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Atcbf1 Gene Enhances Salt Tolerance in Potato (*Solanum tuberosum* L.)

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ABSTRACT

CBF1 (C-repeat/DRE Binding Factor 1), isolated from *Arabidopsis thaliana*, was transformed into potato plants (*Solanum tuberosum* L.) using the CaMV 35S promoter to test for salt tolerance. Two transgenic lines were selected using genomic PCR and northern hybridization. The plants showed normal vegetative growth in *in vitro* culture. To examine *CBF1* gene (*AtCBF1*) function, transgenic lines and a null-transgenic line were planted in a continuously aerated water culture system in a controlled environment. Plant growth and physiological traits contributing to the tolerance were observed in saline (50 mM NaCl) and non-saline (distilled water) environments. Growth performance of transgenic and null-transgenic plants in the control environment did not differ significantly. However, the transgenic plants showed better growth and osmotic adjustment than null-transgenic plants in the saline environment. The transgenic plants accumulated more than 20% biomass and 2-fold higher osmotic adjustment than null-transgenic plants. The results are discussed in relation to the possible role of the *AtCBF1* gene with respect to salt tolerance of potato plants with some unique physiological traits.

Keywords: cbf, salt, tolerance, increase, potato, plant
Abbreviations: ***AtCBF1***, *Arabidopsis thaliana* C-repeat/DRE Binding Factor 1; **GUS,** beta-glucuronidase; **PCR**, polymerase chain reaction; **RT-PCR**, Real Time Polymerase Chain Reaction; **SDS**, sodium dodecyl sulfate

INTRODUCTION

Extreme ecological changes induced by climate change aggravate abiotic stress, causing considerable reductions in crop yields. Therefore, enhancing our understanding of abiotic stress responses, stress tolerance, and the physiological mechanisms underlying these responses using agronomically important traits is one of the most important goals of plant science research. Abiotic stresses such as drought and salinity impose major constraints that limit crop productivity around the world. In general, salt tolerance in plants is achieved through the action of multiple traits that are important for plant growth and physiology, such as traits involved in ion and osmotic homeostasis (Hirayama and Shinozaki 2010; Kaye *et al.* 2011). Similarly, gene transformation can be efficiently applied to enhance salt tolerance when there is a clear link between target gene functioning and the growth and physiological traits of the plant in the presence of salinity (Kaye *et al.* 2011; Tognetti *et al.* 2012).

However, there are big gaps in our understanding of how gene function affects the physiology of salt tolerance. Furthermore, plants commonly overcome salt stress using multiple physiological traits related to water and ion relations (Siddiqui *et al.* 2008). Some physiological traits related to homeostasis can be examined to determine how target genes function in transgenic plants to confer salt tolerance. These physiological traits for salt tolerance could play a critical role in linking target genes and phenotypic responses (Edmeades *et al.* 2004; Yin *et al.* 2004). It is well known that gene transformation technology has great potential to enhance salt tolerance in plant (Apse and Blumwald 2002; Sinclair *et al.* 2004).

CBF1 (C-repeat/DRE Binding Factor 1) is a transcriptional activator that binds C-repeat/DRE, a *cis*-acting DNA regulatory element, which may induce several genes related to improved plant tolerance of salinity. It has been reported that constitutive overexpression of *CBF/DREB* genes greatly enhance salt tolerance in *Arabidopsis thaliana* (Jaglo-Ottosen *et al.* 1998; Kasuga *et al.* 1999), *Brassica napus* (Jaglo-Ottosen *et al.* 2001), and *Prunus avium* (Kitashiba *et al.* 2004). Downstream genes identified by microarray analysis in plants transformed with *CBF* include genes encoding transcription factors, phospholipase C, desaturase, enzymes required for the biosynthesis of sugars, LEA proteins, antifreeze proteins, KIN (cold inducible) proteins, osmoprotectant biosynthesis protein, protease inhibitors, and others (Ryu *et al.* 2009). The use of the *CBF* gene to enhance salt tolerance is limited due to the lack of clear-cut evidence involving the functioning of this gene at the whole-plant level. Previous *in vitro* studies have suggested that the *CBF* gene has great potential for enhancing salt tolerance in plants. Therefore, the present study was undertaken to evaluate *AtCBF1* gene functioning using key growth and physiological traits that indicate the levels of salt tolerance of whole plants in a controlled environment.

