

RESEARCH PAPER

Enhanced *in vitro* drought tolerance of *Solanum tuberosum* and *Solanum commersonii* plants overexpressing the *ScCBF1* gene

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Abstract

M.T. Pino, A. Ávila, A. Molina, Z. Jeknic, and T.H.H. Chen. 2013. Enhanced *in vitro* drought tolerance of *Solanum tuberosum* and *Solanum commersonii* plants overexpressing the *ScCBF1* gene. Cien. Inv. Agr. 40(1):171-184. Cultivated potato crops are sensitive to drought stress, reducing yield and tuber quality when the soil water potential drops to -0.3 MPa. However, drought not only affects plant growth and physiological activity, but this stress also induces biochemical and molecular changes in the cellular gene expression profile, triggering genes that play a direct role in plant protection and gene regulation. The genes involved in regulation include C-repeat Binding Factors/Dehydration responsive element binding (CBF/DREB) transcription factors, which increase cold, drought and salt tolerance in different plant species. The aim of this research was to evaluate whether the overexpression of the *ScCBF1* gene from *Solanum commersonii* enhances drought stress tolerance in transgenic *Solanum tuberosum* and *S. commersonii* plantlets grown *in vitro* and induces drought adaptation mechanisms, such as osmoprotectors and genes for osmotic adjustment and membrane stability. Drought conditions were simulated through the addition of polyethylene glycol (PEG4000) to hormone-free MS medium. The vegetative growth, root development, proline content, and *ScCBF1*, $\Delta 1$ -pyrroline-5-carboxylate synthetase (*P5CS*) and Dehydrins like genes (*DNH10*) expression were evaluated. The constitutive *in vitro* overexpression of *ScCBF1* in both potato species showed better overall plantlet growth and root development under drought stress and higher proline levels in the stems and leaves. A significant increase in *DNH10* expression was also associated with drought stress. In summary, the expression of *ScCBF1* in potatoes induces responses associated with drought adaptation mechanisms, resulting in better overall plant growth.

Key words: *CBF* gene, drought stress, *in vitro*, potato, *Solanum commersonii*, *Solanum tuberosum*.

Introduction

The cultivated potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world, with an annual production of approximately 325

million tons (FAO, 2012). Although grown in many different climates, potato crops are sensitive to drought stress. Low levels of water availability during potato crop growth significantly reduce yield, shortening the production period and limiting geographical distribution. Drought stress is particularly harmful during tuber formation, affecting tuber number, size and quality (Ekanayake

and Midmore, 1992). Drought not only affects tuber production but also primarily reduces vegetative growth, shoot length, leaf size and leaf number (Weisz *et al.*, 1994; Deblonde and Ledent, 2001). In addition to stomata closure, drought stress reduces gas exchange through reductions in the transpiration and photosynthetic rates (Ekanayake and Midmore, 1992; Dalla Costa *et al.*, 1997; Deblonde and Ledent, 2001; Kiziloglu *et al.*, 2006). The vulnerability of potato to drought is attributed to the shallow root system of this crop (50 to 80 cm deep), which does limit water uptake (Ekanayake and Midmore, 1992; Dalla Costa *et al.*, 1997). Drought tolerance could also be attributed to several genotype characteristics. However, the length and capacity of the root system are critical. The root/shoot dry weight ratio increases as plant water stress increases due to relative reductions in the shoot dry weight (Chazen and Neumann, 1994; Iwama, 2008). Drought also induces biochemical and molecular changes (Shinozaki and Yamaguchi-Shinozaki, 2007). The synthesis of compatible solutes, such as proline, soluble sugars, and glycine betaine, plays a crucial role in osmotic adjustments. Under osmotic stress, there is an increase in the concentration of free proline, which acts as an osmotic agent, protecting plants from dehydration (Kavi Kishor *et al.*, 2005; Szabados and Savoure, 2010). It has been suggested that proline participates in multiple roles in plant stress, stabilizing proteins and membranes and providing a source of carbon, nitrogen and energy during cell rehydration (Kavi Kishor *et al.* 2005; Szabados and Savoure, 2010). Proline has been associated with drought tolerance and other abiotic stresses in several plant species, such as rice (Su and Wu, 2004) and potato (Knipp and Honermeier, 2006). A study in different potato genotypes showed the accumulation of free proline in the leaves under drought stress (Bansal and Nagarajan, 1986). However, a recent study showed that the proline concentration increased earlier in drought susceptible varieties than in drought tolerant varieties, suggesting that proline accumulation is only a varietal characteristic in potatoes (Schafleitner *et al.*, 2007). Several genes are

either up or down regulated in response to osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007; Yang *et al.*, 2010). There are genes that directly protect against environmental stresses and genes that play a role in gene expression regulation and signal transduction (Shinozaki and Yamaguchi-Shinozaki, 2007). The genes that play a role in gene expression regulation and signal transduction include transcription factors such as C-repeat Binding Factors/Dehydration responsive element binding (*CBF/DREB*) genes (Liu *et al.*, 1998). The CBFs bind to the cold/dehydration responsive regulatory motif (CRT/DRE) and are present in the promoter of many drought and cold responsive genes, such as those associated with osmoprotectant, cold-responsive (*COR*) and late-embryogenesis-abundant (*LEA*) proteins (Liu *et al.*, 1998; Shinozaki and Yamaguchi-Shinozaki, 2007). In Arabidopsis plants, *AtCBF3* overexpression increased $\Delta 1$ -pyrroline-5-carboxylate synthetase (*P5CS*) transcript accumulation and consequently increased the proline concentration (Gilmour *et al.*, 2000). A similar response has been associated with CBF overexpression in some species, such as tomato (Hsieh *et al.*, 2002), tall fescue (Zhao *et al.*, 2007) and potato (Pino *et al.*, 2008). Studies in wheat have reported an association between the *CBF* genes and *LEA* expression upon abiotic stress (Kume *et al.*, 2005; Kobayashi *et al.*, 2005). Dehydrins like genes (*DHNs*) are members of the *LEA* protein family, and the most abundant class of dehydrins, such as the barley dehydrins *DHN1*, *DHN2*, *DHN3*, *DHN4*, *DHN6*, and *DHN9*, is induced in response to drought or ABA (Allagulova *et al.*, 2010). Some studies in potato and tobacco have also demonstrated an association of *CBF* overexpression with *LEA* protein expression. For example, the overexpression of *AtCBF* in potato substantially increased *Solanum tuberosum* Protein inhibitor (*StPI*) and *DHN10* transcript levels more than ω -9 stearoyl-ACP desaturase (ω -9) and *DHN2* (Pino *et al.*, 2008).

