

# *AtCBF1* Overexpression Confers Tolerance to High Light Conditions at Warm Temperatures in Potato Plants

Leonardo Storani<sup>1</sup> · Carlos E. Hernando<sup>2</sup> · Roberto J. Staneloni<sup>2</sup> · Edmundo Ploschuk<sup>1</sup> ·  
Matias L. Rugnone<sup>1</sup> · Gustavo G. Striker<sup>1</sup> · Jorge J. Casal<sup>1,2</sup> · Ariel Chernomoretz<sup>2,3</sup> ·  
Marcelo J. Yanovsky<sup>1,2</sup>

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**Abstract** We characterized transcriptional responses of potato plants to multiple abiotic stresses and used this information to identify potential mechanisms through which overexpression of the stress related transcription factor *CBF1* from *Arabidopsis thaliana* (*AtCBF1*) confers multiple stress tolerance. Most transcriptional changes were specific to each condition, but genes involved in phenyl-propanoid biosynthesis were affected by all abiotic stresses evaluated. Interestingly, over-expression of *AtCBF1* in potato plants not only conferred tolerance to low temperatures, as previously reported, but also to high-light conditions at 22 °C, suggesting that it confers multiple stress tolerance by enhancing the ability of plants to cope with an excess of radiant energy. Finally, we found that transcriptional changes triggered by abiotic stress were much larger than those resulting from *AtCBF1* over-expression in potato, revealing that overexpression of an heterologous

transcription factor causes minor alterations in the plant transcriptome in comparison to transcriptional changes triggered by abiotic stresses.

**Resumen** Caracterizamos respuestas transcripcionales de plantas de papa a múltiples estreses abióticos y utilizamos esta información para identificar mecanismos potenciales a través de los cuales la sobreexpresión del factor de transcripción *CBF1* relacionado con agobio de *Arabidopsis thaliana* (*AtCBF1*) confiere tolerancia múltiple al estrés. La mayoría de los cambios transcripcionales fueron específicos para cada condición, pero se afectaron los genes involucrados en la biosíntesis de fenil-propanoides por todos los estreses abióticos evaluados. Interesantemente, la sobreexpresión del *AtCBF1* en plantas de papa no solo confirieron tolerancia a bajas temperaturas, como se ha reportado previamente, sino

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✉ Marcelo J. Yanovsky  
myanovsky@leloir.org.ar

Leonardo Storani  
lstorani@agro.uba.ar

Carlos E. Hernando  
chernando@leloir.org.ar

Roberto J. Staneloni  
rstaneloni@leloir.org.ar

Edmundo Ploschuk  
ploschuk@agro.uba.ar

Matias L. Rugnone  
mrugnone@agro.uba.ar

Gustavo G. Striker  
striker@ifeva.edu.ar

Jorge J. Casal  
casal@ifeva.edu.ar

Ariel Chernomoretz  
achernomoretz@leloir.org.ar

<sup>1</sup> Instituto de Fisiología y Ecología Vinculado a la Agricultura, IFEVA, CONICET-UBA, Av. San Martín 4453, CPA1417DSE Buenos Aires, Argentina

<sup>2</sup> Fundación Instituto Leloir - Instituto de Investigaciones Bioquímicas de Buenos Aires, Av. Patricias Argentinas 435, C1405BWE Buenos Aires, Argentina

<sup>3</sup> Departamento de Física, FCEN, UBA, Pabellón 1, Ciudad Universitaria, 1428 Buenos Aires, Argentina

también a condiciones de alta luminosidad a 22 °C, lo que sugiere múltiple tolerancia al estrés mediante el aumento de la habilidad de las plantas para hacer frente a un exceso de energía radiante. Finalmente, encontramos que los cambios transcripcionales disparados por el agobio abiótico fueron mayores que aquellos que resultaron de la sobreexpresión de *AtCBF1* en papa, revelando que la sobreexpresión de un factor heterólogo de transcripción causa alteraciones menores en el transcriptoma de la planta en comparación a cambios transcripcionales disparados por estreses abióticos.

**Keywords** *CBF1* · Potato · Transgenic plants · High light · Drought · Low temperature · Gene expression profiles

## Introduction

The increase in food demand caused by the continuous growth of the population, along with the absence of additional areas with optimal environmental conditions for crop production, is leading to an expansion of agricultural activities in marginal regions (Khush 2001; Rosegrant and Cline 2003; Tilman et al. 2002). In addition, man-induced global climate changes are leading to repeated episodes of drought or extreme temperatures in geographical regions where those abiotic stresses were uncommon. Therefore, understanding the molecular and physiological mechanisms underlying the adaptive responses of plants to abiotic stress conditions is crucial. This knowledge is required not only to facilitate the development of novel cultivars with higher levels of stress tolerance, but also to evaluate and minimize potential environmental and health related risks associated with these biotechnological innovations.

The mechanisms leading to stress tolerance are extremely complex. Some species are adapted to live in environments where intense stress is always present, so they have constitutive adaptations (Bohnert et al. 1995). Others display physiological and molecular adaptations which allow them to tolerate harsh conditions only if they were previously exposed to anticipatory stress-associated signals (generally milder stress conditions). This process, known as acclimation, involves homeostatic alterations that contribute to improving the adjustment of organisms to changes in external conditions (Thomashow 2001). Thus, an acclimation period can confer tolerance to extreme environmental conditions that might otherwise be lethal, without the cost of expressing constitutive adaptive responses.

Acclimation to both drought and low temperature conditions is mediated through specific as well as common signaling pathways, which result in a large reprogramming of the plant transcriptome (Shinozaki and Yamaguchi-Shinozaki 2000; Shinozaki et al. 2003). In addition to increasing the expression of genes encoding hydrophilic proteins that prevent membrane damage, and molecular chaperons that

stabilize proteins against freezing or dehydration-induced denaturation, low temperatures and water deficits also induce the expression of genes encoding scavengers of reactive oxygen species (ROS) (Cushman and Bohnert 2000; Mittler 2002; Zhu 2002). These molecules are formed when electrons transport chains become fully reduced as a consequence of an excess of radiant energy that cannot be processed through the photosynthetic apparatus. This can be due to a decrease in photosynthetic activity caused by drought induced stomatal closure, or to a reduction in enzymatic activity associated with low temperature conditions. Indeed, ROS scavengers are critical to avoid photo-oxidative damage to membranes in response to abiotic stress (Foyer and Allen 2003).

Physiological and molecular responses to drought and low temperatures are mediated by both ABA-dependent as well as by ABA-independent signaling pathways (Qin et al. 2011). An important regulatory element found in the promoters of drought and cold regulated genes is the sequence known as CRT/DRE (C-repeat/Dehydration Responsive Element), composed of a core CCGAC element (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). The DREB family of transcription factors bears homology to AP2/ERF DNA-binding proteins and recognizes CRT/DRE sequences. Members of the DREB2 family are expressed in response to drought, and confer tolerance to it (Liu et al. 1998). On the other hand, *DREB1b*, *DREB1c*, and *DREB1a*, also known as *CBF1*, *CBF2* and *CBF3*, are induced in response to cold exposure and activate the expression of cold-regulated genes (COR) upon binding to CRT/DRE elements present in their promoters. Interestingly, over-expression of *CBF1* induces constitutive expression of COR genes and enhances freezing tolerance in *Arabidopsis thaliana* in non-acclimated plants (Jaglo-Ottosen et al. 1998). In addition, over-expression of any member of the DREB1 family of transcription factors from *Arabidopsis thaliana*, induces tolerance to multiple abiotic stresses in other species (Khan 2011). For example, expression of *Arabidopsis CBF1* confers tolerance to chilling, oxidative, and drought stress in tomato (Hsieh et al. 2002a, b).

*Solanum tuberosum*, a cultivated tuber-bearing potato plant, is sensitive to low temperature conditions as well as to drought (Chen and Li 1980), and an important biotechnological aim is to develop transgenic potato plants capable of tolerating multiple stresses. The first aim of the work described here was to identify the genes whose expression is affected by different abiotic stress conditions in potato (i.e. to define the “potato abiotic stress transcriptome”), in order to determine convergent targets of different stress associated signaling pathways that could be manipulated to trigger multiple stress tolerance in this species. Several studies that analyze the potato transcriptome in response to drought, cold as well as other stresses such as high temperatures and salt, have already been conducted (Carvallo et al. 2011; Gangadhar et al. 2014; Hancock et al. 2014; Kondrak et al. 2012; Rensink et al.

2005a, b; Vasquez-Robinet et al. 2008; Zhang et al. 2014). Here we analyzed the potato transcriptome in response to an additional environmental stress, high light conditions at warm temperatures (22 °C), and compared the effect of this stress to that of drought and cold conditions.

