ASSESSING DROUGHT TOLERANCE IN *CBF1* TRANSGENIC CULTIVATED POTATO LINES (*SOLANUM TUBEROSUM*); AND A SOCIOLOGICAL SURVEY OF COLLEGE STUDENTS' ATTITUDES TOWARDS GENETICALLY MODIFIED POTATOES

By

Nicole Laurel Nichol

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ABSTRACT

ASSESSING DROUGHT TOLERANCE IN *CBF1* TRANSGENIC CULTIVATED POTATO LINES (*S. TUBEROSUM*); AND A SOCIOLOGICAL SURVEY OF COLLEGE STUDENTS' ATTITUDES TOWARDS GENETICALLY MODIFIED POTATOES

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Drought prone areas have been increasing around the world, and due to climate change, it is expected that these areas will only further increase and become more severe. Therefore, the need for drought tolerant crops is imperative. The potato (*Solanum tuberosum*) is the fourth most important food crop in the world, and increasing this crop's ability to tolerate drought stress could aid in feeding our growing global population. Transforming the *CBF1* gene into plants has been shown to increase the plants' freezing, drought and saline stress tolerance. *CBF1* genes from *Arabidopsis thaliana*, *Solanum commersonii* and *Solanum tuberosum* have been transformed into cultivated potato lines under the stress inducible promoter *COR15a*. The *AtCBF1* lines were evaluated using an electrolyte leakage assay and field trials. Two of the lines, E74.8 and E74.9 showed the lowest percent of electrolyte leakage and were able to maintain a yield similar to the wild type control under drought stressed field conditions. The *ScCBF1* and *StCBF1* lines were evaluated using an *in vitro* osmotic stress assay. Two of the *ScCBF1* lines and five of the *StCBF1* lines were able to outperform the wild type controls.

A questionnaire was developed to study the effect of information on college students' attitudes towards genetically modified potatoes. It was found that those whom received the information were more approving of GM potatoes for human consumption, and felt they were safer, than those in the control group. However, the information had no effect on their willingness to consume foods with GM potato ingredients.

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Literature Review

The Potato

The potato, *Solanum tuberosum* is the cultivated potato that is consumed by people worldwide. It is the fourth largest food crop in production following the grains of rice (*Oryza sativa*), wheat (*Triticum aesitvum*) and maize (*Zea mays*) (FAOSTAT, 2009). Potatoes are grown on every continent, except Antarctica, with over 18 million hectares under production in 2009, valued at over $55 billion (FAOSTAT, 2009). Potatoes offer many vitamins and minerals in addiction to its' starchy calories including substantial levels Vitamin C, potassium and fiber (Kolbe and Stephan-Beckmann, 1997).

The cultivated potato is in the *Solanaceae* family along with tobacco (*Nicotianum tabacum*), tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*). *Solanum tuberosum* is believed to have a single origin from a wild potato progenitor *S. brevicaule* in southern Peru (Spooner *et al.*, 2005). From that point potatoes spread throughout the Andes and up into Central America and southern North America. The potato has adapted to a wide range of climates, altitudes and latitudes. In the mid-16th century there is record of potatoes being transported by Spanish explorers to the Canary Islands (Hawkes and Francisco-Ortega, 1993). From there potatoes were able to make their way across Europe and became a cheap food crop for many people. There are approximately 190 wild and cultivated species of potatoes divided into four clades (Spooner *et al.*, 2008). Part of the division of species is based on the ploidy level of the plant ranging from diploid to hexaploid.

The potato is a perennial plant that is mostly self-pollinated but cross-pollination can occur. The plant produces flowers that can range from white to blue (Cutter and Harris, 1992). Many potato cultivars are not able to produce the small green berry fruit due to male sterility, failure to flower and other factors. The tuber that is the edible portion of the plant is morphologically a swollen stem, called a stolon, and grows underground (Cutter and Harris, 1992).

The potato has five growth stages. In stage I, the seed tubers break dormancy and sprouts grow from the eyes of the tuber. The seed tuber utilizes the starch and other compounds within it to initiate sprout growth. In stage II, the plant begins its vegetative growth including leaves, vines, roots and stolons. At this point the plant is able to conduct photosynthesis to obtain its' energy. In stage III, the stolons begin to swell initiating tuber formation. This process can occur when the plant flowers. In stage IV, the tubers bulk up by accumulating water, nutrients and carbohydrates translocated from the roots and leaves. In the final stage, the plant begins to senesce with the leaves turning yellow. At this point the tubers mature by thickening and hardening the skin.

Seed potatoes that are generally used in commercial production are clonally propagated from small or cut tubers. The advantage to using clonally propagated seed tubers, instead of true seed produced from sexual reproduction, is that they are genetically identical and will produce a monoculture crop. True seed is heterozygous and can vary widely in its traits and yield. The disadvantage in using seed tubers is that disease transmission can easily be carried over to from year to year. Sexual reproduction is generally used for crop improvement through breeding strategies, rather than commercial production (Dean, 1994).

There are, however, some challenges that come with breeding potatoes through sexual reproduction. The first challenge is the varying ploidy levels, and the second is the variation in endosperm balance number (EBN). *S. tuberosum* is an autotetraploid (2*n* = 4x = 48) with an EBN of four. The EBN hypothesis was developed to predict the interspecific and interploidy crosses of *Solanum* species (Peloquin *et al.*, 1989). The actual assignment of the EBN value is based on the crossing of *Solanum* species to standard tester species (Carputo *et al.*, 2003). In general diploid species have an EBN of one or two, triploid species are two, tetraploids are either two or four, and pentaploids and hexaploids are 4. Species with the same EBN value can cross freely, and viable seed is produced when the maternal to paternal EBN ratio is 2:1 (Hawkes *et al.*, 1994). To overcome differences in EBN values 2*n* gametes are used. The most common mechanisms of 2*n* gamete formation in potato are first-division restitution and second-division restitution (Peloquin *et al.*, 1989). First-division restitution results in the failure of the chromosomes to move to the opposite poles during meiosis I. In second-division restitution the cell plate fails to form during meiosis II.

Plant Biotechnology

The genetic engineering of plants over the last several decades has mainly taken advantage of tissue culture and the plant pathogen *Agrobacterium tumefaciens*. The ability to introduce a gene that is not found in one individual plant's genome from a separate genome has significantly widened the gene pool. Since potato can be difficult to breed due to the varying ploidy and EBN values, biotechnology has opened up new avenue for introducing genes of interest.

Potatoes were among the early crop plants to be successfully transformed by the *Agrobacterium*-mediated technique (An *et al.*, 1986). *Agrobacterium tumefaciens* is a soil-born bacterium that causes crown gall disease in dicotyledonous plants (Smith and Townsend, 1907). In the 1970's scientists found that a large plasmid within *A. tumefaciens* was the causative agent for producing tumors and was essential to its' virulence (Van Larebeke *et al.*, 1974). They named this plasmid the tumor inducing or Ti plasmid. It was later found that a particular region of the plasmid's DNA is transferred into the nuclear genome of the host plant's cell (Thomashow *et al.*, 1980). This region was then named the Transfer DNA or T-DNA. The Ti plasmid also contains genes known as Virulence, or *vir*, genes and these genes assist bacterium in integrating the T-DNA into the plant host's genome. Plant biotechnology harnesses *Agrobacterium*'s natural ability to insert foreign genes into a plant's nuclear genome. In order for *Agrobacterium*-mediated transformation to be successful, the tumor causing genes are removed from the Ti plasmid. This gives the plant cells a chance to produce a fertile plant. The binary vector system was then developed where one plasmid contains the T-DNA with the gene of interest and the other plasmid contains the *vir* genes (Hoekema *et al.*, 1983). This system increases the efficiency and allows for easier manipulation of the plasmids.

One example of a valuable gene being introduced into a cultivated potato variety is the late blight resistant gene from the wild species *S. bulbocastanum*. Potato late blight, *Phytophthora infestans* L., is one of the most devastating pests in potato. Late blight wiped out the potato crop between 1845 and 1852 in Ireland, and led to the infamous Irish potato famine. A gene for late blight resistance was cloned from *S. bulbocastanum* and was inserted into the genome of a susceptible cultivar, cv. Katahdin (Song *et al.*, 2003). The transformed Katahdin then conferred resistance to late blight. For years breeders had been trying to cross *S.*

bulbocastanum with a susceptible cultivated variety but the differences in ploidy level and EBN values were making it necessary to use a bridging species. Somatic hybrids of *S. bulbocastanum* and *S. tuberosum* were created using PEG-mediated fusion (Helgeson *et al.*, 1998). However, the main advantage to genetic engineering is that only one or a few genes of interest will be inserted instead of the other remaining portion of a genome as it occurs in sexual reproduction.

One of the many areas of potato cultivation that could benefit from the use of biotechnology is abiotic stress tolerance. There are several species of potato that are known to be freezing tolerant, such as *S. acaule* and *S. commersonii*, as they grow in the higher elevations of the Andes (Li, 1977). It has been found in some plants that freezing and drought tolerance are related in the plant's ability to produce membrane-stabilizing proteins. However, *S. tuberosum* is sensitive to several abiotic stresses, and this can result in crop loss.

CBF1 and Abiotic Stress Tolerance

Due to the sessile nature of plants, they have adapted many strategies to combat the stresses and damage that can occur during their life cycle. Plants have evolved methods to overcome both biotic and abiotic stresses. Some abiotic stresses a plant can be faced with include freezing, heat, drought, flood, and saline conditions. Because many crop plants have been developed in a particular location, usually under ideal conditions, they can struggle when moved to different regions or are faced with varying environmental conditions. Plant biotechnology offers options to increase a crop plant's abiotic stress tolerance by introducing novel genes.

One group of genes that has received much attention for abiotic stress tolerance are the CBFs/DREBs (C-repeat Binding Factor/Dehydration Responsive Element Binding). The

transcription factors that were first isolated from *Arabidopsis* were *CBF1* (Stockinger *et al.*, 1997) and *DREB1A* and *DREB2A* (Liu *et al.*, 1998). To date *CBF*/*DREB* genes have been found in every higher plant that has been examined including: barley (Choi, 2002), rice (Dubouzet *et al.*, 2003), canola, rye, tomato (Jaglo *et al.*, 2001) wheat (Kume *et al.*, 2005), soybean (Li *et al.*, 2005), blueberry (Naik *et al.*, 2007), grape (Xiao *et al.*, 2008), tobacco (Park *et al.*, 2001), pepper (Hong and Kim, 2005), and potato (Rensink *et al.*, 2005).

The Discovery of CBF

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 hr of cold treatment and can stay induced for up to two weeks. It was also found that some of the *COR* genes were induced by drought (Hajela *et al.*, 1990). *COR15a* was found to enhance chloroplast and plasma membrane dehydration tolerance by stabilizing the membranes (Steponkus *et al.*, 1998). *CBF1* was then discovered as the element that binds to the C-repeat involved with the promoter of two of the *COR* genes.

In *Arabidopsis* there are six members of the CBF family, with *CBF1, CBF2* and *CBF3* being cold-induced (Gilmour *et al.*, 2004). The CBF proteins are members of the AP2/ERBP family of transcription factors. The three cold-induced CBFs are major regulators in the cold acclimation process in *Arabidopsis*. CBF transcripts are detected within 15 min of exposure to low temperatures and peak around two hrs (Gilmour *et al.*, 1998). Studies in *Arabidopsis* show 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). It also gives the plant the ability to turn on the cold acclimation pathway without having to be exposed to cold temperatures.

The CBF Regulon

Microarray technology allowed for the identification of hundreds of genes that are responsive to low temperature in *Arabidopsis* and other plant species. Some of the genes that are found to accumulate under low temperature treatment include enzymes involved in the synthesis of sucrose and galactinol, both membrane protective sugars (Vogel *et al.*, 2004). Other genes outside of the CBF regulon have also been found in these microarray studies, implying there are other transcription factors that have a role in cold acclimation.

One of the known upstream inducers of the CBF regulon is the inducer of CBF expression 1 (*ICE1*). It is a MYC-like transcriptional activator that binds to the Myc recognition site of the *CBF3* promoter. In the *ice1* mutant, *CBF1* and *CBF2* expression was only slightly decreased in the beginning of the cold treatment, but several of the CBF target genes had decreased expression (Chinnusamy *et al.*, 2003). Overexpression of *ICE1* increases the expression of *CBF2* and *CBF3*, however, only during cold treatment. This indicates there may be upstream factors needed to activate CBF genes. *HOS1* is negative regulator of cold acclimation and targets *ICE1* for ubiquination (Dong *et al.*, 2006).

Several other regulators of the CBF genes have also been identified; *CAMTA3* and *LHY/CCA1*. *CAMTA3* has been identified as a positive regulator of *CBF2* (Doherty *et al.*, 2009). It is a member of a calmodulin-binding transcription factor family. The *camta3* mutant had a 50 and 40% reductions of *CBF2* and *CBF1* expression, respectively, under low temperature. *LHY/CCA1* are involved in circadian clock gene regulation (Mikkelsen and Thomashow, 2009).

It was shown that the expression of *CBF1-3* genes were highest when transferred to the cold during the light period (Fowler *et al.*, 2005).

Negative regulators of the CBF regulon have also been identified. *CBF2* has actually been shown to be a negative regulator of *CBF1* and *CBF3* (Novillo *et al.*, 2004). The overexpression of *Myb15* and *ZAT12* have also been shown to reduce the expression of *CBF1-3* at low temperatures (Vogel *et al.*, 2004). Interestingly both of these transcripts are cold induced, however, a knockout of *Myb15* does not change the expression of *COR15* or *rd29a* genes.

The CBF Pathway in Other Plant Systems

The CBF proteins have been shown to be highly conserved across both cold acclimating and non-acclimating plants. The region that is most highly conserved in the amino acid sequence is within the AP2/EREBP DNA binding domain (Jaglo *et al.*, 2001). Because the *Arabidopsis* CBF (*AtCBF*) genes have been so well studied, many have introduced *AtCBF* genes into other plant systems. The overexpression of any of the cold induced CBF genes in *B. napus* increased the plant's freezing tolerance without cold acclimation (Jaglo *et al.*, 2001). Overexpression of *AtCBF3* has also been shown to increase the freezing tolerance in tobacco and potato, along with *AtCBF1* in potato (Kasuga *et al.*, 2004; Pino *et al.*, 2007; Pino *et al.*, 2008). In tomato, the overexpression of *AtCBF3* or the tomato's CBF1 gene (*LeCBF1*) does not increase the freezing tolerance of the plant (Zhang *et al.*, 2004b). This suggests that there is a significant difference in the CBF pathways between *Arabidopsis* and tomato. One issue that has been observed across many of the plant species is that when a CBF gene is overexpressed negative phenotypes appear. These include dwarfed growth, delayed flowering, and shorter petioles (Kasuga *et al.*, 2004;

Gilmour *et al.*, 2000). However, this issue can mostly be overcome by using an inducible promoter such as *COR15* or *rd29a*.

Recently the transcriptomes and CBF regulons of *S. commersonii* (a freezing tolerant wild potato species), *S. tuberosum* (cultivated non-freezing tolerant potato) and *Arabidopsis thaliana* were studied (Carvallo *et al.*, 2011). It was found that both potato species have CBF regulons composed of hundreds of genes. However, there were sizeable differences in the sets of genes that were a part of the low temperature transcriptome, but the data did not clearly point to any specific genes that may be the key differences in why *S. commersonii* is freezing tolerant and *S. tuberosum* is not.

CBF genes have also been indicated to increase a plant's tolerance to other abiotic stress other than freezing (Vogel *et al.*, 2004). Transgenic CBF plants either using a constitutive or an inducible promoter have been studied under drought and salinity stress (Zhang *et al.*, 2004a). Depending on the CBF gene used and its origin, the CBF gene can confer tolerance to cold, drought, saline and/or abscisic acid. There has been an increasing interest in drought tolerant crop plants as drought prone areas have been increasing around the world, and due to climate change, it is expected that these areas will only further increase and become more severe (Gornall *et al.*, 2010). Therefore, the need for drought tolerant crops is imperative. Drought, saline soil, freezing temperatures and high temperatures can all negatively affect the yield of potatoes (Byun *et al.*, 2007). A variety of potato that could maintain a high yield and good agronomic traits under both optimal and drought conditions would increase the areas where potatoes could be grown. The world's population is also expected to increase over the next few decades, especially in rural areas where they are less capable of fighting drought (Gornall *et al.*, 2010). Drought tolerant potatoes could aid in feeding the growing population.

In the following two chapters the role of *AtCBF1*, *ScCBF1*, and *StCBF1* under the stress-inducible promoter *COR15a* in *S. tuberosum* was evaluated in field and *in vitro* studies. The final chapter studied college students' attitudes towards genetically modified potatoes.

