ABSTRACT

BAGHERZADI, LALEH. Characterization of Transgenic Tomato Plants Expressing the C-domain of Calreticulin from *Zea mays.* (Under the direction of Dr. Heike Sederoff).

Calcium plays an important role in plants and animals. It is involved in multiple signal transduction pathways as a second messenger. The endoplasmic reticulum (ER) is an intracellular organelle which plays a critical role in many cellular processes. It is involved in Ca^{2+} storage and release, as well as protein synthesis and folding. The lumen of the ER contains many proteins which carry out various functions. Calreticulin (CRT), is an ER protein, and functions as a major Ca^{2+} binding protein and key component of the calreticulin/calnexin cycle for folding newly synthesized proteins and glycoproteins. Changes in subcellular calcium (Ca^{2+}) concentrations involving plant signal transduction pathways have been reported in response to drought and osmotic stress. Expressing CBP (Calcium Binding Peptide) in transgenic *Arabidopsis* plants has led to increased drought and salt tolerance. This research aims to investigate the role of ER localized Calcium in the drought-stress response of tomato (*Lycopersicum esculentum*). The ***hypothesis*** of this research is that expression of the high-capacity Ca^{2+}-binding peptide (CBP) from *Zea mays* CRT in tomato plants will increase the biomass and yield under normal growth conditions, and their tolerance to abiotic stresses. The ***specific aims*** were to: (1) Assess the physiological and morphological effects of the expression of this specific transgene in tomato under controlled conditions; (2) Analyze the transgenic tomato plants in response to drought stress and identify regulatory pathways involved in their stress resistance.

Transgenic tomato plants expressing the C-domain of the maize calreticulin gene fused with green fluorescent protein (GFP) driven under the control of the constitutive 35S promoter were characterized..

The integration of the transgene in T_4 and T_5 generation plants was confirmed. Moreover, semi-quantitative RT-PCR analysis showed similar levels of transcript abundance in the leaves of GFP-CBP lines. Expression of the fusion protein was also confirmed in transgenic lines by using antibodies against GFP. Results from the evaluation of transgenic tomato lines (T_4 and T_5 generation) under normal conditions revealed a difference in the number and the size of fruits. Transgenic lines showed a higher number of fruits per plant and a lower average fruit weight compared with that in control lines. Overall, transgenic tomato lines showed a higher biomass and an average seed weight compared with that in control lines.

Transgenic lines expressing the CBP *Zea mays* Calreticulin were tested under drought stress conditions. Based on the measured parameters, drought had a significant effect on the performance and the biomass of both transgenic and control tomato plants. However, phenotypic and physiological characterization of tomato transgenic lines (expressing C-domain of *Zea mays* Calreticulin) showed no significant difference compared with control lines under drought stress conditions.

Characterization of Transgenic Tomato Plants Expressing the C-domain of Calreticulin from *Zea mays*

by
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DEDICATION

“Keep your dream alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination and dedication.”

Gail Devers

This thesis is dedicated to:

My father, Behrooz Bagherzadi and my mother, Mahin Zargarzadeh whose love of reading and respect for education was instilled in me from early childhood and created this opportunity for me.

My twin sister Haleh and my younger sister Negin who are in my heart; friends to my spirit and golden threads in the fabric of my life.

This work is also dedicated to my lovely husband, Renzo Shamey, without whose caring support it would not have been possible to complete the work. I give my deepest expression of love and appreciation for the encouragement that he provided me and the sacrifices he made during my graduate program. Thanks Renzo, you are the best!

Finally to my son, Sean Araz Luca Shamey, who is a blessing and a true gift to our lives.

BIOGRAPHY

Laleh Bagherzadi was born in Azerbaijan in 1980. From early age biology fascinated and interested her in research and this led to biology, and chemistry, experiments often in the kitchen! She started attending agriculture and biotechnology conferences before entering college and enjoyed interacting with scientists and discussing issues with them. After obtaining her Bachelor of Science degree in Agronomy, she got married and moved to the United State in 2005. She loves to learn and hopes to continue to learn.

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THE ROLE OF CALCIUM IN SIGNALING AND DEVELOPMENT

Introduction

The goal of this study is to assess and analyze the functional role of Ca^{2+} in the ER lumen by expressing the Calcium-Binding Peptide (CBP) from maize Calreticulin in tomato plants (chapter 2) and also provide an insight about how the CBP expression affects the response to drought in CBP expressing tomato plants (*Lycopersicon esculentumvar. microtom*) (chapter 3).

Calcium is a ubiquitous secondary messenger [1]. The cytosolic calcium concentration is associated with plant development, growth and stress responses [1]. Calcium plays an important role in the sensing/signal transduction system in plants and animals [2]. Several Ca^{2+}-binding proteins have been reported in plants, such as calmodulin, calcineurin B-like proteins (CBL), and Ca^{2+}-dependent protein kinases [2]. Calreticulin (CRT) protein located in the ER contains a high affinity Ca^{2+} binding site (P-domain) and a low affinity high capacity Ca^{2+} binding site (C-domain) [2]. The variety of Ca^{2+} binding proteins in plants suggests that intracellular Ca^{2+} levels genera through calcium release and storage are tightly regulated [3]. It has been reported that calreticulin affects calcium homeostasis in the ER [4]. These data suggest that Calreticulin plays an important role in modulating ER calcium in higher plants [4]. An overview of the main structure and function of Calreticulin, as well as its role as a regulator in calcium homeostasis is given in this chapter.

Significant role of CRTs in plants' growth and development as well as in plants' response against abiotic stresses has been reported in literature reviews [5]. Drought is the main

abiotic stress which results in reduced plant growth and decreased crop yield in agriculture [6]. A summary of drought studies including the impact of drought on plant growth and development is also highlighted in this chapter.

Signal Transduction Pathway

Signal transduction is the mechanism that activates adaptive responses through perceiving environmental signals and transmitting to cellular machinery. Understanding these mechanisms is fundamentally important to expand our knowledge about how plants respond to environmental signals [1].

The first step of signal transduction pathway starts with perception, then transduction of the signal and last response to the specific signal. Second messengers are molecules in which transmit the signal into the cell and trigger a response. In some cases these molecules activate protein phosphorylation cascade [2]. This activation could lead to regulation of specific transcription factors that control specific stress responsive genes. Sometimes plant hormones (abscisic acid, ethylene) as a result of activation of stress responsive genes could be generated; in turn these molecules could be involved in initiation of second round of signaling [2].

Single molecule sensors might perceive the signals and regulate branches of signaling cascades which are instigated with one type of stress; on the other hand it is also possible that multiple sensors might perceive the initial signals. Beside primary sensors, there are secondary sensors known as secondary messengers which can initiate another cascade of

signaling. These sensors are different than primary sensors in time and space as well as the specificity of perception of signals [1].

Calcium as a Secondary Messenger

One of the important components of signal transduction pathways are second messengers. Calcium is a second messenger which plays an important role in signal transduction pathway [3]. The important role of second messengers such as, calcium, cAMP, cGMP, IP_3 and 1, 2-diacylglycerol has been elucidated in animal systems; although signaling mechanisms associated with calcium-calmodulin and phosphoinositide have also been reported in plants [7].

Increased concentrations of intracellular free calcium have been reported as a result of numerous chemical and physical stimuli [3]. Plasma membrane contains a variety of channels. It has been reported that after stimuli occur, calcium is released to the cytosol through the opening of calcium channels in the plasma membrane [8]. The increased concentration of $[Ca^{2+}]_{cyt}$ either via influx from plasma membrane or release from internal calcium stores initiates the phosphorylation cascade within the cell [9-6].

The change in cytosolic calcium levels has been detected and measured using calcium-activated photoprotein aequorin after the activities of intercellular enzymes, pumps or channels are altered in plants [10].

Calcium Storage and Release

The free calcium level in the cytosol is under strict biochemical and physiological control. Generally cells tend to keep the concentration of cytosolic calcium low (100-200 nM) and this is referred to resting or quiescent state [11-8]. However, upon stimulation the calcium concentration increases and this results in calcium acting as second messenger which brings the cellular response to the stimuli. This increase in calcium concentration could arise from outside the cell or from an internal pool [11].

The concentration of calcium in the intracellular organelles is higher than that in the cytosol and is in the micromolar to millimolar range (0.1 to 1 mM) in cell walls, vacuoles, and the endoplasmic reticulum (ER), whereas in the cytosol calcium is maintained in the nanomolar range (100-200 nM) [12]. This higher level of calcium concentration is due in part to the activity of Ca^{2+}-ATPases, which pump the calcium against a concentration gradient into the storage compartments to maintain the low cytosolic calcium concentration. On the other hand intracellular calcium releases occur through the activity of calcium release channels [13]. In animals one mechanism has been reported that involves calcium release into the cytosol. This mechanism involves interaction of IP_3 with specific IP_3 receptors (IP_3R) and inducing channel opening and allowing the efflux of calcium into the cytosol [14]. However, in plants transient increases in cytosolic Ca^{2+} concentrations, possibly through the release of Ca^{2+} from the ER by cADP-ribose activated channels have been reported [15].

Calcium Binding Proteins

Calcium sensors or calcium binding proteins play a key role by sensing and recognizing the increased level of calcium concentration in the cytosol [16].

Calcium sensors are small proteins that bind to calcium [11]. This binding causes a conformational change that either alters their interaction with other proteins or changes their activity [17]. Calcium binding proteins (CaBPs) function as intracellular calcium receptors or storage proteins. This requires the presence of calcium binding sites with selectivity for calcium in presence of other cations. Most importantly calcium sensors require EF hands in their structures. This helix-loop-helix motif termed EF-hand, binds calcium with high affinity. The EF-hand is highly conserved and mostly exists in pairs to stabilize of the protein structure [17].

Calmodulin (CaMs), Calmodulin like proteins, Calcium dependent protein kinases (CDPKs), and Calcineurin B-like proteins are the major family of calcium sensors which include EF-hands in their protein structure. Phospholipase D (PLD), Annexins, and Calreticulin have been categorized as calcium binding proteins without EF hands [11].

Calreticulin in Plants

Calreticulin is a multifunctional protein which is located in the lumen of the endoplasmic reticulum [4]. Calreticulin acts as a molecular chaperone that may play a role in protein folding process, retention and degradation of misfolded proteins [4]. Other important function of this protein is the involvement in calcium signaling that can regulate intracellular calcium homeostasis [4].

CRT was first detected in the ER by studying rabbit skeletal muscle [18], and in 1989 the gene was cloned [19]. However, CRT has been also identified in plant cells. CRT was first purified as a Ca^{2+} binding protein in spinach leaves [20].

Chen et al., isolated the cDNA clones from barley in 1994 [21], and additional work led to isolation of cDNA from tobacco by Denecke et al. in1995 [22], maize by Dresselhaus et al., in 1996 [23], and Chinese cabbage by Lim et al., in 1996 [24]. In 1997, Nelson et al isolated cDNA from Arabidopsis [25], while Coughlan et al. found the clones in Castor bean in 1997 [26], which was followed by Li and Komatsu‘s work on rice in 2000 [27], and most recently CRT was isolated in wheat by Jia et al. in 2008 [5].

Molecular Structure of Calreticulin

In plants, CRTs have so far been characterized to consist of three distinct structural domains and each domain is responsible for carrying out different functions [28]. The first domain of Calreticulin is the globular N-domain, the middle region is the P-domain and the last portion with a highly acidic region is the C-domain. There is also a signal sequence at the N - terminus and an ER retention motif at the C-terminus [28]. **Fig. 1.1.** shows the structural model of Calreticulin protein.

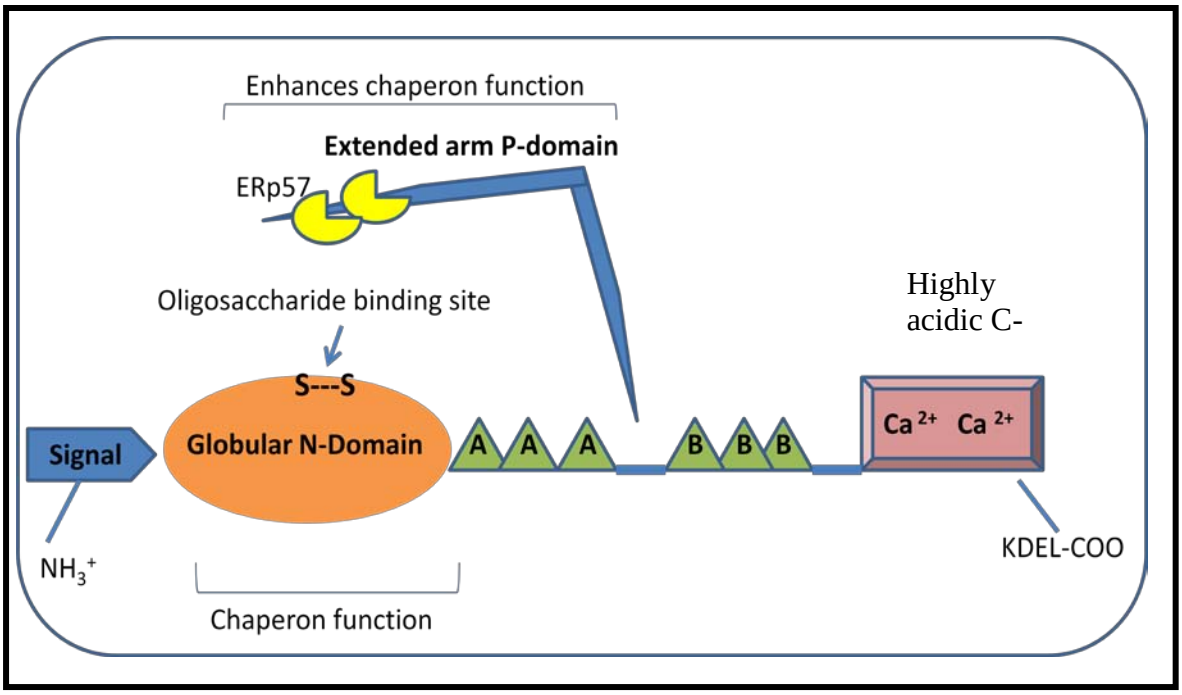

Figure 1.1. Structure of Calreticulin protein. The figure shows a schematic representation of the three main domains of the Calreticulin protein. The protein contains putative nuclear localization signal (N-domain), the proline rich domain of the low capacity/high affinity calcium binding domain (P-domain) and the acidic region of the high capacity/low affinity calcium binding domain (C-domain). Also a signal sequence at the N-terminal (ss) and C-terminal KDEL ER retrieval signal is indicated. The location of the disulphide bridge in the N domain of Calreticulin is also highlighted. Modified figure from Jia et al., (2008) [5].

