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Field Assessment of *AtCBF1* Transgenic Potato Lines (*Solanum tuberosum*) for Drought Tolerance

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Abstract Drought prone areas have been increasing around the world and it is expected that these areas will continue to expand and become more severe due to climate change. Increasing the drought stress tolerance of cultivated potato (*Solanum tuberosum*) could aid in feeding the growing global population. The *Arabidopsis CBF1* gene (*AtCBF1*), which has been shown to increase drought tolerance in other plants, was transformed into a cultivated potato line under the control of the stress inducible promoter *COR15a*. The expression of the *AtCBF1* transgene was verified by RT-PCR and the transformed lines were evaluated in field trials to assess agronomic performance under sub-optimal water management. Despite expression of the *AtCBF1* gene, none of the transgenic lines out-performed the control cultivar under drought-stressed conditions. Abiotic stress responsive genes from cultivated potato and wild related species may yield more promising results thus *CBF1* genes from *S. tuberosum* and *S. commersonii* will be transformed into the potato cultivar Desiree and will be field tested for drought tolerance.

Resumen Las áreas con riesgo de sequía se han estado incrementando alrededor del mundo y se espera que estas superficies continuarán en expansión volviéndose más severas debido al cambio climático. El aumento a la tolerancia al agobio hídrico de la papa cultivada (*Solanum tuberosum*) pudiera ayudar en la alimentación de la población global en crecimiento. El gen de *Arabidopsis* CBF1 (AtCBF1) que se ha demostrado que aumenta la tolerancia a la sequía en otras plantas, se introdujo en una línea de papa cultivada bajo el control del promotor de inducción de agobio COR15a. La expresión del transgen AtCBF1 se verificó mediante RT-PCR y se evaluaron las líneas transformadas en ensayos de campo para analizar el comportamiento agronómico bajo manejo subóptimo de agua. A pesar de la expresión del gen AtCBF1, ninguna de las líneas transgénicas superó en comportamiento a la variedad testigo bajo condiciones de agobio hídrico. Genes de respuesta de agobio abiótico de papa cultivada y de especies silvestres relacionadas pudieran rendir resultados más promisorios, de manera que los genes CBF1 de *S. tuberosum* y *S. commersonii* serán incorporados a la variedad de papa Desiree y serán probados en el campo para tolerancia a sequía.

Keywords *Arabidopsis* · *COR15a* · Field trials · Abiotic stress

Introduction

Cultivated potato *(Solanum tuberosum)* is the third largest food crop in production, following rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (FAOSTAT 2010). Because it is more adaptable to different climates than rice and wheat and provides more nutrition per acre than grain crops, potato is an important component of global agriculture (FAO 2008). However, with the potential for increasing areas of drought stress due to climate change (Gornall et al. 2010) it will be necessary to develop potato cultivars with a greater drought tolerance.

Developing drought tolerant commercial potato cultivars is difficult due to the lack of useful variation for this trait. Some wild potato species are native to arid regions, such as *S. hjertingii* found in the southwestern United States and Mexico (USDA, ARS, National Genetic Resources Program 2012). However, capturing traits from wild species in commercial cultivars is difficult due to varying ploidy and

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endosperm balance number (EBN) among wild and cultivated species as well as the transfer of undesirable traits from the wild species. Plant transformation is an alternative approach for introducing genes of economic value into cultivated varieties and potato has been successfully transformed with a variety of genes (Kuhl et al. 2007; Cooper et al. 2009; Bhaskar et al. 2010; Zarka et al. 2010).