The expression of the CBF regulon increases cold, salinity and drought tolerance in different plant species (Jaglo *et al.*, 2001; Hsieh *et al.*,

2002; Kasuga *et al.*, 2004; Yang *et al.*, 2010). Recent studies have confirmed the existence of the CBF regulon in *Solanum commersonii*, a wild potato species highly tolerant to abiotic stress. *S. commersonii* CBF genes induce similar genes to those induced through CBF transcription factors from *Arabidopsis* (Pennycooke *et al.*, 2008; Carvallo *et al.*, 2011). Therefore, the CBF transcription factors of *S. commersonii* might play a central role in the response to low temperatures and drought stress in potatoes. The aim of this research was to evaluate whether the overexpression of the *ScCBF1* gene, cloned from *S. commersonii*, could enhance drought stress tolerance in genetically modified (GM) plants of *Solanum tuberosum* and *Solanum commersonii* grown under *in vitro* drought stress. To establish possible mechanisms for drought tolerance, the free proline content was measured. The expression of *P5CS* and *DNH10* was also evaluated, as both genes play a role in osmotic adjustment and membrane stability.

Materials and methods

Constructs and plant transformation

A 750 bp fragment containing the coding region of *ScCBF1* (GenBank: EU849672.1) was cloned into the PstI (SbfI) site of the binary transformation vector pCAMBIA1304. The resulting plasmid, Z45, containing the *ScCBF1* coding sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was transformed into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformation was conducted in a cultivated potato non-drought tolerant *S. tuberosum* (cultivar Cardinal) and a wild potato drought tolerant *S. commersonii* Dun (PI 243503 clone 13), according to Pino *et al.* (2008). The plantlets were further selected for hygromycin resistance (5 and 10 mg L⁻¹). Hygromycin-resistant rooted plantlets were analyzed for *ScCBF1* integration through PCR and Southern analyses. The 35S promoter forward primer 35S-P.001 (5'-cacgtcttcaaagcaagtg-3') and the *ScCBF1* gene

reverse primer (5'-gctagcttagatagaataattccataag-3') were used to verify the integration of the 35S-*ScCBF1* operon. The PCR reactions were run in an XP Thermal cycler (Bioer Co., Zhejiang, R.P China) for 35 cycles at 94 °C for 40 s, 55 °C for 40 s and 72 °C for 60 s.

Plant material and drought stress treatment

In this study, wild type (WT) and three GM lines (Lines 6, 21, 22) were used for *S. commersonii*, and WT and three GM lines (Lines 11, 15, 19) were used for *S. tuberosum*. The drought conditions were simulated *in vitro* through the addition of polyethylene glycol 4000 (PEG) mol. wt. 4000 (PhytoTechnology Laboratories., Shawnee Mission, KS, USA) to hormone-free MS medium. Briefly, MS (Murashige and Skoog, 1962) medium containing 20 g L⁻¹ sucrose and no hormones was supplemented with 0, 4 or 8% PEG, generating water potential (MPa) values of -0.021, -0.362 and -0.478, respectively. The media pH was adjusted to 5.7, and the media were solidified with 7.5 g L⁻¹ agar. Glass vessels (200 mL) containing 20 mL of the medium were used. *In vitro* drought stress treatments were performed based on the results of preliminary studies showing that potato vegetative growth was negatively affected when the soil water potential dropped to -0.3 MPa and that stomatal conductance and net photosynthesis were reduced when the soil water potential dropped to -0.55 MPa. The soil water potential was maintained between 0 and -0.03 MPa in irrigated potatoes and other species (Martínez and Moreno, 1992; Molinos *et al.*, 2004; Schafleitner *et al.*, 2007). The water potential of all media was measured using a Vapor Pressure Osmometer Vapro 5520 (Wescor, Inc., Logan, Utah, USA). The measurements were repeated until reaching reliable consistent values.

Experimental conditions

Nodal segments (5-6 mm) from 30-day-old plantlets were cut from the middle third of the

stem (excluding the shoot tips and basal regions) and used as explants. Three explants for each vessel (experimental unit) were cultured. *In vitro* plantlets were grown at 24 °C in a growth chamber with a 16 h light/8 h dark photoperiod provided by a photon flux of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool white fluorescent tubes. The 50-day-old plantlets (without sub culturing) were evaluated for vegetative growth, root development, proline quantification and gene expression.

Evaluation of vegetative growth

The plantlets were removed from the *in vitro* PEG-based medium. The shoots were cut from the roots, and the plantlet height was measured as the length of the main stem from base to the tip. The foliage (stems and leaves) was weighed, collected and stored in liquid N₂ at -80 °C until further analysis.

Evaluation of root characters

The roots were cut from the shoots and washed to remove any residual medium and preserved in 70% alcohol. The samples were measured for root length, root diameter and total root area. The roots were scanned using an EPSON Perfection V700 Photo Scanner (Epson America Inc., Long Beach, CA, USA), and the root characters were analyzed using the WinRhizo-Pro image analysis system (Regent Instruments Inc., Quebec, Canada).

Proline analysis

The free proline content was determined from the stems and leaf tissue in each genotype under each drought stress treatment. The stem and leaf tissues were collected, pulverized in liquid N₂ and stored at -80 °C. The proline analyses, using 20-30 mg lyophilized tissue per sample, were performed as in Gilmour *et al.* (2000). The absorbance was determined at 515 nm using a spectrophotometer SpectroQuest-2800 (UNICO. Dayton, NJ, USA).

The resulting values were compared with a standard curve constructed using known amounts of L-proline (Sigma-Aldrich, SG, Switzerland).

