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Genetic engineering for dehydration-stress tolerance in cucumber (*Cucumis sativus* L.) by expressing the Arabidopsis thaliana-transcriptional regulators *CBF1* and *CBF3* and the *mannose-6-phosphate reductase* gene *M6PR* from celery (*Apium graveolens* L.)

presented by

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of the requirements for the

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ABSTRACT

Genetic engineering for dehydration-stress tolerance in cucumber (*Cucumis sativus* L.) by expressing the *Arabidopsis thaliana*-transcriptional regulators *CBF1* and *CBF3* and the mannose-6-phosphate reductase gene M6PR from celery (*Apium graveolens* L.)

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Salinity and drought conditions are major factors affecting plant productivity and distribution worldwide. To engineer resistance to dehydration stress in cucumber (*Cucumis sativus* L.), transgenic cucumber were generated with genes associated with enhanced abiotic stress tolerance: the mannose-6-phosphate reductase (*M6PR*) gene from celery for mannitol production, and the *CBF1/DREB1b* and *CBF3/DREB1a*, abiotic stress-associated transcriptional regulators from *Arabidopsis thaliana*. T_0 transgenic *M6PR* cucumbers produced detectable mannitol, indicating functionality of the M6PR gene in cucumber. However, mannitol accumulation in the T_1 progeny was highly variable making this trait-difficult to work with. Eleven lines of cucumber were produced with the *CBF* genes, integration and expression was verified in the T_0, T_1 and T_2 generation. Under greenhouse conditions, T_1 and T_2 *CBF*-cucumber plants accumulated elevated levels of proline and soluble sugars, a signature for *CBF* expression in *Arabidopsis*, indicating ability of the *CBF* gene to induce stress related responses in cucumber. Proline and soluble sugars accumulation were highly correlated, suggesting

coordinated regulation in the transgenic plants. In the absence of salt or drought stress, the *CBF* cucumbers showed equivalent growth compared to the nontransgenic controls. In the presence of salt and drought stress, transgenic plants had less reduction in growth. Plant performance and fruit production was evaluated under field conditions. Prior to salinity-stress, transgenic and nontransgenic cucumber lines grew equivalently. *CBF*-cucumber plants accumulated significantly higher levels of compatible solutes in leaves (proline and soluble sugars) and roots (proline) compared to the nontransgenic controls. Transgenic plants also had elevated levels of K^+ and Ca^{++} ions and a decreased Na^+/K^+ ratio in root tissues, suggesting a wider range of adaptive responses in the transgenic plants than has been reported previously. In the absence of salinity, *CBF* lines had less fresh weight than the nontransgenic controls; however, dry weight and fruit yield were equivalent to the nontransgenics. In the presence of salinity stress, *CBF*-transgenic plants showed significantly less reduction in fresh weight, dry weight, fruit number and fruit weight. These results suggest that expression of the *CBF/DREB* in cucumber, a species known for sensitivity to salinity and drought conditions, may offer an effective approach to enhance salinity and drought tolerance.

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Chapter I

Literature Review

Introduction

Environmental factors that impose water-deficits stress place a major limitation on plant productivity (Bray, 1994; Bohnert and Jensen, 1996). Water deficit is intrinsic to most abiotic forms of stress, such as drought, salinity and freezing temperatures (Bohnert and Jensen, 1996). Deleterious effects can be manifested as a reduction in transpiration and photosynthesis, a reduction in growth rate due to reduced cell enlargement, and reduction in the synthesis of metabolites and structural compounds (Zhang *et al.,* 1999). Cellular water deficit also disrupts membrane integrity, which causes loss of cellular water potential and denaturation of cellular proteins (Bray, 1997). To overcome these limitations and to improve production efficiency of plants, development of dehydration stress-tolerant crops is essential (Khush, 1999). In this literature review, I will summarize causes and effects of dehydration stresses in plants and efforts to develop dehydration stress tolerance in plants via conventional breeding and genetic engineering approaches.

I- Causes and effects of plant water stress

Water is the driving force of living organisms; it works as a medium for the biochemical activities in all living cells (Xiong and Zhu, 2002) and is involved in biosynthesis and assembly of molecules into organized structures (Tanford, 1978). In plant cells, water potential (Ψ_w), is responsible for generating the needed turgor pressure for cell expansion. The water potential of a given cell is composed of pressure potential (Ψ_P, which reflects the physical pressure generated by cell wall) and osmotic potential

(Ψ_S which is generated by the solute concentration inside the cell). Plant water deficit results from inability of plants to acquire their water needs, resulting in loss of turgor and/or osmotic stress. This could be due to the unavailability of water under drought and freezing temperatures, or the presence of highly negative osmotic pressure, due to high salt concentrations in the growing environment. Cellular water deficit, if prolonged, could be lethal to plant cells, interfering with basic metabolic pathways and changes in membrane shape and integrity (Bray, 1997).

The ability of plants to respond to and survive water deficit is a complex phenomenon, which requires adjustment at the molecular, cellular and whole plant level (Greenway and Munns, 1980; Ingram and Bartels, 1996; Zhu *et al.,* 1997). At the molecular level, osmotic stress will trigger cascades of signals involving Ca^{++} and reactive oxygen molecules as primarily signals to activate pathways critical for plant survival under the stress conditions (Knight *et al.,* 1997; Knight and Knight, 2001). At the cellular level, responses include metabolic adjustment to produce compatible solutes (Cherry, 1989), activation of transporters at the plasma and vacuolar membranes for ion sequestration or exclusion (Blumwald and Poole, 1985; Shi *et al.,* 2000), and activation of enzymes involved in detoxification of free radicals (Mittova *et al.,* 2002; Bor *et al.,* 2003; Mittova *et al.,* 2004; Badawi *et al.,* 2004). At the whole plant-level, responses include closure of plant stomatal apparatus coupled with an inhibition of vegetative growth and increase in root growth (Maggio *et al.,* 2003).

I-A- Drought effects on plants

Drought is a serious environmental factor that affects plant production worldwide. For example, it is estimated that about 25% of the total cultivated areas with rice in the world is under rain-fed; the shortage of water from one season to another is a serious threat to yield stability (Babu *et al.*, 2001). Drought stress occurs when the rate of water uptake from the soil is less than plant transpiration rate (Bonhert and Jensen, 1996). One of the first responses of plants to dehydration stress is triggered by the increase of ABA concentration, which generates a cascade of signals that leads to a decline in stomatal conductance to minimize transpiration and to keep it in balance with water absorption from soil (Zeevaart and Creelman, 1988; Chandler and Robertson, 1994). At this stage the plant can still maintain turgor, and partial stomatal closure can occur several times on daily basis, especially during mid-day. If unfavorable conditions continue for a long time, then the stomatal apparatus loses the ability to compensate for the lack of water and stomatal conductance declines sharply (Quarrie, 1989). High abscisic acid (ABA) concentrations in plant tissues under drought conditions contribute to reduction of leaf area and plant height, and pollen abortion (Quarrie, 1989). Persistence of drought stress eventually causes a dramatic reduction in all processes contributing to plant yield and reduction in plant growth in general (Cherry, 1989). Persistence of drought conditions eventually forces the plant to concentrate on survival and water conservation mechanisms (Cherry, 1989).

ABA application or an endogenous transient increase in ABA due to drought causes cytosolic pH changes and membrane depolarization; this increases the concentration of free cytosolic Ca^{2+} ions in guard cells in response to transient drought perception (Leung

and Giraudat, 1998). Free cytosolic Ca^{++} activates cyclic adenosine 5'-diphosphate ribose (cADPR), which plays a major role in ABA response (Allen and Schroeder, 1998). Another major player that is activated due to dehydration stress is phospholipase C (PLC), responsible for releasing inositol 1,4,5-triphosphate (IP3), which in turn mediates the release of Ca^{2+} ions into the cytosol (Hirayama *et al.*, 1995). Recent reports conducted on Arabidopsis cell suspensions suggests that this transient increase in IP3 is independent of ABA but still requires Ca^{2+} binding (Takahashi *et al.*, 2001).

I-B Salinity effects on plants

Stress caused by high salinity in soil or in the irrigation water is a serious factor limiting the productivity of major agricultural crops as the majority of the agriculturally important plants species are sensitive to high salt concentrations (McWilliam, 1986; Zhang and Blumwald, 2001). Soil salinity affects about 5% of all cultivated land, approximately 77 million ha (Jain and Selvaraj, 1997; Tester and Davenport, 2003). Areas that are affected with salinity are increasing; for example, 1/3 of the irrigated land worldwide is currently affected by salinity (Tester and Davenport, 2003). Salinization of soil is expected to reach up to 30% in the next 25 years and up to 50% of the arable land by the year 2050 (Wang *et al.*, 2003).

Plant response to salt-induced water deficit depends on several factors including genotype, length and severity of water loss, stage of development, and environmental factors such as temperature and humidity (Bray, 1994). High salinity causes both hyperosmotic and hyperionic stress effects, which if sufficiently severe, could result in plant death (Bohnert *et al.*, 1999; Hasegawa *et al.*, 2000). The plant cell membrane serves

as an impermeable barrier to macromolecules and also most molecules of low molecular mass. Thus, high salt conditions can lead to increased extracellular solute concentration, which causes a flux of water out of the cells, resulting in a decrease in turgor pressure and an increase in concentrations of intracellular solutes (Lichtentaler, 1995).

In addition to the lack of water, exposure to high salinity leads to "toxic sodium effect" where by excess toxic Na^+ in the cytoplasm causes a deficiency of essential ions such as K^+ and Ca^+ (Bohnert and Jensen, 1996; Hasegawa *et al.,* 2000). High concentration of Na^+ ions in the cytosol causes metabolic toxicity; this is in part due to the ability of sodium ions to compete with K^+ ions for binding sites for several enzymes (Tester and Davenport, 2003). High Na^+ and Cl^- concentrations also disrupt enzyme function, protein synthesis, structure and solubility and membrane structure and function (Blum, 1988). It also has been suggested that accumulation of salts in older leaves reduces supply of hormones to the growing tissues, which contribute to poor growth in salt stressed conditions (Munns, 1993).

To survive such conditions, some plants have developed mechanisms to deal with excess sodium ions either by compartmentation of the toxic ions into the vacuole (Apes *at al.,* 1999; Blumwald *et al.,* 2000; Zhang and Blumwald, 2001; Zhang *et al.,* 2001) or by exclusion from the cell (Shi *et al.*, 2000; Zhu, 2002; Shi *et al.,* 2003). Interestingly, halophyte (plants that normally grow in saline areas and can tolerate up to 0.5 M NaCl concentration before suffering injuries) are able to use ions from the surrounding environment for osmotic adjustment by internally distributing them in a way to keep the Na^+ ions away from the cytosol (Zhu, 2000). Alternatively, some plants tend to accumulate compatible solutes in order to overcome high salinity problem (Tarczynski *et*

al. 1993; Bohnert and Jansen, 1996; Sakamoto and Murata, 2000), while other species have the ability to activate proteins that are involved in damage repair in plant cells (Ingram and Bartels, 1996; Campbell and Close, 1997).

High salinity conditions are also responsible for generating reactive oxygen radicals which, if not dealt with, could lead to unbalance in cellular O_2 processing (Rental and Knight, 2004). Strong correlation was found between the ability of plants to adapt to high salinity levels and the increased activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Mittler and Zilinskas, 1994; Liu *et al.,* 1999; Mittova *et al.,* 2002; Bor *et al.,* 2003; Mittova *et al.,* 2004; Badawi *et al.,* 2004).

In general, the process of adaptation to salinity is coupled with activation of different signaling pathways in plants that lead to changes in gene expression (Hasegawa *et al.,* 2000; Xiong *et al.,* 2002; Zhu, 2002; Shinozaki *et al,* 2003; Seki *et al.,* 2003). For example, imposing dehydration stress conditions on *A. thaliana* resulted in the activation of transcriptional factors that are involved in plant adaptation to salt-induced-dehydration-stress (Stockinger *et al.,* 1997; Jaglo-Ottosen *et al.,* 1998; Liu *et al.,* 1998; Kasuga *et al.,* 1999; Haake *et al.,* 2002; Chinnusamy *et al.,* 2003). Microarray data from Arabidopsis plants grown under drought and salinity revealed the presence of 277 upregulated transcripts (5 fold) under drought conditions and 194 cDNAs that are induced under salinity conditions (Seki *et al.,* 2002); the upregulated transcripts, 128 are strictly induced under drought conditions, 119 transcript are also induced under salinity conditions (Seki *et al.,* 2002). Not surprisingly, it was found that about 51% of the drought induced transcripts are also induced under high-salinity conditions; similarly,

about 72% of the high-salt induced transcripts are also induced under drought conditions (Seki *et al.*, 2002).

These results strongly indicate the correlation between drought and salinity signaling mechanisms in plants, which also could explain why many genes encoding for late embryonic abundant proteins (LEA), heat-shock proteins (HSp), osmoprotectant biosynthesis, carbohydrate metabolic enzymes, detoxification enzymes, transporters, ion channels and membrane modification enzymes are activated under both high-salinity and drought stresses (Thomashow, 1999; Knight and Knight, 2001; Shinozaki and Yamaguchi-Shinozaki, 2003; Seki *et al.*, 2002; Shinozaki *et al.*, 2003). The same thing is also true for many genes coding for transcriptional regulators, mitogen activated protein kinases (MAPKs), and phosphatases that are involved in regulating plant response to high salinity and dehydration (Thomashow, 1999; Knight and Knight, 2001; Shinozaki *et al.*, 2003).

II- Improvement of dehydration stress tolerance in plants

II-A Conventional breeding for drought and salt tolerance

Drought and salt response, and apparent tolerance of a species, vary according to the type, concentration and distribution of salts in the root growing zone, duration of stress, and developmental stage of the plant (Jain and Selvaraj, 1997). These are also influenced by other environmental factors such as temperature, humidity, reduced oxygen in poorly drained or structured soil, and elevated CO_2 in the surrounding environment (Pasternak, 1987). Traditional breeding strategies to develop drought and salt tolerant plants has had only limited success, probably due to a combination of difficulties in establishing

selection conditions and the complexity of the resistance mechanisms (Flowers and Yeo, 1995). For example, when evaluating yield performance of a crop under saline conditions, one should consider the variation in salinity levels within a field, the possibility of interaction between salinity level and other environmental factors such as soil fertility, drainage quality and water loss due to transpiration (Flowers, 2004). Thus, using yield components as main criteria for selection requires a long period and multiple locations for testing, and evaluation (Blum, 1989).

Quesada *et al.*, (2002) attributed the lack of success in breeding for salt tolerance to the quantitative nature of most of the processes involved in salt tolerance. In maize, Ribaut *et al.* (1997) measured several yield components (grain yield, ear number, kernel number and kernel weight) in plants growing in three different water-stress regimes. They found that the correlation between the grain yield under well-watered and severe stress conditions was very low (0.31). Therefore, selection based on yield component values would not be effective. They also reported that no major quantitative trait loci (QTL) expressing more than 13% of the phenotypic variance were detected for any of the studied traits. Ribaut *et al.* (1997) concluded that there were inconsistencies in the QTL genomic positions across the three different water regimes. Working with interspecific crosses between salt-sensitive tomato plants (*Lycopersicon esculentum*) and salt-tolerant *L. pimpinellifolium* plants, Foolad (1999) reported the presence of a weak correlation (0.22) between seed germination rate and the percentage of plant survival under salt stress. The overall results indicated that salt tolerance during seed germination was independent of salt tolerance during vegetative growth (Foolad, 1999). In general, QTL

that are linked to salt and drought tolerance at one developmental stage are not necessarily linked to tolerance at other stages (Cushman and Bohnert, 2000).

In spite of these complexities, a number of salt-tolerant varieties of crops such as wheat and rice have been developed (Shannon and Noble, 1990; Forster, 2001). For example, crossing durum wheat and the wild relative *Aegilops tauschii*, increased K^+/Na^+ discrimination in the synthetic hexaploid hybrid wheat (Pritchard *et al.*, 2002). This increase in K/Na discrimination was significantly correlated with fresh weight accumulation in wheat plants under salt-stressed conditions. In rice, Senadhira *et al.*, (2002), reported the production of dihaploid lines of rice from crossing of two Indica breeding lines, one of which is superior in yield, the other is superior in salinity tolerance. Field trials conducted for 5 years revealed that some of the dihaploid lines performed better than other cultivars grown in saline-prone lands. Some lines showed several desirable traits such as high yield, salinity tolerance, and early maturation.

Interestingly, the use of *in vitro* tissue culture and somaclonal variation techniques has resulted in development of salt-tolerant plants (Safarnejad *et al.*, 1996; Boscherini *et al.*, 1999). In alfalfa, Safarnejad *et al.* (1996) isolated somatic clones of alfalfa, which showed increased salt tolerance, greater accumulation of proline, and a greater increase in glutathione reductase compared to the parental line. In tomato, Boscherini *et al.* (1999) identified a somatic clone that showed enhanced tolerance to salinity compared to wild type when tested at different NaCl concentrations (0, 75, 150, 300 mM NaCl). Leaf and flower necrosis was observed only in wild type plants. Plants coming from the somatic clone retained higher leaf turgor compared to the wild type when tested at 150 mM NaCl.

II-B Genetic engineering for dehydration stress

Differences in gene expression profiles among dehydration stress sensitive and tolerant plants indicate that the ability to withstand these unfavorable conditions is conferred by genetically encoded mechanisms (Bray, 1994). A variety of approaches have been used in order to engineer enhanced salinity and drought tolerance in different plant species (Table 1-1).

II-B-1 Genes involved in Na^+ ion exclusion from the cytoplasm

One way for plants to avoid Na^+ ion toxicity is to exclude Na^+ from the cytosol. Ion transport across membranes in plant cells (plasma membrane and tonoplast) is driven by proton gradients generated by proton pumps located at the different membranes (Sze *et al.*, 1999). The main pump in the cell plasma membrane is the plasma membrane H^+-ATPase (PM H^+-ATPase), which is responsible for generating the gradient between the cytosol and the extracellular environment, making the cytosol more basic and the outer environment more acidic (Palmgren, 1998). At the vacuole, there are two major pumps that are responsible for generating an acidic pH inside the vacuole; those pumps are the vacuolar H^+-ATPase (V-ATPase) and the vacuolar H^+ pumping pyrophosphatase (H^+-PPase) (Sze *et al.*, 1999). Plant cells use the electrochemical gradient that is generated by the different pumps to load/unload different materials into or out of the cytosol.

In the case of sodium compartmentation, Na^+ ions are loaded from the cytosol directly into the vacuole before it reaches a critical toxic concentration. The presence of

Table 1. Genetic Engineering for dehydration stress tolerance in plants:

A. Ion exclusion/sequestration/balance.

Function/gene	Species	Resistance phenotype	Conditions of stress treatment	I	C	G	F	References
Plasma membrane Na^+/H^+ antiporter, SOS1	Arabidopsis	Survival rate; germination; callus formation; chlorophyll content	Salinity: Irrigation with 50 mM NaCl for 4D then 100 mM for 4D, then 150 mM for 4D and finally with 200 mM NaCl for 4D		X			Shi *et al.* (2003)
Tonoplast Na^+/H^+ antiporter, AtNHX1	Arabidopsis	Plant survival	Salinity: Irrigation with 0, 50, 100, 150 or 200 mM NaCl for 16D		X			Apes *et al.* (1999)
	Canola	Plant height and weight; seed yield; oil quality	Salinity: Irrigation with 0, 10 and 200 mM NaCl for 10D			X		Zhang *et al.* (2001)*
	Tomato	Plant survival	Salinity: Hydroponic-culture 200mM NaCl for 28D			X		Zhang and Blumwald, (2001)*
	Rice	Seedlings survival under high salinity conditions	Salinity: Seedlings were transferred to hydroponic-culture (300mM NaCl) for 3D		X			Ohta *et al.* (2002)
	Wheat	Biomass production, vegetative growth, germination rates; grain yield	Salinity: Seedlings in pots and irrigated with MS medium with 150 mM NaCl Salinity: Hydroponic-culture (170-300 mM NaCl) Salinity: Germination (petri dishes) supplemented with 0-300 mM NaCl Salinity: Field trail (in soil with EC_e of 1.2, 10.6 and 13.7 dSm^{-1}) soild were irrigated 4 4times during the growing season to 70% field capacity.	X X		X	X	Xue *et al.* (2004)
K^+/H^+ antiporter, HKT1	Wheat	Low membrane depolarization; Na uptake in plant tissue; K/Na ratio; Na content in root tissue	Salinity: Seedlings were transferred to hydroponic-culture (200mM NaCl) for 4-5D		X			Laurie *et al.* (2002)
K^+/Na^+ selectivity, HAL1	Tomato	Rooting and calli formation capacity; plant height; leaves no; fresh and dry weight;	Salinity: *In vitro* roots and calli formation on media with 0, 125 and 175 mM NaCl for 28D Salinity: seedlings in pots were capillary irrigated with 0, 75 and 150 mM NaCl for 20D	X	 X			Gisbert *et al.* (2000)
	Tomato	Fruit yield	Salinity: Irrigated with (35mM NaCl)			X		Rus *et al.* (2001)
	Melon	Root formation; fresh and dry weight	Salinity: *in vitro* assay on medium supplemented with 100 mM NaCl	X				Bordas *et al.* (1996)
Osmotin-like protein	Potato	Root/shoot/nodes number and length.	Salinity: growth on medium with 100mM NaCl	X				Evers *et al.* (1999)

