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Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) confers multiple stress tolerance and enhances organ growth

Wei Tang*, Thomas M. Charles and Ronald J. Newton
*Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC, 27858-4353, USA (*author for correspondence; e-mail tangw@mail.ecu.edu)*

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Abstract

Transcription factors play an important role in regulating gene expression in response to stress and pathogen tolerance. We describe here that overexpression of an ERF/AP2 pepper transcription factor (*CaPF1*) in transgenic Virginia pine (*Pinus virginiana* Mill.) confers tolerance to heavy metals Cadmium, Copper, and Zinc, to heat, and to pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis*, as by the survival rate of transgenic plants and the number of decreasing pathogen cells in transgenic tissues. Measurement of antioxidant enzymes ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) activities demonstrated that the level of the enzyme activities was higher in transgenic Virginia pine plants overexpressing the *CaPF1* gene, which may protect cells from the oxidative damage caused by stresses, compared to the controls. Constitutive overexpression of *CaPF1* gene enhanced organ growth by increasing organ size and cell numbers in transgenic Virginia pine plants over those in control plants.

Introduction

To survive, plants have to deal with various biotic and abiotic environmental stress conditions, which cause adverse effects on the growth and productivity of plants (Thomashow, 1999; Queitsch *et al*., 2000; Vailleau *et al*., 2002; Gong *et al*., 2003;). To adjust to changes in the environment, plants trigger rapid defense responses via a number of signal transduction pathways (Kasuga *et al*., 1999). Stress is perceived and transduced through a chain of signaling molecules that ultimately affect regulatory elements of stress-inducible genes to initiate the synthesis of different classes of proteins including transcription factors, enzymes, molecular chaperons, ion channels, and transporters or alter their activities (Tarczynski *et al*., 1993; Shi *et al*., 2003; Mukhopadhyay *et al*., 2004) Such cascading events controlled by a battery of genes and their intricate regulation help the system to overcome the unfavorable conditions (Lincoln *et al*., 2002; Huckelhoven *et al*., 2003). Alterations in the expression of genes coding for transcription regulators greatly influence plant stress tolerance (Stockinger *et al*., 1997; Uno *et al*., 2000). To understand the process of development of plants and their response to environmental stresses, it is imperative to know the function of crucial genes and phytohormones and their regulation during different phases of the life cycle (Sheveleva *et al*., 1997; Liu *et al*., 1998; Mukhopadhyay *et al*., 2004). It has been proved that ABA plays a unique role in stress response in that it mediates adaptation to a variety of common abiotic stresses, such as drought, high salinity, low/

high temperatures and oxidative stress (Zeevaart and Creelman, 1988). The adaptive processes include changes in gene expression patterns, which ultimately lead to biochemical, cellular, and physiological changes.

Transcriptional factors regulating the stress-responsive gene expression play important roles in stress adaptation (Stockinger *et al*., 1997; Liu *et al*., 1998; Kasuga *et al*., 1999). In Arabidopsis, transcription factor families ERF/AP2, bZIP/HD-ZIP, Myb, WRKY, and several classes of zinc-finger proteins, each containing a distinct type of DNA-binding domain, have been reported to play a role in plant stress responses by regulating the expression of stress-responsive gene under different stress conditions (Rushton and Somssich, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Transgenic plants expressing tomato (*Lycopersicon esculentum*) ethylene-response factor (*ERF*) *Pti4* demonstrated increased resistance to the fungal pathogen *Erysiphe orontii*, and increased tolerance to the bacterial pathogen *Pseudomonas syringae* pv tomato (Gu *et al*., 2002). A R2R3-MYB gene, AtMYB30, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack (Vailleau *et al*., 2002). Expression of the antiapoptotic baculovirus *p35* gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease (Lincoln *et al*., 2002). In another case, overexpression of two Arabidopsis ERF/AP2 genes, CBF1/DREBP1B and DREBP1A, resulted in enhanced tolerance to drought, salt, and freezing (Jaglo-Ottosen *et al*., 1998). These transcription factors bind the stress-responsive *cis*-elements and activate the expression of target genes (Kasuga *et al*., 1999), which regulate biochemical and physiological aspects of stress conditions.

We present here a functional study on the application of an ERF/AP2 pepper transcription factor (*CaPF1*) gene (Yi *et al*., 2004) in tolerance to heavy metals Cadmium, Copper, and Zinc ($CdCl_2$, $CuCl_2$, or $ZnCl_2$), to heat stress, and to pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis*, by an overexpression analysis of *CaPF1* in transgenic Virginia pine (*Pinus virginiana* Mill.). The results demonstrated the usefulness of a pepper transcription factors in transgenic Virginia pine, a gymnosperm tree species. These results could be valuable for future research on engineering stress tolerance based on transcription regulation with different transcription factor sources in gymnosperm species, especially in conifers.

Materials and methods

Plasmid construct and plant transformation

Plasmid vector pMBP-1-CaPF1 (Figure 1A) provided by Dr. Doil Choi (Yi *et al*., 2004) was introduced into competent cells of *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium*-mediated transformation and production of transgenic Virginia pine were performed as described before (Tang *et al*., 2001; Tang and Newton, 2004a).

DNA and RNA blot hybridization

Genomic DNA was extracted from 1.0 g transgenic Virginia pine cell cultures and plantlets, respectively, using a Genomic DNA Isolation Kit (Sigma Chemical, St. Louis, MO, USA) by following the manual. Southern blot analysis was conducted as previously described by Tang *et al*. (2004) except that the *CaPF1* fragment was used as the probe. Total RNA was isolated from 1.5 g transgenic cell cell cultures and plantlets and ground in liquid nitrogen using RNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA) following manufacturer's protocol. Fifteen micrograms of RNA was separated by agarose-gel electrophoresis (Sambrook *et al*., 1989). Electrophoresis and northern blotting of RNAs were performed as described by Tang and Newton (2004a). The *CaPF1* probe corresponding to the *CaPF1* gene were labeled by Digoxigenin (DIG) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). Equal loading of RNA samples was verified with a control of tobacco 25S rRNA.

Plant material, growth conditions, and stress treatments

Mature seeds of Virginia pine (*Pinus virginiana* Mill.) were kindly provided by the Christmas Tree Genetics Program of North Carolina State University. Establishment of callus cultures and regeneration of transgenic Virginia pine plants was as described by Tang and Newton (2004b). Heavy

metals treatment was carried out as described by Gong *et al*. (2003). Different concentrations (0.5, 1, and 1.5 mM) of $CdCl_2$, $CuCl_2$, $ZnCl_2$ (Sigma Chemical, St. Louis, MO, USA), were used for treatment. Bacterial pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis* treatments were carried out as described by Yi *et al*. (2004). Measurement of fresh weight and dry weight were as described by Tang and Newton (2004b). Heat stress tests were performed according to Kim *et al*. (2004) with some modification. Three-week-old callus cultures, shoots, and plantlets were exposed to 46 or 48 °C for 5 h, respectively, and then returned to the normal growth temperature