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Chapter 2

Evaluation of *AtCBF1* Transgenic Potatoes

Research Objectives

This research evaluates the *CBF1* gene from *Arabidopsis thaliana* transformed into MSE149-5Y under the stress inducible promoter *COR15a*. The objectives were:

1. Evaluate the efficacy of the transgenic potato plants in their ability to decrease electrolyte ion leakage induced by freezing temperature treatments compared to the wild type control.
2. Evaluate the agronomic performance of the transgenic potato plants under irrigated and rain-fed conditions in repeated field trials.
    a. Evaluate the 100 cwt./acre yield of the potatoes.
    b. Evaluate the specific gravity of the potatoes.
    c. Evaluate the percent glucose and sucrose of the potatoes.
3. Study the gene expression of the transgene and other stress-induced genes from RNA isolated from leaf tissue samples collected in the field.

Materials and Methods

Plant Material

The potato line MSE149-5Y had been previously transformed with the pSPUD74 construct using *Agrobacterium* mediated transformation (K. Zarka, personal communication; Li *et al.*, 1999). MSE149-5Y is a round yellow fleshed breeding line from Michigan State University. The

construct pSPUD74 contains the *Arabidopsis thaliana CBF1* (*AtCBF1*) gene (GenBank accession AY667247.1) under the inducible promoter, also from *A. thaliana*, *COR15a* (GenBank accession U01377.1; Figure 2.1). The transformations resulted in four independent lines: E74.8, E74.9, E74.14 and E74.16. Potato lines transformed with *AtCBF1* under either the *35S* constitutive promoter or the *rd29A* inducible promoter (GenBank accession D13044) were graciously donated by Dr. Chen's laboratory (Oregon State University, Corvallis, OR; Table 2.1). The Oregon State University (OSU) lines were transformed into a long russet cultivar, Umatilla Russet (Pino *et al.*, 2007). The potato lines were maintained in tissue culture by nodal propagation either in 25 x 150 mm culture tubes or GA-7 Magenta boxes (Magenta Corp, Chicago, IL) in modified Murashige and Skoog (MS) media (4.3 g/L MS salts, 30 g/L sucrose, 1.4 mM sodium phosphate, 1.1 μM thiamine, 0.55 mM myo-inositol, pH 6.0, 8 g/L Agar). The cultures were maintained at 25 ± 3°C, with a 16 hr photoperiod.

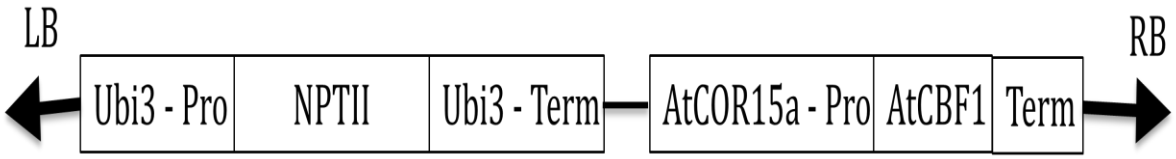

Figure 2.1 Schematic of pSPUD74 construct. The *CBF1* gene from *Arabidopsis thaliana* is directed by the stress-inducible promoter *AtCOR15a*.

Table 2.1 Transformed lines from MSU and OSU.

Experimental Lines	Promoter:Gene of Interest	Experiments
MSE149-5Y	Wild Type Control	Field Trials 2007-2010, Electrolyte Leakage
E74.8, E74.9, E74.14, E74.16	*COR15a:AtCBF1*	Field Trials 2007-2010, Electrolyte Leakage
OR1.2, OR1.11, OR1.15	*35S:AtCBF1*	Field Trial 2010, Electrolyte Leakage
OR2.1, OR2.3, OR2.6	*rd29A:AtCBF1*	Field Trial 2010, Electrolyte Leakage

Electrolyte Leakage Assay

Plant Growth and Temperature Treatment

Seed tubers of MSE149-5Y, E74.8, E74.9, E74.14 and E74.16 that were harvested in the Fall of 2007 were cut into seed pieces and planted in 6" round plastic pots filled with 70% peat moss, 21% perlite, 9% vermiculite (v/v) (Suremix, Michigan Grower Products Inc., Galesburg, MI). A total of eight pots were planted for each line. The plants were placed in the greenhouse and watered daily. Forty days after planting (DAP), the plants were moved to a growth chamber (PGR15, Conviron, Winnipeg, Manitoba, Canada) at 22°C under a 16 hr photoperiod, 60% humidity, 100-130 µmol m^{-2} s^{-1} fluorescent plus incandescent lighting for seven weeks; watered daily. The pots were placed in a completely randomized block design in the greenhouse and growth chamber.

After the seven wks, four pots of each line were moved into a walk-in cooler set at 4°C for 24 hrs, with fluorescent lights, to allow the plants to begin to acclimate. The other four pots remained in the previous growth chamber at 22°C.

Electrolyte Leakage Assay

Following the temperature acclimation treatment, young fully expanded leaves were excised from the plant and transferred to 16 x 100 mm glass culture tubes placed on ice. Two – three leaves from each plant were placed in one tube. There were three tubes for each line (MSE149-5Y, E74.8, E74.9, E74.14 and E74.16), for each acclimation treatment (4 and 22ºC), and for each freezing temperature treatment (control, -2, -2.5, -3, -3.5, -4, -4.5, -5, -5.5, -6ºC), for a total of 60 tubes per line. When all leaves had been transferred, all of the tubes, except the

control tubes, were moved to a -2ºC antifreeze bath (master bath) in a complete randomized design, and incubated for 60 min. The control tubes were kept on ice throughout the experiment and were covered with Saran wrap. An additional four antifreeze baths were set at temperatures starting at -2ºC, and descended by 0.5 degrees for each bath.

After 60 min, a small ice pellet (approximately 14 x 20 mm) was added to each test tube (including the control tubes), and then all tubes were plugged with foam. All of the test tubes were then incubated for an additional 60 min. After the second 60 min incubation, three tubes for each line from the 4ºC and three tubes from the 22ºC treatments were moved to the second antifreeze bath also set at -2ºC and incubated for 40 min. Meanwhile, the master bath temperature was then lowered to -2.5ºC. After 20 min, six tubes (representing the lines and acclimation treatments) were moved into the third bath set at -2.5ºC. After each set of tubes had incubated at its test temperature for 60 min, the tubes were placed in racks on ice. This process continued for all temperatures tested (-2, -2.5, -3, -3.5, -4, -4.5, -5, -5.5, -6ºC). All of the tubes were then placed at 2.5ºC to thaw overnight.

The following day, 3 mL of deionized water was added to each test tube with the thawed leaves and incubated with shaking at room temperature for three hours. Following the shaking, the liquid was transferred to a new test tube without the leaves. The electrical conductivity (L_1) of the water in the new tubes was measured using a conductivity meter (YSI model 35 with cell #3403, k = 1.0/cm), rinsing the meter with deionized water between samples. The older test tubes still containing the leaves were then autoclaved for 15 min to release all of the ions remaining in the leaves. The water in the new tubes was then returned to the old tubes and incubated with shaking at room temperature for three hours. The final ion concentration (L_2) was then measured again with the same conductivity meter. Percent of the total electrolyte

leakage at each test temperature was calculated by $(L_1 \div L_2) \times 100$. ANOVA analysis and mean separations using Fisher's LSD ($\alpha = 0.05$) were conducted on the percent leakage values using PROC GLM to compare the lines within each temperature treatment using SAS software (release 9.20; SAS Institute, Cary, NC).

The assay was later repeated in a similar manner for the Umatilla *CBF1* lines. The modifications were that the lines were originally propagated from tissue culture then transplanted into soil, they were not acclimated to 4ºC prior to the experiment and the temperatures for freezing were only tested down to -5ºC.

Field Trials of *AtCBF1* Transformed Lines

Four years of field trials were conducted at both the Michigan State University Montcalm Research Farm (Entrican, MI) and on the campus of Michigan State University's Crop and Soil Science Farm (East Lansing, MI) between 2007 and 2010. Every year, at both locations, tubers of MSE149-5Y (the wild type control), E74.8, E74.9, E74.14 and E74.16 were planted in a randomized complete block design with four replications. Each plot was 3 m in length with 1 m spacing between rows. Each plot contained 10 seed pieces with red skinned potato plants separating each plot. Guard rows were planted on the flanking sides of each plot. The Montcalm Research Farm plot (MRF) was maintained under irrigated conditions, and the Michigan State University plot (MSU) was maintained under rain fed/dry land conditions. Both plots were maintained using best management practices of fertilizer and pesticide applications. In the 2010 season, the six OSU lines (Table 2.2) were included in the field trial.

Table 2.2. OSU and MSU's designation of OSU *AtCBF1* lines.

OSU's designation (Pino *et al.*, 2007)	**MSU's designation**
35S:*AtCBF1* Line 2	OR1.2
35S:*AtCBF1* Line 11	OR1.11
35S:*AtCBF1* Line 15	OR1.15
rd29A:AtCBF1 Line 1	OR2.1
rd29A:AtCBF1 Line 3	OR2.3
rd29A:AtCBF1 Line 6	OR2.6

Throughout the season leaf tissue samples were collected before flowering, during flowering and during tuber bulking for later RNA isolation. The leaf tip of a young, fully expanded leaf was excised using the cap of a 1.5 mL Eppendorf tube and immediately placed in liquid nitrogen. During the first sampling, a white flag was placed at the base of the plant to mark the plant for later tissue sampling. Three samples were collected from the first three replicates for each line, and this was repeated at both locations. All of the frozen samples were then stored at -80ºC until used for RNA isolation.

Two weeks before harvest the plots were treated with a foliar applied vine desiccant (Rely®280, Bayer CropScience, Research Triangle Park, NC). At harvest, tubers were collected in bags marked with the location, line and replicate. A tag was also included in each bag indicating the location, line, and replicate. Each line was graded for yield and specific gravity.

Recently harvested tubers from each line were used for sugar analysis in 2008 and 2009. Tubers were peeled and cut into 1 x 2" pieces then fed into a commercial electric juicer (Waring Products 6001C, Torrington, CT) until 30 mL were obtained for each line, from each field

location. The sucrose and the glucose concentrations of the lines were determined by Techmark Inc. (Lansing, MI) using the YSI 2700D Bioanalyzer (Yellow Springs, OH).

Statistical analysis for the yield, specific gravity and sugar profiles were conducted using ANOVA and LSD for means separation ($\alpha = 0.05$) on SAS software (release 9.20; SAS Institute, Cary, NC).

Gene Expression Analysis Using RT-PCR

Frozen leaf tissue stored at -80°C was ground to a fine powder using liquid nitrogen and total RNA was then isolated using the RNeasy Plant Mini-Kit (Qiagen Inc., Valencia, CA). The RNA was then DNase treated using RQ1 RNase-Free DNase (Promega Corp., Madison, WI) to remove any residual DNA. 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 (Life Technologies Corp., Carlsbad, CA). Each 20 µL reaction contained 2 µL 10X RT Buffer, 0.8 µL of 25X dNTP mix (100mM), 2 µL 10X RT random primers, 1 µL MultiScribe™ Reverse Transcriptase, 4.2 µL of nuclease free water, and 10 µL of template RNA. The reaction occurred according to the manufacturer's directions of 10 min at 25ºC, 2 hrs at 37ºC, 5 min at 85ºC and held at 4ºC. Eight microliters of cDNA were then used as template in 50 µL PCR amplifications each containing: 10 µL 5X Green GoTaq® Reaction Buffer, 1 µL dNTP mix 10mM, 0.25 µL GoTaq® DNA Polymerase (Promega, Madison, WI), 1 µL of each primer, and 34.75 µL of nuclease free water. The PCRs were carried out under the following conditions: 94ºC of four min, 30 cycles of 94ºC for 60 secs, 60ºC for 90 secs, 72ºC for 90 secs, a final extension for four min at 72ºC and held at 4ºC. The gene specific primers for 18S,

AtCBF1, *DHN10* and *Solanum tuberosum* galactinol synthase 3 (*StGolS3*) are listed in Table 2.3. Control reactions were carried out using RNA isolated from OR1.11 and water replacing template cDNA.

Table 2.3. Primer sequences used in RT-PCR analysis of *AtCBF1* transgenic lines.

Primer	Sequence	Source
18S F	GGGCATTCGTATTTCATAGTCAGAG	(X67238.1, Primer-BLAST, www.ncbi.nlm.nih.gov)
18S R	CGGTTCTTGATTAATGAAAACATCCT	
AtCBF1 F	CTCCGATTACGAGCCTCAAG	(AT4G25490, Primer-BLAST, www.ncbi.nlm.nih.gov)
AtCBF1 R	ATCGTCTCCTCCATGTCCAG	
DHN10 F	GCTAAACCCCAAAAAAAAACTCATT	(Pino *et al.*, 2007)
DHN10 R	GTCCAAAAGACGAGTACATTCAC	
StGolS3 F	AGCCATGGAGGTACACTGGA	(M. A. Carvallo, personal communication, 2008)
StGolS3 R	TTGTCAGCTTCAACTTCACCA	

Results

Electrolyte Leakage Assay

To evaluate the efficacy of the transgenic potato plants in their ability to decrease electrolyte ion leakage an electrolyte leakage assay was used. When a plant confers freezing or drought stress, the cells are maintaining their ions. If more than 50% of a plant's ions are lost due to a freezing or drought stress, this is considered lethal for the plant. In this study three sets of experiments were carried out. The first two sets both involved the four MSE transgenic lines with the *AtCBF1* gene under the stress-inducible *AtCOR15a* promoter and the wild type control line MSE149-5Y. In the first experiment the plants where kept at 22°C and in the second experiment the plants were kept at 4°C for 24 hrs prior to the experiment. In the third experiment the OSU transgenic lines with the *AtCBF1* gene under either under the constitutive 35S promoter or the stress-inducible promoter *rd29a*. For the OSU lines they were only used after being kept at 22°C due to a previous study by Pino *et al* (2007) showing little difference in the % of ion leakage when the plants were kept at room temp. or cold acclimated at 2°C for two weeks.

In the first experiment on the MSE lines, all of the lines lost more than 50% of their ions at each temperature treatment. In the 0°C control treatment, minimal ions were lost for each line (Figure 2.2). At the -2°C treatment, E74.14 lost significantly fewer ions than the WT control line and E74.8. However, with a % leakage at 50.5% the plant would not survive. At the -2.5°C treatment, E74.14 lost significantly less ions than all the other lines, but was well over 50% leakage (69.7%). At the -3°C treatment, E74.14 and E74.16 lost significantly more ions than the other lines, but all were near 100% leakage.

When the lines were cold treated at 4°C prior to the freezing treatments, the lines E74.8 increased in freezing tolerance to -2°C and E74.9 to -2.5°C. All the remaining lines did not increase in freezing tolerance. At the -2 and -2.5°C treatment, E74.8 and E74.9 lost significantly fewer ions than the other lines and their losses were less than 50% (Figure 2.2). However, only E74.9 lost less than 50% at the -2.5 (44.9%). At the -3°C treatment, all of the lines lost more than 50% of their ions, with E74.8 losing the least amount of ions.

For the OSU lines it had previously been determined by Pino *et al*. (2007) with acclimation at 2°C for two weeks all six lines increased their freezing tolerance to -5°C. In this experiment the OSU lines were maintained at 22°C (non-acclimated). The 35S lines OR1.11 and OR1.15 were able to maintain 50% or more of their ions at -2.5°C, with all of the other lines except OR2.6 able to maintain 50% or more of their ions at only -2°C (Figure 2.3). At the -2°C treatment, only OR2.6 had an ion leakage greater than 50%. The remaining OSU lines were not significantly different from each other at this treatment. At the -2.5°C treatment, only the 35S lines OR1.11 and OR1.15 were able to maintain more than 50% of their ions and were significantly different from the remaining lines. At the -3°C treatment, all of the lines had a percent leakage greater than 50%.