I. *In vitro*, C. Growth chamber, G. Greenhouse, F. Field

Table 1. B. Osmotic adjustment/dehydration stress tolerance

Function/gene	Species	Resistance Phenotype	Conditions of stress treatment	I	C	G	F	References
Proline biosynthesis	Tobacco	Root biomass; leaf-water potential	Drought: Water withhold for 10D Salinity: 400mM NaCl 21D			X X		Kishor *et al.* (1995)
P5CR	Tobacco	Photo oxidative damage	Salinity: *In vitro* seed germination on medium with 200 mM NaCl for 42D	X				Hong *et al.* (2000)
(Pyrroline-5-Carboxylate-Reducase)	Rice	Shoot and root biomass	Salinity: *In vitro* seed germination 100 mM NaCl for 10D Drought: Withhold irrigation for 6D then 1D irrigation *4 cycles*	X	X X			Zhu *et al.* (1998)
	Soybean	Seedling survival	Drought: Water withhold for 4D			X		Ronde *et al.* (2000)
PDHase (proline dehydrogenase)	Arabidopsis	Minutes to lodging of inflorescence and recovery; ion - leakage	Salinity: Hydroponic-culture for 30 min. in 600mM NaCl		X	X		Nanjo *et al.* (1999)
Mannitol biosynthesis	Tobacco	Fresh weight and seedling height	Salinity: Hydroponic-culture 250mM NaCl			X		Tarczynski *et al.* (1993)
MtlD (mannitol-1-phosphate dehydrogenase)	Tobacco	Dry weight	Salinity: Hydroponic-culture 150mM NaCl for 28D Drought: Water withheld for 15 D			X X		Karakas *et al.* (1997)
	Wheat	Fresh and dry weight; plant height; number of tillers; root length	Salt/osmotic stress: Calli formation with 1Mpa PEG or 100 mM NaCl for 60D Drought: Irrigation with 50 (stress) or 150 ml (non-stress) H_2O every 4D Salinity: 150 mM NaCl for 20D	X	X X			Abebe *et al.* (2003)
	Eggplant	Seed germination; successful regeneration, seedling height; fresh and dry weight	Salinity: *In vitro* assays on medium with 200mM NaCl or 7.5% PEG	X				Prabhavathi *et al.* (2002)
M6PR (mannose-6-phosphate reductase)	Arabidopsis	Fresh and dry weight	Salinity: 300mM NaCl for 15D		X			Zhifang and Loescher (2003)
Glycine betaine biosynthesis	Arabidopsis	Germination and survival	Salinity: *In vitro* germination and growth on medium with 300mM NaCl Salinity: Hydroponic-culture 200mM NaCl 10D	X	X			Hayashi *et al.* (1997)
CodA (Coline oxidase)	Rice	Less photo-inhibition; faster recovery of stress	Salinity: Hydroponic-culture 150mM NaCl 7D		X			Sakamoto *et al.* (1998)
	Brassica juncea	Germination percentage; length, fresh and dry weight of seedlings	Salinity: germination/ growth on medium with 0, 50, 100, 150 and 200mM NaCl for 12D Salinity: 3 day old seedlings on medium with 0, 50, 100, 150 and 200mM NaCl for 10D	X	X			Prasad *et al.* (2000)
BetB, Betaine aldehyde dehydrogenase	Tobacco	Plant height	Salinity: Hydroponic-culture 200mM NaCl 10D			X		Holmstrom *et al.* (1994)
	Tobacco	Fresh weight; photosynthesis inhibition	Salinity: irrigation with 200mM NaCl for 14 D			X		Holmstrom *et al.* (2000)
BADH Betaine aldehyde dehydrogenase	Carrot	Proliferation and growth of cell culture; plant survival	Salinity: *In vitro* cell culture assay on medium with 0-300 mM NaCl for 14D Salinity: 100, 200, 300, 400 or 500 mM NaCl solution for 30D	X	X			Kumar *et al.* (2004)
	Rice	Photosynthesis inhibition	Salinity: Hydroponic-culture 100mM NaCl for 7D			X		Kishitani *et al.* (2000)
	Tomato	Ion leakage test; *in vitro* root formation	Salinity: *In vitro* root formation on medium with 90-120 mM NaCl	X				Jia *et al.* (2002)
BetA (Betaine aldehyde dehydrogenase)	Cabbage	Total chlorophyll content; relative water content; leaf water potential; leaf osmotic potential	Salinity: *In vitro* leaf-disc bioassay on media with 150 and 300mM NaCl. Salinity: 0, 150 and 300 mM NaCl	X		X		Bhattacharya *et al.* (2004)
	Maize	Faster germination; ion leakage test; Chlorophyll fluorescence; roots biomass; Plant biomass; grain yield	Drought: *In vitro* seed germination at 20% (w/v) PEG Drought: *In vitro* seedlings assay with 10% (w/v) PEG Drought: 21 D water withheld at 10 leaf stage to 15% relative water content Drought: 21 D water withheld to 15% relative water content	X X		X	X	Quan *et al.* (2004)*
D-Ononitol biosynthesis IMT1,	Tobacco	Photo-inhibition; faster recovery; plant survival	Salinity: Hydroponic-culture 250mM NaCl 18D Drought: Withhold irrigation for 8D		X X			Sheveleva *et al.*, (1997)
Fructans biosynthesis SacB	Sugar beet	Fresh and dry weight of leaves and roots (storage and fibrous)	Drought: Irrigated to 20% soil capacity or well-watered for 32D		X			Pilom-Smits *et al.* (1999)
Trehalose biosynthesis TPS1 (trehalose-1-phospho trasferase)	Tobacco	Germination assay	Osmotic assay: germination assay on medium with 3-6% PEG for 7D Dehydration: dehydration for 2D Drought: water withhold for 24D then followed by 1D irrigation	X	X X			Lee *et al.* (2003)
	Rice	Fresh and dry weight; photo-oxidative damage	Salinity: Hydroponic-culture 100mM NaCl for 28D Drought: withhold irrigation for 5D then irrigation for 2D *2 cycles*		X X			Garg *et al.* (2002)
T6PS/T6PP	Rice	Plant and root growth; Chlorophyll fluorescence; plant survival	Salinity: Hydroponic-culture (100mM NaCl) 2D Drought: withhold irrigation for 12D		X X			Jang *et al.* (2004)
Polyamines biosynthesis Arginine decarboxylase, ADC	Rice	Seedling height and fresh weight	Salinity: seedlings treated with 150 mM NaCl for 2D	X				Roy and Wu (2001)
	Rice	Fresh weight	Drought: 2 month old plants irrigated with 20% PEG solution for 6D			X		Capell *et al.* (2004)
S-adenosylmethionine decarboxylase SAM-DC Spermidine biosynthesis	Rice	Seedling height and fresh weight	Salinity: seedlings treated with 150 mM NaCl for 2D	X				Roy and Wu (2002)
	Arabidopsis	Ion leakage; oxidative-stress tolerance	Drought: Water withhold for 15 D Salinity: *In vitro* on medium with 75mM NaCl for 35D Osmotic strass: *In vitro* on medium with 200mM D-sorbitol	 X X	X			Kasukabe *et al.* (2004)

I. *In vitro*, C. Growth chamber, G. Greenhouse, F. Field

Table 1. C. Stress induced proteins.

Function/gene	Species	Resistance Phenotype	Conditions of stress treatment	I	C	G	F	References
HVA1 (LEA protein)	Rice	Ion leakage test; plants height; fresh and dry weight	Salinity: Irrigation with 200 mM NaCl for 8D then 2D irrigation "3 cycles" Drought: Withhold irrigation for 7D then 3D irrigation "3 cycles"			X X		Rohila *et al.* (2002)
	Rice	Faster germination, growth and recovery; plant height and root weight	Osmotic: Seed germination on 200 mM mannitol or 100 mM NaCl for 5D Salinity: Plants in trays with 200mM NaCl 10D then with 50mM NaCl for 30D Drought: Water withhold for 5D then 2D water "2 cycles"	X		X X		Xu *et al.* (1996)
	Wheat	Fresh and dry weight; total dry matter; water-use efficiency	Drought: seedlings were either watered with 100 or 500 ml H_2O every other day for 10 weeks, this represent water-stressed and well-watered treatment			X		Sivamani *et al.* (2000)
	Tomato	Fresh and dry weight of callus	Salinity: *In vitro* assay (root formation and callus formation from different explants) on medium with 125 or 175 mM NaCl for 28D Salinity: Hydroponic-culture with 75mM NaCl for 30D.	X	X			Gisbert *et al.* (2000)
HVA2 (LEA protein)	Tomato	Callus proliferation; roots formation; seedlings fresh and dry weight	Salinity: *In vitro* cell culture assay on medium with 0-200 mM NaCl for 30D	X				Arrillaga *et al.* (1998)
Heat Shock Protein, **DnaK/Hsp70**	Tobacco	CO_2 fixation rate; stomatal closure	Salinity: plants were treated with 0, 300 or 600 mM NaCl for 3D		X			Sugino *et al.* (1999)
Pathogen related protein **PR10**	Canola	Germination assay	Salinity: seeds germination on 0 and 75 mM NaCl	X				Srivastava *et al.* (2004)
Superoxide dismutase	Tobacco	Enhanced photosynthetic efficiency; oxidative stress tolerance	Salinity: irrigation with 300 mM NaCl for 10D Drought: withhold irrigation for 10D Osmotic: irrigation with 10% (W/V) PEG		X X X			Badawi *et al.* (2004)
	Rice	Electron-transport rate; oxidative stress tolerance	Salinity: plants were treated with 100 mM NaCl for 10D		X			Tanaka *et al.* (1999)
GS2 (Glutamine synthetase)	Rice	Plant survival; photo-inhibition	Salinity: Hydroponic-culture with 150mM NaCl for 14D		X			Hoshida *et al.* (2000)

I. *In vitro*, C. Growth chamber, G. Greenhouse, F. Field

Table 1. D- Transcription factors and other proteins involved in gene regulation.

Function/gene	Species	Resistance Phenotype	Conditions of stress treatment	I	C	G	F	References
DREB1A/CBF3	Arabidopsis	Plant survival	Drought: water withheld for 14D Salinity: 2 hrs. hydroponic-culture with 600mM NaCl then transfer to pots.		X X			Kasuga *et al.* (1999)
	Tobacco	Ion leakage test and photosynthetic activity	Drought: in pots 14 D water withheld		X			Kasuga *et al.* (2004)
	Wheat	Delay in welting symptoms	Drought: in trays 10D water withheld			X		Pellegrineschi *et al.* (2004)
DREB1B/CBF1	Tomato	Plant survival; rapid stomatal closure; oxidative stress tolerance	Drought: transgenic and non-transgenic plants were grown in the same pot and water withheld for 28D then watering for 7D		X			Hsieh *et al.* (2002)
	Tomato	Plant survival; plant fresh weight, fruit weight and number; seed number	Salinity: plants were irrigated with 200 mM NaCl for 28D. Drought: water withhold 28D			X X		Lee *et al.* (2003)*
CBF4	Arabidopsis	Plant survival	Drought: water withhold for 9D then watered for 4D		X			Haake *et al.* (2002)
OsDREB1A	Arabidopsis	Plant survival	Salinity: 2 hrs. hydroponic-culture with 600mM NaCl then transferred to pots for 3 weeks.		X			Dubouzet *et al.* (2003)
AtSTZ	Arabidopsis	Higher survival rate after stress and re-hydration; less ion leakage	Drought: water withheld for 14D.		X			Sakamoto *et al.* (2004)
AtGSK1 (protein kinase)	Arabidopsis	Plant survival; root growth	Salinity: irrigation with 300mM NaCl for 10D			X		Piao *et al.* (2001)
AtMYC2 and AtMYB2	Arabidopsis	Ion leakage test	Drought: seedling transferred to hydroponic-culture supplemented with various mannitol concentration for 2hrs.		X			Abe *et al.* (2003)
ABF3 (ABRE Binding Factor)	Arabidopsis	Reduced transpiration; plant survival	Drought: water withheld for 12D then irrigated		X			Kang et al. (2002)
AtRabG3e	Arabidopsis	Tolerant to oxidative stress; higher fresh weight	Salinity: plants were irrigated with 200 mM NaCl for 14D. Drought: seedling assay on agar plates supplemented with 500mM sorbitol for 7D	X		X		Mazel *et al.* (2004)
MsAlfin1	Alfalfa	Calli formation; plant growth and survival	Salinity: *in vitro* calli formation on medium supplemented with 171 mM NaCl Salinity: hydroponic-culture with 128mM NaCl for 17D	X	X			Winicov and Bastola, (1999)
		Enhanced root growth and mass	Salinity: in pots filled with perlite and irrigating with 171mM NaCl for 20D		X			Wincov, (2000)
Tsi1	Tobacco	Chlorophyll content in leaves	Detached leaves floated over 400mM NaCl for 3 D	X				Park *et al.* (2001)
EhCaBP (Ca-binding protein)	Tobacco	Enhanced seed germination; dry weight;	Salinity: seed germination on medium with 50, 100 and 200 mM NaCl for 21D Osmotic stress: seed germination on medium with 200 mM mannitol for 21D	X	X			Pandey *et al.* (2002)
OSISAP1 (Zn-finger protein)	Tobacco	Relative fresh weight; germination %	Salinity and Osmotic stress: seedlings were wrapped in paper towels and soaked with either 250 mM NaCl solution for 4D or mannitol (300 and 400 mM) for 8D	X				Mukhopadhyay *et al.* (2004)
OsCDPK7 (Ca-dependent protein kinase)	Rice	Seedling survival	Salinity: Hydroponic-culture with 200mM NaCl 1D Drought: water withhold for 5D		X X			Saijo *et al.* (2000)
	Rice	Seedling survival	Salinity: Hydroponic-culture with 200mM NaCl 1D Drought: water withhold for 5D		X X			Saijo *et al.* (2001)
CaN (Calcineurin)	Tobacco	Seedling survival; seedling fresh weight	Salinity: germination on media with 250mM NaCl for 4D then transfer to fresh MS Salinity: hydroponic-culture with 200mM NaCl for 7D	X	X			Pardo *et al.* (1998)

I. *In vitro*, C. Growth chamber, G. Greenhouse, F. Field

large, acidic membrane-bound vacuoles in plant cells allows cells to efficiently compartmentalize excess Na^+ ions into the vacuole by the vacuolar Na^+/H^+ antiporter (Blumwald and Poole, 1985). The difference in the H+ is initially established by the H^+-ATPase pump (Blumwald *et al.*, 2000). In salt tolerant species, an increase in transcript level of Na^+/H^+ antiporters was observed upon exposing plants to high salt levels (Tester and Davenport, 2003). Apse *et al.* (1999) overexpressed the *A. thaliana AtNHX1* gene (coding for a vacuolar Na^+/H^+ antiporter) in *Arabidopsis* and showed that transgenic plants were able to tolerate up to 200 mM NaCl treatment (Table 1-1A). Tomato plants overexpressing the *AtNHX1* gene accumulated 20-28 fold more sodium in their vegetative tissues compared to wild type plants (Zhang and Blumwald, 2001) and *AtNHX1*-overexpressing canola plants accumulated up to 6% of their dry weight as sodium compared to almost 0.01% in non-transgenic plants (Zhang *et al.*, 2001).

Salt exclusion can be facilitated by the use of the *SOS* (*S*alt *O*verly *S*ensitive) genes, which encode a plasma membrane Na^+/H^+ antiporter (*SOS1*), a serine/threonine protein kinase (*SOS2*) and a myristoylated Ca^{2+}-binding protein (*SOS3*). Identification of those genes has also furthered our understanding of Ca^{2+} signaling in plant response pathways to salinity. Sudden change in Na+ ion concentration in the cytosol is immediately coupled with a transient change in the Ca^{2+} concentration in the cytosol; this transient change in cytosolic Ca^{2+} is known as the Ca signature (Knight *et al.*, 1997). The calcium ions then bind to the myristoylated Ca^{2+}-binding protein, encoded by *SOS3*, which then mediates downstream responses (Liu and Zhu, 1998; Ishitani *et al.*, 2000; Zhu, 2002). *SOS3* interacts with and activates the *SOS2*, which is a serine/threonine protein kinase (Halfter *et al.*, 2000; Liu *et al.*, 2000; Zhu, 2002). Both SOS3/SOS2 proteins are

responsible for regulating the plasma membrane Na^+/H^+ antiporter, which is encoded by the *SOS1* gene in *Arabidopsis* (Shi *et al.*, 2000).

Overexpression of the plasma membrane Na^+/H^+ antiporter gene *SOS1* in a mutated strain of yeast lacking the Na^+/H^+ antiporters had a slight but significant increase on yeast survival on medium supplemented with 100 mM NaCl. Co-expression of *SOS3/SOS2* as well as *SOS1* genes in the same mutated strain of yeast had a dramatic effect on yeast survival on medium supplemented with NaCl (Zhu, 2002). Moreover, overexpression of *SOS1* gene in *Arabidopsis* significantly improved plant salt tolerance (Shi *et al.*, 2003). Shi *et al.*, (2003) reported that transgenic plants overexpressing *SOS1* gene accumulated less Na^+ in their tissues than their non-transgenic counterparts (Table 1-1A).

Recently, there have reports indicating that the SOS2 protein kinase may have multiple effects on other genes (Cheng *et al.*, 2004; Qiu *et al.*, 2004). Qiu *et al.*, (2004) demonstrated that the tonoplast Na^+/H^+ transporter in *Arabidopsis* is also one of the targets of the *SOS* regulatory pathway, which means that there might be more branches to the *SOS* pathway. Interestingly, Cheng *et al.* (2004) showed that SOS2 protein kinase also regulates the vacuolar H^+/Ca^{2+} antiporter CAX1. Co-expression of *SOS2* specifically activated *CAX1* gene in yeast. Using the yeast two-hybrid assay, SOS2 was found to interact with the N terminus of CAX1.

II-B-2 Genes that control osmolyte and compatible solute content in plants

An important adaptation to osmotic stress is the ability to accumulate compatible solutes (e.g., proline, glycine-betaine, alcohol sugars, fructans and trehalose) in the cytoplasm under dehydration-stress conditions (Bohnert and Jansen, 1996). Compatible

solutes, which are sometimes called osmoprotectants, are non-toxic organic metabolites of low molecular weight that act to raise the osmotic potential of the cell, or to stabilize membranes or macromolecular structure (Bohnert and Jensen, 1996). Engineering strategies for developing salt stress tolerance has been performed with genes encoding production of proline, glycine betaine, mannitol, fructans, and trehalose (Holmberg and Bulow, 1998; Taiz and Zeiger, 1998).

Several groups have demonstrated that proline accumulating in response to water or salt stress can act as an osmoprotectant in plant cells under salt stress (Kishor *et al.*, 1995; Taiz and Zeiger, 1998; Zhu *et al.*, 1998; Hong *et al.*, 2000; Ain-Lhout *et al.*, 2001). Mali and Mehta (1977) were among the first to report on the rapid accumulation of proline in rice varieties upon exposure to drought stress. Water stress-tolerant rice plants showed a 5.4 fold increase in free proline compared to 1.2 fold increases in sensitive varieties. Increased proline accumulation in response to dehydration stresses has been observed in numerous species, and has been reviewed extensively in the literature (Cherry, 1989). One of the first attempts to engineer plants to overproduce proline came from Kishor *et al.* (1995), which who overexpressed in tobacco the moth bean Δ1-pyrroline-5-carboxylate synthetase (P5CS), a bifunctional enzyme that catalyzes the conversion of glutamate to proline. The transgenic plants accumulated 10 to 18 fold more proline than the non-transgenics. Proline accumulation was accompanied by an increase in salinity tolerance measured as increased germination percentage and seedling fresh weight when grown in 200 mM NaCl (Table 1-1B). Zhu *et al.* (1998) also reported similar results in rice (*Oryza sativa* L) when overexpressing the same enzyme under a stress inducible promoter. Moreover, Ronde *et al.* (2000) overexpressed the Δ1-pyrroline-5-carboxylate

reductase, (in antisense orientation) in soybean plants. Antisense-soybeans failed to survive a 6-day drought stress at 37 C° in contrast to wild type plants that survived the treatment. Similarly, removal of the feedback inhibition of P5CS enzyme resulted in an increased accumulation of proline in plant tissue that correlated with an increased tolerance to osmotic stresses (Hong *et al.*, 2000).

Glycinebetaine is a common compatible solute in many different organisms including certain plant species (Sakamoto and Murata, 2000). Betaine *in vitro* acts as an osmoprotectant by stabilizing the structure of proteins and the highly ordered structure of membranes against the adverse effects of water deficit conditions such as high salinity and extreme temperature (Gorham, 1996). Many plant species grown in saline and arid areas accumulate glycinebetaine in response to drought and salinity (Grumet and Hanson, 1986; Saneoka *et al.*, 1995; Sakamoto and Murata, 2000). Hayashi *et al.* (1997) achieved enhanced salt-tolerance in *Arabidopsis* by overexpressing the soil bacterium *Arthrobacter globiformis codA* gene (choline oxidase, a key enzyme in glycine-betaine production). Overexpression of the *E. coli betA* gene (which encodes choline dehydrogenase) in tobacco confers salt-tolerance (Holmstrom *et al.*, 2000). Growth of control plants was totally inhibited by watering with 200 mM NaCl solution, whereas transgenics were not affected (Table 1-1B). Jia *et al.*, (2002) transformed tomato plants with the *Atriplex hortensis*-BADH gene, which encodes betaine aldehyde dehydrogenase, to convert betaine aldehyde into glycine-betaine. Transgenic tomato plants grew normally under 120mM NaCl and exhibited enhanced root development compared to non-transgenic plants (Table 1B). More recently, cabbage plants overexpressing the bacterial *betA* gene, exhibited higher tolerance to NaCl stress compared to nontransformed plants

(Bhattacharya *et al.*, 2004). Transgenic cabbage plants showed better growth response and greater stability in maintaining plant water relations at high levels of salinity.

Sugar alcohols also work as osmolytes in plant cells exposed to salt stress (Cherry, 1989; Tarczynski *et al.*, 1993; Bohnert and Jensen, 1996; Bohnert *et al.*, 1999) and can also serve as scavengers for reactive oxygen species (Halliwell *et al.*, 1988). Tarczynski *et al.* (1993) first reported stress protection of transgenic tobacco plants by overexpressing the osmolyte sugar alcohol mannitol (Table 1-1B). Since then several reports have been published indicating that overexpression of genes involved in production of sugar alcohols in plants can confer stress tolerance (Karakas *et al.*, 1997; Liu *et al.*, 1999; Zhifang and Loescher, 2003). Karakas *et al.* (1997) transformed tobacco plants with a gene encoding mannitol-1-phosphate dehydrogenase (*MtlD*). Transgenic plants were not affected under salt stressed conditions that caused a dry weight reduction of 44% in the nontransgenic controls. More recently, Tilahun *et al.* (2003) showed that overexpression of the bacterial gene *mtlD* in wheat resulted in plants that were more tolerant to high salt stress. When subjected to 150 mM NaCl, transgenic T2 wheat plants showed 50% reduction in fresh weight and 30% reduction in dry weight compared to 77% and 73% reduction in fresh and dry weight respectively in non-transgenic wheat (Table 1B). In contrast to using the bacterial gene *mtlD*, Everard *et al.* (1997) isolated a gene encoding mannose-6-phosphate reductase (*M6PR*), key enzyme for mannitol production from celery. Zhifang and Loescher (2003) introduced the *M6PR* into *Arabidopsis* plants and showed that transgenic *Arabidopsis* plants were able to grow normally and complete normal development and seed production in the presence of 300 mM NaCl. Similarly, expression of the *E. coli GutD* gene (encoding for gluctiol-6-

phosphate dehydrogenase) a key enzyme for biosynthesis of the sugar alcohol sorbitol, caused increased accumulation of sorbitol in transgenic maize, and enhanced salt-tolerance compared to non-transgenics (Liu *et al.*, 1999).

Fructans are soluble polymers of fructose that are produced by approximately 15% of flowering plant species. Accumulation of fructans in the cell vacuole helps maintain water potential gradients of cells by raising the osmotic potential of the cell (Pilon-Smits *et al.*, 1995). Pilon-Smits *et al.* (1999) overexpressed a gene from *Bacillus subtilis* (*SacB* gene) to produce bacterial fructans in sugar beet. The growth of transgenics was significantly enhanced under drought conditions compared to the nontransgenics, as measured by higher dry leaf and root weights.

The disaccharide trehalose (α-D-glucopyranosyl-1, α -D-glucopyranoside) is present in a large variety of microorganisms and plants where it can serve as a reserve carbohydrate and as an osmoprotectant (Vogel *et al.*, 2001). The occurrence of trehalose has also been documented in several desiccation-tolerant plants (Muller *et al.*, 1995). It protects membranes and proteins in cells exposed to salt-stress induced dehydration (Penna, 2003). Pilon-Smits *et al.* (1998) showed that transgenic tobacco plants overexpressing the *Escherichia coli OtsA* and *OtsB* genes (encoding trehalose-6-phosphate-synthase and trehalose-6-phosphatase respectively) responded better to dehydration stresses compared to non-transgenics. Under drought conditions the transgenics yielded 30-39% more dry weight compared to non-transgenics. They also reported that detached leaves from transgenic tobacco plants had a higher capacity to retain water than the wild type. Using the *OtsA* and *OtsB* genes under salt inducible

promoter, Garg *et al.* (2002) reported successful production of transgenic rice plants with an elevated tolerance to 100 mM NaCl (Table 1-1B).

II-B-3 Genes that encode stress induced proteins such as the LEA (late embryogenesis abundant) and COR (cold regulated) proteins

Another important group of genes that play a role in plant adaptation and resistance to dehydration-stress induced conditions are known as LEA (late embryogenesis abundant) and COR (cold regulated) genes. LEA proteins were first identified and characterized in cotton as a set of proteins that accumulate in embryos at the late stage of seed development when seeds are undergoing the dehydration process necessary for long term survival in a dormant state (Dure *et al.*, 1981). Transcription of genes encoding LEA proteins is also activated in other tissues such as leaves subjected to osmotic stresses (Zhang *et al.*, 2000). LEA proteins are divided into 5 major groups according to sequence homology (Swire-Clack and Marcotte, 1999). Group 1 proteins that might play a role in binding or replacement of water, group 2 and 4 proteins that may play a role in maintaining protein and membrane structure under severe dehydration, and finally group 3 and 5 that are thought to have a role in ion sequestration in plant cells (Swire-Clack and Marcotte, 1999). The class 2 LEA proteins (also known as the *lea* D11 family) are dehydrins that accumulate in plant cells in response to dehydration stresses and low temperatures. The dehydrins, which range from 82 to 575 amino acids in length, share several conserved domains (Close, 1997). The first, named the K domain, is an α-helix domain composed of 15 amino acids (EKKGIMDKIKEKLPG) which exists in single or multiple copies. The second domain is the S-segment, consisting of a stretch of 6-10 Ser

residues. The third is the Y-segment (T/VDEYGNP), located at the N-terminus (Close, 1996).

It has been hypothesized that LEA proteins play a role in desiccation tolerance during seed development and in response to dehydration and salinity stress (Hoekstra *et al.*, 2001 and Close, 1997). This role is probably achieved through maintenance of protein or membrane structure, sequestration of ions, binding of water, and function as molecular chaperones (Bray, 1997). Two classes of LEA proteins have been shown to have a direct functional role in salt and dehydration tolerance in plants. Rice plants transformed with the HVA1 gene from barley (a group 3 LEA protein) showed an increased tolerance to dehydration and salinity (Table 1-1C), compared to the non-transgenics (Xu *et al.*, 1996). Improved salinity tolerance was also reported in yeast cells expressing the tomato LEA protein LE25, a group4 LEA protein (Imai *et al.*, 1996). Another group of LEA proteins that has a role in water binding or replacement is the LEA1 group; yeast cells overexpressing the LEA1 protein, Em, had enhanced growth when subjected to medium with high osmolarity (Swire-Clark and Marcotte, 1999).