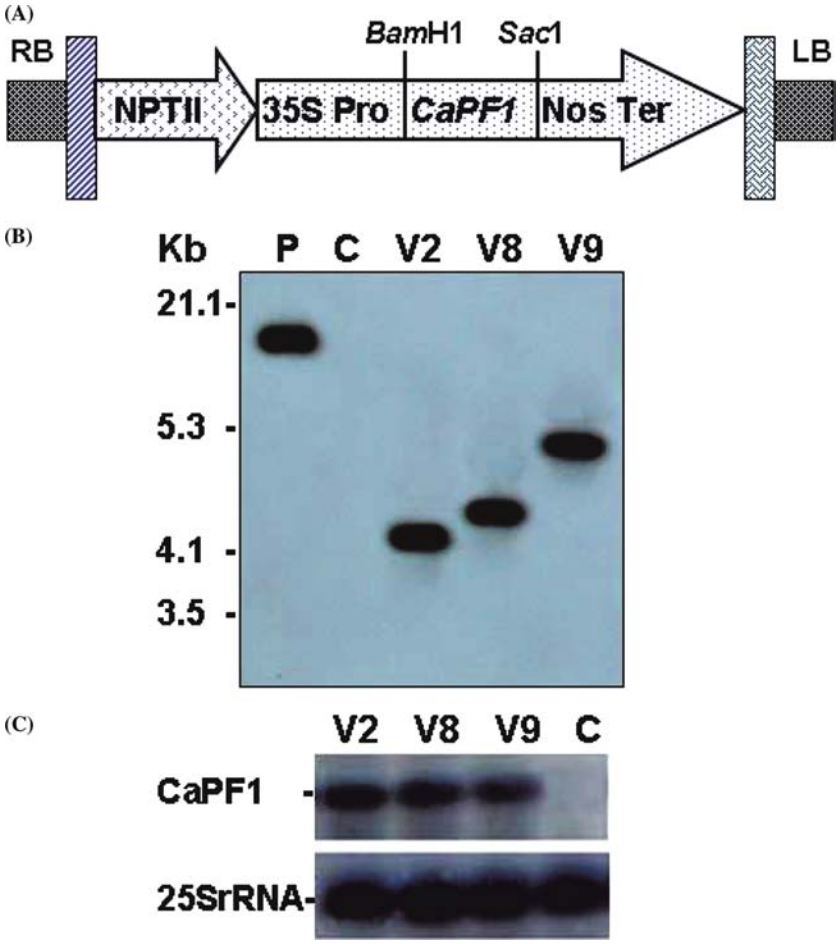


Figure 1. Linear plasmid T-DNA map, Southern, and northern hybridization analysis. (A) Plasmid vector pMBP-1-CaPF1 T-DNA indicating the localization of two genes (*NPTII*, Neomycin phosphotransferase II; *CaPF1*, *Capsicum annuum* pathogen and freezing tolerance-related protein 1), a promoter (35Spro, cauliflower mosaic virus 35S promoter), one terminator (nosTer, terminator from the nopaline synthase gene), and T-DNA borders (LB left border, and RB right border). Arrows indicate gene translation orientation. The probe used in Southern blot analysis of transgenic plants is the restriction enzymes *Bam*HI and *Sac*I fragment of *CaPF1*. (B) Southern blot analysis of transformed plants. Plasmid and genomic DNA were digested with the restriction enzyme *Bam*HI that recognize sites inside the T-DNA and flanking plant genomic DNA. Lane P: plasmid DNA of pMBP-1-CaPF1 (5 pg); lane C: DNA from non-transformed plant (20 μg); lanes V2, V8, and V9: DNA from transgenic plants derived from cell lines V2, V8, and V9 (20 μg). (C) Northern blot analysis of total RNA extracted from non-transformed plant and transgenic plants derived from cell lines V2, V8, and V9. RNA (15 μg) was prepared as described in the experimental protocol section from a non-transformed plant (line C, control) and transgenic plants of transgenic cell lines V2 (line V2), V8 (line V8), and V9 (line V9), and were hybridized (at 65 °C) with the 1.3-kp *CaPF1* probe corresponding to the *CaPF1* gene, which were labeled with Digoxigenin (DIG) (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA). The integrity and the amount of RNA applied to each lane were verified by hybridization of the RNA to a probe for 25SrRNA (lower panel).

(25 °C). Survival rate was determined and photographs were taken 3 weeks after treatments.

Determination of antioxidant enzyme activity

Measurement of antioxidant enzymes ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) activities were as described by Tang and Newton (2004b). One gram of needle tissues were homogenized under ice-cold conditions in 3 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4 °C. The homogenates were centrifuged at 10 000 × *g* for 20 min and the supernatant fraction was used for the assays.

Cell size and number measurement

Cell size and number measurement were carried out as described by Mizukami and Fischer (2000). For comparison of cell size and numbers, the distal portion of the 10th needle epidermis was analyzed because it has cells that are uniform in size. For statistical analysis, images were digitized with a UMAX scanner (UMAX Technologies, Fremont, CA) and were analyzed by using the NIH IMAGE program (http:/rsb/info.nih.gov/nih-image) (Mizukami and Fischer, 2000). Statistical assays were carried out by using the General Linear Model procedure of SAS (SAS, Cary, NC) and one-way ANOVA at 95% confidence interval.

Scanning electron microscopy

For scanning electron microscopy, callus cultures with shoots and needles were washed in distilled water for 30 min, followed by drying on filter paper at 25 °C for 30 min. Samples fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4 °C, dehydrated in a graded ethanol series, and washed in a graded amyl acetate series. They were then critical-point dried in CO_2 and mounted on copper stubs coated with a thin layer of gold in a cold sputtering system. Samples directly examined and photographed using a Quanta 200 environmental scanning electron microscope (FEI Company, Hillsboro, Ore) at low vaccum mode. The vaccum was 0.94 Torr, the diameter of beam was 5 Spot Size, and the accelerating voltage was 15.0 kV.