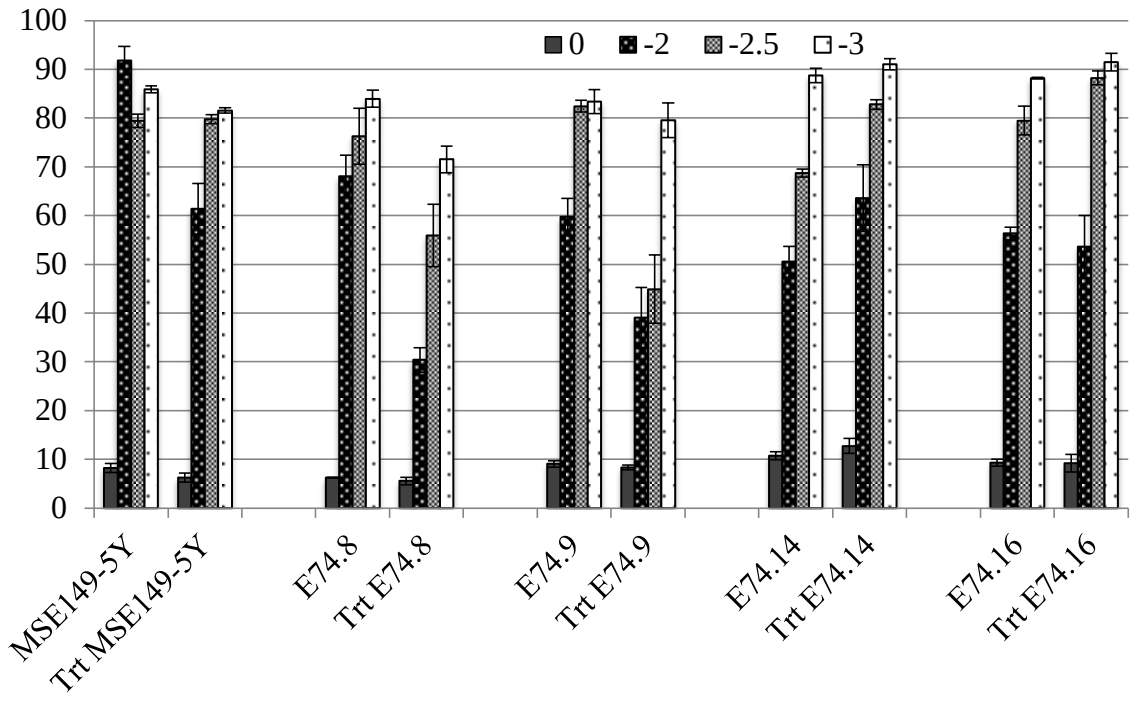

Figure 2.2 Electrolyte Leakage Assay for the MSE lines. Each line was tested twice, once with no cold treatment and once with a 4°C cold treatment for 24 hrs (labeled Trt) prior to the experiment. MSE149-5Y is the WT control. All of the temperature treatments were analyzed by ANOVA using Fishers LSD (α=0.05) between the lines within the non-treated and cold treated experiments. In each of the temperature treatments, none of the non-treated lines were able to maintain a percent leakage less than 50%. A percent leakage of 50% or more is considered lethal. At the 0°C control treatment; E74.14 was significantly different from E149-5Y. E74.8 was significantly different from all of the lines and had the greatest % leakage. At the -2°C treatment; E74.14 had significantly less ions loss than E74.8 and E149-5Y. At the -2.5°C treatment; E74.14 has significantly less ion loss than all of the other lines. At the -3°C treatment; E74.14 and E74.16 had significantly more ion loss than the other three lines. For the cold treated lines at the 0°C control treatment; E74.16 lost significantly more ions than E149-5Y and E74.8. E74.16 lost significantly more ions than all of the other four lines. At the -2°C treatment; E74.8 and E74.9 showed an increase in freezing tolerance at this temperature having a % leakage less than 50%. Both lines were significantly different from the other three lines. At the -2.5°C treatment; both the E74.8 and E74.9 were again statistically significant from the other three lines. However, only E74.9 maintained a % leakage less than 50%. At the -3°C treatment; all of the lines had a % leakage far greater than 50%. E74.8 had a significantly less % leakage than all of the other lines. E74.14 and E74.16 % leakages were significantly greater than the other three lines.

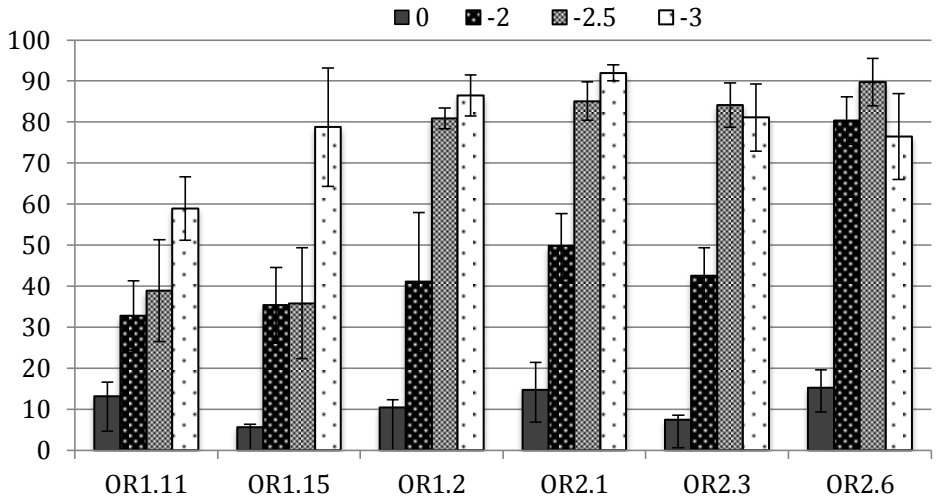

Figure 2.3 Electrolyte Leakage Assay for OSU Lines. All of the temperature treatments were analyzed by ANOVA using Fishers LSD (α=0.05). At the 0°C control treatment; OR1.15 had statistically the lowest % leakage compared to the other five lines. At the -2°C treatment; all of the lines except OR2.6 had a % leakage less than 50%. At the -2.5°C treatment; only OR1.11 and OR1.15 maintained a % leakage less than 50% and both were significantly less than the other four lines. At the -3°C treatment, all of the lines had a % leakage greater than 50% and only OR1.11 and OR2.1 were significantly different from each other.

Field Trials of *AtCBF1* Transformed Lines

In order to test the agronomic performance, using best practices, of the MSU *AtCBF1* lines under non-irrigated conditions, seed potatoes were planted in two field locations from 2007-2010, and the OSU lines were planted in 2010. The Montcalm Research Farm (MRF) and was one location and was maintained under irrigated conditions. Michigan State University's Crop and Soil Science Farm (CSSF) was the second location and was maintained under rain fed conditions. Each year the potatoes were planted in early to mid-May and were harvested approximately 125 days later in the fall. All four of the years the CSSF plot experienced drier conditions than the MRF plot (Figure 2.4). It is standard recommendations for potatoes to receive between 20-24 inches of water in a growing season. These conditions were maintained at the MRF plot with the utilization of irrigation. The CSSF

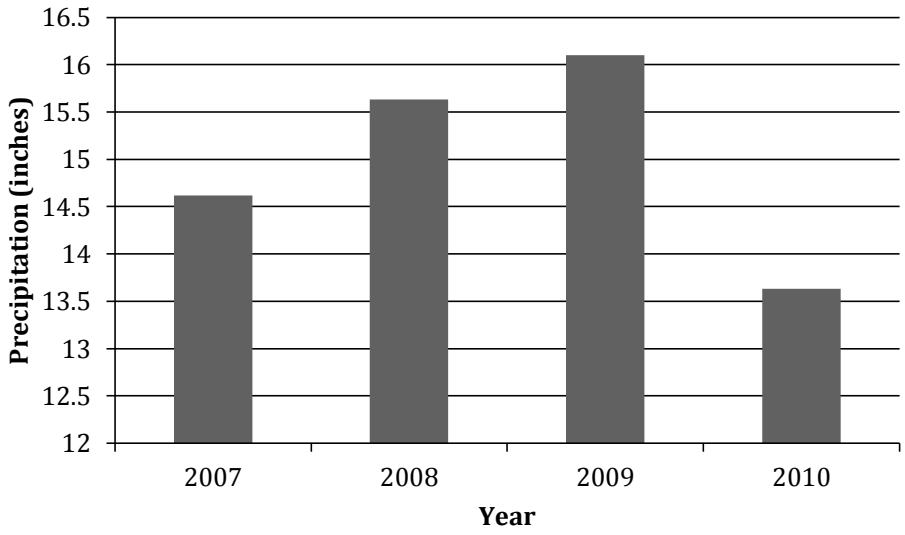

Figure 2.4 Total Precipitations at the CSSF. The total amount of precipitation measured at the CSSF plot during the 125 day growing season (on average mid-May to mid-September). These conditions are below the standard practice of 20-24 inches of water for potato crops.

plots received far less water, thus creating an environment for short-term drought events to compare the drought tolerance of the lines.

In the 2007 and 2010 field seasons the MRF field had significantly greater 100 cwt./acre yield and specific gravity than the CSSF field (Table 2.4 and 2.5). In 2008, there was no significant difference in yield; however, MRF had a significantly lower specific gravity. In 2009, CSSF had a significantly greater yield than MRF, but there was no significance in the specific gravity.

Table 2.4 100 cwt./acre yield ANOVA between the two field locations for each growing season. The different letters denote statistically significant values within the year ($\alpha = 0.05$).

Year	MRF – Irrigated	CSSF – Non-irrigated	LSD
2007	195.37 - A	118.33 - B	16.76
2008	276.4 - A	261.7 - A	36.80
2009	79.6 – B	441 – A	46.68
2010	287.8 – A	183.59 - B	56.93

Table 2.5 Specific gravity ANOVA between the two field locations for each growing season. The different letters denote statistically significant values within the year ($\alpha = 0.05$).

Year	MRF – Irrigated	CSSF – Non-irrigated	LSD
2007	1.066 - A	1.056 - B	0.0018
2008	1.073 - B	1.076 - A	0.0016
2009	1.068 - A	1.058 - A	0.0104
2010	1.069 - A	1.064 - B	0.0037

To compare the yield and specific gravity of each line the years were combined for ANOVA of each location. OR2.6 was dropped from the analysis due to a significant loss of plants at the CSSF location. The wild type control line, MSE149-5Y, had the greatest 100 cwt./acre yield mean at the CSSF location (Table 2.6). It was significantly greater than all the transgenic lines except E74.8 and E74.9. The OSU lines had a significantly lower yield than the MSU lines. There was little difference between the lines for specific gravity with OR1.11 being significantly less than E74.16, E74.8 and E74.9 at the CSSF location. There was more difference in specific gravity at the MRF location (Table 2.7). All of the OSU lines, except OR2.3, were greater than all of the MSU lines. There were no significant differences between the MSU lines for specific gravity. All of the OSU lines were significantly greater in yield than all of the MSU transgenic lines. The WT control line was only significantly less than OR1.11 and significantly greater than E74.16.

In 2008 and 2009 tuber samples from each line, and from each location, were submitted for analysis of the percent glucose and sucrose. Between 2008 and 2009 there was a significant difference in both percents of glucose and sucrose (Table 2.8). The percent glucose was highest in 2009; however, the percent sucrose was highest in 2008. The combined percent glucose in

Table 2.6 2007-2010 CSSF 100 cwt./acre yields and specific gravity; means with the same letter are not significantly different ($\alpha = 0.05$).

Line	100 cwt./acre Yield Mean (LSD = 70.51)	Specific Gravity Mean (LSD = 0.009)
MSE149-5Y	313.7 - A	1.061 - AB
E74.8	248.4 - AB	1.063 - A
E74.9	256.2 - AB	1.063 - A
E74.14	231.1 - B	1.058 - AB
E74.16	212.7 - B	1.062 - A
OR1.11	106.1 - C	1.053 - B
OR1.15	105.9 - C	1.058 - AB
OR1.2	105.5 - C	1.056 - AB
OR2.1	66 - C	1.055 - AB
OR2.3	51.34 - C	1.054 - AB

Table 2.7 2007-2010 MRF 100 cwt./acre yield and specific gravity, means with the same letter are not significantly different ($\alpha = 0.05$).

Line	100 cwt./acre Yield Mean (LSD = 72.63)	Specific Gravity Mean (LSD = 0.009)
MSE149-5Y	277.88 - BC	1.068 - C
E74.8	205.4 - CD	1.070 - BC
E74.9	213.38 - CD	1.065 - C
E74.14	212.31 - CD	1.068 - C
E74.16	205.06 - D	1.069 - BC
OR1.11	384.75 - A	1.083 - A
OR1.15	350 - AB	1.082 - A
OR1.2	350.5 - AB	1.080 - A
OR2.1	336.5 - AB	1.081 - A
OR2.3	333 - AB	1.078 - AB

2008 and 2009 was within range for a chipping variety. The combined percent sucrose was high in both years for a chipping variety. The percent glucose remained the same for each line in both years at the CSSF under rain fed conditions (Tables 2.9 and 2.10). In 2008, there was a significant increase in both the percent glucose and sucrose in the tubers at the CSSF location. In

2009, the percent glucose at CSSF was lower than at the MRF location, however, the percent sucrose increased at the CSSF similar to 2008.

Table 2.8 2008 and 2009 t-test of sugar analysis; means with the same letter are not significantly different ($\alpha = 0.05$).

Year	% Glucose means (LSD = 0.0021)	% Sucrose means (LSD = 0.0033)
2008	0.0052 - B	0.6332 - A
2009	0.0105 - A	0.2945 - B

Table 2.9 2008 sugar analysis of tubers; means with the same letter are not significantly different ($\alpha = 0.05$).

Line	MRF		CSSF	
	% Glucose	% Sucrose	% Glucose	% Sucrose
MSE149-5Y	0.0034 - C	0.477 - C	0.012 - B	0.851 - BC
E74.8	0.0026 - A	0.411 - AB	0.005 - A	0.789 - AB
E74.9	0.0024 - A	0.462 - BC	0.006 - A	0.759 - A
E74.14	0.0032 - BC	0.389 - A	0.005 - A	0.802 - AB
E74.16	0.0028 - AB	0.490 - C	0.01 - B	0.901 - C

Table 2.10 2009 sugar analysis of tubers; means with the same letter are not significantly different ($\alpha = 0.05$).

Line	MRF		CSSF	
	% Glucose	% Sucrose	% Glucose	% Sucrose
MSE149-5Y	0.016 - A	0.222 - B	0.012 - A	0.396 -B
E74.8	0.012 - BC	0.191 - D	0.005 - B	0.367 - D
E74.9	0.011 - C	0.215 - C	0.006 - B	0.353 - E
E74.14	0.015 - AB	0.181 - E	0.005 - B	0.373 - C
E74.16	0.013 - ABC	0.228 - A	0.01 - A	0.419 - A

RT-PCR

To study the gene expression of the transgene and other stress-induced genes, RNA was isolated from leaf tissue samples collected in the field from the transgenic and WT lines. Reverse transcriptase PCR (RT-PCR) was then employed to semi-quantitatively measure the gene expression of the transgene and two other stress inducible genes native to potato. *Arabidopsis CBF1* (*AtCBF1*), dehydrin 10 (*DHN10*), and *S. tuberosum* galactinol synthase 3

(*StGolS3*) genes along with 18S ribosomal control were used. The samples that were collected at the MRF location are designated with the letter M and the samples collected at the CSSF location are designated with the letter C. The times when the samples were collected are designated by numbers following the letters (1 = pre-flowering, 2 = flowering, 3 = tuber bulking). In 2008, the transgene *AtCBF1* was expressed in all of the samples except E74.9 M3, E74.14 C2, and E74.16 M3; and was not found in the WT control MSE149-5Y (Figure 2.5). In the E74.8 line the three highest expressing samples were M1, M2 and C2. In the E74.9 line the two highest expressing samples were C1 and C3. In the E74.14 lines the three highest expressing samples were M1, M2 and C3. In the E74.16 the least expressing sample was C3. The stress-induced gene *DHN10* was found to be expressing at slightly varying levels across all the lines. In the WT control line (MSE149-5Y) the two highest expressing samples were C1 and C2. In the E74.8 line the three highest expressing samples were M1, M2, and C2. In the E74.9 line the two least expressing samples were M3 and C3. In the E74.14 line the two least expressing samples were M3 and C2. In the E74.16 line the least expressing sample was C3. The other stress-induced gene *StGolS3* was expressed in most of the samples except MSE149-5Y C3, E74.8 C1 and C3, E74.9 C1 and C3. In the WT line all of the samples were expressing at similar levels. In the E74.8 line the three highest expressing samples were again M1, M2 and C2. In the E74.9 line the three highest expressing samples were also M1, M2 and C2. In the E74.14 line the three highest expressing samples were M1, M2 and C1. In the E74.16 line the two least expressing samples were M3 and C3. The 18S ribosomal control was consistently expressed across all the lines and samples.

In 2010 for the MSU lines the *AtCBF1* transgene was expressed in most of the transgenic samples except E74.9 M1, M2, C2, and E74.14 C2 and all of the WT control lines (Figure 2.6). In the E74.8 line the two highest expressing samples were C1 and C2. In the E74.9 line C1 was

the only expressing sample. In the E74.14 line M1 and M2 samples were the highest expressing. In the E74.16 line the highest expressing sample was M2. The *DHN10* gene was found to be expressing in most of the samples except E74.9 M2 and C2, E74.14 C1 and C2, and E74.16 M2 and C2. In the WT line all of the samples were expressing at a similar level. In the E74.8 line, C1 and C2 had slightly higher expression than the other two samples. In the E74.9 line C1 had higher expression over M1. In the E74.14 line, M1 and M2 had similar expression. In the E74.16 line M1 and C1 had similar expression. The *StGolS3* gene was expressed in all of the lines at varying degrees. In the WT line, C1 and C2 had the highest expression. In the E74.8 line, C1 and C2 also had the highest expression. In the E74.9 line, C1 had the highest expression. In the E74.14 line M2 had the highest expression. In the E74.16 line, C1 had the highest expression. The 18S ribosomal control was consistently expressed across all the lines and samples.

In 2010 for the OSU lines, the *AtCBF1* transgene was not detected in the 35S lines (OR1.11-OR1.2, Figure 2.7). *AtCBF1* expression was not found in any of the *rd29A* lines OR2.1 and OR2.3. The expression of *AtCBF1* was weak for all of the expressing samples. The *DHN10* gene was found to be expressing in most of the samples except OR1.11 M1, C1 and C2, and OR1.2 C2. In the OR1.15 line, M2 was the highest expressing. In OR1.2 the expression was similar across the three samples. The expression in OR2.1 and OR2.3 was weak but similar across the samples. The *StGolS3* gene was expressed in all of the samples at varying degrees except OR2.1 M1. In OR1.11, OR1.15 and OR2.1 C1 and C2 were the highest expressing samples. In OR1.2 C1 was the highest expressing sample. In OR2.3 M2 was the least expressing sample. The 18S ribosomal control was consistently expressed across all the lines and samples.