In addition, several groups have recently reported that over-expression of *A. thaliana* *CBF1* and *CBF3* genes in potato plants confers tolerance to salt stress (Behnam et al. 2006), freezing (Movahedi et al. 2012; Pino et al. 2007, 2008), high temperatures (Dou et al. 2015) and drought (Movahedi et al. 2012), but the physiological and molecular mechanisms through which CBF genes confer abiotic stress tolerance in potato plants are not well understood. Potato plants have three *CBF* genes in tandem, which are orthologues of Arabidopsis *CBF1*, *CBF2* and *CBF3* and, indeed, the potato *CBF1* orthologue is also induced in response to cold treatments (Pennycooke et al. 2008). Interestingly, overexpressing the *AtCBF3* gene in potato plants results in the up-regulation of some genes that are part of the Arabidopsis CBF regulon, but other genes induced by *AtCBF3* in potato encode proteins whose orthologues have not been identified as part of the Arabidopsis CBF regulon (Carvalho et al. 2011). Whether these genes responding to the overexpression of the *AtCBF3* gene in potato but not in Arabidopsis also respond to other stress treatments in potato is not known. Therefore, a second aim of this project was to determine whether a “potato stress-transcriptome” could be used to obtain clues on the mechanisms underlying multiple stress tolerance in transgenic potato plants over-expressing an *AtCBF1* gene.

Finally, genetically modified organisms (GMOs) are subject to intense monitoring by governmental regulatory agencies, both in relation to environmental bio-safety, as well as in terms of potential effects on human health. Therefore, the characterization of potentially unintended effects associated with the transgenic event analyzed is likely to be an important step of the regulatory process. In this particular case, we considered “unintended” effects all those changes in the expression of genes whose transcripts levels are affected by overexpression of a stress related transcription factor, but do not respond to stress treatments in wild type plants. Recent work conducted to characterize “unintended” effects associated with the over-expression of an abiotic-transcriptional regulator suggests that this strategy may not necessarily cause extensive “unintended” transcriptional alterations (Abdeen et al. 2010). However, this result might be biased given that a later study evaluated the effect of over-expressing an *A.thaliana* transcription factor in *A.thaliana*. Here we used a combination of approaches to characterize the effect of overexpressing *Arabidopsis thaliana* *CBF1* in a heterologous species. This approach allowed us to narrow down the transcriptional changes that are likely to be considered “unintended” effects to a small subset of genes, which should facilitate the evaluation of the potential risks associated with these transgenic

plants. In addition, we also observed that the transcriptional changes induced by different abiotic stresses were much larger than those caused by *AtCBF1* over-expression in potato. This indicates that overexpression of an heterologous transcription factor in a crop species only causes limited effects on the plant transcriptome, limiting the extent of potential “unintended” effects.

## Materials and Methods

### Plant Material and Growth Conditions

Potato plants (*Solanum tuberosum* cv. Désirée) were grown under sterile conditions in glass bottles containing Murashige and Skoog (MS, Sigma) medium solidified with 0.8 % phytoagar (Sigma). Plants were propagated in vitro and grown under 16 h light (50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  cool-white fluorescent illumination) / 8 h dark cycles, at 22 °C. The analysis of physiological and molecular responses of plants to different stress treatments was conducted on plants transferred to plastic pots containing a mixture of perlite:vermiculite:peat moss (1:1:1), and acclimated for 1 month under the same light and temperature conditions in which the treatments were applied.

### Stress Treatments

To analyze the effect of low temperature conditions, plants were first grown under long days (16 h light/8 h darkness) with fluorescent illumination (50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 22 °C. After 1 month, half of the plants were exposed to 4 °C under similar light conditions, while the remaining plants were kept at 22 °C. The effect of high light stress was evaluated on plants grown first under long day conditions in a growth chamber illuminated with high pressure sodium lamps at 50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , at 22 °C. After 1 month, half of the plants were exposed to 800  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at 22 °C, while the remaining plants were kept under 50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at 22 °C. For drought stress, plants were grown under natural radiation (1450  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at midday) in a greenhouse in 3 l pots supplemented with daily irrigation until water soil capacity was saturated. After 1 month under this condition, irrigation was interrupted for half of the plants while the remaining plants continued with the previous daily irrigation regime.

### Anthocyanin and Chlorophyll Measurement

For anthocyanin determination, petioles from the third completely expanded leaf were harvested and placed in 1.5 ml plastic tubes containing 1 ml of 1 % HCl methanol, and kept in darkness for 2 days at 4 °C. The amount of anthocyanin was determined spectrophotometrically, measuring absorbance at 530 nm and correcting for chlorophyll absorption

(Mancinelli et al. 1991). For chlorophyll determination, leaf discs of 20 mm<sup>2</sup> were placed in 1.5 ml plastic tubes containing 1 ml of N,N-dimethylformamide and incubated for 3 days in darkness at 4 °C. Chlorophyll levels were measured spectrophotometrically, determining absorbance at 647 and 660 nm, and calculating chlorophyll concentration according to Moran and Porath (Moran and Porath 1980).

### Leaf Area, Chlorophyll Fluorescence, Photosynthetic Rate and Leaf Conductance Measurements

Leaf area was determined using a Li-3100C Area Meter (Li-Cor Inc., Lincoln, NE, USA). Chlorophyll fluorescence measurements were determined using the Fluorescence Monitoring System 2 (FMS-2, Hansatech Instruments Ltd, Kings Lynn, UK), and Fv/Fm ratios were measured in dark adapted leaves. This parameter is known to correlate with the number of functional PSII reaction centers, so that it can be used to quantify photoinhibition (Somersalo and Krause 1989). Maximum photosynthetic rates were measured using a Li-6200 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA), with a 0.25-L chamber attached to a regulated portable power (QB1 205LI-670, Quantum Devices Inc., Barneveld, WI) at 1500 μmol m<sup>-2</sup>.s<sup>-1</sup> of red light. Leaf conductance was measured using the Leaf Porometer Model SC-1 (Decagon Devices Inc., Pullman, WA, USA).

### Transpiration and Desiccation Tolerance Measurements

Transpiration was estimated from water consumption measurements, which were based on changes in pot weight recorded on a daily basis. The response to short term desiccation was measured analyzing changes in fresh weight of detached leaves (Verslues et al. 2006) that were kept under continuous illumination (50 μmol.m<sup>-2</sup>.s<sup>-1</sup>) at 22 °C for 24 h.

### Development of *AtCBF1* Overexpressing Potato Plants

The coding sequence of the *Arabidopsis thaliana* *CBF1* gene was amplified by PCR and ligated into the binary vector pCHF3 (Fankhauser et al. 1999). The resulting plasmid pCHF3.*AtCBF1*, which contains the coding sequence under the control of CaMV 35S promoter and the Rubisco terminator, was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Potato plants (*Solanum tuberosum* Cv. Désirée) were transformed with the pCHF3.*AtCBF1* as previously described (Beaujean et al. 1998).

### Molecular and Phenotypic Characterization of Transgenic Plants

To confirm the presence of the transgene, 11 independent lines were transferred to plastic pots containing a mixture of

perlite:vermiculite:peat moss (1:1:1), and allowed to grow for 3 weeks. DNA was isolated using the CTAB method and was used to confirm the presence of the transgene by PCR. The primers used for genotyping were: 1224 (5'-CGCCAGGGTTTTCCAGTCACGAC-3') and 1233 (5'-AGCGGATAACAATTTTCACACAGGA-3'), which recognizes the promoter and terminator regions of the pCHF3. The expression of *AtCBF1* transgene was then measured in PCR confirmed lines. For this, RNA was isolated from mature leaves using the RNeasy® Plant Mini Kit (Qiagen). Reverse transcription was done using SuperScript™ III Reverse Transcriptase (Invitrogen®). Expression was evaluated using semi-quantitative RT-PCR. The actin gene was used as a housekeeping gene to normalize expression levels across samples.

For phenotypic analysis, *AtCBF1* over-expressing and WT potato plants were grown in 300 ml plastic pots under long day conditions for 3 weeks. Total height, as well as length of specific internodes, was measured with a ruler. Potato yield was also measured in plants grown for 4 months in 3 l plastic pots kept under the same conditions. For the later we assessed tuber number as well as tuber weight per plant.

### Expression Profiling

Total RNA extracted from young leaves belonging to two different potato plants were pooled for each biological replicate, and two biological replicates were obtained and characterized for each experimental condition. Five micrograms of RNA from each sample were processed and hybridized to GeneChip® Tomato Genome Arrays (Affymetrix, Inc.), according to the manufacturer's instructions. Microarray signals were made comparable by scaling the average overall signal intensity of all probe sets to a target signal of 250. Data were analyzed using MAS5. Microarray data was analyzed using different statistical approaches described below. Microarray results were confirmed for a subset of genes and conditions using qRT-PCR. For this, total RNA was isolated using the RNeasy® Plant Mini Kit (Qiagen). Then, RNA samples were subjected to DNase treatment with RQ1 RNase-Free Dnase (Promega), and to retrotranscription using SuperScript™ III Reverse Transcriptase (Invitrogen®) and oligo-dT. Synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche) using the Mx3000P® QPCR System (Stratagene). Data were analyzed by MxPro™ QPCR Software (Stratagene). We quantified the relative amount of RNA of the *CHS*, *PAL1*, and *PORb* genes using the *Ef1* gene as housekeeping control (Nicot et al. 2005). The list of the primers used is detailed in the ESM\_18.

### ANOVA

One way ANOVA was performed on the resulting data to identify genes regulated by different abiotic stress factors.

