

RESEARCH PAPER

# A comparison of the low temperature transcriptomes and CBF regulons of three plant species that differ in freezing tolerance: *Solanum commersonii*, *Solanum tuberosum*, and *Arabidopsis thaliana*

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## Abstract

*Solanum commersonii* and *Solanum tuberosum* are closely related plant species that differ in their abilities to cold acclimate; whereas *S. commersonii* increases in freezing tolerance in response to low temperature, *S. tuberosum* does not. In *Arabidopsis thaliana*, cold-regulated genes have been shown to contribute to freezing tolerance, including those that comprise the CBF regulon, genes that are controlled by the CBF transcription factors. The low temperature transcriptomes and CBF regulons of *S. commersonii* and *S. tuberosum* were therefore compared to determine whether there might be differences that contribute to their differences in ability to cold acclimate. The results indicated that both plants alter gene expression in response to low temperature to similar degrees with similar kinetics and that both plants have CBF regulons composed of hundreds of genes. However, there were considerable differences in the sets of genes that comprised the low temperature transcriptomes and CBF regulons of the two species. Thus differences in cold regulatory programmes may contribute to the differences in freezing tolerance of these two species. However, 53 groups of putative orthologous genes that are cold-regulated in *S. commersonii*, *S. tuberosum*, and *A. thaliana* were identified. Given that the evolutionary distance between the two *Solanum* species and *A. thaliana* is 112–156 million years, it seems likely that these conserved cold-regulated genes—many of which encode transcription factors and proteins of unknown function—have fundamental roles in plant growth and development at low temperature.

**Key words:** *Arabidopsis*, CBF regulon, freezing tolerance, low temperature transcriptome, *Solanum* species.

## Introduction

Plants differ greatly in their abilities to cope with freezing temperatures (Levitt, 1980). Those plants that have adapted to temperate environments generally have considerable freezing tolerance and can cold acclimate; that is, they increase in freezing tolerance in response to low non-freezing temperatures. In contrast, plants that have adapted

to tropical and subtropical climates generally have little, if any, freezing tolerance and do not cold acclimate. Understanding the molecular basis for why some plants can cold acclimate and survive freezing temperatures whereas others cannot is a fundamental goal of cold acclimation research.

In recent years, considerable effort has been directed at determining the role of cold-regulated genes in freezing tolerance. In *Arabidopsis thaliana*, it has been established that a cascade of changes in gene expression, involving alterations in the transcript levels for hundreds of genes, is initiated within minutes of exposing plants to low temperature (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Vogel *et al.*, 2005). Moreover, it has been established that some of these cold-regulated genes contribute to freezing tolerance. The best understood freezing tolerance pathway is the CBF cold response pathway (Van Buskirk and Thomashow, 2006; Chinnusamy *et al.*, 2007). Among the first wave of cold-induced genes are three—*CBF1*, *CBF2*, and *CBF3* (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Medina *et al.*, 1999), also known as *DREB1b*, *DREB1c*, and *DREB1a*, respectively (Shinwari *et al.*, 1998)—that encode members of the AP2/ERF family of DNA-binding proteins (Riechmann and Meyerowitz, 1998). The CBF transcription factors bind to the CRT/DRE DNA regulatory element present in the promoters of cold-regulated genes and induce their expression. Constitutive overexpression of either *CBF1*, *CBF2*, or *CBF3* in *A. thaliana* results in expression of the CBF regulon and brings about an increase in freezing tolerance without a cold stimulus, indicating that the CBF regulon has a fundamental role in cold acclimation (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2004; Kasuga *et al.*, 2004).

Changes in gene expression have been demonstrated to occur in response to low temperature in a wide range of plant species, including both freezing-tolerant and freezing-sensitive plants. Moreover, CBF cold response pathways have been demonstrated to exist in plants that cold acclimate, such as *Brassica napus* (Jaglo *et al.*, 2001), poplar (Benedict *et al.*, 2006), and barley (Choi *et al.*, 2002; Xue, 2002), as well as in plants that do not cold acclimate, such as tomato (Hsieh *et al.*, 2002; Zhang *et al.*, 2004) and rice (Dubouzet *et al.*, 2003). In each of these particular plant species there is at least one *CBF* gene that is induced in response to low temperature, and constitutive overexpression of *CBF* genes brings about changes in gene expression without cold treatment. However, whereas CBF overexpression increases the freezing tolerance of *A. thaliana* (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998), *B. napus* (Jaglo *et al.*, 2001), poplar (Benedict *et al.*, 2006), and potato (Pino *et al.*, 2007), it does not result in increased freezing tolerance in tomato (Hsieh *et al.*, 2002; Zhang *et al.*, 2004) and rice (Dubouzet *et al.*, 2003). The reason for this difference is not clear, but it may involve differences in the composition of the CBF regulons. For instance, in *A. thaliana* and poplar, the CBF regulons include ~85 (Vogel *et al.*, 2005) and 63 (Benedict *et al.*, 2006) cold-induced genes, respectively, whereas the CBF regulons of tomato (Zhang *et al.*, 2004) and rice (Dubouzet *et al.*, 2003) comprise only ~10 cold-induced genes.

Given the demonstrated role of cold-regulated genes in plant cold acclimation, it is reasonable to think that differences in gene expression are likely to contribute to differences in the ability of plants to cold acclimate. Such

thinking motivates comparative transcriptome studies using plants that differ in freezing tolerance to identify genes with critical roles in freezing tolerance. However, drawing conclusions from such studies requires consideration of the evolutionary distances between the plants being examined and their respective responses to low temperature. For instance, the divergence time between species with cold transcriptome data ranges from ~20 million years ago (MYA; *A. thaliana*–*Brassica*) to ~200 MYA (monocot–dicot) (Yang *et al.*, 1999). In addition, tomato and rice are not only freezing-sensitive, but are also chilling-sensitive. This adds another factor that could affect gene expression and complicate interpretations of comparative studies.

With these thoughts in mind, a comparative transcriptome study was conducted using the common cultivated potato, *Solanum tuberosum*, and the wild potato *Solanum commersonii*. These plants are closely related evolutionarily. Assuming the neutral substitution rate to be  $\sim 7 \times 10^{-9}$  (Moniz de Sa and Drouin, 1996), the divergence time between these two species is likely to be only ~3 million years as the synonymous substitution rate between these two species is ~0.04 (see Materials and methods). Both plant species are chilling tolerant, but whereas *S. commersonii* can cold acclimate, *S. tuberosum* cannot. When *S. commersonii* and *S. tuberosum* plants are grown at warm temperature, their freeze-killing temperatures are about  $-4.5$  °C and  $-3$  °C, respectively (Chen and Li, 1980), but after 2 weeks of low temperature treatment (2 °C), the killing temperature of *S. commersonii* decreases to about  $-11.5$  °C while that of *S. tuberosum* remains at about  $-3$  °C (Chen and Li, 1980). The results presented here indicate that this difference in freezing tolerance is not due to ‘macro-scale’ differences in gene regulation in response to low temperature or the size of their CBF regulons, but reveal rapid evolution of the CBF pathways in the two plant species that may contribute to their differences in freezing tolerance. The analyses also led to the identification of 40 groups of putative orthologous genes (pOGs) that are cold-induced and 13 pOGs that are cold-repressed in *A. thaliana*, *S. commersonii*, and *S. tuberosum*. Given the relatively large evolutionary distance between these three plant species—they diverged 112–156 MYA (Yang *et al.*, 1999; Bell *et al.*, 2005)—it seems likely that these genes, many of which encode proteins of unknown function, have fundamental roles in plant low temperature biology.