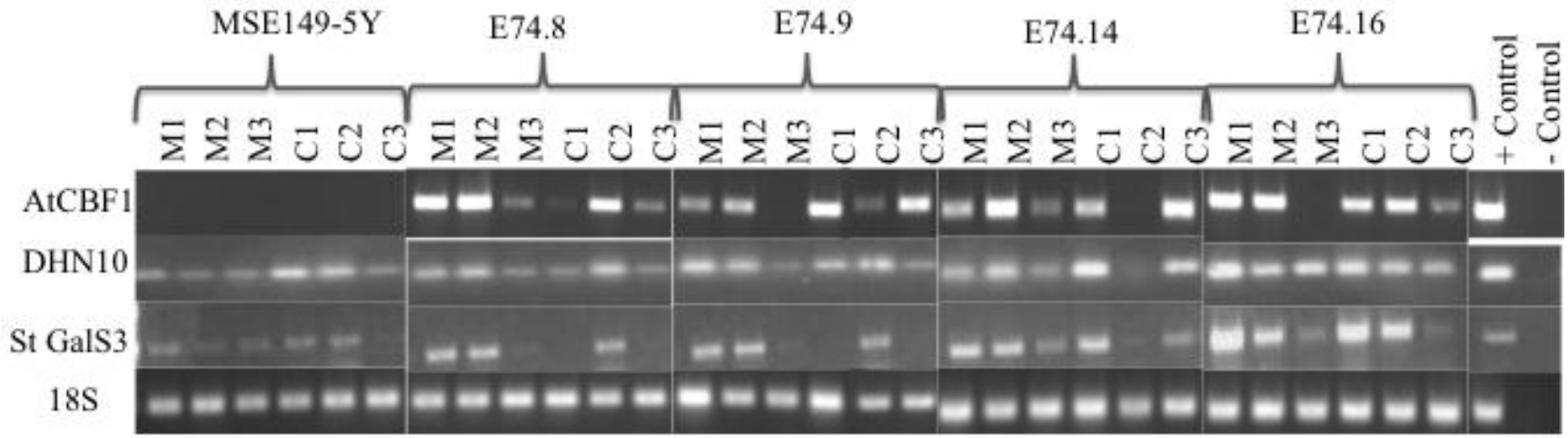

Figure 2.5 Reverse Transcriptase PCR from the 2008 field trial. The gene expression of four different genes was visualized by RT-PCR from RNA isolated from fresh leaf tissue collected in the field. The lane designations of M1, M2… C1, C2 denote the location from which the sample was taken from (M = MRF, C =CSSF) and the number signifies the timing of the sampling (1 = pre-flowering, 2 = flowering, 3 = tuber bulking). The *AtCBF1* gene is the transgene in the E74.8 – E74.16 lines and was expressing in all of the samples except E74.9 M3, E74.14 C2, and E74.16 M3; and was not found in the WT control MSE149-5Y. The *DHN10* gene is a stress induced potato gene and was found to be expressing at slightly varying levels across all the lines. The *S. tuberosum* galactinol synthase 3 gene, *StGolS3*, has also been found to be a stressed induced gene and downstream of the CBF regulon. It was being expressed in most of the samples except MSE149-5Y C3, E74.8 C1 and C3, E74.9 C1 and C3. The *18S* gene was the ribosomal control gene used in 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.

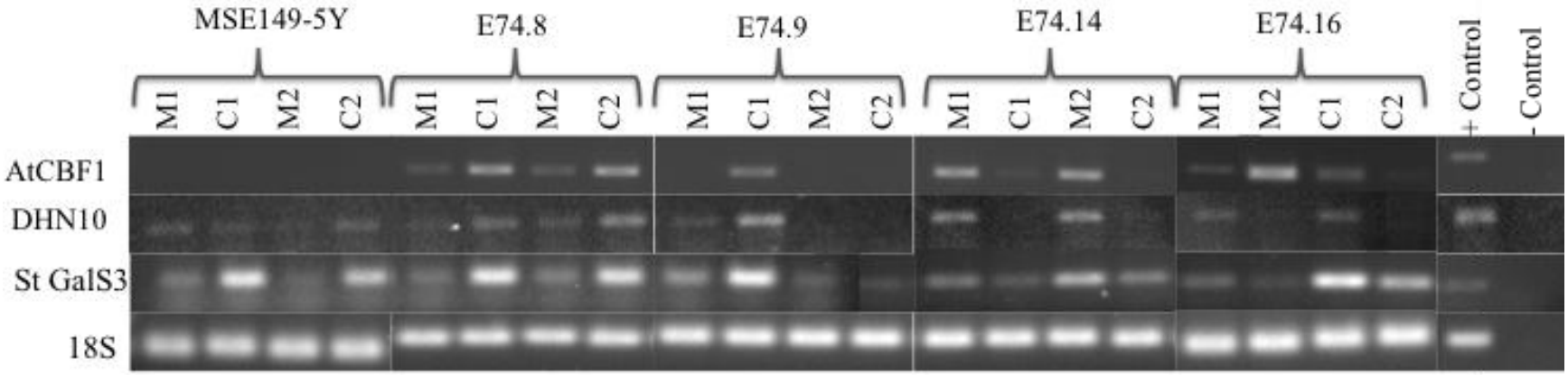

Figure 2.6 Reverse Transcriptase PCR from the MSU lines in the 2010 field trial. The gene expression of four different genes was visualized by RT-PCR from RNA isolated from fresh leaf tissue collected in the field. The *AtCBF1* transgene was expressed in most of the transgenic samples except E74.9 M1, M2, C2, and E74.14 C2 and all of the WT control lines MSE149-5Y. The stressed induced *DHN10* gene was found to be expressing in most of the samples except E74.9 M2 and C2, E74.14 C1 and C2, and E74.16 M2 and C2. The other stress induced gene *StGolS3* was expressed in all of the lines at varying degrees. The *18S* gene was the ribosomal control gene used in all of the lines. 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 reaction.

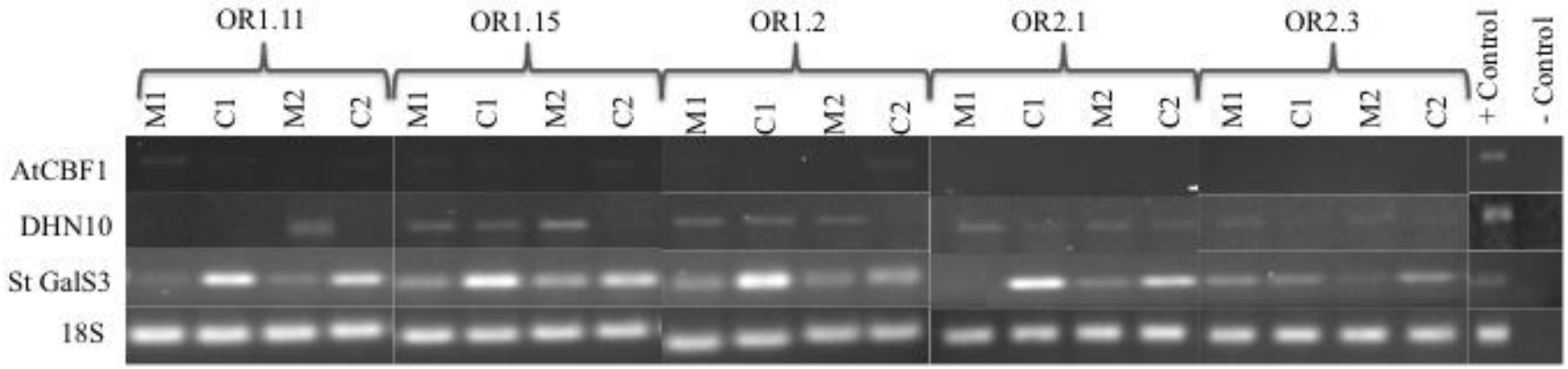

Figure 2.7 Reverse Transcriptase PCR from the OSU lines in the 2010 field trial. The gene expression of four different genes was visualized by RT-PCR from RNA isolated from fresh leaf tissue collected in the field. The *AtCBF1* transgene was not detectable in the 35S samples (OR1.11 – OR1.2). *AtCBF1* expression was not found in any of the *rd29A* lines OR2.1 and OR2.3. The stressed induced *DHN10* gene was found to be expressing most of the samples except OR1.11 M1, C1 and C2, and OR1.2 C2. The other stress induced gene *StGolS3* was expressed in all of the samples at varying degrees except OR2.1 M1. The *18S* gene was the constitutive control gene used in all of the lines. 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.

Discussion

Previous studies have shown that plants transformed with CBF genes are able to increase their freezing tolerance as studied by electrolyte leakage assays (Jaglo-Ottosen *et al.*, 1998; Pino *et al.*, 2007). The transgenic MSU lines were only able to increase their freezing tolerance after they had been cold acclimated for 24 hr. E74.8 and E74.9 were the only two lines that were able to increase their freezing tolerance to -2.5°C and -3°C, respectively. E74.14 and E74.16 did not perform any better than the WT control, even when cold acclimated. The OSU transgenic lines were not cold acclimated and had a similar freezing tolerance increase to that of the cold acclimated MSU transgenic lines. The overexpressed OSU lines had the lowest percent ion leakage, along with OR2.3 (under the inducible promoter), at -2°C. Surprisingly, OR1.2 performed similarly to the inducible promoter lines at the -2.5°C treatment. OR2.6 was the only line that was not able to increase its freezing tolerance. It had previously been shown by Pino et al. that with cold-acclimation, all of the lines increased their freezing tolerance to -5°C (2007). Since the OSU lines were not cold acclimated in this study, they can only be compared to the MSU lines that were not cold acclimated. In this comparison the OSU lines OR1.11, OR1.15, OR1.2 and OR2.3 showed more freezing tolerance than the MSU lines. It has also been shown in *B. napus* that the plants were able to increase freezing tolerance without cold acclimation when the CBF gene was overexpressed (Jaglo *et al.*, 2001). It is encouraging to see that at least two of the MSU lines under inducible promoters were able to increase their freezing tolerance when cold acclimated.

In order to truly test the transgene's ability to confer abiotic stress tolerance, the MSU transgenic lines were subjected to agronomic field trials for four years. There have been few

studies of transgenic crops tested for abiotic stress tolerance in the field (Waterer *et al.*, 2010; Dunwell, 2000; Schafleitner *et al.*, 2007). Field trials are, however, the best way to determine if a line (transgenic or not) is going to have the preferred agronomic traits and produce the needed yield. From 2007-2010 the rain-fed only plot was planted at the Crop and Soil Science Farm (CSSF) in East Lansing, MI and the control plot was planted at the Montcalm Research Farm (MRF) in Entrican, MI. At the MRF site, irrigation was administered to the plot according to best management practices. At the CSSF site, the plot was only rain-fed. Because of limited resources, no roof or other modification to keep the field dry was implemented. The weather in East Lansing consisted of irregular rainfall through out the field trials and induced a short-term drought stress on the plot. It is standard practice for potato fields to receive between 20 to 24" of water during a growing season. As seen in Figure 2.4, the CSSF location did not receive any more than 16" in a season. Each year the plants at CSSF appeared water-stressed with leaf curl in July, and wilting with early senescence in August.

To indirectly test if the plants at the CSSF location were experiencing an osmotic stress, the sugar profiles of the tubers were analyzed in 2008 and 2009. In 2008 the percent glucose and the percent sucrose were elevated at the CSSF location compared to the MRF location across all the lines. In 2009, only the percent sucrose was elevated at the CSSF location. The percent glucose levels at the MRF location were elevated in 2009 compared to 2008. Elevated sugar levels have been associated with water deficit stress (Geigenberger *et al.*, 1997). There was no correlation between the transgenic lines and the WT control in the glucose and sucrose levels in the tubers in either year. Interestingly, the % glucose at the CSSF location remained the same between 2008 and 2009. The % sucrose level at CSSF was much higher for all of the lines and this may be related to the fact that 2008 (15.63') had slightly less rain then 2009 (16.1") and that

a majority of the rain in 2009 fell in the early part of the season, and was very dry later during the tuber bulking period of growth.

In 2007 and 2010, MRF had a much greater yield overall, than at CSSF. In 2008, there was no significant difference in yield between the two locations. At CSSF, when all of the yields had been combined from all four seasons, the WT control E149-5Y had the highest yield, even under the dry conditions. However, E74.8 and E74.9 did not have a significantly less yield. As noted previously, E74.8 and E74.9 were also the only two lines that increased their freezing tolerance in the electrolyte leakage assay. The yields for the OSU lines were the lowest overall, but they had only been grown in the field in 2010. Remarkably, the inducible OSU lines had even lower yields than the constitutively expressed OSU lines. This result goes against the notion that using an inducible promoter reduces negative phenotypes (Kasuga *et al.*, 2004). At the MRF location, the OSU lines did the opposite in terms of yield and had greater yields than the MSU lines. This may be the result of including the 2009 yield data, in which the MSU lines yielded significantly less than the other three years. However, seeing that the OSU lines could perform as well as the MSU WT, this suggests that it was not the transgenic insertion that decreased the yield under the stressed conditions. Even the overexpressing OSU lines were able to give good yields at the MRF location. For this limited field trial of the OSU lines, it appears they are not able to increase their drought tolerance and still perform to the agronomic standards. There seemed to be a correlation between the electrolyte leakage data and the field data for the MSU lines, however, the OSU results contradict the electrolyte leakage data from both this study and Pino 2007. It would be interesting to see if the data trend would hold up over repeated field trials of the OSU lines.

The specific gravity of all the lines combined tended to be higher at the MRF location except in 2008. Most of the transgenic lines had specific gravities that were not significantly different from each other and the WT at the CSSF location. Only OR1.11 had specific gravity lower than E74.8, E74.9 and E74.16. At the MRF location the OSU lines had significantly higher specific gravities than the MSU lines. Only OR2.3 was not significantly different from E74.8 and E74.16. In general the water stress slightly decreased the specific gravity in the MSU lines, and more dramatically decreased the specific gravity in the OSU lines.

Only the gene expression data from 2008 and 2010 was available as all of the RNA samples from 2007 and 2009 were used up in attempts to optimize Northern hybridizations. The expression of the *AtCBF1* transgene was variable across the lines and between the locations. There was little correlation between high gene expression in samples from the CSSF location. In 2008, the *DHN10* stress induced gene was expressed across all of the lines, including the WT, at both locations. High expression of *DHN10* was seen in correlation with the expression of *AtCBF1* in E74.8 E74.14 and E74.16. The gene also had slightly higher expression in the WT in the first two CSSF samplings. The *StGolS3* gene was most closely correlated to the *AtCBF1* expression in all of the lines. In 2010, *AtCBF1* was expressed more in the CSSF samples than the MRF samples. However, in E74.14 and E74.16 the *AtCBF1* expression was the highest in the MRF samples. In E74.9 the only sample that was positive for *AtCBF1* expression was the first one from CSSF. The expression of *DHN10* was mostly correlated to the *AtCBF1* expression levels. The only sample that did not fit this model was the M2 from E74.16, where there was strong *AtCBF1* expression but no *DHN10* expression. The *StGolS3* gene was expressed across all of the lines, and in the majority of the samples the level of its expression would mirror the expression of *AtCBF1*. There is no strong evidence from the RT-PCR data that correlates with

any lines' performance in the field or in the electrolyte leakage assay. In some lines and for some samples the gene expression correlated with the field location, but in other samples there was no correlation.

Some of the factors that may have contributed to the irregularity of *AtCBF1* gene expression in the transgenic lines may have been that the gene can become desensitized to the stress and activation by mechanical agitation (Zarka *et al.*, 2003). It was shown with CBF2 that when the transgenic plant was kept at a low temperature for an extended period of time, that the transcript levels would decrease and eventually be undetectable. The plants would need time to recover at a warm temperature, then placed back in a cold temp treatment before the transcript could be seen. This may be the case with the MSU transgenic lines in that they are constantly being exposed to several abiotic stresses (water deficit, heat, etc.) and that the transcript level may not be detectable at the time the sample was collected. The fact that CBF genes have been found to be activated by mechanical agitation may also have been the reason for the gene to have turned on in samples from both locations and at varying levels within lines and locations.

Overall it was found that E74.8 and E74.9 are the best candidates out of all the lines tested. Both of these lines showed that they could decrease electrolyte ion leakage induced by freezing temperature treatments by a couple degrees Celsius and were able to maintain a yield similar to the WT under water stressed conditions. However, it should be noted that the yields at the CSSF location were generally lower than at the MRF, and thus under ideal locations the transgenic lines would not be the best candidates. Further field trials with the OSU transgenic lines could reveal more about their ability to confer abiotic stress tolerance. Another way to improve this type of research would be to better optimize the tissue sampling in the field to better improve the gene expression data. As always, incorporting different genes under new and

different promoters may also lead to a transgenic potato that can confer abiotic stress tolerance and maintain yield.