Many genes have been identified that increase plant tolerance to one or more abiotic stresses and these can be classified as functional or regulator proteins (Shinozaki and Yamaguchi-Shinozaki 2007). Examples of functional proteins would include: stomatal density and water channel proteins (Sade et al. 2010; Yoo et al. 2010), late embryogenesis abundant proteins (LEAs) (Cheng et al. 2002; Babu et al. 2003) and enzymes for osmolyte biosynthesis (Waditee et al. 2005; Yamada et al. 2005; Park et al. 2007). Examples of regulator proteins would include: transcription factors from the basic leucine zipper (bZIP) family (Uno et al. 2000; Hsieh et al. 2010), the myeloblastosis (MYB) family (Abe et al. 2003; Jung et al. 2008; Rahaie et al. 2010) and the APETALA2 (AP2) family (Stockinger et al. 1997; Liu et al. 1998; Oh et al. 2009), protein kinases (Umezawa et al. 2004; Mizoguchi et al. 2010; Ying et al. 2011) and abscisic acid biosynthetic genes (Iuchi et al. 2001; Xiong et al. 2001; Umezawa et al. 2006). One group of genes that has received significant attention is the *CBF/DREB* genes (C-repeat Binding Factor/Dehydration Responsive Element Binding) from the AP2 family of transcription factors. Early studies on cold acclimation in *Arabidopsis* revealed four cold-regulated (*COR*) genes: *COR6.6, COR15, COR47,* and *COR78* (Hajela et al. 1990), with *COR6.6, COR15a* and *COR78* encoding hydrophilic polypeptides (Thomashow 1998). The *COR* gene transcripts accumulated after 4 h of cold treatment and could stay induced for up to 2 weeks. It was also found that some of the *COR* genes were induced by drought (Hajela et al. 1990) and that *COR15a* enhanced chloroplast and plasma membrane dehydration tolerance by stabilizing the membranes (Steponkus et al. 1998). The transcription factors *CBF1, DREB1A* and *DREB2A*, which bind to the promoter region of the *COR* genes, were then isolated from *Arabidopsis* (Stockinger et al. 1997; Liu et al. 1998). Cold-induced expression of the *CBF1* gene activates the expression of a major regulon of *COR* genes resulting in structural/biochemical changes and altered photosynthetic capabilities that impact freezing tolerance (Guy 1990; Pino et al. 2008).

Six *CBF* genes have been found in *Arabidopsis* and among them *CBF1, CBF2* and *CBF3* are cold-induced and are major regulators in the cold acclimation process in *Arabidopsis* (Gilmour et al. 2004). Studies in *Arabidopsis* have shown that when any of the three cold-induced *CBFs* are expressed using a constitutive promoter, the *CBF* target genes are turned on even at warm temperatures (Jaglo-Ottosen et al. 1998; Gilmour et al. 2000) and the cold acclimation pathway is activated without exposure to cold temperatures. To date *CBF*/*DREB* genes have been found in every higher plant that has been examined including: barley (*Hordeum vulgare*) (Choi 2002), rice (Dubouzet et al. 2003), canola (*Brassica sp.*), rye (*Secale cereale*), tomato (*Solanum lycopersicum*) (Jaglo et al. 2001), wheat (Kume et al. 2005), soybean (*Glycine max*) (Li et al. 2005), blueberry (*Vaccinium corymbosum*) (Naik et al. 2007), grape (*Vitis vinifera*) (Xiao et al. 2008), tobacco (*Nicotiana tabacum*) (Park et al. 2001), pepper (*Capsicum annuum*) (Hong and Kim 2005), and potato (Rensink et al. 2005).

The CBF proteins have been shown to be highly conserved across both cold acclimating and non-acclimating plants with the most highly conserved region falling within the AP2/EREBP DNA binding domain (Jaglo et al. 2001). The *Arabidopsis* CBF genes (*AtCBF*) have been introduced into several plant systems to study the effect on freezing, drought and salinity tolerance (Jaglo et al. 2001; Kasuga et al. 2004; Pino et al. 2007, 2008). Transgenic tomato plants expressing *CBF* using either a constitutive or an inducible promoter have also been studied under drought and salinity stress leading to the conclusion that *CBF* genes can confer tolerance to these stresses in tomato (Hsieh et al. 2002; Lee et al. 2003) which is a close relative of potato.

In order to study the effect of the *AtCBF1* gene in cultivated potato, four transgenic lines containing the *AtCBF1* gene under the control of the stress-inducible promoter *COR15a* (Hajela et al. 1990; Baker et al. 1994) were evaluated in 4 years of field trials. Gene expression data and agronomic performance data under irrigated and non-irrigated conditions are presented.