## Materials and methods

### *Plant material, growth conditions, and RNA extraction*

Transgenic lines of *S. commersonii* and *S. tuberosum* cv. Umatilla that constitutively expressed the *A. thaliana* *CBF3* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter were generated as described by Pino *et al.* (2007). Wild-type and transgenic plants were grown in an Enconair growth chamber (‘Bigfoot’ GC-20, Enconair Ecological Chambers Inc., Winnipeg, Manitoba, Canada) maintained under a 16 h photoperiod,  $350 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity at 25 °C. Three biological replicates for each wild-type species were grown for 3 weeks under these conditions (each biological replicate consisted of three plants). Eight hours after dawn, wild-type plants were transferred to an environmentally controlled cold room maintained at 2 °C

under a 16 h photoperiod with  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, and leaf tissue was harvested after 2, 8, 24, and 168 h. Warm controls were maintained at 25 °C under a normal growth photoperiod and tissue was harvested at 2, 8, and 24 h in the light for use as reference control samples. In the case of the 168 h cold samples, the 24 h warm control was used as the reference because the 168 h warm plants were already flowering. The leaf tissue of transgenic lines was collected 8 h after dawn.

For the mechanical agitation treatment, 3-week-old plants of both species grown under continuous light in magenta vessels were secured together in a cardboard box and dropped ~15 cm every 2 s for 15 min. Samples were collected at 0, 15, and 30 min after mechanical treatment. Two replicates of two plants from each species were randomly selected from each vessel and immediately frozen in liquid nitrogen. A control without agitation was included.

Total RNA was isolated from leaf tissue using RNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA). For quantitative real-time PCR (qRT-PCR) experiments, samples were treated with RNase-free DNase (Qiagen) using the on-column DNase digestion method provided by the manufacturer.

#### qRT-PCR

An amount of RNA that fell within the linear range for all genes tested (generally 100–250 ng) was reverse transcribed using a reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The 20  $\mu\text{l}$  final reaction was diluted to 200  $\mu\text{l}$ . A 1  $\mu\text{l}$  aliquot of each cDNA was used in a qRT-PCR, with the addition of 0.4  $\mu\text{M}$  of each primer and Fast SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) to make a final reaction volume of 10  $\mu\text{l}$ . The qRT-PCRs were performed using a FAST 7500 Real Time PCR System (Applied Biosystems). The primers used are listed in Supplementary Table S1 available at *JXB* online. Relative expression was calculated using the potato 60S gene [expressed sequence tag (EST) clone STMCK67] as reference. This gene was confirmed not to change in levels under the test conditions used. Standard curves for each gene were included in each qRT-PCR run as a measure of efficiencies. Data were analysed by analysis of variance (ANOVA) using SAS program 9.1 (SAS Institute Inc., Cary, NC, USA) with mixed procedures; when appropriate, least significant difference was used for multiple comparisons.

#### RNA labelling and hybridization of potato microarrays

cDNA microarray experiments were conducted using the 10K potato cDNA microarray (TIGR, [http://www.jcvi.org/potato/sol\\_ma\\_microarrays.shtml](http://www.jcvi.org/potato/sol_ma_microarrays.shtml)). RNA (20  $\mu\text{g}$ ) was labelled by the indirect labelling aminoallyl method. The slides were hybridized using the indirectly labelled aminoallyl probes hybridization method ([http://www.jcvi.org/potato/sol\\_ma\\_protocols.shtml](http://www.jcvi.org/potato/sol_ma_protocols.shtml)). To avoid bias due to dye-related differences, labelling dyes for each sample pair (cold/warm or transgenic line/wild type) were swapped in one of the three independent hybridizations (three biological replicates for cold treatments and two or three transgenic lines for the CBF regulon experiment).

#### Data processing and analysis

The TIFF images were quantified using Genepix 3.0 (Axon Instruments, Union City, CA, USA). The software automatically flags spots that cannot be found in one of the channels. Spots with aberrant shapes were checked manually and flagged as bad. Spots with lower signal intensity than the background (spots with  $\geq 55\%$  of the pixels with lower signal intensities than background) were also flagged as bad. All these 'bad' flagged clones were excluded from further analysis.

The data were normalized using the print tip loess method in the LIMMA package (Smyth, 2004). cDNA clones were regarded

as differentially expressed if their modulated *P*-values from LIMMA were  $< 0.05$ . The modulated *P*-values were derived from false discovery rate (FDR)-corrected raw *P*-values (Storey and Tibshirani, 2003). An average fold-change value was calculated for the two duplicates of each clone on the array. In cases where one duplicate of a clone did not pass the threshold *P*-value and/or was flagged as low quality, the value of the other duplicate was used if it had a qualifying *P*-value and was not flagged as low quality.

Hierarchical clustering was generated with Cluster (Eisen *et al.*, 1998) using normalized log ratios. Gene Ontology (GO) designations for *S. tuberosum* EST clones were obtained from [http://jcvi.org/potato/sol\\_ma\\_microarrays.shtml](http://jcvi.org/potato/sol_ma_microarrays.shtml).

#### *Arabidopsis thaliana* cold-regulated and CBF regulon genes

A list of cold-regulated *A. thaliana* genes was generated from a large number of previously published microarray experiments (Vogel *et al.*, 2005; Kilian *et al.*, 2007) and new experiments (unpublished arrays have been submitted to Array Express) that used the Affymetrix ATH1 gene chip to monitor transcript levels (Supplementary Table S2 at *JXB* online). The cel files from previous experiments (submission numbers ME00320 and ME00325) were downloaded from TAIR (<http://www.arabidopsis.org>), imported into R, and analysed using Bioconductor Affy and SimpleAffy packages (Gautier *et al.*, 2004; Wilson and Miller, 2005). Eighty Affymetrix cel files that passed quality control analysis were used to identify cold-regulated transcripts in the Columbia ecotype (Gentleman *et al.*, 2005). Arrays were RMA (robust multichip average) normalized and the expression values for 15 220 probes that were present in at least half of the arrays by MAS5.0 present calls were retained for further analysis (Irizarry *et al.*, 2003). Probes were analysed for differential expression between low temperature treatment and controls using LIMMA (Smyth, 2004). Contrasts between cold-treated Columbia samples and controls were performed for early cold response (0.5, 1, 2, and 3 h), 24 h cold response, and 168 h cold response. Differentially expressed probes were identified using LIMMA's decide tests function to select probe sets with a  $\geq 2$ -fold difference between treatment and control with a significance after FDR adjustment of  $< 0.05$ . Probe sets were mapped to *A. thaliana* transcripts using the ath1121501.db Bioconductor package (<http://www.bioconductor.org/packages/2.5/data/annotation/html/ath1121501.db.html>). Transcripts were identified as differentially expressed in each category: early cold response, 24 h cold response, and 7 d cold response. This cold-regulated gene list of 1151 cold-induced and 1095 cold-repressed transcripts was used for subsequent analysis (Supplementary Table S3 at *JXB* online).

To identify CBF-regulated transcripts, contrasts between arrays of CBF2-overexpressing seedlings and wild-type seedlings that were grown at warm temperature were made (Vogel *et al.*, 2005). LIMMA's decide test was performed to identify differentially expressed transcripts with a fold-change  $> 2$ , and a BH (Benjamini and Hochberg)-adjusted *P*-value of  $< 0.05$ . Probe sets were mapped to *A. thaliana* transcripts using the ath1121501.db Bioconductor package (<http://www.bioconductor.org/packages/2.5/data/annotation/html/ath1121501.db.html>). Transcripts appearing in both the cold-regulated gene list and the CBF-regulated gene list were designated members of the *A. thaliana* CBF regulon.