Results and discussion

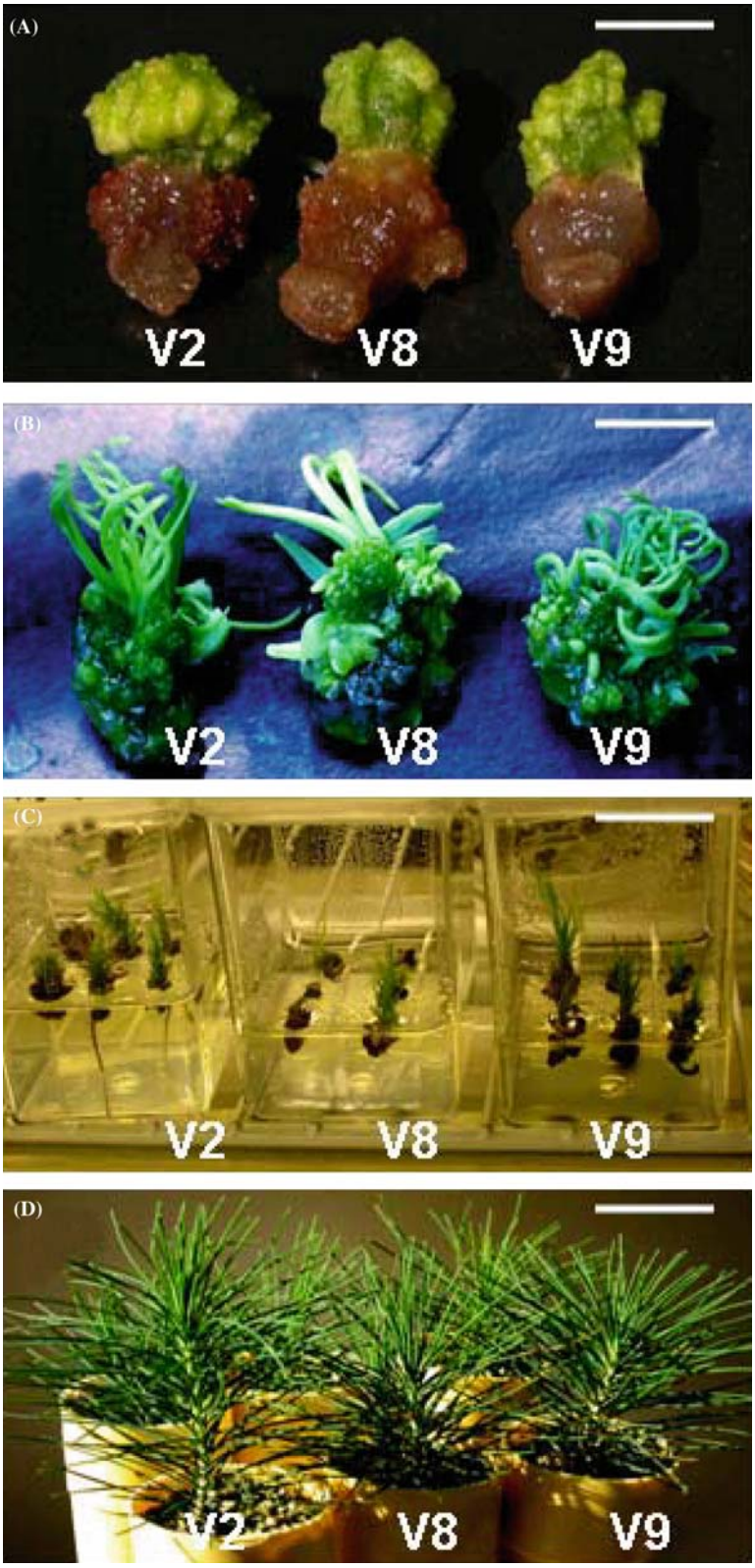
Production of transgenic plants

Virginia pine is an important Christmas tree and forest species. Because of its high-efficiency *in vitro* regeneration ability, Virginia pine has great potential to become a useful model plant for basic and applied research purposes in gymnosperm species. Here, we report on the use of an ERF/AP2 pepper transcription factor (*CaPF1*) (Yi *et al.*, 2004) in Virginia pine. To generate transgenic cultures and plantlets, 66 callus cultures derived from single Virginia pine embryos were infected with the *Agrobacterium tumefaciens* strain EHA105 containing pMBP-1-CaPF1 (Figure 1A) carrying the neomycin phosphotransferase gene (*NPTII*) and the ERF/AP2 pepper transcription factor (*CaPF1*). Seventy-five independent Virginia pine callus lines that were resistant to kanamycin were generated. After the T-DNA insert was confirmed by Southern blot (Figure 1B) and northern blot analyses (Figure 1C), 3 transgenic callus lines (V2, V8, and V9) with 1 copy of the transgene were selected as candidates to induce shoot formation and to generate transgenic plantlets (Figure 2A–D) for stress tolerance experiments. These transgenic callus lines, clusters of shoots, and plantlets were transferred into fresh media tri-weekly for 12 weeks to produce more callus cultures, clusters of shoots, and plantlets.

CaPF1 *overexpression confers heavy metal tolerance*

To assess the heavy metals tolerance of transgenic callus cultures, shoots, and plantlets in Virginia pine, three transgenic lines (V2, V8, and V9) with one copy of the transgene were used for stress tolerance. Virginia pine calli and shoots were cultured for 6 weeks on TE medium containing

Figure 2. Transgenic plants regenerated from kanamycin-resistant callus cultures in Virginia pine. (A) Kanamycin-resistant calli derived from a cotyledon of mature zygotic embryos for 6 weeks (bar = 1.1 cm). (B) Clusters of shoots derived from transgenic calli on a selective medium for 6 weeks (bar = 1.4 cm). (C) Rooting of transgenic shoot on rooting medium for 6 weeks (bar = 2.5 cm). (D) Transgenic plants established in soil in the greenhouse for 3 months (bar = 3.2 cm).


(A)
V2
V8
V9
(B)
V2
V8
V9
(C)
V2
V8
V9
(D)
V2
V8
V9

different concentrations (0.5, 1, and 1.5 mM) of $CdCl_2$, $CuCl_2$, or $ZnCl_2$ to assess their survival under heavy metals stress. Figure 3A–C showed the morphology of *CaPF1* transgenic callus cultures (Figure 3A), shoots (Figure 3B), and plantlets (Figure 3C, D) under heavy metal cadmium stress (1.5 mM $CdCl_2$), as well as the pBI121 transgenic callus, shoots, or plant control and the non-transgenic callus, shoots, or plant control. Assessment of survival rates demonstrated that transgenic callus, shoots, and plants were tolerant to heavy metals $CdCl_2$ (Figure 4A), $CuCl_2$ (Figure 4B), or $ZnCl_2$ (Figure 4C), compared to the controls.

Effect of CaPF1 *overexpression on heat tolerance*

To examine whether *CaPF1* overexpression affected heat tolerance, we determined the survival rates of *CaPF1* transgenic calli, shoots, and plants after a brief exposure to 46 and 48 °C (lethal temperature conditions). Three-week-old callus cultures (Figure 5A–C) and shoots (Figure 5D–F) on TE medium were exposed to 48 °C for 5 h with the lids on and then returned to the normal growth temperature for 3 weeks. *CaPF1* transgenic calli and shoots survived with much higher frequency than the controls. Heat experiments with three-month-old plants demonstrated that *CaPF1* transgenic plants conferred tolerance to heat stress (Figure 5G). Survival rates were determined after 3 weeks of recovery. The results demonstrated that *CaPF1* transgenic plants had more than 60% survival rate and controls had less than 10% survival rate (Figure 5H). Our results also indicated that *CaPF1* transgenic Virginia pine callus, shoots, and plants have improved heat tolerance.

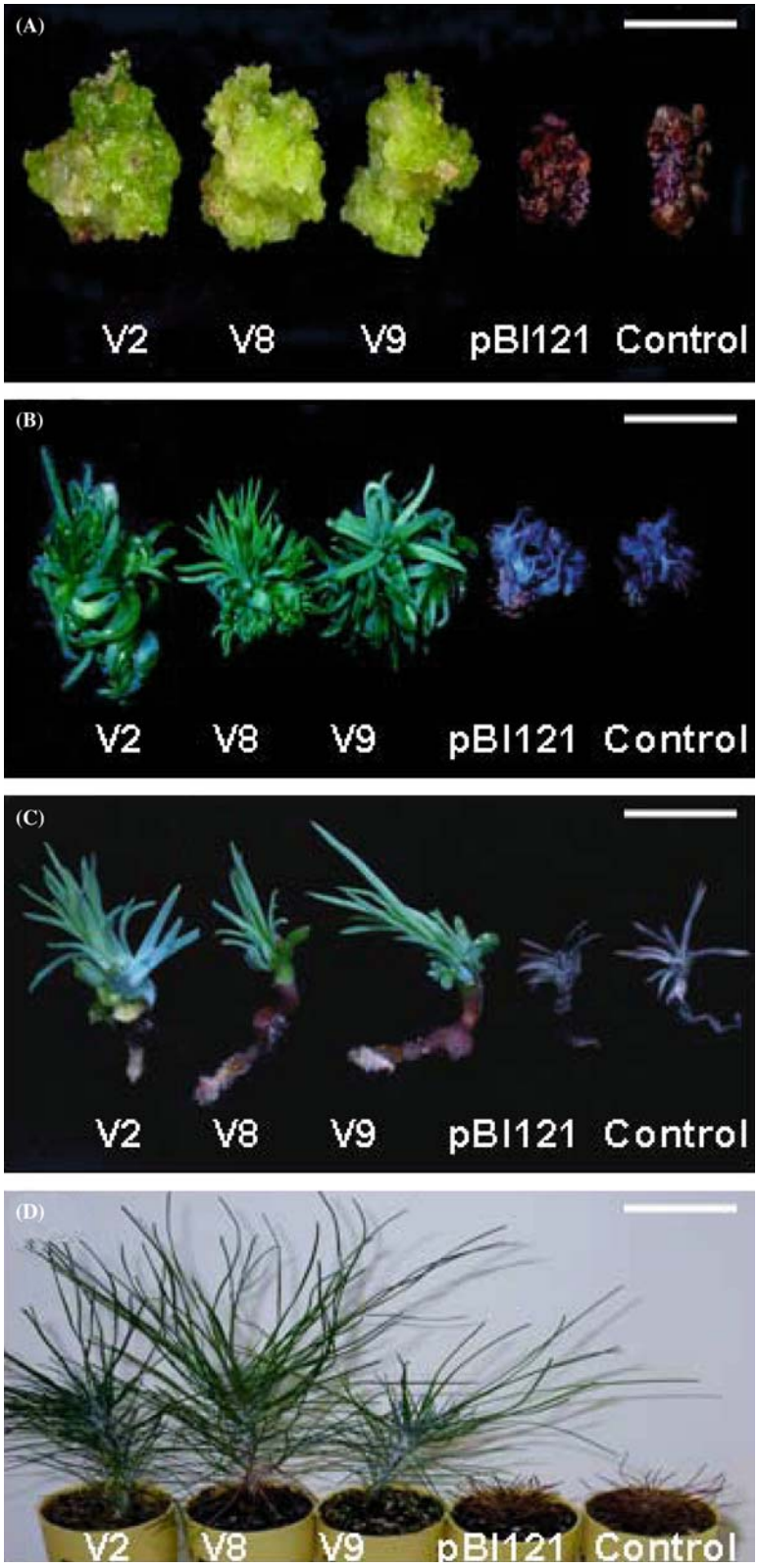
CaPF1 *overexpression inhibits bacterial pathogen growth*

To determine if the *CaPF1* transgenic cell lines could inhibits bacterial pathogen growth in Virginia pine as observed in Arabidopsis and tobacco (Yi *et al.*, 2004), we have investigated influence of *CaPF1* transgenic cell cultures on growth of bacterial pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis*. Compared to the control (Figure 6A), retarded growth of bacterial pathogen was conferred by *CaPF1* overexpression in transgenic cell lines V2 (Figure 6B), V8 (Figure 6C), and V9 (Figure 6D). Determination of number of bacterial pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis* 3, 6, and 9 days after inoculation demonstrated that numbers of *Bacillus thuringiensis* (Figure 6E) and *Staphylococcus epidermidis* (Figure 6F) were decreased in *CaPF1* transgenic cell cultures, compared to the controls.