MATERIALS AND METHODS

Plant, bacteria, and culture condition

The potato cultivar *Solanum tubersosum* var. 'Superior' was employed in this study. Tuber tissues of this cultivar were inoculated on MS medium (MS salts, 30 g L^{-1} sucrose, and 8 g L^{-1} phytagel, pH 5.6–5.8) and maintained in a growth room at 23°C under a 14 h light/10 h dark photoperiod. Binary vector *p*CAMBIA2300 containing *CBF* under the control of the CaMV (*Cauliflower mosaic virus*) 35S promoter was used to construct *p*CAMBIA2300/35S-CBF. This vector construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404. The *Agrobacterium* was grown on YEP medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, and 2 mM $MgSO_4$, pH 7.2) at 28°C for 48 h prior to transformation.

Received: 6 August, 2012. Accepted: 25 december, 2012.

Original Research Paper

Agrobacterium-mediated transformation and transgenic plants

For the transformation of *p*CAMBIA2300/35S-CBF1 into potato plants, full-length *Arabidopsis thaliana* CBF1 cDNA, obtained by RT-PCR, was inserted into the *Hin*d III site of *p*CAMBIA2300. The recombinant DNA was transferred into the *Agrobacterium* strain using the freeze-thaw method described by Holster *et al.* (1978), and the plants were transformed as described by Yeo *et al.* (2000). Regenerated transgenic plants were transplanted into a soil mixture for propagation. Null-transgenic plants were obtained by transformation with the *p*BI121 vector containing only the *GUS* gene.

Genomic DNA extraction and PCR analysis

Genomic DNA was extracted from the transgenic plants, null-transgenic plants, and wild-type plants with CTAB buffer using the method of Graham (1994). The purified DNA was resolved by electrophoresis on a 1% agarose gel in TBE buffer with subsequent transfer in 20X SSC to a Hybond-N^+ membrane (Amersham, USA). Filters were hybridized with random primer-labeled cDNA probes according to Church and Gilbert (1984) and washed with 0.2X SSC/0.1% SDS at 65°C. The blots were exposed to X-ray films with intensifying screens at -70°C.

RNA preparation and northern blot analysis

To test the expression of *AtCBF1* in transgenic potato plants, total RNA was extracted using Tri-Reagent (MRC, USA). For northern blot analysis, RNA was electrophoresed in a 1.2% denaturing formaldehyde/MOPS agarose gel. The northern blot was transferred to a Hybond-N^+ membrane (Amersham, USA) using the capillary transfer method, with 20X SSC as the transfer buffer. Prehybridization was performed for 15 min at 65°C in hybridization solution (1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, and 7% SDS) before hybridization solution containing radio-labeled cDNA probe was added, followed by hybridization overnight at 65°C in a Hybaid oven (Hybaid, USA). The probe was labeled using the random oligonucleotide priming method (Amersham, USA). Stringent membrane washing was performed with 0.2X SSC/0.1% SDS at 65°C. The washed membrane was then exposed to X-ray film at -70°C.

Plant growth and salt stress

Young seedlings of transgenic and wild-type plants were grown in test tubes containing MS medium. The seedlings were transferred from *in vitro* culture to a water culture system when the seedlings had four or five foliar leaves. A continuously aerated water culture system was used, and the system was maintained in a growth chamber (13 h day at 25 ± 2°C and 290 mmole m^{-2} s^{-1} PAR, 11 h night at 18 ± 2°C). An optimal nutrient solution for potato was employed in the culture system, i.e., 379 g KNO_3, 325 g $Ca(NO_3)_2$, 80 g $NH_4H_2PO_4$, 215 g $MgSO_4 \cdot 7H_2O$, and 23 g Fe-EDTA in 1 L water. Plants were arranged in a completely randomized design within a plot.