Plants exposed to low non-freezing temperature undergo a phenomenon known as cold acclimation, a process that is necessary for many plant species to survive freezing temperatures. Thomashow and co-workers identified a group of genes, designated as *CO*ld *R*esponsive (*COR*), that are induced upon cold acclimation (Gilmour *et al.*, 1992; Lin and Thomashow, 1992; Hovarth *et al.*, 1993). The *COR* genes were also identified by other groups and are also known as *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible). *COR* genes comprise four families, each of which is composed of two genes that are physically

linked in the genome in tandem array (Thomashow, 1999). *COR15*, 78, 6.6 and 47 encode hydrophilic polypeptides. The COR47 hydrophilic polypeptide belongs to group 2 LEA proteins (Thomashow, 1999).

COR genes may help plant cells to tolerate potentially damaging effects of dehydration associated with freezing-induced drought injury (Steponkus *et al.*, 1998). As temperatures drop below 0 °C, ice formation initiates in the intercellular spaces of plant tissues, resulting in a drop of water potential outside the cell. The water potential gradient will facilitate the movement of unfrozen water. At –10 °C, more than 90% of the osmotically active water moves out of the cell causing dehydration injuries (Thomashow, 1999). Over expression of the *COR15a* gene in *Arabidopsis thaliana* protoplasts resulted in a small, but significant increase in protoplast survival upon freezing over the range of –4.5 to –7.0 °C (Artus *et al.*, 1996). *COR15a*, which encodes a chloroplast-targeted polypeptide, enhances the freezing tolerance of chloroplasts by protecting membranes from freeze-induced dehydration.

II-B-4 Genes involved in transcriptional regulation of stress response mechanism in plants

II-B-4a The CBF system

In general, plant responses to abiotic stresses and water deficits are multigenic, where cascades of biochemical and cellular changes are necessary for plants to adapt to environmental changes (Bohnert and Jensen, 1996). Thus induction of cascades of responses may be more effective in increasing stress tolerance than single gene changes. In some cases, individual changes had only a modest effect on stress tolerance. For

example, introduction of glycinebetaine into *Arabidopsis*, *Brasica napus* and tobacco (Huang *et al.*, 2000) caused only a small increase in stress tolerance. Although transgenic plants accumulated 8-18 fold more glycinebetaine than the non-transgenic controls, there was only a moderate improvement in salt tolerance in tobacco plants as measured by fresh weight of shoots. In addition, only *B. napus* showed a slightly better photosynthetic rate in response to salinity (Huang *et al.*, 2000). Overexpression of the cold induced *COR15a* gene in *Arabidopsis thaliana* did not improve the freezing tolerance at the whole plant level (Artus *et al.*, 1996).

An alternative approach to the induction of a single gene is to induce an array of adaptive plant responses through the use of key transcription factors (Thomashow, 1999). The *COR* genes are characterized by the presence of a common *cis*-acting element within their promoter region that confers stress-induction. Baker *et al.* (1994) reported that the 5' region of the *Arabidopsis thaliana cor15a* gene includes a *cis*-acting element that confers cold-, drought-and ABA-regulated gene expression. Yamaguchi-Shinozaki and Shinozaki (1994) identified a 9 bp *cis*-acting element (TA**CCGAC**AT) at the promoter region of the *COR78/RD29A* gene (*COR78* and *RD29A* are alternative designations of the same gene); and named it drought responsive element (DRE). The DRE stimulated gene expression in response to low temperature, dehydration, and high salinity in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). Stockinger *et al.* (1997) identified a 5 bp DNA regulatory element (**CCGAC**) at the promoter of the *COR* gene family and designated it as the C-repeat (CRT). The CRT element, which also occurs in the DRE element, was found to be essential for *COR* gene transcription in response to low temperature (Stockinger *et al.*, 1997).

Using the yeast one-hybrid system, Stockinger *et al.* (1997) isolated an *A. thaliana* cDNA clone encoding the transcription factor *CBF1* [CRT (C-repeat)/DRE (Drought Response Element) Binding Factor]. CBF1 is a 24 kDa protein, with a nuclear localization domain and an activation domain; it also has an AP2 domain that has a DNA-binding site. Transcripts of the *CBF* gene family increase dramatically within 15 minutes after transferring *Arabidopsis* plants to low non-freezing temperature. This increase is followed by an increase in *COR* gene transcripts (Gilmour *et al.*, 1998), indicating that *CBF* gene expression is an early step in the *COR* gene transcriptional cascade. Similarly, the Shinozaki group cloned two cDNAs encoding for DREB [DRE (Drought Response Element) Binding] proteins *DREB1a* and *DREB2b* and showed that expression of *DREB1a* was activated by low temperature, while *DREB2b* transcript was activated by dehydration (Liu *et al.*, 1998). *CBF1*, *CBF2* and *CBF3* are alternative designation of *DREB1b*, *DREB1c* and *DREB1a*.

II-B-4b Regulation of CBF

Gilmour *et al.* (1998) proposed that due to the rapid induction of *CBF* genes upon cold treatment (about 15 minutes) and the lack of a CRT/DRE element in the promoter of *CBF/DREB1* genes, another protein that regulates expression must be present in warm conditions. This protein would bind to the *CBF* promoter and induce *CBF* expression upon cold treatment. This hypothetical protein was designated as *I*nducer of *C*BF *E*xpression, *ICE* (Gilmour *et al.*, 1998; Thomashow, 2001; Thomashow *et al.*, 2001). Upon exposure of plants to low non-freezing temperatures, a modification in ICE or in

another associated protein would occur, this would allow ICE to bind to *CBF* promoters and upregulate *CBF* expression.

A breakthrough in understanding cold and freezing tolerance mechanisms was achieved when Chinnusamy *et al.* (2003) isolated and identified *ICE1*, an upstream transcription activator that positively upregulates transcription of the *CBF* gene family. *ICE1* gene encodes a 53.5 kD nuclear localized MYC-like basic-Helix-Loop-Helix (bHLH domain) transcription factor. ICE1 has an acidic domain near the N-terminus with a typical bHLH DNA binding domain and a dimerization domain near the C-terminus. ICE1 binds to a *cis*-element (CANNTG) about 1 kb upstream of the *CBF3* promoter. Zarka *et al.*, (2003) found that there are at least two regulatory elements in the promoter region of *CBF2, I*nducer of *C*BF *E*xpression *r*egion 1 and 2 (ICEr1 and ICEr2 respectively), including the core sequence (CANNTG). Results from Chinnusamy *et al.* (2003) indicate that there are 5 MYC recognition sites at the promoter of *CBF3*. Knockout and overexpression experiments revealed the role of the *ICE1* gene in chilling and freezing tolerance in *Arabidopsis* plants. Mutated *Arabidopsis* plants (*ice1*) were impaired in their response to chilling and freezing temperatures, while *ICE1*-overexpressing plants were significantly more chilling and freezing tolerant than wild type (Chinnusamy *et al.*, 2003).

Interestingly, although *ICE1*-overexpressing plants were chilling and freezing tolerant, they did not show an elevation in *CBF3* gene expression level in warm temperature; an increase in *CBF3* expression was only observed under cold temperature (Chinnusamy *et al.*, 2003). In *ice1*-mutants, the expression levels of *CBF1* and *CBF2* were similar to wild type plants, although there was some delay in expression at 1 and 3

hours of cold treatment (Chinnusamy *et al.*, 2003). Results from Chinnusamy *et al.* (2003) indicate that expression of members the *CBF/DREB1* gene family might be regulated differently due to the observation that members of the *CBF/DREB1* family are differentially induced under different conditions (Haake *et al.*, 2002) and that members of *CBF/DREB1* family are differentially expressed under the same stress condition (Novillo *et al.*, 2004). For example, screening of the Arabidopsis genome revealed the presence of another CBF homolog, *CBF4*, which has a 63% amino acid similarity to the CBF gene family (Haake *et al.*, 2002). The expression of *CBF4/DREB1d* is not induced under low temperatures, but is rapidly induced in response to drought and ABA treatment.

Recently, Novillo *et al.* (2004) investigated the contribution of each *CBF/DREB1* member in cold adaptation. Using knockout mutants of *CBF2*, Novillo *et al.* (2004) found the surprising results that *cbf2* plants had an elevated level of *CBF1* and *CBF3* transcript even under warm temperature. This resulted in induction of target genes and increased freezing, dehydration, and salinity tolerance (Novillo *et al.*, 2004). Northern blot analysis revealed a delay in expression between the different members of the *CBF* gene family, with *CBF1* and *CBF3* expression preceding the expression of *CBF2*. These results are consistent with the fact that the transcript level of *CBF2* is almost 5 times higher than the transcript levels of *CBF1* and *CBF3* under warm condition (Fowler and Thomashow, 2002). Novillo *et al.*, (2004) concluded that *CBF2* might act as a negative regulator of both *CBF1* and *CBF3*. Under warm condition, the steady state of *CBF2* transcript would negatively regulate the expression of *CBF1* and *CBF3*, to make sure that their expression is tightly controlled. Upon cold exposure, *ICE* and other proteins would be activated to

rapidly increase the expression of *CBF1* and *CBF3*, resulting in increased environmental-induced dehydration tolerance (Novillo *et al.*, 2004).

II-B-4c Effect of overexpression of CBF/DREB on the plant transcriptome

Examination of gene expression changes allows for comparison between the effects of cold treatment and CBF overexpression. Transcriptome-profiling experiments in *Arabidopsis*, using Affymetrix-Gene-Chip (8297 genes) revealed changes in transcript level of 306 genes (about 4% of the total genes tested) in response to cold (Fowler and Thomashow, 2002). Of these 306 genes, 218 genes showed a 3-fold or greater transcript increase and 88 genes showed a two-fold transcript decrease in *Arabidopsis* plants treated at 4 C° (Fowler and Thomashow, 2002). Of the upregulated genes, more than 70% increased transiently, with expression reaching the highest level during the first 24 hours of cold treatment, followed by a dramatic decline in transcript level (Fowler and Thomashow, 2002). Transiently-upregulated genes were classified into several groups, transcription factors (CBF1 and 3, other AP2 domain containing proteins, zinc finger proteins, MYB proteins, MADS box containing proteins, and other ethylene responsive element binding factors); cell metabolic regulation (e.g., carbohydrate and osmolyte biosynthesis along with other genes involved in starch catabolism); transporters (water channels, ion and sugar transporters); cellular communication (protein kinases and proteases); cellular defense and detoxification mechanisms (LEA proteins and other enzymes involved in radicals detoxification); and finally a class of proteins with unknown function (45 genes). The remaining genes were categorized as long-term upregulated genes. Long-term upregulated genes continue to accumulate several fold

(compared to plants growing in warm temperature) even when tested after 7 days (Fowler and Thomashow, 2002). This group of genes also included transcription factors, genes involved in metabolic pathways, transporters, cell signaling, cell maintenance and detoxification as well as 20 genes of unknown function. Genes that were down regulated by cold included genes involved in photosynthesis and metabolism, signal transduction, heat shock and transcriptional regulation (Fowler and Thomashow, 2002).

Seki *et al.* (2002) also reported that more than 40% of transcripts induced under cold temperature (53 transcript were induced under cold treatment in total) are also induced under both drought and high salinity, among those are different members of the *COR* genes (*COR15a*, *COR47* and *COR78*). Sequencing of the promoter region of the 22 genes that are induced under drought, cold and high salinity conditions indicated the presence of the CRT/DRE element in 16 genes and 8 of which also had the ABRE element in their promoter region (Seki *et al.*, 2002).

Comparison of transcriptome profiling between warm-grown Arabidopsis plants constitutively expressing *CBF1*, *CBF2* or *CBF3* and control nontransgenic plants revealed that not all genes upregulated under cold temperature are also upregulated when overexpressing *CBF*. Thus, the CBF regulon represents only a part of the genes that are cold upregulated (Fowler and Thomashow, 2002). It was found that 60 genes (more than 80% of those genes are transiently upregulated) upregulated under cold were not upregulated in plants overexpressing any of the *CBF* genes; those were suggested to be *CBF*-independent. Of the 41 genes upregulated in *CBF* overexpressing plants, 30 were also upregulated by cold, which makes them the members of the *CBF* regulon (Fowler and Thomashow, 2002). Genes that were upregulated include genes were known to have

CDT/DRE elements, such as *COR6.6*, *COR15b*, *COR47*, *COR78/RD29*. Interestingly, genes that may be involved in osmolyte accumulation such as galactinol synthase for galactinol and raffinose production and *P5CS* for proline production were also increased in response to *CBF* expression (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Seki *et al.*, 2001; Seki *et al.*, 2002).

It is known that many of the genes that are induced by the *CBF* genes in response to dehydration induced stress conditions are also induced by the application of abscisic acid ABA (Yamaguchi-Shinozaki and Shinozaki, 1994; Ishitani *et al.*, 1997; Liu *et al.*, 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). Thomashow (1999) suggested that ABA concentration increases transiently in response to cold non-freezing temperature. This non-accumulative response helped establish the argument that ABA doesn't play a role in cold acclimation, and that cold acclimation occurs via two separate, ABA-dependent and ABA-independent pathways. The ABA-independent pathway includes the *CBF/DREB1* genes (Liu *et al.*, 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki *et al.*, 2003). More recently, Knight *et al.* (2004) showed that the activation of the *CBF* pathway could also occur *via* the ABA pathway; 100 μM ABA treatment for 1 hour was sufficient to increase the transcript and the protein level of the *CBF1*, *CBF2* and *CBF3* genes. Knight *et al.* (2004) suggested that the *CBF1-3* genes, which are activated by cold and ABA treatment, might give them a distinctive role in plant adaptation under both freezing and drought conditions, in contrast to *CBF4* which is strictly activated under drought and ABA treatment (Haake *et al.*, 2002). This possibly adds another layer of complexity to signaling in plants under abiotic stress conditions.

II-B-4d Effect of CBF/DREB-overexpression on plant metabolome

The *CBF/DREB* gene families are transcriptional activators that directly or indirectly work as master switches in regulating transcript in plants in response to dehydration-inducing conditions (Thomashow, 2001). Interests in exploring large scale changes in plant metabolome, as a direct result of *CBF/DREB* expression, has been recently investigated in Arabidopsis (Cook *et al.*, 2004).

Cook *et al.* (2004) included three ecotypes of *Arabidopsis thaliana*, differing in tolerance to freezing temperatures [Cape Verde Islands-1 (Cv-1), Wassilewskija-2 (Ws-1), and Columbia (CM]; the first is less freezing tolerant than the latter two. Using a GC–time-of-flight MS method to assess large scale changes in metabolic profile, at least 325 low molecular weight compounds (carbohydrates, amines, organic acids, and other polar molecules) increased 2-fold or more in Ws-plants in response to 14 days of cold treatment. Of those 325 compounds, 114 increased at least 5-fold higher compared to the control non-cold acclimated plants. On the other hand, in the Cv-1 ecotype, only 269 compounds increased in response to cold acclimation, of which 244 were common with the Ws-1 ecotype. The finding that only 53 out of the 269 compounds had at least 5-fold increase in Cv-1 cold acclimated plants compared to 114 in the freezing tolerant Ws-1 ecotype may explain why these ecotypes differ in their ability to withstand freezing temperatures.

Cook *et al.* (2004) went on to compare the metabolome profile of *CBF3/DREB1a* overexpressing Arabidopsis plants and cold acclimated-wild-type Col plants. These experiments revealed that of the 325 metabolites that increased in response to low temperature, 256 also significantly increased in *CBF3/DREB1a* overexpressing plants. Of

those compounds, 102 increased at least 5-fold in the CBF3/DREB1a overexpressing plants. These results clearly demonstrate the similarity between the metabolome of *CBF3*-expressing plants and the metabolome of cold-acclimated wild-type plants. Cook *et al.* (2004) suggested that the dramatic increase in proline and low-molecular-weight soluble carbohydrates is a signature of the *CBF* regulon.

II-B-4e Conservation of the CBF/DREB system among plant species

CBF1/DREB1b homologs have been identified in *Arabidopsis thaliana* (Stockinger *et al.*, 1997; Haake *et al.*, 2002), canola (Jaglo *et al.* 2001), barley (Choi *et al.*, 2002), tomato (Jaglo *et al.* 2001), rice (Choi *et al.*, 2002; Dubouzet *et al.*, 2003), strawberry and sour cherry (Owens *et al.*, 2002), suggesting that the CBF/DREB system is highly conserved throughout the plant kingdom, including both dicots and monocots.

Jaglo *et al.* (2001) reported the presence of other *CBF/DREB1* homologs in *Arabidopsis* (*DREB1e* and *DREB1f*), and other plants. Canola (*Brassica napus*), which like Arabidopsis a member of the Brassica family, encodes two different CBF-like proteins that share approximately 76% homology to the *Arabidopsis CBF1* gene; similarly, *BnCBF1* and *BnCBF2*, accumulated in canola plants within 30 minutes after transferring plants into cold nonfreezing temperature (Jaglo *et al.*, 2001). The expression of the *BnCBF1* and *BnCBF2* was followed by accumulation of the *B. napus-COR15a* ortholog, *Bn115*.

Jaglo *et al.* (2001) also reported the presence of CBF homologs in the more distantly related, chilling sensitive species tomato (*Lycopersicon esculentum* L). Similar to the Arabidopsis *CBF1*, *CBF2* and *CBF3* genes, Zhang *et al.* (2004) found that *LeCBF1*,

LeCBF2 and *LeCBF3*, are organized in tandem and all lack introns. Interestingly, *LeCBF1* was induced only in response to cold non-freezing conditions after about 30 minutes, reaching maximum induction after 2 hours, while *LeCBF2* and *LeCBF3* were not induced under cold. *LeCBF1*, *LeCBF2* and *LeCBF3* responded very weakly to mechanical agitation, drought, salinity and ABA treatment (Zhang *et al.*, 2004). Owens *et al.* (2002), reported that *CBF1*-orthologs also exist in sour cherry (*Prunus cerasus* L.) and strawberry (*Fragaria X ananassa* Duchesne). The putative orthologs of sour cherry *PcCBF1* and strawberry *FaCBF1* shared about 48% similarity at the amino acid level to the *Arabidopsis* CBF1 protein (Owens *et al.*, 2002).

Distantly related plants such the monocots rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rice (*Oryza sativa*) also have *CBF*-like homologues (Jaglo *et al.*, 2001; Choi *et al.*, 2002). The rye and wheat polypeptides had 30% and 34% sequence homology, respectively, to the Arabidopsis CBF1 polypeptide. Choi *et al.* (2002) identified a sequence, *OsCBF3,* from the rice genome database similar to the *CBF3* gene from Arabidopsis. This in turn was used to screen a barley BAC library, leading to identification of a barley *CBF3* ortholog. Expression of the barley *CBF* gene, *HvCBF3*, was found to be cold induced (Choi *et al.*, 2002).

II-B-4f Engineering dehydration-stress tolerance in plants using the CBF/DREB system

Jaglo-Ottosen *et al.* (1998) achieved a breakthrough by demonstrating that overexpression of a transcription factor in plants would result in activation a cascade of genes directly/indirectly involved in abiotic stresses tolerance. Transgenic, non-

acclimated *Arabidopsis* plants overexpressing the *CBF1* gene were more freezing tolerant than their non-acclimated control counterpart as determined by electrolyte leakage (a test used to determine membrane damage) and whole plant freezing test assays. EL_{50} values (the freezing temperature that results in release of 50% of tissue electrolytes) indicated that *CBF1* overexpressing *Arabidopsis* plants were significantly more tolerant to freezing than wild type (3.3 °C difference). Similarly, Kasuga *et al.* (1999) overexpressed *DREB1A* in *Arabidopsis* and showed that *DREB1A* enhanced tolerance to drought, salt and freezing in transgenic plants: 69.2% of the transgenics survived a 14 day dehydration treatment, whereas no wild type plants survived (Table 1-1D). When exposed to –6 C° for 2 days, followed by 5 days exposure to 22 C°, less than 10% of the nonacclimated control plants survived while more than 75% of the transgenics survived. Moreover, 78.6% of the transgenics were able to survive a treatment of dipping in 600 mM NaCl solution for 2 h, before transplanting into pots, while only 17.9% of the wild-type plants survived this treatment (Kasuga *et al.*, 1999).

Arabidopsis plants overexpressing *CBF4* gene also were tolerant to freezing and drought stresses (Haake *et al.*, 2002). Freezing tests of nonacclimated plants revealed that while only 1% of wild type plants survived freezing at –10°C for 20 hours, the range of survival was 52-100% in the transgenic plants, depending on the expression level of *CBF4*. The same trends were achieved when plants were subjected to 9 days of dehydration; only 2% of the wild type plants survived compared to 87% of transgenic plants (Table 1-1D).

Transgenic Arabidopsis plants overexpressing *CBF1* (Jaglo-Ottosen *et al.*, 1998) exhibited numerous physiological changes associated with CBF expression and increased

dehydration stress resistance including increased membrane stability, increased compatible solute accumulation and the ability to scavenge for reactive oxygen species (Jaglo-Ottosen *et al.,* 1998; Kasuga *et al.,* 1999; Gilmour *et al.,* 2000; Hsieh *et al.* 2002a). Non-acclimated transgenic *Arabidopsis* plants had lower EL_{50} values in response to freezing temperatures than their nontransgenic counterparts indicating that *CBF/DREB*-overexpressing plants suffered less membrane damage.

CBF/DREB-overexpressing plants also accumulated higher levels of osmolytes e.g., (proline and soluble sugars) compared to the nontransgenic controls (Jaglo-Ottosen *et al.,* 1998; Kasuga *et al.,* 1999; Gilmour *et al.,* 2000; Hsieh *et al.* 2002b). *CBF3*-overexpressing *Arabidopsis* lines had approximately 3-fold higher level of total soluble sugars and 5-fold higher proline levels compared to the nontransgenics (Gilmour *et al.,* 2000). This increase in proline was accompanied with an increase in the transcript level of Δ-pyrroline-5-carboxylate synthetase (*P5CS*), a key enzyme in proline synthesis. Haake *et al.* (2002) also reported similar results when overexpressing the *CBF4* gene in *Arabidopsis*, a gene that is only activated under drought and ABA treatment.

In canola, non-cold acclimated plants overexpressing the Arabidopsis-*CBF1*, *CBF2* or *CBF3* genes had EL_{50} value of –6 C° while their nontransgenic counterparts had a value of -3 C° (Jaglo *et al.,* 2001). The EL50 values were –12.7 C° and –8.1 C°, for the transgenic and the nontransgenic controls, when plants were cold acclimated before performing the electro-leakage test (Jaglo *et al.,* 2001). Similarly, constitutive expression of the *Arabidopsis-CBF* genes in canola plants resulted in activation of *Bn115* and *Bn28* (orthologs to the Arabidopsis *COR15*a and *COR6.6* genes) without cold acclimation. Thus, canola appears to have a cold-response pathway that is very close to Arabidopsis

Arabidopsis *CBF* genes also can confer increased dehydration stress resistance to other less closely related species (Hseih *et at.*, 2002a; Hseih *et al.*, 2002b; Owens *et al.*, 2002; Kasuga *et al.*, 2004). Kasuga *et al.* (2004) used the stress-inducible rd29A promoter to drive the expression of *DREB1A/CBF3* gene in tobacco plants. Tobacco plants expressing *DREB1A/CBF3* under the dehydration inducible promoter rd29A were more drought tolerant compared to wild type plants and had higher photosynthetic activity under drought and cold-nonfreezing temperature. Similarly, tomato plants overexpressing the Arabidopsis *CBF1* gene were more dehydration stress tolerant than wild type plants; 83.3% of the transgenic plants survived a 4 week drought treatment while less than 6% of the nontransgenics survived this treatment (Hseih *et al.*, 2002b). The transgenic plants had 3-4 fold more proline under nonstressed conditions compared to the nontransgenic controls. Proline levels increased under stress conditions in both transgenic and nontransgenic plants but were 30-60% higher in plants overexpressing the *Arabidopsis-CBF1* gene (Hseih *et al.*, 2002b). Interestingly, whereas overexpression experiments of *LeCBF1* in *Arabidopsis* resulted in accumulation of *COR* gene transcripts and increased the EL_{50} values by –2.5 °C in the overexpressing lines, no differences in the electro-leakage were observed in transgenic tomato lines overexpressing *AtCBF1*, *AtCBF3* or *LeCBF1* (Zhang *et al.*, 2004).