One of the important and common characteristics of plant CRTs is glycosylation in the N-domain region [29]. The globular N-domain is highly conserved. It has been suggested that conserved cystein residues in the N-domain play an important role in forming a disulfide-bridge which is critical for the proper folding of CRT [29].

However, potential differences subsist among CRT isoforms as shown in **Fig. 1.2.** For plant CRT1 and CRT2 isoforms the most conserved glycosylation site is located near position 50-

60 in the N-domain; however, for CRT3 isoforms this site is located near position 96 in the same region of N-domain [30] as shown in **Fig. 1.2.**

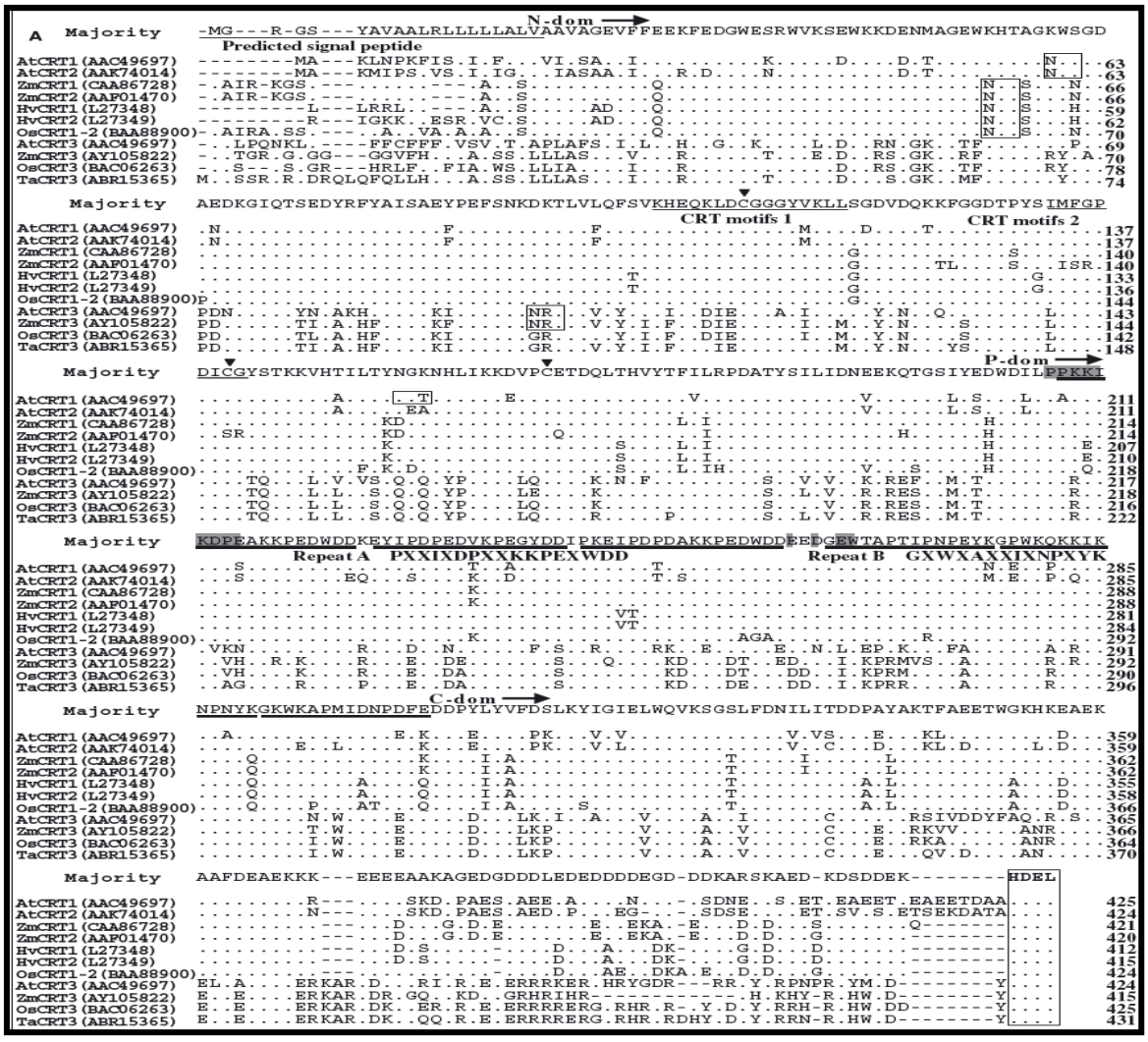

Figure 1.2. Sequence alignment of several plants Calreticulin. The signals peptide and the CRT motifs 1 and 2 are highlighted. The highly conserved Cys residues are shown as solid triangles. N-glycosylation sites are indicated as boxed regions. Four amino acid residues (Glu, Asp, Glu, Trp) critical for the chaperone activities are presented in gray shades. The Bold underline represents the A and B triplicate repeats. An ER retention sequence is indicated with bold text. The three main domains of Calreticulin protein are indicated with arrows. Figure from Jia et al., (2009) [30].

The P-domain starts with a putative nuclear targeting sequence (PPKXIKDPX) as shown in **Figure. 1.2**. This domain is followed by two triplicate repeated motifs, known as repeat A and B. In plant CRTs the sequences of these motifs are PXXIXDPXXKKPEXWDD and GXWXAXXIXNPXYK respectively, whereas in animal CRTs these sequences for A and B are PXXIXDPDAXKPEDWDE and GXWXXPXIXNPXYK [4]. Although the two repeated motifs are conserved but they are not identical in plants and animals.

The P-domain forms an extended arm structure [30]. One of the distinct characteristics of this arm structure is the interaction with other chaperones. Four amino acid residues (Glu, Asp, Glu, Trp) are critical for chaperone activity in animal CRTs [30]. These amino acids are also found at the tip of the arm structure in plant CRTs as highlighted in **Fig. 1.2.** [30]. The C-domain of Calreticulin is less conserved in comparison with other domains but is highly acidic [31]. In plant CRTs an ER retention motif at the C-terminus is HDEL whereas in animal CRTs this motif contains KDEL [31]. The C-domain binds to calcium with low affinity (K = 0.3-2 mM) and high capacity (20-50molCa^{2+}/molprotein) [31]. The signal peptide sequence is at the N-terminus and an ER retention motif signal is at C-terminus [30]. The disulphide bridge and conserved calreticulin family are located in the N-domain as shown in **Fig. 1.2.** In the P domain repeats A and B, located in the P-domain are also shown in **Fig. 1.2.**

Diverse Functions of Calreticulin

Calreticulin participates in many cellular processes including calcium storage and release, regulation of calcium signaling and intracellular calcium homeostasis, modulation of gene

expression, and chaperoning in the ER in plants and animals [32]. However, calreticulin also plays an important role in cell adhesion, wound healing and apoptosis in animals [32]. Coppolino et al., (1997) showed that calreticulin interacts with the cytoplasmic domain of integrin and this association could control integrin-mediated cell adhesion [33]. Integrin is an important mediator for cell adhesion to extracellular ligands. Integrin interacts with several signaling proteins and could participate in many cellular regulations such as cell shape, growth and differentiation [34].

Another interesting function of animal calreticulin was reported in relation to specifically modifying gene expression by binding to nuclear hormone receptors [35]. From both *in vivo* and *in vitro* approaches it has been shown that calreticulin can bind to the DNA-binding domain of steroid receptors and can prevent their interaction with DNA in *vitro* [35].

Because a large number of studies have examined the localization of the calreticulin outside of the ER [35], but there is still a considerable degree of controversy in the literature concerning the cellular localization of the protein. As a result it is difficult to explain the exact role for calreticulin outside of the ER in plants. In animals, cell adhesion and other functions of the calreticulin outside of the ER have been suggested [4].

Because of its low affinity but high capacity calcium binding to the C-domain, it has been suggested that calreticulin plays an important role in calcium homeostasis and signaling. The main function of calreticulin inside the endoplasmic reticulum is shown in **Fig. 1.3.** Calreticulin has been indicated to be involved in calcium homeostasis by binding to calcium with high capacity and low affinity.

Mesaeli et al., (1999) demonstrated that a calreticulin gene knockout is lethal to embryonic cells and loss of Calreticulin reduces calcium release via the $InsP_3$ (inositol 1, 4, 5-triphosphate) receptor in Calreticulin–deficient cells in mice ($crt^{-/-}$) [36]. However, in another study, Nakamura et al., (2001) examined the function of calreticulin as a regulator of Ca^{2+} homeostasis using calreticulin-deficient mouse embryonic fibroblasts and showed that in cells without Calreticulin, the ER had a lower capacity for calcium storage [31]. While the role of Calreticulin has been widely studied in animals, studies on plant Calreticulin are less frequent. However, an important function of *Arabidopsis* Calreticulin (AtCRT1a and AtCRT3) has been reported by studying $crt^{-/-}$ mouse fibroblasts [37]. Both AtCRT1 and AtCRT3 restored the ER calcium level and putative chaperone deficiencies in the CRT-deficient mouse fibroblasts [37]. Moreover, Jin et al., (2009) have reported that the C-terminal part of AtCRT3 was crucial for the Calreticulin protein to retain the defective BRI1-9 in the endoplasmic reticulum. bri1 *Arabidopsis* plants (a dwarf *Arabidopsis* mutant caused by retention of a defective brassinosteroid receptor in the ER) showed a dwarf phenotype. It was shown that EBS2 encodes for *Arabidopsis* CRT3 and loss of function *ebs2* mutations compromise ER retention of bri1 and suppress its dwarfism [38].

Persson et al., (2003) investigated the role of calreticulin in the endoplasmic reticulum (ER) in plant cells [39]. To examine how altered expression of CRT affects Ca^{2+} uptake and release, the ER-enriched membrane fractions from NT1 cells were used. Compared to control plants, transgenic plants showed 2-fold increase in ATP-dependent $^{45}Ca^{2+}$ accumulation in the ER enriched fraction when calreticulin increased by 2.5 fold [39]. It was also demonstrated that by using heat shock promoter an ER-targeted GFP-CBP peptide

constructed as translational fusion of the GFP gene to a sequence derived from the C-domain of maize Calreticulin, the intracellular Ca^{2+} levels could be manipulated. It was suggested that Calreticulin plays an important role in modulating ER calcium in higher plants. Transgenic plants expressing C-domain of maize Calreticulin (ER-CBP) survived longer than heat-shocked ER-GFP control plants when transferred to Ca^{2+} depleted medium. It was suggested that CBP expressing lines could store more calcium and use the restore calcium under calcium depleted medium [40].

Calreticulin is also involved in chaperon activity [41]. Many chaperon molecules are heat shock proteins [42]. The question may arise as to why cells need chaperones. Studies have shown that the function of chaperones is to prevent aggregation and misfolding of proteins [42]. Also, the role of chaperon proteins has been investigated during stress conditions. Castiglioni et al., (2008) have reported that expression of cold shock proteins (CSPs) from bacteria (E.coli) to maize promoted stress adaptation and improved vegetative performance under water deficit conditions [43].

Calreticulin as a molecular chaperone has been suggested to associate with membrane-anchored calnexin, in promoting folding of newly synthesized glycoproteins [41].

Both proteins are monomeric and are members of the legume lectin family [44]. Based on the crystallographic data from the structure of the Calreticulin, it has been suggested that the N-domain is a globular domain with homology to lectin which contains a glucose binding site and a disulfide bridge [44]. The P-domain with an extended arm is connected to a globular N-domain. The P-domain also interacts with ERp57 (an ER, PDI-like protein) to assist the

disulfide exchange reactions in misfolded proteins [45]. **Fig. 1.4.** shows the structure of the calreticulin and calnexin in relation to chaperon activity [45].

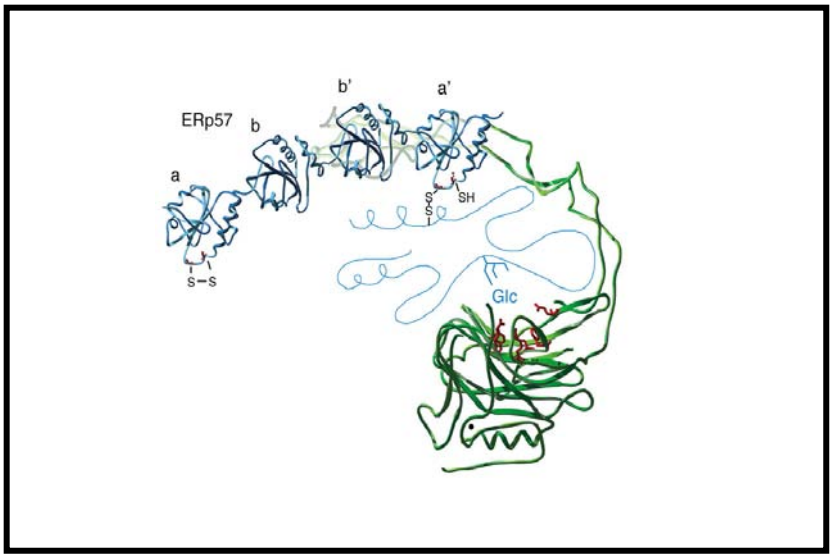

Figure 1.2. Model for the interaction of a folding glycoprotein with Calreticulin. The P-domain and N-domain are involved in protein folding by interaction with glycoproteins. The association of ERp57 with the P-domain and folding glycoproteins through the disulfide bond is shown [45].

One of the special features of the ER is the co-translational addition of N-glycans to proteins [46]. Glc3Man9GlcNAc2-core glycans are transferred to the newly synthesized proteins. As soon as the N-glycan is added to the proteins, Glucosidase I will remove the first (terminal) glucose from the glycoprotein, followed by Glucosidase II, which will remove the second glucose and eventually the third glucose. After the first two glucoses are removed, the

glycoprotein becomes a substrate for chaperone proteins like Calnexin (CNX) and Calreticulin (CRT) [46].

These two chaperones are lectins meaning that these proteins can bind to carbohydrate structures [47]. These lectin-like chaperones bind monoglucosylated N-glycans and their roles are to keep proteins in a folding competent state. Studies have shown that inhibitors of N-glucosidase which prevent glycoprotein binding to Calnexin and Calreticulin cause delays in the folding of the glycoproteins [47]. After glycoproteins are folded correctly, the last glucose unit from the N-glycan will be removed by Glucosidase II. This removal is a signal for correct folding and enables initiation of a pathway(s) for the proteins to exit the ER. However, removing the terminal mannose from the middle branch of N-glycans by a 1, 2-mannosidase provides a signal for folding failure, thus glucose will be added and the glycoprotein will be back to the cycle for further processing [46].