#### Inference of putative orthologous groups (pOGs)

A list of pOGs between the putative unique transcripts (PUTs) sequences assembled from ESTs of *S. tuberosum* (PlantGDB, <http://www.plantgdb.org/>, version 157a) and *A. thaliana* protein sequences was obtained from TAIR.

pOGs have been previously established using protein sequences from four plant species with complete genomes (*A. thaliana*, TAIR6; *Populus trichocarpa*, v1.1; *Oryza sativa japonica*, version 2;

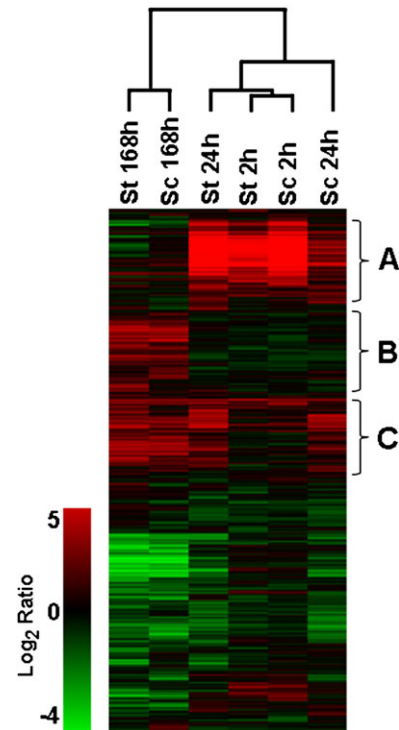
and *Physcomitrella patens*, version 1.1) (Hanada et al., 2008). From all these four genomes, a best matching protein for PUTs was identified with BLAST (Altschul et al., 1997) using only matches with E-values  $<10^{-5}$ . A PUT was assumed to be in the pOG of its best matching protein from these four species only if the evolutionary distance between PUTs and the rice member(s) and PUTs and the *A. thaliana* member(s) in the pOG is less than the distance between the rice and the *A. thaliana* members. The evolutionary distances of all sequences were calculated using the protdist program in the Phylogeny Inference Package (PHYLIP) with Gamma correction (Felsenstein, 2005).

## Results

### Identification of cold-regulated genes in *S. commersonii* and *S. tuberosum*

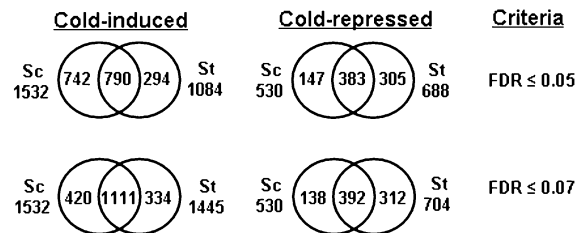
The low temperature transcriptomes of *S. commersonii* and *S. tuberosum* cv. Umatilla were compared using the TIGR potato cDNA array (10K, version 4) which has ESTs for ~10,000 genes. Plants were grown for 3 weeks at 25 °C, transferred to 2 °C for 2, 24, and 168 h, and the RNA was isolated and analysed (there were three biological replicates for each time point). As a first assessment of the similarities and differences between the low temperature transcriptomes of *S. commersonii* and *S. tuberosum*, a heat map presenting the transcript levels for all probes at 2, 24, and 168 h without regard for fold-change or statistical significance was prepared (Fig. 1). The results indicated that the transcript levels for the vast majority of the genes represented on the array either increased or decreased at one or more time points during the experiment in both *S. commersonii* and *S. tuberosum*. Moreover, the expression patterns for both species were similar. For instance, the group of genes designated as cluster A were induced early in both species—at 2 h and 24 h—and returned to non-induced levels by 168 h; cluster B genes were unaffected by low temperature during the first 24 h of treatment, but were highly induced at 168 h; and cluster C genes were little affected after 2 h of cold treatment, but were induced at 24 h and 168 h. Nevertheless, the patterns were far from identical, with both qualitative and quantitative differences clearly evident.

Next two filtering criteria were applied to the data: a 2-fold change in transcript level and an FDR of  $\leq 0.05$ . Using these criteria, ~15–20% of the ESTs were considered cold-regulated in each species. In *S. commersonii* and *S. tuberosum*, respectively, ~13% and ~10% of the ESTs were cold-induced and 5% and 6% were cold repressed at one or more of the time points tested. Transcripts for 1532 and 1084 ESTs increased in *S. commersonii* and *S. tuberosum*, respectively, of which 790 increased in both plant species (Fig. 2A); transcripts for 530 and 688 ESTs decreased in *S. commersonii* and *S. tuberosum*, respectively, of which 383 decreased in both plant species (Fig. 2A). However, the apparent differences in gene expression for the cold-induced genes were very sensitive to the filtering criteria used. For instance, if the fold-change criterion was kept at 2, but the statistical significance was slightly relaxed to FDR  $\leq 0.07$ , then 1531 and 1445 ESTs increased in levels in *S. commersonii* and

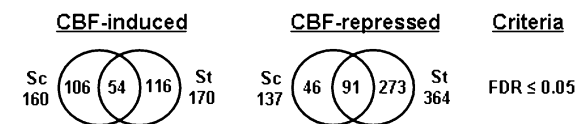


**Fig. 1.** Hierarchical clustering of *S. commersonii* (Sc) and *S. tuberosum* (St) transcripts at 2, 24, and 168 h of cold treatment at 2 °C. Data are shown as the average log ratio from three biological replicates. The figure shows all spots on the array (including bad flagged spots) prior to statistical selection.

### A Cold-regulated ESTs



### B CBF-regulated ESTs



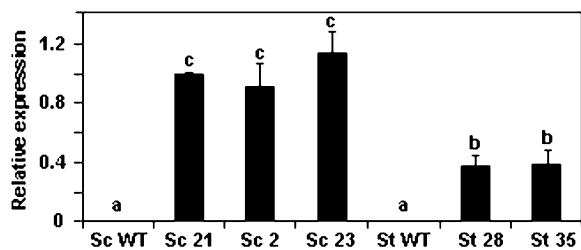
**Fig. 2.** Cold- and CBF-regulated ESTs in both *S. commersonii* (Sc) and *S. tuberosum* (St). (A) The number of total ESTs that were either cold-induced or cold-repressed in Sc or St using 2-fold change and either FDR  $\leq 0.05$  or FDR  $\leq 0.07$ . The results from 2, 24, and 168 h of cold treatment at 2 °C were combined. A given EST was counted only once regardless of the number of time points at which it was determined to be cold-regulated. (B) CBF regulons in Sc and St. CBF-regulated genes were selected as being differentially expressed in 35S::AtCBF3 transgenic lines compared with non-transformed plants with a 2-fold change cut-off and FDR  $\leq 0.05$ .

*S. tuberosum*, respectively, with an overlap of 1111 transcripts (Fig. 2A). Thus, by making a small change in the statistical significance level, the overlap in cold-induced genes increased from 52% to 73%.

In sum, the expression profiling experiments indicated that in *S. commersonii* and *S. tuberosum* plants, gene expression was altered in response to low temperature to similar degrees with similar kinetics and that there was considerable overlap in the genes that were cold-regulated in both plant species. The responses, however, were clearly not identical, indicating divergence in the cold regulatory programmes of the two closely related species.