Materials and Methods

Plant Material MSE149-5Y, a Michigan State University potato breeding line that is highly amenable to *Agrobacterium*-mediated transformation, was transformed with the pSPUD74 construct according to Li et al. (1999). The construct pSPUD74 contains the *AtCBF1*gene (GenBank accession AY667247.1) under the *Arabidopsis COR15a* inducible promoter (GenBank accession U01377.1; Fig. 1). Only one shoot was recovered from any single explant to ensure independent transformation events. Putative transformants that rooted on selective media were confirmed by PCR. The lines selected for this research were designated as: E74.8, E74.9, E74.14 and E74.16. These transgenic potato lines were maintained in tissue culture by nodal propagation in GA-7 Magenta boxes (Magenta Corp, Chicago, IL) on modified Murashige and Skoog (MS) media (4.3 $g \cdot L^{-1}$ MS salts, 30 $g \cdot L^{-1}$ sucrose, 1.4 mM sodium phosphate, 1.1 μM thiamine, 0.55 mM myo-inositol, pH 6.0, 8 $g \cdot L^{-1}$ agar). The stock

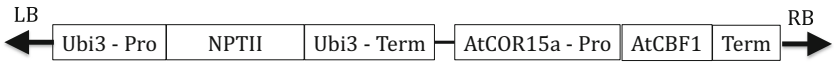

Fig. 1 Schematic of pSPUD74 construct. The *CBF1* gene from *Arabidopsis thaliana* is directed by the stress-inducible promoter *AtCOR15a*

cultures were maintained at 25±10 °C, with a 16 h photoperiod.

Greenhouse-produced minitubers were planted at the MSU Lake City Research Center (LRC, Lake City, MI) for tuber increase in 2006 and 2008 and harvested tubers were used to plant the research plots at both locations from 2007 to 2010. The Lake City increase plots are isolated from commercial potato production areas and are tested and rogued to prevent virus accumulation. Field year 1 or field year 2 seed was used for the research plots. After washing and grading, all harvested seed was packed in paper bags and stored at 4 °C until April when it was moved to room temperature and prepared for planting. For both the increase and research plots, seed pieces of approximately 2.0–2.5 oz were used and no pre-planting treatments were made.

Field Trials of AtCBF1 Transformed Lines Agronomic performance of the four *AtCBF1* lines and the non-transgenic control (MSE149-5Y) was evaluated in field trials with irrigation at the Montcalm Research Center (MRC) and without irrigation at the Michigan State University Campus Farm (Campus) for 4 years 2007–2010. Both locations were maintained using best management practices for fertilizer and pesticide applications. Planting and harvest dates as well as the total amount of precipitation/irrigation for each location during each growing season are presented in Table 1. Experiments were planted in a randomized complete block design with four replications. Plots were 3 m long with 0.86 m between- row spacing and 0.3 m within- row spacing. Plant phenotype observations were made for each of the plots in the trials pre-flowering, at flowering and at tuber bulking.

After harvest, each plot was analyzed for total yield, tuber size distribution and specific gravity. ANOVA and LSD for mean separation (α=0.05) were conducted for total tuber yield and specific gravity using SAS software (release 9.20; SAS Institute, Cary, NC).

Gene Expression Analysis Using Reverse Transcriptase-PCR (RT-PCR) To verify the expression of the *AtCBF1* transgene, RNA was isolated from leaf tissue of the AtCBF1-transgenic lines and MSE149-5Y (control). Leaf tissue was collected in 2008 at both field locations from the same plants at three different time points: pre-flowering (1), flowering (2) and tuber bulking (3). Harvested tissue was immediately frozen in liquid nitrogen and later used for total RNA extraction (RNeasy Plant Mini-Kit, Qiagen Inc., Valencia, CA) and DNase treatment (RQ1 RNase-Free DNase, Promega Corp., Madison, WI). RNA was quantified using a Nanodrop 8000 spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE). cDNA was obtained by reverse transcription of 100–200 ng/μL of total RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Life Technologies Corp., Carlsbad, CA). Eight microliters of the cDNA reactions were then used as template in 50 μL PCR amplifications using GoTaq® DNA Polymerase as directed by the manufacturer (Promega, Madison, WI). Gene specific primers for *AtCBF1* were 5′-CTCCGATTACGAGCCTCAAG-3′ and 5′-ATCGTCTCCTCCATGTCCAG-3′ and gene specific primers for the 18S gene were 5′- GGGCATTCGTATTTCATAGTCAGAG-3′ and 5′- GGTTCTTGATTAATGAAAACATCCT-3′. PCR cycling conditions were: 94 °C for 4 min, 30 cycles of 94 °C for 60 s, 60 °C for 90 s, 72 °C for 90 s and a final extension for