Furthermore, if the proteins are not folded correctly, EDEM (ER mannosidase) will target misfolded proteins in a process referred to as ER-associated degradation (ERAD). Misfolded proteins will be degraded in the cytosol by the proteasome [48]. **Fig. 1.4**. shows CRT/CNX cycle in protein folding.

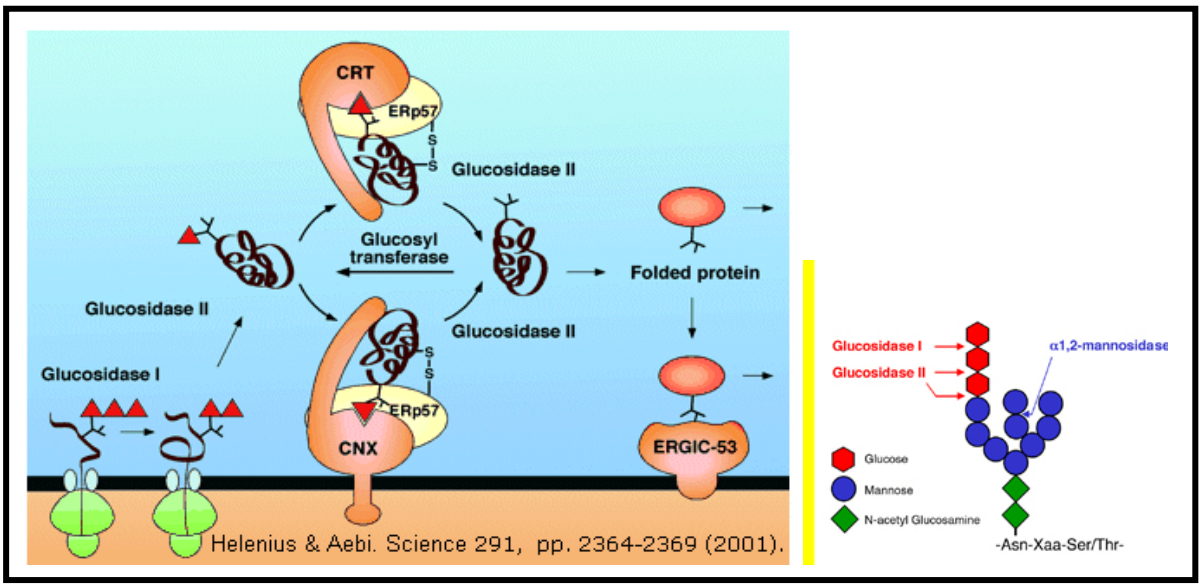

Figure 1.3. Calnexin/Calreticulin cycle in protein folding. Right: the newly synthesized protein and N-glycans attached to the side-chain of the protein. Through the cycle, Glucosidase I and Glucosidase II remove the glycan residue from the protein and the protein will enter to the cycle. Left: Calreticulin/Calnexin cycle through the glycosylation process. If protein folds correctly, it will remove the ER; however, misfolded protein will be recognized by 1, 2-mannosidase and glycan will be added to the misfolded protein. Recognized misfolded protein will return to the cycle for further processes. Parodi et al., (2000) [47].

Calreticulin in plants exhibits a similar structure and basic function compared to that in animals, however, the role of Calreticulin in plants is less elucidated.

The role of maize Calreticulin expression in tomato plants under drought stress conditions was investigated and the results are discussed in chapter 3 of this thesis. Published studies on drought and the impact of drought on plants will also be discussed in this chapter. The effect of drought on plant growth and its effect on water relations and plant photosynthesis will also be examined. In addition, morphological, physiological and molecular mechanisms of drought resistance in plants will also be described.

Effect of Drought on Plants

Plant Growth and Crop Yield

Several studies have reported that drought stress results in a severely reduced germination rate [49]. Kaya et al., (2006) have studied seeds of sunflower for tolerance to salt and drought. It was reported that inhibition of germination of sunflower seedlings were due to the osmotic effect than salt toxicity [49]. Studies on Alfalfa (*Medicago sativa*) have shown a reduction in germination potential, hypocotyls length, shoot and root fresh and dry weights using polyethylene glycol-induced water deficit [50].

Many factors are involved in growth in plants. Growth is accomplished through the cell division, cell elongation and differentiation. Cell division is less reduced than cell expansion under drought conditions. [51]. Under drought stress cell elongation is inhibited due to loss of turgor in plant tissues [6] as shown in **Fig. 1.5.**

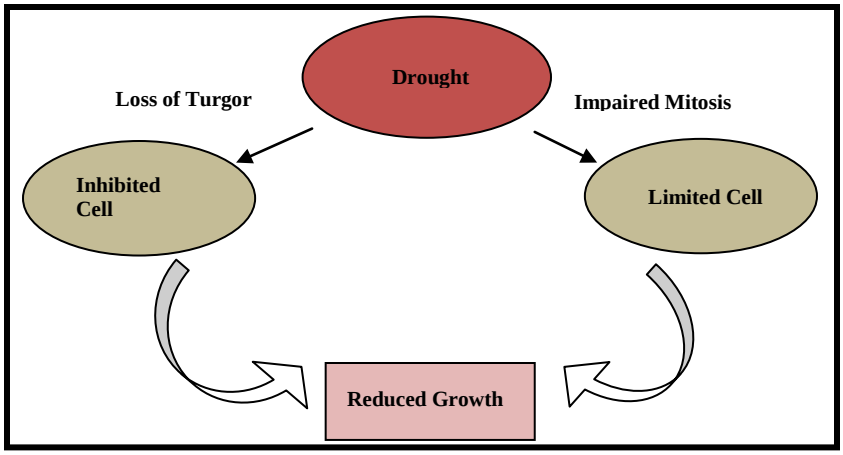

Figure 1.4. A proposed model for reduced growth mechanisms of plants under drought. Plant's growth is reduced under drought as a result of inhibition in cell elongation and limited cell division during drought. Cell division is affected due to impaired mitosis under drought stress. Reduced cell elongation, expansion and cell division can lead to reduced plant's growth. Adapted from Faroog et al., (2009) [6].

Yield integrates many of the physiological processes in a complex way [6]. Although several studies have reported that drought induces a yield reduction in crop species, it is difficult to interpret how plants accumulate and display the changing physiological processes [52].

Drought and Water Relations

Plant water relation is influenced by leaf water potential, stomata resistance, rate of transpiration, leaf temperature and relative water content [53]. Studies on applying water stress on wheat and rice plants have reported lower relative water content in plants with water stress than the ones without stress. Selected cultivars were subjected to four levels of water stress at vegetative stage. The exposure of plants to drought led to decrease in leaf water potential, relative water content and transpiration rate however the leaf temperature was increased [53].

Water use efficiency is calculated by dividing net photosynthesis rate by transpiration rate. Abbate et al., (2004) reported that wheat plants under limited water supply had greater water use efficiency than well watered plants which could be due to the closure of stomata to reduce transpiration rate [54]. Costa et al., (1997) reported early season drought stress in potato significantly reduced water use efficiency leading to decreased growth [55].

Drought and Photosynthesis

The main effects of drought on plants are decreased leaf expansion, impaired photosynthetic machinery, and leaf senescence [56].

The role of drought-induced stomatal closure is very important. In order for plants to prevent water loss, plants tend to close their stomata [57]. This as a result decreases in leaf turgor or water potential. By closing the stomata, CO_2 uptake is limited in leaves and as a result, CO_2 concentration in the chloroplast decreases and could limit the photosynthesis rate under drought stress conditions [57]. However there is a debate whether drought mainly limits photosynthesis through the closure of stomata, but it has been reported that stomata closure is generally accepted to cause decreased photosynthesis under mild drought [58]. Stomatal response has been also reported often more closely linked to soil moisture than leaf water status. Abscisic acid, a phytohormone, regulates stomatal responses [59]. Under water stress, chemical signaling becomes an important factor for plant adaptation. Root-sourced signals are transported through the xylem to leaves and as a result it decreases leaf growth and reduces water loss. However stomata conductance is not controlled through the soil moisture alone but there is a complex interaction of factors that play an important role in stomata response under drought stress [59].

Drought conditions also cause a decline in Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) activity which as a result, limits photosynthesis [60]. Rubisco activity is controlled either by reaction with CO_2 and Mg^{2+} (which carbamylates a lysine residue in catalytic site) or by binding inhibitors within the catalytic site. This inhibitor binding are essential for activity of the Rubisco. Tight binding inhibitors decrease the activity of Rubisco in the light [61]. Parry et al., (2002) have reported that at night, 2-carboxyarabinitol-1-phosphate (CA1P) is formed which inhibits catalytic activity of Rubisco by tightly binding to it [62]. it was suggested that in tobacco plants under drought conditions, the decrease of

Rubisco activity is not primarily the result of changes in the activation by CO_2 and Mg^{2+}, but rather due to the presence of tight binding inhibitors such as 2-carboxy-d-arabinitol-1-phosphate (CA1P) [62].

Parry et al., (1997) reported that total measured Rubisco activity from extracted leaves increased when tight-binding inhibitors were removed by using buffer containing 200 mol m^{-3} SO_4^{2-} which displaces any bound CA1P [63]. Other than the mentioned factors affecting photosynthetic rate under drought condition, adenosine trisphosphate (ATP) synthesis and impaired photophosphorylation has been reported that limits photosynthesis. In a study by Tezara et al., (1999) it was shown that stress decreases CO_2 assimilation and the amounts of ATP in the leaves of sunflower (*helianthus annuus L.*) [64].

Furthermore, under drought conditions, the export rate of sucrose from source to sink organs is affected, presumably as a result of decreased photosynthetic rates [65]. Limited photosynthesis and sucrose accumulation in leaves may hamper the rate of sucrose export to the sink tissues which affects the reproductive development [66]. Komor et al., (2000) reported that the root to shoot dry matter ratio was high in perennial cotton under drought stress, showing a preferential accumulation of starch and dry matter in roots as an adaptation to drought [65].

Mechanisms of Drought Resistance

Drought affects plants at the cellular, tissue and organ level and causes damage or adaptation reactions [67]. However further work is required to facilitate the understanding of plants defense mechanisms against water deficit. To that end, here some of the main published

studies pertaining to morphological, physiological and molecular mechanisms of plants under water deficit conditions are described.

Escape and Avoidance Strategy

Plant's escape strategy is one of the first responses under drought stress [68]. Shortened life cycle or growing season is the mechanisms that would allow plants to reproduce sooner and to avoid the period of stress. On the other hand the avoidance strategy consists of mechanisms that would be able to reduce water loss [68]. Two main strategies in plants to control and balance water loss is by controlling stomatal closure and maintaining water uptake through productive and extensive root systems [69]. Root systems play an important role under drought stress conditions. Deep root systems allow a plant access to water deep in the soil [69]. A study on chickpea has shown the importance of roots in coping with terminal drought, because root length and density of chickpea plants was higher in drought-tolerant genotypes [70]. Rodrigues et al., (1995) reported the effect of water deficit on *Lupinus albus L*. in which significant increase in the fine root length density and slight increase in the fine root dry weight was observed [71].

In summary, the response mechanisms of plants under drought stress involve escape or avoidance by reducing the growth duration or avoid stress by maintaining high water potential in tissue either by improving water uptake (root structure) or reducing water loss (through stomata closure) or by reducing the leaf surface area by producing smaller leaves [6].

Drought and Physiological Mechanisms

Osmotic adjustment, antioxidation and osmoprotection have been reported as mechanisms in plants for drought resistance [72]. Even though several mechanisms have been reported for the physiological basis of plants under drought stress, the molecular mechanisms underlying the physiological functions are not fully understood.

Osmotic adjustment is a common stress tolerance strategy [73]. Under drought, plants overproduce different types of compatible organic solutes. Compatible solutes are highly soluble and nontoxic even at high concentrations and protect plants simply through osmotic adjustment, detoxification or reactive oxygen species and stabilize the structure of proteins under stress conditions [74].

Examples of molecules used or produced by plant cells to compensate for osmotic pressure include soluble sugars, proline, calcium, and potassium. Under water deficit conditions, the osmotic potential of the cell is lowered as a result of solute accumulation; this process maintains turgor of the cell, since accumulation of solutes attracts water into the cell [75]. In the study by Mohammadkhadni et. al., (2008) it was reported that free proline levels increased (from 1.56 to 3.13 times) in response to drought stress in two maize cultivars. It was suggested that proline plays a role in minimizing the damage caused by dehydration. However increased level of proline was observed in shoots than roots of maize cultivars [76].

Growth Regulators in Plants

Phytohormones or plant growth regulators are substances that affect physiological processes in plants at very low concentrations; auxins, gibberellines, cytokinins, ethylene and abscisic

acid are the major components which play an important role under stress conditions [77]. Studies have reported the decreases of auxins and cytokinin, and increases in abscisic acid and ethylene under drought [78]. Among those hormones, abscisic acid is known as a stress hormone and its production has been reported under variety of stress conditions specifically drought. Sharp et al., (1994) have reported that the increase in abscisic acid accumulation under drought can regulate gene expression and can regulate stomatal closure through the cross talk with cytokinins in order to reduce water loss under water deficit. Moreover, the increased abscisic acid under stress conditions like drought could alter the relative growth rates such as root to shoot dry weight ratio or decrease in leaf area [78].

Drought and Molecular Mechanisms in Plants

Changes in gene expression (either up or down regulation) have been reported under stress conditions. Under drought conditions various genes at the transcriptional level are induced and the products of induced genes function in drought tolerance [79].

Proteins in Response to Drought

Aquaporins are one of the most important proteins which play an important role in water transport. These proteins are located in the plasma membrane and abundantly expressed in roots [80]. Javot et al., (2001) reported 25 to 30% reduction in hydraulic conductivity of the root cortex by comparing *Arabidopsis* knockout mutants of PIP2; 2 (an abundantly expressed aquaporin isoforms) to the wild type [81].

Another group of stress proteins involved in drought tolerance in plants is heat shock proteins, dehydrins and late embryogenic abundant proteins. All these proteins are involved in stabilizing and protection of other proteins or protein structures during the stress conditions [82]. Wechsberg et al., (1994) have studied one class of late embryogenesis-abundant (LEA) proteins during development and water stress in *Ranunculus sceleratus* L. achenes seeds. When seeds were placed at 21 DPA in polyethylene glycol (PEG) at -1.5 MPa the seed moisture content was reduced and this was accompanied by accumulation of 31 KDa protein. This protein was no longer detected when the seeds were transferred to water. The data indicated depends on the degree and duration of the water stress, accumulation of dehydrin-like proteins also changes in *R. sceleratus* plants [82].