#### Identification of CBF regulon genes in *S. commersonii* and *S. tuberosum*

Genes that comprise the CBF regulons of *S. commersonii* and *S. tuberosum* were defined as those that were determined to be cold-regulated in the experiments described above and correspondingly induced or repressed in response to constitutive overexpression of the *A. thaliana* *CBF3* gene (*AtCBF3*) in transgenic plants grown at warm temperature. CBF-regulated genes were identified by transforming plants with the *A. thaliana* *CBF3* gene placed under control of the constitutive CaMV 35S promoter (Fig. 3) and using the potato microarrays to identify the genes that were differentially expressed in the transformed plants using the lines shown in Fig. 3. Using a 2-fold change and FDR  $\leq 0.05$  as cut-off criteria, it was determined that of the 1532 cold-induced ESTs of *S. commersonii* and 1048 cold-induced ESTs of *S. tuberosum*, 160 (10%) and 170 (16%), respectively, were members of the CBF regulon (Fig. 2B). Of these, 54 ESTs were members of the CBF regulon in both *S. commersonii* and *S. tuberosum*. With regard to the 530 cold-repressed ESTs of *S. commersonii* and 688 cold-repressed ESTs of *S. tuberosum*, 137 (26%) and 364 (51%), respectively, were CBF regulon members (Fig. 2B). Of these, 91 were members of the CBF regulons of both *S. commersonii* and *S. tuberosum*



**Fig. 3.** *AtCBF3* transcript levels in *S. tuberosum* (St) and *S. commersonii* (Sc) transformed with 35S::*AtCBF3*. *AtCBF3* transcript levels were determined by qRT-PCR in three Sc and two St 35S::*AtCBF3* transgenic lines grown at 25 °C. qRT-PCR was performed using 100 ng of total RNA per sample. Relative expression was calculated using the potato 60S gene (clone STMCK67) as an internal reference. Relative expression of Sc 21 was set to 1 and the other lines were adjusted accordingly. The letters a, b, and c indicate statistically significant differences (ANOVA,  $P < 0.05$ ,  $n=2$ ). Error bars indicate the standard deviation. WT, non-transformed plants.

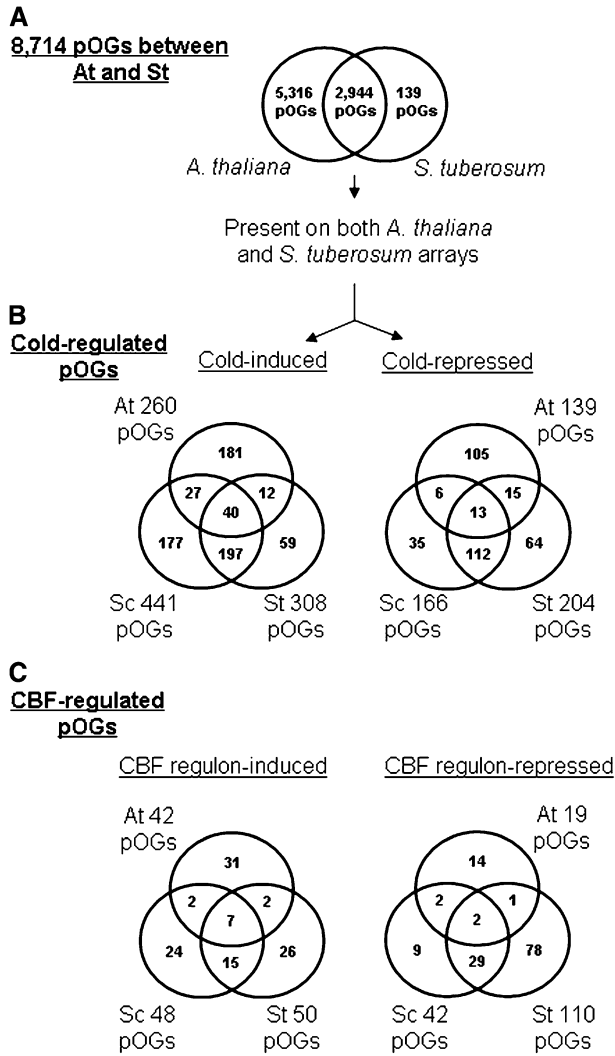
(Fig. 2B). These results indicate that many of the CBF-regulated genes in *S. commersonii* are also members of the CBF regulon of *S. tuberosum*, but, additionally, that there is considerable divergence in the CBF regulatory programmes between the two species.

#### Identification of cold-regulated genes conserved in both *Solanum* species and *A. thaliana*

The microarray results identified 790 ESTs that were cold-induced in both *S. commersonii* and *S. tuberosum*, and another 383 ESTs that were cold-repressed in both potato species (Fig. 2A). A question raised was whether *A. thaliana* had orthologues of these potato ESTs and, if so, whether they too were cold-regulated. Given the relatively large evolutionary distance between *A. thaliana* and *Solanum* species [they diverged 112–156 MYA (Yang *et al.*, 1999; Bell *et al.*, 2005)], orthologous genes that are commonly responsive to cold conditions are likely to be conserved components of a fundamental cold response programme in dicots. To address this issue, pOGs between *S. tuberosum* ESTs and *A. thaliana* genes were first defined (see Materials and methods). A total of 8714 pOGs (Fig. 4A; Supplementary Table S4 at *JXB* online) were identified including 18 420 *S. tuberosum* ESTs (26% of the total) and 14 647 *A. thaliana* protein-coding genes (55% of the total). The low number of *S. tuberosum* ESTs assigned to pOGs is probably due to the fact that the pOGs were constructed using protein-encoding genes while the *S. tuberosum* ESTs not only include protein-encoding regions of mRNAs, but also probably included untranslated regions of mRNAs and transcripts from RNA genes. In addition, some of the ESTs may represent lineage-specific genes (Yang *et al.*, 2009; Lin *et al.*, 2010).

Of the 8714 pOGs between *S. tuberosum* and *A. thaliana*, 2944 (34%) (Fig. 4A) had at least one *S. tuberosum* EST present on the potato array and at least one *A. thaliana* gene represented on the ATH1 Affymetrix gene chip. These 2944 pOGs could thus be tested for conserved cold-regulated genes, though the estimate of conservation would be a minimal value as not all members of a given pOG were represented on both arrays. Analysis of the potato gene expression data indicated that 441 and 308 pOGs included at least one cold-induced gene from *S. commersonii* and *S. tuberosum*, respectively, and that, of these, 237 pOGs had at least one cold-induced gene from both species (Fig. 4B). Similarly, 166 and 204 pOGs included at least one cold-repressed gene in *S. commersonii* and *S. tuberosum*, respectively, with 125 pOGs having at least one cold-repressed gene in both species (Fig. 4B).

To determine whether *A. thaliana* had cold-regulated genes that belonged to these same pOGs, it was first necessary to develop a list of *A. thaliana* cold-regulated genes. This was accomplished by analysing the microarray data from previously published and new experiments comparing warm- and cold-treated plants (see Materials and methods). This resulted in the identification of 1151 cold-induced genes and 1095 cold-repressed genes



**Fig. 4.** Comparison of cold- and CBF-regulated pOGs of *S. tuberosum* (St), *S. commersonii* (Sc), and *A. thaliana* (At). (A) A total of 8714 putative orthologous groups (pOGs) were identified between At and St. The Venn diagram shows pOGs with at least one At gene on the ATH1 chip, at least one potato clone on the potato cDNA array, or at least one gene from each species in both arrays (overlap). (B) Overlaps of cold-induced and cold-repressed pOGs in Sc, St, and At are shown from the pOGs present in both arrays in A. (C) Comparison of CBF regulon pOGs in Sc, St, and At. Overlaps of CBF-induced and CBF-repressed pOGs are shown based on the pOGs present in both arrays.