Table 1 Planting/harvesting dates and precipitation/irrigation totals for the Montcalm Research Center (MRC) and the Michigan State University Campus Farm (Campus), 2007–2010

	Location	
	MRC	Campus
2007		
Planting date	5/17/07	5/18/07
Harvest date	9/24/07	9/21/07
Days after planting (DAP)[a]	130	126
Total precipitation (inches)[b]	23.47	14.62
2008		
Planting date	5/21/08	5/21/08
Harvest date	9/3/08	9/4/08
Days after planting (DAP)[a]	105	106
Total precipitation (inches)[b]	26.26	15.63
2009		
Planting date	5/15/09	5/7/09
Harvest date	9/20/09	9/19/09
Days after planting (DAP)[a]	128	135
Total precipitation (inches)[b]	22.62	16.1
2010		
Planting date	5/20/12	5/26/12
Harvest date	9/25/10	9/26/10
Days after planting (DAP)[a]	128	123
Total precipitation (inches)[b]	21.58	13.63

[a] DAP=the number of days after planting that tubers were harvested

[b] Total precipitation at MRC=rainfall+irrigation

4 min at 72 °C. Reverse transcriptase PCR (RT-PCR) products were separated and visualized on a 1 % agarose gel stained with ethidium bromide.

Results and Discussion

Gene Expression Based on RT-PCR results, the *AtCBF1* gene was expressed in the transgenic lines and not in MSE149-5Y (control) (Fig. 2). The *18S* ribosomal gene transcript was used as a control and was detected in all lines. Although the *AtCBF1* gene was expressed it did not reflect induction due to stress as would be expected of a gene under the control of a stress-inducible promoter (*COR15a*). This was likely due to temporal stresses in the field such as, short dry periods prior to irrigation and mechanical agitation which has been shown to activate the *CBF* genes (Zarka et al. 2003).

Yield and Specific Gravity The weather at the Campus location consisted of irregular rainfall throughout the growing season which induced a short-term drought stress on the plots. Standard management practices for potatoes in Michigan require the plants to receive 20 to 24″ of water during a growing season. During these experiments, the campus location did not receive any more than 16″ per season (Table 1) and plants at the campus site appeared water-stressed with leaf curl in July, and wilting with early senescence in August. During the 2007 and 2010 growing seasons, the Campus site experienced the most drought stress (Table 1).

