unknown.

Under AC, all genes were grouped in 27 and 30 modules in cmm1T and cmm6-6, respectively (Figure 5A). The largest module ('turquoise' in both cmm1T and cmm6-6) consisted of 4,525 and 6,748 genes, respectively, whereas the smallest ('orange' in cmm1T and 'skyblue' in cmm6-6) contained 37 and 74 genes, respectively. The module-trait relationships revealed that temperature causing 50% lethal injury (LT50) under AC condition was correlated with the 'royal blue' cluster in cmm1T (r = 0.89), the 'light cyan' cluster in cmm6-6 (r = 0.81), and the 'red' module in both clones (r = 0.72 in cmm1T and r = 0.77 in cmm6-6) (Figure 5A). The cmm1T specific module 'royal blue' contained 198 genes (Supplemental Table S9). Among them, a gene involved in secondary metabolisms (an acyl-transferase similar to *AT2G39980*) was identified. By contrast, the cmm6-6 specific module 'light cyan' had 299 genes (Supplemental Table S9), including a heat shock transcription factor (*HSFs*) similar to *AtHSFA4C* and a stress-responsive gene similar to *RESPONSIVE TO DEHYDRATION22* (*AtRD22*). Since cmm1T and cmm6-6 are both able to cold acclimate, we examined the shared 'red' module genes. The 'red' cluster comprises 1,320 genes in cmm1T and 1,216 in cmm6-6 (Figure 5B). Out of 53 common genes, 36 had known annotations. Most were involved in "RNA regulation", "protein synthesis and modifications" and "miscellaneous functions" (Supplemental Table S10). Notably, *CBF3* and *CBF4* (MM > 0.65) were located in this module and both were induced in cmm1T and cmm6-6. Two additional key transcription factors involved in the modulation of cold-related genes were identified in this co-expression network (MM > 0.65): a MYB-related (similar to *AT3G04030*, *MYR2*) and a WD-40 protein (similar to *AT5G67320, HOS15*) involved in histone deacetylation in response to abiotic stress.

DISCUSSION

In potato, exposure to cold represents an abiotic stress that can significantly impact crop growth, yield, and economics. While the cultivated genepool for potato lacks much diversity for cold tolerance, some wild potato species, most notably *S. commersonii,* harbor genes for both

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cold tolerance and cold acclimation. *S. commersonii* is, therefore, a species of interest to potato breeders and provides a tractable system to study the regulation of cold adaptation. Here we described for the first time the transcriptomic response of two *S. commersonii* clones contrasting in cold tolerance but both able to cold acclimate. We further analyze the molecular mechanisms involved in cold tolerance and cold acclimation.

Co-expression network analysis reveals major differences in transcriptome reprogramming of NAC-stressed cmm1T and cmm6-6

Our analysis indicated that most of cmm1T-specific genes in the 'salmon' module (correlated with the phenotypic trait) were involved in lignin synthesis and modifications, lipid metabolism, stress responsive genes and protein transport. For example, a laccase (similar to *AtLAC4*, *AT2G38080*), which is involved in lignin biosynthesis, was found suppressed in cmm1T. Gall et al. (2015) reported that reduction in lignin deposition in cell walls not only increases its permeability but also enhances its elasticity. Recently, we also showed that other *LACs* such as *LAC3, LAC12* and *LAC13* were repressed by microRNAs (miRNA408) under NAC, confirming that a fine-tuning expression of laccases is important to determine cold tolerance in cmm1T (Esposito et al. 2020a; Esposito et al. 2020b). These features may allow cell walls to withstand growing ice crystals, thus reducing damages. *AtPRX72,* which encodes a peroxidase, was reported by Herrero et al. (2013) to be implicated in lignin biosynthesis. Consistent with the possibility that lignin deposition is reduced under cold stress, our analyses discovered a differentially expressed peroxidase gene similar to *AtPRX72*, the suppression of which in cmm1T was significantly associated with the cold tolerance. By contrast, an aspartic protease gene (similar to *ASPG1-OE*), which encodes a protein associated with the endomembrane system, was induced in cmm1T. Yao et al. (2012) showed that water loss was dramatically reduced in *A. thaliana* mutant lines ectopically overexpressing the *ASPG1-OE*. Presumably, accumulation of aspartic protease results in reduced free water that might be transformed into ice, which in turn can rupture the plasmatic membrane. Also induced in cmm1T was the acyl carrier protein gene *(ACP),* which encodes an essential cofactor carrying acyl chains of different lengths. ACP participates in cycles of condensation, reduction and dehydration. Evidence from *in vitro* and *in vivo* studies indicated that ACP isoforms are specific for enzymes involved in fatty acid biosynthesis, suggesting that they act by changing the fatty acid composition of cell membranes and leaves (Huang et al. 2017). Tang and colleagues (2012) confirmed a link