CaPF1 *overexpression increases antioxidant enzyme activity in transgenic plants*

To assess the regulation of *CaPF1* overexpression on antioxidant enzymes in transgenic Virginia pine under stress conditions, APOX, GR, and SOD activities were evaluated in *CaPF1* transgenic Virginia pine (Tables 1–3). Compared to non-transgenic and pBI121 transgenic plants, APOX activity was higher in *CaPF1* transgenic Virginia pine following stress (Table 1). It was observed that GR activity was not decreased in V2, V8, and V9 plants, but decreased in the controls (Table 2). SOD is a key enzyme for detoxification of superoxide anions and, therefore, its activity was measured as another parameter of oxidative stress. The level of SOD activity was higher in V2, V8, and V9 plants than in the controls (Table 3). The enzyme activity of APOX, GR, and SOD was rapidly reduced under stress conditions in controls, but the activity of these enzymes was relatively stable in *CaPF1* transgenic Virginia pine plants (Tables 1–3). These results demonstrated that *CaPF1* overexpression enhanced abiotic (heavy metals and heat) and biotic (bacterial pathogens) stress tolerance by regulating antioxidant enzyme activities under different stress conditions.

Figure 3. Heavy metal Cadmium tolerance of *CaPF1* overexpressing transgenic Virginia pine plants. (A) Virginia pine calli were cultured for 6 weeks on TE medium containing 1.5 mM $CdCl_2$ to assess their survival under heavy metal cadmium stress (bar = 1.5 cm). (B) Virginia pine shoots were cultured for 6 weeks on TE medium containing 1.5 mM $CdCl_2$ (bar = 2.1 cm). (C) Regenerated Virginia pine plantlets were cultured for 6 weeks on TE medium containing 1.5 mM $CdCl_2$ (bar = 2.5 cm). (D) Virginia pine plants were watered for 6 weeks with 1.5 mM $CdCl_2$ solution (bar = 3.5 cm). Photographs were taken at the end of the 6 week treatment. V2, V8, and V9: calli, shoots, or plants of transgenic cell lines V2, V8, and V9; pBI121: pBI121 transgenic callus, shoots, or plants; Control: non-transgenic callus, shoots, or plants.


(A)
V2
V8
V9
pBI121
Control
(B)
V2
V8
V9
pBI121
Control
(C)
V2
V8
V9
pBI121
Control
(D)
V2
V8
V9
pBI121
Control

CaPF1 *overexpression reduces lipid peroxidation*

To determine if *CaPF1* overexpression could reduce oxidative damage under abiotic (heavy metals and heat) and biotic (bacterial pathogens) stress conditions in Virginia pine, thiobarbituric acid reactive substances (TBARS) content was evaluated in V2, V8, and transgenic V9 and the control plants.

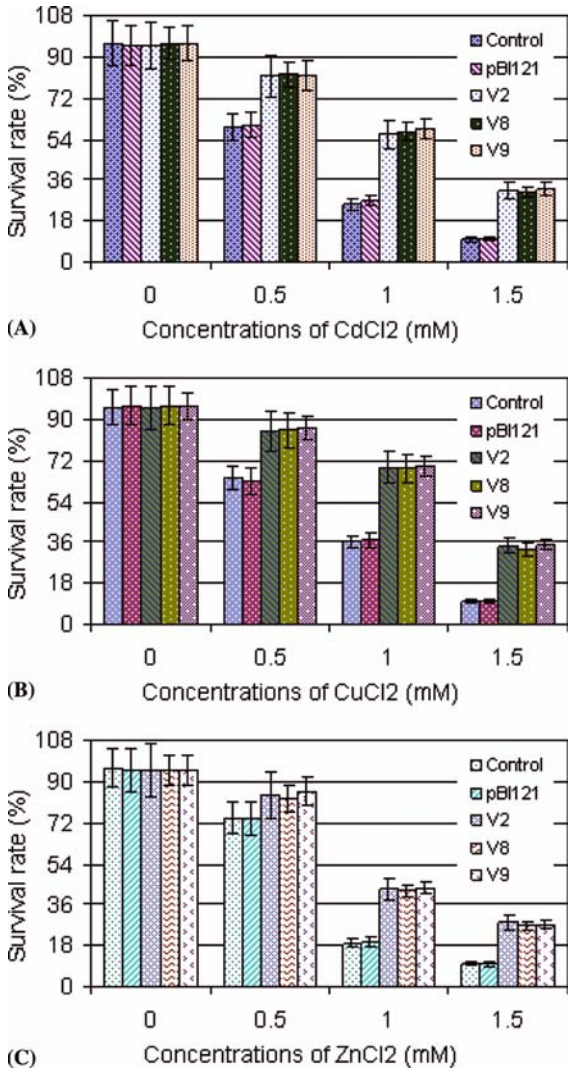
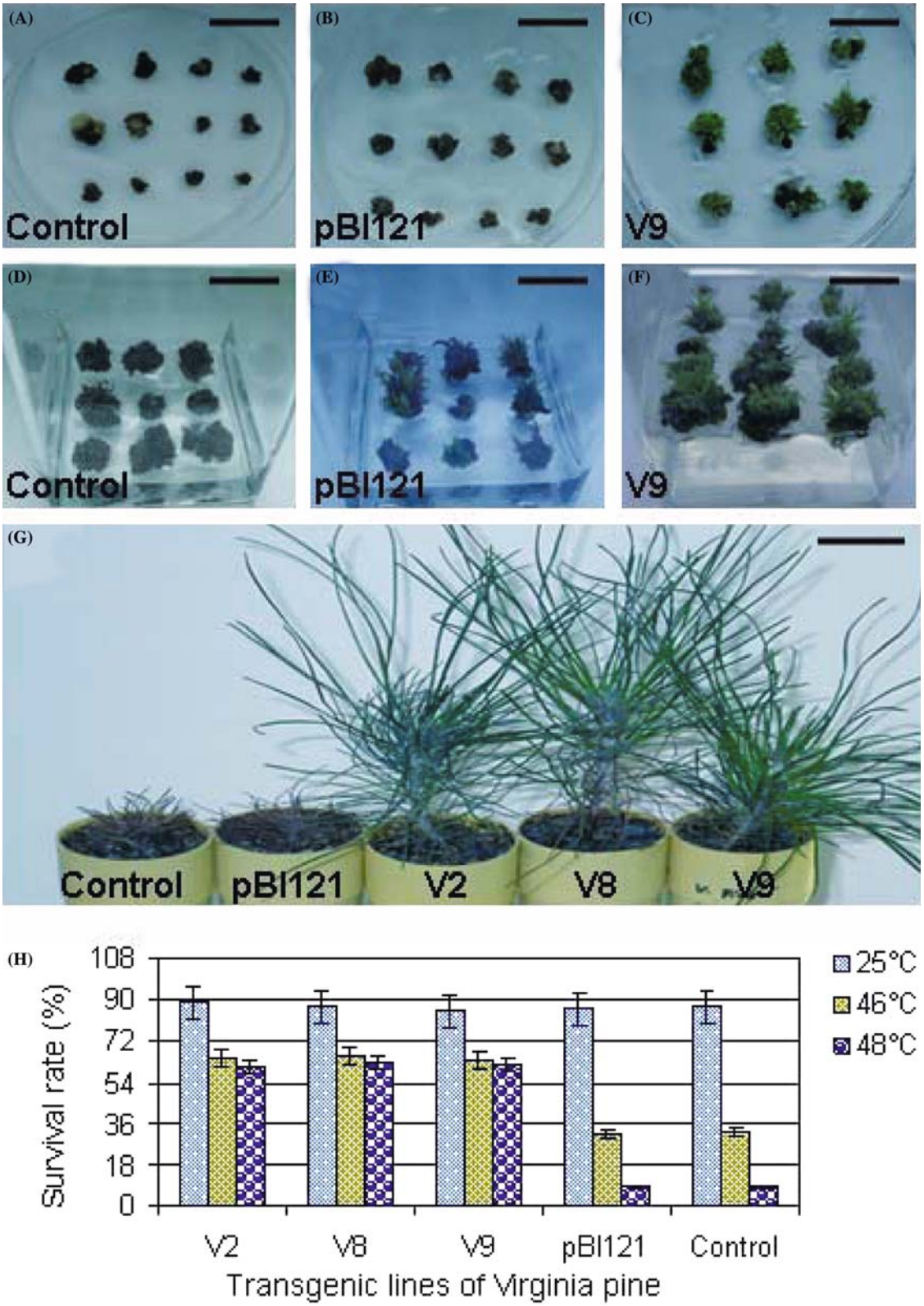