ANOVA was also used to identify genes whose expression was up or down regulated in transgenic plants overexpressing *AtCBF1* compared to non transgenic WT controls. Multiple testing was taken into account by converting p-values to q-values (Storey and Tibshirani 2003). Genes considered for further analysis had a q-value of less than 0.1 and a 2 fold change in expression compared to their corresponding controls, and had a present call in all samples from at least one experimental condition. BLASTP was used to determine the identity of each gene and, whenever possible, the closest Arabidopsis homologue was established.

### Hierarchical Clustering

Global expression analysis of *AtCBF1-OX* plants exposed to abiotic stress was performed using the statistical programming framework R (Gentleman et al. 2004). In order to produce a complete-linkage hierarchical clustering dendrogram, a metric based on absolute correlation levels between sample expression profiles was considered. The obtained ordination of samples was robust against the use of different linkage methods. Moreover, sample profiles built upon the whole set of transcripts or just a non-specific filtered subset of genes produced identical results.

### Linear Model Analysis

To analyze transcriptional responses of *AtCBF1-OX* plants exposed to abiotic stress, the Bioconductor Limma package was used to fit a linear model to the expression value for each gene, and to assess the significance of differential expression between different experimental conditions (Smyth 2004). The Benjamini and Hochberg's method was used to control the false discovery rate (fdr) at a 0.05 level (Benjamini and Hochberg 1995). Additionally, a minimal fold change level of two was required for a gene to be dubbed as differentially expressed.

### Template Matching Analysis

A feature selection method based on a method known as template matching (Pavlidis and Noble 2001) was implemented in R scripts, in order to identify genes with patterns of expression strongly correlated to ideal responders to genetic background, temperature or light stress conditions main effects in *AtCBF1-OX* plants exposed to multiple stresses. The number of genes associated to each one of these templates, with a correlation larger than a given absolute R threshold value, was used as a proxy for the extent of global transcriptional changes induced by the corresponding condition, at that statistical significance level.

### Multi Response Permutation Procedure

The MRPP analysis (Reiss et al. 2010) was performed using the R package *Vegan* (Dixon 2009). The MRPP analysis assess for group mean differences, testing for unusually low average intra-group dissimilarities compared against random group assignment of sampling units. In the same spirit of analysis of variance procedures, if two groups are different, the average of the within-group dissimilarities ought to be less than the average dissimilarities between random collections of samples. The statistic of the test,  $\delta$ , was calculated as a weighted sum of within-group average distances. Its associated statistical significance was estimated through random shuffling group memberships. Finally, the effect size parameter  $A = 1 - \delta_{\text{observed}} / e_{\text{expected}}$ , allowed us to establish a quantitative comparison between the magnitude of the transcriptional changes induced by differences in genomic backgrounds, and those associated with environmental stress conditions.

## Results and Discussion

### Global Analysis of the Potato Transcriptome to Multiple Abiotic Stresses Reveals Convergent Effects on the Phenyl-Propanoid Biosynthetic Pathway

To identify common as well as specific molecular mechanisms underlying responses of potato plants to low temperatures, drought, and high light stress, we conducted a global analysis of the potato transcriptome. For this, we used Affymetrix tomato arrays that have already been successfully used to evaluate gene expression in potato (Bagnaresi et al. 2008) and conducted the experiments using tissue culture potato plantlets transferred to plastic pots containing a mixture of perlite:vermiculite:peat moss. The plants were grown for 1 month in a growth chamber under a 16 h light ( $50 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) / 8 h dark cycle at 22 °C and then one group of plants was exposed for 1 day to a sharp decrease in temperature from 22 to 4 °C keeping the same light conditions, while another group of plants was exposed to an increase in light intensity from 50 to  $800 \mu\text{mol.m}^{-2}.\text{s}^{-1}$  at warm temperatures (22 °C). Then, the transcriptome of both groups of plants was compared to that of plants remaining under control conditions (i.e. low light and warm temperatures). In addition, we also analyzed the transcriptome of potato plants that were grown in the greenhouse with daily irrigation, and compared it to the transcriptome of plants that were grown identically for 1 month but received no additional water for the last 2 days before they were harvested.

Out of approximately 10,000 genes present in the array, 7982 genes showed signal intensities above background levels in at least one environmental condition, revealing that Affymetrix tomato arrays were indeed useful to evaluate the

transcriptome of potato plants. Using a one way analysis of variance we identified 1427 genes with a statistically significant stress treatment effect ( $q$ -value<0.1), and a three-fold (or more) increase or decrease in expression under at least one condition, compared to expression levels observed in plants not exposed to stressful conditions (Fig. 1, ESM\_1). The number of stress regulated genes increased to 3138 if we also considered genes whose expression increased or decreased at least two fold. Thus, this analysis indicates that up to 40 % of the potato genes evaluated show significant changes in expression under the abiotic stress conditions tested here. Many of the gene families identified as stress regulated in our experiments have previously been identified as stress regulated in studies conducted with field grown potato plants, but we should be cautious in extrapolating the results obtained here with plants derived from tissue cultured plantlets to those taking place in field grown plants under conventional conditions.

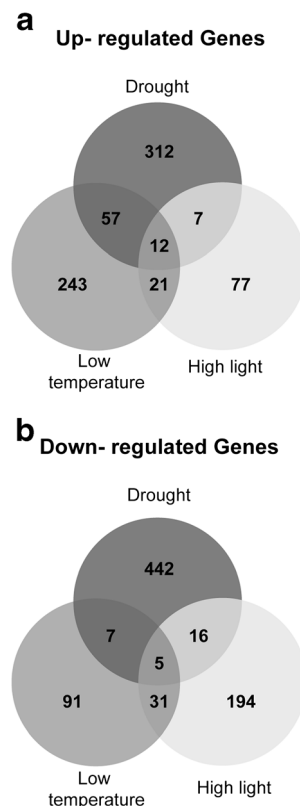
Genes preferentially up-regulated in response to a decrease in water availability included several members of the homeodomain, NAC, bZIP, zinc finger and MYB families of transcription factors (ESM\_1 and ESM\_2), which have previously been implicated in transcriptional responses to drought in general (Ariel et al. 2007; Gollmack et al. 2011; Nakashima et al. 2012; Uno et al. 2000) as well as specifically in potato (Kondrak et al. 2012; Vasquez-Robinet et al. 2008). In addition to transcription factors, the list of strongly drought up-regulated genes included genes encoding metabolic enzymes

such as the proline dehydrogenase *ERD5*, or signaling factors such as phosphatases, known to mediate ABA signaling in other species (ESM\_1 and ESM\_2). Genes down-regulated under drought were mostly involved in protein translation (ESM\_1 and ESM\_2). In addition, several genes associated with cell wall elongation and chloroplast function were also down-regulated in these water stressed plants (ESM\_1 and ESM\_2).

A significant overlap was found between genes up-regulated in response to drought, and those up-regulated in response to low temperature, which was twice the overlap expected by chance ( $p=1.6 \times 10^{-28}$ , hypergeometric distribution) (Fig. 1a). Some of these genes encoded transcriptional regulators of the b-ZIP gene family, which are likely involved in ABA signaling (ESM\_3). In addition, genes involved in sugar metabolism, mainly starch degradation and sucrose synthesis, were also found among those simultaneously up-regulated in response to both stress treatments (ESM\_1 and ESM\_3). Interestingly, a common consequence of plant exposure to drought or low temperature conditions is a reduction in leaf water potential and, therefore, the enhanced expression of genes involved in starch degradation and sucrose synthesis might have an osmotic function helping to maintain cell turgor under water limiting conditions. Interestingly, we found an aquaporin related gene whose expression was strongly up-regulated in response to low temperatures but was strongly down-regulated in response to drought (ESM\_4). The opposite behavior of this gene most likely reflects the different adaptive strategies activated by these two stresses to overcome a similar physiological problem. Indeed, while reduced leaf water potential under drought is caused by limited water availability, dehydration under low temperature conditions is triggered by reduced rates of water transport into cells. Therefore, the down-regulation of this aquaporin could be involved in minimizing water loss from cells under drought, while its up-regulation in response to low temperatures most likely facilitates water flow into cells, helping to maintain high water potentials within cells in both cases.