Despite the preliminary greenhouse experiments in which the transgenic *AtCBF1* lines showed wilting tolerance (data not shown), the same transgenic lines did not demonstrate drought tolerance in the field studies. At the MRC site, the yields of the transgenic lines did not differ significantly from the control with the exception of E74.16 in 2007 (Table 2). Yields of the transgenic lines at the Campus site were not significantly different from the control in 2008 and 2010 but some of the transgenic lines yielded significantly less than the control in 2007 and 2009 (Table 2). For both irrigated and non-irrigated trials, size distribution comparisons between MSE149-5Y and the *CBF1* lines were generally non-significant (data not shown) with the exception of E74.8 which had significantly lower percentages of A size tubers (diameter of 2–3.25 in.) and significantly higher percentages of B size tubers (diameter<2 in.) at both sites in 2007 and at the irrigated MRC site in 2008 and 2009. E74.9 also had a significantly lower percentage of A size tubers and higher percentage of B size tubers at the non-irrigated 2007 plot but was not significantly different from MSE149-5Y for any other year/location combination. There were no significant differences for specific gravity between the control and transgenic lines at either site during any of the 4 years of trials with the exception of 2007 in which the specific gravity was significantly lower in the control than in the transgenic lines (Table 2). If the expression of the *AtCBF1* gene increased drought tolerance, we would expect to see greater yields in the transgenic lines compared to the control (MSE149-5Y) under drought conditions. As this was not the case, we conclude that in these plants the *AtCBF1* gene either does not increase drought tolerance or does not increase drought tolerance sufficiently enough to impact field performance. These results conflict with those of Hsieh et al. (2002) which reported increased drought tolerance despite abnormal phenotypes in tomato lines expressing the *AtCBF1* gene under the control of a constitutive promoter. However, the current study was limited to only four transgenic lines and data collected from field studies, whereas Hsieh et al. (2002) included 22 transgenic tomato lines and utilized data from greenhouse trials. These experimental differences may account for the contradictory conclusions regarding the impact of *CBF1* genes on drought tolerance. Although field trials are the best way to determine if a line (transgenic or not) has the expected agronomic traits and produces an acceptable yield, only a few transgenic crops have been tested for abiotic stress tolerance in the field (Dunwell 2000; Schafleitner et al. 2007; Waterer et al. 2010). Small changes in the plant physiology/biochemistry observed

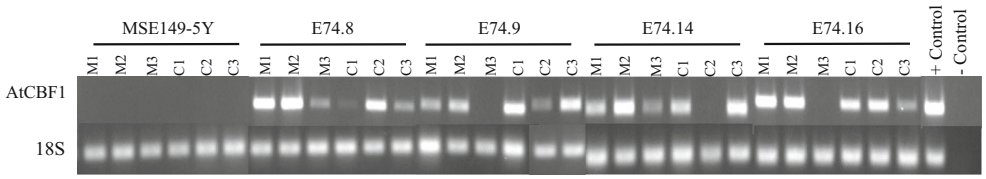

Fig. 2 Reverse Transcriptase PCR from the 2008 field trial. RT-PCR was used to verify the expression of the *AtCBF1*transgene in the leaf tissue of MSE149-5Y (*control*) and the transgenic lines. *Letter/number* designations above the lanes designate the location from where the sample was taken (*M*=MRC; *C*=Campus) and the timing of the sampling (*1*=pre-flowering, *2*=flowering, *3*=tuber bulking). The *18S* gene expression was used as an internal control for all of the samples. The positive control for each primer set was RNA isolated from OR1.11 (35S:*AtCBF1*) grown in tissue culture and the negative control was water used in place of cDNA in the reactions

Table 2 Yield and specific gravity for *AtCBF1* transgenic potato lines at the Montcalm Research Center (MRC) and the Michigan State University Campus Farm (Campus), 2007–2010

	Yield[a]				Specific gravity[b]			
	MRC		Campus		MRC		Campus	
2007								
E149-5Y	8.4	A	5.0	A	1.066	A	1.050	B
E74.14	5.2	AB	3.1	B	1.068	A	1.059	A
E74.16	4.2	B	3.1	B	1.066	A	1.057	A
E74.8	5.2	AB	3.0	B	1.065	A	1.058	A
E74.9	5.7	AB	3.2	B	1.067	A	1.058	A
2008								
E149-5Y	9.0	A	9.5	A	1.071	A	1.076	A
E74.14	8.5	A	7.6	A	1.075	A	1.075	A
E74.16	8.4	A	7.2	A	1.076	A	1.077	A
E74.8	7.3	A	7.8	A	1.072	A	1.077	A
E74.9	7.6	A	6.6	A	1.074	A	1.076	A
2009								
E149-5Y	3.6	A	17.3	A	1.070	A	1.062	A
E74.14	2.2	A	10.4	B	1.067	A	1.062	A
E74.16	1.7	A	9.4	B	1.073	A	1.059	A
E74.8	2.2	A	13.8	AB	1.076	A	1.064	A
E74.9	2.1	A	14.1	AB	1.072	A	1.063	A
2010								
E149-5Y	11.9	A	2.5	A	1.064	A	1.048	A
E74.14	9.2	A	4.8	A	1.061	A	1.051	A
E74.16	10.0	A	4.0	A	1.064	A	1.051	A
E74.8	9.3	A	3.7	A	1.065	A	1.050	A
E74.9	9.8	A	4.4	A	1.065	A	1.052	A

[a] Total yield in kg/plot

[b] Specific Gravity: weight in air/weight in air - weight in water

in controlled laboratory experiments may not translate to abiotic stress tolerance in a field trial.