When seedlings reached the stage at which several fully expanded leaves and vigorous roots were present, salt stress was imposed by supplementing the nutrient solution with 50 mM NaCl. Salt stress was then maintained for 15 days. Nutrient solution lacking NaCl was used for the control.

Plant growth responses under salt stress

Plants were sampled after 15 days of stress treatment. Leaf samples were harvested just before initiation of the NaCl treatment and after the treatment was terminated. After harvesting, the leaves were immersed in distilled water for 8 h to make them turgid and were then frozen in liquid nitrogen, followed by thawing, in order to express cell sap. After the expression of cell sap, the remaining tissues were dried at 85ºC for 2 days before dry weights were measured. The relative growth rates were obtained using classical plant growth analysis (Hunt 1990).

Ion analysis and osmotic adjustment

Osmotic potential (OP) of the expressed cell sap was determined using a vapor pressure osmometer (Wescor 5520, Utah, USA). Osmotic adjustment was calculated using the difference between the OP of plants under NaCl treatment (stressed) and plants grown without NaCl treatment (unstressed). Concentrations of potassium and sodium in the cell sap were measured with an inductively coupled plasma (ICP) emission spectrometer (Plasma 400, The Perkin Elmer Corp., USA).

RESULTS

The *AtCBF1* gene was inserted into potato plants and was constitutively expressed under the control of the CaMV 35S promoter (**Fig. 1**). To confirm the integration of the *AtCBF1* gene into the potato genome, kanamycin-resistant transgenic potato plants were analyzed using PCR. The PCR products showed the expected band pattern in transgenic plants, displaying a 750 bp PCR fragment that was lacking in the wild type (**Fig. 2**).

The expression of *AtCBF1* in transgenic plants was detected by northern blot analysis. *AtCBF1* mRNA expression was not observed in wild-type or null-transgenic plants because *p*BI121 in both types of plants only contained *GUS* sequences (**Fig. 3**). However, two transgenic lines displayed the expected *AtCBF1* mRNA signal, while the null-transgenic and wild-type lines did not display the genomic PCR band or the mRNA expression signal of *AtCBF1*. Both transgenic lines showed similar patterns of *AtCBF1* gene expression.

The transgenic plants displayed significantly better growth performance in the saline environment compared to wild type (**Figs. 1, 5**). However, the growth of the transgenic plants in the non-saline environment did not differ considerably from that of the wild type. Furthermore, the transgenic plants accumulated 20% more biomass in the saline environment compared to wild type. Moreover, the transgenic plants showed greater relative growth rates under both stress and unstressed conditions compared to wild type (**Fig. 4**). These data suggest that the *AtCBF1* gene might contribute to certain physiological traits related to salt tolerance, contributing to plant growth in both stress and stress-free conditions.

The transgenic plants showed significantly greater ability to perform salinity-induced osmotic adjustments than wild-type plants (**Fig. 5**), with transgenic plants performing more than twice the level of osmotic adjustment compared to that of the wild type. However, the osmotic potential of expressed sap from turgid leaf tissue was -249.33 ± 24.60 mmol kg^{-1} for transgenic plants and -272.00 ± 23.06 mmol kg^{-1} for wild-type plants when the plants were grown in NaCl-free nutrient solution. By contrast, the transgenic plants exhibited considerably lower osmotic potentials during the imposition of salt stress compared with wild type. Under salt stress, the turgid osmotic potential in transgenic plants was -527.33 ± 12.46 mmol kg^{-1}, while in wild-type plants, the level was -388 ± 9.29 mmol kg^{-1}. Salt-induced osmotic adjustment, which predominantly occurred in transgenic plants, improved the ability of the plants to grow in the saline environment due to a decrease in osmotic potential.

The transgenic plants contained increased sodium concentrations and decreased potassium levels in the saline environment compared to the wild type. At the end of the experiment, the sodium concentration in the expressed sap of transgenic plants was 579.83 ± 35.23 ppm, with 13,747.53± 483.60 ppm potassium, while the expressed sap of wild-type plants contained 319.21 ± 19.82 ppm sodium and 10543.53 ± 208.45 ppm potassium. Wild-type plants showed higher potassium: sodium ratio than transgenic plants (**Fig. 6**), suggesting that salinity-induced changes in the K^+/Na^+ ratio may not be a critical factor for growth reduction in a saline environment.