On the other hand, more than 200 genes were affected by the cold treatment but not (or to a much lesser extent) by water deficit (Fig. 1a). An interesting subset of genes in this class included *LATE ELONGATED HYPOCOTYL 1* (*LHY*) and several of its homologs, such as *REVEILLE1* (*RVE1*) and *RVE8/LCL5* (ESM\_5). *LHY* is a single MYB transcription factor that is part of the circadian clock in plants (Schaffer et al. 1998). *LHY* acts by directly promoting or repressing the expression of core-clock components, as well as many clock output genes (Carre and Kim 2002). Interestingly, *LHY* has recently been shown to control the circadian regulation of *CBF1-3* expression and to mediate the cold induction of their expression in Arabidopsis. Furthermore, double mutants of *LHY* and its closest homolog *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) display increased sensitivity to

**Fig. 1** Venn diagrams showing common and specific effects of different abiotic stress treatments on gene expression in potato plants. **a** Up-regulated genes and **b** Down regulated genes



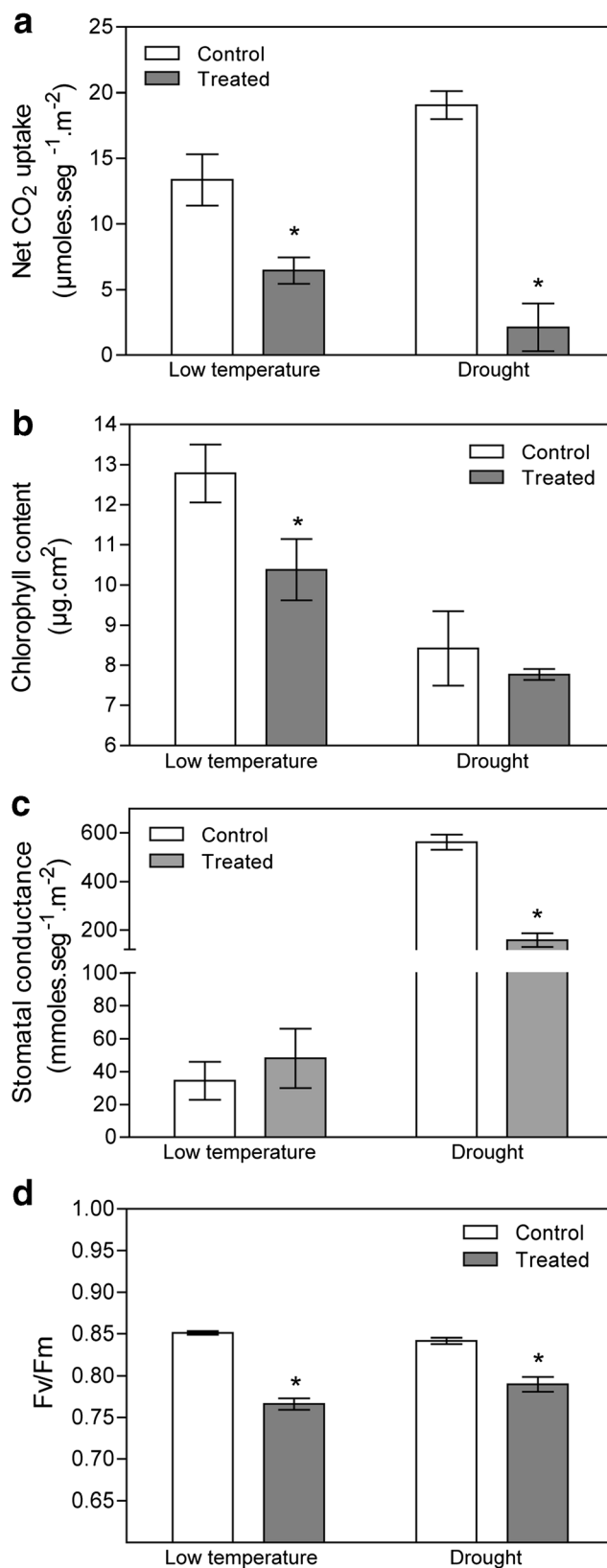
freezing temperatures (Dong et al. 2011). Thus, the observation of increased expression of *LHY* and several of its homologs in response to cold suggests that this gene family may have an important role mediating temperature responses in the plant kingdom, helping to keep the cold transcriptome under circadian control.

Among genes down-regulated in response to low temperature conditions we found many associated with mitochondrial electron transport. This might be associated with increased electron flow through the alternative oxidase pathway, which might function to minimize the production of reactive oxygen species under low temperature conditions. Indeed, an almost ten-fold increase in expression was observed for a gene encoding an alternative oxidase homologue (*AOX*) in response to the low temperature treatment (see probe ID Les.4223.1.S1\_at S1). Interestingly, AOX enzymes function to prevent the formation of an excess reactive oxygen species in the mitochondria under stressful environmental conditions, but may also have more pervasive effects, including some that are extramitochondrial (Fiorani et al. 2005).

As expected, all the above mentioned transcriptional changes were associated with rapid changes in physiological processes. Both drought and low temperature conditions triggered a reduction in photosynthetic activity (Fig. 2a). This response was associated with a reduction in chlorophyll levels in plants exposed to low temperatures but not in those exposed to drought (Fig. 2b). On the other hand, plants exposed to drought (but not those under low temperature conditions) displayed reduced leaf conductance (Fig. 2c). Both treatments triggered a reduction in the Fv/Fm ratio (Fig. 2d), which indicates photo-oxidative damage most likely generated by absorbed light that exceeds the photosynthetic capacity of plants exposed to these abiotic stresses.

Since both low temperature and drought conditions increase oxidative stress leading to photo-inhibition, we expected to find a large overlap between genes induced by high light stress, compared to those induced in response to drought and low temperatures. To specifically evaluate the effect of an excess of radiant energy, potato plants grown in a growth chamber under low light conditions ( $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 1 month were transferred to an identical growth chamber under high light conditions ( $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), keeping them at warm temperatures ( $22^\circ\text{C}$ ) and well irrigated. Interestingly, a strong and fast hyponastic response was observed in plants exposed to high light conditions (Fig. 3a), which most likely contributed to minimize light exposure. In spite of this light

avoidance response, this treatment caused a decrease in the Fv/Fm ratio (Fig. 3c) as well as in photosynthetic activity (Fig. 3b). However, no changes in overall chlorophyll levels



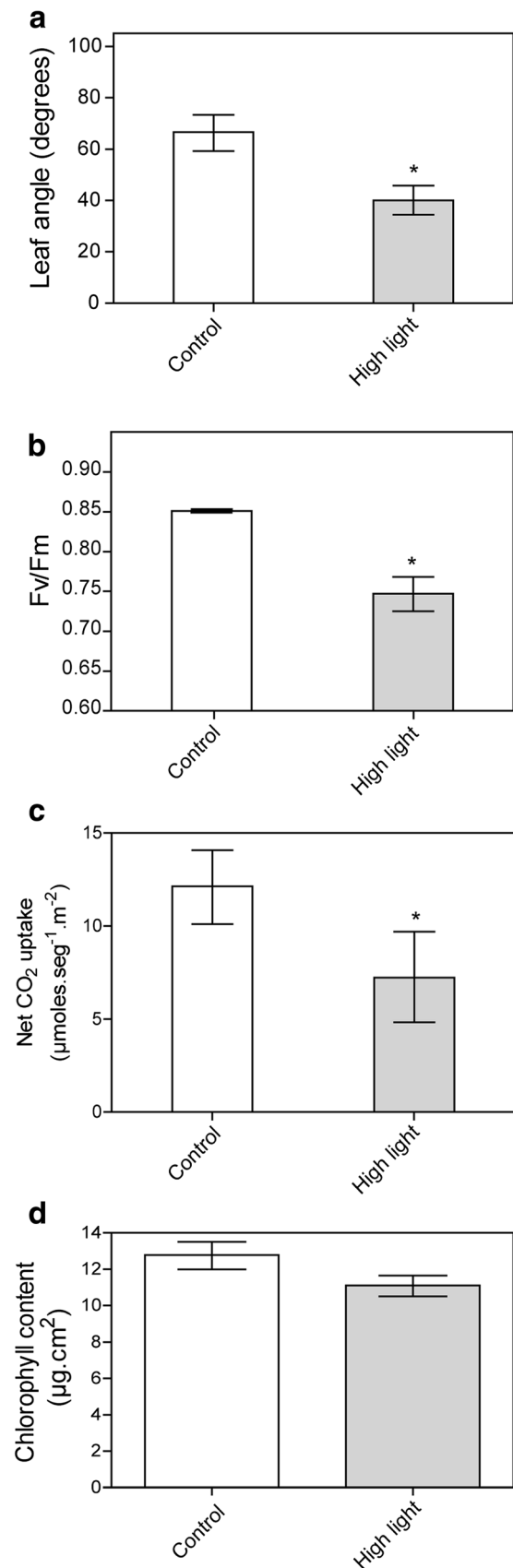
**Fig. 2** Physiological parameters measured in potato plants exposed to drought and low temperature stresses. **a** Photosynthetic rate. **b** Leaf chlorophyll content. **c** Stomatal conductance. **d** Fv/Fm. In **(a)** data are mean $\pm$ SD of four biological replicates. In **(b)** and **(d)** data are mean $\pm$ SD of three biological replicates. In **(c)** data are mean $\pm$ SD of five biological replicates. Asterisk indicates significant differences between treatments ( $p < 0.05$ )

**Fig. 3** Physiological parameters measured in potato plant under high light stress. **a** Angle between the third leaf and the stem. **b** Fv/Fm. **c** Photosynthetic rate. **d** Chlorophyll content. In **(a)** and **(b)** data are mean  $\pm$ SD of three biological replicates. In **(c)** and **(d)** data are mean  $\pm$ SD of five biological replicates. Asterisk indicates significant differences between treatments ( $p < 0.05$ )

were observed during the first 24 h under high light conditions (Fig. 3d). Thus, these data shows both convergent as well as stress specific responses to high light conditions compared to those seen in response to drought and cold stress. Interestingly, from the 1427 genes whose expression increased or decreased more than three-fold under stress conditions, only 17 had a simultaneous response in the same direction (12 up-regulated and 5 down-regulated) under the three stress treatments evaluated (Fig. 1). Among the 12 genes that were up-regulated, five were associated with secondary metabolism, particularly with the production of phenolic compounds such as anthocyanins and flavonoids (Table 1). Anthocyanins are colored pigments, which can protect plants against ultraviolet light (Steyn et al. 2002). Among the genes associated with key enzymes in this pathway that were up-regulated more than three fold in all stress treatments, we found a gene encoding a *chalcone synthase* (*CHS*), an observation that was confirmed by RT-qPCR (Fig. 4a). Extending the analysis to those genes that showed at least a two-fold change in expression in the same direction under all conditions revealed a similar enrichment in genes involved in phenylpropanoid biosynthesis (10 out of 30 genes) (Table 1). Another key rate limiting enzyme in this pathway that was up-regulated more than two fold under all three stress treatments is *phenyl alanine ammonia lyase* (*PAL*) (Fig. 4b). Although the statistical significance of the high-light effect was slightly above the threshold for this particular gene (*ESM\_1*), the effect was confirmed by RT-qPCR. Thus, these results suggest that the synthesis of protective pigments is likely to be the main common mechanism underlying adaptation to different abiotic stresses in potato plants. Consistent with this hypothesis, we found an increase in the amount of anthocyanins in response to the three stresses evaluated in this work (Fig. 4c).