Gene expression analysis

Total RNA was isolated from leaf tissue using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) according to Chomczynski and Sacchi (1987). For the quantitative real-time PCR (qRT-PCR) analysis, 1 μg of RNA was reverse transcribed using a Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The qRT-PCR reactions were performed in 25 μL of LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics; Mannheim, Germany), containing 10 ng of cDNA and 0.3 nM of each primer. Primers were designed to generate the 63-bp *ScCBF1* fragment (F: 5-tatggcggaggactaatgc-3, R: 5-tccacataatgatctcccatttc-3), the 102-bp *P5SC* fragment (F: 5-cgatccacaat cagagctaattc-3, R: 5-gcagtcataaccaccttcca-3) (Schafleitner *et al.*, 2007), the 496-bp *StC118 (DNH10)* fragment (F: 5-gctaaacccccaaaaaaactcatt-3, R: 5-gtgaatgtactctgtcttttgac-3) (Pino *et al.*, 2008) and the 100-bp potato 60S fragment (F: 5-aacaatggcggctaagaaga-3, R: 5-tgccataaacctttttgt-3) (Carvalho *et al.*, 2011). The qRT-PCR reactions were performed using a real-time PCR LightCycler 2.0 System (Roche Diagnostics, Mannheim, Germany). Specific primers for the potato 60S gene [expressed sequence tag (EST) clone STMCK67] were used as the internal control for the normalization of the RNA steady-state level. The relative gene expression in the wild-type (WT) lines at 24 °C (0 time) was set to 1, and the other values were adjusted accordingly. For each gene, a calibration curve was generated for each gene based on serial dilutions (10^{-1} to 10^{-3} $\mu\text{g mL}^{-1}$) of the templates. The purity of amplified products was verified through melting curve analyses. The initial amount of transcript in each sample was calculated from the standard curve using the default (fit point/arithmetic)

methods in the LightCycler Software Version 3 (Roche Diagnostics, Mannheim, Germany) and normalized to the values of the internal control. The data represent the means of three experimental replicates over time (n=9).

Experimental design and statistical analysis

A randomized complete block design was used. Wild type and three GM lines per gene construct and potato species were evaluated for three drought stress treatments. For each genotype and drought stress treatment, three independent experiments over time, using three replicate samples per experiment, were conducted, and each replicate consisted of an experimental unit of three plantlets. A total of 27 plantlets were evaluated for each genotype and drought stress treatment. The data were statistically analyzed using analysis of variance (ANOVA) and mean separation (Duncan's Multiple Range) tests, performed using the SAS Program VERSION 9.1.3 2003 (SAS Institute Inc., Cary, NC, USA).

Results and discussion

Transgenic lines overexpressing the ScCBF1 gene showed significantly better plantlet growth and survival under drought stress

One of the first responses to water deficit is reduced plant growth, due to the inhibition of cell expansion. Water limitation reduces the force, or cell turgor pressure, required for cell expansion and reduces leaf expansion and stem and root growth (Spollen *et al.*, 1993; Iwama, 2008). It has been reported in potatoes and other plant species that soil water potential lower than -0.3 MPa reduces plant growth and biomass (Molinos *et al.*, 2004; Schafleitner *et al.*, 2007). The results of this study showed that plant growth was adversely affected through *in vitro* drought stress treatments ($P \leq 0.0001$) when the water potential dropped to -0.36 MPa (with 4% PEG). Only GM

lines overexpressing the *ScCBF1* gene showed better overall plant growth in terms of reduction in the stem length and total foliage weight (Table 1). *In vitro* drought stress adversely affected the shoot height in both potato species, and the WT lines were more affected, showing 70% shoot height reduction after treatment with 4% PEG and an 80% height reduction when the water potential dropped to -0.48 MPa (8% PEG). The shoot height of the GM lines was less affected. In GM *S. tuberosum* (Lines 11, 15 and 19), the shoot height was inhibited 30% after treatment with 4% PEG and 55% after treatment with 8% PEG. Similar results were observed for total foliage weight (stems + leaves), showing that the GM lines were significantly less affected under *in vitro* drought stress compared with the WT lines ($P \leq 0.0001$). In GM *S. tuberosum* Line 19, a 52% reduction in the total foliage weight was observed with 4% PEG treatment and a 67% reduction was observed with 8% PEG (-0.48 MPa), while the WT lines showed severe growth inhibition, with a 92% reduction after 8% PEG treatment. *In vitro* drought stress adversely affected plantlet survival in both potato species. Although all GM lines showed 100% plantlet survival (0, 4, and 8% PEG), WT *S. commersonii* showed a 15% reduction in survival after 8% PEG treatment ($P = 0.0168$), and WT *S. tuberosum* showed a 20% reduction in survival after 4% PEG treatment and a 40% reduction in survival after 8% PEG treatment ($P \leq 0.0001$). These results suggest that *in vitro* screening is sufficient to discriminate drought tolerance in terms of biomass development and plantlet survival, similar to previous studies showing that *in vitro* screening is a useful technique for the initial screening to select drought tolerant genotypes in different plant species, such as sunflower (Punia and Jain, 2002), *Rubus* (Orlikowska *et al.*, 2009), and potato (Gopal and Iwama, 2007). Gopal and Iwama (2007) reported the general effect of water stress on *in vitro* plant growth, which was similar to the observations obtained under field conditions, in terms of the reduction of stem and foliage development, root number and root dry

weight. Previous studies in cultivated potatoes have shown that along with overall plant growth inhibition, other major physiological mechanisms are also affected with low soil water potential. Indeed, after 10 days under drought stress, the soil water potential dropped to -0.55 MPa, the stomatal conductance was reduced to 89%, and net photosynthesis was reduced to 86% (Martínez and Moreno, 1992).

ScCBF1 overexpression in potatoes increased root development through the elongation of existing roots.

Several studies have shown that the roots continue elongating under conditions of low water potential, suggesting that this differential response in roots and shoots could be an adaptation to drought stress (Westgate and Boyer, 1985; Spollen *et*

Table 1. *In vitro* drought stress effects on overall plantlet growth, expressed as a percentage of shoot height reduction, plant survival and foliage weight loss for *Solanum commersonii* (Sc.WT), GM plantlets (Lines 6, 21, 22), *Solanum tuberosum* cv 'Cardinal' (St.WT) and GM (Lines 11, 15, 16). *In vitro* potato plantlets were grown under different PEG4000 concentrations (0, 4 and 8%). The average values of three different experiments are shown (n=27)