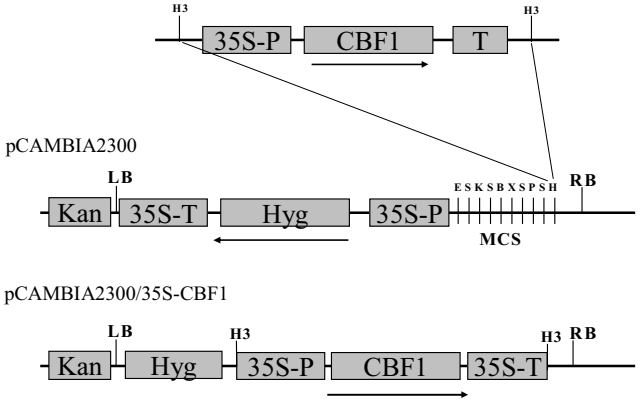

Fig. 1 Construction of *p*CAMBIA2300 vector conveying *At*CBF1 to the potato plants.

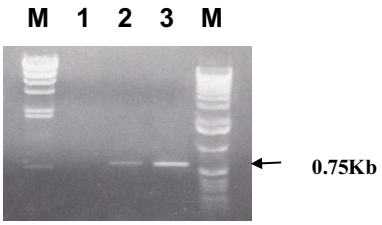

Fig. 2 RT-PCR of genomic DNA for *At*CBF1 transgenic potato plant. Sense primer was hit on CaMV 35S promoter and anti sense primer was on *At*CBF1 gene. Lanes 1, NtX control; 2, *p*BI121 vector; 3-4, CBFp1-p2.

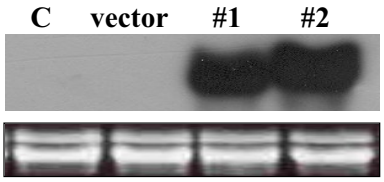

Fig. 3 mRNA expression of the *At*CBF1 gene in the transgenic potato plants by northern blot analysis. Total RNA was isolated from transgenic tobacco lines and electrophoresed (lower panel). Transferred RNA onto the membrane was hybridized with [α-^{32}P] dCTP labeled *PsTP* cDNA (upper panel). Lanes 1, Wild type: 2, Control plant transformed with binary vector containing the *GUS* gene only; 3-4, CBFp1-p2.

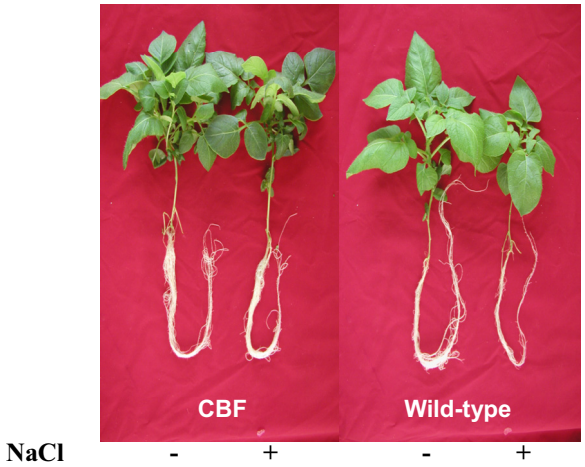

Fig. 4 Growth of *At*CBF1 transgenic and wild type potato plants in with (+) and without (-) 50 mM NaCl treatment for 15 days.