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between *ACP* and cold tolerance in transgenic tobacco lines overexpressing the *ACP* gene. Notably, GO terms associated with these *ACP* processes were not found for the cold-sensitive clone cmm6-6. Also noteworthy are genes involved in RNA regulation. Two transcription factors, *CBF3* and *CBF4*, were differentially expressed and induced in cmm1T. These genes were also responsive in cmm6-6 but with contrasting kinetics, suggesting a different regulation of *CBF* genes in the two clones. Compared to *CBF3* and *CBF4*, *CBF1* and *CBF2* did not change their expression following non-acclimated conditions in both clones. This is in agreement with the theory that the induction of *CBF3* may repress the expression of *CBF2*. Our data are also consistent with Novillo et al. (2004), who reported that *CBF2* negatively regulates the expression of *CBF1* and *CBF3* in *Arabidopsis*. CBFs are key players in plant cold tolerance mechanisms. In *Arabidopsis*, three duplicated *CBFs* (*AtCBF1/DREB1b*, *AtCBF2/DREB1c* and *AtCBF3/DREB1a*) regulate the expression of more than 100 cold responsive (*COR)* genes that impart freezing tolerance (Maruyama et al. 2004; Vogel et al. 2005). In crop plants, *CBFs* are widely distributed and often duplicated (Stockinger et al. 2007; Knox et al. 2010; Aversano et al. 2015). In *S. commersonii,* in particular, comparative analyses indicated that the *CBFs* underwent rapid expansion via duplication processes, which led to the presence of four paralogs (*CBF1*, *CBF*2, *CBF*3, and *CBF*4) and two pseudo-genes (*ψCBF2* and *ψCBF3*) arranged in tandem (Aversano et al. 2015). Reports on the functional role of each CBFs in *S. commersonii* are still scanty. Pino et al. (2008) demonstrated that the over-expression of *AtCBF1* in transgenic lines of *S. commersonii* further increases the freezing tolerance in response to low temperature. Later, Carvallo et al. (2011) reported that in transgenic *S. commersonii* and *S. tuberosum* constitutively expressing *AtCBF3*, 160 cold-induced genes were up-regulated in the former species and only 54 in the latter. These data reveal a rapid evolution of the *CBF* pathways in the two plant species that may contribute to their freezing tolerance differences.

Besides CBFs, it is worthy to note that other genes involved in the cold signaling pathway were affected in our *S. commersonii* clones. For example, *HOS1*, known to be a negative regulator of the INDUCED CBF EXPRESSION 1 (*ICE1*) gene, was repressed in cmm1T but induced in cmm6-6. This probably explains why *CBF3* and *CBF4* were down-regulated in cmm6-6 compared with cmm1T. Taken together, our observations suggest that molecular mechanisms involved in the regulation of cold signaling pathway might explain the contrasting phenotypes of cmm1T and cmm6-6.

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RNAseq reveals genotype-specific and common genes associated with cold tolerance under acclimated conditions (AC)