A subset of genes involved in maintaining redox homeostasis was strongly induced under high light stress but not in response to the other stress treatments (*ESM\_1* and *ESM\_6*). This finding suggests that plants exposed to drought and low temperature conditions only had a moderate oxidative stress and, thus, the increment in the synthesis of protective pigments was likely an anticipatory response to protect plants from further oxidative damage rather than an immediate response triggered by strong oxidative damage.

In addition to the subset of genes that were commonly up-regulated by all three stresses, five genes were down-regulated at least three-fold under all stress conditions (Table 2). This





**Table 1** Genes up-regulated in the three stresses analyzed

Affy ID	Description	Fold change (treatment/control)		
		Drought	Low temperature	High Light
Fold change >3				
Les.1968.1.A1_at	No hits found	5.01	10.88	20.74
Les.2672.1.S1_s_at	Putative acid phosphatase [ <i>Lycopersicon esculentum</i> ]	9.14	11.82	3.93
Les.3650.1.S1_at	Chalcone synthase 1; Naringenin-chalcone synthase 1	3.83	8.50	63.55
Les.4472.1.S1_at	Hydroxycinnamoyl CoA quinate transferase	4.03	21.87	3.78
LesAffx.16884.1.S1_at	PREDICTED: ripening-related protein-like [ <i>Vitis vinifera</i> ]	4.46	17.08	3.40
LesAffx.23563.1.A1_at	Putative transformer serine/arginine-rich ribonucleoprotein	4.61	5.16	3.16
LesAffx.34276.1.S1_at	Cinnamoyl-CoA reductase, putative [ <i>Ricinus communis</i> ]	6.59	9.02	6.80
LesAffx.34276.2.S1_at	Cinnamoyl-CoA reductase, putative [ <i>Ricinus communis</i> ]	6.86	7.82	4.09
LesAffx.61398.1.S1_at	PREDICTED: hypothetical protein [ <i>Vitis vinifera</i> ]	3.56	35.19	9.38
LesAffx.63776.1.S1_at	Flavonoid 3-glucosyl transferase [ <i>Solanum tuberosum</i> ]	3.26	8.16	31.90
LesAffx.68320.1.S1_at	Chalcone isomerase-like protein [ <i>Populus trichocarpa</i> ]	4.64	11.05	20.95
LesAffx.8748.1.A1_at	Putative acid phosphatase [ <i>Lupinus luteus</i> ]	6.05	6.15	7.52
Fold change >2				
Les.2063.1.A1_at	No hits found	2.13	4.86	3.71
Les.2278.1.S1_at	Flavanone 3 beta-hydroxylase [ <i>Solanum tuberosum</i> ]	2.64	4.33	4.36
Les.2403.1.S1_at	Flavonoid glucosyltransferase UGT73E2 [ <i>Antirrhinum majus</i> ]	2.03	9.54	4.94
Les.2988.1.S1_at	Cinnamic acid 4-hydroxylase [ <i>Solanum tuberosum</i> ]	3.30	11.51	2.01
Les.3085.1.S1_at	Flavonol synthase [ <i>Solanum tuberosum</i> ]	2.68	3.34	4.98
Les.3319.3.S1_at	Putative allantoinase [ <i>Solanum tuberosum</i> ]	9.75	3.43	2.40
Les.3357.1.S1_at	POP1; transporter [ <i>Arabidopsis thaliana</i> ]	2.39	6.68	2.12
Les.4356.2.S1_at	Pyruvate, phosphate dikinase	20.33	5.83	2.52
Les.4452.1.S1_at	Putative anthocyanin permease [ <i>Solanum lycopersicum</i> ]	2.36	5.83	76.76
Les.4617.1.S1_at	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase;	2.30	2.19	6.34
Les.702.1.S1_at	S-adenosylmethionine decarboxylase 2 [ <i>Solanum lycopersicum</i> ]	2.63	17.22	2.99
LesAffx.18686.1.S1_at	Phosphatase [ <i>Arabidopsis thaliana</i> ]	9.67	2.78	2.82
LesAffx.3698.3.S1_at	Cytochrome P450 NADPH-reductase [ <i>Petunia x hybrida</i> ]	2.86	14.44	3.33
LesAffx.63609.2.S1_at	Cysteine-type peptidase, putative [ <i>Ricinus communis</i> ]	2.46	4.47	2.21
LesAffx.63984.1.S1_at	CYP98A33v1 [ <i>Nicotiana tabacum</i> ]	2.09	28.37	3.79
LesAffx.66657.1.S1_at	Glutaredoxin [ <i>Populus trichocarpa</i> ]	5.14	2.27	4.51
LesAffx.67629.1.S1_at	Serine-threonine kinase [ <i>Persea americana</i> ]	2.70	6.45	2.60
LesAffx.9229.1.S1_at	Alpha/beta hydrolase, putative [ <i>Ricinus communis</i> ]	2.93	2.10	6.77

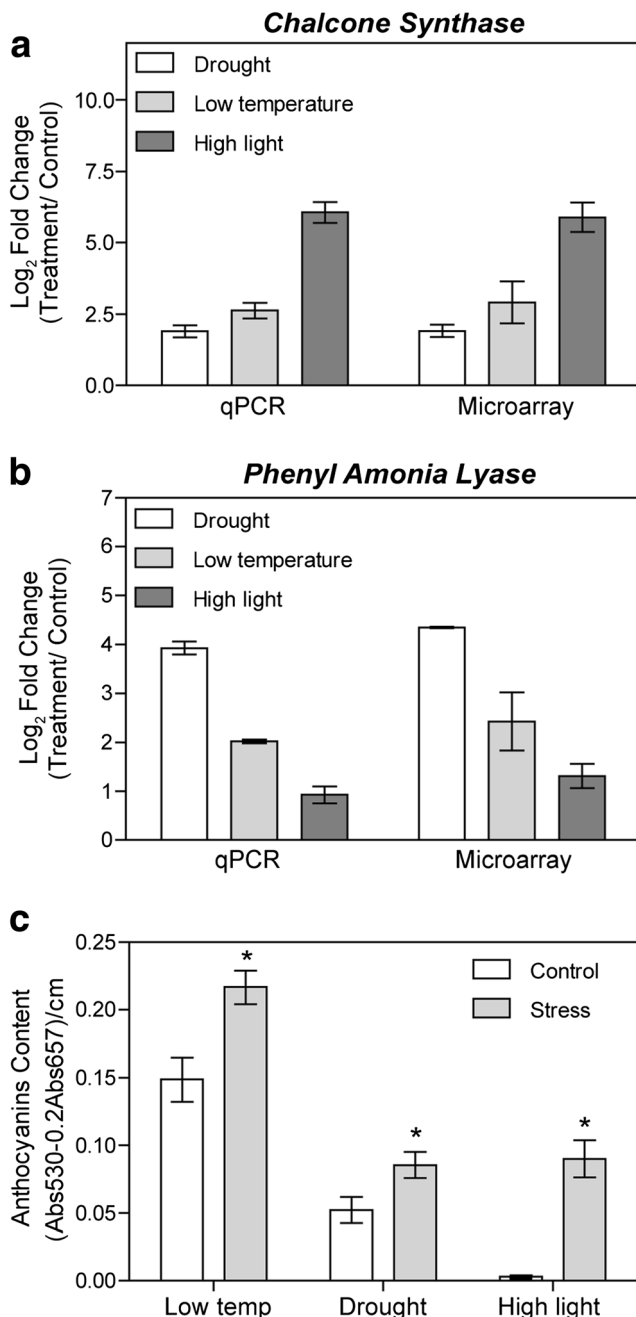
The first column displays the probe ID code, the second column displays the gene description, and the third column displays the Fold Change values

group increased to 19 genes if we considered those genes down-regulated at least two-fold in response to all stresses. A distinct functional category was not identified among these genes, but we found genes encoding an expansin, an aquaporin and a cell wall remodeling enzyme, which might be associated with a reduction in cell elongation and leaf area normally observed in response to these stresses (ESM\_7). Finally, light signals acting through specific photoreceptors, such as phytochromes and cryptochromes, have been shown to modulate responses to drought, low temperature and high light stresses (Carvalho et al. 2011; Yu et al. 2010). Interestingly, the three abiotic stresses evaluated here triggered a reduction in the expression of *CRYPTOCHROME INTERACTING*

*bHLH 1 (CIB1)* (ESM\_8), a bHLH transcription factor shown to mediate light regulation of flowering time in *Arabidopsis* (Liu et al. 2008). Thus, it will be interesting to test whether *CIB1* plays a role modulating stress responses in potato or other plants.