(Supplementary Table S3 at *JXB* online). Of the 2944 pOGs that had at least one gene from *A. thaliana* represented on the ATH1 array, 260 had at least one cold-induced gene and 139 had at least one cold-repressed gene (Fig. 4B).

The results presented in Fig. 4B show that 40 pOGs included one or more cold-induced gene from each plant species and that 13 pOGs included one or more cold-repressed gene from all three species. The 40 conserved cold-induced pOGs included genes with a wide range of functions (Table 1). A number of the encoded proteins had previously been associated with ‘stress’, including two LEA (late embryogenesis

abundant) proteins [LEA14 (Choi *et al.*, 2002) and ERD10 (early responsive to desiccation) (Kiyosue *et al.*, 1994)], ELIP1 (early light-inducible protein 1) (Hutin *et al.*, 2003), HSP60 (Rikhvanov *et al.*, 2007), sucrose synthase 1 (Dejardin *et al.*, 1999), and protein phosphatase 2C (Umezawa *et al.*, 2009). In addition, four transcription factors—Agamous-like 20 (AGL20)/Suppressor of Overexpression of CO (SOC1) (Lee *et al.*, 2000), ADOF1 (Lijavetzky *et al.*, 2003), HSFA8 (Chawade *et al.*, 2007), and NAC019 (Jensen *et al.*, 2010)—and nine proteins of unknown function were found to be conserved. As for the 13 conserved cold-repressed pOGs, six were associated with photosynthesis; they were either proteins located in the chloroplast or enzymes involved in photosynthesis (Table 2). Low temperature has been shown to down-regulate the expression of photosynthesis-related genes in a number of plant species (Wisniewski *et al.*, 2008; Zhang *et al.*, 2009).

#### Identification of the CBF regulon genes conserved in both *Solanum* species and *A. thaliana*

The *A. thaliana* CBF regulon was defined as those genes for which transcript levels either increased or decreased at least 2-fold (FDR  $\leq 0.05$ ) in response to constitutive overexpression of *CBF2* at warm temperature in *A. thaliana* transgenic lines (Vogel *et al.*, 2005) and were also included in the list of cold-induced or cold-repressed genes described above. This resulted in the assignment of 169 cold-induced genes and 58 cold-repressed genes to the *A. thaliana* CBF regulon (Supplementary Tables S5, S6 at *JXB* online). These genes, as well as the genes that were determined to comprise the CBF regulons of *S. commersonii* and *S. tuberosum* (Fig. 2B), were then assigned to pOGs (Fig. 4C). An inspection of the resulting Venn diagrams indicated that of the 22 cold-induced CBF regulon pOGs conserved in both *Solanum* species, only seven were members of the *A. thaliana* CBF regulon, and that of the 31 cold-repressed CBF regulon pOGs conserved in both *Solanum* species, only two were members of the *A. thaliana* CBF regulon. The seven conserved cold-induced CBF regulon pOGs encoded ELIP1, LEA14, ERD10, COR47, ADOF1, RD26, SUS1, and an invertase/pectin methylesterase inhibitor family protein. The two conserved cold-repressed CBF regulon pOGs encoded a phosphoethanolamine *N*-methyltransferase and  $\beta$ -galactosidase. These data indicate that there is considerable divergence in the CBF regulons of *A. thaliana* and the two *Solanum* species.

#### Conservation of rapidly induced transcription factors in both *Solanum* species and *A. thaliana*

In *A. thaliana*, there are a number of genes encoding transcription factors that are induced rapidly in response to low temperature with similar kinetics to *CBF1*, *CBF2*, and *CBF3*. These genes include *CZF1*, *ZAT10*, and *RAV1* (Vogel *et al.*, 2005). Like *CBF1*, *CBF2*, and *CBF3*, these