Consistent with their common ability to cold acclimate, cmm1T and cmm6-6 showed similar behavior as indicated by number of DEGs, percentage of up and down-regulated genes and modules detection following acclimation. For example, the 'red' module correlated with the temperature causing 50% lethal injury (LT50) for both clones. In this module, both *CBF3* and *CBF4* (MM > 0.65) were identified and both were induced in cmm1T and cmm6-6; by contrast, *CBF1* and *CBF2* were not affected. A WD-40 protein (similar to *AT5G67320*, *HOS15*) involved in histone deacetylation was also found in the same module. It has been shown that loss of function *Arabidopsis* mutants of this latter gene were hypersensitive to freezing (Zhou et al. 2008). More recently, Park and colleagues (2018) reported a mechanism of gene de-repression through which *HOS15* promotes the degradation of histone deacetylase HD2C in a cold-dependent manner, inducing switches in chromatin structure and facilitating recruitment of *CBFs* to the *COR* gene promoters. Our results suggest that *HOS15* might be essential for similar activation of *CBF3* and *CBF4* in both cmm1T and cmm6-6 during cold stress under AC. Along with the activation of *HOS15,* the repression of *HOS1* in both clones is also intriguing. It is known that *HOS1* interacts with the cold activator *ICE1.* According to the proposed mechanism, under non-stressful temperatures *HOS1* ensures low levels of *ICE1*; as a consequence, the cold signaling pathway is not induced. By contrast, in response to cold, the mRNA level of *HOS1* rapidly and transiently decreases, alleviating the *ICE1* degradation: this allows the cold responses through *CBF3* (Lee et al. 2012; McGregor et al. 2015). We hypothesize that the down-regulation of *HOS1* may be important also for the subsequent activation of *CBF4.*

Differences in cold stress response between the two clones were also identified. Under AC, cmm1T modulates the expression of genes involved in secondary metabolism as revealed by annotation of DEGs and by genes belonging to the 'royal blue' module. Of particular note are up-regulated genes involved in anthocyanin production and accumulation, including an acyl-transferase gene (augustus_masked_scaffold6708_abinit_gene_0_10, similar to *AT2G39980*). This observation is consistent with previous observations that phenolic compounds, including flavonoids and especially anthocyanins, are commonly induced by low temperatures (Janska et al. 2010; Kovinich et al. 2014). Anthocyanins are fundamental for plant physiological processes, and their abundance in *S. commersonii* leaves has been associated with a greater tolerance to cold stress (D'Amelia et al. 2017,). Our data suggest anthocyanins have positive impacts on cold

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tolerance in cmm1T and may be acting directly as cell osmoregulators (Chalker-Scott, 2002) and/or indirectly by enhancing photosynthesis (Steyn et al. 2002). On the other hand, in response to cold stress under AC, cmm6-6 displayed enhanced expression of genes involved in abiotic stress response, RNA regulation and signaling. Among them, a member of heat shock transcription factor (*HSFs*) (similar to AT5G45710, *HSFA4C*) showed fivefold up-regulation relative to the control. *HSFs* are transcriptional regulators that mediate the activation of a large set of genes induced by high temperature or other stress conditions. They exist in multiple copies (e.g., 21 genes in *Arabidopsis,* 24 in tomato (Scharf et al.2012), 27 in poplar (Zhang et al. 2016), 40 in cotton (Wang et al. 2014), 27 in willow (Zhang et al. 2015), 25 in rice (Scharf et al. 2012), and 56 in wheat (Xue et al. 2014). Wang et al. (2014) revealed remarkable differences in transcriptional regulation of *HSFs*, concluding that HSF proteins have undergone considerable functional diversification. Among the families identified in *Arabidopsis*, the function of the HSFA4 group is not well known. It has been reported that HSFA4A of wheat and rice increases cadmium tolerance (Shim et al. 2009), whereas HSFA4 from tomato activates heat stress-induced genes and interacts with HSFA5*.* In our study, the induction of the *HSFA4C* gene under AC might contribute to the cold tolerance of cmm6-6. Similarly, a related *HSF* gene (similar to *AtRD22*) was induced in cmm6-6. *AtRD22* is induced in response to different stresses, including drought and exogenous ABA stress (Harshavardhan et al. 2014). *RD22* has been reported to be up-regulated by stress conditions in rice (Ding et al. 2009), rice (Wang et al. 2007) and maize (Gan et al. 2011), consistent with an important role in cold stress response in *S. commersonii*.