Owens *et al.* (2002), overexpressed the *A. thaliana-CBF1* gene in strawberry and tested the plants for their ability to withstand freezing temperature compared to nontransgenic plants. The EL_{50} values indicated that *CBF1* expressing strawberry plants were significantly more tolerant to freezing temperatures than wild type (-10.3 °C in the transgenic compared to -6.4 °C in wild type).

III- The objectives of this dissertation

The above examples clearly indicate that various types of genes can be used to engineer dehydration stress tolerance in plants. The objective of this work was to investigate the possibility to engineer enhanced dehydration stress tolerance in cucumber plants following two approaches. Cucumber plants are known to be sensitive to salinity and drought (Mass and Hoffman, 1977). Salinity and drought has been reported to have strong negative effects on cucumber plants, especially on seed germination and seedling emergence, leaf expansion rate, photosynthesis, fruit set, as well as fruit growth rate and fruit quality (Chartzoulakis, 1994; Navazio and Staub, 1994; Ho and Adams, 1994; Tazuki, 1997; Serce *et al.*, 1999; Drozdova *et al.*, 2004). At the time I started this work, there was no published data on the evaluation of genetically engineered plants for enhanced salt or drought tolerance under field conditions. To our knowledge, there are only two published reports, both of which came in late 2004 that evaluated genetically engineered plants for enhanced dehydration stress tolerance under field conditions (Table 1-1). Quan *et al.* (2004) reported enhanced grain yield production by transgenic maize plants expressing a gene for betaine aldehyde dehydrogenase following drought stress period of 21 days, and Xue *et al.* (2004) tested wheat plants expressing the *Arabidopsis* tonoplast H^+/Na^+ antiporter gene for their ability to grow in saline soil and reported higher grain production in the transgenic plants compared to the nontransgenic controls. The aim of this thesis is to:

1-Investigate and test the possibility to improve dehydration-stress tolerance in cucumber (*Cucumis sativus* L.), plants by following two strategies

(a) Induction of mannitol production in cucumber plants by using the celery *M6PR* gene, or

(b) Induction of an array of adaptive responsive mechanisms that are associated with dehydration-stress tolerance in plants by expressing the *A. thaliana*-transcriptional regulators *CBF/DREB1* genes in cucumber plants.

2- Evaluate and validate the performance and fruit yield of transgenic cucumber plants for their ability to withstand dehydration stress under greenhouse and field conditions.

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Chapter II

Expression of the *Arabidopsis thaliana* transcriptional regulators, *CBF1* and *CBF3*, confers dehydration stress tolerance in cucumber (*Cucumis sativus* L.) plants.

Introduction

Environmental factors that impose dehydration stress affect species distribution and plant productivity (Bray, 1994; Bohnert and Jensen, 1996). As the driving force of living organisms, water serves as a medium for the biochemical activities in living cells, is involved in biosynthesis and assembly of molecules into organized structures, and is responsible for generating the needed turgor pressure for plant cell expansion (Tanford, 1978; Xiong and Zhu, 2002). When plants fail to acquire sufficient water, the resultant water deficit causes loss of turgor and/or osmotic stress. Dehydration stress can be caused by several abiotic stresses including drought, freezing temperatures and salinity.

Soil salinity is estimated to affect about 77 million ha (Jain and Selvaraj, 1997; Tester and Davenport, 2003) and may reach up to 50% of the total arable land by the year 2050 (Wang *et al.*, 2003), making it a priority to find ways to alleviate this problem. Similarly, due to climate changes worldwide, a long-term trend of higher temperatures with a decrease in rainfall is expected to negatively impact agricultural production, especially in arid and semiarid regions (Hillel and Rosenzweig, 2002). These trends, along with the need to increase food production for a continuously growing world population will increase drought- and salinity-affected areas.

In general, plant responses to changing environmental conditions are mediated by alterations in gene expression (Guy *et al.*, 1985; Greenway and Munns, 1980; Bohnert and Jensen, 1996; Ingram and Bartels, 1996; Zhu *et al.*, 1997). The first group of genes whose expression is altered in response to dehydration-inducing conditions include those encoding transcriptional factors (TF), mitogen activated protein kinases (MAPKs), dephosphorylation enzymes, and chromatin remodeling proteins (Thomashow, 1999; Knight and Knight, 2001; Shinozaki *et al.*, 2003). Regulation of the first group of genes generates signals that lead to adaptive responses including the induction of genes involved in biosynthesis of osmolytes and compatible solutes, late embryogenesis abundant (LEA) proteins, transporters, and detoxification enzymes (Shinozaki and Yamaguchi-Shinozaki, 1997; Seki *et al.*, 2001; Fowler and Thomashow, 2002; Seki *et al.*, 2002). Thus, induction of response-cascades has been suggested as an effective approach to enhance dehydration stress tolerance (Thomashow, 1999).

Study of processes that lead to freezing and drought tolerance in *Arabidopsis thaliana* revealed critical information about plant response to dehydration stress conditions. Exposure of Arabidopsis plants to low, non-freezing temperatures or to drought stress conditions leads to increased expression of a group of genes, the *CO*ld *R*esponsive (*COR*) and *R*esponsive to *D*rought (*RD*) genes that are induced under both cold acclimation and drought conditions (Gilmour *et al.*, 1992; Hovarth *et al.*, 1993; Lin and Thomashow, 1992; Thomashow, 1999). The *COR* and *RD* genes are characterized by the presence of a *cis*-acting element (CCGAC) within their promoter regions known as the CRT/DRE element [CRT (C-repeat)/DRE (Drought Response Element)], which confers responsiveness to low temperature, dehydration and salinity (Baker *et al.*, 1994;

Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al.*, 1997). The CRT/DRE element is bound by the CBF/DREB (CRT Binding Factor/DRE binding) transcription factors which are characterized by a putative nuclear localization domain, an activation domain and an AP2 DNA binding domain (Stockinger *et al.*, 1997; Liu *et al.*, 1998).

Transgenic *Arabidopsis* plants overexpressing *CBF1* showed elevated expression of target *COR* transcripts and were more tolerant to freezing stress than their non-transgenic counterparts as determined by electrolyte leakage and whole plant assays (Jaglo-Ottosen *et al.*, 1998). Similarly, transgenic *Arabidopsis* plants overexpressing *DREB1a* had enhanced drought and salinity resistance (Kasuga *et al.*, 1999). Transgenic plants also exhibited several physiological changes associated with increased dehydration stress resistance including an increase in proline and total soluble sugars accumulation compared to their nontransgenic counterparts (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Jaglo *et al.*, 2001; Seki *et al.*, 2001; Seki *et al.*, 2002; Haake *et al.* 2002; Kasuga *et al.* 2004). More recently, elevated expression of *CBF* was shown to cause large-scale-metabolome changes in Arabidopsis including marked increase in soluble sugars and amino acids leading to the suggestion that increased accumulation of some soluble carbohydrates (galactinol, glucose, raffinose and fructose) and proline is a signature of the *CBF* regulon (Cook *et al.*, 2004).

Induction of *CBF* genes also confers increased dehydration stress resistance to other less closely related species. Tobacco plants expressing *CBF3/DREB1A* had higher photosynthetic activity under drought conditions compared to wild type plants (Kasuga *et al.*, 2004); tomato plants overexpressing the *Arabidopsis-CBF1/DREB1B* gene showed elevated tolerance to drought (Hseih *et al.*, 2002a, b) and salinity (Lee *et al.*, 2003); and

strawberry plants expressing the *Arabidopsis CBF1/DREB1B* gene had less membrane damage in response to freezing temperatures than their nontransgenic counterparts (Owens *et al.*, 2002).

In the present work, we sought to investigate and test the possibility to improve dehydration-stress tolerance in cucumber (*Cucumis sativus* L.) by expressing the *A. thaliana* transcriptional activators *CBF1* and *CBF3*. Cucumber is known to be sensitive to salinity and drought especially in arid and semi-arid areas (Mass and Hoffman, 1977).

Materials and methods

Plant constructs and transformation

The *Agrobacterium* plant transformation constructs containing *CBF1* or *CBF3*, under control of the CaMV 35S promoter were kindly provided by M. F. Thomashow (Jaglo-Ottosen *et al.*, 1998). Cucumber transformation was performed using the following procedure derived from the methods of Tabei *et al.* (1998). Cucumber seeds, *cv.* Straight 8 (Hollar Seeds, Rocky Ford, Co.) were decoated and surface sterilized for 15 min with 15 % Clorox solution (1% sodium hypochlorite solution) with 1-2 drops of Tween 20 (Sigma-Aldrich, St. Louis, MO). Seeds were rinsed several times with sterilized distilled water and placed overnight in the dark on hormone-free MS basal salt mixture (Murashige and Skoog, 1962) supplemented with 30g/l sucrose and 2.5 g/l Phytagel (Sigma-Aldrich, St. Louis, MO). Cotyledon explants were prepared by separating from the embryo, removing the outer edges, and cutting into 4-6 explants. Explants were co-cultivated with *Agrobacterium tumefaciens* strain C58 (Deblaere *et al.*, 1985) , by dipping in a 1/20 diluted overnight culture for 10 min. Explants were blotted on sterilized filter paper then cultured onto C1 shoot induction medium [MS basal salt mixture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mg/l benzyl amino purine (BAP) (Sigma-Aldrich, St. Louis, MO), 1 mg/l abscisic acid (ABA) (Sigma-Aldrich, St. Louis, MO), 30 g/l sucrose and 2.5 g/l Phytagel]. Plates were wrapped in aluminum foil and incubated in the dark on culture shelves at 25 C°. Three days later, explants were rinsed several times with sterilized H_2O, blotted on filter paper and cultured onto C2 medium [C1 medium supplemented with 400 mg/l Timentin® (GlaxoSmithKline, Research Triangle Park, NC)]. A week later, explants were transferred on to a selection

medium C3 [C2 medium supplemented with 75 mg/l kanamycin (Sigma-Aldrich, St. Louis, MO)] and were monitored for 3-4 weeks for shoot growth and elongation. Emerging shoots were then placed onto selection medium C4 [C3 medium supplemented with 8 g/l agar (Sigma-Aldrich, St. Louis, MO)]. Shoots were continuously grown on media supplemented with 75 mg/l kanamycin (Sigma-Aldrich, St. Louis, MO) for selection. Shoots were rooted in 250 ml Magenta boxes (Magenta Corp, Chicago, IL) containing 50 ml of MS medium with 30 g/l sucrose and 7 g/l agar.

PCR-verified transgenic plants were transferred into 10 cm pots filled with Baccto soil mix (Michigan Peat Co., Houston TX) and kept in the growth chamber for 2-3 weeks (16/8 h light, temperature was maintained at 22 C°, Relative humidity was maintained around 50%). Acclimated shoots were transferred to the greenhouse for 2-3 weeks before transferring into 3.6 l pots filled with Baccto soil mix for seed production. Transgenic *CBF1* or *CBF3*-cucumber plants were self pollinated to produce fruits in the greenhouse; 6-8 weeks old fruits were harvested and seeds were extracted, dried and stored in a dry area.

DNA and RNA isolation, PCR, and Northern blot analysis

DNA was extracted from 200 mg young leaf samples using the Wizard DNA purification kit (Promega, Cat # A7951, Madison, WI). For RNA isolation, young leaf tissues were collected and immediately frozen and ground in liquid nitrogen and extracted using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA). RNA quantity and quality was determined by measuring absorbance at 260 nm and by gel electrophoresis (Sambrook and Russell, 2001). Quantitation of RNA was performed with

a Molecular Imager FX Pro multi-imager system (Bio-Rad® Laboratories, Hercules, CA). PCR was carried out using *CBF1*- and *CBF3*-specific primers (Stockinger *et al.*, 1997). Northern hybridization analysis was conducted using ^{32}P labeled *CBF1* and *CBF3* cDNA full length probes following the procedure of Sambrook and Russell (2001).

Salinity experiments

T_1 segregating progeny of transgenic T_0 plants were planted in the greenhouse and screened by PCR for the presence of the introduced *CBF* genes at the 2-3-leaf stage. Transgenic seedlings, non-transgenic T_1 segregant progeny, (Azygous) and wild type 'Straight 8' (WT) plants were transplanted into 15cm clay pots filled with vermiculite (Therm-o-Rock East Inc, Grade no. 3A, Washington, PA). To minimize evaporation, the soil surface was covered with plastic disks that fitted at the top of the clay pots. In the first greenhouse experiment, two families (A4 and B3) were tested with three levels of NaCl (0 mM, 100 mM and 200 mM) in a randomized complete block design with three replications. Salt treatment was initiated seven days after seedling transplant with a stepwise increase of 50mM NaCl at two day intervals to reach 200mM NaCl. Plants were fertilized with 300 ppm 20:20:20 fertilizer once a week throughout the experiment. Young leaves (3-4 cm diameter) were collected every four days for sugar and proline analysis as described below.

In the second and third greenhouse experiments, 10 and 9 *CBF*-families were tested (Table, 1), at three NaCl levels (0, 50 and 100 mM), in a randomized complete block design with six replications. Salt treatment was initiated 15 days after transplanting, with a stepwise increase of 50 mM NaCl at three day intervals to reach 100mM NaCl. Total

soluble sugars and proline were measured at time 0 (immediately before the salt treatment) and 15 days post initiation of salt treatment (dps). Growth parameters (total above ground fresh and dry weight, plant height and number of leaves) were measured at 15 dps. Statistical analysis was performed using Microsoft Windows® EXCEL and SAS® 9.1.2 programs.

Drought experiments

T_2 segregating progeny of transgenic families were planted in the greenhouse and screened by PCR for the presence of the *CBF* gene at the 2-3-leaf stage. Transgenic seedlings, non-transgenic segregants, and wild type 'Straight 8' plants were transplanted into 3.6 l pots filled with Baccto soil mix (Michigan Peat Co., Houston TX). A split plot design with water as the main effect was used with three replications in experiment 1 and four replications in experiments 2 and 3. Genotypes were assigned randomly within each main plot. Drought treatment was carried out by withholding water until the first sign of wilting (about nine days in experiments 1 and 2 and 12 days in experiment 3) followed by one day of irrigation to full soil saturation. In the first experiment, this cycle was repeated three times, in the second experiment this cycle was repeated twice and in the third experiment this cycle was performed one time. Plant heights were determined after each cycle of drought; total above-ground fresh and dry weight was determined at the end of the experiment. Leaf tissue samples were collected immediately before the beginning of the treatment, as well as after each drought cycle for proline and sugar analysis.

Sugar and proline analyses

One young leaf (3-4 cm diameter) was collected from each plant at each sample date, freeze-dried for 48 hr, ground, and split into two aliquots, one for proline and one for sugar analysis. Proline analysis was carried out using the procedure described by Troll and Lindsey, (1955) (Appendix A). Total soluble sugar analysis was performed by the phenol/sulfuric acid method of Dubois *et al.*, (1956) (Appendix A).

Chlorophyll fluorescence

Components of chlorophyll fluorescence were quantified using a portable Plant Efficiency Analyzer fluorometer (Hansatech, Norfolk, UK). Measurements were performed in the greenhouse, using attached leaves. Three leaves, 7-9 cm in diameter, and well-exposed to sun light, were chosen per plant for sequential measurements. After 30-40 min dark adaptation period, minimal fluorescence (F_0), maximal fluorescence (Fm), variable fluorescence (Fv) and fluorescence efficiency (Fv/Fm) were measured immediately before the beginning of the drought treatment, and after 2, 6 and 12 days of drought.

Results

Introduction and expression of the Arabidopsis *CBF1* and *CBF3* transgenes in cucumber plants

Thirteen transgenic cucumber plants were produced, six with *CBF1* and seven with *CBF3*. Presence and expression of *CBF* genes was verified in the T_0 plants by PCR and northern blot analysis, respectively (data not shown). Successful transfer of the *CBF1* and *CBF3* genes into T_1 and T_2 progeny was verified using PCR analysis; χ^2 analysis of segregating progeny was consistent with single gene insertion in each case (Table 2-1). Analysis of *CBF* expression in transgenic T_2 plants showed varying transcription levels among the different lines (Figure 2-1).

Transgenic cucumber plants expressing *CBF* genes have elevated levels of proline and total soluble sugars in leaves compared to the non-transgenic controls

Greenhouse experiments were performed to evaluate the response of *CBF1* and *CBF3*-transgenic cucumber plants to salt stress. Seeds were only available for two lines, A3 and B4. In the absence of salt stress, *CBF*-expressing cucumber plants accumulated significantly higher levels of both free proline (up to 5 fold higher) and total soluble sugars (2 fold higher), compared to the nontransgenic controls (Figure 2-2A, D; ANOVA, $P< 0.01$ and $P<0.05$ for proline and soluble sugars, respectively). Under salt stress conditions, *CBF*-cucumber plants accumulated higher levels of proline and soluble sugars compared to the non-stressed conditions (ANOVA, $P<0.01$). The azygous (non-

Table 2-1. Segregation analysis of T_1 transgenic cucumber lines expressing the Arabidopsis *CBF1* and *CBF3* genes. Presence or absence of the gene was determined by PCR analysis

Line	**Gene construct**	**T_1 segregation (trans: non)**	**χ^2 (3:1)**
A1	*35S::CBF1*	46 : 11	0.71 ns
A3	*35S::CBF1*	49 : 13	0.30 ns
A4	*35S::CBF1*	51 : 16	0.09 ns
A5	*35S::CBF1*	54 : 14	0.15 ns
A6	*35S::CBF1*	55 : 19	0.01 ns
B1	*35S::CBF3*	44 : 12	0.21 ns
B4	*35S::CBF3*	46 : 10	1.14 ns
B5	*35S::CBF3*	49 : 10	1.63 ns
B6	*35S::CBF3*	47 : 9	0.69 ns
B7	*35S::CBF3*	40 : 12	0.02 ns

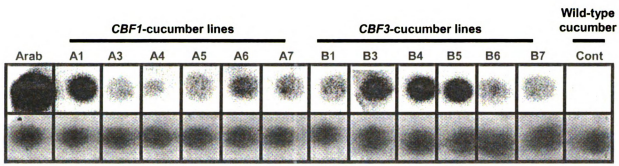

Figure 2-1. Expression of *A. thaliana* transcriptional regulators *CBF1* and *CBF3* genes in T2 transgenic cucumber lines. One young leaf was collected from each of four PCR-positive plants/line and pooled for RNA extraction. 15 ug total RNA was directly blotted onto the membrane. The *A. thaliana* positive control was "cold acclimated for 3 hrs. at 4 C" before isolating RNA. Top panel: hybridization with *CBF1* probe. Bottom panel: hybridization with *eIF4a* as a loading control.

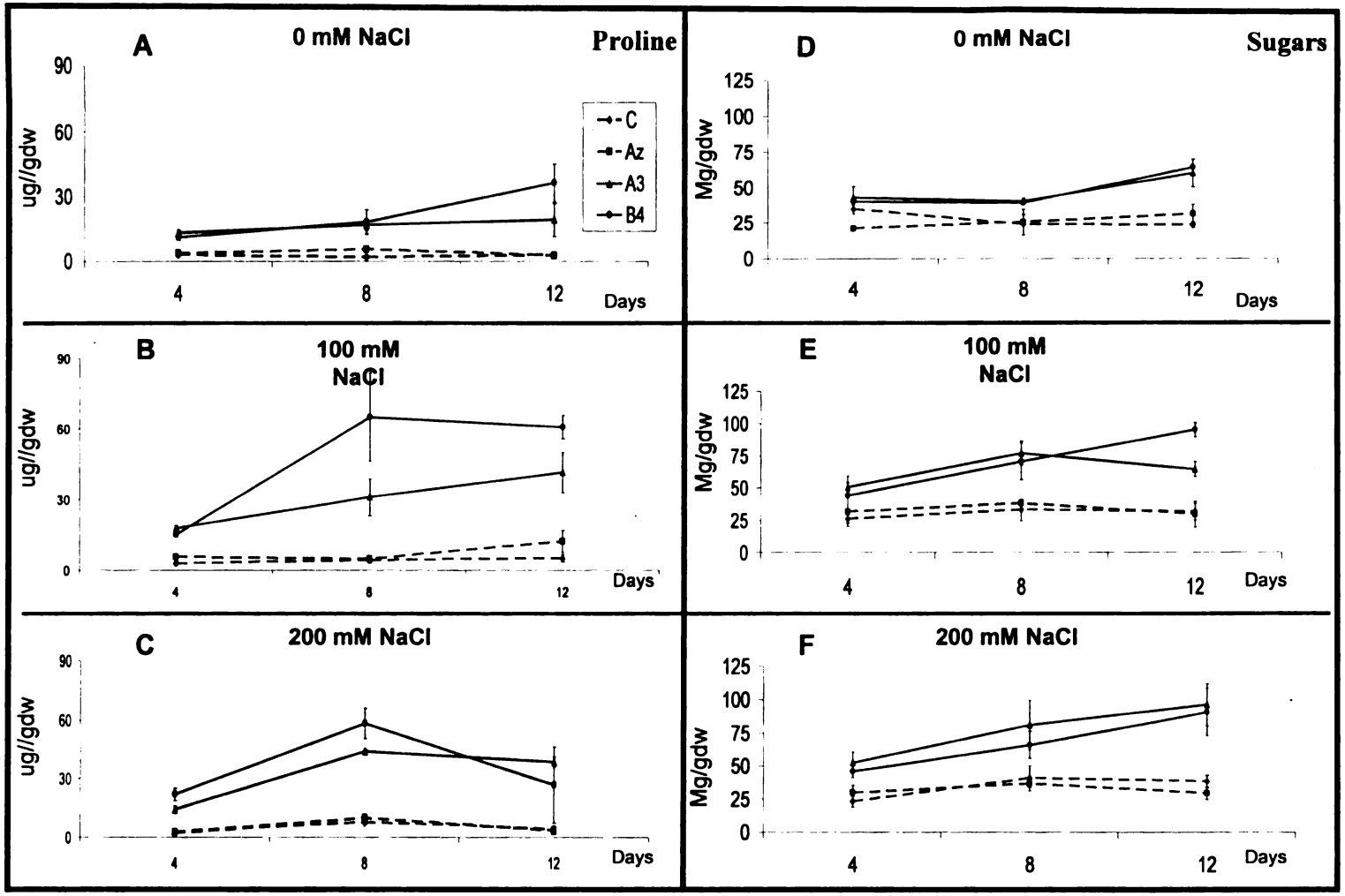

Figure 2-2. Free proline content (A-C) and total soluble sugars (D-F) of two transgenic lines (A3, B4), non-transformed control (C) and nontransgenic segregants (AZ) under control and salt treated conditions. Data are the mean of 3 replicates with 3 plants/replicate/treatment ± SE. Leaf tissues were collected 4, 8 and 12 days post initiation of salt treatment.

transgenic segregant) and wild-type plants had equivalent levels of proline and soluble sugars accumulation, indicating the observed differences from *CBF*-plants were due to the presence of *CBF* genes. Exposure of cucumber plants to 200 mM NaCl did not cause an increase in either proline or sugar concentration (Figure 2-2C, F), compared to the 100 mM NaCl level (Figure 2-2 B, E). In addition, plants irrigated with 200 mM NaCl exhibited severe leaf discoloration and necrosis; therefore, 100 mM NaCl was the maximum concentration used in subsequent experiments.

Additional *CBF* families (10 and 9 families) were tested in the second and third experiments, equivalent results were obtained in both experiments. Significant differences were observed between transgenic and nontransgenic families for proline and soluble sugar accumulation prior to the initiation of salt treatment; on average, *CBF*-expressing cucumber plants had 24.5 ± 3.1 mg/gdw soluble sugar and 8.9 ± 0.4 μg/gdw proline, compared to an average of 15.8 ± 1.2 mg/gdw soluble sugars and 2.3 ± 0.3 μg/gdw proline in the nontransgenic controls (ANOVA, $P<0.01$ and $P<0.05$ for proline and soluble sugar, respectively). Fifteen days post initiation of salinity treatment, proline and total soluble sugar accumulation was significantly greater in the transgenic families compared to the nontransgenic controls at all salt levels (Figure 2-3A and B; Table 2-2). There were no significant differences between the *CBF1* and *CBF3* lines (Table 2-2).