| Species | PEG4000 (%) | Genotype | Shoot height reduction (%) | Total foliage weight loss (%) | Plant survival (%) | |
|-----------------------|-------------|---------------|----------------------------|-------------------------------|--------------------|----------|
| <i>S. commersonii</i> | 0 | Sc.WT | 0.0 | 0.0 | 100.0 | |
| | | Line 6 | 0.0 | 0.0 | 100.0 | |
| | | Line 21 | 0.0 | 0.0 | 100.0 | |
| | | Line 22 | 0.0 | 0.0 | 100.0 | |
| | 4 | Sc.WT | 64.5 | 85.1 | 100.0 | |
| | | Line 6 | 35.1 | 45.0 | 100.0 | |
| | | Line 21 | 32.1 | 60.0 | 100.0 | |
| | | Line 22 | 32.2 | 57.2 | 100.0 | |
| | 8 | Sc.WT | 82.8 | 91.2 | 85.2 | |
| | | Line 6 | 50.5 | 87.1 | 100.0 | |
| | | Line 21 | 60.9 | 89.2 | 100.0 | |
| | | Line 22 | 49.0 | 80.2 | 100.0 | |
| | | | PEG Treatment | P≤0001 | P≤0001 | P=0.0642 |
| | | | Genotypes | P≤0001 | P≤0001 | P=0.0440 |
| | | PEGXGenotype | P≤0001 | P≤0001 | P=0.0168 | |
| <i>S. tuberosum</i> | 0 | St.WT | 0.0 | 0.0 | 100.0 | |
| | | Line 11 | 0.0 | 0.0 | 100.0 | |
| | | Line 15 | 0.0 | 0.0 | 100.0 | |
| | | Line 19 | 0.0 | 0.0 | 100.0 | |
| | 4 | St.WT | 72.9 | 81.2 | 80.0 | |
| | | Line 11 | 50.3 | 67.1 | 100.0 | |
| | | Line 15 | 42.4 | 67.0 | 100.0 | |
| | | Line 19 | 44.5 | 52.4 | 100.0 | |
| | 8 | St.WT | 84.8 | 91.7 | 60.0 | |
| | | Line 11 | 56.5 | 78.6 | 100.0 | |
| | | Line 15 | 59.4 | 69.8 | 100.0 | |
| | | Line 19 | 51.5 | 67.5 | 100.0 | |
| | | | PEG Treatment | P≤0001 | P≤0001 | p=0.0008 |
| | | | Genotype | P≤0001 | p=0.0015 | P≤0001 |
| | | PEGXGenotypes | P≤0001 | p=0.0039 | P≤0001 | |

al., 1993). A field trial evaluating 250 potato genotypes using “the pulling resistance of roots selection-criterion” showed a positive correlation between the maximum length and root dry weight under drought stress (Ekanayake and Midmore, 1992). The potato GM lines showed differences in the root development compared with that in the WT lines ($P \leq 0.0001$) in terms of root length, root diameter and total root area (Table 2). For example, in Line 15, the root length increased from 39.4 cm (0% PEG) to 48.2 cm (4% PEG), while the total root area increased from 3.5 cm² (0% PEG) to 4.0 cm² (4% PEG). Similar results were observed in *S. commersonii*, where the root length for GM Lines 6, 21 and 22, grown on 4% PEG, was between 47 and 58 cm, while the root length for the WT lines was significantly shorter at 22 cm (Figure 1). However, when the water potential dropped to -0.478 MPa (8% PEG), the root growth was severely affected, while in GM *S. tuberosum* lines, the root length was between 25 and 30 cm, and in the WT lines, the root length was reduced to 14 cm. In addition, the root area

was reduced 2-3 cm² in the GM lines and 0.9 cm² in the WT lines. These results show that a PEG medium-water potential lower than -0.4 MPa drastically reduces root development, similar to previous studies showing that a soil water potential lower than -0.3 MPa reduces overall potato plant growth (Molinis *et al.*, 2004; Schafleitner *et al.*, 2007). These results showed that drought tolerant lines increased root development through the elongation of the existing roots and the expansion of the root area, similar to the results of previous studies in different plant species (Westgate and Boyer, 1985; Iwama, 2008).

The proline content increased under in vitro drought stress and ScCBF1 gene overexpression

Several plant species cope with drought stress through osmolyte accumulation, such as soluble sugars and free proline, which act as osmotic agents, protecting plant cells from dehydration (Szabados and Savoure, 2010). In different Andean

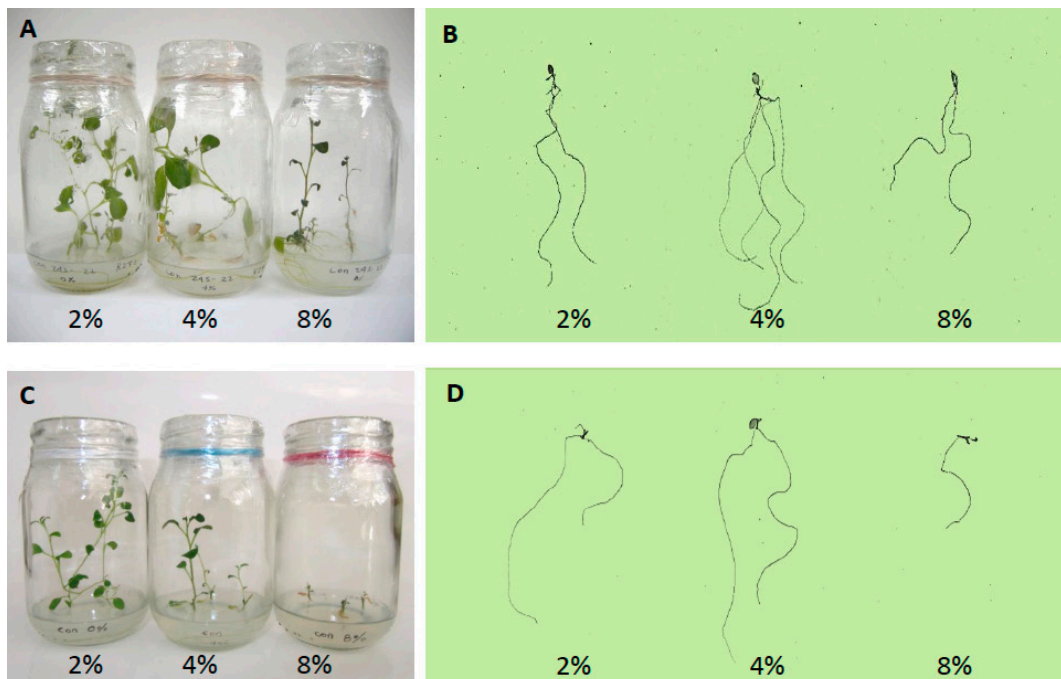


Figure 1. *In vitro* drought stress screening in potato plantlets, showing the overall growth and root development in GM *Solanum commersonii* Line 22 (A, B) and WT plants (C, D). *In vitro* potato plantlets were grown under different PEG4000 treatments (0, 4 and 8%).