Figure 4. Survival rates of *CaPF1* transgenic Virginia pine plants under different concentrations (0.5, 1, and 1.5 mM) of $CdCl_2$ (A) $CuCl_2$ (B), and $ZnCl_2$ (C) stresses. Survival rates were evaluated at the end of the 6-week treatment. V2, V8, and V9: plants derived from transgenic cell lines V2, V8, and V9; pBI121: pBI121 transgenic plants; Control: non-transgenic plants. Experiments were repeated three times, and each replicate consisted of 30–90 plants. Data represent the mean = SE.

TBARS were measured as markers of lipid peroxidation in *CaPF1* transgenic and the control plants. The total amount of TBARS was considered in each case. There is a higher amount of TBARS content in the control, compared to the *CaPF1* transgenics (Table 4). Therefore, *CaPF1* overexpression reduced oxidative damage under the abiotic (heavy metals and heat) stress conditions.

Heavy metals are directly implicated in the generation of oxidative stress in the plant-surrounding environment. Cadmium, Copper, and Zinc are some of the major industrial pollutants that show phytotoxicity even at low doses (Groppa *et al*., 2001). These metals cause oxidative stress, growth inhibition, and plant death (Stroinski *et al*., 1993; Foyer *et al*., 1994). On the other hand, Copper and Zinc are essential elements for plants, but are strongly phytotoxic at high concentrations. They may alter cell membranes by peroxidative degradation of polyunsaturated fatty acids evidenced by accumulation of lipid peroxidative products (Lobreaux *et al*., 1995; Gawel *et al*., 1996). Oxidative stress is a phenomenon which has been implicated as one of the main agents causing cellular damage in all aerobic organisms exposed to a wide variety of stress conditions (Foyer *et al*., 1994; Kovtun *et al*., 2000). Plant cells can be protected against this oxidative damage by a broad spectrum of radical-scavenger systems, including APOX, GR and SOD (Foyer *et al*., 1994; Roxas *et al*., 1997; Groppa *et al*., 2001). Moreover, plants possess a number of strategies to cope with heavy metals toxicity including regulation of metal uptake by the root system (Lobreaux *et al*., 1995), phytochelatin synthesis (Gawel *et al*., 1996) and prevention of free radical-induced cellular damage by production of antioxidants. Heat stress is another abiotic factor

Figure 5. Heat tolerance of *CaPF1* overexpressing callus, shoots, and plants. (A–C) Callus cultures on shoot formation medium exposed to 48 °C for 5 h with the lids on and then returned to the normal growth temperature for 3 weeks. (D–F) Shoots on shoot formation medium exposed to 48 °C for 5 h and then returned to the normal growth temperature for 3 weeks. (G) Plants exposed to 48 °C for 5 h and then returned to the normal growth temperature for 3 weeks. (H) Survival rates of plants exposed to 25 °C (control), 46 and 48 °C for 5 h and then returned to the normal growth temperature for 3 weeks. Experiments were repeated four times (n = 15 each), and the small bars represent the standard errors. The photographs were taken 3 weeks after heat treatment.


(A)
Control
(B)
pBI121
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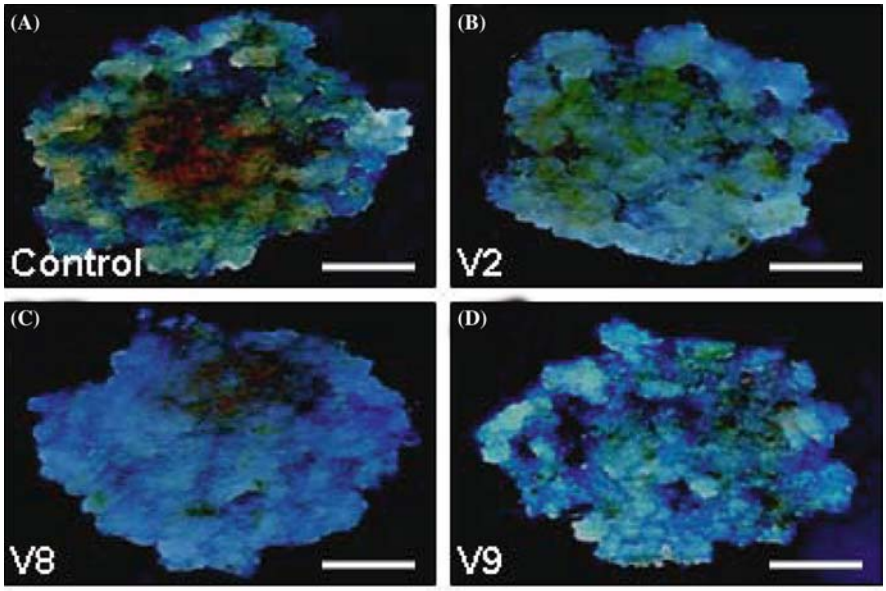
V9
(D)
Control
(E)
pBI121
(F)
V9
(G)
Control
pBI121
V2
V8
V9
(H)
Survival rate (%)
108
90
72
54
36
18
0
V2
V8
V9
pBI121
Control
Transgenic lines of Virginia pine
25°C
46°C
48°C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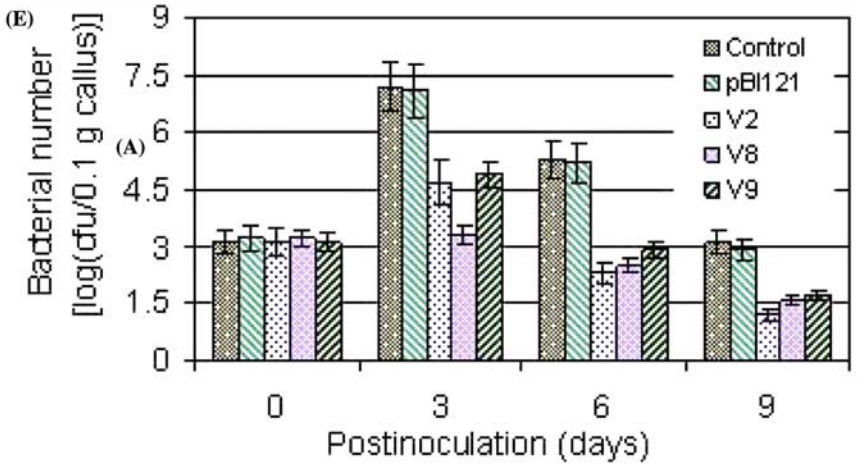
(E)
(A)
Bacterial number
[log(cfu/0.1 g callus)]
9
7.5
6
4.5
3
1.5
0
0
3
6
9
Postinoculation (days)
Control
pBI121
V2
V8
V9