The transgenic cucumber plants showed a progressive increase in proline and soluble sugar accumulation at both 50 and 100 mM NaCl levels, while proline and sugars levels in the nontransgenic controls did not increase above 50 mM NaCl (Figure 2-3). The

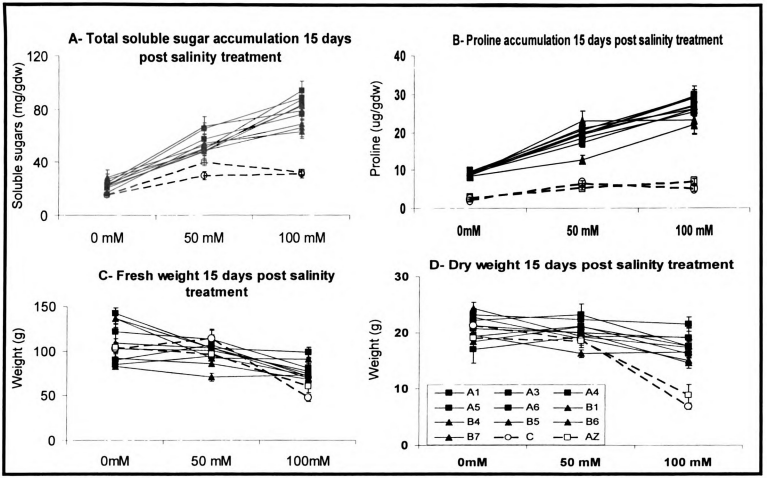

Figure 2-3. Total soluble sugar, proline content, above ground fresh weight and dry weight of ten transgenic lines (*CBF1* lines A1, A3, A4, A5, and A6 and *CBF3* lines B1, B4, B5, B6, and B7), non-transformed control (C) and azygous segregants (AZ) 15 days post initiation of 50 or 100 mM NaCl treatment. Data are pooled from two experiments with 5 and 6 replicates, respectively and with two plants/replicate/treatment/experiment. All genotypes were tested in both experiments except B7, which was only tested in Experiment 1. Equivalent results were obtained in both experiments. Transgenic lines are indicated by closed symbols, nontransgenic lines by open symbols.

Table 2-2. Levels of soluble sugar, free proline, above ground fresh weight and dry weight, and plant height in *CBF*-transgenic and control lines 15 days post initiation of NaCl treatment.

	0 mM NaCl			50 mM NaCl			100 mM NaCl		
	CBF1 [1]	*CBF3* [2]	Non-trans[3]	*CBF1*	*CBF3*	Non-trans	*CBF1*	*CBF3*	Non-trans
Sugars	28.3 ± 2.6	32.2 ± 5.2	14.5 ± 0.9**	53.7 ± 3.4	54.5 ± 5.9	34.7 ± 3.0*	85.9 ± 5.6	71.9 ± 7.3	31.6 ± 2.8**
Proline	9.7 ± 0.6	8.9 ± 0.7	2.7 ± 0.3**	19.2 ± 1.4	19.2 ± 2.2	5.9 ± 0.6**	27.4 ± 1.2	25.5 ± 3.0	5.9 ± 0.7 **
Fresh weight	108.3 ± 9.8	111.3 ± 8.3	103.5 ± 5.2	103.6 ± 7.7	99.9 ± 5.3	105.3 ± 9.2	79.8 ± 7.7	72.9 ± 5.6	53.5 ± 5.5 *
Dry weight	20.5 ± 1.5	21.3 ± 1.7	20.1 ± 0.7	21.2 ± 1.2	19.1 ± 0.9	18.6 ± 1.1	18.3 ± 1.6	16.6 ± 1.1	8.9 ± 0.8 **
Height	145.2 ± 10.1	152.4 ± 9.2	137.0 ± 6.8	133.5 ± 9.6	140.4 ± 6.8	141.4 ± 10.7	117.0 ± 11.2	114.7 ± 7.9	114.8 ± 5.7

Data are pooled from two experiments with six replicates/treatment and 12 genotypes for experiment 2 and five replicates/ treatment and 11 genotypes for experiment 3. All genotypes were tested in both in both experiments except B7 line, which was only tested in Experiment 1. Equivalent results were obtained in both experiments.

Each value is the mean ± SE.

*, ** Non-transgenic lines are significantly different from the transgenic lines by ANOVA orthogonal contrasts, $P< 0.05$ and $P< 0.01$, respectively.

1- Mean of five *CBF1* lines (A1, A3, A4, A5, and A6).

2- Mean of five *CBF3* lines (B1, B4, B5, B6, and B7).

3- Mean of wild type Straight 8 and non-transgenic segregant progeny.

amount of accumulation of the two types of compounds within a given genotype was highly correlated (r^2=0.89; Figure 2-4A), indicating that they are part of a coordinated response. Salinity treatment caused a marked increase in soluble sugar and proline accumulation in *CBF*-plants beyond what would be expected from an additive effect of the two separate components, *CBF* in the absence of stress, and salt stress in the absence of *CBF* (Figure 4B, C arrows). Thus, the effect of the *CBF* gene on solute accumulation increased in response to salt stress.

Given the increased accumulation of compatible solutes in 35S:*CBF* plants in response to salinity stress, *CBF* transcript levels were compared in the presence or absence of salinity stress (Figure 2-5). The lack of differences is consistent with expected results for expression driven by the constitutive CaMV35S promoter, and this also indicates that there were not differences in *CBF* mRNA stability associated with salt stress.

CBF-expressing cucumber plants showed less reduction in fresh and dry weight under salt stress conditions

Growth data were collected at the end of experiments 2 and 3. At 0 mM NaCl, there were no significant differences between *CBF*- transgenic families and the nontransgenic controls for height, and above ground fresh and dry weight (Figure 2-3 C, D; Table 2-2). At 50 mM NaCl, neither the *CBF* nor the nontransgenic lines showed significant differences in growth. At 100 mM NaCl, nontransgenic controls exhibited a significant reduction in fresh weight (48%), dry weight (56%), and height (16%) relative to non-salt stressed plants. While 100 mM NaCl also affected growth of the transgenic cucumber

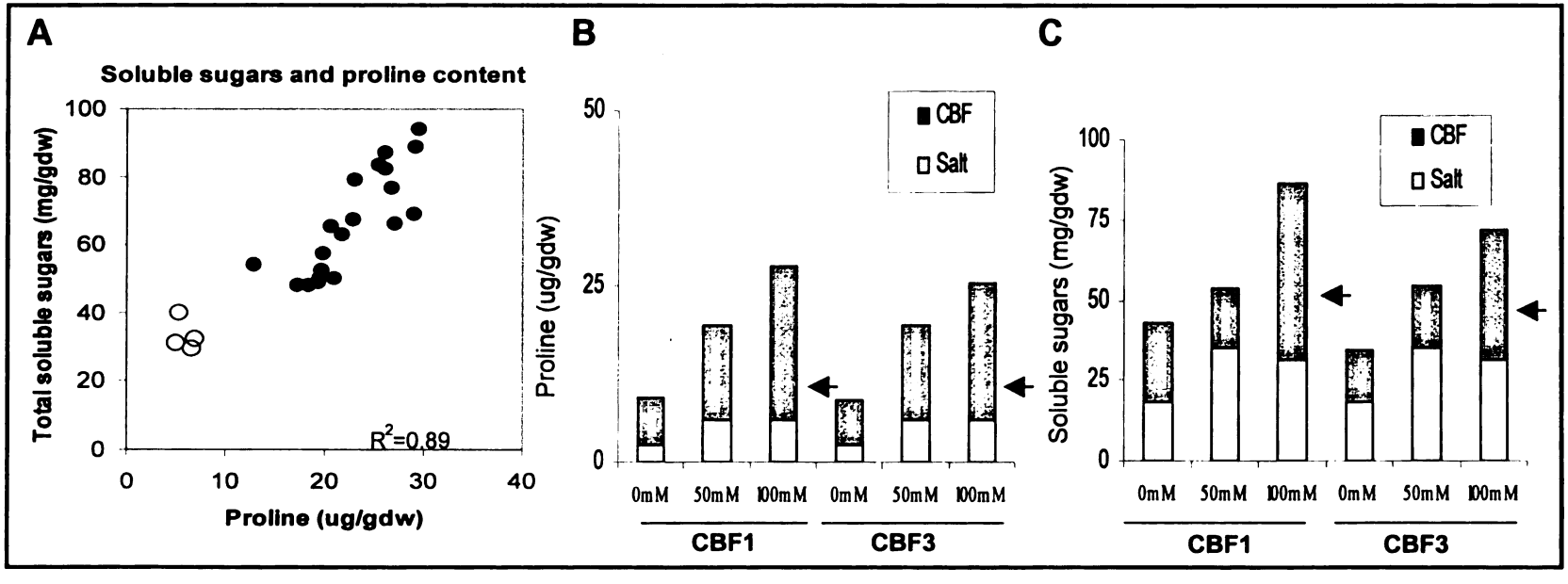

Figure 2-4. A- Correlation between soluble sugar and proline content in cucumber plants 15 days post initiation of 50 and 100 mM NaCl treatment. Open symbols represent non-transgenic controls, closed symbols represent transgenic lines. Data are from experiments 2 and 3 as described earlier. B and C- Relative contribution of salinity and CBF. White bars indicates contribution of salinity in the absence of CBF to proline (B) and soluble sugars (C) accumulation. The contribution of CBF in the absence of salinity is marked by arrows.

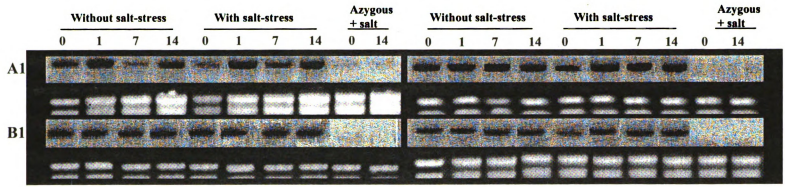

Figure 2-5. Expression of *A. thaliana*-transcriptional regulators *CBF1* and *CBF3* genes in four T2 cucumber expressing lines and non-transgenic azygous lines growing in the presence or absence of 100 mM NaCl for 2 weeks. One young leaf was collected from each of three PCR-positive plants/line/treatment at 0, 1, 7 and 14 days post initiation of salt stress. 15 ug total RNA was loaded in each lane. The top panels: hybridization with *CBF1* probe. The bottom panel: EtBr stained RNA samples.

lines (a reduction of 30%, 16%, and 22% in fresh weight, dry weight, and height respectively), growth inhibition was significantly less severe relative to the nontransgenic controls for fresh weight (ANOVA, $P< 0.05$) and dry weight (ANOVA, $P< 0.01$). Plants expressing *CBF1* or *CBF3* genes had almost twice the dry weight as the nontransgenic controls. *CBF1* and *CBF3* lines performed equivalently (Table 2-2). Growth in the presence of 100 mM NaCl appears to be correlated with levels of soluble sugars and proline levels ($r^2 = 0.79$ and 0.78, respectively).

Transgenic CBF-cucumber plants have elevated level of compatible solutes and less reduction in photosynthetic capacity in response to drought stress

CBF1 and *CBF3*-expressing cucumber plants were also tested under drought stress conditions. The same trends were observed in all three experiments. Under well-irrigated conditions, significant differences in proline levels between the *CBF*-expressing families and the nontransgenic controls were observed while significant differences in soluble sugar were observed later in the experiment (Figure 2-6A, C; ANOVA, $P<0.01$). After the first cycle of drought (until the first symptoms of wilting of lower leaves), *CBF*-expressing cucumber plants accumulated approximately twice the amount of soluble sugar and approximately 5-fold higher levels of proline than their nontransgenic counterparts (Figure 2-6B, D; ANOVA, $P<0.01$). Although after a second cycle of drought proline and soluble sugar levels remained higher in the *CBF*-plants than the non-transgenics, soluble sugar level in the transgenic plants did not increase compared to the first cycle and levels of proline declined (Figure 6D). Moreover, the wilted lower leaves did not recover after the second re-irrigation. No differences in proline or soluble sugar

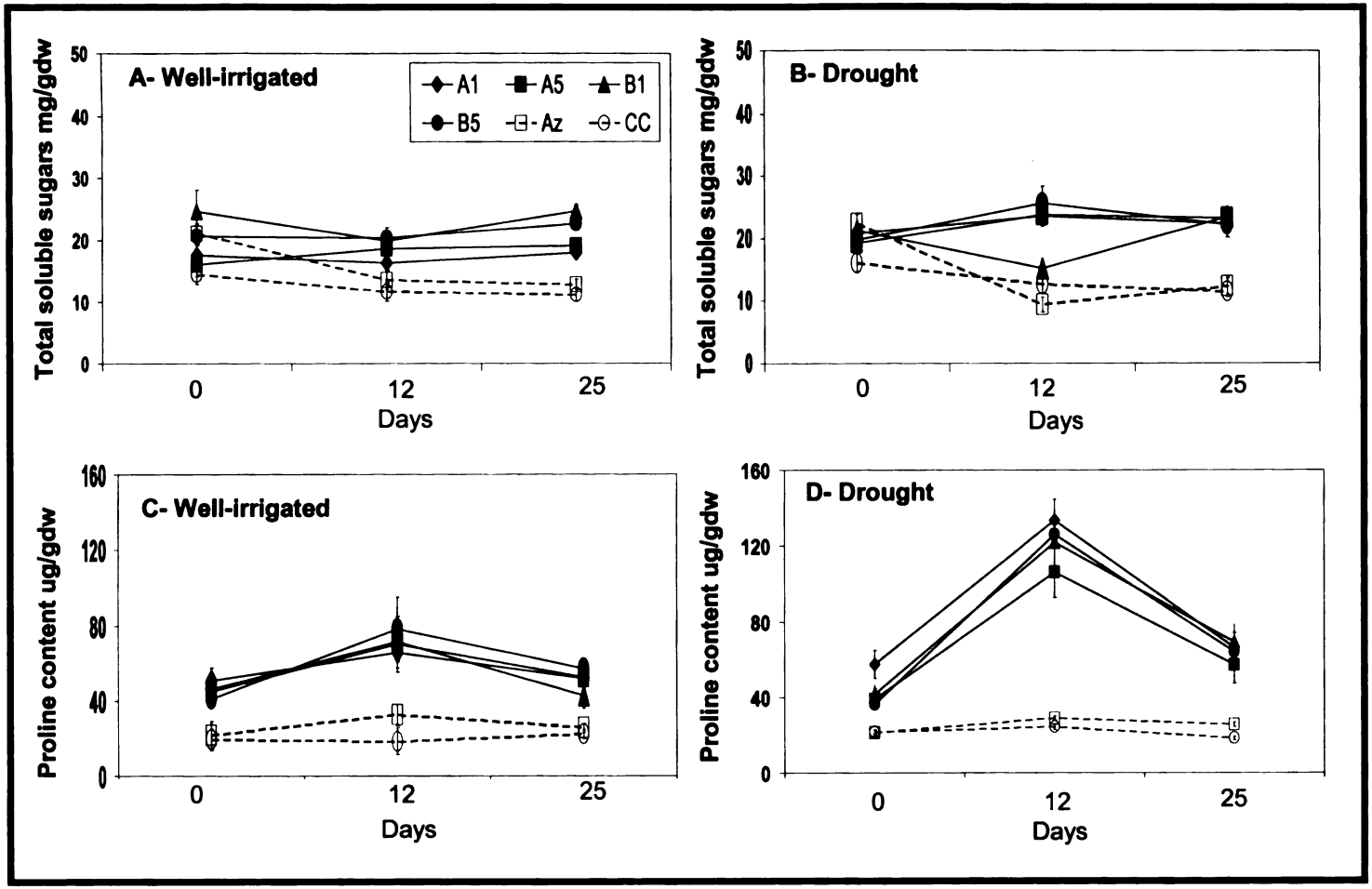

Figure 2-6. Total soluble sugar and free proline content of *CBF1* and *CBF3*-expressing cucumber lines and non-transgenic controls under well-irrigated and drought conditions. Data are the mean ± SE of 3 replicates with 3 plants/replicate/treatment. Leaf tissues were collected 0, 12 and 25 days post initiation of treatment.

levels were observed between the azygous and the wild-type plants. Chlorophyll fluorescence was used to determine the maximum photochemical efficiency in cucumber plants under drought stress conditions. Measurements were taken immediately before the beginning of drought treatment and after 2, 6 and 12 days of drought in experiment 3.

There were no significant differences between the *CBF*-transgenic cucumber plants and the nontransgenic controls under well irrigated conditions. Significant differences were detected after 12 days of drought treatment (Figure 2-7), at which time stressed plants began to show the first obvious signs of wilting in the lower leaves for both the transgenic and the nontransgenic controls. The photosynthetic activity of the upper, non-wilted leaves of *CBF*-expressing plants had 50% higher fluorescence value than the nontransgenic controls, reflecting greater stability of photosystem II (PSII) under drought stress conditions (Figure 2-7).

CBF-expressing cucumber plants showed less growth reduction under drought stress conditions

Growth parameters (plant height, above ground fresh and dry weight) were collected at the termination of the drought experiments. Under well-irrigated conditions, no significant differences were observed between CBF-transgenic families and the nontransgenic controls for height, above ground fresh weight and dry weight (Figure 2-8). In response to a cycle of drought, transgenic-CBF lines showed significantly less reduction in plant height and dry weight than did the nontransgenic controls (average reduction of 45% and 46% for height and dry weight vs. 25% and 15%, for CBF lines; ANOVA, $P < 0.05$). The CBF-transgenic lines did not show significantly less reduction in

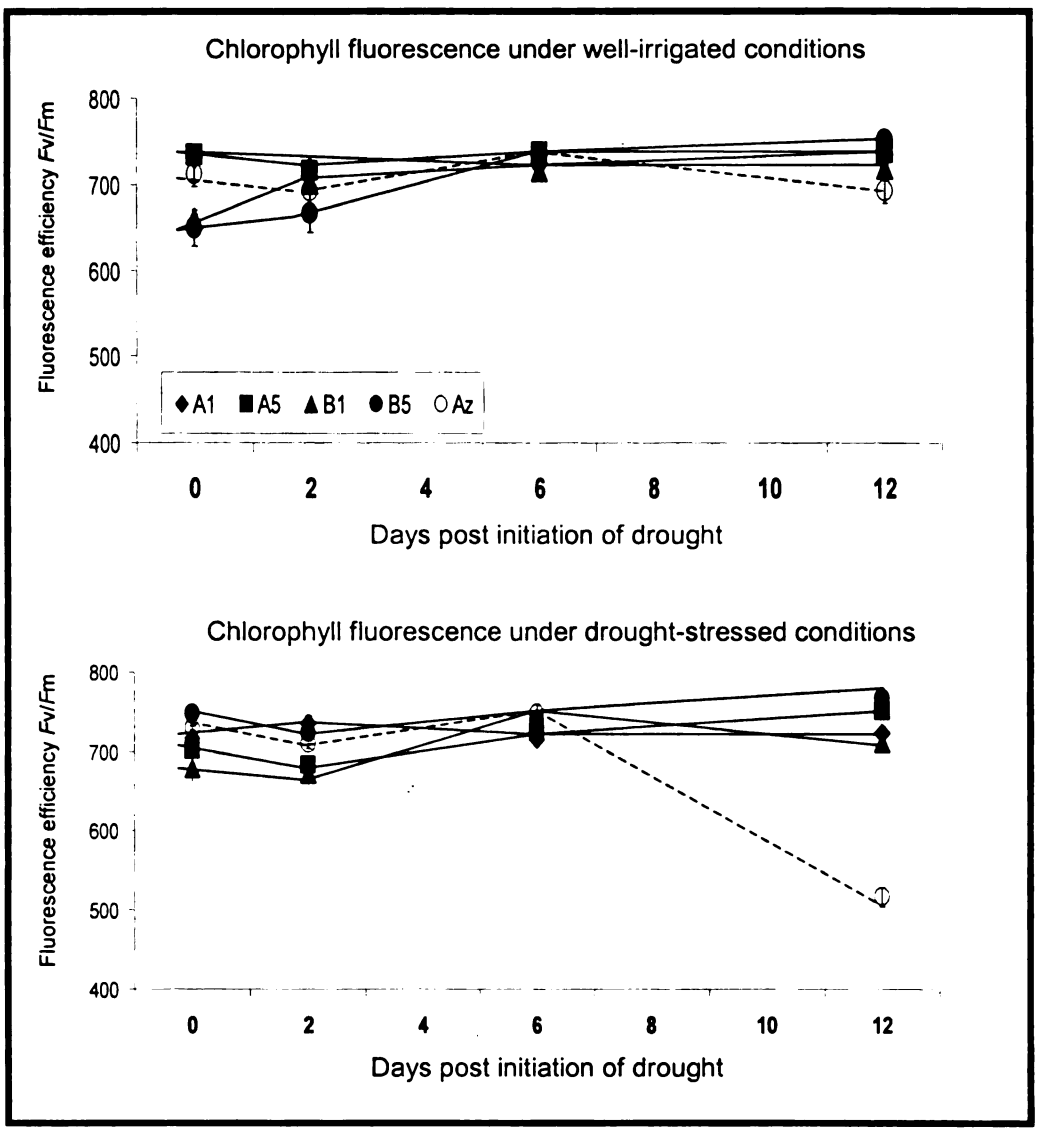

Figure 2-7. Chlorophyll fluorescence (*Fv*/*Fm*) in *CBF*-expressing cucumber plants and azygous non-transgenic plants, under well-irrigated and drought-stressed conditions. Each data point represents the mean of four replicates with three plants/replicate/treatment ± SE.

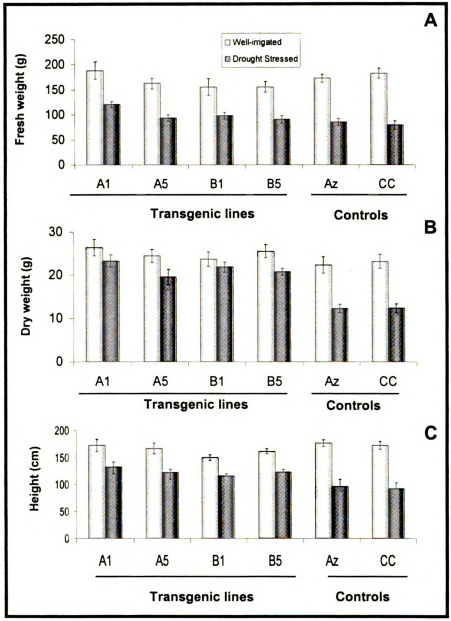

Figure 2-8. Effect of drought conditions on above ground fresh weight (A), dry weight (B) weight, and plant height (C) of *CBF1*, and *CBF3*-transgenic lines, non-transformed controls (CC), and non-transgenic segregants (AZ). Measurements were recorded after one cycle of drought stress. Light bars: well-irrigated-plants; dark bars: drought stressed-plants. Values are the mean ± SE of three replicates/treatment with 3 plants/replicate.

fresh weight (39%) than the nontransgenic controls (50%) (ANOVA, $P < 0.12$). Equivalent trends were observed after two cycles of droughts; with significantly less reduction in height (48% vs. 29%) and dry weight (45% vs. 24%) for the CBF lines compared to the nontransgenic controls.

Discussion

Several lines of transgenic cucumber plants expressing *CBF1/DREBb* and *CBF3/DREBa* genes under the control of the constitutive *CaMV35S* promoter were produced and tested for physiological changes and response to dehydration stresses. Previous studies with the *CBF* gene family demonstrated that overexpression of *CBF* in *Arabidopsis* caused significant increase in accumulation of compatible solutes, especially soluble sugars and proline (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Seki *et al.*, 2001; Seki *et al.*, 2002; Haake *et al.* 2002; Cook *et al.*, 2004).

Under nonstressed conditions, cucumber plants expressing *CBF1* and *CBF3* genes had higher soluble sugar and proline levels than the nontransgenic controls; these levels continued to increase throughout the experiment, indicating that the heterologous-*CBF1* and *CBF3* genes induce pathways in cucumber similar to those in *A. thaliana.* The levels of increase in soluble sugar accumulation (2-3 fold) and proline (5-fold) were comparable to that observed in *CBF*-overexpressing *Arabidopsis* plants (Gilmour *et al.*, 2000; Gilmour *et al.*, 2004). Similar findings were also observed when overexpressing *CBF/DREB* genes in the heterologous species, tomato and tobacco (Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b; Lee *et al.*, 2003; Kasuga *et al.*, 2004).