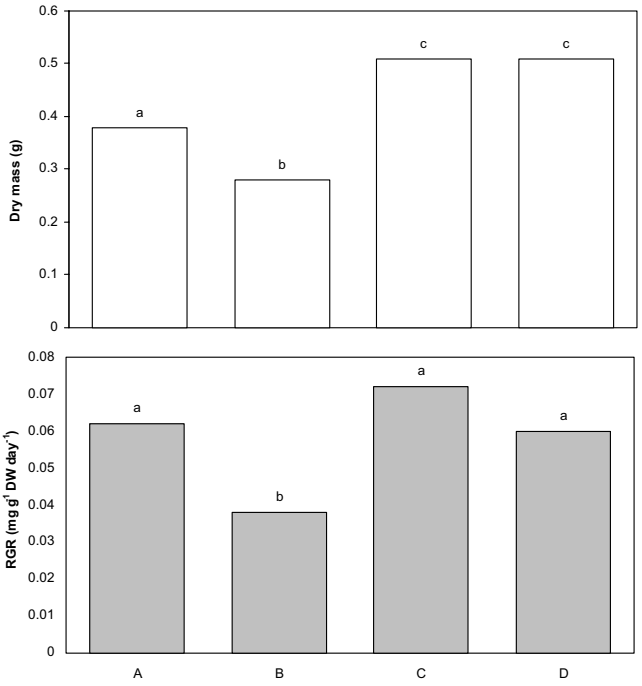

Fig. 5 Relative growth rate and biomass accumulation of AtCBF1 transgenic and wild type potato plant in saline and non-saline environment. Note: On X-axis A= CBF1 with salinity, B=wild type with salinity, C= CBF1 with salinity D= wild type without salinity. Similar letters on bar graphs = non-significant difference (paired *t*-test).

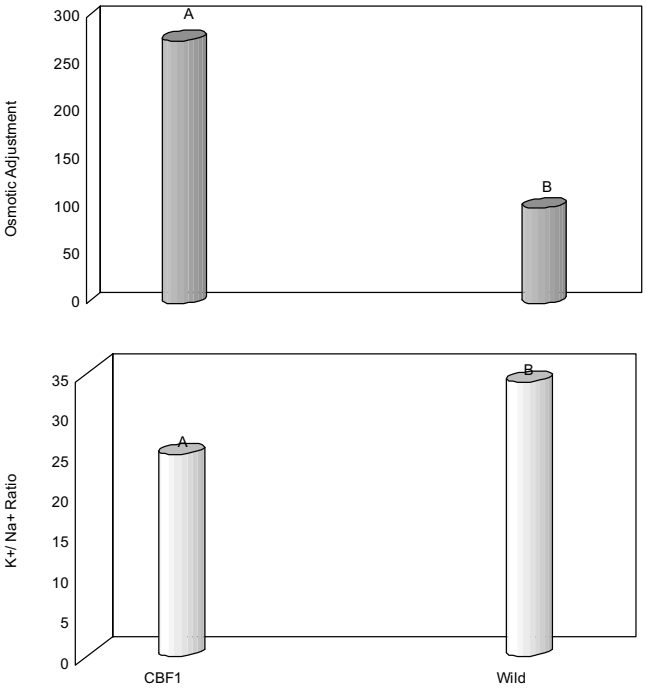

Fig. 6 Osmotic adjustment and K+/ Na+ ratio of AtCBF1 transgenic and wild type potato plant. Note: Similar letters on bar graph represent non-signifcant difference (paired *t*-test).

DISCUSSION

The *AtCBF1* gene was successfully transformed in potato plants showing over expression. *CBF* genes are considered "master switches" that activate the expression of genes, enhancing freezing tolerance in *Arabidopsis* plants (Thomashow *et al.* 2001). This suggested the possible role of this gene in the water-deficit stress response. Hence, it was presumed that the *Arabidopsis CBF* gene may also function under high salt conditions. Therefore, this study was undertaken to determine the response of plants transformed with

this gene in both salt stress and normal environments. It was postulated that an absolute and relative reduction in growth would be seen in these plants in both saline and non-saline environments. Our results, however, contradict the finding that overexpression of *AtCBF3* led to dwarfism in transgenic *Arabidopsis* plants (Kasuga *et al*. 1999). The dwarfism observed in that study could be prevented by replacing the constitutive 35S promoter driving *AtCBF3* with the stress-inducible promoter RD29A (Kasuga *et al*. 1999; Smirnoff and Bryant 1999). However, the transgenic potato plants produced in the current study did not exhibit growth retardation, even though the cauliflower mosaic virus 35S promoter was used to drive the *CBF1* gene. These results indicate that the *CBF1* gene may have different modes of action than CBF3, which might affect growth and development in the potato plant under optimal growth conditions. Vigorous growth of the transgenic plants in an optimal environment indicates that *AtCBF1* did not modify the growth of the potato plant and clearly did not elicit dwarfism.