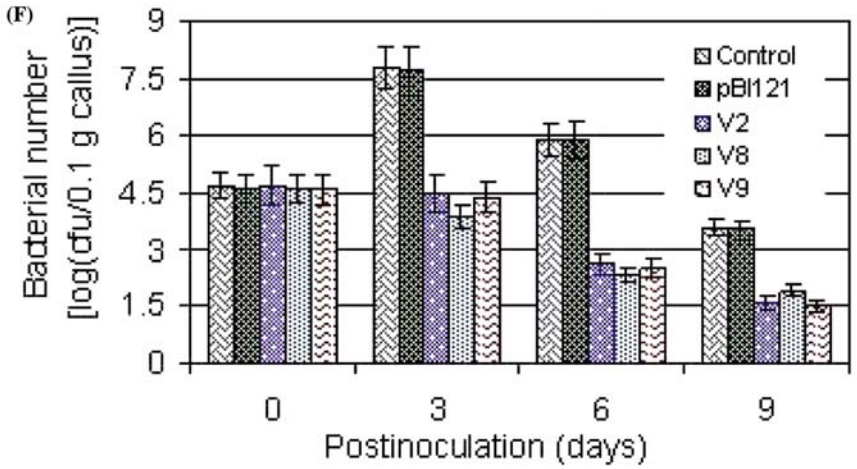
(F)
Bacterial number
[log(cfu/0.1 g callus)]
9
7.5
6
4.5
3
1.5
0
0
3
6
9
Postinoculation (days)
Control
pBI121
V2
V8
V9

Figure 6. Bacterial pathogen tolerance of *CaPF1* transgenic Virginia pine cell cultures. Photographs were taken 6 days after pathogen inoculation. (A) pBI121 transgenic cell cultures. (B) *CaPF1* transgenic cell line V2. (C) *CaPF1* transgenic cell line V8. (D) *CaPF1* transgenic cell line V9 (A–D bars = 1.2 cm). (E) Bacterial *Bacillus thuringiensis* tolerance of *CaPF1* transgenic Virginia pine cell cultures. (F) Bacterial *Staphylococcus epidermidis* tolerance of *CaPF1* transgenic Virginia pine cell cultures. Reduced bacterial growth in *CaPF1* transgenic Virginia pine cell cultures (V2, V8, and V9) was observed, compared with pBI121 transformed (pBI121) and non-transgenic control (control). Plants were infected with 2×10^5 CFU/ml solution of *Bacillus thuringiensis* or *Staphylococcus epidermidis*, and bacterial numbers were determined at 0, 3, 6, and 9 days after inoculation, respectively. Data are presented as means ± SE. One data out of three independent experiments with similar results is shown.

that results in oxidative stress. In Virginia pine, the enzyme activity of APOX, GR, SOD, and TBARS was rapidly reduced under stress conditions in controls, but the activity of these enzymes was relatively stable in *CaPF1* transgenic Virginia pine plants (Tables 1–4). These results demonstrated that *CaPF1* overexpression enhanced abiotic (heavy metals and heat) stress tolerance by regulating antioxidant enzyme activities and lipid peroxidation under stress conditions.

Overexpression of *CaPF1* clearly resulted in tolerance against bacterial pathogens *Bacillus thuringiensis* and *Staphylococcus epidermidis* stresses (Figure 6). In our study, *CaPF1* functions as a positive regulator in the regulation of stress tolerance. We found that *CaPF1* reduced growth of bacterial pathogens after *CaPF1* transgenic cell cultures have been inoculated with *Bacillus thuringiensis* and *Staphylococcus epidermidis* (Figure 6). This observation is consistent with an earlier observation that *CaPF1* transgenic *Arabidopsis* conferred resistance against *P. syringae* pv tomato DC3000 that infects wild-type *Arabidopsis* Col-0 and *CaPF1* transgenic tobacco plants resulted in tolerance to *P. syringae pv tabaci* that infects wild-type tobacco (Yi *et al.*, 2004). There may be two possible reasons why *CaPF1* conferred resistance to bacterial pathogens. First, *CaPF1* protected cells under stress condition by increasing antioxidant enzymes APOX, GR, and SOD

Table 1. Antioxidant enzyme ascorbate peroxidase (APOX), glutathione reductase (GR), and superoxide dismutase (SOD) activities of Virginia pine plants.

Treatments	APOX activity (U/g FW)				
	Control	pBI121	V2	V8	V9
Without stress	6.2 ± 1.3a	6.3 ± 2.1a	6.2 ± 1.4a	6.4 ± 1.5a	6.7 ± 2.1a
1.5 mM $CdCl_2$	3.7 ± 1.4b	3.6 ± 2.0b	6.0 ± 1.3a	6.3 ± 1.4a	6.5 ± 2.2a
1.5 mM $CuCl_2$	3.5 ± 1.5b	3.4 ± 2.2b	6.3 ± 1.5a	6.2 ± 1.6a	6.6 ± 2.1a
1.5 mM $ZnCl_2$	3.4 ± 1.6b	3.5 ± 2.3b	6.1 ± 1.2a	6.1 ± 1.7a	6.4 ± 2.3a
Heat (48 °C)	3.8 ± 1.8b	3.9 ± 2.4b	6.4 ± 1.6a	6.7 ± 1.8a	6.8 ± 2.5a
2×10^5 CFU/ml *Bacillus thuringiensis*	3.9 ± 1.7b	3.8 ± 2.1b	6.8 ± 1.7a	6.9 ± 1.9a	6.7 ± 2.4a
2×10^5 CFU/ml *Staphylococcus epidermidis*	3.8 ± 1.9b	3.7 ± 1.9b	6.7 ± 1.8a	6.8 ± 1.7a	6.9 ± 2.1a

Enzyme activity was determined 6 weeks after treatment by different stresses, respectively. Each treatment was replicated three times. Data represent the mean ± SE. Values followed by different letters are significantly different ($\alpha = 0.05$) by ANOVA.

Table 2. Antioxidant enzyme glutathione reductase (GR) activity of Virginia pine plants.

Treatments	GR activity (U/100 g FW)				
	Control	pBI121	V2	V8	V9
Without stress	9.5 ± 1.4a	9.3 ± 2.1a	9.6 ± 1.2a	9.4 ± 1.2a	9.7 ± 2.5a
1.5 mM $CdCl_2$	5.5 ± 1.4b	5.3 ± 2.1b	9.6 ± 1.2a	9.4 ± 1.2a	9.7 ± 2.4a
1.5 mM $CuCl_2$	5.6 ± 1.1b	5.5 ± 2.2b	9.5 ± 1.3a	9.5 ± 1.3a	9.6 ± 2.6a
1.5 mM $ZnCl_2$	5.4 ± 1.2b	5.6 ± 2.3b	9.2 ± 1.2a	9.5 ± 1.2a	9.6 ± 2.5a
Heat (48 °C)	5.8 ± 1.7b	5.9 ± 2.5b	9.3 ± 1.7a	9.7 ± 1.5a	9.8 ± 2.7a
2×10^5 CFU/ml *Bacillus thuringiensis*	5.3 ± 1.5b	5.4 ± 2.1b	9.2 ± 1.4a	9.3 ± 1.3a	9.6 ± 2.6a
2×10^5 CFU/ml *Staphylococcus epidermidis*	5.6 ± 1.1b	5.1 ± 1.4b	9.2 ± 1.6a	9.1 ± 1.0a	9.8 ± 2.1a

Enzyme activity was determined 6 weeks after treatment by different stresses, respectively. Each treatment was replicated three times. Data represent the mean ± SE. Values followed by different letters are significantly different ($\alpha = 0.05$) by ANOVA.

activities. Second, *CaPF1* reduced oxidative damage derived from bacterial pathogen stress by decreasing TBARS. However, the molecular mechanism by which *CaPF1* regulates stress tolerance remains to be elucidated further. It would be most helpful to study the structure and functions of components that are associated with stress-response-signaling pathways. The multiple stress tolerance of transgenic plants overexpressing *CaPF1* is similar to that of transgenic plants overexpressing AtNDPK2 (Moon *et al.*, 2003). Our results suggest an important role for *CaPF1* in the environmental stress associated with oxidative stress. These results strongly indicate that *CaPF1* may be an important upstream signaling component of the abiotic stress and pathogen-mediated signaling cascade in plants. Multiple stress tolerance can arise from *CaPF1* overexpressed transgenic Virginia pine that is associated with increased expression of a number of antioxidant

Table 3. Antioxidant enzyme superoxide dismutase (SOD) activity of Virginia pine plants.