Accumulation of soluble sugar was highly correlated with proline accumulation, indicating that syntheses of these compounds are coordinately regulated in the *CBF*-expressing plants. In *Arabidopsis*, the promoters of genes for key enzymes involved in proline and sugar biosynthesis (e.g., *P5CS* and *galactinol synthase*) have binding sites for CBF and have been shown to be upregulated in response to *CBF* expression (Seki *et al.*, 2001; Seki *et al.*, 2002; Fowler and Thomashow, 2002; Gilmour *et al.*, 2000; Vogel *et al.*,

2005). While orthologous genes in cucumber may also include CBF-binding sites, direct transcriptional regulation by *CBF* would not fully explain the observed increase in proline and sugars in *CBF*-cucumbers in response to salt stress. Exposure to salinity increased the levels of soluble sugar and proline in *CBF*-plants beyond a simple additive effect of the individual contributions of salinity and *CBF*. The increase in proline and sugar accumulation in response to salt stress was not a direct result of salt-induced increase in *CBF*-transcript levels, indicating that the enhanced accumulation, is at least in part downstream of *CBF* per se. It is possible that post- transcriptional or translational regulation of key enzymes that are critical for production and accumulation of sugars and proline is affected. Alternatively, the initial induction of dehydration stress-related responses by *CBF* may result in cascades of signals that indirectly, lead to changes in transcription rate of key proline and sugar biosynthetic genes.

Transcriptional profiling in *CBF*-overexpressing *Arabidopsis* plants indicated activation of several classes of genes including genes encoding transcriptional regulators (e.g., AP2 containing proteins, zinc-finger containing proteins, MYB-family transcriptional activators), genes involved in stress-signaling (MAP-kinases, calcineurin and calcineurin-like proteins), and genes involved in metabolism and catabolism (Fowler and Thomashow, 2002, Sike *et al.*, 2001; Maruyama *et al.*, 2004; Vogel *et al.*, 2005). The responding genes including transcriptional factors could be clustered into groups whose expression increased or decreased at different time periods following transfer to the cold, suggesting sequential induction (Fowler and Thomashow, 2002, Sike *et al.*, 2001; Maruyama *et al.*, 2004; Vogel *et al.*, 2005). These observations indicate involvement of multiple regulatory systems which can be initially triggered by *CBF*. Indeed, not all of

the upregulated genes include the CDT/DRE element in their promoters, suggesting that these genes may be secondarily regulated by one of the *CBF*-induced transcription factors (Fowler and Thomashow, 2002; Vogel *et al.,* 2005). The up-regulation of these genes could eventually affect other pathways in plants that could also induce production of compatible solutes. Thus, *CBF*-induced pathways may influence subsequent responses, perhaps priming the *CBF*-cucumbers to allow for enhanced response to stresses.

There appears to be a limitation, however, to the increase in proline and sugar in response to stress, even in the *CBF*-plants. In our conditions, the transgenic cucumber plants did not accumulate higher levels of soluble sugars and proline when treated with 200 mM NaCl or when imposing a second drought cycle. This may indicate a limitation in the adaptive responses that can be induced by the *CBF/DREB* genes, or limitation of the ability of a species to respond to the unfavorable stress conditions. In non-transgenic plants, accumulation of soluble sugar and proline did not increase beyond levels obtained with the 50 mM salt treatment, or with imposition of drought, while levels of soluble sugars and proline continued to increase in the *CBF*-expressing cucumber plants (at 100 mM NaCl and after 2 cycles of drought), suggesting that the *CBF* increased the range of response in cucumber plants to a higher limit.

These differences in range of response were also reflected in plant growth. In the presence of 50 mM NaCl, no significant differences in above ground fresh and dry weight and plant height were observed among *CBF*-transgenic and nontransgenic lines, indicating that at 50 mM NaCl, wild-type cucumber plants were able to adapt and adjust to this salinity level. In the presence of 100 mM NaCl, the cucumber plants expressing the Arabidopsis-*CBF1* and *CBF3* genes showed significantly less reduction in above-

ground fresh and dry weight, compared to the nontransgenic controls. These results coupled with the drought stress results, indicate that *CBF* allowed for increased salt and drought stress resistance in cucumber.

Moreover, at the time of first wilt of lower leaves, *CBF*-expressing cucumber plants also exhibited less reduction in *Fv*/*Fm* (a measure of stability of photosystem II) in response to drought stress, than did the nontransgenic controls, indicating an additional physiological effect of the *CBF* transgene. Similar effects were also reported in tomato and tobacco plants expressing *CBF1/DREBb* (Hseih *et al.*, 2002; Lee *et al.*, 2003; Kasuga et al., 2004). Increased photosynthetic stability may result from altered expression of CBF-target genes, or an indirect effect of increased osmoprotectant. The presence of higher levels of proline has been reported to correlate with higher protection of photosystem II (De-Ronde *et al.*, 2004). Similarly, greater stability of PS II was also reported with the production of the compatible solute glycine betaine (Hayashi *et al.*, 1997; Sakamoto *et al.*, 1998; Kishitany *et al.*, 2000; Holmstom *et al.*, 2000; Quan *et al.*, 2004), trehalose (Garg *et al.*, 2002; Jang *et al.*, 2003), and mannitol (Loescher *et al.*, personal communication), or by overexpressing the yeast invertase gene in tobacco plants, which results in accumulation of glucose and fructose up to 8-fold (Fukushima *et al.*, 2001).

Several studies with *CBF*-overexpressing *Arabidopsis* plants have reported negative impacts on plant growth (Lui *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000). Severity of growth retardation was positively correlated with *CBF* expression levels (Liu *et al.*, 1998; Gilmour *et al.*, 2000), and was minimized the by use of a stress-inducible promoter (Lee *et al.*, 2003, Kasuga *et al.*, 2004; Pellegrineschi *et al.*, 2004). Additional

phenotypic differences in plants constitutively overexpressing *CBF3*, included darker leaves, shorter petioles, and delayed bolting in *Arabidopsis* (Gilmour *et al.*, 2000), and shorter internodes and less fruit and seed production in *CBF*-overexpressing tomatoes (Hsieh *et al.*, 2002). Expression of the *CBF* gene in cucumber did not have visible retard growth under the conditions tested. The lack of negative effects in this study could be due to differences in expression levels of *CBF1* and *CBF3* genes in *35S:CBF*-cucumber plants compared to the levels *35S:CBF* Arabidopsis, or to differences in the nature of the downstream responses in cucumber in response to *CBF* expression.

Despite enhanced stress resistance in tomato, efforts to clone *COR* homologs from tomato were not successful, even under low stringency conditions, indicating the absence of *COR* genes as a *CBF* target in that species (Hsieh *et al.*, 2002). Furthermore, microarray analysis of Arabidopsis and tomato plants expressing *CBF* genes, revealed the presence of marked differences in induced gene expression between these species, including the failure to observe predicted orthologs to Arabidopsis *CBF*-regulon genes. These results further suggests that responses may differ in heterologous systems, and may have differential impacts on growth and development.

In conclusion, the present study demonstrates that expression of *CBF* in cucumber, a species known for sensitivity to salinity and drought conditions, may offer an effective approach to enhance salinity and drought tolerance. Our results shows that *35S:CBF*-expressing cucumber plants were able to adapt to a higher range of dehydration-induced stresses than did their nontransgenic counterparts without apparent costs on plant growth. This increase in stress resistance was also accompanied by coordinated physiological responses including accumulation of compatible solutes and maintenance of photosystem

II stability. Further studies to evaluate plant performance as well as fruit production under field conditions are needed to begin to assess agricultural potentials.

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Chapter III

Cucumber (*Cucumis sativus* L.) plants expressing the *Arabidopsis thaliana*-transcriptional regulators, *CBF1* and *CBF3*, are more tolerant to salinity stress under field conditions.

Introduction

Stress caused by high concentrations of NaCl in soil or irrigation water negatively influences productivity of major agricultural crops (McWilliam, 1986; Zang and Blumwald, 2001). Statistical assessments of naturally salt-affected areas worldwide vary, but in general it is estimated that close to 1 billion hectares (approximately 7% of the world's land area) have naturally saline soil (Ghassemi *et al.*, 1995). In addition to the naturally affected areas, it is estimated that 77 million hectares have become salt affected due to extensive agricultural practices, mainly in irrigated areas worldwide. It has been estimated that these numbers will increase to affect up to 50% of the total arable land by the year 2050 (Wang *et al.*, 2003). Moreover, the demand to increase food production for the continuously growing world population will result in the need for more land for agricultural production, leading to an increase in salinity affected areas (Ghassemi *et al.*, 1995). Economic damage due to soil salinization has been difficult to assess, but Ghassemi *et al.* (1995) estimated that economic damage due to soil salinization at the Colorado River Basin is about 750 million US dollars/year and was about 330 and 208 million US dollars/year for the Punjab area and the Murray–Darling Basin in Australia.

Excess NaCl in soil and irrigation water causes hyperosmotic and hyperionic stress effects, which if sufficiently severe can result in plant death (Bohnert *et al.*, 1999; Hasegawa *et al.*, 2000). The hyperosmotic effect results from concentration of extracellular solutes, which causes a flux of water out of the cell, a decrease in the osmotic potential within the cell, and in the cellular turgor pressure (Lichtentaler, 1995). The hyperionic effect resulting from exposure to high salinity leads to "toxic sodium effect", whereby excess Na^+ in the cytoplasm causes an unbalance of other essential ions such as K^+ and Ca^+ (Bohnert and Jensen, 1996; Hasegawa *et al.*, 2000). High concentration of Na^+ ion in the cytosol causes metabolic toxicity, in part due to the competition between K^+ and Na^+ for binding sites for several enzymes (Tester and Davenport, 2003). High Na^+ and Cl^- concentrations interfere with enzyme function, protein synthesis, structure and solubility, and membrane fluidity and function (Blum, 1988).

Traditional breeding programs to develop salinity tolerance in plants have had modest success due to difficulties in establishing selection criteria, limited availability of sources of genetic resistance, quantitative nature of resistance, and the variety of mechanisms involved in salinity tolerance (Flowers and Yeo, 1995; Quesada *et al.*, 2002). For example, when evaluating yield performance of a crop under saline conditions, many factors can influence performance, including variation in salinity levels within a field, or possibility of interaction between salinity level and other environmental factors such as soil fertility, drainage quality, and water loss due to transpiration (Flowers, 2004). Thus, using yield components as main criteria for selection requires a long period and multiple locations for testing, and evaluation (Blum, 1989). Furthermore, results from several

groups indicate that QTL linked to salinity tolerance at a given developmental stage may differ from those linked to tolerance at another developmental stage (Greenway and Munns, 1980; Foolad, 1999; Cushman and Bohnert, 2000; Quesada *et al.*, 2002; Foolad, 2004).

Physiological and molecular studies aimed toward understanding plant response to salinity stress have indicated the complexity of this phenomenon, where an entire cascade of biochemical and cellular changes is necessary to adapt to high salinity stress (Bohnert and Jensen, 1996). Gene expression analysis, in the model plant *A. thaliana* under different dehydration inducing conditions (drought, salinity and freezing temperatures), revealed changes in expression patterns of several groups of genes. One of the first groups that shows immediate changes are those that encode transcription factors (TFs), mitogen activated protein kinases (MAPKs), dephosphorylation enzymes, and chromatin remodeling proteins (Thomashow, 1999; Knight and Knight, 2001; Hasegawa *et al.*, 2000; Xiong *et al.*, 2002; Zhu, 2002; Shinozaki *et al*, 2003; Seki *et al.*, 2003; Vogel *et al.*, 2005). This primary response is followed by activation of multiple mechanisms that are essential for plant adaptation to dehydration stresses (Bohnert and Jensen, 1996; Ingram and Bartels 1996; Campbell and Close, 1997).

The multigenic nature of plant response to salinity suggests that induction of multiple adaptive mechanisms at the same time might be a good strategy to engineer salinity tolerance in plant species. The *CBF/DREB* [*CRT* (C-repeat) Binding Factor /*DRE* (Drought Response Element Binding)] gene family encodes transcriptional activators that work as master switches in regulating plant response to dehydration-inducing conditions (Thomashow, 1999; Shinozaki *et al.*, 2003). Expression of the *CBF* gene family is

activated in response to low temperatures, drought or high salinity (Stockinger *et al.*, 1997; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999; Haake *et al.* 2002).

Transgenic *Arabidopsis* plants overexpressing *CBF/DREB* genes showed elevated levels of resistance to dehydration stresses relative to their nontransgenic counterpart, as determined by electrolyte leakage and whole plant test assays (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999). Similarly, in growth chamber trials, transgenic *Arabidopsis* plants overexpressing *CBF3/DREB1a* had enhanced resistance to drought and salinity stresses (Kasuga *et al.*, 1999; Kasuga *et al.*, 2004); expression of *CBF1/DREB1b* gene in tomato increased the resistance levels to salinity and drought (Hsieh *et al.*, 2002; Lee *et al.*, 2003); and expression of either *CBF1/DREB1b* or *CBF3/DREB1a* genes in cucumber plants reduced their susceptibility to salinity and drought stress compared to their nontransgenic control counterparts (Tawfik and Grumet, 2001 and 2003). In general, the increase in dehydration stress resistance is accompanied by increased membrane stability and/or accumulation of compatible solutes, especially proline and soluble carbohydrates (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Jaglo *et al.*, 2001; Seki *et al.*, 2001; Seki *et al.*, 2002; Haake *et al.* 2002; Hseih *et al.*, 2002b; Tawfik and Grumet, 2003; Kasuga *et al.* 2004). This elevation in compatible solute accumulation in *CBF/DREB* expressing transgenic plants, especially in soluble sugars and proline, has been described as a signature for *CBF/DREB* expression (Cook *et al.*, 2004).

Despite these successful examples which clearly demonstrate the potential value of the *CBF/DREB* system in increasing dehydration stress tolerance in plants, enhanced tolerance has not yet been demonstrated in field conditions. Indeed, Flowers (2003) stated that "after years of research using transgenic plants to alter salt tolerance, the value of this

approach has yet to be established in the field". To our knowledge, there are only two published reports that evaluated genetically engineered plants for enhanced dehydration stress tolerance under field conditions. Quan *et al.* (2004) reported enhanced grain yield production by transgenic maize plants expressing a gene for betaine aldehyde dehydrogenase following drought stress period of 21 days. Xue *et al.* (2004) tested wheat plants expressing the *Arabidopsis* tonoplast H^+/Na^+ antiporter gene for their ability to grow in saline soil and reported higher grain production in the transgenic plants compared to the nontransgenic controls.

In this work we sought to evaluate the performance of *CBF*-expressing cucumber plants under salinity stress in field conditions (Tawfik and Grumet, 2001; Tawfik and Grumet, 2003). Cucumber is known to be sensitive to salt (Mass and Hoffman, 1977). Salinity delays seed germination and seedling emergence, decreases leaf expansion rate and water potential, and decreases plant photosynthesis and yield (Chartzoulakis, 1994; Tazuki, 1997). Previously, we demonstrated that cucumber plants expressing *CBF1* and *CBF3* genes had elevated levels of resistance to salinity and drought conditions in greenhouse conditions. In the current study, *CBF1* and *CBF3*-expressing cucumber plants were tested for their ability to withstand continuous irrigation with 100 mM NaCl for 25 days under field conditions. Transgenic *35S:CBF* cucumber plants had higher levels of proline and soluble sugars in leaves and accumulated higher levels of K^+ and Ca^{++} ions in roots relative to non-transgenic controls in the presence or absence of salinity stress. In response to salinity, the CBF-cucumber lines exhibited significantly less reduction in growth and fruit yield than did the non-transgenic controls, demonstrating the potential effectiveness of CBF in conferring salt stress resistance in the field.

Materials and methods

Plant materials

The transgenic *35S-CBF1* and *35S-CBF3* cucumber lines were produced as described in chapter II. Of the 13 *CBF*-expressing lines, four lines were selected for the field trial; two lines expressing *CBF1*, A1 (high level of *CBF1* expression) and A5 (low level of *CBF1* expression); and two lines expressing *CBF3* gene, B1 (low level of *CBF3* expression) and, B5 (high level of *CBF3* expression) (Chapter II). Two types of controls were included: wild-type parental cultivar "Straight 8" (Hollar Seeds, Rocky Ford, Co.) and azygous sibling progeny of the transgenic *CBF*-cucumber lines. T_2 segregating azygous progeny and commercial "Straight 8" seeds were planted in trays (51 x 40 x 6.5 cm, 32 cells/tray, Hummert ™ International, Earth City, Mo) in the greenhouse and screened by PCR for the presence of the *CBF* genes at the 2-3-leaf stage. DNA was extracted from 200 mg young leaf samples of seedlings using the Wizard DNA purification kit (Promega, Cat # A7951, Madison, WI). PCR was carried out using *CBF1*- and *CBF3*-specific primers (Stockinger *et al.*, 1997). Non-transgenic segregants from the different *CBF*-lines were pooled for the azygous control plots.

Field salinity experiment

The field experiment was arranged in a split plot design, with four replications with salt treatment as the main plot and genotype as the sub-plot. Six genotypes were tested [four T_2 *CBF*-families, parental Straight 8 (wild-type WT) and azygous segregant T_2 progeny (Az)], with 6 plants per subplot. To allow for regulation of salinity levels, seedlings were transplanted into 50 x 30 x 15 cm plastic bags filled with 22.5 kg

playground sand (Sandastic Co., IL) and perforated with drainage holes along the edges. Plants were spaced 60 cm apart along rows of 1.5 m wide black plastic mulch; between row spacing was 2.1 m. Two control "Straight 8" plants separated each plot. The experimental plots were surrounded by two border rows of control "Straight 8" plants on all sides. Border plants were directly transplanted into the soil.

Irrigation was applied manually every other day using a 750 liter water tank connected to a tractor. The plants were allowed to acclimate for three weeks before starting salt stress treatment. Two salinity levels were used (0 mM and 100 mM NaCl), and stepwise salt application was carried out with an increase of 25 mM every other day until reaching the 100 mM NaCl level. Once the desired salinity level was reached, irrigation was applied daily, until run through, (approximately 2.0 l/day). Plants were fertilized with 150 ppm 20:20:20 (N: P: K) fertilizer twice a week throughout the experiment.

All measurements and leaf sampling were conducted using the middle four plants of each plot. Sampling for sugar and proline content was done four times during the experiment, at one-week intervals starting just before the initiation of salt application. Two 3-4 cm diameter leaves were collected from the main stem of each plant; sampled leaves of a given plot were combined for proline and sugar analysis. Samples were taken early in the morning and placed immediately in liquid nitrogen. Growth measurements (number of nodes on the main stem, number of branches, and number of male and female flowers) were recorded just before initiation of the salinity treatment. Fruit were harvested three times, at 12, 18, and 24 days post initiation of salinity treatment. Twenty four days post initiation of salt treatment, vines were harvested and above ground fresh

and dry weight was measured. After removing the above ground parts, the sand surrounding the roots was washed away with water; roots were removed and then rinsed several additional times with water before being placed in plastic bags on ice. Once in the lab, roots were washed several times with deionized water, blotted on paper towels, and then stored at -80 C° until further analysis.

Soluble sugars, proline and mineral analysis

Leaf samples collected from the field were placed in 13x100 mm glass tubes and freeze-dried for 48 hr. Samples from a given plot were pooled, ground, and split into two aliquots for proline and sugar analyses. Root samples were freeze dried for 48 hr. Samples of a given plot were pooled, ground with a morter and pestel in liquid nitrogen, and split into aliquots for proline, sugar and mineral analysis. Proline and soluble sugars analyses were carried out following the procedure described by Troll and Lindsley (1955) and Dubois *et al.,* (1956), respectively. Mineral analyses were performed on ashed root samples prepared by placing 2.0 g of freeze dried roots into ceramic crucibles and incinerating at 500 °C for 16 hrs to insure complete ashing. After cooling, ash weight was determined. Weighed samples of approximately 100 mg ash were added to 25 ml of 3N HNO_3 digestion solution. The samples were incubated for 1 hour at room temperature and the solution filtered through 90mm x 100, No.2, Whatman® filter paper (Whatman® International Ltd, Maidstone, England) into labeled vials. The concentration of Na^+, K^+ and Ca^{++} ions were determined by the MSU soil analysis laboratory using a D.C. Plasma Emission atomic analyzer (Pye Unicam SP9).

Results

CBF-expressing cucumber plants and the nontransgenic controls had equivalent in growth under field conditions before salinity treatment

Six genotypes were tested [two T_2 *CBF1*-families (A1 and A5), two *CBF3*-families (B1 and B5), parental "Straight 8" (wild-type WT) and azygous segregant T_2 progeny (Az)]. Prior to initiation of salinity treatment, several growth parameters were measured (Table 3-1). All lines performed equivalently; no significant differences were observed in vine length, number of nodes, number of branches, or number of male and female flowers between transgenic cucumber plants and the nontransgenic controls (Table 3-1).

Transgenic cucumber plants expressing *CBF* genes have elevated level of proline and total soluble sugars compare to the non-transgenic controls

In the absence of salinity treatment, transgenic *CBF*- cucumber plants accumulated significantly higher levels of proline (5-10 fold) compared to the nontransgenic controls, and then levels increased throughout the growing season (Figure 3-1). As the season progressed levels of soluble sugars also were significantly higher in the non-salt stressed *CBF*-cucumber plants compared to their nontransgenic counterparts. When irrigated with 100 mM NaCl CBF-cucumber plants accumulated significantly higher levels of proline and total soluble sugars compared to both non salt stressed conditions. CBF lines accumulated higher levels of proline and soluble sugars than salt stressed nontransgenic controls (ANOVA, $P< 0.01$). Twenty one days following initiation of salinity, the *CBF1* and *CBF3*-transgenic cucumber families, on average, had more than 15 fold higher levels of proline and 4 times higher levels of total soluble sugars compared to the nontransgenic

Table 3-1. Number of nodes, branches, male and female flowers of T_2 *35S:CBF1* lines (A1 and A5), T_2 *35S:CBF3* lines (B1 and B5), and non-transgenic cucumber lines (azygous and wild type) prior to initiation of salinity treatment in the field, 3 weeks post transplanting.

Parameters	*CBF1*-families		*CBF3*-families		Nontransgenic controls	
	A1	A5	B1	B5	Azygous	Wild-type
Vine length (cm)	84.9 ± 1.6	87.3 ± 1.3	87.9 ± 1.8	83.0 ± 1.9	85.5 ± 2.2	87.7 ± 2.4
Number of nodes	12.3 ± 0.3	13.2 ± 0.3	14.2 ± 0.3	13.0 ± 0.3	13.3 ± 0.3	12.9 ± 0.3
Number of branches	6.4 ± 0.2	7.1 ± 0.2	7.4 ± 0.2	7.0 ± 0.3	7.2 ± 0.2	6.9 ± 0.2
Male flowers	11.0 ± 0.5	12.0 ± 0.5	11.5 ± 0.5	10.8 ± 0.4	11.2 ± 0.4	10.5 ± 0.3
Female flowers	2.2 ± 0.1	3.1 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	3.1 ± 0.2	3.0 ± 0.2

Values are the mean ± S.E of 8 replicate plots with 4 measured plants/plot.