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Chapter 3

Evaluation of *StCBF1* and *ScCBF1* Transgenic Potatoes

Research Objectives

This research evaluates the *CBF1* gene from *Solanum tuberosum* and *S. commersonii* transformed into cv. MSE149-5Y and cv. Desiree under the stress inducible promoter *COR15a*. The objectives were:

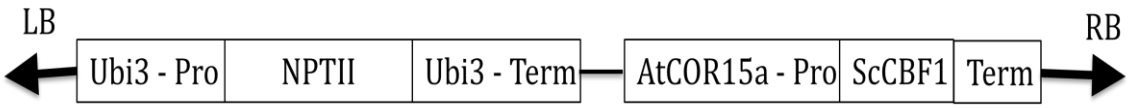
1. Genetically engineer MSE149-5Y and Desiree with two separate constructs of *COR15a:StCBF1* and *COR15a:ScCBF1*.
2. Evaluate the transgenic plants under osmotic stress to study the transgene's ability to confer stress tolerance.

Materials and Methods

Construction of Plasmids for Transformation

Two constructs were created for this study: pSPUD89 and pSPUD 90. The pSPUD89 vector contains the *Solanum commersonii CBF1* (GenBank: EU849672.1) gene and the *Arabidopsis thaliana COR15a* promoter (GenBank: U01377.1, Baker *et al.*, 1994) in a vector with a pBINPLUS (van Engelen *et al.*, 1995) backbone with the *nptII* selectable marker (Figure 3.1A). The pSPUD90 vector has all of the same characteristics as pSPUD89 except it contains the *S. tuberosum CBF1* (GenBank: EU849677.1) gene (Figure 3.1B). Both of the *S. commersonii* and

A.



B.

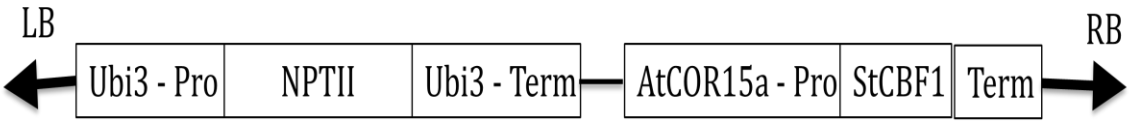


Figure 3.1 Schematic of p SPUD89 and pSPUD90 constructs. A. Contains the *CBF1* from *S. commersonii*. **B.** Contains the *CBF1* from *S. tuberosum*. In both constructs the stress-inducible promoter AtCOR15a directs the genes.

S. tuberosum genes were provided by Dr. Chen's laboratory (Oregon State University, Corvallis, OR) in glycerol stocks of the plasmids pZJ/ScCBF1 and pZJ/StCBF1. Both of the *CBF1* genes were excised from their respective plasmids by digesting the plasmids with *Hind*III and *Bam*HI (New England Biolabs Inc., Ipswich, MA) according to the manufacturers directions. The genes were then PCR amplified using the following primers: *ScCBF1* forward primer 5'-CCA GCT GGC AGG AAG AAG TTT CG-3', reverse primer 5'-GCC ATG TAA GCA TCA GCT TCC ACA-3', *StCBF1* forward primer 5'- CCA GCT GGC AGG AAG AAG TTT CG-3', reverse primer 5'- TCT GCA CAT TGA GGT GGA GGT AGC A-3'. Two µL of the respective PCR amplified *CBF1* genes were used in 50 µL PCRs each containing: 10 µL 5X Green GoTaq® Reaction Buffer, 1 µL dNTP mix 10mM, 0.25 µL GoTaq® DNA Polymerase (Promega, Madison, WI), 1 µL of each primer 10mM, and 34.75 µL of nuclease free water. The PCR amplifications were carried out under the following conditions: 94ºC of four min, 30 cycles of

94ºC for 60 secs, 60ºC for 90 secs, 72ºC for 90 secs, a final extension for four min at 72ºC and held at 4ºC. The PCR products were run on a 1% agarose gel and the bands were excised and the DNA was isolated using the QIAquick Gel extraction kit (Qiagen, Valencia, CA). The isolated DNA was then digested with *Sac*I and *Hind*III according to the manufacturer's directions for three hrs (New England Biolabs Inc.).

The pSPUD14 vector containing the *COR15a* promoter was digested with *Hind*III (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's directions for three hrs then heat inactivated at 65°C for 20 min. The digested DNA was run on a 1% agarose gel to confirm the digestion and to isolate the sample using the QIAquick Gel extraction kit (Qiagen). The isolated *COR15a* gene and either the *ScCBF1* or *StCBF1* genes were ligated together with T4 ligase (New England Biolabs Inc.). The ligation was run on a 1% agarose gel and the band that was at the appropriate size of approximately 1700 bp was excised and isolated using the QIAquick Gel extraction kit (Qiagen).

The pSPUD77 vector (a pBINPLUS plasmid with a pBI121 based terminator) was digested with *Hind*III (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's directions for three hrs then heat inactivated at 65°C for 20 min. Four microliters of purified *COR15a* ligated to the respective *CBF1* gene and one μL of the digested pSPUD77 were ligated with T4 ligase (New England Biolabs Inc.). The reaction was incubated at room temp. for 12 min, and then transformed into *E. coli* DH5α Competent Cells (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. After the cells had been plated out on LB Kan (50 μg/mL) plates and colonies had been selected, the plasmids were isolated and purified with the Wizard *Plus* SV Miniprep kit (Promega, Madison, WI) according to the manufacturer's directions.

The plasmids pSPUD89 and pSPUD90 were transformed into *Agrobacterium tumefaciens* GV3101 (Holsters *et al.*, 1980) by collecting 50 µL of *Agrobacterium* cells growing on a LB Kan plate and added the cells to one mL of ddH_2O. The cells were vortexed briefly and centrifuged at maximum speed for one min. The supernatant was discarded and the cells were washed with one mL of ddH_2O, mixed by pipetting and centrifuged at maximum speed for one min. The wash step was repeated two more times. The supernatant was discarded leaving 100 µL of cells and water. One microliter of purified plasmid was added to the cells and mixed by gently pipetting up and down. The cells and plasmid mixture were transferred to a chilled cuvette and were electroporated using the Ec2 setting (2.5 kV/cm) on the MicroPulser Electroporator (Bio-Rad Laboratories, Hercules, CA). One milliliter of LB liquid was added to the cuvette and the mixture was transferred to a 1.5 mL Eppendorf tube. The tube was incubated at 30°C with shaking for one hr and then aliquots of the liquid were plated out on TY Kan plates (5 g/L Bacto-tryptone, 3 g/L Yeast extract, 0.5 g/L $CaCL_2$-$2H_2O$, 15 g/L Agar, 50 µm/mL Kanamycin). The plates were incubated at 30°C for 48 hrs.

Transformation

Transgenic *Sc/StCBF1* potato lines were generated using an *Agrobacterium* mediated transformation protocol adapted from (Cearley and Bolyard, 1997). Using a single *Agrobacterium* colony of either pSPUD89 or pSPUD90 to inoculate three milliliters of TY media with 50 µm/mL Kanamycin (TY+Kan). The culture was grown overnight at room temp. with 200 rpm shaking. The culture was then transferred to 50 mL of TY+Kan and grown overnight at room temp with 200 rpm shaking. The next day stem internodes of the Michigan

State University breeding line MSE149-5Y and the cultivar Desiree were obtained from material maintained in tissue culture as described in Chapter 2. The stems were cut into 0.5 – 1.0 cm explant pieces, and placed in liquid MS medium (4.3 g/L MS Basal Salts, 20 g/L sucrose).

The *Agrobacterium* cultures were spun down for 15 min. at 3000 rpm. The supernatant was discarded and the pellet was re-suspended in 25 mL of MS media. The absorbance of the culture was measured at 600 nm and the culture was diluted to obtain an OD between 0.6 and 0.8. The stem explants were placed in the diluted *Agrobacterium* cultures and incubated for 20 min at room temp. The *Agrobacterium* culture was discarded and the explants were placed on ZIG media plates (4.3 g/L MS Basal Salts, 20 g/L sucrose, 6 g/L Agar, 9.1 μM zeatin riboside, 0.057 μM, IAA, 0.577 μM GA_3, pH 5.7). The plates were sealed with parafilm and incubated at 22°C with a 16/8 hr photoperiod (60-80 $uEm^{-2}s^{-1}$) under four layers of cheesecloth. After four days of co-culture, the explants were washed in sterile dH_2O with Timentin (300 μg/mL) then placed on ZIG plus antibiotic plates (ZIG+Ab; see ZIG media above with the addition of 300 μg/mL Timentin and 50 μg/mL of Kanamycin). The plates were sealed with parafilm and incubated under the same conditions above with the cheesecloth. Every 10-16 days the explants were moved to new ZIG+Ab plates until shoots appeared. After the appearance of shoots the cheesecloth was removed. When the shoots were approximately two centimeters long, they were cut above the callus and placed on rooting media (4.3 g/L MS salts, 30 g/L sucrose, 1.4 mM sodium phosphate, 1.1 μM thiamine, 0.55 mM myo-inositol, pH 6.0, 8 g/L Agar, 50 μg/mL Kanamycin). After rooting was obtained the plantlets were maintained in tissue culture in the general MS prop media without antibiotics.

Molecular Characterization

For all of the transformed lines, DNA was isolated from fresh leaf tissue using the DNeasy Plant kit (Qiagen) following the manufacturer's directions. For each line approximately 200 mg of fresh leaf tissue was frozen in liquid nitrogen and ground to fine powder using a mortar and pestle. In the final step, the DNA was eluted using 50 µL of the provided Buffer AE. PCR was used to confirm the presence of the transgene in each of the lines. The forward primer 5'-AGC TGA GAA AGC TGC GGC GT-3' and the reverse primer 5'-CAG CTG GCC TTT TTG GGT TAT TCG A-3' were used for the lines transformed with pSPUD89. The forward primer 5'-GCC GCT GAG GCT GCC GAA AT-3' and the reverse primer 5'-GCG GCC GCT GAA AAC GCA T-3' were used for the lines transformed with pSPUD90. Each 50 µL PCR amplifications contained: 10 µL 5X Green GoTaq® Reaction Buffer, 1 µL dNTP mix 10mM, 0.25 µL GoTaq® DNA Polymerase (Promega), 1 µL of each primer 10mM, and 34.75 µL of nuclease free water. The PCRs were carried out under the following conditions: 94ºC of four min, 30 cycles of 94ºC for 60 secs, 60ºC for 90 secs, 72ºC for 90 secs, a final extension for four min at 72ºC and held at 4ºC. The PCR products were run on a 1% agarose gel using ten µL of the PCR product.

Osmotic *in vitro* Assay

To study the efficacy of the transgene's ability to improve the plants ability to tolerate osmotic stress, increasing concentrations of agar were used in media for an *in vitro* assay as described by Gopal *et al.* (2008). Three concentrations of agar were used: six, eight, and ten g/L. The media was made up also containing 4.3 g/L MS salts and 30 g/L sucrose with a pH of 5.7. Ten milliliters of media were poured into 25 x 150 mm glass culture tubes. A node from tissue

culture grown plantlets of each transgenic line was placed in each of the three concentrations of agar with three replications for each line, for a total of nine nodes from each line. MSE149-5Y and Desiree nodes were also included as wild type controls. The cultures were incubated at 25°C with a 16/8 hr photoperiod (60-80 $uEm^{-2}s^{-1}$) for 25 days. Over those 25 days the cultures were observed for roots that penetrated the media (days to rooting). A line was declared to have rooting when a root could be seen by the naked eye, and the root was penetrating the media. On the 25^{th} day the plantlets were gently removed from the media and the shoot height and root lengths were recorded. Leaf tissue samples were also collected and immediately frozen in liquid nitrogen.

Total RNA was isolated from the leaf tissue samples using the Spectrum Plant Total RNA kit (Sigma-Aldrich Corp., St. Louis, MO) following the manufacturer's directions for protocol A. The RNA was then DNase treated using 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 as described in Chapter 2. Eight microliters of cDNA were then used as template in 50 µL PCR reactions as described above. The primers for *ScSBF1* (pSPUD89) and *StCBF1* (pSPUD90) listed above were used and primers for 18S (Table 2.3) were used as the control. Ten microliters of each reaction product was run on a 1% agarose gel.

Results

pSPUD89 and pSPUD90 Transformations

The *Agrobacterium*-mediated transformation using the GV3101 stain was effective in producing transgenic potato lines with either of the *Sc/StCBF1* genes. Six shoots were collected from 50 MSE149-5Y explants transformed with pSPUD89. Four of these shoots rooted in the 50 µg/mL Kanamycin rooting media. Sixteen shoots were collected from 50 MSE149-5Y explants transformed with pSPUD90; eight of these shoots rooted. All of the putative transgenic plants in the MSE149-5Y background were denoted as E89 or E90 followed by shoot number. For some lines, a letter denoting that the shoot was collected from the same explant but a different callus piece follows the shoot line. Sixty-two shoots were collected from 100 Desiree explants transformed with pSPUD89; 26 of these shoots rooted. Fifty-two shoots were collected from 100 Desiree explants transformed with pSPUD90; eighteen of these shoots rooted. All of the putative transgenic plants in the Desiree background were denoted as Des89 or Des90 followed by the shoot number and sometimes letter. All of the putative lines that were carried through appeared phenotypically normal in the culture tubes.

For the E89 lines, all four were PCR positive for DNA amplification of the 353 bp *ScCBF1* fragment. One line was later lost due to contamination. There were five PCR positive lines for DNA amplification of the 345 bp *StCBF1* fragment out of the E90 lines (63%). There were eleven PCR positive lines out of the Des89 lines (42%) and nine PCR positive out of the Des90 lines (50%) (Table 3.1).

Table 3.1 PCR positive transformed lines for pSPUD89 and pSPUD90.

MSE149-5Y PCR positive transformed lines		**Desiree PCR positive transformed lines**	
E89.2B	E90.8	Des89.1	Des90.1A
E89.2D	E90.9	Des89.5	Des90.3B
E89.2E	E90.14	Des89.18	Des90.8
	E90.15	Des89.27	Des90.11
		Des89.29	Des90.15
		Des89.32	Des90.18
		Des89.37	Des90.20
		Des89.43	Des90.24
		Des89.45	
		Des89.47	
		Des89.48	

Osmotic *in vitro* Assay

To study the transgene's ability to confer stress tolerance in the transformed lines, an *in vitro* osmotic assay was employed. In this assay three concentrations of agar were used: 6, 8 and 10 g/L. Typically 6 g/L are used in tissue culture and the increased concentrations of agar created increased levels of osmotic stress for the plantlets. All of the transgenic lines were tested at each of the agar concentration levels. The effect of the agar concentrations on each of the transgenic lines varied widely. This first parameter that was measured was days to rooting, or how long it took each plant to develop roots that penetrated the media. In the days to rooting analysis only Des89.32, Des89.45, E89.2B, Des90.24 and the WT control Desiree took longer to root as the concentration of agar increased (Figure 3.2). E90.14 and E90.15 rooted faster in the 8 and 10 g/L concentrations than the 6 g/L concentration. For all of the remaining lines there was no significant difference between the concentrations in the number of days it took for the lines to root. Within the 6 g/L Des89.1, Des89.37, Des90.1A and Des90.11 took significantly more days to root than the WT control Desiree. Only E89.2E took significantly more days to root than the

WT control E149-5Y. None of the remaining lines took significantly less days to root than the WT controls. Within the 8 g/L, Des90.15 took longer to root than Desiree, and E90.9 took fewer days to root than E149-5Y. None of the other lines were significantly different from the WT controls. Within the 10 g/L, only E89.2B took longer to root than E149-5Y. None of the other lines were significantly different from the WT controls.

The root lengths of Des89.32, Des89.47, Des89.48, E89.2B, E89.2D, and E90.9 significantly decreased as the agar concentrations increased (Figure 3.3). The root lengths of Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20, E90.14 and E90.15 significantly increased as the agar concentration increased. For all of the remaining lines there was no significant difference between the concentrations for root lengths. Within the 6 g/L, all of the Desiree transgenic lines, except Des89.27, had significantly shorter roots than Desiree. None of the E149-5Y transgenic lines had either significantly shorter or longer roots than the WT control. Within the 8 g/L, only Des89.45, Des90.8, and Des90.15 had roots that were not significantly shorter than the WT control; all of the other lines had significantly shorter roots. Within the 10 g/L, only Des89.18, Des89.27, Des90.20, and Des90.3B had roots that were not significantly shorter than the WT control. None of the E149-5Y transgenic lines were significantly different from the WT control.