**Table 1.** Forty cold-induced pOGs from *S. tuberosum* (St), *S. commersonii* (Sc), and *A. thaliana* (At)

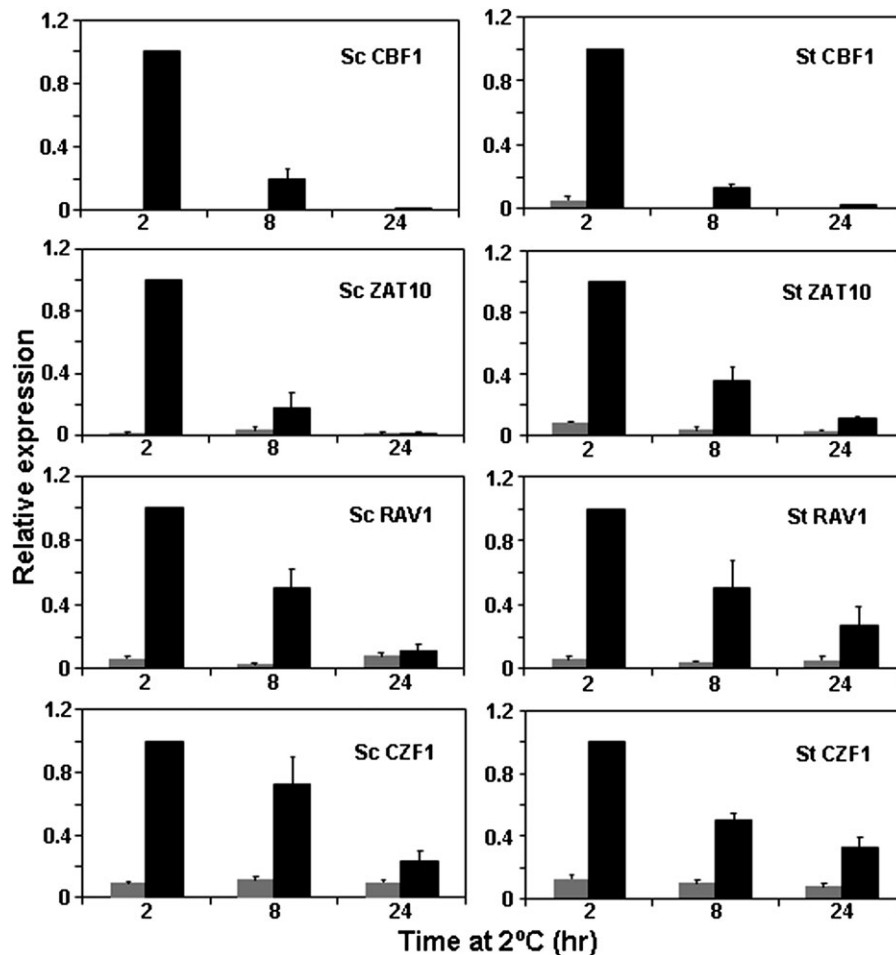
St up	Sc up	At up	At description
STMDJ69	STMGE83, STMDJ69	AT2G18900	Transducin family protein/WD-40 repeat family protein
STMIW06	STMIW06	AT4G25990	Chloroplast import apparatus CIA2-like
STMIU11	STMIU11	AT5G60680, AT2G28400	Unknown protein, unknown protein
STMCG52	STMGV17, STMCG52	AT2G45660	AGL20 (Agamous-like 20)
STM CN22	STM CN22	AT5G65280	GCL1 (GCR2-like 1); catalytic
STMEK16	STM IQ63, STMEK16	AT3G12670	EMB2742 (embryo defective 2742)
STMIV71, STM IY51	STMIV71, STM IY51	AT1G27760	Interferon-related developmental regulator family protein/FRD protein family
STMJG77, STM IY74	STM IY74	AT5G01880	Zinc finger (C3HC4-type RING finger) family protein
STMEP26	STMEP26	AT5G26920	Calmodulin binding
STMEO27	STMEO27	AT2G33210, AT3G23990	Chaperonin, putative HSP60 (heat shock protein 60)
STMIU32, STM ID24	STMIU32, STM ID24	AT3G53230	Cell division cycle protein 48 putative (CDC48)
STM IQ26, STM JI56, STM GA34	STM IQ26, STM JI56, STM GA34	AT1G01470	LEA14 (late embryogenesis abundant 14)
STMDH66, STM JL22	STM JL22	AT4G35940	Unknown protein
STMEI36, STMEQ55	STMEI36	AT1G31660	Unknown protein
STMET41	STMET41	AT1G25400	Unknown protein
STMGF95	STMGF95	AT1G51700	ADOF1 ( <i>Arabidopsis</i> dof zinc finger protein 1)
STM IY82	STM IY82	AT1G52890, AT4G27410	ANAC019 ( <i>Arabidopsis</i> NAC domain-containing protein 19), RD26 (responsive to desiccation 26)
STMDO86	STMD S75, STMD O86	AT3G16810	APUM24 ( <i>Arabidopsis</i> pumilio 24)
STM EW81, STM CB90	STM EW81, STM CB90	AT5G62360, AT5G62350	Invertase/pectin methylesterase inhibitor family protein invertase/pectin methylesterase inhibitor family protein (DC 1.2 homologue)
STMHE19	STMHE19	AT5G20830	SUS1 (sucrose synthase 1)
STM DP77	STM DP77	AT4G29780	Unknown protein
STM GH65, STM JJ17	STM HG34, STM JJ17	AT4G30290, AT5G48070	ATXTH19, ATXTH20 (xyloglucan endotransglucosylase hydrolase 19 and 20)
STMHA92	STMHA92	AT1G67970	AT-HSFA8 ( <i>Arabidopsis thaliana</i> heat shock transcription factor A8)
STMHS29	STMHS29	AT1G42440	Unknown protein
STMGG79	STMGG79	AT3G55510	Unknown protein
STM CX87	STM CX87	AT5G16010	3-Oxo-5- $\alpha$ -steroid 4-dehydrogenase family protein/steroid 5- $\alpha$ -reductase family protein
STMDU38	STM HN39, STMD U38	AT1G80270	DNA-binding protein putative
STMHO64	STMHO64	AT2G17270	Mitochondrial substrate carrier family protein
STM IH78, STM GL16	STM IH78, STM GL16	AT4G27940	Mitochondrial substrate carrier family protein
STMED50	STMED50	AT4G33905, AT2G14860	Peroxisomal membrane protein 22 kDa putative
STMGR56	STMGR56	AT4G28450	Transducin family protein/WD-40 repeat family protein
STMIP59	STMIP59	AT1G32860	Glycosyl hydrolase family 17 protein
STMHT66	STMHT66	AT4G31140	Glycosyl hydrolase family 17 protein
STMIO48	STMIO48	AT4G00640	Unknown protein
STM GU17	STM GU17	AT4G12000	Unknown protein
STMHS17	STMHS17	AT3G11410, AT1G07430	ATPP2CA ( <i>Arabidopsis</i> protein phosphatase 2CA), protein phosphatase 2C putative
STMJO29	STMJO29	AT1G20450, AT1G20440, AT1G76180	ERD10/LTI45 (early responsive to dehydration 10), COR47 (cold regulated 47), ERD14 (early responsive to dehydration 14)
STMJO47, STMIX48	STMJO47, STMIX48	AT3G22840, AT4G14690	ELIP1 (early light-inducible protein) chlorophyll binding, ELIP2 (early light-inducible protein 2) chlorophyll binding
STMHO88, STMHT73	STMHO88, STMHT73	AT5G07990	TT7 (transparent testa 7)
STMGJ81	STMGJ81	AT1G53645	Hydroxyproline-rich glycoprotein family protein

genes are also induced in response to mechanical agitation. To explore the conservation of low temperature regulatory programmes, it was of interest to determine whether *S. tuberosum* and *S. commersonii* had potential orthologues of *CZF1*, *ZAT10*, and *RAV1* and, if so, whether they were induced quickly in response to low temperature and in

response to mechanical agitation. Among the potato ESTs, potential orthologues of these genes were identified and their regulation was tested. The results indicated that all three of these genes were quickly induced in response to low temperature (Fig. 5) and mechanical agitation (Fig. 6) in both potato species.

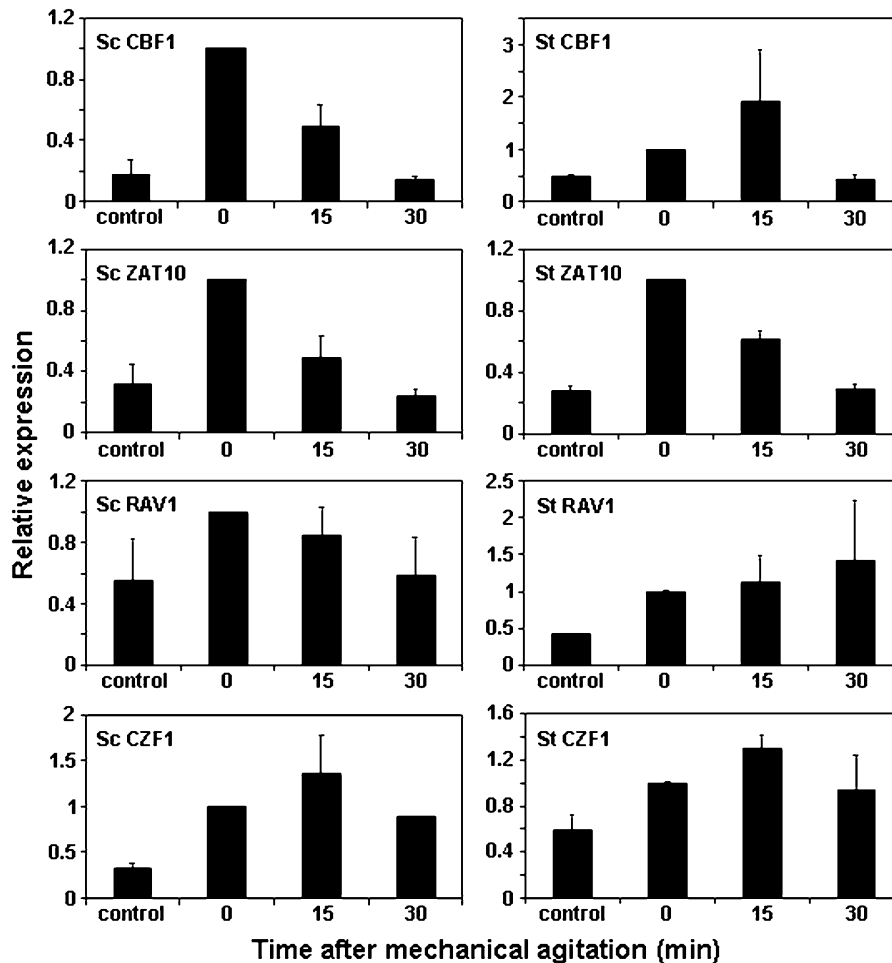
**Table 2.** Thirteen cold-repressed *pOGs* from *S. tuberosum* (*St*), *S. commersonii* (*Sc*), and *A. thaliana* (*At*)