Table 2. *In vitro* drought stress effects on plantlet root development for *Solanum commersonii* (Sc.WT), GM plantlets (Lines 6, 21, 22), *Solanum tuberosum* cv Cardinal (St.WT) and GM plantlets (Lines 11, 15, 16). *In vitro* potato plantlets were grown under different PEG4000 concentrations (0, 4 and 8%). The average values of three different experiments are shown (n=27)

| Species | PEG4000 (%) | Genotype | Total root length (cm) | Root diameter (mm) | Total root area (cm ²) | |
|-----------------------|-------------|---------------|------------------------|--------------------|------------------------------------|--------|
| <i>S. commersonii</i> | 0 | Sc.WT | 53.42 | 0.259 | 4.13 | |
| | | Line 6 | 31.93 | 0.190 | 1.90 | |
| | | Line 21 | 36.45 | 0.236 | 2.71 | |
| | | Line 22 | 42.71 | 0.233 | 3.13 | |
| | 4 | SC.WT | 22.14 | 0.263 | 1.83 | |
| | | Line 6 | 57.69 | 0.204 | 3.70 | |
| | | Line 21 | 46.95 | 0.268 | 3.95 | |
| | | Line 22 | 50.57 | 0.195 | 3.10 | |
| | 8 | Sc.WT | 16.28 | 0.253 | 1.30 | |
| | | Line 6 | 19.14 | 0.190 | 1.14 | |
| | | Line 21 | 16.24 | 0.197 | 1.00 | |
| | | Line 22 | 18.31 | 0.222 | 1.33 | |
| | | | PEG treatment | P≤0001 | P=0.0015 | P≤0001 |
| | | | Genotype | P≤0001 | P≤0001 | P≤0001 |
| | | PEGXGenotype | P≤0001 | P≤0001 | P≤0001 | |
| <i>S. tuberosum</i> | 0 | St.WT | 35.34 | 0.300 | 3.32 | |
| | | Line 11 | 36.28 | 0.228 | 2.60 | |
| | | Line 15 | 39.42 | 0.281 | 3.45 | |
| | | Line 19 | 35.84 | 0.207 | 2.33 | |
| | 4 | St.WT | 23.98 | 0.258 | 1.94 | |
| | | Line 11 | 38.03 | 0.250 | 3.04 | |
| | | Line 15 | 48.12 | 0.267 | 4.04 | |
| | | Line 19 | 38.01 | 0.252 | 3.09 | |
| | 8 | WT | 13.88 | 0.219 | 0.96 | |
| | | Line 11 | 24.71 | 0.240 | 1.86 | |
| | | Line 15 | 27.94 | 0.233 | 3.04 | |
| | | Line 19 | 30.11 | 0.243 | 2.29 | |
| | | | PEG treatment | P≤0001 | P=0.004 | P≤0001 |
| | | | Genotypes | P≤0001 | P=0.002 | P≤0001 |
| | | PEGXGenotypes | P≤0001 | P≤0001 | P≤0001 | |

potato clones, free proline is accumulated under drought stress, increasing earlier in drought susceptible varieties than in drought tolerant plants (Schafleitner *et al.*, 2007). Because previous studies reported proline accumulation in GM Arabidopsis plants overexpressing the *CBF3* gene (Gilmour *et al.*, 2000) and in GM tomato plants overexpressing the *AtCBF1* gene under water deficit (Hsieh *et al.*, 2002), the proline content was

also evaluated in the present study. Overall, the proline concentration increased approximately 1.5- and 2.5-fold under *in vitro* drought stress (Figure 2). In *S. tuberosum*, the increase in the proline concentration was higher than in *S. commersonii* (Figure 2B), showing significant differences among *in vitro* drought stress treatments ($P \leq 0.0001$) and genotypes ($P \leq 0.0001$). GM *S. tuberosum*, Lines 11, 15 and 19, showed increased proline content

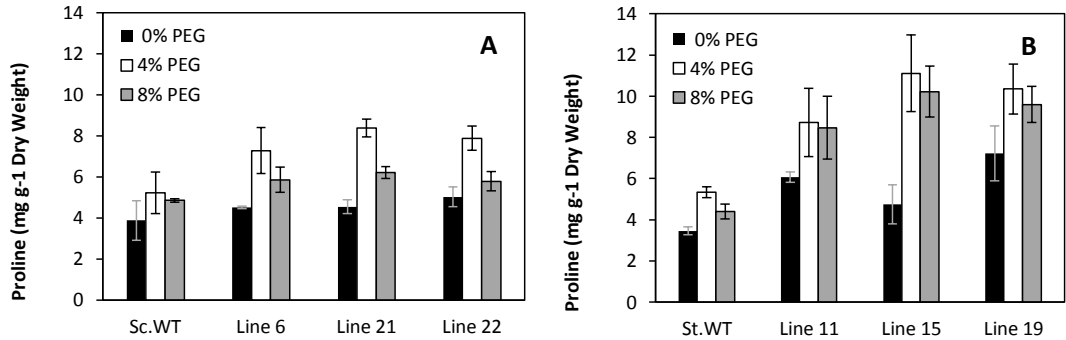


Figure 2. Effect of *in vitro* drought stress on free proline accumulation: (A) in GM *Solanum commersonii* Lines 6, 21, and 22 and WT plants, and (B) in GM *Solanum tuberosum* cv. Cardinal Lines 11, 15, and 19 and WT plants. The average values of three different experiments are shown (n=9). The bars indicate the standard deviation. Significant differences were observed in both potato species, between GM lines and WT plants ($P \leq 0.0001$).

compared with the non-drought stressed WT plants. For example, in Line 15, the free proline was 11.1 mg g⁻¹ dry weight, and in WT plants, the free proline was significantly lower at 5.3 mg g⁻¹ dry weight. Similar results were observed in *S. commersonii* (Figure 2A).

Differential expression of ScCBF1, pyrroline-5-carboxylate synthetase (P5CS) gene and dehydrin-like gene (DNH10) was observed in both potato species upon drought stress

Similar to previous studies reporting that CBF/DREB gene expression is associated with the expression of *P5CS* and *DNH*-like genes under abiotic stress (Gilmour *et al.*, 2000; Kobayashi *et al.*, 2005; Kume *et al.*, 2005), in both potato species, the constitutive overexpression of the *ScCBF1* gene was also associated with *P5CS* and *DNH10* transcript accumulation under *in vitro* drought stress (Figure 3).

The overexpression of the *ScCBF1* gene in GM lines for both potato species was 1.8- and 3.3-fold higher than that in control WT plants (Figure 3A and Figure 3B). This slight increase might reflect the fact that the majority of GM lines showed only one insertion site in the Southern analysis (data not shown). GM *S. tuberosum* Line 15 showed two insertion sites and increased transcript accumulation. Despite the *ScCBF1*

expression level, the GM lines showed better plantlet growth and root development than the WT lines in both potato species. Notably, in the GM lines, the *ScCBF1* transcript increased upon *in vitro* drought stress, reflecting the presence of endogenous potato *CBF* homologs, which are also induced under abiotic stress (Gilmour *et al.* 2000; Carvallo *et al.*, 2011).