#### Potato Plants Overexpressing the *Arabidopsis thaliana* *CBF1* Gene Showed Enhanced Tolerance to High Light Conditions at Warm Temperatures

Overexpression of the CBF family of transcription factors (DREB1s) has been shown to confer tolerance to multiple abiotic stresses in several species (Khan 2011). In potato in



**Fig. 4** Effect of abiotic stress on anthocyanin biosynthesis, *CHS* and *PAL* expression. **a** *Chalcone Synthase* expression analyzed by microarrays and qRT-PCR under three different abiotic stresses in potato plants. **b** Expression level of *Phenyl Ammonia Lyase* analyzed by microarrays and qRT-PCR under three different abiotic stresses. **c** Anthocyanin content in petioles of plants exposed to drought and low temperature stresses. Data are mean±SD of five biological replicates. Asterisk indicates significant differences between treatments ( $p < 0.05$ )

particular, *AtCBF1* overexpression has been shown to confer freezing, drought and salinity stress tolerance (Behnam et al. 2007; Movahedi et al. 2012), while over-expression of *AtCBF3* confers heat stress tolerance (Dou et al. 2015). Since low temperature conditions and drought affect plant growth enhancing susceptibility to photoinhibition, we

hypothesized that tolerance to multiple stresses in plants overexpressing *CBF1* could be associated with enhanced tolerance to high light stress. To test this possibility we developed transgenic potato plants overexpressing the *Arabidopsis thaliana CBF1 (AtCBF1)* gene, and characterized tolerance of these plants to low temperatures, drought and high light stress.

Eleven independent transgenic lines were obtained, which were positive for a PCR reaction detecting the *AtCBF1* transgene (Fig. 5a). These 11 independent lines also displayed detectable levels of the *AtCBF1* mRNA by RT-PCR (Fig. 5b). Two lines, *AtCBF1-OX-6* and *AtCBF1-OX-11*, were selected for further studies. Transgenic and wild type (WT) plants were grown in a growth chamber under long day conditions, and plant height, internode length, and tuber yield were measured (ESM\_9). Previous work has shown that ectopic expression of *AtCBF1* in tomato or potato produces growth retardation and dwarfism (Pino et al. 2007; Zhang et al. 2004). In agreement with this observation, these plants showed a strong (*AtCBF1-OX\_6*) or intermediate (*AtCBF1-OX\_11*) dwarf phenotype (Fig. 5c and ESM\_9). In contrast, although it was reported that the constitutive expression of the *AtCBF1* gene causes a reduction in tuber yield (Pino et al. 2007), we found that tuber yield was similar in WT and in our two transgenic lines, at least under the growth conditions we used for this evaluation (ESM\_9).

To evaluate the response of these plants to low temperature stress, WT and transgenic plants overexpressing *AtCBF1* were transferred from 22 to 4 °C. The Fv/Fm ratio decreased after 1 day at 4 °C in both genotypes, but the reduction was significantly less severe in the transgenic plants overexpressing *AtCBF1*, revealing an increased tolerance to low temperature conditions in these plants (ESM\_10). Indeed, after exposure to low temperature conditions for 2 weeks, chlorotic spots and wilting were observed in WT plants, while transgenic plants did not show any of these symptoms (ESM\_10).

To evaluate desiccation tolerance, leaves from transgenic and WT plants were cut and kept under continuous illumination at 22 °C, while fresh weight was evaluated during a 75 h period (ESM\_11). During the first hours, leaves from WT plants lost more water than leaves from transgenic plants, indicating enhanced drought sensitivity, which was coupled to a slower stomatal closure in comparison to the transgenic plants. We also evaluated drought tolerance with another assay that mimics more realistic drought stress conditions. For this, WT and *AtCBF1* transgenic plants were grown for 1 month with daily irrigation in a growth chamber under long day conditions at 22 °C. After 1 month, irrigation was interrupted for half of the plants. Notoriously, 10 days after interruption of irrigation, leaf wilting was observed in WT plants but not in *AtCBF1-OX* transgenic plants (ESM\_11d). This differential response to drought was associated with an earlier stomatal closure in the transgenic plants with respect to the WT plants, which allows them to maintain a better plant

**Table 2** Genes down-regulated in the three stresses analyzed

Affy ID	Description	Fold change (control/treatment)		
		Drought	Low temperature	High light
Fold change >3				
Les.173.1.S1_at	Putative aquaporin PIP-type [ <i>Lycopersicon esculentum</i> ]	10.48	3.60	3.93
Les.5638.1.S1_at	Putative basic helix-loop-helix protein BHLH7 [ <i>Lotus japonicus</i> ]	18.00	5.37	3.99
Les.65.1.S1_at	Gibberellin 20-oxidase-2; 20ox-2 [ <i>Lycopersicon esculentum</i> ]	15.31	5.58	4.09
Les.810.1.S1_at	GDSL-lipase protein [ <i>Capsicum annuum</i> ]	6.28	3.26	6.13
LesAffx.23465.1.S1_at	Zeamatin precursor, putative [ <i>Ricinus communis</i> ]	4.27	5.30	4.92
LesAffx.53857.1.S1_at	Glutaredoxin [ <i>Populus trichocarpa</i> ]	18.62	27.56	10.16
Fold change >2				
Les.1546.1.A1_at	Lustrin A-like [ <i>Oryza sativa Japonica Group</i> ]	2.13	17.10	5.21
Les.210.1.S1_at	Xyloglucan endo-transglycosylase precursor	3.32	2.53	4.45
Les.2360.1.S1_at	Nitrite reductase [ <i>Nicotiana tabacum</i> ]	3.58	2.19	3.42
Les.3733.1.S1_at	Alpha-expansin [ <i>Cicer arietinum</i> ]	21.21	2.61	4.87
Les.4099.1.S1_at	Phosphoenolpyruvate carboxylase kinase 2	3.66	2.10	2.70
Les.5850.1.S1_at	NADPH:protochlorophyllide oxidoreductase	2.81	3.48	18.59
Les.641.1.S1_at	ERD3 (early-responsive to dehydration 3) [ <i>Arabidopsis thaliana</i> ]	3.94	2.56	2.19
LesAffx.51979.1.S1_at	Actin depolymerizing factor 5 [ <i>Gossypium hirsutum</i> ]	2.03	2.02	2.22
LesAffx.53857.2.S1_at	Glutaredoxin [ <i>Populus trichocarpa</i> ]	2.43	2.36	2.67
LesAffx.57251.1.S1_at	Fasciclin-like arabinogalactan protein 4 [ <i>Gossypium hirsutum</i> ]	5.30	2.83	2.55
LesAffx.61034.1.S1_at	LOL1 (LSD ONE LIKE 1); DNA binding [ <i>Arabidopsis thaliana</i> ]	6.03	4.02	2.21
LesAffx.64171.1.S1_at	LOB domain-containing protein, putative [ <i>Ricinus communis</i> ]	93.82	2.23	3.28
LesAffx.71242.2.S1_at	Transcription factor, putative [ <i>Ricinus communis</i> ]	3.71	2.90	4.47

The first column displays the probe ID code, the second column displays the gene description, and the third column displays the Fold Change values