The shoot heights of Des89.32, Des89.47, Des89.48, E89.2B, E89.2D, and E90.9 significantly decreased as the agar concentration increased (Figure 3.4). The shoot heights of Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20, Des90.24, E90.14 and E90.15 significantly increased as the agar concentrations increased. For all of the remaining lines there was no significant difference between the concentrations for shoot heights. Within the 6 g/L only Des90.3B and Des89.43 had significantly shorter shoots than the WT control. None of the other

lines were significantly different than the WT controls. Within the 8 g/L only Des90.18 was not significantly shorter than the WT control, and only E89.2D's shoots were significantly shorter than the WT control. None of the other lines were significantly different from the WT controls. Within the 10 g/L only Des89.18 and Des89.20 were not significantly shorter than Desiree. E89.2B, E89.2D and E90.9 were significantly shorter than E149-5Y. None of the other lines were significantly different from the WT controls.

Reverse transcriptase PCR was performed on the RNA that was isolated from all of the leaf tissue samples that were collected from each line at each agar concentration. The primers for either the *Sc* or *StCBF1* genes were used. Only E89.2B and Des90.20 from the 10 g/L concentration were positive for gene expression (Figure 3.5).

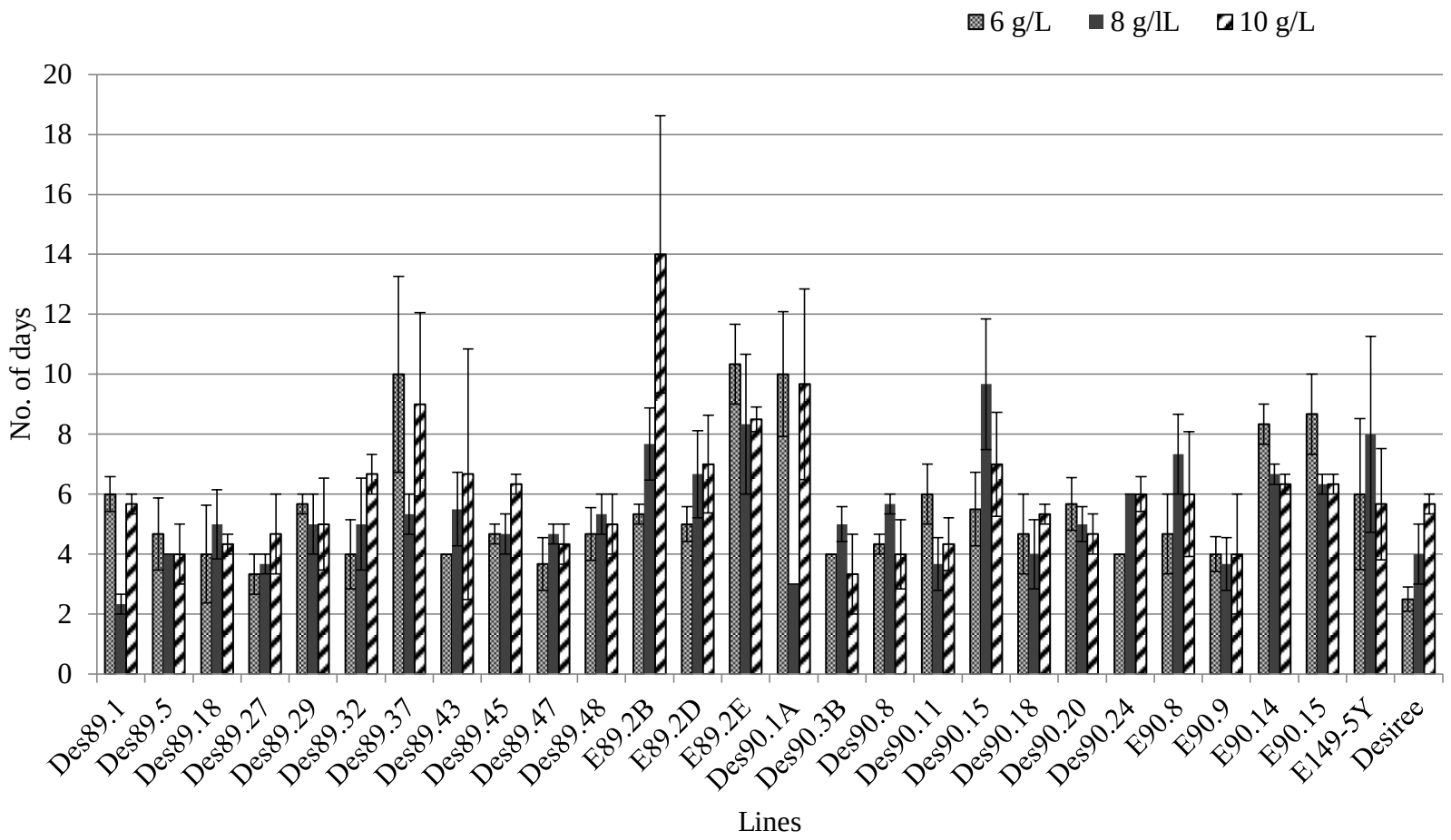


Figure 3.2 Days to rooting of transgenic lines in osmotic *in vitro* assay. Each of the *Sc/StCBF1* transgenic lines were placed in standard MS media with 3 different concentrations of agar: 6, 8 and 10 g/L. There were three replications of each line, for each concentration, and the means and standard errors were calculated for each line. For the majority of the lines there was no significant difference between the agar concentrations. Des89.32, Des89.45, E89.2B, Des90.24 and the WT control Desiree took longer to root as the concentration of agar increased and E90.14 and E90.15 rooted faster in the 8 and 10 g/L than in the 6 g/L concentration.

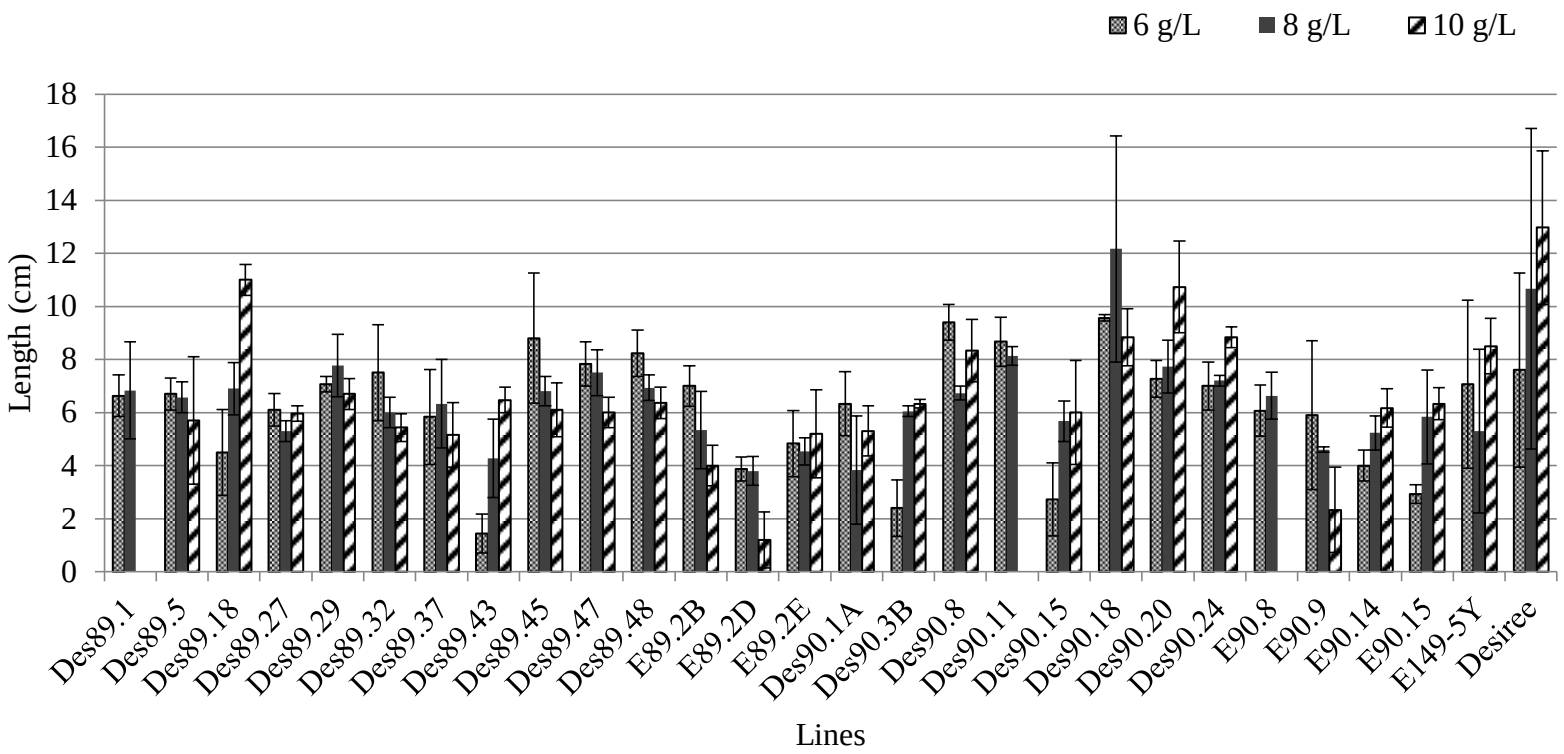


Figure 3.3 Root Length of transgenic lines in osmotic *in vitro* assay. For the majority of the lines there was no significant difference between the agar concentrations. Des89.32, Des89.47, Des89.48, E89.2B, E89.2D, and E90.9's root lengths significantly decreased as the agar concentrations increased. Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20, E90.14 and E90.15's root lengths significantly increased as the agar concentration increased.

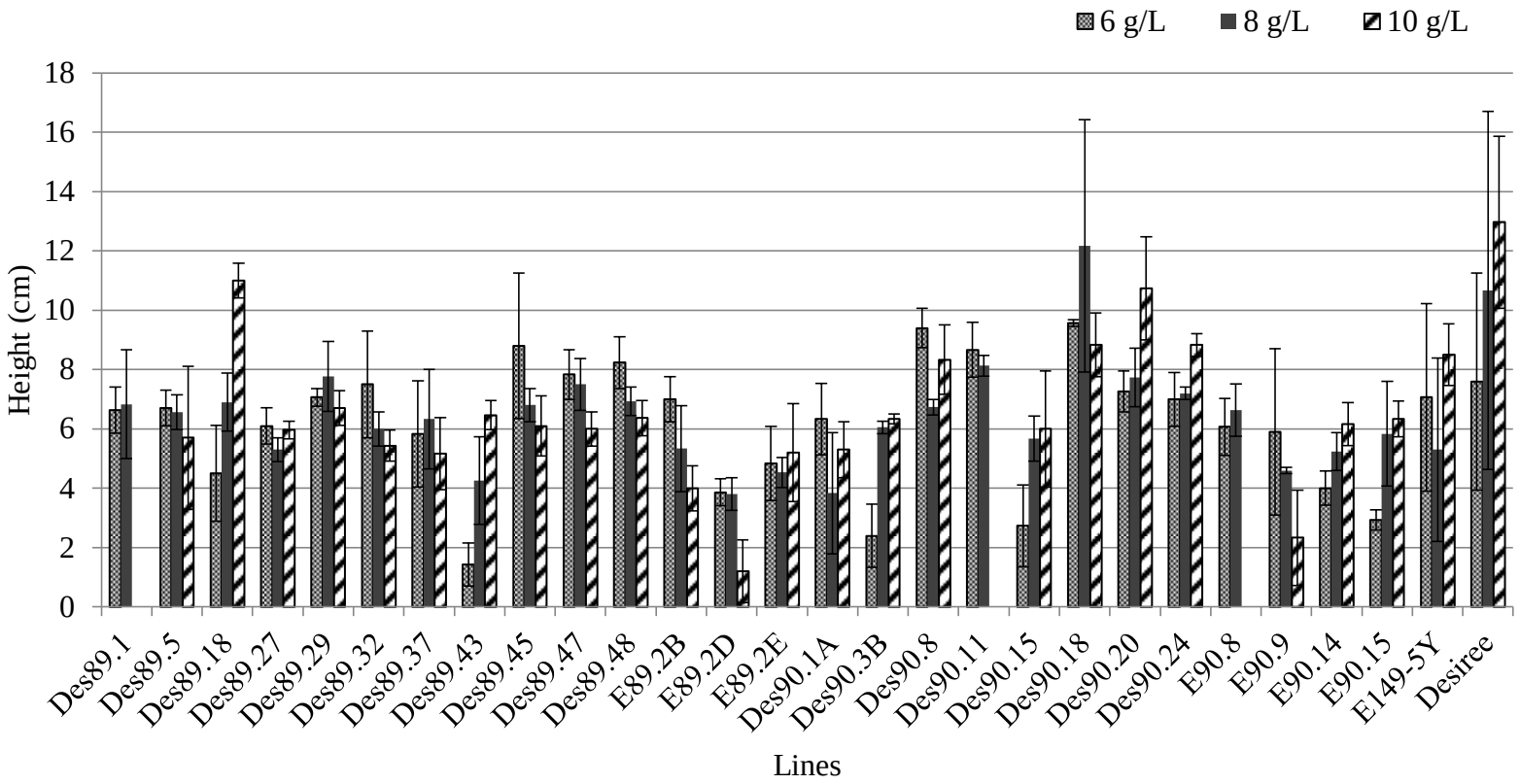


Figure 3.4 Shoot heights of transgenic lines in osmotic *in vitro* assay. For the majority of the lines there was no significant difference between the agar concentrations. Des89.32, Des89.47, Des89.48, E89.2B, E89.2D, and E90.9 had significantly shorter shoots as the agar concentration increased. Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20, Des90.24, E90.14 and E90.15 had significantly taller shoots as the agar concentrations increased.

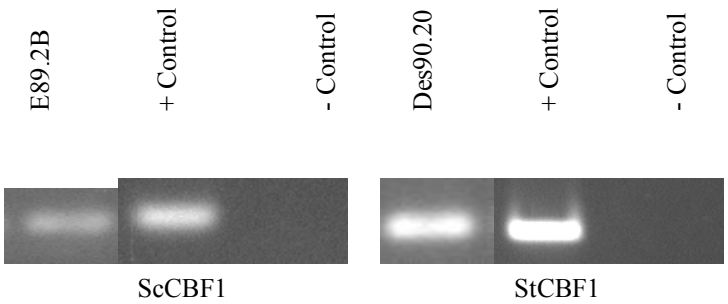


Figure 3.5 RT-PCR of *Sc/StCBF1* transgenes from osmotic *in vitro* assay. RNA was isolated from leaf tissue from all of the lines at each of the agar concentrations. Only E89.2B and Des90.2 at the 10 g/L concentration were positive for expression of their respective transgenes.

Discussion

In total there were nine PCR positive lines for the transgene in the MSE149-5Y background and 20 in the Desiree background. One of the projects that emerged during this research was optimizing the *Agrobacterium*-mediated transformation of *S. tuberosum*. Throughout this process the protocols for transformation and a regeneration system were optimized using the protocol that was adapted from Cearley and Bolyard (1997) and using the GV3101 *Agrobacterium tumefasciences* strain. These changes lead to increased transformation efficiency and regeneration of the cv. Desiree, and slightly increased the transformation and regeneration of cv. MSE149-5Y.

The osmotic *in vitro* assay that was adopted from (Gopal *et al*., 2008) was used to test the efficacy of the transgene in the transgenic lines. Des89.32 and E89.2B consistently performed poorly in the three parameters of number of days to rooting, root length, and shoot height. Des89.47, Des89.48, E89.2D, and E90.9 also performed poorly in both the root length and shoot height measurements. E90.14 and E90.15 consistently outperformed the WT and other lines in all three parameters. Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20 also outperformed the

WT and other lines in the root length and shoot height measurements. This assay is a crude tool to evaluate lines, but Des89.32 and E89.2B could easily be removed from further studies if space were an issue. Interestingly, E89.2B was one of the only two lines that had a positive result in the RT-PCR. It may be that the high expression of the transgene in this plant is having negative effects on its growth and development. The best candidate lines that stand out from this assay are: Des89.18, Des89.43, Des90.3B, Des90.15, Des90.20, Des90.24, E90.14 and E90.15. These lines were able to increase their root lengths and shoot heights as the agar concentrations increased. Des90.20 was the other line that was positive for the transgene expression and this line did well in the root and shoot measurements.

The issue with using the results of the root and shoot measurements is that it cannot be determined if the transgene was responsible for increasing the root length and shoot height, or if the increased root length caused the shoot height to increase. The only line that one can make a weak conclusion is Des90.20 where there was transgene expression present, as measured by RT-PCR. The expression of the transgene may be conferring tolerance to the osmotic stress and allowing the plant to grow well under such conditions. Further analysis of all the lines in osmotic stress studies in growth chambers and in the field would help to validate the results of this *in vitro* study.

It is hypothesized that using either the *S. commersonii* or the native *S. tuberosum* CBF1 genes will confer more abiotic stress tolerance rather than using the *AtCBF1* gene in cultivated potatoes. Based on this preliminary data it is too early to tell if there are some potential candidate lines that may outperform the E74.8 and E74.9 lines that have been previously studied. There are, however, many other candidate genes out there that have been identified in both

potatoes and other plants that may hold more promise in bringing increased abiotic stress tolerance.

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Chapter 4

A Study of the Effect of Information on College Students' Attitudes Towards Genetically Modified Potatoes.