The expression of *P5CS* and *DNH10* was also examined, as both genes play a role in osmotic adjustment and membrane stability, and the expression of these genes is up regulated through *CBF* genes (Gilmour *et al.*, 2000). Previous studies have revealed that GM tobacco plants, overexpressing *P5CS*, accumulated more proline than the control plants, showing enhanced tolerance to osmotic stress (Kavi Kishor *et al.*, 2005). Other studies have suggested that *P5CS* overexpression is associated with significantly better root development (Kavi Kishor *et al.*, 2005). The GM potato Lines 15 and 21 showed significantly larger total root areas and accumulated higher proline content in the stems and leaves. In addition, the GM lines showed 1.5-fold increased *P5CS* transcript accumulation compared with control WT plants (Figure 3C and Figure 3D). However, this level of transcript accumulation is low with respect to previous results reported in *Arabidopsis* plants, where *AtCBF3* overexpression resulted in increased proline content (5-fold higher compared

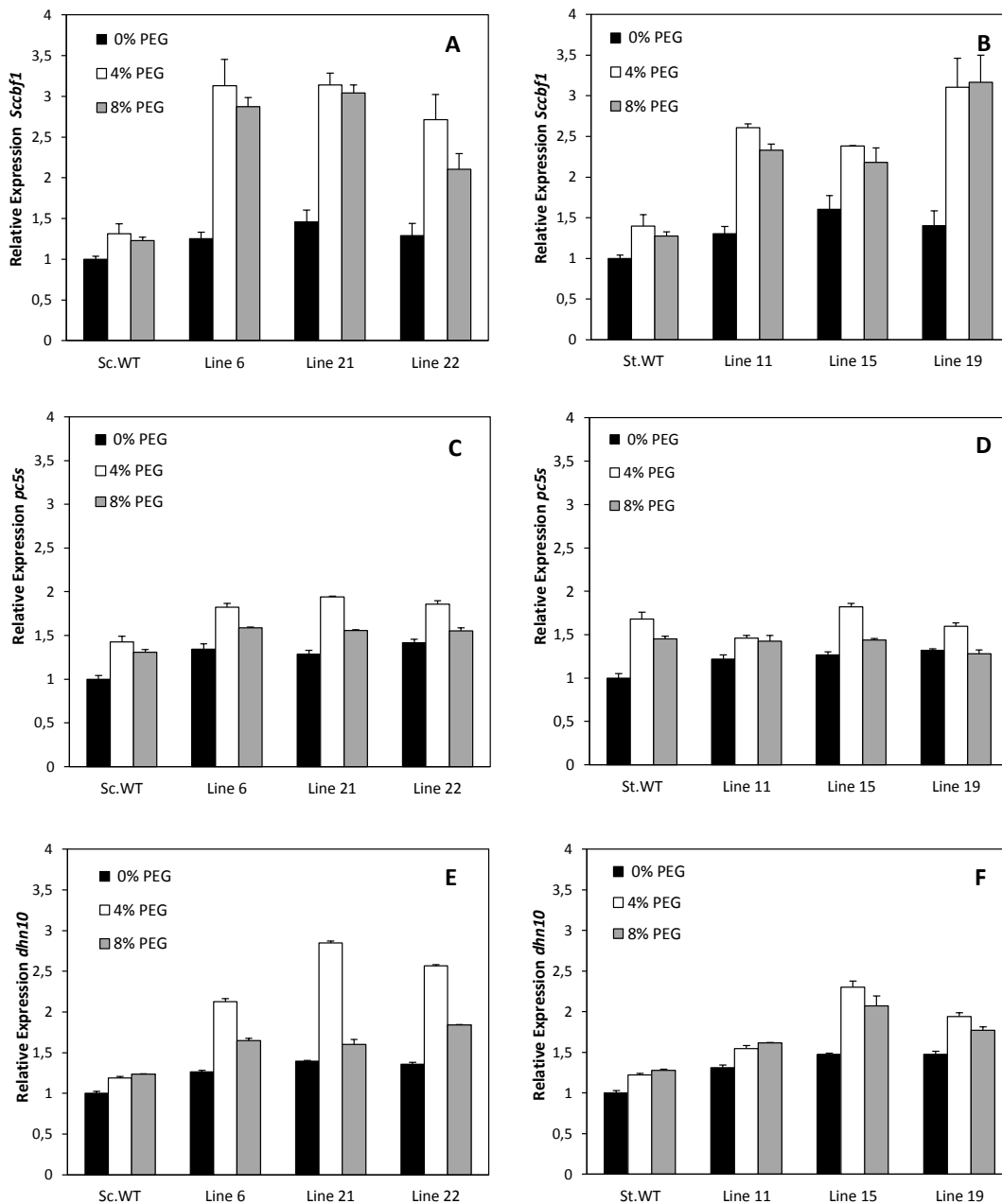


Figure 3. Effect of drought stress on the relative gene expression of *ScCBF1*, pyrroline-5-carboxylate synthetase (*P5CS*) and *Dehydrins (DNH10)* genes. The transcript levels were determined through qRT-PCR analysis in GM *Solanum commersonii* Lines 6, 21 and 22 and WT plants (A, C, D) and in GM *Solanum tuberosum* Lines 11, 15 and 19 and WT plants (B, D, F). Potato plantlets were grown under *in vitro* drought stress at 24 °C, with a 16 h:8 h day:night photoperiod, in hormone free MS medium containing 0, 4 and 8% PEG4000. The qRT-PCR analysis was performed using 100 ng of total RNA per sample. The relative expression of each transcript was normalized using the potato 60S gene as an internal reference. For *Solanum commersonii*, the relative expression in WT plants at 0% PEG was set to 1, and the other values were adjusted accordingly. For *Solanum tuberosum*, the relative expression in WT plants at 0% PEG was set to 1, and the other values were adjusted accordingly. The average values of three different experiments are shown (n=9). The bars indicate the standard deviation. Significant differences were observed for *ScCBF1* and *DNH10* expression in both potato species and between GM lines and WT plants ($P \leq 0.0001$).

with control WT plants) and 4-fold higher *P5CS* transcript accumulation (Gilmour *et al.*, 2000). This difference might partially reflect the fact that in *Arabidopsis* plants, maximum expression was reported at the beginning of the stress treatment (24 hours), and subsequently, *P5CS* transcript accumulation was significantly reduced (Gilmour *et al.*, 2000). In both GM potato lines, the gene expression analysis was conducted at the end of the experiment (50 days under *in vitro* drought stress). Thus, the differences in the observation might also reflect the *P5CS* probes used. Indeed, a previous study in tomatoes overexpressing the *AtCBF1* gene showed that proline concentrations were higher in GM tomato plants than in WT plants, but no significant differences in the *P5CS* expression between WT and GM plants were observed, and the authors attributed this difference to the *P5CS* probe used (Hsieh *et al.*, 2002).