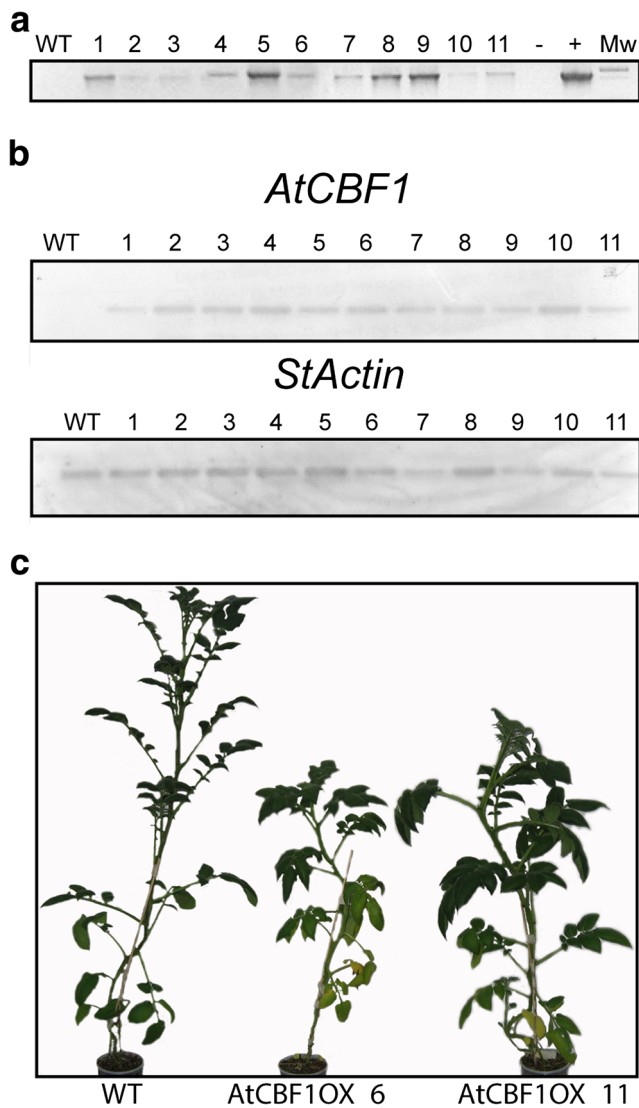
water status as well as to keep more water in the soil for a longer period of time (ESM\_11). In addition, transgenic plants over-expressing *AtCBF1* seemed to be more tolerant to drought stress as a consequence of reduced photo-inhibition. As shown in ESM\_11e, drought caused a reduction in Fv/Fm in both genotypes, but this reduction was less intense in the *AtCBF1* transgenic plants. This was also associated with a milder decrease in photosynthetic activity in the *AtCBF1* transgenic compared to WT plants in response to drought (ESM\_11).

Finally, to test if some of the enhanced tolerance to drought and low temperature was due to an improved ability to tolerate an excess of radiant energy, we conducted experiments where the only environmental variable modified was light intensity. The Fv/Fm ratio decreased similarly in all genotypes in plants transferred for 24 h from low ( $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) to high light conditions ( $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) (Fig. 6a). However, after a few days, chlorotic spots were detected in WT but not in *AtCBF1-OX* plants (Fig. 6f). This response was associated with a decrease in photosynthetic rate (Fig. 6b), which was stronger in WT than in transgenic plants. In addition, while WT plants showed a strong hyponastic response to high light stress, this response was strongly attenuated in the transgenic plants

(Fig. 6c). WT plants showed a reduction in leaf area after 30 days compared to the transgenic plants grown under the same conditions (Fig. 6d), most likely as a consequence of a decrease in the amount of intercepted radiation in WT plants, together with a decrease in photosynthetic rate (Fig. 6b). Anthocyanin content was constitutively higher in the transgenic plants under control conditions, allowing transgenic plants to overcome the stress imposed by the high light treatment better than WT plants (Fig. 6e). This difference in anthocyanin content between genotypes was maintained under the stress treatment (Fig. 6e).

### Transcriptional Changes Associated with *AtCBF1* Overexpression in Potato Plants

To better understand the molecular mechanisms behind the multiple stress tolerance observed in *AtCBF1* transgenic plants, we compared the transcriptional changes triggered by *AtCBF1* overexpression with those induced in response to low temperature, high light, and drought stress in potato plants. For this, we first identified genes whose expression was either up or down regulated by *AtCBF1* under control conditions, i.e. in well watered plants grown at warm temperatures, under



**Fig. 5** Molecular and phenotypic characterization of *AtCBF1*-OX transgenic potato plants. **a** Southern-PCR of 11 independent transgenic lines. **b** RT-PCR for *AtCBF1* in potato transgenic plants. **c** One month old plants grown in 300 ml plastic pots

moderate/low light intensity conditions. We found 138 genes that showed at least a two-fold increase in expression, and 187 genes with a two-fold decrease in mRNA levels in *AtCBF1* over-expressing plants compared to WT plants (ESM\_12). Interestingly, 45 of the 138 genes up-regulated by *AtCBF1* over-expression were also up-regulated in response to at least one of the stress treatments analyzed before. In addition, 34 of the 187 genes down-regulated in *AtCBF1* transgenic plants were also affected by at least one of the stress treatments analyzed. This observation suggests that over-expression of *AtCBF1* confers multiple stress tolerance by modulating the expression of a subset of abiotic stress-responsive genes.

Several genes that could be responsible for the enhanced stress tolerance observed in the potato *AtCBF1* transgenic

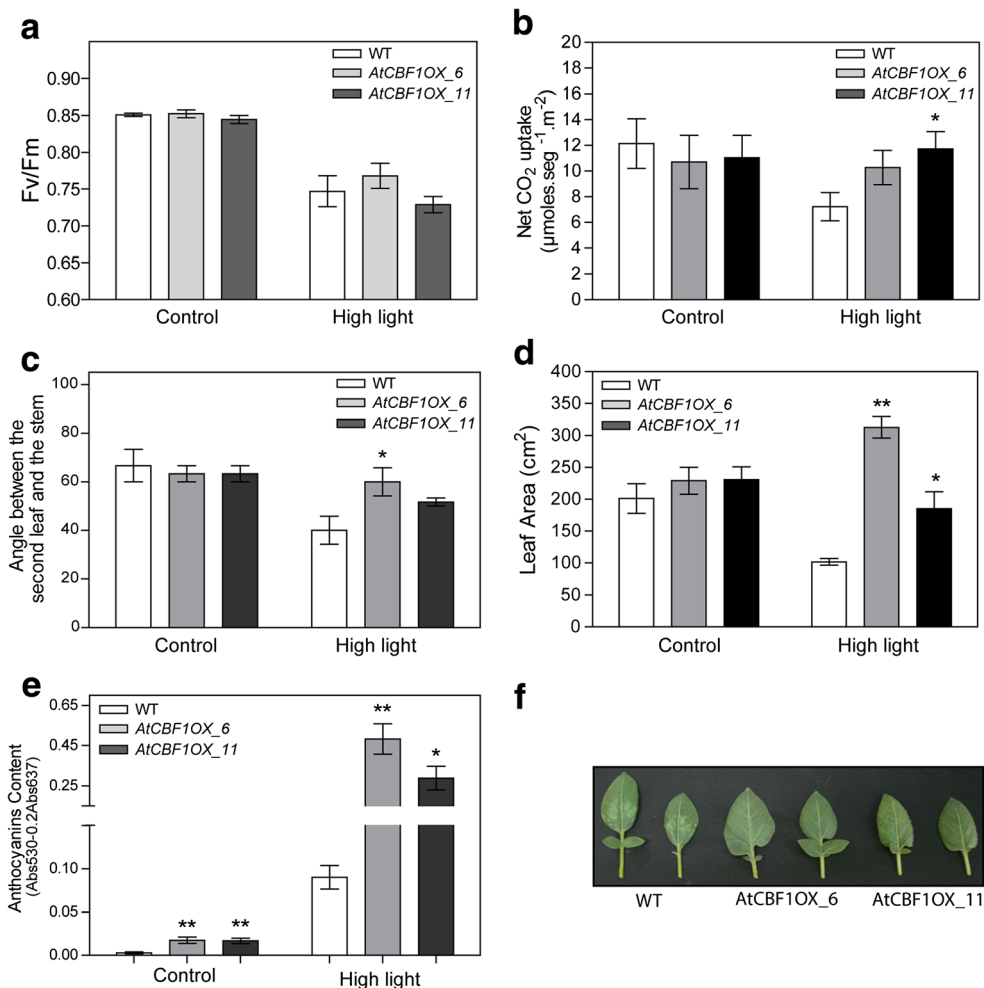
plants were identified. One of the genes that was strongly up-regulated by ectopic overexpression of *AtCBF1* was a homolog of *ANI*, a transcription factor recently shown to confer enhanced abiotic stress tolerance (Ben Saad et al. 2010; Huang et al. 2008). In addition, genes that are known components of the CBF1 regulon, such as *ERD10* and *LEA 14*, were also up-regulated in the potato *AtCBF1* transgenics, suggesting that the stress tolerance phenotypes are linked to known activities of CBF1 (Carvalho et al. 2011; Gilmour et al. 2004) (ESM\_13). Interestingly, a gene that could explain some of the enhanced tolerance to high light stress is that encoding *NADPH:PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B (PORB)*. Reduced levels of the *PORB* gene product are usually associated with photoinhibition caused by chlorophyll degradation (Frick et al. 2003). Expression of this gene was strongly reduced in WT plants in response to high light, but this reduction was attenuated in the transgenic plants (ESM\_14), and observation that was confirmed by RT-qPCR (ESM\_14).

Finally, potato plants have *CBF1* orthologue, and therefore it was interesting to determine if the expression of this gene was responding to the stress treatments evaluated here and/or if overexpression of *AtCBF1* had any effect on the expression of the potato *CBF1* orthologue. Unfortunately, expression levels of the potato *CBF1* orthologue in our microarray data were below the threshold required to consider expression data as reliable for further statistical analysis and, therefore this gene was not present in our lists of expressed genes. However, an inspection of the raw data revealed that the signal intensity of the microarray probe associated with the potato *CBF1* gene indeed increased more than ten times in wild type as well as in potato plants expressing the *AtCBF1* gene in response to cold conditions (data not shown), as previously reported (Pennycooke et al. 2008). Under warm conditions, however, expression of the potato *CBF1* orthologue was extremely low and it was therefore not possible to determine whether the expression of the endogenous gene was affected to any significant extent by overexpression of its Arabidopsis orthologue.