Introduction

The social and scientific discussions on genetically modified (GM) foods and the public's opinion of them has been on going for decades. In Europe these discussions have led to tighter regulations and an overall negative public opinion of GM crops that reached a high in the 1990's (Gaskell *et al.*, 2004). In the United States there is no regulation on the labeling of GM foods like there is in Europe, but there are some U.S. consumer groups that are calling for a change. If the labeling requirements were to change, what kind of information would benefit the consumers the most and how would that affect their buying practices? In the present study, college students were given a questionnaire to study the effects of information on their attitudes towards genetically modified potatoes.

Literature Review

Public Perceptions and Attitudes

In 2004, Hallman *et al.* found that in general American consumers are unaware of GM food and the topics related to human health. The respondents expressed a desire for GM foods to be labeled, however, they admit to having never sought after information on GM foods (Hallman *et al.*, 2004). They also found that Americans have little knowledge of the general facts of genetic engineering technology. The respondents were in general split on their approval of GM

crops. Another group found that with a telephone survey in 2002, 34% of Americans were against GM food (Ganiere, 2006).

Onyango (2004a) studied consumers' willingness to consume GM foods. He found that males, Caucasians, Southerners, and those with some college education were more willing to consume GM foods. When the consumers were informed of the alleged risks associated with GM food, their willingness to consume GM food significantly decreased. A related study found that minorities and women were less approving of GM technology (Pudurl, 2005). They also found that those with increased formal education were more accepting of plant-based GM technology.

The consumers' willingness to pay for GM products with a direct consumer benefit was studied (Onyango, 2005). They found that consumers were more apt to choose products with direct health, environmental and production related benefits. They also found that consumers had more positive attitudes towards products that were plant-based instead of animal or bacterial based. Another study found that consumers were more likely to choose nutritionally enhanced food products if they had been developed through plant-plant methods as opposed to animal-plant methods (Onyango, 2004b).

Tegene *et al.* (2003) studied the willingness of consumers to pay based on food labels. They had three products that they used: vegetable oil, tortilla chips, and potatoes. Participants also were given information packets that included either the industry, environmental (Greenpeace) or independent 3rd party perspectives. All of the information packets had points on scientific impact, human impact, financial impact and environmental impact. They found that negative information had more of an impact than positive information, thus the consumers were

not willing to pay higher prices when they received more negative information. The lowest bids overall were placed on GM potatoes.

Information Treatments

Developing effective communication strategies to inform the public on genetic engineering of food is critical for successful commercialization of the technology. Qin and Brown (2007) used two different information formats to explore the public's understanding of, interest in, and attitudes towards genetically modified salmon. They found that those that read the "consequences" format learned more, were more interested, and expressed a higher level of confidence in judgment than those that read the "perspective" format. They also found that women were less approving of GM salmon than men. Through their pre and post measurements they found that reading the information (either format) led to a positive change in attitude toward the GM salmon.

Scholderer and Frewer (2003) used technology driven information formats to study changes in consumer's attitude towards GM foods in four European countries. Compared to the control group that received no information, the treatment groups' attitudes all decreased in their preference for GM foods. They concluded that the technology driven information strategy is not what is needed to convince consumers of the benefits to GM foods. Another study by Frewer *et al.* (2003) studied the trust consumers have when information is provided by different sources regarding GM food. Again they were in four European countries and provided the participants with either product specific information or balanced/general information on genetic engineering in food production. The participants were asked to evaluate beer or yogurt. There was very little difference in the attitudes as it correlates to the type of information or the source of the

information. The participants' trust depended on their pre-measured attitudes of GM foods. Huffman *et al*. (2007) studied the effects of prior beliefs and learning on the consumers' acceptance of GM foods. They found that uninformed participants were more susceptible to information from interested and third parties. Informed participants were not significantly affected by new information.

A study of Turkish high school and university students' attitudes towards biotechnology was recently conducted (Usak *et al.*, 2009). They found that there was a strong statistical correlation between the level of biotechnology knowledge and the sub dimensions of attitudes towards biotechnology. They found no differences in attitudes between genders. Attitudes towards purchasing GM foods were negative, despite an appreciation of agricultural biotechnology.

GM Potatoes and Acrylamide

In 1995 Monsanto released a new potato variety that had been genetically modified to contain the *Bt cry3a* gene, making the potato resistant to an important potato pest – the Colorado Potato Beetle (Thornton, 2003). The potatoes were marketed under the brand *NewLeaf*® and over the course of several years the potatoes went under several other modifications to make them resistant to two potato viruses. Initially growers quickly adapted the new technology and were pleased to reduce their insecticide costs. The difficulties that can come with the new technology, however, became apparent in the potato processing industry. Potatoes were one of the first genetically modified (GM) foods that still looked like it came from potatoes when consumers saw it on their plate. Because of this, industry wanted to make sure that the GM potatoes remained separate for the non-GM throughout the processing chain. This division in the

processing plants was costing the processors more than they intended, and they thus passed this cost on to the growers (Thornton, 2003). By 1999 the percent of potato acreage that was planted as *NewLeaf*® significantly decreased, and by 2001 Monsanto shut down their potato biotechnology program. Since then, there have not been any genetically modified potatoes released for production and consumption in the U.S.

Acrylamide is a chemical compound that is both used in the chemical industry and can be naturally formed in some foods when cooked. In 2002, Swedish scientists found that acrylamide could naturally form in foods with high carbohydrate levels when they were baked at high temperatures (Vinci *et al.*, 2011). It was later found that acrylamide levels form at significant levels when the amino acid asparagine is present with reducing sugars from carbohydrates and the food is heated to a high temperature. Elevated levels of acrylamide have been found in coffee, baked goods, roasted and processed potatoes. Acrylamide has been shown to be a neurotoxin and carcinogenic compound in rodents, and is thus a possible human carcinogen (Friedman *et al.*, 1995). In 2005, the World Health Organization (WHO) along with the Food and Agriculture Organization of the United Nations (FAO) recommended that the carcinogenicity of acrylamide in humans continue to be studied and that appropriate efforts to reduce acrylamide concentrations in food should continue (WHO/FAO, 2005).

Genetically modifying cultivated potatoes is one approach scientists have taken. In 2008 a group of scientists reported altering the asparagine levels in potatoes by incorporating tuber-specific silencing of two genes in the asparagine biosynthesis pathway (Rommens *et al.*, 2008). This transgenic method was also novel in that it employed all-native (only potato) genes in the genetic modification. They concluded that if these low-asparagine potatoes replaced the conventional potatoes, people could reduce their acrylamide consumption by 30%. In 2010, the

same group reported another method to reduce over all acrylamide levels by silencing another gene that reduces the levels of reducing sugars in the tubers during cold storage (Ye *et al.*, 2010).

The purpose of this study is to investigate the effect of information on college students' attitudes towards a genetically modified food. The low-acrylamide potatoes were used as the example of a genetically modified crop that provides a direct benefit to the consumer. In the treatment group, the students are provided with information on the new findings of acrylamide's potential harmful effects. The students are also provided with some information on GM crops in general, and on the specific low-acrylamide GM potatoes. The control group is not introduced to the acrylamide issue and is only provided with the same information on GM crops in general and some very basic information on genetically modified potatoes. It is hypothesized that the treatment group will have a more favorable attitude towards the GM potatoes knowing they provide a direct consumer benefit.

Methods and Materials

Questionnaire Design and Administration

Two versions of the questionnaire were developed and managed via surveymonkey.com. The control questionnaire was slightly shorter as it did not contain any reading or questions on acrylamide. The treatment questionnaire included information and questions on acrylamide and also contained more detail in the GM potato reading about low-acrylamide potatoes. The treatment group was also asked of their likelihood of seeking out more information on acrylamide and GM potatoes.

Participants were recruited from Integrative Studies of Social Sciences classes at Michigan State University in the spring of 2011. The students were asked to voluntarily respond

to the questionnaire that would be sent to them via email. It was explained that the questionnaire would be completely anonymous and the outcome would have no effect on their grade. In order to randomly divide up the respondents between the two versions of the questionnaire the students were instructed in the email to click on the first link if the last digit of their phone number was between 0-4 and to click on the second link if the last digit was between 5-9.

Measurements

The majority of the questions used a 5-point Likert scale with 3 being neutral. Some questions only needed a yes or no response, while a few others only used four levels. The demographic questions were used to gather typical demographic data in addition to some more detailed data on the student's educational background. The full questionnaires can be found in Appendix A.

Pre and Post Measurements

For the pre-measurements respondents were asked about their potato consumption, both fresh and processed potatoes. They were asked if they have heard of genetically modified (GM) crops, and to self rate their knowledge of GM crops. The treatment group was also asked if they have heard of acrylamide and to self rate their knowledge of acrylamide. Each section contained a short reading passage and the respondents were asked to rate the difficulty and trustworthiness of the reading. Only the treatment group was asked the post measurements of their likelihood of seeking out more information on acrylamide and GM potatoes.

Data Analysis

SAS 9.2v was used to analyze the results (Cary, NC). The PROC GLM program with the control and treatment groups as a class was used to compare the two groups for each of the questions. To explore the many effects of each of the sub-classes, t-tests using the least significant difference (LSD) at $\alpha = 0.05$ were used for each question.

Results

Respondents

A total of 113 completed the survey for the treatment group and 130 for the control group. Similar percentages of males and females responded to both the treatment (30.4% male, 69.6% female) and control (34.6% male, 65.4% female) questionnaires, and there was no significant difference between the two groups. In both groups the majority of the respondents (86.7% treatment, 82.3% control) were 20 years old or younger. This younger age is also reflected in the majority of respondents being of either freshman or sophomore standing. There was no significant difference in the distribution of GPA between the two groups. There was also no significant difference between the two groups in the racial/ethnic distribution (Table 4.1). There was a higher percentage of Asians that responded to the questionnaire than the percentage at the University level.

Table 4.1 Distribution of race/ethnicity between the treatment and control groups, and the total student population at Michigan State University.

Responses	Treatment group	Control group	MSU
American Indian/Alaskan Native	2.7%	1.6%	0.4%
Asian	8.8%	11.6%	4.3%
Black or African American	5.3%	6.2%	6.5%
Chicano	0.9%	0.8%	No data
Hawaiian/Pacific Islander	2.7%	0%	0.1%
Hispanic	2.7%	3.9%	3.3%
White/Caucasian	82.3%	77.5%	69.5%
Other	3.5%	6.2%	3.6%

Effect of Information: Treatment versus Control

There was no significant difference between the two groups on whether they have heard of genetically modified (GM) crops and their self rated knowledge of GM crops. Both groups had mostly heard of GM crops (Yes: 93% treatment, 85.3% control) and said they were "knowledgeable." The control group did differ in their ratings of the difficulty and trustworthiness of the reading passage. The control group found the GM crops reading slightly more difficult and less trustworthy than the treatment group. Both groups "approved" of GM crops for human consumption. The control group said GM foods were slightly more risky than the treatment group. Both groups were "willing" to consume foods with GM ingredients and were "confident" in their understanding of how GM crops are tested and regulated.

For the GM potato portion of the questionnaire there were significant differences in the content of the reading passages. The control group had a very short reading:

> "Each year the average American consumes 126 pounds of potatoes. Currently in the U.S., none of the potatoes grown for human consumption are genetically modified. However, scientists are currently developing GM potatoes."

The treatment group had a longer reading that contained more content on acrylamide as they had been previously read a passage and answered a few questions on acrylamide:

> **"**Each year the average American consumes 126 pounds of potatoes. Currently in the U.S., none of the potatoes grown for human consumption are genetically modified. In response to the findings of elevated levels of acrylamide in cooked potatoes, scientists have developed a GM potato that results in significantly lower levels of acrylamide when cooked. This GM potato was modified by moving two potato genes into an area of the potato genome where they are able to "turn down" the genes responsible for making the amino acid asparagine (the amino acid that when present with starch produces acrylamide). The scientists state that if the GM Low-Acrylamide potatoes replaced conventional potato varieties, people could reduce their acrylamide consumption by 30%."

The treatment group did find their reading more difficult than the control group. Both groups found the reading trustworthy, but the treatment group found the GM potato reading slightly less trustworthy than the GM crops reading. Both groups were "confident" in their understanding of how the GM potatoes are developed; however, the control group was "neutral" in their understanding of the effects of GM potatoes on consumer's health. The control group was less approving of GM potatoes for human consumption and said GM potatoes are less safe compared to the treatment group. There was no difference between the groups as they were both "willing" to consume foods with GM potato ingredients, said it was "important" to be informed about GM potatoes, and "neutral" in their interest to receive more information on GM potatoes.

Male/Female Differences

The males had heard of GM crops less than the females, but both groups reported to be "knowledgeable" of GM crops. Both groups rated the difficulty and trustworthiness of the reading as "easy" and "trustworthy." The females, however, were less approving of GM crops for human consumption, less willing to consume foods with GM ingredients and less confident

in understanding how GM crops are tested and regulated (Table 4.2). Both males and females deemed the risk of GM food in terms of effects on human health "risky."

For the GM potato section there were no significant differences between males and females for all of the questions. The females had slightly increased their approval of GM potatoes from their approval of GM crops, and were slightly more willing to consume foods with GM potato ingredients.

Within the control group, males were more approving of GM crops, and were more willing to consume foods with GM and GM potato ingredients. There was no difference between the two groups on their rating of the risk of GM crops and GM potatoes. Within the treatment group there were no significant differences between males and females in their approval, assessment of risk or willingness to consume either GM crops or GM potatoes.

Race

Chicanos and Others have heard of GM crops less than Hawaiian/Pacific Islanders, Hispanics, and White/Caucasians. Chicanos are more willing to consume foods with GM ingredients than Others, American Indian/Alaskan Natives, Asians, and Black/African Americans. Hawaiian/Pacific Islanders were less confident in understanding how GM crops are tested and regulated than Others. American Indian/Alaskan Natives, Black/African Americans, and Hawaiian/Pacific Islanders are less willing to consume GM potatoes than Chicanos. Chicanos felt it was less important to be informed on GM L-A potatoes than Black/African Americans and Others. Chicanos were also the least interested in receiving more info than Asians, Black/African Americans, Hawaiian/Pacific Islanders, White/Caucasians and Others.

White-Males

Within the control group, white males and white females were more willing to consume foods with GM or GM potato ingredients than minority females. Within the treatment group, white males and white females were more approving of GM crops than minority males and females. Minority males said GM crops are more risky than minority females, white males and white females. White males and white females are more willing to consume foods with GM ingredients than their minority counterparts. White males were more approving of GM potatoes than minority males and minority females. White males said GM potatoes were less risky than minority males. White males were more willing to consume foods with GM potato ingredients than minority males.

Other Class Effects

Consumption of Potatoes

There was no significant difference in potato consumption between the control and treatment groups or between males and females. Within the control and treatment groups those that never eat baked or mashed potatoes said the risk of GM food and GM potatoes in terms of effects on human health was more risky than those that eat baked or mashed potatoes 2-4 times/week. Within the control group those that eat processed potatoes daily were more willing to consume foods with GM potato ingredients than those that only eat processed potatoes 2-4 times/year or never. Within the treatment group, those that eat processed potatoes daily were more approving of GM crops and were more willing to consume foods with GM ingredients those that eat them 2-4 times/year. Those that only eat processed potatoes 2-4 times/year said GM crops are more risky than those that eat them 2-4 times/week. Those that eat processed

potatoes 2-4 times per year were less approving of GM potatoes for human consumption, and were less willing to consume foods with GM potato ingredients than those that eat them daily, 2-4 times/week and 2-4 times/month.

Number of College Level Biology Courses Taken

There were no significant differences between the control and treatment groups or between males and females for the number of biology courses taken. Those that had no college biology courses declared themselves "less knowledgeable" about GM crops than those that have had 3 or more classes. Those that have had no college biology courses found the GM crops reading more difficult than those with 5 or more classes. Those that had 2 or less classes found the reading less trustworthy, and were less confident in understanding how GM potatoes are developed than those with 5 or more classes. Those with 5 or more classes said GM potatoes are safer than those with 4 or less classes (Table 4.3). Those with 5 or more classes said it was more important to them to be informed and were more interested in receiving more information than those with 2 or less classes. Within the treatment group, those than have taken 3 or more biology courses said the GM potatoes were less risky than those that have taken 2 or less biology courses.

Self-Rated Knowledge of GM Crops

There were no significant differences between the control and treatment groups or between males and females in their self-ratings on their knowledge of GM crops. Within the control group those that were "very knowledgeable" were more approving than those that were "knowledgeable," "not very knowledgeable" or "not at all" for approval of GM crops and GM potatoes for human consumption. Those that were very knowledgeable and knowledgeable were

more neutral on the risk of GM food in terms of effects on human health. Those that were very knowledgeable were also more neutral on the risk of GM potatoes. The two knowledgeable groups were also more willing to consume foods with GM ingredients than the less knowledgeable groups. The very knowledgeable were more willing to consume products with GM potato ingredients than those claimed to be not knowledgeable at all.