Drought and Signaling Events

In plants stress-induced signaling requires sensing of the stress that leads to activation of defense and acclimation pathways [83]. Moreover specific responses to stress include the accumulation of metabolites like betaines or heat shock proteins (known as protective proteins). Chemical signals involves calcium, calcium dependent proteins, reactive oxygen species and plant hormones and it is hypothesized that they act through signal transduction pathways to activate genomic re-programming which allows adaptation to environmental conditions [84].

Calcium known as a second messenger plays an important role in signal transduction cascades [85]. It was reported that calcium can improve water stress tolerance [86]. Abdul Jaleel et al., (2007) have reported that addition of $CaCl_2$ to drought stressed *Catharanthus*

roseus plants lowered the proline oxidase activities (proline oxidase catalyzes the conversion of proline to glutamate in which it reduces the concentration of proline) compared to control plants. It was also reported that calcium increased the level of Glycine betaine content. This accumulation of Glycine betaine through the $CaCl_2$ could indicate its role as an osmoprotection under drought condition [86].

Calreticulin in Response to Stress Conditions

Enhanced expression levels of Calreticulin mRNA and protein in response to stress conditions such as cold, salt and phytohormones have been reported [30]. This increased expression of calreticulin could be involved in modulating gene expression of other metabolic pathways and as a result plants could respond and adapt to the stress condition.

Calreticulin plays an important role in cellular processes; as role in calcium storage and release and as a chaperone in folding newly synthesized proteins [30]. Calreticulin's role in response to abiotic stress has been investigated. To elucidate the function of the calreticulin in response to drought, Jia et. al., (2008) studied full length wheat calreticulin *Ta*CRT (*Triticum aestivum L.*), over expressed in tobacco (*Nicotiana benthamiana*) plants [5]. TaCRT-over-expressing plants showed drought resistance comparing with control plants under water deficit condition. Transgenic tobacco plants showed higher water use efficiency, water retention ability, relative water content and lower degree of membrane damage under water deficit conditions comparing to wild type control plants. It was concluded that wheat calreticulin is involved in the drought stress responses [5].

Although current data indicate that by over-expressing calreticulin in transgenic tobacco plants, enhanced resistance to drought is obtained, the molecular mechanism still remains unknown [5].

A common characteristic and strategy of plants to deal with stress conditions and cope with unfolded proteins is to increase the production of chaperon molecules and folding enzymes in the ER. This would lead to an increased number of folding proteins in the ER and prevent unfolding of proteins [25]. Over-expression of Calreticulin (as an important chaperon) could result in increased amounts of chaperon protein and increase the ratio of correctly folded proteins in the ER under various stress conditions [25].

Calreticulin as a calcium binding protein plays an important role in regulating the calcium storage and release in endoplasmic reticulum in plants [27]. As a regulator of calcium homeostasis, Li et al., (2008) have demonstrated the function of calreticulin in mutant *Arabidopsis* plants in response to calcium and salinity stress. Arabidopsis crt3 mutant showed sensitivity to calcium depleted media at germination stage. The primary roots of crt3 mutant seedling were shorter than wild type and even loss of chlorophyll was observed in crt3 mutant under 7mM EGTA media while wild type seedlings remained green [27].

However the crt3 mutants exhibited lager rosettes under salt stress conditions compared to wild type plants. Neither single nor double crt1, crt 2 mutants showed an altered response to salt stress. It was shown that calreticulin mutant crt3 in Arabidopsis is involved in the plants stress response to calcium depletion and plays an important role in calcium homeostasis and salinity stress [27].

Based on the other functions of Calreticulin in the ER, (regulating calcium homeostasis and modulating calcium signaling), it is likely that over-expression of calreticulin could increase the calcium capacity to rapidly store or release calcium from the ER. Overexpression of maize CRT in Arabidopsis lines, led to increased total calcium and higher levels of chlorophyll and seed yield compared to control wild type and 35S:GFP lines [87]. Arabidopsis transgenic lines (over-expressing maize Calreticulin) also showed increased root growth and better survival under intermittent drought stress through the up-regulation of *CIPK*6 [87]. Calcium plays an important role in signal transduction pathways by acting as a second messenger. It is likely that under stress conditions the cytosolic concentration of calcium increases, either via uptake from the apoplast or via calcium channels located in the membrane of the organelles. Calcium could bind to a variety of proteins in the cytosol, such as calmodulin which could lead to changes gene expression. By considering the fact that several signaling pathways are regulating plant stress responses, overexpression of calreticulin could activate specific signal transduction pathways which could as a result alleviate the plant response to drought stress.

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Characterization of Transgenic Tomato Plants expressing the C-domain of *Zea mays* Calreticulin

Introduction

Calcium is an essential mineral for plant growth and development. It plays an important role as a second messenger [1]. Cytosolic calcium concentrations are associated with stress responses [2]. The level of calcium in the cytosol is tightly regulated. High levels of free calcium have been reported in subcellular organelles (0.1-10 mM) [3]. Under normal conditions the cytosolic concentration of calcium remains in the range of 10-200 nM. This is called “resting” calcium [3]. Increased levels of the free calcium in the cytosol have been reported resulting from external stimulation [4]. Studies have reported an increase in calcium concentration due to stress, especially due to salt stress [5]. However, a sudden increase in the calcium concentration, triggered by stress, could be toxic if it persists for a long period of time [5]. As a result plants have evolved ways to take up excess calcium and store it in the organelles such as ER or vacuole [5]. Calreticulin’s role as a regulator of calcium homeostasis has been investigated [6]. Calreticulin is a multi-functional protein located in the lumen of the ER [7]. The C-domain of Calreticulin has high-capacity but low affinity binding to calcium, and expressing this domain in the ER has been reported to increase calcium storage [8]. It has been reported that calreticulin affects calcium homeostasis in the ER in tobacco cells *in vitro* and in *vivo* [9].

In addition, over-production of a maize CRT in tobacco cell suspension cultures improved the growth of cells exposed to a high calcium medium [9]. Wyatt et al., (2002) reported the

expression of the C-domain of Calreticulin in *Arabidopsi*s plants [10]. It was demonstrated that induction of an ER-targeted GFP-CBP peptide constructed as a translational fusion of the green fluorescent protein (GFP) gene to a sequence derived from the maize calreticulin C-domain, the intracellular Ca^{2+} levels could be manipulated. It was also reported that the plants expressing ER-targeted CBP survived longer than ER-GFP control plants when transferred to Ca^{2+} depleted medium [10]. These data suggest that the C-domain of Calreticulin plays an important role in modulating ER calcium in higher plants. Nevertheless, the current knowledge of Calreticulin and its functions in plant physiology is still limited.

Our goal in this study is to find out how expression of CBP (calcium binding peptide) from *maize* Calreticulin could affect the plant's response under normal and stress growth conditions in tomato plants. Based on the previous work [11], our lab generated transgenic tomato lines by transformation of wild type tomato plants (*Lycopersicum esculentum*) with the binary plasmids (pBIN2311-GFP-CBP), which carries the C-domain of the *Zea mays* Calreticulin (CBP) gene and GFP fusion under the control of the constitutive Cauliflower Mosaic virus 35S promoter (CaMV 35S) using *Agrobacterium tumefaciens*-mediated transformation. Control plants were transformed with the same binary vector which carries the green fluorescent protein (GFP) coding sequence, but lacks the CBP coding sequence (35S:GFP). The transgenic lines expressing an ER-targeted fusion gene are referred to as CBP plants because they contain the calcium binding peptide gene. Ten independent lines of the primary transformants expressing 35S:GFP-CBP were regenerated and exhibited stable expression of the gene in the T_1 generation. Homozygous lines from the T3 generation were identified and selected for further study [11].

This chapter documents the phenotypic effects of the change in gene expression in the 35S:GFP-CBP expressing plants compared to the wt and the 35S:GFP plants under stress-free "normal" growth conditions. The presence of the maize CBP transgene in tomato plants was confirmed by PCR using maize CBP-specific primers. The *CBP* mRNA expression was analyzed in selected 35:GFP-CBP lines (T_4 generation) for transcript abundance. Semi-quantitative RT-PCR showed no difference in levels of transcript abundance in the leaves of independent CBP expressing lines (35S:GFP-CBP).

Additionally, protein levels of the CBP transgene was analyzed using Western blot. Using an anti-GFP antibody, the CBP expressing lines (35S:GFP-CBP) showed the expression of the GFP-CBP fusion protein. The expression and localization of the CBP-GFP was analyzed in CBP expressing lines using the confocal laser scanning microscope. The GFP-CBP protein was expressed in the root and the leaf of the CBP expressing (35S:GFP-CBP) lines.

Interestingly, two plants from the selected line (35S:GFP-CBP-4) showed no presence of the transgene and this was confirmed by RNA and protein analysis. Because these plants contain an increased chloroplast number, but lacked the gene of interest, the offspring of these plants were selected as controls for this project.

In tomato plants, the guard cell chloroplast number is related to the ploidy level of the plant [12]. Using the chloroplast counting approach, CBP expressing tomato plants were shown to have a higher number of chloroplasts in the guard cells compared to wild type and GFP vector control plants [12] indicating that these plants are polyploids. Therefore, to obtain polyploid plants as controls, wild type plants were treated with Surflan A.S (herbicide) [13]. We found that the CBP expressing plants had a higher average fresh and dry root and shoot

weight than control plants under non-stress conditions. Based on the results, total seed dry weight in CBP expressing lines was also found to be higher compared to control lines. The experiments evaluating the expression of the GFP-CBP fusion protein in the CBP expressing plants (35S:GFP-CBP) and the detailed phenotypic characterization of these plants will be documented in the section below.

Results

Identification of 35S:GFP-CBP Expressing Lines

Using the approach reported in a previous study [11], plasmid construct lines were generated by inserting 370 bp of C-domain of maize Calreticulin together with mGFP gene and the HDEL ER retention sequence into the binary plasmids under the control of the constitutive CaMV 35S promoter (pBIN2311-GFP-CBP). Control GFP construct was generated by removing the CBP gene from the plasmids and leaving only the green fluorescent protein (GFP) coding sequence. Homozygous lines were identified and T_3 generation tomato plants were used for the study. The construct lines were screened for GFP-CBP expression. Preliminary analysis showed that the GFP-CBP construct was expressed in roots, leaves and tomato fruits.

Here, we used, T_3 generation CBP expressing plants (35S:GFP-CBP) generated by Khodakovskaya and Sederoff (unpublished results) et al. [11], to phenotypically characterize the plants that expressed the maize C-terminal CBP in tomato. For the phenotypic analysis, three independent GFP-CBP CBP expressing lines (35S:GFP-CBP-1, 35S:GFP-CBP-2 and 35S:GFP-CBP-4) were selected, along with 35S:GFP expressing vector control lines, wild

type and sibling control plants (transgenic plants which showed polyploidy but did not show the gene of interest). CBP expressing lines (35S:GFP-CBP) as well as control lines used in this study are shown in **Table 2.1.**

Table 2.1. CBP expressing 35S:GFP-CBP tomato lines and control lines. T_4 and T_5 generation of CBP expressing lines (35S:GFP-CBP) along with control lines (wild type, 35S:GFP and lines) were used in this study. Range of the plants per line is shown in each generation. Two plants from line 35S:GFP-CBP-4 (4-4-6 and 4-1-5) which were negative for maize CBP gene were identified from T_4 generation and used as a sibling control in T_5 generation.

Selected line Types	T3 generation	T4 generation/used in this study # of the plants/line		T5 generation/used in this study # of the plants/line
35S:GFP-CBP–1	GFP-CBP-1-1 GFP-CBP-1-3 GFP-CBP-1-5	GFP-CBP-1-1-(1-3) GFP-CBP-1-3-(1-4) GFP-CBP-1-15-(1-3)		GFP-CBP-1-15-1-(1-2) GFP-CBP-1-2-1-(1-3) GFP-CBP-1-3-2-(1-3)
35S:GFP-CBP– 2	GFP-CBP-2-1 GFP-CBP-2-2 GFP-CBP-2-8	GFP-CBP-2-1-(1-2) GFP-CBP-2-2-(1-4) GFP-CBP-2-8-(1-3)		GFP-CBP-2-8-2-(1-5) GFP-CBP-2-2-2-(1-5)
35S:GFP-CBP- 4	GFP-CBP-4-1 GFP-CBP-4-3 GFP-CBP-4-4	GFP-CBP-4-1-(1-7) GFP-CBP-4-3-(1-4) GFP-CBP-4-4-(1-6)		GFP-CBP-4-1-7-(1-5) GFP-CBP-4-4-1-(1-4)
Wild type /Control	WT-1 WT-2 WT-3	WT-1-(1-3) WT-2-(1-4) WT-3-(1-2)		WT-1-1-(1-5)/treated with Surflan WT-2-1-(1-5)/treated with Surflan
35S:GFP/Control	GFP-7 GFP-8 GFP-14	GFP-7-(1-3) GFP-8-(1-2) GFP-14-(1-3)		GFP-7-1-(1-8)/treated with Surflan GFP-14-1-(1-4)/treated with Surflan
Siblings/Control	----------	GFP-CBP-4-1-5/identified plant GFP-CBP-4-4-6/identified plant		4-1-5-(1-10)/used as sibling control 4-4-6-(1-10)/used as sibling control

Genomic DNA from the CBP expressing tomato lines as well as the 35S:GFP vector control lines was extracted and analyzed by PCR to confirm the integration of the transgene using gene-specific primers (**Fig. 2.1.)**, CBP expressing tomato plants showed the C-domain of the

maize Calreticulin fragment confirming the presence of the transgene whereas control plants (wild type and 35S:GFP) showed no such band. Actin was used as a control (band size 206 bp).