Plant Phenotype One issue observed across many plant species is that when a *CBF* gene is overexpressed, negative phenotypes appear including: dwarfed growth, delayed flowering, and shorter petioles (Gilmour et al. 2000; Kasuga et al. 2004; Pino et al. 2007, 2008). Although previous research has shown that this issue can be overcome by using an inducible promoter (Lee et al. 2003), the *AtCBF1* lines in this study had slightly altered phenotypes (slightly shorter petioles, a slightly more compact canopy and leaves with a blue-green hue, (data not shown) despite the use of the stress-inducible *COR15a* promoter. This was observed in both irrigated and non-irrigated plots suggesting that simply being planted in the field produced enough stressors to activate the stress-induced COR15α promoter. Reduced sprouting (longer dormancy) during storage was also observed in some of the transgenic lines (data not shown) and is a trait that will need to be analyzed in future experiments.

One aspect of potato cultivation that could benefit from the use of biotechnology is drought tolerance and the *CBF* genes are potential targets for this technology. The *AtCBF1* potato lines that were evaluated in this study did not show any yield advantage over the non-transgenic control under any growing conditions. Incorporating different genes under new and different promoters may lead to a transgenic potato that can confer abiotic stress tolerance and maintain yield.

The wild potato species, *S. commersonii*, grows at higher elevations in the Andes and is freezing tolerant (Li 1977). Because the *CBF* genes have been shown to enhance both freezing and drought tolerance (Jaglo-Ottosen et al. 1998; Hsieh et al. 2002; Lee et al. 2003; Pino et al. 2007), the *S. commersonii CBF1* gene is of interest for drought tolerance studies. *S. commersonii* and *S. tuberosum* CBF1 genes share 92 % homology whereas CBF1 genes from both potato species share only 45 % homology with *A. thaliana*. Recently, the transcriptomes and CBF regulons of *S. commersonii*, *S. tuberosum* and *A. thaliana* were studied (Carvallo et al. 2011). Both potato species had CBF regulons composed of hundreds of genes but there were sizeable differences in the sets of genes that were a part of the low temperature transcriptome. However, the data did not identify any specific genes that would account for the variation in freezing tolerance between *S. commersonii* and *S. tuberosum*. Additional constructs have been made containing the *S. commersonii CBF1* gene (provided by Dr. Tony H.H. Chen, Oregon State University) under the control of the *Arabidopsis COR15a* promoter and have been transformed into the potato cultivar Desiree to determine if this *CBF* gene will provide useful levels of drought tolerance in cultivated potato. We will also transform Desiree with three other genes that have been shown to increase abiotic stress tolerance: an IPT gene (isopentenyltransferase) under the control of a SARK promoter (senescence associated receptor protein kinase) (Rivero et al. 2007), a RING-H2 gene named Xerico (really interesting new gene, zinc finger motif with histidine at the 5th position of the motif) (Ko et al. 2006) and a M6PR gene (mannose 6-phosphate reductase) (Zhifang and Loescher 2003). These plants will be evaluated in greenhouse and laboratory trials.

There has been an increasing interest in drought tolerant crop plants due to the expectation of increased area and severity of drought around the world (Gornall et al. 2010). Abiotic stresses such as drought, saline soil, freezing temperatures and high temperatures can all negatively affect the yield of potatoes (Byun et al. 2007). A potato variety that could withstand these stresses while exhibiting high yields and expected agronomic traits would maintain and/or increase the areas where potatoes could be grown and aid in feeding the growing world-wide population.

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