Treatments	SOD activity (U/100 mg FW)				
	Control	pBI121	V2	V8	V9
Without stress	8.5 ± 1.3a	8.3 ± 2.1a	8.6 ± 1.3a	8.4 ± 1.7a	8.7 ± 2.5a
1.5 mM $CdCl_2$	3.7 ± 1.4b	3.1 ± 2.3b	8.4 ± 1.7a	8.1 ± 1.9a	8.5 ± 2.1a
1.5 mM $CuCl_2$	3.6 ± 1.1b	3.5 ± 2.2b	8.5 ± 1.5a	8.3 ± 1.6a	8.6 ± 2.6a
1.5 mM $ZnCl_2$	3.4 ± 1.2b	3.6 ± 2.1b	8.2 ± 1.9a	8.5 ± 1.7a	8.4 ± 2.2a
Heat (48 °C)	3.9 ± 1.7b	3.8 ± 2.5b	8.4 ± 1.8a	8.7 ± 1.4a	8.8 ± 2.4a
2×10^5 CFU/ml *Bacillus thuringiensis*	3.3 ± 1.5b	3.4 ± 2.4b	8.3 ± 1.4a	8.8 ± 1.5a	8.9 ± 2.3a
2×10^5 CFU/ml *Staphylococcus epidermidis*	3.9 ± 1.5b	3.7 ± 1.0b	8.7 ± 1.8a	8.7 ± 1.9a	8.9 ± 2.4a

Enzyme activity was determined 6 weeks after treatment by different stresses, respectively. Each treatment was replicated three times. Data represent the mean ± SE. Values followed by different letters are significantly different ($\alpha = 0.05$) by ANOVA

Table 4. Thiobarbituric acid reactive substance (TBARS) changes of Virginia pine plants.

Treatments	TBARS (mmol/g FW)				
	Control	pBI121	V2	V8	V9
Without stress	115.5 ± 12.4a	115.3 ± 12.1a	116.6 ± 13.2a	116.4 ± 12.2a	117.7 ± 12.5a
1.5 mM $CdCl_2$	215.5 ± 21.4b	213.3 ± 23.1b	126.6 ± 12.2a	123.4 ± 13.2a	125.7 ± 13.5a
1.5 mM $CuCl_2$	205.6 ± 19.1b	208.5 ± 20.2b	124.5 ± 13.3a	127.5 ± 12.3a	127.6 ± 12.6a
1.5 mM $ZnCl_2$	221.4 ± 21.2b	225.6 ± 21.3b	116.2 ± 12.7a	116.5 ± 14.1a	117.6 ± 12.9a
Heat (48 °C)	233.9 ± 21.7b	231.8 ± 24.5b	138.4 ± 14.8a	135.7 ± 16.4a	138.8 ± 12.4a
2×10^5 CFU/ml *Bacillus thuringiensis*	225.3 ± 21.5b	224.7 ± 22.1b	136.2 ± 15.4a	132.3 ± 16.3a	137.6 ± 15.6a
2×10^5 CFU/ml *Staphylococcus epidermidis*	221.6 ± 19.1b	221.1 ± 21.0b	131.2 ± 16.0a	131.1 ± 21.1a	131.8 ± 19.8a

TBARS was determined 6 weeks after treatment by different stresses, respectively. Each treatment was replicated three times. Data represent the mean ± SE. Values followed by different letters are significantly different ($\alpha = 0.05$) by ANOVA.

Table 5. Determination of fresh weight (F.W.), cell size, and cell number of Virginia pine cell lines 6 weeks after culture of shoot formation.

Cell lines	Organ growth			
	F.W. (mg)/100 shoots	Mean F.W. (mg)/shoot	Cell size (% of the control)	Cell number/Unit (% of the control)
Control	2121.5 ± 12.4a	21.2 ± 1.2a	100.0 ± 0.0a	100.0 ± 0.0a
pBI121	2121.6 ± 13.1a	21.2 ± 1.3a	100.0 ± 0.0a	100.0 ± 0.0a
V2	4215.4 ± 12.2b	42.1 ± 1.2b	62.5 ± 1.9b	187.6 ± 2.9b
V8	4217.3 ± 13.5b	42.1 ± 1.3b	67.3 ± 1.7b	198.6 ± 2.6b
V9	4218.6 ± 12.7b	42.1 ± 1.2b	63.1 ± 1.8b	218.8 ± 2.7b

Percentages of results from V2, V8, and V9 transgenic needles to those from control needles are shown. Each treatment was replicated three times. Data represent the mean ± SE. Values followed by different letters are significantly different ($\alpha = 0.05$) by ANOVA.

genes, including APOX, GR, and SOD. Thus, we suggest that *CaPF1* mediates multiple stress tolerance by signaling the expression of genes involved in antioxidant and protective functions.

CaPF1 *overexpression enhances shoot growth*

To understand *CaPF1* function in plant organ formation, we have examined the effects of *CaPF1* on shoot growth and development. We observed that the size of *CaPF1* transegnic shoots was larger than the controls (Table 5; Figure 7A–D, at the same age). Scanning electron microscopy (Figure 7E–H) confirmed the visual observation in *CaPF1* transgenic cell lines (V2, V8, and V9) and the controls (the non-transgenic control). Determination of cell size and number per unit demonstrated that the controls had larger cell size (Figure 7I) and the V2, V8, and V9 transgenic cell lines had increased the cell number (Figure 7J–L).