One plant from CBP expressing line 4, (35S:GFP-CBP-4, plant 4-4-6), was found to be negative for the presence of the transgene (Lane 1, white box and gray arrow) in the presence of control Actin as shown in **Fig. 2.1.**

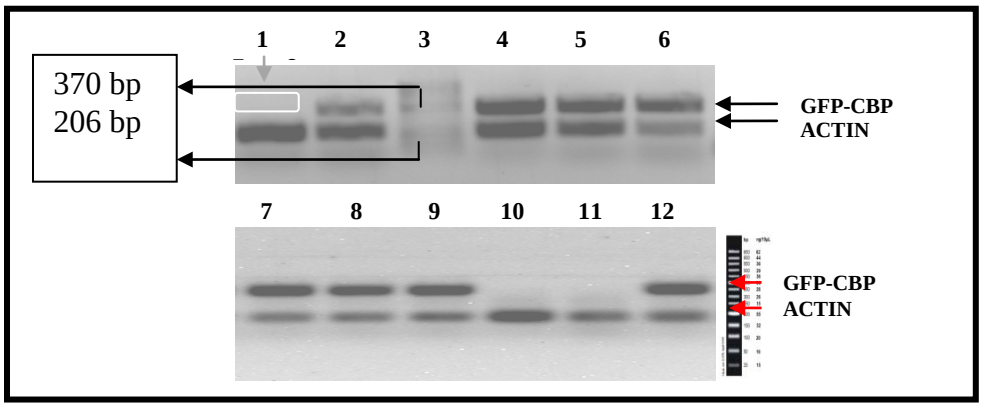


Figure 2.1. Detection of the maize CBP gene in CBP expressing tomato lines. The ethidium bromide-stained agarose gel showed amplification by the polymerase chain reaction (PCR) of the maize CBP gene (370-bp fragment; *arrow* on the right) in CBP expressing tomato plants (35S:GFP-CBP line 1, 2 and 4), while no band was observed in wild type and 35S: GFP control plants. From left to right, Lane 1-CBP expressing line 4 (35S:GFP-CBP- 4, plant (4-4-6)). Lane 2-CBP expressing line 1(35S:GFP-CBP-1, plant (1-3-2)). Lane 3- 50bp Mini DNA ladder. Lane 4-CBP expressing line 1 (35S:GFP-CBP-1, plant (1-3-2)). Lane 5-CBP expressing line 2 (35S:GFP-CBP-2, plant (2-8-1)). Lane 6- CBP expressing line 2 (35S:GFP-CBP-2, plant (2-2-2)). Lane 7- CBP expressing line 4 (35S:GFP-CBP-4, plant (4-4-2)). Lane 8- CBP expressing line 4 (35S:GFP-CBP-4, plant (4-4-3)). Lane 9-CBP expressing line 4 (35S:GFP-CBP -4, plant (4-4-4)). Lane 10- wild type control. Lane 11-35S:GFP vector control. Lane 12-CBP expressing line 2 (35S:GFP-CBP-2, plant (2-1-1)). Actin used as control (206-bp fragment; arrow on the right).

The expression of the *CBP* gene was analyzed in selected lines (T_4 and T_5 generation) for transcript abundance as shown in **Fig. 2.2 (A-C)**. Semi-quantitative RT-PCR showed no difference in the levels of transcript abundance in the leaves of the independent 35S:GFP-CBP expressing lines. No band was observed in wild type or 35S:GFP control plants. From T_4 generation, the CBP expressing plant (35S:GFP-CBP -4, plants 4-4-6) showed no RNA expression for the gene of interest as shown in **Fig. 2.2.A**. The offspring of this plant, referred to as siblings, were used as control for further analysis in T_5 generation as shown in **Fig. 2.2.B.** In presence of actin, CBP expressing line 1 and 2 (35S:GFP-CBP-1 and 35S:GFP-CBP-2) showed the same transcript abundance for maize *CBP* mRNA, whereas no band was observed in sibling or wild type plants **Fig. 2.2.B**.

Furthermore, based on the semi-quantitative RT PCR analysis, no difference was observed in transcript abundance among selected plants from the same line (35S:GFP-CBP-1 and 35S:GFP-CBP-2) as shown in **Fig. 2.2.C**. Actin was used as an internal loading control.

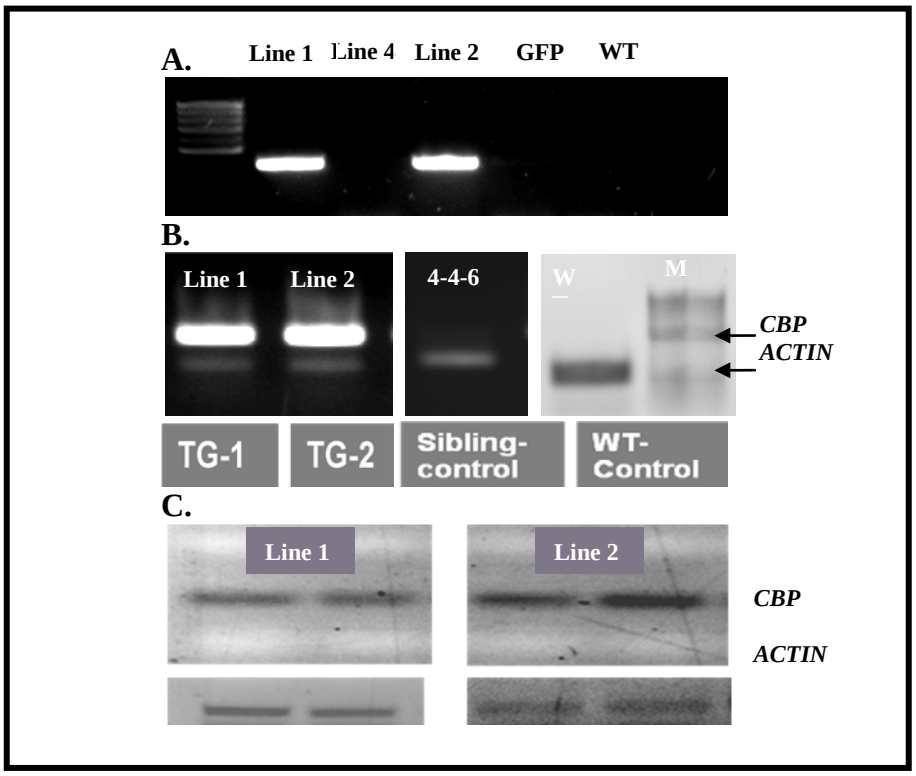


Figure 2.2. Semi-quantitative PCR-mediated CBP mRNA expression analysis. Semi-quantitative RT PCR of *CBP* expression in leaf tissues of 2 months old plants from 35S:GFP-CBP-1, 35S:GFP-CBP-2 and 35S:GFP-CBP-4 with 35S:GFP and wild type control plants were analyzed. A. T_4 generation lines were tested for transcript abundance. CBP expressing line 1and 2 (35S:GFP-CBP-1 and 35S:GFP-CBP -2) showed the band (band size 370 bp), while no band was observed in wild type and 35S:GFP control plants. CBP expressing line 4 (35S:GFP-CBP-4, plant 4-4-6), represents a candidate transformant but no target fragment was detected. B. Semi-quantitative RT PCR expression of T_5 generation plants from CBP expressing line 1 and 2 (35S:GFP-CBP-1 and 2) which showed the same maize *CBP* mRNA expression level; sibling control (plant 4-4-6) and wild type control plant showed no band. M: Molecular 50bp ladder. C. Same transcript abundance was observed between the selected plants from the same CBP expressing lines 1and 2, (35S:GFP-CBP-1 and 2). Plants (1-3-2 and 1-15-1) from CBP expressing line1 (35S:GFP-CBP - 1) and plant (2-8-1 and 2-2-2) from CBP expressing line 2 (35S:GFP-CBP 2) were used. Actin was used as an internal loading control.

To test for the presence of the CBP-GFP fusion protein in the 35S:GFP-CBP plants, western blot analysis was performed with specific anti-GFP antibody (Genscript, NJ). The maize CBP protein is 41 KDa long and the GFP is 27KDa long. Consequently, we found a 41 KDa band in CBP expressing lines 1 and 2 (35S:GFP-CBP-1, 35S:GFP-CBP-2) as shown in **Fig. 2.3.** No bands were seen in the wild type plants, indicating that the anti-GFP antibody was specific. Total protein from three independent 35S:GFP-CBP CBP expressing lines (1, 2 and 4), the 35S:GFP vector control lines and wild type control tomato plants was extracted and separated by SDS-PAGE. GFP-CBP fusion proteins and GFP control lines were visualized by using antibodies against GFP. **Fig. 2.3.** shows the expression of the fusion protein in CBP expressing lines as well as vector control 35S:GFP lines. There was protein degradation observed in all CBP expressing lines (35S:GFP-CBP-1, 2) probably because of high expression level on the selected plants. CBP expressing tomato line 4, (35S:GFP-CBP-4, plant 4-4-6) was negative to presence of fusion protein. No band was detected on the DNA and mRNA level as shown in **Fig. 2.1** and **Fig. 2.2.**

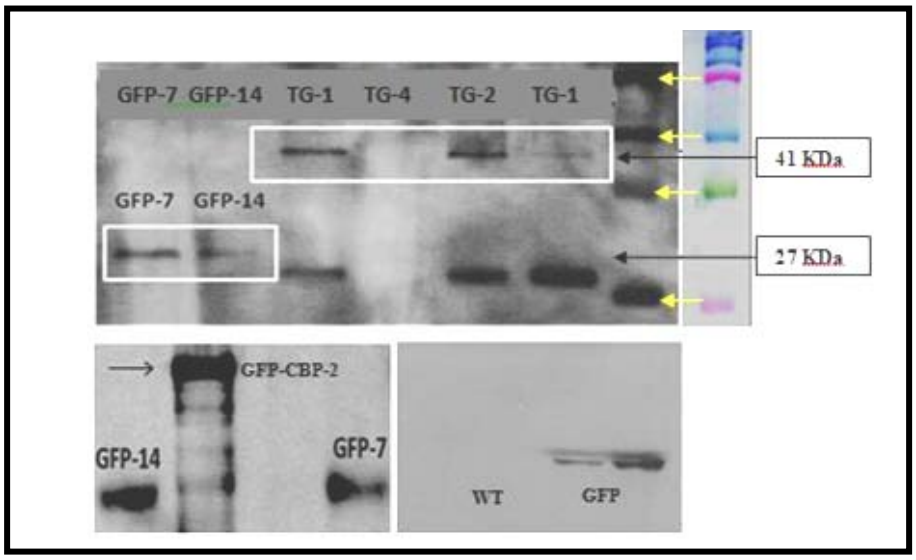


Figure 2.3. Western-blot analysis of GFP-CBP protein in 35S:GFP-CBP plants and control plants. Total protein was extracted from the leaves of CBP expressing and control tomato plants and separated by SDS-PAGE. The protein of interest was transferred onto a PVDF membrane, probed with specific anti-GFP antibody. Based on the western blot analysis tomato plants expressing GFP fusion protein under the 35S promoter showed 27 kDa size band (GFP-7, GFP-14), while CBP expressing plants from line 1, 2 under the control of 35S promoter showed 41 kDa size band. However, no band was observed in plant 4-4-6 from CBP expressing line 4. Wild type plant was used as a negative control. Plants (1-3-4 and 1-1-5) from CBP expressing line 1 (35S:GFP-CBP-1) and plant (2-2-8) from CBP expressing line 2 (35S:GFP-CBP-2) showed breakdown products (25 kDa size band). A Molecular-mass standard (Precision Plus Protein Standards, Kaleidoscope^{TM} Bio-Rad, Bethesda, MD) was used.

Expression Analysis of GFP-CBP Constructs

Tomato lines expressing 35S:GFP-CBP (1, 2 and 4), as well as the 35S:GFP line, which was transformed with the same vector but lacked the maize CBP sequence, were analyzed based on their GFP expression pattern. The GFP expression was visible in shoots, leaves and roots of transgene expressing in 35S:GFP-CBP-1 and 35S:GFP-CBP- 2 but not in wild type plant as shown in **Fig. 2.4.**

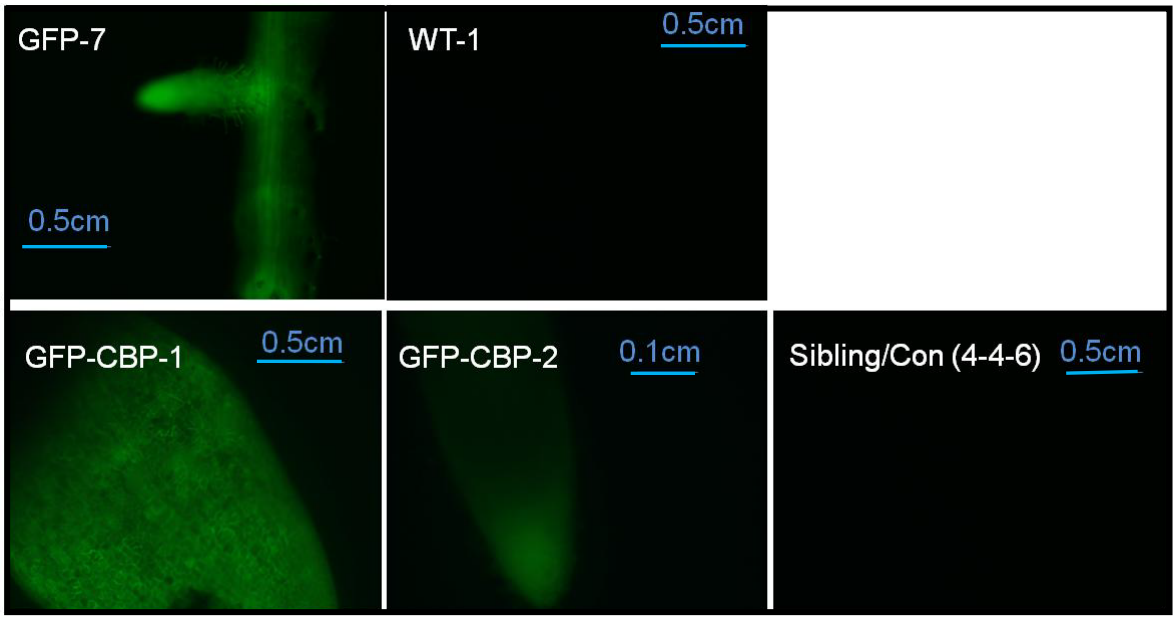


Figure 2.4. Expression analysis of the GFP in CBP expressing lines. GFP expression was only visible in 35S:GFP-CBP lines 1 and 2 and in 35S:GFP control plants, but not in wild type plants. Expression pattern was observed in roots and leaves of transgene, expressed in tomato lines, however CBP expressing line 4 (35S:GFP-CBP-4, plant 4-4-6) which was negative to presence of transgene also showed no GFP expression pattern.

Ploidy Level Analysis of the 35S:GFP-CBP Lines vs. 35S:GFP and Wild type Tomatoes

Ellul et al., (2003) have reported an increase in ploidy level of transgenic plants using Agrobacterium-mediated transformation in tomato cotyledons (*Lycopersicon esculentum* Mill). It was reported that this process depends on the genotype and procedure used for the transformation [14]. Moreover, Sigareva et al., (2004) stated the use of mannose selection protocol for tomato reduced the adverse effects on the ploidy level of transgenic tomato plants [15]. In addition, Hamaoka et al., (1991) analyzed microspore- derived plants produced through another culture in *Brassica campestris* [16]. The number of chloroplasts in the guard cells was reported to be clearly related to the ploidy level. The number of

chloroplasts in the cells was increased after the chromosomes were artificially doubled with colchicine [16].