### Transcriptional Profiles as an Aid for Risk Assessment of Transgenic Plants Expressing a Heterologous Transcription Factor

The first generation of transgenic plants authorized for cultivation in the field were genetically modified with single genes controlling simple traits, such as herbicide tolerance, or enhanced resistance to different pests (Shah et al. 1986; Vaeck et al. 1987). In contrast, the next generation of transgenic plants includes those that over-express transcription factors that can simultaneously affect, directly or indirectly, many different traits, contributing to increasing crop yield by modulating

**Fig. 6** Tolerance to high light stress in *AtCBF1*-OX transgenic potato plants. **a** Fv/Fm. **b** Photosynthetic rate and chlorophyll content in stress treated plants relative to control plants. **c** High light avoidance measured as the angle between the third leaf and the stem. One month old WT and transgenic potato plants were transferred from  $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  to  $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 1 day before measurements. **d** Leaf area of 2 month old plants. **e** Anthocyanin content in petioles of plants exposed to high light stress. **f** Leaves from transgenic and WT plants exposed to high light conditions. In **d**, **e** and **f**, plants were grown under control or high light conditions ( $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 1 month. \* and \*\* indicate significant differences between *AtCBF1*-OX plants and WT controls under each condition ( $p < 0.05$  and  $p < 0.01$  respectively)

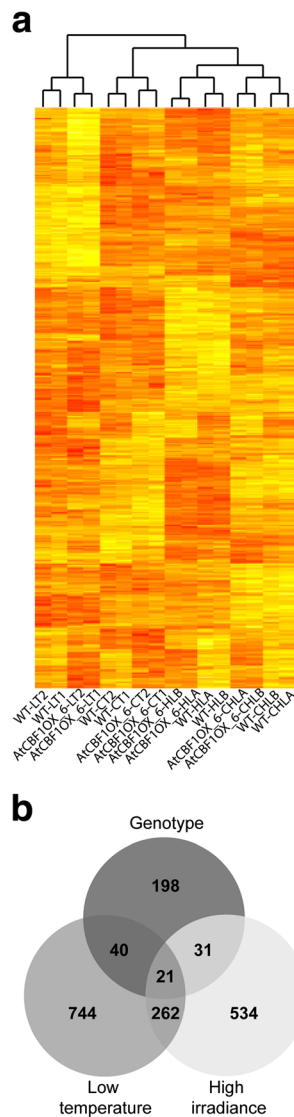


developmental programs or tolerance to abiotic stresses (Preuss et al. 2012). An important issue that needs to be addressed by the governmental regulatory agencies controlling the cultivation of genetically modified (GM) plants in the field is how to assess the environmental and health related risks associated with this next generation of transgenic plants, and which risk management procedures should be implemented (Ricroch et al. 2011).

The demonstration of “substantial equivalence” with their non-genetically modified counterparts is a key regulatory requirement for commercialization of GM plants (Craig et al. 2008). Non-targeted omic approaches have been useful to evaluate this issue (Ricroch et al. 2011). In particular, many studies have shown that first generation GM plants are usually substantially equivalent to their non-GM counterparts in terms of their chemical composition, analyzed with metabolomic approaches, and also in terms of their transcriptome, analyzed through DNA microarrays (Ricroch et al. 2011). However, GM plants over-expressing regulatory transcription factors are likely to target many different genes and pathways and, therefore, differences in their

transcriptional profiles compared to their non-GM counterparts are expected. Then, it is important to evaluate the extent of those changes both quantitatively and qualitatively in order to obtain adequate information to estimate and evaluate environmental and/or health risks associated with this generation of GM plants.

To further evaluate the overlap between *AtCBF1* and stress regulated genes in potato plants we analyzed the effect of *AtCBF1* not only under control conditions, but also the transcriptome of WT and *AtCBF1* overexpressing plants exposed to low temperatures or high light conditions (Fig. 7). We performed a global analysis of transcriptional profiles to quantitatively compare the magnitude of changes caused by overexpression of the *AtCBF1* transcription factor in relation to those induced by changes in environmental conditions normally experienced by plants on a daily and/or seasonal basis. The dendrogram structure resulting from hierarchical clustering of sample expression profiles revealed that biological variability, dissimilar genotypic backgrounds, differences in control conditions and, finally, high light stress and low temperature induced transcriptional changes present an increasing level of global dissimilarity (Fig. 7a). Particularly relevant to this



**Fig. 7** Transcriptome analysis of wild type and *AtCBF1*-OX transgenic plants exposed to abiotic stresses. **a** Global transcriptomic analysis using gene expression heatmaps. The transcriptional profiles of a non-specifically filtered subset of 2184 genes were considered. Sample names were built using the following keywords: WT, CBF1, for wild type and *AtCBF1*-OX genotype samples respectively, CHL, HL for control high-light, and high-light samples respectively, CT, LT for control temperature and low-temperature samples respectively. Numbers 1 and 2 indicated for the CT and LT samples, as well as the letters *A* and *B* indicated for the CHL and HL samples represent the two different biological replicates obtained for each treatment. Complete-linkage hierarchical clustering was used to reorder samples and genes according to relative similarities to highlight global patterns of gene expression. **b** Venn diagram for gene sets differentially modulated (Benjamini-Hochberg adjusted *p*-values below the 0.01 level and a fold-change greater than 2) under the three main effects considered in this work: genomic background, light, and temperature treatments

study was the finding that the magnitude of changes associated with environmental stress treatments was greater than the one linked to genotypic effects. This was in line with the observation that a linear model analysis of differential

expression reported 290, 848, and 1067 differentially expressed genes (adjusted *p*-value  $q=0.01$ ) associated to genomic background, light, and temperature main effect factors respectively (Fig. 7b).

Similar results were also obtained using a template matching analysis of expression profiles. For a given statistical significance level, light stress treatment committed the largest set of genes, which was approximately 20 % larger than the one induced by temperature stress, and up to three times larger than the one associated with genetic background differences (ESM\_15). Finally the multi response permutation test procedure (MRPP)- a multivariate non-parametric procedure for testing the hypothesis of no difference between two or more groups -, indicated that the transcriptional changes triggered by low temperature and high light stresses were approximately five times larger than those associated with *AtCBF1* over-expression (ESM\_16). These results, taken together, strongly indicate that over-expression of a key stress-related transcription factor has modest effects on the transcriptome compared to the effect triggered by fluctuations in environmental conditions.

Along a quantitative assessment of induced global transcriptional changes, an extremely important step needed for risk assessment of transgenic plants over-expressing a transcription factor is to identify unintended effects associated with this genetic modification. Given that the aim of over-expressing *AtCBF1* is to enhance abiotic stress tolerance, those genes that were regulated by *AtCBF1* but not by abiotic stress constitute a cluster of unintended target genes. To further evaluate the overlap between *AtCBF1* and stress regulated genes in potato plants we analyzed the effect of *AtCBF1* not only under control conditions, but also the transcriptome of WT and *AtCBF1* over-expressing plants exposed to low temperatures or high light conditions. An overlap analysis of the set of genes showing statistically significant differential changes under the three main effects was used to identify genes regulated by *AtCBF1* but not by abiotic stresses, genes regulated by high light and/or low temperature but not by *AtCBF1*, as well as genes regulated by *AtCBF1*, high light and low temperatures (Fig. 7b and ESM\_17). The cluster of genes regulated only by *AtCBF1* included 198 genes. Although some of these genes might be regulated by abiotic stress under conditions not evaluated here, or at different times during the course of exposure to stress, a significant proportion of this subset of genes may represent true non-stress related targets of *AtCBF1* activity. Therefore, this subset of genes should be the focus of the analysis aimed at evaluating potential environmental or health related hazards associated with transgenic plants over-expressing *AtCBF1*, before these or similarly GM plants are authorized for cultivation in the field and eventually for commercial release.

## Conclusions

The use of a non-targeted genomics approach allowed us to identify genes simultaneously regulated by different abiotic stresses in potato plants. This data set can help identifying novel candidate genes that could be used to develop stress tolerant potato plants. In addition, our results show that comparison of an “abiotic stress transcriptome” with that of GM plants over-expressing a stress related transcription factor is a useful strategy to determine potential mechanisms through which particular transcription factors confer stress tolerance. Indeed, in this case we provide strong evidence that tolerance to multiple abiotic stresses in potato plants over-expressing *AtCBF1* is due, at least in part, to an increased ability to tolerate radiant energy in excess of the maximal amount that can be channeled through the photosynthetic process. Since this tolerance to multiple abiotic stresses may be due to increased anthocyanins levels, and these metabolites are produced at the expense of diverting carbon away from TCA intermediates, it will be important to evaluate carbon metabolism and sugar composition in the tubers of these or similar transgenic plants before they can be used for commercial purposes (Payyavula et al. 2012).

Finally, we also show that a comparative genomics strategy can be used to identify unintended targets, i.e. genes affected by the over-expression of a stress related transcription factor, which are not regulated by stress in wild type potato plants. This approach should help narrow down the list of genes on which bio-safety risk assessments should focus before this or similar GM plants are authorized for cultivation in the field.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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