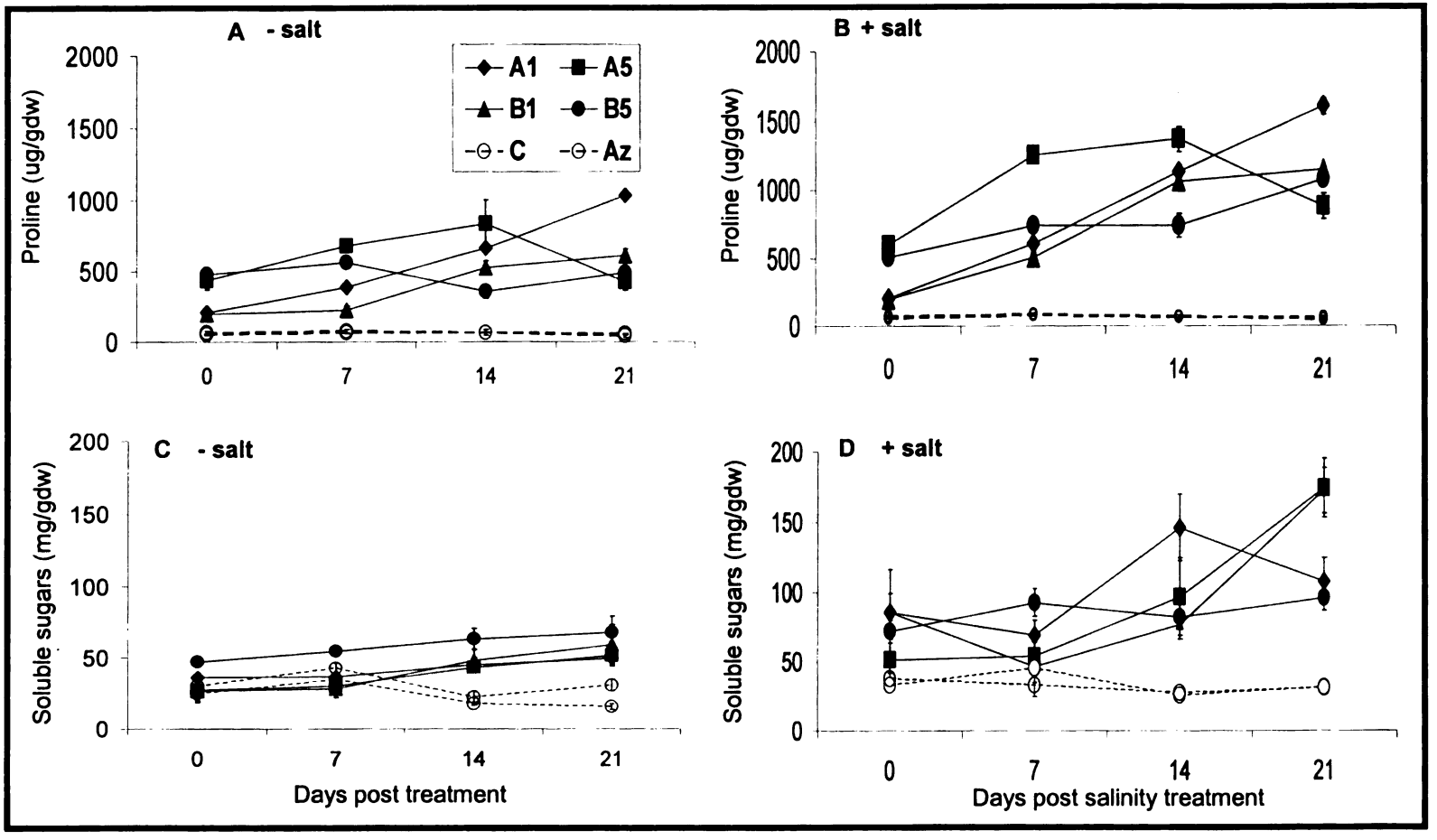

Figure 3-1. Accumulation of proline ug/gdw (A, B) and soluble sugars (C, D) in cucumber plants growing in the field in the absence (A and C) or presence (B and D) of 100 mM NaCl. Data are mean ± SE of 4 replicates/ treatment with four plants/replicate. Transgenic cucumber lines expressing *CBF1* or *CBF3* genes are represented with solid symbols and solid line, nontransgenic controls are represented by open symbols and dotted line.

counterparts. Salinity did not cause a significant increase in levels of compatible solute accumulation in the azygous or the parental "Straight 8" wild-type controls. No significant differences were found between *CBF1* vs. *CBF3*-cucumber lines (ANOVA, $P< 0.76$). Azygous plants did not differ from parental "Straight 8" plants.

Accumulation of compatible solutes was also tested in root samples at the termination of the experiments, 24 days post initiation of salinity stress treatment. In the absence and presence of salinity stress, *CBF*-cucumber roots accumulated higher levels of proline than their nontransgenic counterparts (ANOVA, $P<0.05$; Figure 3-2 A). No significant differences in accumulation of soluble sugar were detected between *CBF*-transgenic and the nontransgenic cucumber plants (Figure 3-2 B).

Transgenic cucumber plants expressing *CBF* genes have elevated level of potassium ions in their roots compared to the non-transgenic controls

Analysis of ashed root samples revealed differences in ion composition between the transgenic and the nontransgenic controls. *CBF*-lines exhibited 10-15 fold higher levels of K^+ ions than the nontransgenic controls in the presence and absence of salinity stress (Figure 3-3A). On the other hand, Na^+ levels did not differ significantly between the transgenic and nontransgenic plants and did not increase significantly in response to salinity treatment (Figure 3-3B). Transgenic cucumber plants had markedly lower Na^+/K^+ ratio in roots under both non-salt stress, and salt stress conditions compared to the nontransgenic controls. The average Na^+/K^+ ratio did not change in the transgenic lines in response to salinity stress (Figure 3-3C). Calcium levels also differed in the *CBF* cucumber transgenic lines with approximately 2-fold higher levels than in the

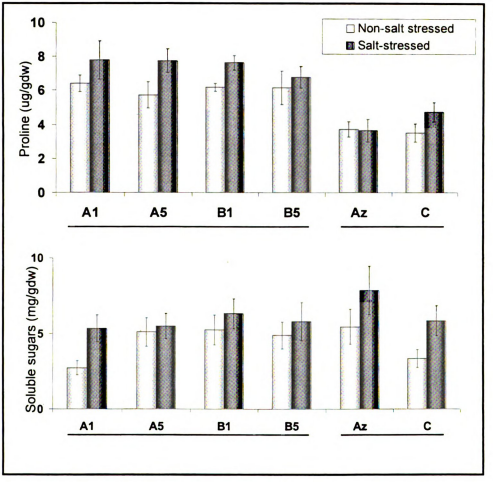

Figure 3-2. Accumulation of proline mg/gdw (A) and soluble sugars mg/gdw (B) in root tissues of cucumber plants growing in the field in the absence (light bars) or presence of 100 mM salinity stress (dark bars). Data represents mean ± SE of four replicates/treatment with four plants/replicate.

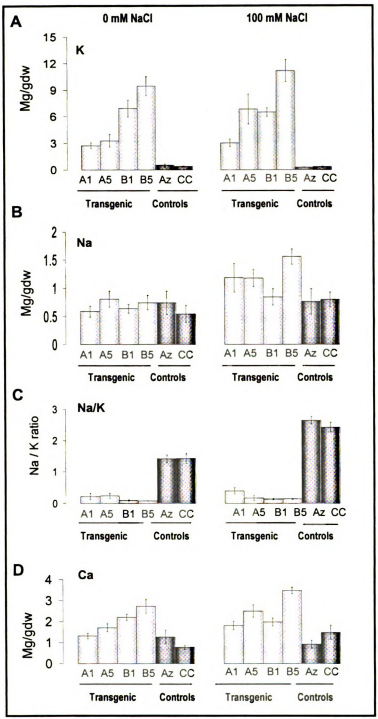

Figure 3-3. Potassium (A), sodium (B), Na/K ratio (C) and calcium content (D) in roots of cucumber plants growing in the field for 24 days, in the presence or absence of salinity treatment. Each value is the mean ± SE of four replicates samples composed of ashed root tissue pooled from four plants/replicate.

nontransgenic lines under both salt stressed, and non-salt stressed conditions (Figure 3-3D).

CBF-cucumber plants had less reduction in fresh and dry weight under salt stress conditions

Under non-salt stressed conditions, *CBF*-expressing cucumber families had lower fresh weight than the nontransgenic controls at the time of harvest; dry weight was equivalent for the CBF and non-transgenic controls (Figure 3-4; Table 3-2). Salinity treated *CBF*-cucumber plants on average did not show a significant reduction in fresh weight (690 g vs. 646 g in the absence or presence of salinity), while nontransgenic cucumber plants had an average reduction of 38% in fresh weight in response to salinity treatment. Similarly, on average, salinity treated *CBF*-expressing plants did not show a significant reduction in dry weight, while azygous and wild type Straight 8 plants had an average reduction of more than 50%.

Transgenic cucumber plants had higher yield under salinity conditions compared to the nontransgenic controls

In the absence of salinity stress, no significant differences were observed in number of fruits or fruit weight between the *CBF*-expressing cucumber plants and the nontransgenic controls (Figure 3-4; Table 3-2). Salinity treated *CBF*-expressing lines did not show significant reduction in fruit number or weight, compared to a 35% reduction in fruit number and 50% reduction in fruit weight for the nontransgenic controls. Salt stressed *CBF1* lines (A1 and A5) had higher yields than the *CBF3* lines (B1and B5).

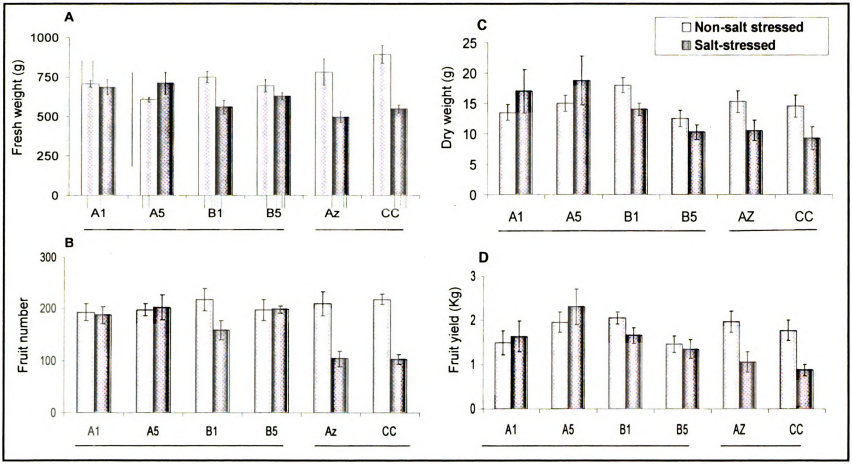

Figure 3-4. Above ground fresh (A) and dry weight (B) accumulation, average fruit number (C) and average fruit yield (D) per plot in cucumber plants growing in the field in the presence or absence of salinity treatment for 24 days. Light bars represent non-salt stressed conditions, dark bars represents salt stressed conditions.

Table (3-2). Fresh weight, dry weight, fruit number and total fruit yield of *CBF*-expressing transgenic families and the nontransgenic control lines 24 days post initiation of salinity treatment.

	0 mM NaCl			100 mM NaCl		
	Transgenic lines		Non-transgenic lines	Transgenic lines		Non-transgenic lines
	CBF1[1]	CBF3[2]		CBF1	CBF3	
Fresh weight (g)	689.7 ± 28.2		837.87 ± 69.8 *	645.9 ± 45.9		521.87 ± 30.1
	657.5 ± 18.4	722.0 ± 38.1		697.7 ± 58.7	594.0 ± 33.1	
Dry weight (g)	201.4 ± 12.4		214.00 ± 11.6	186.8 ± 11.6		103.12 ± 8.9**
	195.50 ± 10.0	207.4 ± 14.8		195.00 ± 14.2	178.6 ± 9.1	
Fruit number	14.7 ± 1.4		14.87 ± 1.8	15.0 ± 2.4		9.87 ± 1.2**
	14.25 ± 1.4	15.25 ± 1.4		17.87 ± 3.8*	12.1 ± 1.1	
Total fruit weight (kg)	1.74 ± 0.2		1.87 ± 0.24	1.74 ± 0.28		0.96 ± 0.4**
	1.72 ± 0.25	1.75 ± 0.15		1.97 ± 0.38*	1.5 ± 0.19	

Data for fresh and dry weight are the mean ± SE of four replicate plots/treatment with 4 plants/plot. Data for fruit yield are the mean ± SE of four replicate plots/treatment with 4 plants/plot totaled over three harvests.

*, ** Non-transgenic genotypes are significantly different from the transgenic lines, or *CBF1* families are significantly different from *CBF3* families, within a salt stress level by orthogonal contrast (P< 0.05, 0.01, respectively).

1 data for *CBF1*-families are pooled from lines A1 and A5; for *CBF3*-families data are pooled from lines B1 and B5.

2 Date are pooled from non-transformed Straight 8 and nontransgenic segregant progeny.

Discussion

The *CBF* gene has been demonstrated to confer dehydration stress resistance in several species in growth chamber and greenhouse studies (Kasuga *et al.*, 1999; Hsieh *et al.*, 2002; Lee *et al.*, 2003; Pellegrineschi *et al.*, 2004; Kasuga *et al.*, 2004). This study tested the performance of the *35S:CBF*-cucumber plants under field conditions. Consistent with greenhouse experiments (Chapter II), field grown transgenic *35S:CBF*-cucumber plants had elevated levels of proline and soluble sugars relative to the nontransgenic controls. *CBF*-cucumber plants progressively accumulated higher levels of the compatible solutes throughout the growing season, even in the absence of salinity stress.

Irrigation with 100 mM NaCl caused significant elevation in soluble sugars and proline levels in the leaves of CBF-cucumber plants while levels of sugar and proline did not change in the nontransgenic controls, indicating enhanced ability of the CBF-lines to respond to salinity stress. The higher levels of proline in roots of the transgenic *CBF*-lines indicate that *CBF* caused osmotic adjustment throughout the plant.

In addition to the increases in compatible solutes, marked changes in ion composition in root tissues also were observed. Levels of K^+ ion in the transgenic lines were about 10-15 fold higher than the nontransgenic controls, in the presence or absence of salt stress; levels of Ca^{++} were approximately two-fold higher. To our knowledge this is the first report that shows the influence of *CBF* expression on ion composition in plants, suggesting an effect on ion transport properties in roots. K^+ and Ca^{++} are major nutrients and are the two most abundantly distributed cations in plant tissue (Devlin and Witham, 1983). In addition to serving as a primary contributor to cell turgor, K^+ is also involved in

many physiological processes in plants (Zhu *et al.*, 1997). Ca^{++} is critical for stabilizing and maintaining cell walls and is involved in various signal transduction pathways (Knight and Knight, 2001).

Exposure of plants to high salinity leads to a "toxic sodium effect", whereby excess Na^+ in the cytoplasm causes a deficiency of essential ions such as K^+ and Ca^{2+}, and competes with K^+ ions for binding sites for several enzymes (Bohnert and Jensen, 1996; Hasegawa *et al.*, 2000; Tester and Davenport, 2003). Living cells tend to accumulate K^+ and exclude Na^+, to maintain sufficient levels of K^+ to perform essential functions that sodium either cannot fulfill or actively inhibits (Epstien, 1998). The increase in K^+ content in *CBF*-cucumber roots was comparable in the presence or absence of salinity stress, suggesting that excess Na^+ ions did not prevent the elevated K^+ accumulation by the *CBF*-cucumber roots. Accumulation of Na^+ did not differ between transgenic and nontransgenic plants, and was not significantly increased in the presence of salinity stress, suggesting selectivity in K^+ ion uptake.

A possible explanation for the enhanced in K^+ and Ca^{++} accumulation by the *CBF*-cucumber roots may be due to differential expression of different transporters and channels responsible for ion uptake from the soil or subsequent movement through the plant. Recent transcriptional profiling of *Arabidopsis* plants overexpressing *CBF/DREB* genes (Seki *et al.*, 2001; Fowler and Thomashow, 2002; Vogel *et al.*, 2005) provided an opportunity to examine global gene expression profile changes due to *CBF/DREB*. Among the upregulated genes are putative and known transporter proteins and membrane channel proteins that might play a role in preferential selectivity to K^+ and Ca^{++} ions over the toxic Na^+ ions (Fowler and Thomashow, 2002; Vogel *et al.*, 2005).

One of the genes that was upregulated in *CBF2* overexpressing *Arabidopsis* plants encodes a Ca^{++}/ATPase transporter, which contains a CRT element in its promoter region (Vogel *et al.*, 2005). Calcium has been shown to maintain or enhance the selective absorption of potassium by plants at high concentration of of sodium (Epstien, 1998), thus such a transporter might facilitate enhanced Ca^{++} and K^+ uptake. Whether a similar *CBF*-inducible transporter is present in cucumber roots, or whether other transporters may be affected, remains to be determined. In general, the samples used for the *Arabidopsis* transcriptional analyses performed to date have been primarily composed of shoot tissue (Vogel *et al.,* 2005). More comprehensive analysis of root tissue may lead to identification of additional relevant genes as possible targets for *CBF/DREB*.

Another possible explanation for K^+ accumulation may be related to the elevated levels of proline accumulation in *CBF*-cucumber roots. It has been observed that in response to several abiotic and biotic stimuli in plants, that there is a correlation between compatible solute accumulation and K^+ ion content (Garcia *et al.,* 1993; Hare and Cress, 1997; Backor *et al.*, 2004). Garcia *et al.* (1993) found that lower Na/K ratios were obtained upon treating rice roots with several osmoprotectant, including proline. Backor *et al.* (2004) recently found that in heavy-metal resistant strains of lichen photobionts (*Trebouxia erici*) levels of proline correlated with ability to block K^+ ion efflux. Garg *et al.* (2002) observed that transgenic rice engineered for trehalose accumulation were able to maintain a higher level of selectivity for K^+ over Na^+ uptake in the roots. Thus expression of *CBF* genes, either directly (through altered gene expression) or indirectly (by changing levels of compatible solutes), might affect processes involved in ion homeostasis in plants.

Prior to initiation of salinity treatment, no significant differences were observed between the transgenic and the nontransgenic plants as measured by vine length, number of nodes, number of branches, female and male flowers. At the end of the experiment, in the absence of salinity, *CBF* lines had less fresh weight than the nontransgenic controls; however, dry weight and fruit number and fruit weight were equivalent to the nontransgenics. These results suggest the possibility to obtain positive effects of *CBF* for stress resistance without a negative impact on fruit yield, although this would need to be verified in more extensive testing situations.

In the presence of salinity stress, *CBF*-transgenic plants showed enhanced tolerance to stress conditions compared to the nontransgenic lines as measured by fresh weight, dry weight, fruit number, and fruit weight. *CBF1*-cucumber lines did not show reduction in yield, compared to 34% reduction in fruit number and more than 50% reduction in fruit weight in the nontransgenic lines. Thus the *CBF* genes conferred increased salt stress tolerance to cucumber plants.

The growing demand to increase food production worldwide, requires a multi-disciplinary approach that will include adding new land to the agricultural production area, the use of low quality saline water and the reuse of drainage waters, as well as developing new salinity tolerant plants capable of adapting to a wider range of dehydration-inducing stresses. Introduction of the CBF gene into cucumber activated a variety of salt adaptive responses including increased in compatible solute accumulation and maintenance of higher K^+/Na^+ balance. The *CBF*-transgenic cucumbers showed enhanced resistance to salinity stress, with minimal or no reduction in growth and fruit

yield in the absence of salt stress, making this approach very promising to engineer dehydration resistance in crops.

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Chapter IV

Introduction of the celery mannose-6-phosphate reductase (M6PR) gene for mannitol production into cucumber (*Cucumis sativus* L).

Introduction

Plant response to unfavorable conditions requires adjustment at the molecular, cellular and whole plant level (Greenway and Munns, 1980; Ingram and Bartels, 1996 and Zhu *et al.*, 1997). One of the many mechanisms that plants have developed to overcome the low osmotic potential associated with salinity and drought conditions, is the ability to accumulate compatible solutes in the cytoplasm (Tarczynski *et al.*, 1993; Bohnert and Jansen, 1996; Shen *et al.*, 1997; Sakamoto and Murata, 2000). Compatible solutes (e.g., proline, sugar alcohols, fructans, trehalose, quaternary ammonia compounds, and tertiary sulfonic compounds), are non-toxic organic metabolites of low molecular weight that can decrease the osmotic potential of cells without interfering with cellular metabolism. The compounds can also serve as osmoprotectants to help stabilize membranes and macromolecular structures (Bohnert and Jensen, 1996, Stoop *et al.*, 1996, Zhang *et al.*, 1999). Thus, attempts to engineer enhanced salinity tolerance in plants have included the use of genes encoding key enzymes for biosynthesis of compatible solutes such as mannitol (Tarczynski *et al.* 1993; Karakas *et al.*, 1997; Abebe *et al.*, 2003; Zhifang and Loescher, 2003), proline (Kishor *et al.*, 1995; Zhu *et al.*, 1998; Ronde *et al.*, 2000), and glycine-betaine (Holmstrom *et al.*, 1994; Hayashi *et al., 1997*; Sakamoto *et al., 1998*; Holmstrom *et al.*, 2000; Kishitani *et al.*, 2000; Jia *et al.*, 2002)

Sugar alcohols such as mannitol, galactitol and sorbitol represent the chemically reduced form of aldoses or ketose sugars (Loescher and Everard, 1996, Loescher and Everard, 2000). It also has been suggested that sugar alcohols may play an important role in scavenging active oxygen species and preventing peroxidation of lipids, which can lead to membrane damage (Halliwell *et al.*, 1988; Smirnoff and Cumbes, 1989; Tarczynski *et al.*, 1993; Bohnert and Jensen, 1996; Stoop *et al.*, 1996; Bohnert *et al.*, 1999). Synthesis of mannitol also was suggested to work as a supplemental mechanism to dissipate reducing power (NADPH) accumulated during the light reactions of photosynthesis (Loescher, 1987).

Mannitol is the most common form of sugar alcohol in nature, and it has been reported in numerous plant species, including many crops such as carrot, parsley, celery, green beans, cabbage, pumpkins, coffee and olive trees (Loescher *et al.*, 1992; Stoop *et al.*, 1996). Plant species that produce mannitol as one of their primary photosynthetic products tend to have a substantial dehydration stress tolerance as in celery, coffee and olive trees (Loescher *et al.*, 1992; Stoop *et al.*, 1996).

A role for mannitol in adaptation to dehydration stress is supported by changes in mannitol production in response to salinity and drought stress. Exposure of celery plants to 300 mM NaCl resulted in a shift in the pool size of sucrose, mannitol and starch towards nearly exclusive accumulation of mannitol (Everard *et al.*, 1994). Similar results were observed when testing celery plants grown in saline hydroponic culture equivalent to 30% sea water (Stoop and Pharr, 1994). No differences in dry weight were observed between salt stressed and non stressed plants, suggesting that the total carbohydrate assimilation was not affected by salinity. Furthermore, salt stress induces expression of

key mannitol biosynthetic enzymes and down regulates mannitol catabolic enzymes (Zamski *et al.,* 1996; Loescher and Everard, 2000; Zamski *et al.,* 2001; Zhifang and Loescher, 2003).

Several studies reported enhanced stress protection of transgenic plants by introducing bacterial genes for sugar alcohol production. Expression of the *Echerichia coli* mannitol-1-phosphate dehydrogenase gene (*mtlD*) in tobacco resulted in the accumulation of mannitol in leaves and roots of transgenic tobacco plants as detected by NMR and mass spectroscopy (Tarczynski *et al.* 1992; Tarczynski *et al.,* 1993; Karakas *et al.,* 1997) The mannitol-accumulating tobacco plants had an elevated level of tolerance to 100 mM NaCl, as indicated by fresh weight, plant height and root biomass. In addition to salt stress resistance, *mtlD*-expressing tobacco plants had higher relative water content in their leaf tissues, in response to drought stress (Karakas *et al.*, 1997). Eggplant (*Solanum melongena* L.) seedlings expressing the *mtlD* gene exhibited increased tolerance to salinity stresses as measured by increased germination rate and higher fresh and dry weight compared to the nontransgenic controls at 200 mM NaCl (Prabhavathi *et al.*, 2002). Transgenic T_2 *mtlD*-wheat plants subjected to 150 mM NaCl showed less reduction in fresh and dry weight than did the non-transgenic wheat (Abebe *et al.*, 2003).

Similarly, expression of the *E. coli GutD* gene encoding glucitol-6-phosphate dehydrogenase, a key enzyme for biosynthesis of the sugar alcohol sorbitol in maize plants, also increased sorbitol accumulation and enhanced salt tolerance compared to the nontransgenic controls (Liu *et al.*, 1999). Rice plants (*Oryza sativa* L.) expressing the *E. coli GutD* and the *mtlD* genes, were able to accumulate both sorbitol and mannitol in

their vegetative tissue and were more salt tolerant than their nontransgenic counterparts (Tilahon et al, 2003).

In addition to the use of the bacterial genes, mannitol biosynthetic genes from celery also have been used to engineer mannitol accumulation (Zhifang and Loescher, 2003). In celery, mannitol is synthesized from fructose-6-phosphate in three steps:-

Fructose-6-P (fructose-6-phosphate), mannose-6-P (mannose-6-phosphate), mannitol-1-P (mannitol-1-phosphate), PMI (phosphomannose isomerase), M6PR (mannose-6-phosphate reductase), Pase (phosphatase)

In celery, fructose-6-phosphate and mannose-6-phosphate are in an equilibrium state; fructose-6-phosphate is converted into mannose-6-phosphate by phosphomannose isomerase (PMI). Conversion of mannose-6-phosphate into mannitol-1-phosphate which is performed by mannose-6-phosphate reductase (M6PR) is the first committed step in the pathway and appears to be the primary site of regulation of mannitol biosynthesis (Everard and Loescher, 1997; Zhifang and Loescher, 2003). Mannitol-1-phosphate is then converted into mannitol by phosphatase enzyme (Pase).