St down	Sc down	At down	At description
STMDV46	STMDV46	AT1G09750	Chloroplast nucleoid DNA-binding protein-related
STMIV36	STMIV36	AT2G39470	PPL2 (PSBP-like protein 2)
STMCX38	STMCX38	AT3G16150	L-Asparaginase putative
STMER63	STMDB57, STMER63, STMDB57	AT3G23730	Xyloglucan:xyloglucosyl transferase putative
STMGO23	STMGO23	AT4G14540	CCAAT-box-binding transcription factor subunit B (NF-YB) (HAP3)
STMCR16, STMCL01, STMCV75, STMIV24, STMCK44	STMCR16, STMCL01, STMCV75, STMIV24, STMCK44	AT1G70410, AT3G01500	Carbonic anhydrase putative, carbonate dehydratase putative, CA1 (carbonic anhydrase 1)
STMEP82, STMCS89	STMCS89	AT1G48600	Phosphoethanolamine <i>N</i> -methyltransferase 2 putative (NMT2)
STMCD65	STMCD65	AT5G56870	BGAL4 (beta-galactosidase 4)
STMGX24	STMGX24	AT5G35790	G6PD1 (glucose-6-phosphate dehydrogenase 1)
STMJH69	STMJH69	AT3G15840	PIF1 (post-illumination chlorophyll fluorescence increase)
STMIM55	STMIM55	AT1G32080	Membrane protein putative
STMDB78, STMCO55	STMCO55	AT1G73330	ATDR4 ( <i>Arabidopsis thaliana</i> drought-repressed 4)
STMJD18	STMJD18	AT4G25260, AT4G12390	Invertase/pectin methyltransferase inhibitor family protein PME1; pectinesterase inhibitor



**Fig. 5.** Transcript accumulation of *S. commersonii* (*Sc*) and *S. tuberosum* (*St*) orthologues of *CBF1*, *ZAT10*, *RAV1*, and *CZF1* in response to low temperature. *Sc* and *St* plants were grown for 3 weeks under a 16 h photoperiod. Eight hours after dawn plants were either transferred to 2 °C (black bars) for 2, 8, and 24 h or kept at 25 °C (grey bars) for the same periods of time. qRT-PCR analysis was performed to determine the transcript levels of *Sc* and *St* genes. Average values of three different experiments are shown. The relative expression level of each transcript was normalized using the potato 60S gene (clone STMCK67) as an internal reference. Relative expression of the *Sc* 2 h cold sample was set to 1 and the other values were adjusted accordingly. Error bars indicate the SE.





**Fig. 6.** Transcript accumulation of *S. commersonii* (Sc) and *S. tuberosum* (St) orthologues of *CBF1*, *ZAT10*, *RAV1*, and *CZF1* in response to mechanical agitation. Three-week-old plants grown in magenta boxes were mechanically agitated for 15 min (see Materials and methods). Tissue was collected from two plants at 0, 15, and 30 min after the mechanical agitation treatment. The control sample received no mechanical agitation. qRT-PCR analysis was performed to determine the transcript levels of Sc and St genes. Average values of two different experiments are shown. The relative expression level of each transcript was normalized using the potato 60S gene (clone STMCK67) as an internal reference. The relative expression of the Sc 0 min sample was set to 1 and the other values were adjusted accordingly. Error bars indicate the SE.

## Discussion

One of the fundamental goals of cold acclimation research is to understand the molecular basis for the differences in freezing tolerance between plant species that are evolutionarily closely related. Here this issue is addressed through a comparative transcriptome analysis of two closely related potato species—*S. commersonii*, which cold acclimates, and *S. tuberosum* (cv. Umatilla), which does not. The present focus on the transcriptome was motivated by the fact that cold acclimation is known to involve changes in gene expression. The goal of the study was to explore whether there might be an obvious ‘macro-scale’ explanation for the differences in these two potato species in their ability to cold acclimate. In short, the expression profiling experiments did not provide such an explanation. Both plants altered gene expression in response to low temperature to similar degrees with similar kinetics. Moreover, there was considerable overlap in the cold-regulated gene sets.

Thus, the differences in freezing tolerance between *S. tuberosum* and *S. commersonii* must trace to specific differences in their responses to low temperature. Indeed, although there was considerable overlap in the genes that were cold-regulated in both plant species, the results point to both qualitative and quantitative differences in the cold-regulated gene sets of the two species (Figs 1, 2). However, at this point, the specific differences observed must be viewed with caution. Some of these differences are likely to be only apparent, as a small change in statistical criteria used for the analysis had a large effect on the percentage overlap of cold-induced genes (Fig. 2). Moreover, the microarray that was used to monitor gene expression was based on ESTs from *S. tuberosum*. Given that there are notable differences in the nucleotide sequences of *S. tuberosum* and *S. commersonii*—the synonymous substitution rate between these two species is  $\sim 0.04$  (see Materials and methods)—it is possible that some of the genes that were observed to be cold-regulated in *S. tuberosum*, but not in *S. commersonii*, might be due to nucleotide mismatches between the *S. commersonii* transcripts and the

probe ESTs (those genes that were found to be cold regulated in *S. commersonii*, but not *S. tuberosum*, would be less likely to be subject to this possible limitation). Future studies will be directed at detailing the qualitative and quantitative differences between the transcriptomes of these two potato species. These efforts will be greatly facilitated by the recently completed sequencing of the potato genome (<http://potatogenomics.plantbiology.msu.edu/>) and the rapidly developing technologies for RNA sequencing.

The present findings are similar to those of Oufir *et al.* (2008) who used the same potato array to compare the low temperature transcriptome of *S. tuberosum* (cv. Desiree), which they showed does not cold acclimate, with that of *S. phureja* CHS (diploid potato), which they showed does cold acclimate. They found that 213 genes were cold-induced and 101 cold-repressed  $\geq 2$ -fold in *S. tuberosum* cv. Desiree and that 92 genes were induced and 159 repressed in *S. phureja*. Only 22 genes were found to be cold-induced and 15 to be cold-repressed in both species.

A second objective of this study was to determine whether macro-scale differences in the composition of the CBF regulons of *S. tuberosum* and *S. commersonii* might account for their differences in ability to cold acclimate. One specific question was whether the CBF regulon of *S. tuberosum* was much smaller than that of *S. commersonii*. As noted earlier, the CBF regulons of freezing-sensitive tomato (Zhang *et al.*, 2004) and rice (Dubouzet *et al.*, 2003) have been reported to be composed of as few as 10 cold-induced genes, whereas the CBF regulon of *A. thaliana* includes at least 85 cold-induced genes (Vogel *et al.*, 2005). The present results, however, indicate that the sizes of the *S. tuberosum* and *S. commersonii* CBF regulons are not appreciably different; the number of cold-induced genes assigned to the regulons was 170 and 160, respectively (Fig. 2B). Moreover, the results indicate that the CBF regulons of both *S. tuberosum* and *S. commersonii* are much larger than that of *A. thaliana*. Indeed, the potato arrays used here included probes for only  $\sim 10\,000$  genes. Thus, if the probes on this array are reflective of the overall genome, it could be that the CBF regulons of these two potato species each comprise nearly a thousand cold-induced genes.