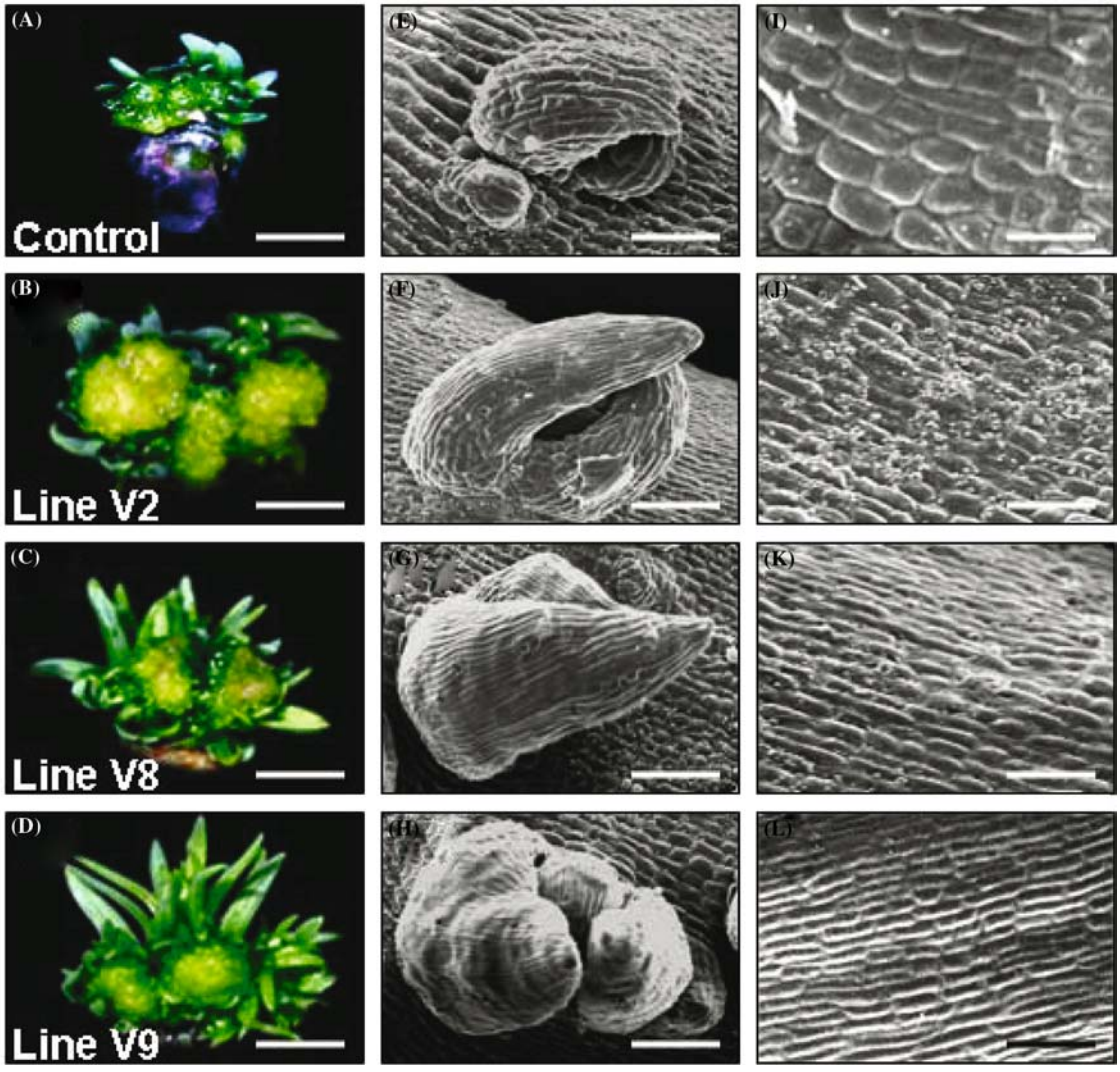

Figure 7. Overexpression of *CaPF1* in transgenic Virginia pine enhanced organ growth. (A–D) Virginia pine shoots of non-transgenic control (A) and shoots derived from *CaPF1* transgenic cell lines V2 (B), V8 (C), and V9 (D), at the same age of culture (A–D bars = 1.2 cm). (E–H) Scanning electron microscopic observation of non-transgenic control (E) and shoots derived from *CaPF1* transgenic cell lines V2 (F), V8 (G), and V9 (H) (E–H bars = 0.5 mm). (I–L) Scanning electron microscopic observation of needle surfaces of non-transgenic control (I) and shoots derived from *CaPF1* transgenic cell lines V2 (J), V8 (K), and V9 (L) (F–J bars = 0.2 mm). Shoots were grown at the same culture conditions and specimens were photographed at the same magnification for A–D, E–H, and I–L, respectively.

We demonstrate that *CaPF1* is an organ size regulator that is necessary and sufficient to control cell number and growth of organ throughout shoot development, but does not alter superficial morphology by increasing cell number in Virginia pine. Our results demonstrated that *CaPF1* regulates the cell number and size of mature organ by increasing cell division and growth.

We have shown that overexpression of the transcription factor *CaPF1* caused outgrowth of shoot and needles in 3 transgenic lines (V2, V8, and V9) in Virginia pine. Determination of cell size and number per unit demonstrated that the controls had larger cell size (Figure 7I) and the V2, V8, and V9 transgenic cell lines had increased the cell number (Figure 7J–L). We demonstrate that *CaPF1* may be an organ size regulator that is sufficient to control cell number and growth of organs throughout shoot development without altering superficial morphology by increasing cell number in Virginia pine.

Therefore, discovery of transcription factors involved in environmental stress responses provides new targets for genetic engineering of tree and other plants for better multiple stress tolerance. The system described in this study is suitable for many applications, especially for functional identification of novel genes derived from genomic research in pine species.

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References

Foyer, C.H., Deascouveries, P. and Kunert, K.J. 1994. Protection against oxygen radicals: important defense mechanism studied in transgenic plants. Plant Cell Environ. 17: 507–523.

Gawel, J.E., Ahner, B.A., Friedland, A.J. and Morel, F.M.M. 1996. Role for heavy metals in forest decline indicated by phytochelatin measurements. Nature 381: 64–65.

Gong, J.M., Lee, D.A. and Schroeder, J.I. 2003. Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis. Proc. Natl. Acad. Sci. USA 100: 10118–10123.

Groppa, M.D., Tomaro, M.L. and Benavides, M.P. 2001. Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs. Plant Sci. 161: 481–488.

Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X., Han, Y. and Martin, G.B. 2002. Tomato transcription factors *pti4, pti5*, and *pti6* activate defense responses when expressed in *Arabidopsis*. Plant Cell 14: 817–831.

Huckelhoven, R., Dechert, C. and Kogel, K.H. 2003. Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*. Proc. Natl. Acad. Sci. USA 100: 5555–5560.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. 1998. Arabidopsis *CBF1* overexpression induces COR genes and enhances freezing tolerance. Science 280: 104–106.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinnozaki, K. and Shinozaki, K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol. 17: 287–291.

Kim, J.B., Kang, J.Y. and Kim, S.Y. 2004. Over-expression of a transcription factor regulating ABA responsive gene expression confers multiple stress tolerance. Plant Biotechnol. J. 2: 459–466.

Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA 97: 2940–2945.

Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R. and Gilchrist, D.G. 2002. Expression of the antiapoptotic baculovirus *p35* gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. Proc. Natl. Acad. Sci. USA 99: 15217–15221.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391–1406.

Lobreaux, S., Thoiron, S. and Briat, J.F. 1995. Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. Plant J. 8: 443–449.

Mizukami, Y. and Fischer, R.L. 2000. Plant organ size control: *AINTEGUMENTA* regulates growth and cell numbers during organogenesis. Proc. Natl. Acad. Sci. USA 97: 942–947.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C. Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. 2003. NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc. Natl. Acad. Sci. USA 100: 358–363.

Mukhopadhyay, A., Vij, S. and Tyagi, A.K. 2004. Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc. Natl. Acad. Sci. USA 101: 6309–6314.

Queitsch, C., Hong, S.W., Vierling, E. and Lindquist, S. 2000. Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. Plant Cell 12: 479–492.

Roxas, V.P., Smith, R.K. Jr, Allen, E.R. and Allen, R.D. 1997. Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat. Biotechnol. 15: 988–991.

Rushton, P.J. and Somssich, I.E. 1998. Transcriptional control of plant genes responsive to pathogens. Curr. Opin. Plant Biol. 1: 311–315.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, New York.

Sheveleva, E., Chmara, W., Bohnert, H.J. and Jensen, R.G. 1997. Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. Plant Physiol. 115: 1211–1219.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 2000. Molecular responses to dehydration and low temperature: differences and cross talk between two stress signaling pathways. Curr. Opin. Plant Biol. 3: 217–223.

Shi, H., Lee, B.H., Wu, S.J. and Zhu, J.K. 2003. Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat. Biotechnol. 21: 81–85.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. 1997. *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94: 1035–1040.

Stroinski, A. and Floryszak-Wieczorek, J. 1993. Effect of cadmium on the host–pathogen system. IV. Influence of cadmium and Phytophtora infestans on membrane permeability of potato tuber. J. Plant Physiol. 142: 575–578.

Tang, W., Luo, H. and Newton, R.J. 2004. Effects of antibiotics on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. Plant Cell Tiss. Org. Cult. 70: 71–81.

Tang, W. and Newton, R.J. 2004a. Regulated gene expression by glucocorticoids in cultured Virginia pine (*Pinus virginiana* Mill.) cells. J. Exp. Bot. 55: 1499–1508.

Tang, W. and Newton, R.J. 2004b. Increase of polyphenol oxidase and decrease of polyamines correlate with tissue browning in Virginia pine (*Pinus virginiana* Mill.). Plant Sci. 167: 621–628.

Tang, W., Sederoff, R. and Whetten, R. 2001. Regeneration of transgenic Virginia pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*. Planta 213: 981–989.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. 1993. Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science 259: 508–510.

Thomashow, M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 571–599.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity. Proc. Natl. Acad. Sci. USA 97: 632–11 637.

Vailleau, F., Daniel, X., Tronchet, M., Montillet, J.L., Triantaphylides, C. and Roby, D. 2002. A R2R3-MYB gene, AtMYB30, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. Proc. Natl. Acad. Sci. USA 99: 10179–10184.

Yi, S.Y., Kim, J.H., Joung, Y.H., Lee, S., Kim, W.T., Yu, S.H. and Choi, D. 2004. The pepper transcription factor *capf1* confers pathogen and freezing tolerance in *Arabidopsis*. Plant Physiol. 136: 2862–2874.

Zeevaart, J.A.D. and Creelman, R.A. 1988. Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39: 439–473.