CONCLUSION

We report for the first time the global pattern of transcriptomic changes of two clones of *S. commersonii* contrasting in their cold tolerance via RNAseq. Under NAC, many genes were affected in the cold susceptible genotype vs. the cold tolerant genotype. By contrast, both clones showed a similar behavior when first cold acclimated. Using WGCNA to elucidate biological gene networks, we document a pivotal role for a *CBF* pathway under both AC and NAC, although the cold acclimation and cold tolerance traits are independently regulated. Particularly interesting was *CBF4*. This gene displayed opposing expression trends (increased *vs.* decreased expression levels in the tolerant cmm1T *vs.* sensitive cmm6-6 under NAC conditions, respectively). By contrast, under AC, *CBF4* was induced in both clones. Our results contribute to advanced

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understanding of the molecular basis of cold response under AC and NAC, facilitating more in-depth analyses of the molecular and physiological mechanisms behind cold tolerance in potato and contributing to future biotechnological applications.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Figure Legends

Figure 1. Total number of expressed transcripts under non-acclimated (NAC) and acclimated (AC) cold stress conditions in cmm1T and cmm6-6.

Figure 2. Gene response results following 30 min of cold treatment at -2C under non-acclimated conditions (NAC). (**A**) Number of total differentially expressed genes (DEGs) in cmm1T and cmm6-6. DEGs that were in common or uniquely expressed (**B**).

Figure 3. Gene response results following 30 min of cold treatment at -2C under acclimated conditions (AC). (**A**) Number of total differentially expressed genes (DEGs) in cmm1T and cmm6-6. (**B**) DEGs in common or uniquely expressed.

Figure 4. Composite visualization of co-expression analysis under NAC. Modules-trait association plots showing module eigengenes associated with the trait in cmm1T and cmm6-6 (**A**). Each row corresponds to different modules (labeled by color): green represents low adjacency (negative correlation); red represents high adjacency (positive correlation). Scatterplot gene significance for weight (GS) versus module membership (MM) for the 'salmon' module in cmm1T and the 'purple' module in cmm6-6 (**B**). Genes showing MM > 0.65 are reported on the right of the dashed line.

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Figure 5. Composite visualization of co-expression analysis under AC. Modules-trait association plots showing module eigengenes associated with the trait in cmm1T and cmm6-6 (**A**). Each row corresponds to different modules (labeled by color): green represents low adjacency (negative correlation); red represents high adjacency (positive correlation). Scatterplot of gene significance for weight (GS) versus module membership (MM) for the 'red' module in cmm1T and cmm6-6 (**B**). Genes showing MM > 0.65 are reported on the right of the dashed line.

Table 1. RNAseq results confirmed by quantitative qPCR in cmm1T and cmm6-6 under NAC (up) and AC (down) conditions.

Non-acclimated condition

	CMM1T			CMM6-6		
	RNAseq (log^2FC)	qPCR-RT (log^2FC)	FDR**	RNAseq (log^2FC)	qPCR-RT (log^2FC)	FDR
CBF1	ns*	ns	-	ns	ns	-
CBF2	ns	ns	-	ns	ns	-
CBF3	4.2	3.5	0.018	-2,23	-1	0.008
CBF4	2.7	2	0.047	-3,5	-2,7	9.9e-16
HOS1	-1,2	-1	0.039	1,8	0,8	0.006
SIZ1	-2,5	-1	0.036	-4	-3	0.022
ZAT12	2	1.7	0.040	-2,8	-2	0.0016
CIPK17	ns	ns	-	ns	ns	-
CIPK23	ns	ns	-	ns	ns	-
COR47	ns	ns	-	ns	ns	-

* not significant. ** False Discovery Rate

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Acclimated condition

	CMM1T			CMM6-6		
	RNAseq (log^{2}FC)	qPCR-RT (log^{2}FC)	FDR[**]	RNAseq (log^{2}FC)	qPCR-RT (log^{2}FC)	FDR
CBF1	ns	ns	-	ns	ns	-
CBF2	ns	ns	-	ns	ns	-
CBF3	2.8	2.4	0,018	3,3	2,5	0,019
CBF4	2.1	1.9	0,019	2,3	1,6	1,1e-16
HOS1	-2,9	-1,8	0,046	-2,2	-1,4	0,047
SIZ1	-0,8	-0,4	0,041	-1	-0,5	0,037
ZAT12	2,8	1,9	0,035	3	2,2	2,1e-14
CIPK17	ns	ns	-	ns	ns	-
CIPK23	ns	ns	-	ns	ns	-
COR47	ns	ns	-	ns	ns	-

* not significant. ** False Discovery Rate

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