We analyzed the ploidy levels of selected CBP expressing plants as well as 35S:GFP controls and wild type plants. The chloroplast numbers of each guard cell in the leaf epidermis of each plant were analyzed. A total of sixty plants were selected for the experiment; the number of chloroplasts was increased in CBP expressing lines whereas in the Wild type it did not increase. **Fig. 2.5.** shows the number of chloroplasts in guard cells of CBP expressing tomato plants. While the average number of the chloroplasts in CBP expressing lines was 6-8, wild type plants showed an average around 4 and 35S:GFP control lines showed an average around 4-6. The grand average of chloroplast numbers in CBP expressing (35S:GFP-CBP) tomato lines as well as in 35S:GFP and wild type control plants is shown in **Fig. 2.6.**

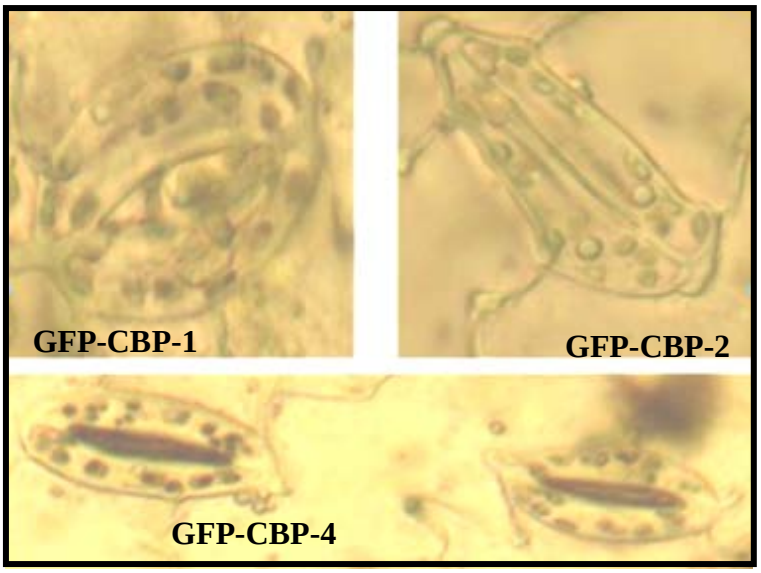
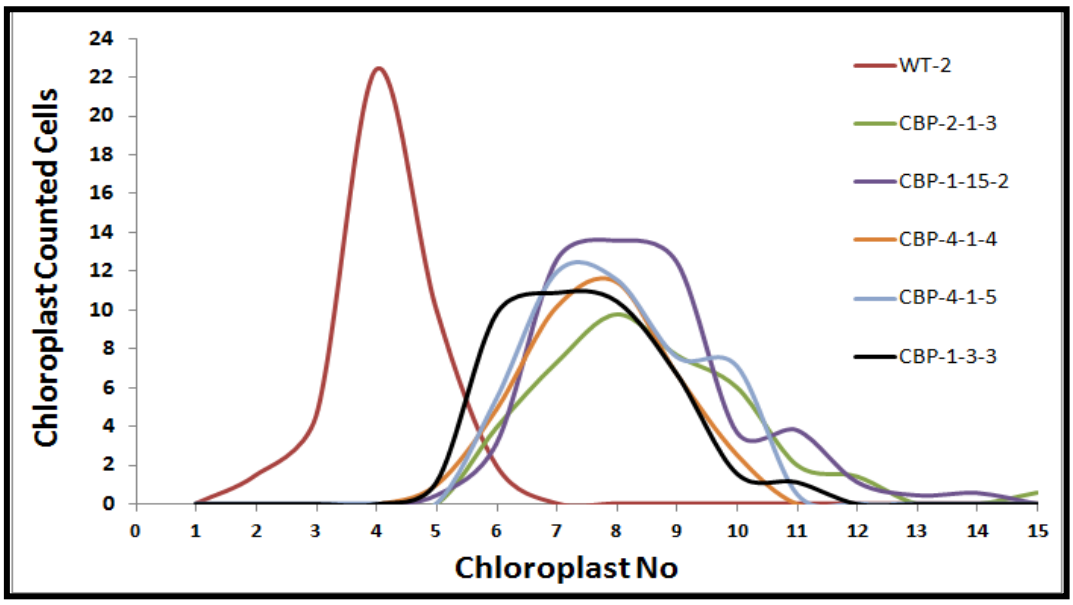


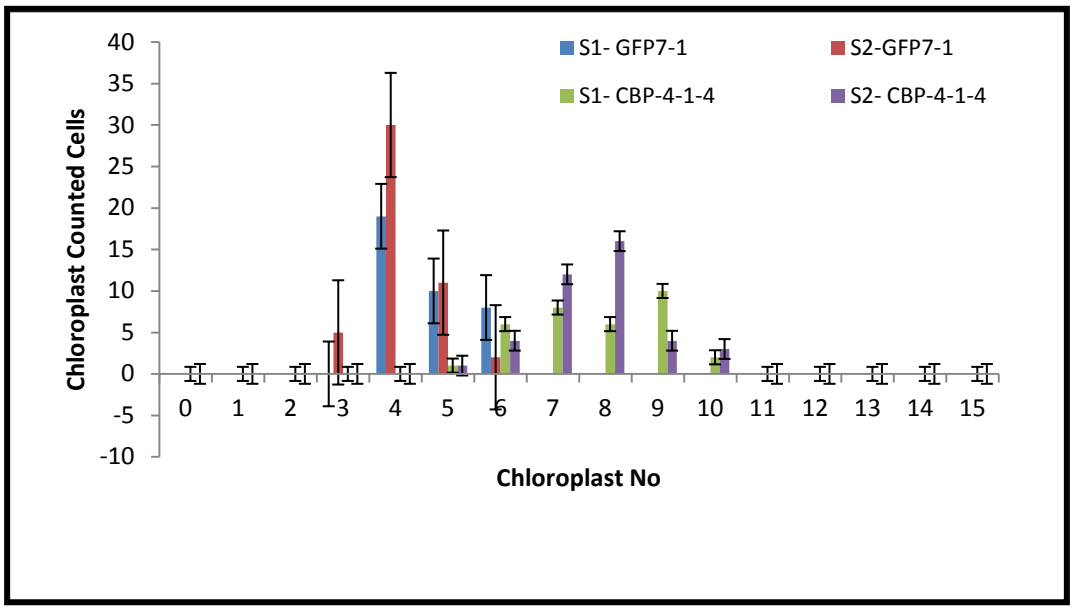
Figure 2.5. Increased chloroplast number in CBP expressing tomato lines. The number of chloroplasts for a guard cell in a leaf of a plant in CBP expressing lines is shown. Two leaves for each plant were selected. A total of sixty plants were selected and 25 guard cells per each leaf were evaluated. The chloroplast number in CBP expressing lines was on average around 6-8.

Figure 2.6. Chloroplast number analysis for CBP expressing and control tomato plants. A. The average chloroplast number per total counted chloroplasts per each cell. Wild type plant shows a grand average of 4, while the selected CBP expressing plants (35S:GFP-CBP) from line 1, 2 and 4 show an average value around 8. **B.** Chloroplast number analysis for CBP expressing and control tomato plants. The number of the chloroplasts in the guard cells for selected CBP expressing line 4 (35S:GFP-CBP-4, plant 4-1-4) and 35S:GFP vector control line, plant 7-1 is shown. For the selected plants two leaves were analyzed (S1 and S2) and based on the data shown the average chloroplast number per chloroplast counted cells for 35S:GFP plant is around 4 whereas for CBP expressing line 4 (35S:GFP-CBP-4), the average ranges between 7-9.

A.


Chloroplast Counted Cells
Chloroplast No
WT-2
CBP-2-1-3
CBP-1-15-2
CBP-4-1-4
CBP-4-1-5
CBP-1-3-3

B.

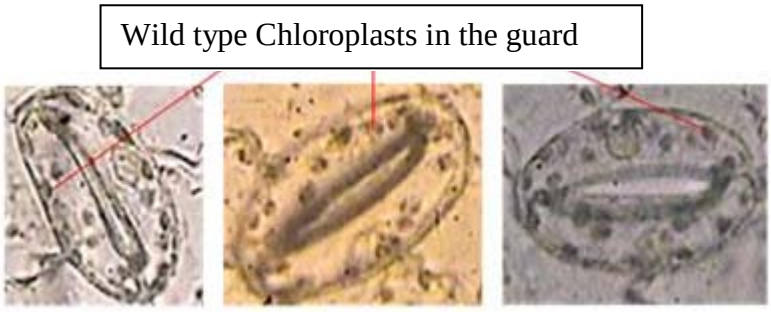

Chloroplast Counted Cells
Chloroplast No
S1- GFP7-1
S2-GFP7-1
S1- CBP-4-1-4
S2- CBP-4-1-4

Because the number of chloroplasts was not the same when comparing CBP expressing lines with control vector and wild type plants, it was beneficial to increase the ploidy level in wild type and 35S:GFP plants in order to have a more suitable control for the phenotypic and morphology analysis of the plants. Wild type seedlings as well as 35S:GFP control seedlings were thus selected and treated with Surflan A.S. [13]. Treated seedlings were analyzed for the induction of chloroplast number. The results showed an increase of chloroplast number in guard cells of the selected wild type plants as shown in **Fig. 2.7.A.**

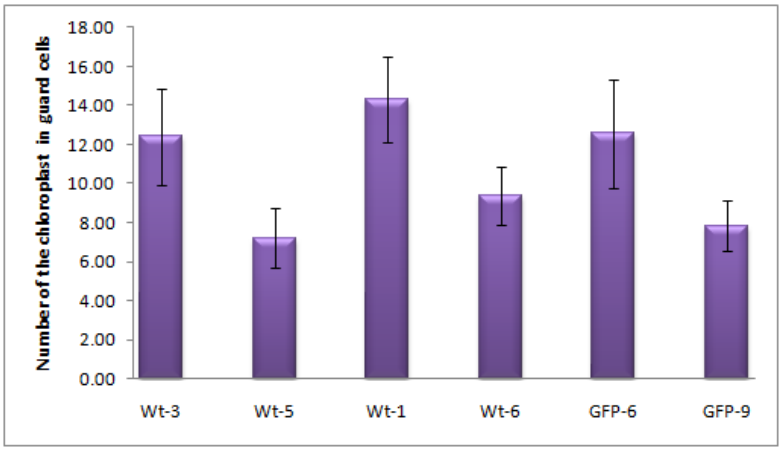
The grand average of chloroplast numbers, shown in the graphs, indicates the induction of the number through the treatment of plants (wild type and 35S;GFP) with Surflan A.S. as shown in **Fig. 2.7.B.** Moreover, **Fig. 2.7.C.** shows the meristem of a wild type seedling covered with Surflan agar solution during the treatment.

Figure 2.7. Induction of chloroplast numbers in wild type and 35S:GFP control tomato plants. A. The guard cells from wild type control plants show increased chloroplast number after treatment with Surflan A.S. **B**. average of chloroplast numbers in wild type and 35S:GFP plants is shown. Ten plants from wild type and ten plants from 35S;GFP lines were selected. The average increased from 4 (before treatment) to 6-14 (after treatment); bars represent Standard deviation**.** (n= 10). **C.** Seedlings were treated just as the first true leaves started to become apparent. The agar/Surflan solution was pipetted onto the emerging meristem of seedlings. Treatments were repeated 3 times with 4 day intervals in between.

A.


Wild type Chloroplasts in the guard

B.


Number of the chloroplast in guard cells
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Wt-3
Wt-5
Wt-1
Wt-6
GFP-6
GFP-9

C.

Phenotypic Analysis of CBP Expressing Tomato Lines

For the phenotypic analysis, three independent CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 35S:GFP-CBP-2 and 35S:GFP-CBP-4) were selected (T5 generation). Additionally, wild type and 35S:GFP vector control lines which were treated with Surflan were used as controls in the phenotypic analysis. Offspring of the T4 generation plants 4-1-5 and 4-4-6 from 35S:GFP-CBP line 4 (referred to siblings here), were also used as control in the analysis since they did not exhibit the presence of the maize CBP gene (based on the analysis of DNA, RNA and protein), but showed an increased number of chloroplasts in their guard cells. All plants were propagated in the phytotron under normal growth conditions. Phenotypic and morphological differences between CBP expressing and control tomato plants were compared.

There was a significant increase in the root and shoots' fresh and dry weight in the 35S:GFP-CBP plants compared to the control samples. The average number of fruits per plant per line was also higher in CBP expressing plants (35S:GFP-CBP) compared to control lines. However, the average fruit weight in CBP expressing lines (35S:GFP-CBP) was lower than that for control lines. Seed production was also increased in tomato CBP expressing lines (35S:GFP-CBP) compared to control lines. Details are given in the following sections.