Several stress-responsive Dehydrins have been associated with CBF overexpression and drought stress. Dehydrins act as chaperons, stabilizing vesicles, proteins, and membrane structures in stressed plants (Close, 1997; Allagulova *et al.*, 2010). However, the precise function of these proteins remains unknown. In wheat, early Dehydrin expression has been associated with drought tolerant genotypes and *CBF* gene expression (Kobayashi *et al.*, 2005; Kume *et al.*, 2005). In *Solanum* species, such as tobacco and potato, *DHN*-like genes have been associated with cold tolerance and CBF gene expression (Pino *et al.*, 2008). A previous study in GM tomato plants overexpressing the *AtCBF1* gene did not show Dehydrin like-gene expression (*TAS14*) (Hsieh *et al.*, 2002). These results could be attributed to the low homology between *Arabidopsis* gene probes and tomato endogenous homologs or unknown tomato *LEA* genes induced through the

heterologous expression of the *CBF1* gene. In the present study, GM *S. tuberosum* (Line 15) and GM *S. commersonii* (Lines 21 and 22) showed the best drought tolerance, in terms of plant growth, total root length and total root area, and 1.5- to 3.0-fold higher *DNH10* expression ($P \leq 0.0001$) compared with WT plants (Figure 3E and 3F). In this study, the majority of GM *S. commersonii* lines showed higher *ScCBF1* and *DNH10* transcript accumulation with drought stress (GM Lines 6, 21 and 22), while in GM *S. tuberosum*, the transcript accumulation was significantly lower.

In summary, *in vitro* screening for drought tolerance showed that the *ScCBF1* gene overexpression in GM potato induces several responses associated with drought adaptation mechanisms, resulting in better overall plant growth, in terms of shoot height, total foliage weight, root development and plantlet survival. An association between *ScCBF1* and *DNH10* transcript accumulation was observed, but no association with the *P5CS* gene was detected, despite the observed effects on the proline content during drought stress. Further studies are needed to evaluate these GM lines in the field, in terms of physiological parameters and tuber production under different watering treatments. In addition, it is necessary to confirm the function of *P5CS* and *DNH10* and other *DNH-like* genes through the genetic transformation of potato. Moreover, the identification of a novel potato stress inducible promoter is needed to increase *ScCBF1* transcript accumulation under drought stress.

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Resumen

M.T. Pino, A. Ávila, A. Molina, Z. Jeknić y T.H.H. Chen. 2013. *Solanum tuberosum* y *Solanum commersonii*, sobre-expresando el gen *ScCBF1*, mejoraron su tolerancia a sequía en condiciones de cultivo *in vitro*.. Cien. Inv. Agr. 40(1):171-184. La papa cultivada es sensible a sequía, tanto el rendimiento como la calidad se reducen cuando el potencial hídrico de suelo baja a -0.3 MPa. La sequía también produce alteraciones fisiológicas, bioquímicas y moleculares, que han sido asociados a cambios en la expresión de genes que actúan directamente en la protección de la planta o regulando otros genes de respuesta. En este último grupo, están los factores de transcripción del tipo CBF/DREB, que han sido asociados a tolerancia heladas, salinidad y sequía en diferentes especies. El objetivo de este estudio fue evaluar si la sobreexpresión del gen *ScCBF1*, clonado desde *Solanum commersonii*, podría mejorar la tolerancia a sequía en plantas genéticamente modificadas de *Solanum tuberosum* and *S. commersonii* bajo condiciones *in vitro*. En conjunto con algunos mecanismos de adaptación a sequía, como osmoprotectores y genes asociados al ajuste osmótico y estabilidad de las membranas. Los tratamientos de sequía fueron simulados *in vitro*, agregando polyethylen-glycol (PEG4000) en medio MS libre de hormonas. Se evaluó crecimiento vegetativo, desarrollo radicular, contenido de prolina, y expresión de los genes *ScCBF1*, *P5CS* y *DNH10*. La expresión constitutiva del gen *ScCBF1* en ambas especies mejoró significativamente el crecimiento vegetativo y radicular bajo sequía-*in vitro*. Además, aumentó significativamente el contenido de prolina en hojas y tallos, y la expresión del gen *DNH10*, sugiriendo que la sobreexpresión constitutiva del gen *ScCBF1* estaría asociada a algunos mecanismos de adaptación a sequía.

Palabras clave: Gen *CBF*, *in vitro*, papas, sequía, *Solanum commersonii*, *Solanum tuberosum*.

References

- Allagulova, Ch.R., F.R. Gimalov, F.M. Shakirova, and V.A. Vakhitov. 2010. The Plant Dehydrins: Structure and Putative Functions. *Biochemistry (Moscow)* 68:945-951.
- Bansal, K.C., and S. Nagarajan. 1986. Leaf water content, stomatal conductance and proline accumulation in leaves of Potato (*Solanum tuberosum* L.) in response to water stress. *Indian J. Plant Physiol.* 29:397-404.
- Carvalho, M., M.T.Pino, Z. Jeknić, C. Zou, C. Doherty, S. Shiu, T. Chen, and M. Thomashow. 2011. 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*. *J. Exp. Bot.* 62:3807-3819.
- Chazen, O., and P.M. Neumann. 1994. Hydraulic signals from the roots and rapid cell-wall hardening in growing maize (*Zea mays* L.) leaves are primary responses to polyethylene glycol-induced water deficits. *Plant Physiol.* 104:1385-1392.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Close, T.J. 1997. Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiol. Plant.* 100:291-296.
- Dalla Costa, L., G. Delle Vedove, G. Gianquinto, R. Giovanardi, and A. Peressotti. 1997. Yield, water use efficiency and nitrogen uptake in potato: influence of drought stress. *Potato Res.* 40:19-34.
- Deblonde, P., and J. Ledent. 2001. Effects of moderate drought conditions on green leaf number stem height, leaf length and tuber yield of potato cultivars. *Eur. J. Agron.* 14:31-41.
- Ekanayake, I.J., and D.J. Midmore. 1992. Genotypic variation for root pulling resistance in potato and its relationship with yield under water-deficit stress. *Euphytica* 61:43-53.