Expression of *AtCBF1* significantly enhanced biomass accumulation in potato plants under salt stress. In this study, transgenic potato plants that expressed *AtCBF1* had greater dry weights and relative high growth rates in the saline environment compared to wild-type plants. The enhanced ability to accumulate biomass confers greater tolerance to salt stress.

The transgenic potato lines had a significant osmotic adjustment capacity, leading to reduced osmotic potentials compared to wild-type plants in the saline environment. This result is in agreement with findings from a previous study, in which the tuber yield of greenhouse-grown potato plants was related to decreased osmotic potentials in leaves when plants were grown in a water culture supplemented with 150 mM NaCl (Shaterian *et al.* 2005). Salinity-induced osmotic adjustment can be achieved through active synthesis, accumulation, and/or transport of inorganic or organic solutes, resulting in lowered water potential to help stressed plants achieve osmotic homeostasis (Siddiqui *et al.* 2008). Furthermore, Apse and Blumwald (2002) proposed the importance of osmotic adjustment *via* the synthesis of non-toxic compatible solutes to enhance salt tolerance using genetic engineering. In maize, salt-induced osmotic adjustment was found to partially contribute to improved plant growth in the presence of salt stress (De Costa *et al*. 2007). Transgenic potato plants have previously been shown to exhibit improved salt tolerance resulting from the incremental accumulation of proline, a well-known compatible solute, produced by the genetic transformation of the plants with pyrroline-5-carboxylate synthetase cDNA (Hmida-Sayari *et al.* 2005). Osmotic homeostasis contributes to salt tolerance by maintaining plant turgor pressure, stomatal conductance, and photosynthesis (Katerji *et al.* 1997; Siddiqui *et al.* 2008), although osmotic adjustment also consumes energy. This process, therefore, represents a trade-off that affects plant growth.

However, in this study, the transgenic plants produced 20% more biomass under salt stress compared to wild-type plants, even though the transgenic plants exhibited twice as much salt-induced osmotic adjustment as wild-type plants. These results indicate that the *AtCBF1* gene may play a significant role in actively increasing cellular solute levels in response to salt stress. Further work is needed to help elucidate this mechanism in detail, including understanding which solutes might be induced by salt stress in response to *CBF1* overexpression.

The transgenic plants accumulated more sodium and potassium than wild-type plants in the saline environment. The potassium: sodium ratio in the wild-type plants was much greater than that of transgenic plants, indicating that the *AtCBF1* gene has no significant role in ion homeostasis under salt stress. However, the ratio of potassium to sodium in a plant growing in a saline habitat is a critical physiological trait that helps the plant successfully adapt to its environment (Apse and Blumwald 2002; Siddiqui *et al*. 2008). Satti and Lopez (1994) found that tomato plants exhibited critically reduced levels of growth when the K^+/Na^+ ratio was below 0.3. In this study, the transgenic potato plants managed to avoid severe ion imbalance; the K^+/Na^+ ratio in these plants was much greater than normal, even though salt stress increased sodium levels while it decreased potassium levels. These results indicate that salt-induced imbalance may not be critical to the reduction of growth exhibited in plants under long-term moderate salinity conditions.

CONCLUSION

Transgenic potato plants containing the *AtCBF1* gene showed significantly greater biomass accumulation and osmotic adjustment than wild-type plants, indicating that the *CBF1* gene plays a significant role in the achievement of salt tolerance through active osmotic adjustments in saline environments.

ACKNOWLEDGEMENTS

This work was financially supported by the research program (PJ008598) of the National Academy of Agricultural Science, RDA, Suwon, Korea.

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