The M6PR gene was fist cloned by Everard *et al.* (1997) from celery plants (*Apium graveolens* L.). Expression of the M6PR gene, in *Arabidopsis thaliana*, a non-mannitol accumulating species, enabled plants to grow and complete their normal life cycle (including seed production) in the presence of 300 mM NaCl (Zhifang and Loescher, 2003).

In this work, I investigated the possible use of the *M6PR* gene to engineer enhanced dehydration stress tolerance in cucumber plants. Transgenic *M6PR*-cucumber plants were produced in our laboratory, analyzed for the presence of M6PR gene by PCR and tested for accumulation of mannitol in leaf tissue.

Materials and methods

Plant constructs, transformation and seed production

The *Agrobacterium* plant transformation construct containing the *M6PR* gene, under the control of the *CaMV 35S* promoter (Zhifang and Leoscher, 2003) was kindly provided by W. H. Leoscher (Michigan State University). Cucumber transformation was performed based as described in Chapter II.

DNA isolation and PCR analysis

DNA was isolated from young leaf samples; 200 mg leaf tissue was ground in liquid nitrogen and extracted using the Wizard DNA purification kit (Promega, Cat # A7951, Madison, WI). DNA quality was determined by gel electrophoresis (Sambrook and Russell, 2001). PCR was carried out according to the procedure of Sambrook and Russell (2001), using *M6PR* specific primers (RG278, forward, CACAGCACACACACCAC and RG279, reverse CACACATTCCCCTCCACA).

ELISA analysis

ELISA test was used to test for the presence of the NPT II protein in the T_0 transgenic plants using NPTII-ELISA® kit (Agdia Inc., Elkhart, IN). One leaf disc of each plant was collected and placed in 96 well ELISA plate and stored in -80 C° until further analysis. Just before starting the ELISA procedure, leaf discs were left at room temperature followed by re-freezing for 2-3 times to ensure cell wall leakage of tissues.

Mannitol analysis

One leaf (6-8 cm diameter; approximate fresh weight 1.5 g) was collected from T_0 and T_1 plants, freeze-dried for 48 hr, ground and placed in 13x100 mm disposable glass tubes (Cat No. 47729-572, VWR international, West Chester, PA). Total soluble carbohydrates were extracted according to the procedure of Loescher *et al.* (1997) (Appendix A). Standards were previously prepared using 0.1 g of fructose, glucose, sucrose, raffinose, mannitol, myo-inositol, all mixed together and dissolved in 100 ml H_2O (this should give 1000 ppm) to establish elution peak time as discussed in more details at appendix A.

Results and discussion

Introduction and expression of the celery (*Apium graveolens*)-*M6PR* gene in cucumber plants

Six transgenic cucumber plants were produced, and the presence of the *M6PR* gene was verified in the T_0 plants by PCR (Figure 4-1). Successful transfer of the *M6PR* gene into T_1 progeny was verified using PCR analysis (Table 4-1). χ^2 analysis of segregating progenies was consistent with a single gene insertion for the six families.

Table 4-1. Segregation analysis of T_1 transgenic cucumber lines expressing the celery-M6PR gene. Presence or absence of the gene was determined by PCR analysis

Line	T_1 segregation (trans: non)	χ^2 (3:1)
M1	29 : 8	0.22 ns
M2	31:18	5.30 ns
M3	42:9	4.6 ns
M4	38:12	0.03 ns
M5	34:8	3.86 ns
M6	22:11	2.75 ns

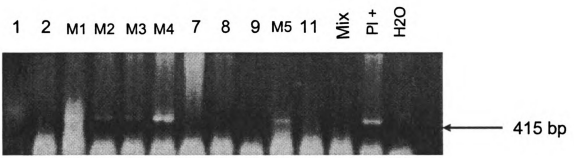

Figure 4-1. PCR analysis of the presence of the M6PR gene in T_0 cucumber plants. Lane 1-11: putative transgenic cucumber plants. Lane 12-14: PCR mix, plasmid control and H_2O. The arrow indicates the expected 415 bp product size.

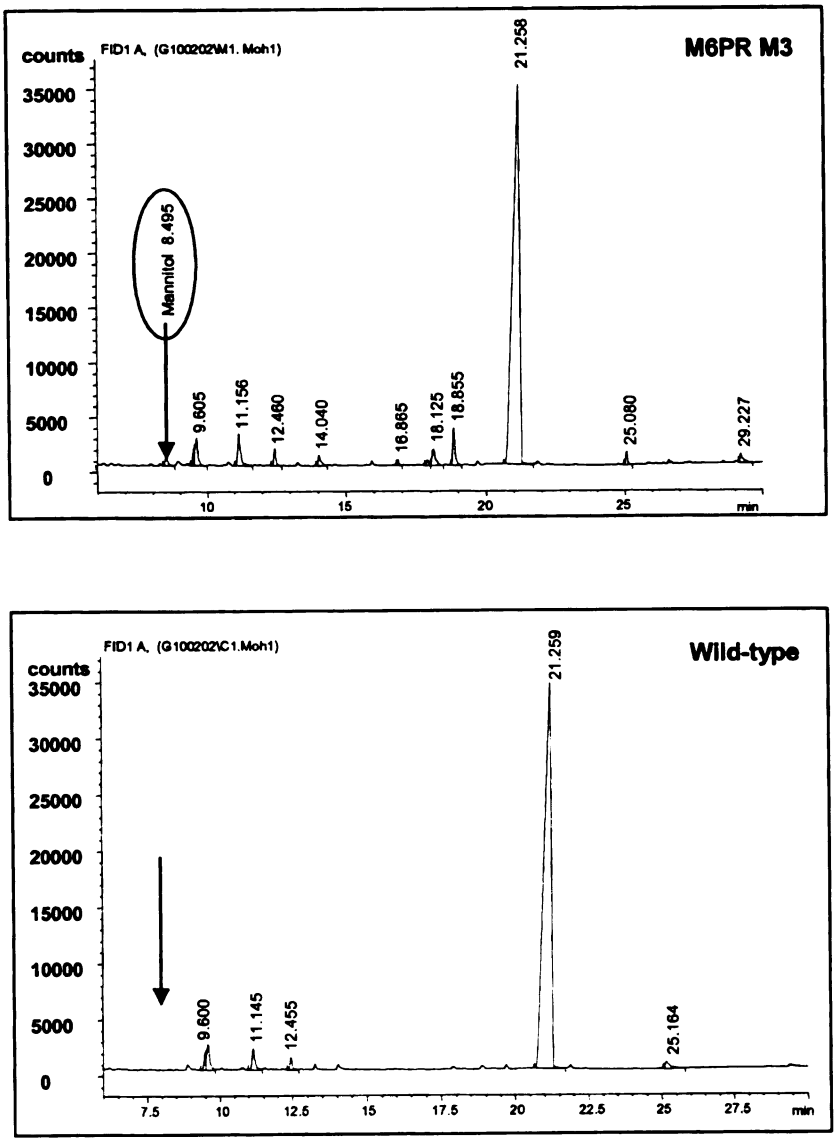

Figure 4-2. Gas chromatography for mannitol analysis in one of the cucumber T_0 plants (M3) (top) and nontransgenic wild-type cucumber plant (bottom). The arrows indicate the 8.5 elution time which represents the expected mannitol peak based on elution time of the mannitol standard. The peak at 8.5 is absent in the wild type cucumber plant.

Transgenic cucumber plants expressing the M6PR gene have elevated level of mannitol in leaves compared to the non-transgenic controls

Mannitol accumulation in the T_0 progeny was tested using gas chromatography (Figure 4-2). All six putative PCR-verified transgenic cucumber plant showed the expected mannitol peak (elution time 8.5 min) indicating mannitol presence.

Mannitol also was observed occasionally in the T_1 M6PR plants (Figure 4-3; for example, M3), the M6PR T_1 plants did not consistently show mannitol accumulation (Table 4-2). Mannitol accumulation in four families was further investigated. To test whether the inconsistency in observed mannitol accumulation was due to catabolism of mannitol or translocation into sink tissue, leaf and sink tissues were collected from greenhouse growing cucumber plants at two different time points; early in the day (before 10:00 a.m) and later in the day (between 3-5:00 p.m). Mannitol was not detectable in majority of samples, and no obvious pattern could be determine relative to old vs. young tissue, leaves vs. flowers or time of the day. Due to the inconsistence in mannitol accumulation of the T1 progeny, this project was not pursued further.

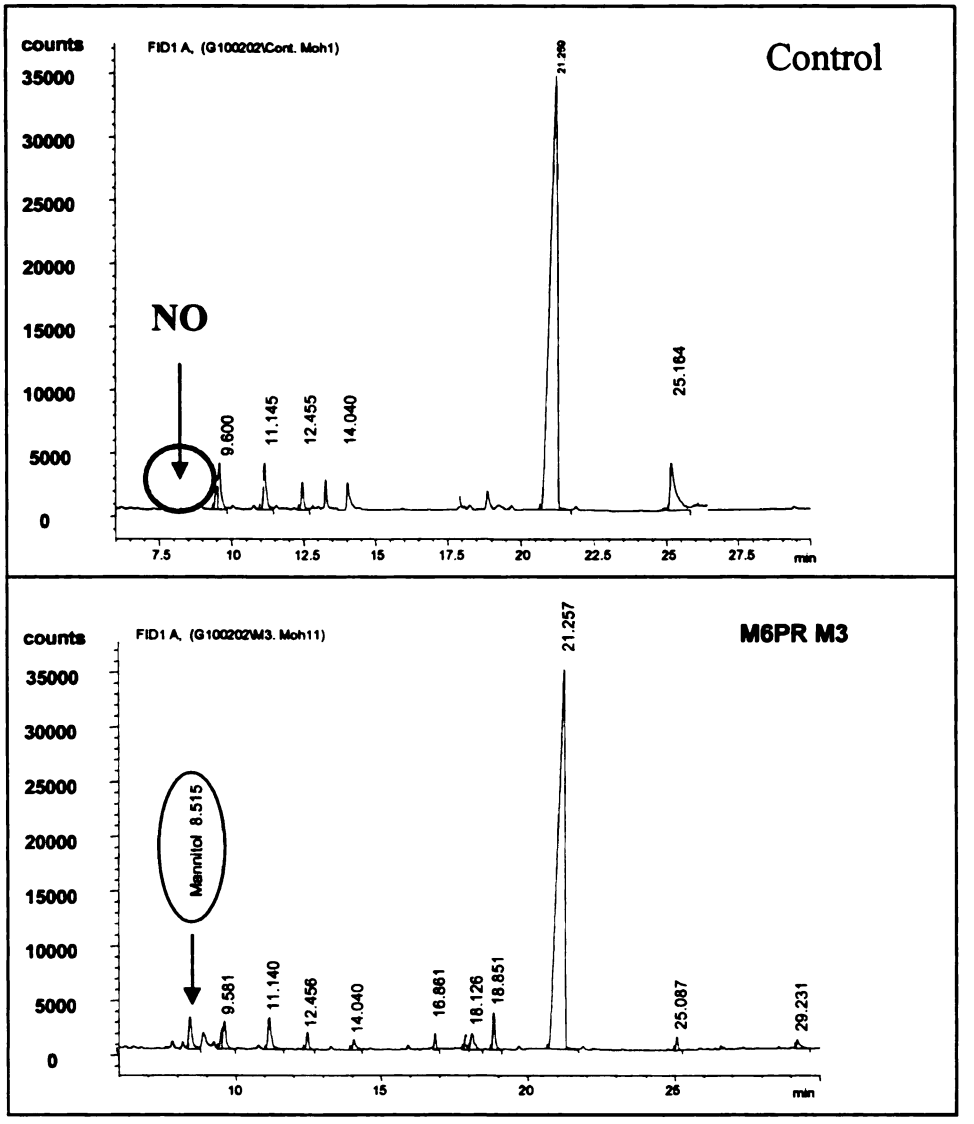

Figure 4-3. Gas chromatography for mannitol analysis in wild type (top) and T_1 plant from family M3 (bottom). The arrows indicate the 8.5 elution time which represent the expected mannitol peak based on elution time of the mannitol standard. The first peak which represent mannitol is absent in the wild type plants and indicated by the black arrow.

Table 4-2. Mannitol accumulation in segregating T_1-M6PR plants. Different tissues were analyzed (young leaves, fully expanded leaves and female flowers) were collected at two time points (before 10:00 a.m and after 3:00 p.m) from M6PR plants growing in the greenhouse.

M6PR lines	PCR positive +:-	Number of plants showing mannitol accumulation					
		Young leaves		fully expanded leaves		Flowers	
		Early	Late	Early	Late	Early	Late
M1	12 : 4	2 : 14	1 : 14	0 : 14	1 : 14	3 : 10	1 : 13
M3	9 : 5	0 : 14	0 : 14	4 : 10	2 : 12	7 : 4	2 : 12
M4	14 : 3	4 : 13	2 : 15	6 : 11	2 : 15	0 : 17	-------
M5	10 : 8	2 : 16	1 : 17	3 : 15	6 : 12	1 : 16	3 : 15

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Conclusions and future work

In the current work, I produced several lines of transgenic cucumber plants expressing the *Arabidopsis thaliana* transcriptional regulators *CBF1/DREBb* and *CBF3/DREBa* genes under the control of the constitutive *CaMV35S* promoter (Chapter 2). Greenhouse experiments indicated that *CBF1* and *CBF3* cucumber plants showed array of adaptive responses to the imposed salinity and drought stress, including the accumulation of compatible solutes (e.g., proline and soluble sugar), less reduction in photochemical efficiency as measured by chlorophyll fluorescence, less growth reduction, and accumulation of higher above ground dry matter compared to non-transgenic controls. These observations are similar to other reports that introduced CBF genes into tomato, tobacco and rice; however, *CBF* homologues have been identified in all of those species. This raises the question of whether cucumber has *CBF* homologues. I tried to look for *CBF* homologues in cucumber by following more than one approach; for example, trying southern hybridization using *CBF1* and *CBF3* as probes with low stringency washing conditions, designing degenerate primers for conserved common motifs among the known *CBF* genes from different species, and finally using the cucumber genomic library to screen for *CBF* homologues. The fact that I could not clone any homologues does not role out the possibility that cucumber may have CBF homologues. One thing to pursue in the future would be expand efforts to identify CBF-homologs from cucumber and ask if they are true functional homologues of the Arabidopsis-*CBF* gene family, by testing for expression, phenotypic and physiological changes in Arabidopsis plants overexpressing these homologues. It would also be

beneficial to understand how these CBF-homologues are regulated in response to different stresses.

Despite the enhanced stress resistance in tomato, expression of predicted *COR* homologs did not increase in response to overexpression of CBF. Given the fact that there are marked differences between *Arabidopsis* and tomato, in gene expression in response to *CBF*-overexpression, and the current failure to observe *CBF*-regulation of predicted tomato orthologs to *Arabidopsis CBF*-regulon genes, further suggests that responses may differ in heterologous systems, which in turn may make finding of *CBF*-target genes in cucumber more challenging. One way to answer this would be by using microarray analysis to compare gene transcription profiles of CBF-expressing and nontransgenic control cucumber plants. The one drawback in this approach is that the microarray chip would be from a different species (*Arabidopsis*). Another alternative could be the generation of subtractive libraries from CBF-transgenic and nontransgenic cucumber plants.

Salinity treatment (50 and 100 mM NaCl) caused a marked increase in soluble sugar and proline accumulation in *CBF*-plants beyond what would be expected from an additive effect of *CBF* and salt stress. The same trend was also observed in some of the work on *CBF*-expressing plants, although it was less pronounced than in our study. The lack of differences in *CBF* levels in the transgenic lines in the presence or absence of salinity indicated the presence of other levels of regulation downstream of *CBF* in cucumber. Recently, Cook *et al.* (2004) showed metabolic changes in *Arabidopsis* in response to cold acclimation and overexpression of *CBF* genes. The idea that *CBF* genes, which encode transcriptional regulators, could activate waves of responses makes it of

interest to monitor metabolomic changes in cucumber plants under different stress conditions. Thus questions here would be what are the metabolic changes in cucumber plants in the presence or absence of salinity? What are the changes in the *CBF*-cucumber line in the presence or absence of salinity? How would these differ at different stress levels? Informations from these experiments might also help identify possible *CBF* target genes. These types of studies could provide insights into the secondary regulation in plants and how this is relate to gene expression profiling.

We also reported for the first time that expression of the CBF system in cucumber plants caused an increase in ion composition of cucumber roots. Cucumber roots accumulated higher levels of K^+ and Ca^{++} ions in the presence or absence of salinity stress, suggesting that excess Na^+ ions did not prevent the accumulation of K^+ to higher levels in the roots of *CBF*-cucumber plants and maybe an increased selectivity in K^+ uptake or due to differential expression of different transporters and channels responsible for ion uptake or movement through the plant. The fact that Ca^{++}/ATPase transporter was one of the upregulated genes in *CBF*-overexpressing plants raises questions about ion transport regulation in *CBF*-expressing plants.

Calcium has been shown to maintain or enhance the selective absorption of potassium over sodium in plants under salt stress (Epstein, 1998). This may be through activation of Na^+/H^+ antiporters at the plant plasma membrane or maybe by activating some high K selective channels. Another question is whether changes in Na^+/K^+ ratio is an indirect side effect of accumulation of compatible solutes in plant tissue? If so then the phenomenon would be similar to that in previous reports (Chapter III). One way to

answer these questions would be by using tissue specific microarray analyses, or more comprehensive analysis of root tissue may lead to identification of additional genes as possible targets for *CBF/DREB*. Another possibility would be to generate subtractive libraries from roots of *CBF*-cucumber plants and compare it with nontransgenic control cucumber plants.

Finally, when we first started this work we had few questions to ask; can *CBF* confer dehydration stress tolerance in a heterologous system? What are the limitation of the transgenic cucumber plants in response to salinity and drought? What are the possible effects of the *CBF* gene on growth and yield data of cucumber plants in the presence or absence of salinity stress? Although my current work was an attempt to answer many of these questions, our findings also raised new questions:

1- What are the metabolic changes in cucumber plants in the presence or absence of salinity and drought? How does it differ in *CBF* cucumber lines in the presence or absence of dehydration stresses?

2- How would these differ at different stress levels? Can these metabolic changes be used to predict possible CBF-target genes in cucumber?

3- What cause the accumulation of higher K^+ levels in roots of *CBF*-cucumber plants; it is due to changes in K^+ uptake, K^+ selectivity, or in ion movement in plant tissue?

4- The changes in the Na^+/K^+ ratio an indirect side effect of accumulation of compatible solutes in plant tissue?

In summary, results demonstrate the potential usefulness of the *CBF/DREB* system in providing elevated levels of dehydration stress tolerance in salt sensitive species such

as cucumber and also raise new questions about the function of CBF in regulating stress tolerance related phenotypes.

Appendix A: Proline, soluble sugar and mannitol protocols

Proline analysis

One leaf (3-4 cm diameters) was collected from each plant, and freeze-dried for 48 hrs and ground to assure uniformity. Each sample was split into two equal weights for proline and sugar analysis. Proline analysis was carried out using the ninhydrin reagent procedure described by Troll and Lindsley, (1955). Tissue samples (0.1-0.2 g dry weight) were placed in 13x100 mm disposable glass tubes (Cat No. 47729-572, VWR international, West Chester, PA) and proline extracted from dried tissue by overnight soaking in 3.0 ml of distilled H_2O followed by heating at 80 C° for 30 min. The supernatant was then transferred into a fresh 13x 100 glass tube and the heating step was repeated with fresh 3 ml of water H_2O for 30 min. Tissues and debris were removed from the samples by centrifuging for 1 min at 2500 g. the supernatant was then transferred into fresh glass tubes. 400 µl of the extract solution was transferred to microcentrifuge tubes containing 400 µl of the ninhydrin reagent (prepared as described below) and 400 µl of glacial acetic acid. One gram of Ninhydren reagent (Sigma-Aldrich, St. Louis, MO) was prepared by dissolved in 16 ml of concentrated phosphoric acid in the dark; then bringing the volume to 40.0 ml with glacial acetic acid (MERCK KGaA, Darmstadt, Germany). The sample tubes were closed and placed in a boiling water bath for an hour. The tubes were removed from the water bath, and cooled to room temperature for 10-15 min. The samples were transferred into a new 13x100 mm disposable glass tube, and 2.5 ml of toluene added to each tube. The tubes were periodically gently shaken by hand for a few second to allow the red color to dissolve in the toluene phase. Absorbance of the toluene phase was measured spectophotometrically at $A515$. The standard curve was prepared by

dissolving 10 mg of L-Proline (Sigma-Aldrich, St. Louis, MO) into 10 ddH_2O ml (1mg/ml, 1000ppm). 1, 2, 4, 6, 8, and 10 µl of the standard was added into a fresh microcentrifuge tube then brought up to 400 µl with water and processed in as described above.

Total soluble sugar analysis

Total soluble sugar analysis was performed by the phenol/sulfuric acid method as described by Dubois *et al.,* (1956). Three ml of 80% ethanol was added to 0.1-0.2 g of the dried samples in 13x100 mm glass tubes and incubated overnight at 4 C°. The next day, total soluble sugars were extracted by boiling the samples for 30 min; the supernatant was transferred into a fresh 13x 100 glass tube and the heating step was repeated with fresh 3 ml of 80% ethanol. Tissues and debris were removed from the samples by centrifuging for 1 min at 2500 g. The supernatant was then transferred into fresh glass tubes followed by adding 3 ml of chloroform (MERCK KGaA, Darmstadt, Germany); samples were mixed by inverting several times and centrifuged for 5 min at 2500 g. The clear upper aqueous phase was transferred into a fresh 13x100 mm disposable glass tubes. 100 µl of the extract solution was added to 1.9 ml H_2O, followed by 50 µl 80% phenol (Sigma-Aldrich, St. Louis, MO); 5 ml of concentrated sulfuric acid was quickly added. Samples were left for 10 min before gently vortexing at low speed. Absorbance of the mix was measured at *A*485.

Mannitol analysis

One leaf (6-8 cm diameter; approximate fresh weight 1.5 g) was collected from T_0 and T_1 plants, freeze-dried for 48 hr, ground and placed in 13x100 mm disposable glass tubes (Cat No. 47729-572, VWR international, West Chester, PA). To total extract soluble carbohydrates, samples were boiled in 3 ml 80% ethanol for 30 min and the supernatant transferred into fresh 13x100 mm glass tubes. The pellet was re-extracted by boiling again with 2 ml 80% ethanol for another 30 min. After the extraction, 5 ml water was added to each sample, and samples were transferred into 15 ml disposable Corning tubes (Corning Inc., Corning, NY). Five ml of chloroform (MERCK KGaA, Darmstadt, Germany) was added to each sample; the samples were capped and shaken by hand and then centrifuged for 5 min at 2500 g. The clear upper aqueous phase was transferred into a fresh 13x100 mm disposable glass tubes and placed in heating blocks at 50 C° in the fume-hood until the samples were completely dried (approximately 2-3 hrs.). One ml of derivatization solution [pyridine (Sigma-Aldrich, St. Louis, MO) kept over NaOH pellets (Sigma-Aldrich, St. Louis, MO)] was added to each tube. The tubes were capped tightly and vortexed until all the dried sugar crystal were dissolved. The tubes were placed in a heating block at 70-80 C° for an hour; samples were vortexed every 30 min, then transferred to a tube rack and allowed to cool to room temperature for 10-20 min. One ml of room temperature hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, MO) was added to each sample. Samples were allowed to stand for 20-30 seconds before adding 100 µl of trifluoroacetic acid (TFAA) (Sigma-Aldrich, St. Louis, MO), followed by brief vortexing. Samples were incubated for an hour at room temperature, then 1.0 ml of clear upper aqueous phase was transferred into GC Vials (Alltech Corporation, Deerfield, IL),

capped tightly and placed in the auto-sampler tray for GC-Series II analysis (Hewlett Packard 5890-II gas chromatography, Palo Alto, CA) which was fitted with a DB-17 capillary column (J &W Scientific, Folsom, Ca, USA). Standards were previously prepared using 0.1 g of fructose, glucose, sucrose, raffinose, inositol, all mixed together and dissolved in 100 ml H_2O (this should give 1000 ppm). Different volumes of the standard mix were transferred into 13x100 mm glass tubes (1.0, 0.5, 0.1, 0.05, 0.025 and 0.01 ml) and were placed in heating blocks at 50 C° in the fume-hood until the samples were completely dried (approximately 2-3 hrs.) followed by the same derivatization steps that was described earlier.