- FAO. 2012. Statistical databases FAOSTAT. Available online at: <http://faostat.fao.org/site/567/default.aspx#ancor> (Website accessed: July 2012).
- Gilmour, S., A. Selbot, M. Salazar, J. Everar, and M. Thomashow. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* 124:1854-1865.
- Gopal, J., and K. Iwana. 2007. *In vitro* screening of potato against water-stress mediated through sorbitol and polyethylene glycol. *Plant Cell Rep.* 26:693-700.
- Hsieh, T., J. Lee, Y. Charng, and M. Chan. 2002. Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. *Plant Physiol.* 130:618-626.
- Iwama, K. 2008. Physiology of the potato: new insights into root system and repercussions for crop management. *Potato Research* 51:333-353.
- Jaglo, K., S. Kleff, K. Amundsen, X. Zhang, V. Haake, J. Zang, T. Deits, and M. Thomashow. 2001. Components of the Arabidopsis C-repeat/Dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.* 127:910-917.
- Kasuga, M., S. Miura, K. Shinozaki, and K. Yamaguchi Shinozaki. 2004. A combination of *Arabidopsis DREB1A* gene and stress inducible rd29A promoter improved drought and low temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* 45:346-350.
- Kavi Kishor, P.B., S. Sangam, M.N. Amrutha, P. Sri Lakshmi, K.R. Naidu, K.R.S.S. Rao, S. Rao, K.J. Reddy, P. Theriappan, and N. Screenivasulu. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implication in plant growth and abiotic stress tolerance. *Current Sci.* 88:424-438.
- Kiziloglu, F.M., U. Sahin, T. Tunc, and S. Diler. 2006. The effect of deficit irrigation on potato evapotranspiration and tuber yield under cool season and semiarid climatic condition. *Journal of Agronomy* 5:284-288.
- Knipp, G., and B. Honermeier. 2006. Effect of water stress on proline accumulation of genetically modified potatoes (*Solanum tuberosum* L.) generating fructans. *J. Plant Physiol.* 163:392-397.
- Kobayashi, F., S. Takumi, S. Kume, M. Ishibashi, R. Ohno, K. Murai, and C. Nakamura. 2005. Regulation by Vrn-1/Fr-1 chromosomal intervals of CBF-mediated Cor/Lea gene expression and freezing tolerance in common wheat. *J. Exp. Bot.* 56:887-895.
- Kume, S., F. Kobayashi, M. Ishibashi, R. Ohno, C. Nakamura, and S. Takumi. 2005. Differential and coordinated expression of Cbf and Cor/Lea genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. *Genes Genet. Syst.* 80:185-197.
- Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transcription pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* 10:1391-1406.
- Martínez, C.A., and U. Moreno. 1992. Expresiones fisiológicas de resistencia a la sequía en dos variedades de papa sometidas a estrés hídrico en condiciones de campo. *R. Bras. Fisiol. Veg.* 4:33-38.
- Molinos, C., A. Villegas, P. Sanchez, G. Alcantar, N. Rodriguez, and L. Ruiz. 2004. Efecto del potencial osmótico y contenido de Ca en el medio de cultivo sobre la distribución de Ca²⁺ y K⁺, producción de biomasa y necrosis apical de vid "R110". *INCI* 29:384-388.
- Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Orlikowska, T., D. Kucharska, and M. Horbowicz. 2009. The reaction of raspberry and blackberry cultivars to drought stress simulated *in vitro* by polyethylene glycol (PEG) 6000. *Acta Hort.* (ISHS) 839:337-342.
- Pennycooke, J.C., H. Cheng, S.M. Roberts, Q. Yang, S.Y. Rhee, and E.J. Stockinger. 2008. The low temperature-responsive, *Solanum* CBF1 genes maintain high identity in their upstream regions in a genomic environment undergoing gene

- duplications, deletions, and rearrangements. *Plant Mol. Biol.* 67:483-497.
- Pino, M.T., J. Skinner, Z. Jeknić, P. Hayes, A. Soeldner, M. Thomashow, and T. Chen. 2008. Ectopic AtCBF1 over-expression enhances freezing tolerance and induces cold acclimation-associated physiological modifications in potato. *Plant, Cell and Environ.* 31:393-406.
- Punia, M., and A. Jain. 2002. *In vitro* selection for drought tolerance in sunflower (*Helianthus annuus L.*). *Nat. J. Plant Improv.* 4:27-30.
- Schafleitner, R., A. Gaudin, R. Gutierrez, C. Alvarado, and M. Bonierbale. 2007. Proline accumulation and real time PCR expression analysis of genes encoding enzymes of proline metabolism in relation to drought tolerance in Andean potato. *Acta Physiol Plant.* 29:19-26.
- Shinozaki, K., and K. Yamaguchi-Shinozaki. 2007. Gene networks involved in drought stress response and tolerance *J. Exp. Bot.* 58:221-227.
- Spollen, W.G., R.E. Sharp, I.N. Saab, and Y. Wu. 1993. Regulation of cell expansion in roots and shoots at low water potentials. *In: Smith J.A.C., and H. Griffiths (eds.). Water deficits, plant responses from cell to community.* Oxford: Bios Scientific Publishers 37-52.
- Su, J., and R. Wu. 2004. Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. *Plant Sci.* 166:941-948.
- Szabados, L., and A. Savoure. 2010. Proline: a multifunctional amino acid. *Trends in Plant Science* 15:89-97.
- Weisz, R., K. Kaminski, and Z. Smilowitz. 1994. Water deficit effects on potato leaf growth and transpiration: utilizing fraction extractable soil water for comparison with other crops. *American Journal of Potato Research* 71:829-840.
- Westgate, M.E., and J.S. Boyer. 1985. Osmotic adjustment and inhibition of leaf, root, stem, and silk growth at low water potentials in maize. *Planta* 164:540-549.
- Yang, S., B.Vanderbeld, J. Wan, and Y.Huang. 2010. Narrowing Down the Targets: Towards Successful Genetic Engineering of Drought-Tolerant Crops. *Molecular Plant* 3:469-490.
- Zhao, J., W. Ren, D. Zhi, L. Wang, and G. Xia. 2007. Arabidopsis DREB1A/CBF3 bestowed transgenic tall fescue increased tolerance to drought stress. *Plant Cell Rep.* 26:1521-1528.