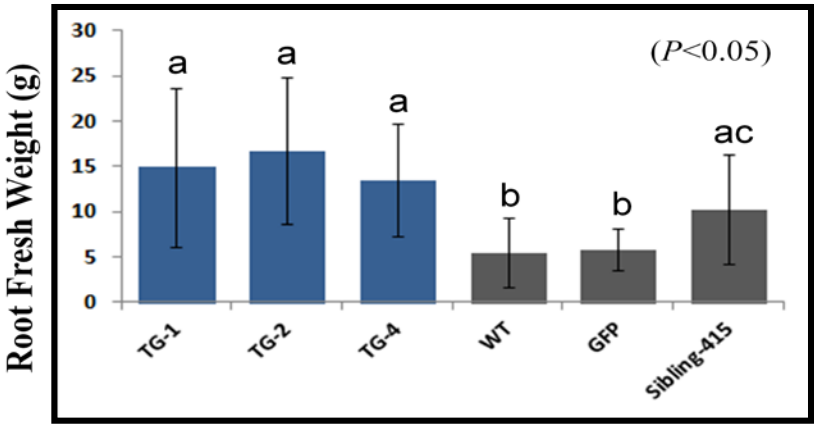
Total Average of Root and Shoot Fresh Weight

The average of fresh root weight of CBP expressing (35S:GFP-CBP) lines and control tomato plants (35S:GFP and wild type) planted in the phytotron, was measured. CBP expressing tomato plants were found to have higher average fresh root weight (15.1 +/- 7.8)

than control plants (7.2 +/- 4.1). T-test analysis the fresh root weight of all the CBP expressing lines (35S:GFP-CBP -1, 2 and 4) showed a significant difference compared to the control wild type and 35S:GFP expressing tomato plants (p<0.05) but did not exhibit a significant difference compared to the sibling control plants (10.3 +/- 6.08, p<0.5). Moreover no significant difference was found between CBP expressing lines (**TG-1:** 14.9 +/- 8.9; **TG-2**: 16.7 +/- 8.05; **TG-4**: 13.5 +/- 6.39). However a significant difference was found between 35S:GFP and wild type control plants compared to the sibling control tomato plants (p<0.05), (**wt**: 5.5 +/- 3.8; **GFP**: 5.8 +/- 2.28; **sibling**: 10.3 +/- 6.08,) as shown in **Fig. 2.8.A.**

The average of fresh shoot weight of 35S:GFP-CBP CBP expressing and control (35S:GFP and wild type) tomato plants was measured as well. Based on the results, the CBP expressing lines 1, 2 and 4 (35S:GFP-CBP -1, 35S:GFP-CBP- 2 and 35S:GFP-CBP -4) had a larger fresh shoot weight than control plants. T-test results indicate that CBP expressing lines were significantly different compared to the wild type and 35S:GFP expressing tomato plants (p<0.05). However, only CBP expressing line 2 showed a significant difference compared to the sibling plants (p=0.02), whereas CBP expressing tomato lines, 35S:GFP-CBP-1 and 35S:GFP-CBP- 4 had no significant difference compared to the sibling tomato plants. Also there was no significant difference among CBP expressing lines. Among control plants, t-test results of 35S:GFP and sibling showed significant differences (p=0.0003), but wild type and sibling plants did not exhibit a significant difference (**TG1**: 46.1 +/- 13.75; **TG-2**: 56.9 +/- 22.38; **TG-4**: 49.4+/- 20.33), (**wt**: 19.5 +/- 15.21; **GFP**: 17.9 +/- 5.21; **sibling**: 33.0 +/- 12.46). **Fig. 2.8.B.** shows the average shoot weight of CBP expressing lines compared to control lines.

A.



B.

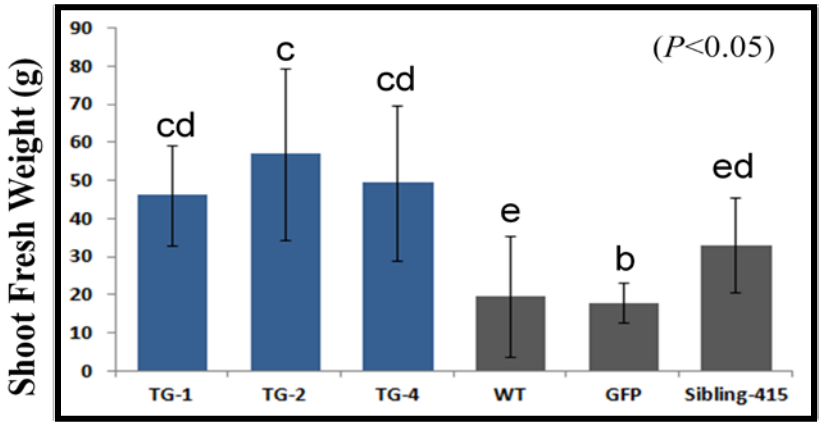


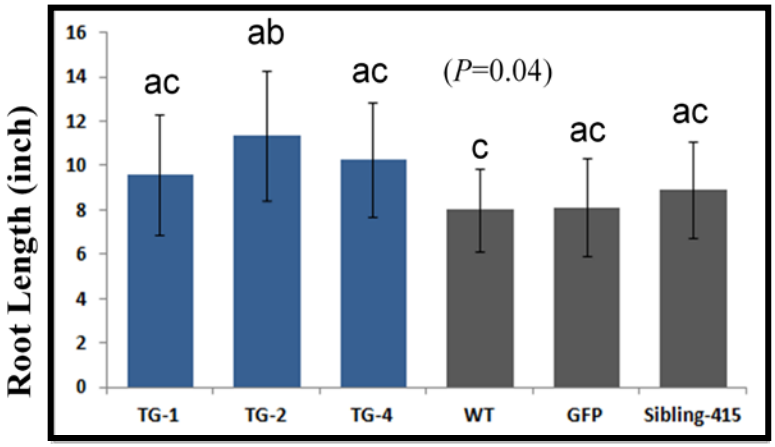
Figure 2.8. Total average of root and shoot fresh weight (g). CBP expressing lines as well as control lines were analyzed for their fresh root and shoot weight (g). **A.** The average of root fresh weight in CBP expressing lines (35S:GFP-CBP) was higher than control wild type and 35S:GFP vector control lines. The difference was significant (p<0.05). **B.** Fresh shoot average weight of CBP expressing lines showed a higher value compared to the control lines (wild type, 35S:GFP and sibling lines) and the difference was significant (p<0.05); however the difference between CBP expressing lines 1and 4 (35S:GFP-CBP -1, 35S:GFP-CBP-4) and sibling control lines was found not to be significant. Bars represent standard deviation; (n= 9 for CBP expressing line1, n= 9 for CBP expressing line 2 and n= 9 for CBP expressing line 4; n= 12 for wild type and 35S:GFP lines and n= 19 for sibling lines).

Total Average of Root and Shoot Length

The average of fresh root length of CBP expressing lines (35S:GFP-CBP) and control tomato plants (wild type, 35S:GFP and sibling plants) was analyzed. CBP expressing tomato plants had higher average fresh root length than control plants. Based on the t-test results only CBP expressing line 2 (35S:GFP-CBP-2) plants were significantly different compared to the wild type (p=0.04). However, no other CBP expressing lines 1and 4, (35S:GFP-CBP-1 and 35S:GFP-CBP-4) showed significant differences compared to all control plants. Moreover, no significant difference was found among CBP expressing lines (35S:GFP-CBP-1, 2 and 4). There was also no significant difference between control plants (Wild type, 35S:GFP and sibling lines); (**TG1**: 9.59 +/- 2.74; **TG-2**: 11.37 +/- 2.92; **TG-4**: 10.27+/- 2.59), (**wt**: 8.0 +/- 1.86; **GFP**: 8.12 +/- 2.23; **sibling**: 8.92 +/- 2.18) . **Fig. 2.9.A.** shows the average root length of CBP expressing lines compared to control lines.

CBP expressing tomato plants (35S:GFP-CBP) had approximately the same average shoot length as the control plants. No significant difference was found between CBP expressing and control plants. T-test results of the CBP expressing lines showed no significant difference between CBP expressing lines (35S:GFP-CBP-1, 2 and 4). Moreover the results of control plants (wild type, 35S:GFP and sibling lines) also indicated no significant difference among control lines. (**TG1**: 5.24 +/- 0.86; **TG-2**: 5.95 +/- 1.25; **TG-4**: 5.29+/- 1.21), (**wt**: 4.85 +/- 1.5; **GFP**: 5.29 +/- 1.47; **sibling**: 6.02 +/- 1.73). **Fig. 2.9.B.** shows the average shoot length of CBP expressing lines compared to control lines.

A.



B.

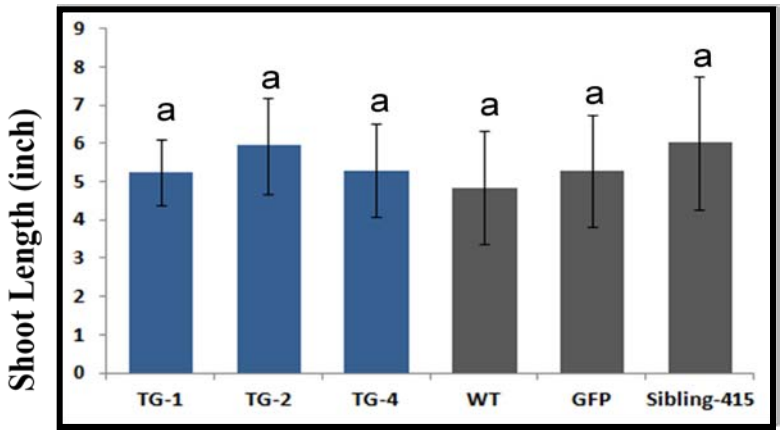


Figure 2.9. Total grand average of root and shoot fresh length. A. CBP expressing tomato lines (35S:GFP-CBP line 1, 2 and 4) showed a higher average of fresh root length compared to control plants (wild type, 35S:GFP and sibling plants); however significant difference was only found between CBP expressing line-2 (35S:GFP-CBP-2) and wild type (p=0.04) but not in any other control lines (35S:GFP and sibling plants). **B.** Average fresh shoot length of CBP expressing lines (35S:GFP-CBP) was the same compared to control lines and no significant difference was detected. Bars represent standard deviation; (n= 9 for CBP expressing line 1, n= 9 for CBP expressing line 2 and n= 9 for CBP expressing line 4; n= 12 for wild type and 35S:GFP lines and n= 19 for sibling lines).

Total Average of Shoot and Root Dry Weight

CBP expressing tomato lines (35S:GFP-CBP) showed a higher total average dry weight (root and shoot dry weight) compared to control plants (wild type, 35S:GFP and sibling lines) and for CBP expressing line 2 (35S:GFP-CBP -2) the difference was significant compared to all control lines ($p<0.05$). There was no significant difference between CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4). Wild type control and 35S:GFP expressing lines also showed no significant difference. Significant difference was found between wild type, and 35S:GFP vector control lines against sibling lines ($p=0.01$). In addition, CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1 and 35S:GFP-CBP -4) showed no significant difference compared to sibling lines. However, t-test results for CBP expressing lines 1and 4 (35S:GFP-CBP-1 and 35S:GFP-CBP-4) showed they are significantly different compared to control wild type and 35S:GFP plants ($p<0.05$). (**TG1**: 12.24 +/- 6.32; **TG-2**: 16.28 +/- 9.94; **TG-4**: 10.08+/- 6.04), (**wt**: 3.35 +/- 2.30; **GFP**: 4.17 +/- 1.67; **sibling**: 7.93 +/- 4.27). **Fig. 2.10.A.** shows the total average weight of CBP expressing and control tomato lines.

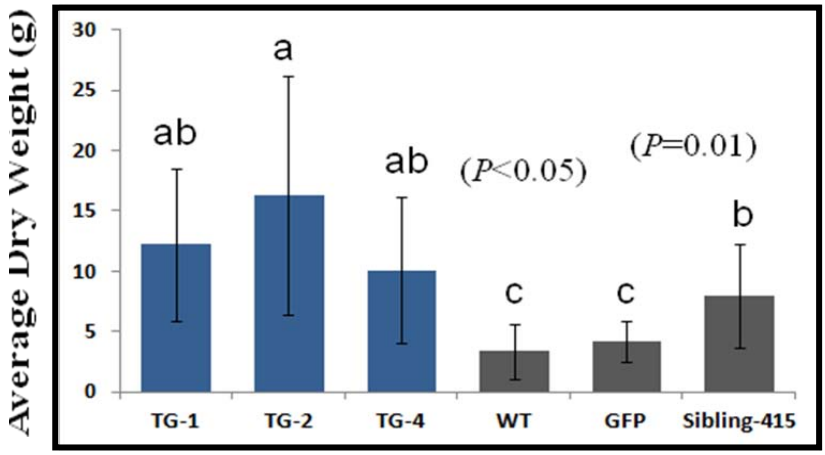
In terms of the dry root weight, CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) had a higher average root dry weight compared to control, wild type, 35S:GFP. There was no significant difference among CBP expressing lines. Wild type and 35S:GFP expressing lines showed no significant difference; however there was a significant difference between 35S:GFP vector control and wild type plants compared to sibling lines ($p=0.01$). In addition, CBP expressing line 2 and 4 (35S:GFP-CBP-2 and 35S:GFP-CBP-4) showed a significant difference compared to wild type and 35S:GFP plants ($p<0.05$), but no significant difference with sibling lines. T-test results showed no significant difference between CBP expressing

line 1(35S:GFP-CBP-1) and all control lines. (**TG1**: 5.68 +/- 6.19; **TG-2**: 5.67 +/- 5.19; **TG-4**: 3.18+/- 1.69), (**wt**: 0.78 +/- 0.49; **GFP**: 1.09 +/- 0.5; **sibling**: 2.65 +/- 2.07). **Fig. 2.10.B.** shows average root weight of CBP expressing lines vs. control tomato lines.

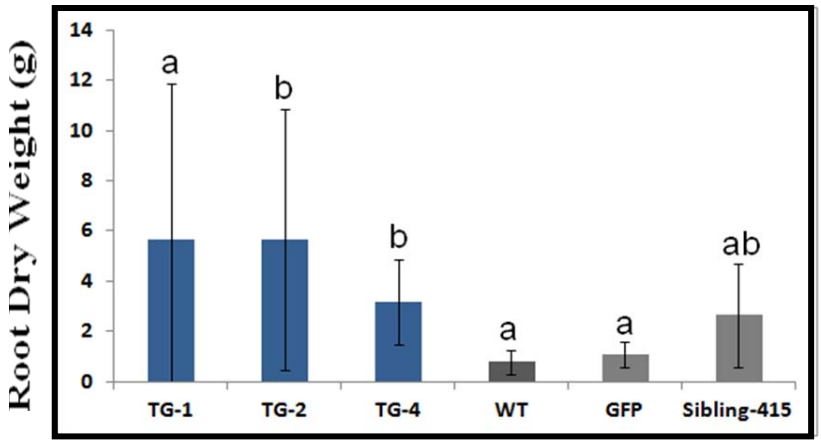
The total shoot dry weight of CBP expressing plants (35S:GFP-CBP-1, 2 and 4) was on average higher compared to control lines (wild type, 35S:GFP and sibling). T-test results showed a significant difference between the CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) and wild type and 35S:GFP control lines ($p<0.05$). However, only CBP expressing line 2 (35S:GFP-CBP-2) showed a significant difference with sibling control plants ($p=0.04$). No significant difference was found among CBP expressing lines. Control lines, wild type and 35S:GFP also showed no significant difference. However there was a significant difference between wild type and 35S:GFP compared to sibling lines ($p<0.05$). (**TG1**: 5.7 +/- 2.60; **TG-2**: 10.6 +/- 6.15; **TG-4**: 7.7+/- 3.63), (**wt**: 2.6 +/- 1.81; **GFP**: 3.1 +/- 1.30; **sibling**: 5.3 +/- 3.30)**. Fig. 2.10.C.** shows average root weight of CBP expressing lines vs. control tomato lines.