Within the treatment group, those that were not knowledgeable at all were less willing to consume products with GM ingredients than those that were knowledgeable. Those that were not knowledgeable at all were less approving of GM potatoes than those that were very knowledgeable. Those that were very knowledgeable said GM potatoes were less risky than those that were not knowledgeable. Those that were not knowledgeable at all were less willing to consume foods with GM potatoes ingredients than the other knowledgeable groups.

Table 4.2 Average of responses separated out by gender and race-gender.

	Male	**Female**	**White-Male**	**White-Female**	**Minority-Male**	**Minority-Female**
Approve GM crops	X		X	X		
Less Approval		X			X	X
GM crops risky	X	X			X	
GM crops safe			X	X		X
Willing to eat GM crops	X		X	X		
Less willing to eat		X			X	X
Approve GM potatoes	X	X	X	X		
Less approval					X	X
GM potatoes risky	X	X			X	X
GM potatoes safe			X	X		
Willing to eat GM potatoes	X	X	X	X		
Less willing to eat					X	X

Table 4.3 Average of responses separated out by self-declared knowledge of GM crops and number of college biology courses.

	Very Knowledgeable of GM crops	**Less Knowledgeable of GM crops**	**5 or more College Biology Courses**	**Less than 5 College Biology Courses**
Approve GM crops	X			
Less Approval		X		
GM crops risky		X		X
GM crops safe	X		X	
Willing to eat GM crops	X			
Less willing to eat		X		
Approve GM potatoes	X			
Less approval		X		
GM potatoes risky		X		X
GM potatoes safe	X		X	
Willing to eat GM potatoes	X			
Less willing to eat		X		

Discussion

The attempt to randomize the respondents between the control and treatment groups was successful as there were no significant differences between the two groups. Unlike Hallman *et al.*, (2004) the students in this study were all aware of GM crops at some level, and many reported that they were very/knowledgeable on the subject.

The effect of the information on the students' attitude was in general favorable towards genetically modified (GM) potatoes. These results contradict the results from studies using European participants whose attitudes towards GM foods became more negative when

information was provided (Frewer *et al.*, 2003; Frewer, 2003). In another study that looked at the consumers' prior beliefs and learning and their relation to acceptance of GM foods, they found that informed participants were not affected by the new information (Hallman *et al.*, 2004). In this study, most of the students claimed they were knowledgeable about GM crops, yet they were receptive to the new information. This may have been that the information provided about the low-acrylamide potatoes was much more novel than the GM crops that are on the market at this time, or that their interest in the possible health effect of acrylamide made them more receptive to the new information. Although the treatment group was more approving of GM potatoes, and said they were less risky than the control group; the two groups were similar in their willingness to consume GM potatoes. The students that have had five or more college level biology classes viewed the GM potatoes as safer and were more approving of them than their peers with less biology courses. They also, not surprisingly, had more confidence in understanding how the GM potatoes were developed. Corresponding to the Turkish student study, these students with a lot of biology education were no more willing to consume GM potatoes than all of their peers (Usak *et al.*, 2009).

One interesting difference early on between the control and the treatment groups was that they did differ in their ratings of the difficulty and trustworthiness of the GM crops reading passage. Both groups had the exact same GM crops reading passages, however, the treatment group had just read and answered questions about acrylamide. This may have affected the treatment group's responses to the reading.

Analogous to Onyango's findings; our study also showed that females and minorities were less approving of GM crops and less willing to consume foods with GM ingredients (Onyango, 2004a). The information treatment increased females' approval of GM potatoes and

willingness to consume, but had no significant effects on the males. It was investigated to see if this study also revealed a "white-male effect." There is a growing body of evidence that men worry less than women, and whites less than minorities on topics from environmental pollution to abortion (Bord and O'Connor, 1997; Wilcox, 1990). Kahan et al. (2007) supports the theory that the white-male effect is an artifact of variance in cultural worldviews. The white-males' insensitivity to risk is based on a defensive response to maintain the cultural identity of the risk insensitive white-male. Indeed it was found that white-males, along with white females, were less sensitive to the perceived risks of GM crops. In the treatment group, minority females were more influenced than minority males by the information and the minority females shifted closer in their responses to the white females.

Overall this study illustrates that providing information to potential consumers about a genetically modified food that provides a direct benefit to the consumer improves the consumers' approval of the food for human consumption. It also reduces the consumer's perception of the risks associated with the GM food. These results can give scientists, marketing groups and policy makers a platform on which to build their information formats they wish to provide to consumers regarding the next wave of genetically modified food crops.

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APPENDIX

Questionnaire

The sections in italics were only included in the treatment group's questionnaire.

1. How often do you eat mashed or baked potatoes?
   A. Daily
   B. 2-4 times/week
   C. 2-4 times/month
   D. 2-4 times/year
   E. Never

2. How often do you eat fried or processed potatoes (i.e. French fries, chips, hash-browns)?
   A. Daily
   B. 2-4 times/week
   C. 2-4 times/month
   D. 2-4 times/year
   E. Never

3. Have you heard of acrylamide?
   A. Yes
   B. No

4. How knowledgeable are you about acrylamide?
   A. Very knowledgeable
   B. Knowledgeable
   C. Not very knowledgeable
   D. Not at all

Acrylamide Reading Passage:
Acrylamide is a compound found in food that contains starch and the amino acid asparagine. It is formed when it is cooked under a high-temperature process (baking, frying, roasting). French fries, potato chips, breakfast cereal and coffee contain elevated levels of acrylamide. Acrylamide has the potential to cause cancer according to national and international food and health organizations. It is recommended by these national and international agencies that consumers avoid foods that have been over-cooked (i.e. dark brown fries, toast) to lower acrylamide consumption.*

*The Food and Drug Administration (FDA), Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO)

Acrylamide Questions:

1. For you, how difficult was it for you to read the passage?

A. Very easy
B. Easy
C. Okay
D. Somewhat difficult
E. Difficult

2. For you, how trustworthy was the reading?

A. Completely trustworthy
B. Somewhat trustworthy
C. Neutral
D. Somewhat untrustworthy
E. Completely untrustworthy

How confident are you in understanding:

3. Where acrylamide is found?

A. Very confident
B. Confident
C. Neutral
D. Somewhat unconfident
E. Not confident

4. How it is formed in food?

A. Very confident
B. Confident
C. Neutral
D. Somewhat unconfident
E. Not confident

5. Acrylamide's potential effects on human health?

A. Very confident
B. Confident
C. Neutral
D. Somewhat unconfident
E. Not confident

6. Overall, how important is it to you to be fully informed about acrylamide?

A. Very important
B. Important
C. Neutral
D. Slightly unimportant
E. Not important at all

7. How interested are you in receiving more information on acrylamide?
   A. Very interested
   B. Interested
   C. Neutral
   D. Slightly uninterested
   E. Not interested at all

Genetically Modified Crops Section:

1. Have you heard of genetically modified crops?
   A. Yes
   B. No

2. How knowledgeable are you about genetically modified crops?
   A. Very knowledgeable
   B. Knowledgeable
   C. Not very knowledgeable
   D. Not at all

GM Crops:

Genetically modified (GM) crops are produced by moving genetic material from one species to another species; for example a corn plant that is resistant to insect damage has been produced by incorporating genetic material from a bacteria into the corn's genetic material. In U.S. agriculture, food from some GM crops has been grown and consumed by animals and humans since the mid 1990's.

In the U.S., GM crops are tested, inspected and regulated by the United States Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). Before a GM crop can be approved for production and consumption, it must be tested for many aspects pertaining to human and animal health impact (i.e. allergies, toxins) and environmental impact.

GM Questions:

1. For you, how difficult was it for you to read the passage?
   A. Very easy
   B. Easy
   C. Okay
   D. Somewhat difficult
   E. Difficult

2. For you, how trustworthy was the reading?
   A. Completely trustworthy
   B. Somewhat trustworthy
   C. Neutral
   D. Somewhat untrustworthy
   E. Completely untrustworthy

3. Overall, how do you feel about the use of GM crops for human consumption?
   A. Strongly approve
   B. Approve
   C. Neutral
   D. Disapprove
   E. Strongly disapprove

4. How risky would you say GM foods are in terms of their effects on human health?
   A. Very risky
   B. Somewhat risky
   C. Neutral
   D. Safe
   E. Very Safe

5. How willing are you to consume foods produced with GM ingredients?
   A. Very willing
   B. Somewhat willing
   C. Neutral
   D. Slightly unwilling
   E. Not willing at all

6. How confident are you in understanding how GM crops are tested and regulated?
   A. Very confident
   B. Confident
   C. Neutral
   D. Somewhat unconfident
   E. Not confident

GM Low-Acrylamide Potato:

Each year the average American consumes 126 pounds of potatoes. Currently in the U.S., none of the potatoes grown for human consumption are genetically modified. In response to the findings of elevated levels of acrylamide in cooked potatoes, scientists have developed a GM potato that results in significantly lower levels of acrylamide when cooked. This GM potato was modified by moving two potato genes into an area of the potato genome where they are able to "turn down" the genes responsible for making the amino acid asparagine (the amino acid that when present with starch produces acrylamide). The scientists state that if the GM Low-Acrylamide potatoes replaced conventional potato varieties, people could reduce their acrylamide consumption by 30%.

GM Potato:

Each year the average American consumes 126 pounds of potatoes. Currently in the U.S., none of the potatoes grown for human consumption are genetically modified. However, scientists are currently developing GM potatoes.

GM Potato Questions:

1. For you, how difficult was it for you to read the passage?
   A. Very easy
   B. Easy
   C. Okay
   D. Somewhat difficult
   E. Difficult

2. For you, how trustworthy was the reading?
   A. Completely trustworthy
   B. Somewhat trustworthy
   C. Neutral
   D. Somewhat untrustworthy
   E. Completely untrustworthy

3. How confident are you in understanding how the GM *Low-Acrylamide* potatoes were developed?
   A. Very confident
   B. Confident
   C. Neutral
   D. Somewhat unconfident
   E. Not confident

4. How confident are you in understanding the potential effects of the GM *Low-Acrylamide* potatoes on consumer's health?
   A. Very confident
   B. Confident
   C. Neutral
   D. Somewhat unconfident
   E. Not confident

5. Overall, how do you feel about the use of GM *Low-Acrylamide* potatoes for human consumption?
   A. Strongly approve
   B. Approve
   C. Neutral
   D. Disapprove
   E. Strongly disapprove

6. How risky would you say GM *Low-Acrylamide* potatoes are in terms of their effects on human health?
   A. Very risky
   B. Somewhat risky
   C. Neutral
   D. Safe
   E. Very Safe

7. How willing are you to consume foods produced with GM *Low-Acrylamide* potato ingredients?
   A. Very willing
   B. Somewhat willing
   C. Neutral
   D. Slightly unwilling
   E. Not willing at all

8. Overall, how important is it to you to know about GM *Low-Acrylamide* potatoes?
   A. Very important
   B. Important
   C. Neutral
   D. Slightly unimportant
   E. Not important at all

9. How interested are you in receiving more information on GM *Low-Acrylamide* potatoes?
   A. Very interested
   B. Interested
   C. Neutral
   D. Slightly uninterested
   E. Not interested at all

How likely are you to seek out the following information regarding acrylamide and GM potatoes?

5. New scientific findings on the impact of acrylamide and GM potatoes on consumer's health:
   A. Very likely
   B. Likely
   C. Neutral
   D. Slightly unlikely
   E. Never

6. New scientific findings on acrylamide and GM potatoes and their impact on the environment:
   A. Very likely
   B. Likely
   C. Neutral
   D. Slightly unlikely
   E. Never

7. How GM potatoes are regulated:
   A. Very likely
   B. Likely
   C. Neutral
   D. Slightly unlikely
   E. Never

8. The process used to of modify the GM potatoes:
   A. Very likely
   B. Likely
   C. Neutral
   D. Slightly unlikely
   E. Never

Demographic data

1. Course in which you were asked to complete this survey

2. How many college level (NOT including AP) biological science classes have you taken (or are currently enrolled in)?
   A. 0
   B. 1-2
   C. 3-4
   D. 5 or more

3. Gender
   A. Female
   B. Male

4. Age:
   A. 20 or less
   B. 21-24
   C. 25 and above

5. Class Standing
   A. Freshman
   B. Sophomore
   C. Junior
   D. Senior
   E. Graduate or other

6. Current GPA
   A. 4.0
   B. 3.5 – 3.9
   C. 3.0 – 3.4
   D. 2.5 -2.9
   E. 2.4 or less

7. Race/ethnicity (check all that apply):
   A. Y. Hispanic
   B. Z. Chicano
   C. AA. American Indian/Alaskan Native

D. Asian
E. Black or African American
F. Hawaiian/Pacific Islander
G. White/Caucasian
H. Other

Table A.1 Demographic responses to GM Questionnaire.

Category	Responses	Treatment group	Control group
Gender	Male	30.4%	34.6%
	Female	69.6%	65.4%
Age	20 or younger	86.7%	82.3%
	21-24	10.6%	12.3%
	25 or older	2.7%	5.4%
Class Standing	Freshman	47.8%	54.6%
	Sophomore	35.4%	25.4%
	Junior	13.3%	16.9%
	Senior	3.5%	3.1%
Current GPA	4.0	5.3%	5.4%
	3.5-3.9	41.6%	36.9%
	3.0-3.4	31%	31.5%
	2.5-2.9	15.9%	22.3%
	2.4 or less	6.2%	3.8%
Race/ethnicity (check all that apply)	American Indian/Alaskan Native	2.7%	1.6%
	Asian	8.8%	11.6%
	Black or African American	5.3%	6.2%
	Chicano	0.9%	0.8%
	Hawaiian/Pacific Islander	2.7%	0%
	Hispanic	2.7%	3.9%
	White/Caucasian	82.3%	77.5%
	Other	3.5%	6.2%

Table A.2 Means of responses to GM Questionnaire for Treatment and Control Groups. Means compared by LSD, $\alpha = 0.05$, * denotes significant difference between the treatment and control

group.

Question	Scale	Means Treatment (n = 113)	Means Control (n = 130)
Mashed or baked potato consumption	1 = Daily	2.8636	3.0385
Fried or processed potato consumption	3= 2-4 times/month 5 = Never	2.5273	2.5116
Heard of acrylamide	1= Yes	1.9231	No Data
Knowledge of acrylamide	1 = Very knowledgeable 4 = Not at all	3.7787	
Acrylamide Reading Passage			
Difficulty of reading	1 = Very easy 5 = Difficult	1.8407	
Trustworthiness of reading	1 = Completely trustworthy 5 = Completely untrustworthy	2.0265	
Confidence in knowing:			No Data
Where acrylamide is found	1 = Very confident 5 = Not confident	2.4071	
How acrylamide is formed in food		2.4860	
Importance to be informed about acrylamide	1 = Very important 5 = Not important at all	2.6814	
Interested in receiving more information on acrylamide	1 = Very interested 5 = Not interested at all	3.1504	
Genetically Modified Crops Reading Passage			
Heard of GM crops	1 = Yes 2 = No	1.0714	1.1473
Knowledge of GM crops	1 = Very knowledgeable 4 = Not at all	2.4779	2.6589
Difficulty of reading	1 = Very easy 5 = Difficult	1.7788	2.0*
Trustworthiness of reading	1 = Completely trustworthy 5 = Completely untrustworthy	1.9823	2.2713*
Approval of GM crops for human consumption	1 = Strongly approve 5 = Strongly disapprove	2.6071	2.8268
Risk of GM food in terms of effects on human health	1 = Very risky 5 = Very safe	2.885*	2.6032
Willing to consume foods with GM ingredients	1 = Very willing 5 = Not willing at all	2.4336	2.5984

Table A.2 cont'd

Confidence in understanding how GM crops	1 = Very confident	2.8407	2.8898

are tested and regulated	5 = Not confident		
GM/Low-Acrylamide (L-A)[t] Potato Reading Passage [t] only the treatment group had "Low-Acrylamide" included in the reading and questions			
Difficulty of reading	1 = Very easy 5 = Difficult	1.8739*	1.3438
Trustworthiness of reading	1 = Completely trustworthy 5 = Completely untrustworthy	2.2054	2.1953
Confidence in understanding how GM L-A potatoes are developed	1 = Very confident 5 = Not confident	2.4867	2.7222
Confidence in understanding effects of GM L-A potatoes on consumer's health		2.6071	3.112*
Approval of GM L-A potatoes for human consumption	1 = Strongly approve 5 = Strongly disapprove	2.5133	2.9127*
Risk of GM L-A potatoes in terms of effects on human health	1 = Very risky 5 = Very safe	3.0991*	2.6032
Willing to consume foods with GM L-A potato ingredients	1 = Very willing 5 = Not willing at all	2.3874	2.624
Importance to be informed about GM L-A potatoes	1 = Very important 5 = Not important at all	2.8571	2.5984
Interested in receiving more information on GM L-A potatoes	1 = Very interested 5 = Not interested at all	3.1593	3.2344
Likeliness to seek out information on:			
New scientific findings on acrylamide and GM potatoes on consumer's health	1 = Very likely 3 = Neutral 5 = Never	3.2394	No Data
New scientific findings on acrylamide and GM potatoes impact on the environment		3.2035	
How GM potatoes are regulated		3.2566	
The process used to modify the GM potatoes		3.2297	