While the difference in freezing tolerance between *S. commersonii* and *S. tuberosum* does not appear to involve a significant difference in size of their CBF regulons, the results suggest that the regulons have diverged considerably (Fig. 2B). The results indicate that of the 160 cold-induced genes that were up-regulated by AtCBF3 overexpression in *S. commersonii*, only 54 were up-regulated by AtCBF3 overexpression in *S. tuberosum* (as alluded to above, those genes that were found to be induced in *S. commersonii*, but not in *S. tuberosum*, are not likely to be artefacts of using ESTs from *S. tuberosum*). One might therefore speculate that the genes that are members of the CBF regulon of *S. commersonii*, but not members of the *S. tuberosum* CBF regulon, have critical roles in freezing tolerance. However, this must not be the case as constitutive overexpression of AtCBF3 has been shown to increase the freezing tolerance of *S. tuberosum* without a cold treatment; whereas the LT<sub>50</sub> for non-acclimated wild-type *S. tuberosum* plants was  $-3.0\text{ }^{\circ}\text{C}$ , non-acclimated transgenic *S. tuberosum* plants

expressing AtCBF3 had an LT<sub>50</sub> of  $-5.0\text{ }^{\circ}\text{C}$  (Pino *et al.*, 2007). Thus, *S. tuberosum* has genes that can impart freezing tolerance, and at least some of these are members of the AtCBF3 regulon.

The basic question thus raised is: why does *S. tuberosum* not increase in freezing tolerance in response to low temperature? At this point it is known that the answer is not simply a matter of *S. commersonii* having CBF genes that are cold-induced and *S. tuberosum* not having such genes. Both *S. tuberosum* and *S. commersonii* have two CBF loci (Pennycooke *et al.*, 2008). At one locus, both *S. commersonii* and *S. tuberosum* have four CBF genes, one of which, CBF1, is induced in response to low temperature. At the second locus, *S. commersonii* has one CBF gene, CBF4, and *S. tuberosum* has two CBF genes, CBF4 and CBF5, and in both species the CBF4 gene is cold-induced. The implication is that the genes that are targeted by the CBF1 and CBF4 proteins of *S. tuberosum* do not include all of those targeted by AtCBF3 and that this difference is the fundamental reason why *S. tuberosum* does not cold acclimate. Transgenic *S. tuberosum* plants that express each of the *S. commersonii* and *S. tuberosum* CBF genes are currently being made to test this possibility.

One note of caution is that the genes that were identified here as members of the *S. tuberosum* AtCBF3 regulon are not likely to be sufficient for freezing tolerance. The first obvious reason is that the arrays that were used to monitor gene expression only included probes for  $\sim 10\,000$  genes. Thus, there are certain to be many genes that comprise the AtCBF3 regulon that are not on the current list and might have roles in freezing tolerance. However, even more important is that the definition of the AtCBF3 regulon used here is likely specifically to exclude the genes with roles in freezing tolerance. For genes to be assigned to the regulon, they must be induced or repressed in response to AtCBF3 overexpression in transgenic plants grown at warm non-acclimating temperatures and also be correspondingly induced or repressed in response to low temperature. Therefore, all of the genes in the present CBF regulon list are cold-regulated genes in wild-type *S. tuberosum*. However, given that AtCBF3 overexpression imparts freezing tolerance and low temperature does not, it would appear that the genes that are regulated by AtCBF3 and impart freezing tolerance are not cold-regulated in *S. tuberosum* and, therefore, would not be included in the present CBF regulon gene list. One might then think that those genes that are induced or repressed in response to AtCBF3 expression, but are not cold regulated, would be among those that are most critical to freezing tolerance. The problem in identifying these critical genes is that AtCBF3 overexpression in *S. tuberosum* (Pino *et al.*, 2007), as is the case in *A. thaliana* (Liu *et al.*, 1998; Gilmour *et al.*, 2000), affects multiple aspects of plant growth and development; AtCBF3-overexpressing plants have a dwarf stature, for instance. Thus, many of the genes that are affected in plants overexpressing AtCBF3 are likely to be secondary effects of CBF expression and not related directly to freezing tolerance.

The third objective of this study was to identify cold-regulated genes that were conserved between the two *Solanum* species and *A. thaliana*. Given the relatively large evolutionary distance between these plants, such genes might have fundamental roles in low temperature biology. Due to the fact that the potato array used includes <25% of the potato genome, the present analysis was significantly limited in scope. Nevertheless, 53 pOGs were identified, 40 of which include cold-induced genes (Table 1) and 13 of which include cold-repressed genes (Table 2). Some of the conserved genes had previously been associated with stress, including genes encoding LEA and ELIP proteins. However, the largest group of conserved genes encoded proteins of unknown function. The study of these genes may reveal novel mechanisms fundamental to plants coping with low temperature. In addition, it was found that the low temperature regulatory programmes of these three species have seven transcription factors in common in addition to *CBF* genes. Moreover, four of these genes—*CBFI*, *ZAT10*, *RAVI*, and *CZFI*—were found to be induced not only in response to low temperature (Fig. 5), but also in response to mechanical agitation (Fig. 6). Thus, not only are the genes conserved, but regulatory mechanisms controlling expression of these genes have conserved elements beyond low temperature *per se*.

Comparative studies of genome-wide transcript profiles have revealed conserved components in core biological processes and divergent expression patterns across species that may be important for taxa-specific processes. For example, transcript profiling data from three highly divergent species including *Caenorhabditis elegans*, *Drosophila melanogaster*, and budding yeast have been used to dissect expression conservation and divergence among orthologous genes (McCarroll *et al.*, 2004). Expression patterns remain conserved among these three species for genes involved in fundamental biological processes such as mitochondrial metabolism, DNA repair, and cellular transport. However, most expression profiles are species specific. Even between relatively closely related species, a substantial number of genes show significant differences in expression levels. In a comparison between *D. melanogaster*, *D. simulans*, and *D. yakuba*, species that diverged ~2.5–5 MYA, ~20–50% of the orthologous genes showed expression differences (Ranz *et al.*, 2003; Rifkin *et al.*, 2003). Similarly, in a comparison of *Saccharomyces cerevisiae* and *S. paradoxus*, species that diverged ~5 MYA, the expression patterns of orthologous genes under various environmental stresses showed extensive differences (Tirosch *et al.*, 2006). In addition, due to a high rate of gene duplication, losses and gains of stress responses have occurred readily among plant duplicate genes that contribute to expression divergence between species (Zou *et al.*, 2009). These findings are largely consistent with the apparent divergence among cold-responsive genes of *S. tuberosum* and *S. commersonii*. Moreover, given the large evolutionary distance between *A. thaliana* and the two *Solanum* species (diverged ~112–156 MYA; Yang *et al.*, 1999; Bell *et al.*, 2005), it was not surprising to find a low degree of conservation of cold-responsive genes between

these species. Indeed, the genes that were found to be conserved over such evolutionary time are likely to be members of a core cold response network that have fundamental roles in the low temperature biology of plants.

## Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primers used in qRT-PCR.

Table S2. Affymetrix cel files used to identify *A. thaliana* cold-regulated genes.

Table S3. *Arabidopsis thaliana* cold-regulated genes.

Table S4. List of 8714 pOGs between *A. thaliana* and *S. tuberosum*.

Table S5. *Arabidopsis thaliana* up-regulated CBF regulon genes.

Table S6. *Arabidopsis thaliana* down-regulated CBF regulon genes.

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