Figure 2.10. Total average dry shoot and root weight of CBP expressing and control tomato plants. A. Total dry weight average of the CBP expressing lines (35S:GFP-CBP-1, 2 and 4) was compared to wild type, 35S:GFP and sibling control plants. CBP expressing plants showed a higher total dry weight compared to control plants. However significant difference was found only between CBP expressing line-2 and all control lines ($p<0.05$). CBP expressing lines 35S:GFP-CBP-1 and 4 showed a significant difference with wild type and 35S:GFP ($p<0.05$) but not with sibling lines. **B.** The total average root dry weight of CBP expressing lines was higher than that for control lines. While CBP expressing 35S:GFP-CBP-1 showed no significant difference compared to control lines, CBP expressing 35S:GFP-CBP-2, and 4 showed a significant difference against wild type and 35S:GFP vector control lines ($p<0.05$) but not against sibling lines. **C.** CBP expressing lines showed a higher total average shoot dry weight compared to control lines. A significant difference was found between CBP expressing line 2 (35S:GFP-CBP-2) and sibling lines ($p=0.04$); however CBP expressing lines 35S:GFP-CBP-1, 2 and 4 showed a significant difference only compared to wild type and vector 35S:GFP control lines ($p<0.05$). Bars represent standard deviation; (n= 9 for CBP expressing line1, n= 9 for CBP expressing line 2 and n= 9 for CBP expressing line 4; n= 12 for wild type and 35S:GFP lines and n= 19 for sibling lines).

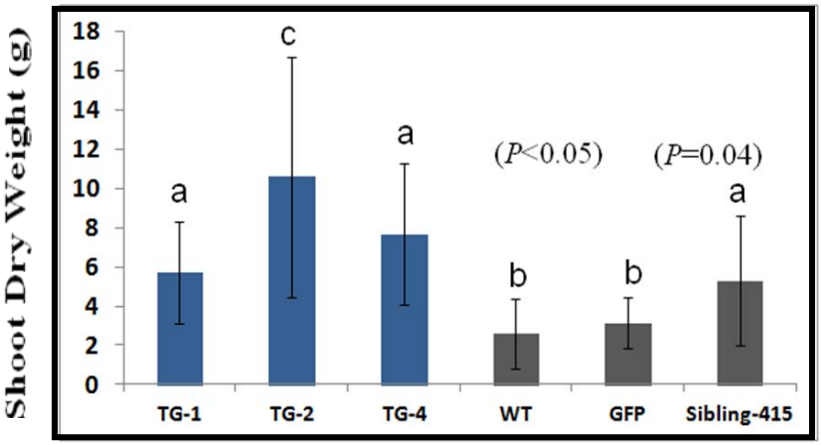
A.


Average Dry Weight (g)
30
25
20
15
10
5
0
a
ab
ab
c
c
b
(P<0.05)
(P=0.01)
TG-1
TG-2
TG-4
WT
GFP
Sibling-415

B.


Root Dry Weight (g)
14
12
10
8
6
4
2
0
a
b
b
a
a
ab
TG-1
TG-2
TG-4
WT
GFP
Sibling-415

C.

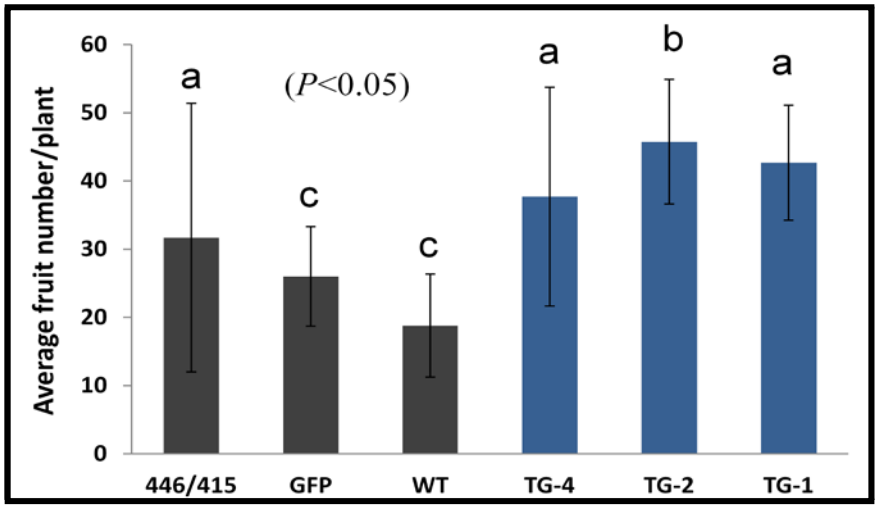

Shoot Dry Weight (g)
18
16
14
12
10
8
6
4
2
0
a
c
a
b
b
a
(P<0.05)
(P=0.04)
TG-1
TG-2
TG-4
WT
GFP
Sibling-415

Analysis of Average Fruit Number and Weight of CBP Expressing Lines

The total average fruit number was higher in CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) compared to control lines (wild type, 35S:GFP and sibling). CBP expressing line 2 (35S:GFP-CBP-2) were found to be significantly different compared to all control lines *(*$p<0.05$). CBP expressing 35S:GFP-CBP-1 and 4 were found to be significantly different compared to wild type and 35S:GFP ($p<0.05$), but not to sibling control lines. There was no significant difference among CBP expressing lines and among control lines as shown in **Fig. 2.11.A**. Data of the total fruit number of CBP expressing lines and control plants treated with Surflan A.S. (T_5 generation) were compared to untreated wild type and 35S:GFP lines from the last generation (T_4 generation).

The comparison could indicate whether the increased chloroplast number in wild type and 35S:GFP control lines resulted in a significant phenotypic change when compared to CBP expressing lines. Based on the average total fruit number, CBP expressing lines exhibited a higher average fruit number compared to all treated and untreated control lines (wild type and 35S:GFP). The data also indicated that control lines treated with Surflan A.S. had no significant difference compared to untreated control lines with respect to the average fruit number. T-test results were found to be significantly different between CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 35S:GFP-CBP-2 and 35S:GFP-CBP-4) and all the treated wild type and 35S:GFP control lines ($p<0.05$) and untreated wild type and 35S:GFP control lines ($p<0.005$) as shown in **Fig. 2. 11. B.** (**TG1**: 42.7 +/- 8.43; **TG-2**: 45.8 +/- 9.13; **TG-4**: 41.3+/- 16.05), (**wt**: 18.8 +/- 7.56; **GFP**: 26.0 +/- 7.29; **sibling**: 31.7 +/- 19.70; **GFP/UN (untreated with Surflan)**: 19.8 +/- 9.0; **wt/UN**: 20.6 +/- 6.0).

A.



B.

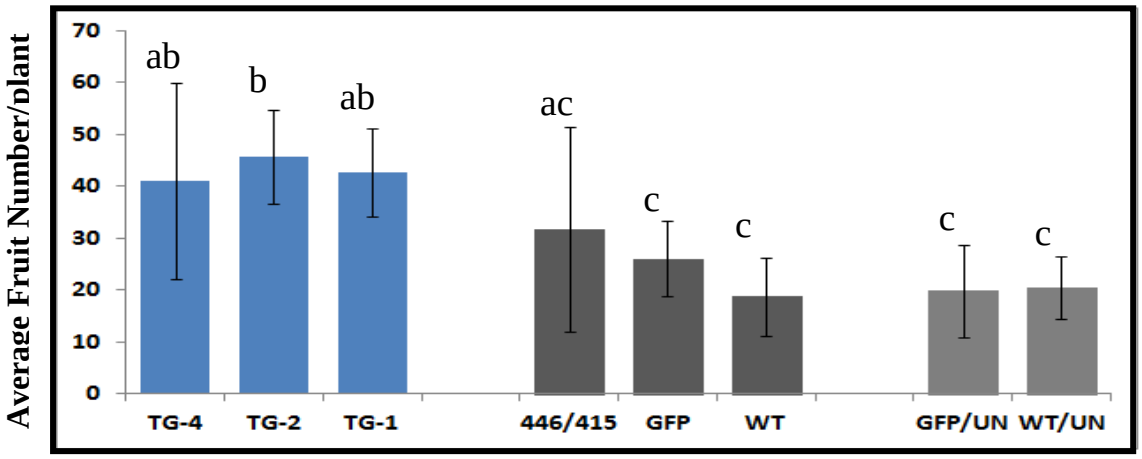
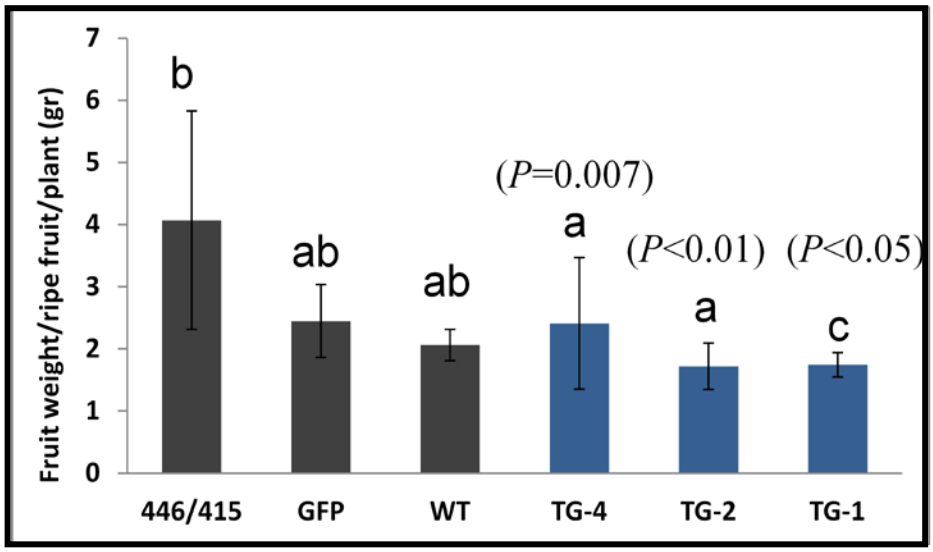


Figure 2.11. Average fruit number of CBP expressing and control tomato plants per plant per line. **A**. CBP expressing lines (35S:GFP-CBP-1, 2 and 4) showed a higeher fruit number compared to control lines (wild type and 35S:GFP) and this difference was significant (p<0.05). However only CBP expressing line 2 (35S:GFP-CBP line-2) showed a significant difference compared to sibling control lines (p<0.05). **B**. The average fruit number of CBP expressing lines was compared to treated control lines (wild type and 35S:GFP) with Surflan A.S. and untreated control lines from the previous generation (wild type and 35S:GFP). The average fruit number in CBP expressing lines was higher than both treated control lines and untreated (UN) control lines and the difference was significant. Bars represent standard deviation; n= 13 for sibling lines, n= 15 for wild type and 35S:GFP, n= 9 for CBP-expressing line1, n= 9 for CBP expressing line 2, n= 9 for CBP expressing line 4.

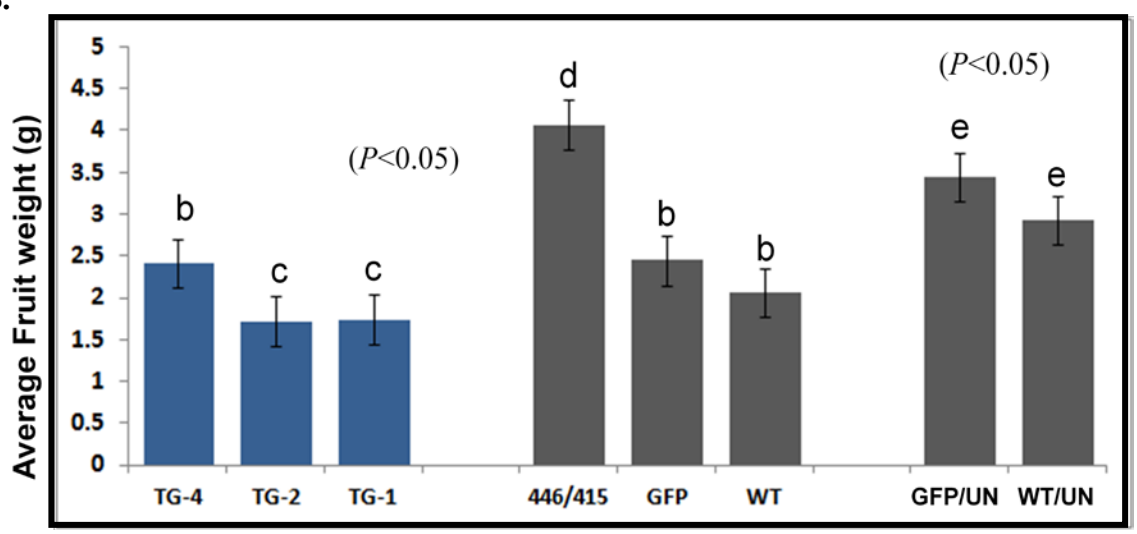
The total average fruit weight in CBP expressing lines 1 and 2 (35S:GFP-CBP-1, 35S:GFP-CBP-2) was lower than control lines (wild type and 35S:GFP, sibling) except for CBP expressing line 4 (35S:GFP-CBP-4), which showed the same average with 35S:GFP control line and slightly higher average than wild type control lines. The difference between control lines and CBP expressing line 1 (35S:GFP-CBP-1) was found to be significant ($p<0.05$). However, a significant difference was found only between CBP expressing line 4 (35S:GFP-CBP-4) and sibling control lines ($p=0.007$) whereas no significant difference was found between CBP expressing line 4 (35S:GFP-CBP-4) and control wild type and 35S:GFP lines. A significant difference was also found between CBP expressing line 2 (35S:GFP-CB-2) and sibling and 35S:GFP control lines ($p<0.01$), but not with wild type control lines. In addition, there was a significant difference between 35S:GFP and wild type compared to sibling lines ($p<0.001$) as shown in **Fig. 2.12.A.** The total fruit weight of CBP expressing lines and control plants (wild type and 35S:GFP) treated with Surflan AS (T_5 generation) was compared to untreated wild type and 35S:GFP lines from the last generation (T_4 generation). The average total fruit weight was lower in CBP expressing lines compared to all treated and untreated control lines. T-test results showed significant differences between CBP expressing lines and untreated control wild type and 35S:GFP lines ($p<0.05$) as shown in **Fig. 2.12.B.** (**TG1**: 1.74 +/- 0.2; **TG-2**: 1.72 +/- 0.37; **TG-4**: 2.41+/- 1.06), (**wt**: 2.06 +/- 0.25; **GFP**: 2.45 +/- 0.58; **sibling**: 4.07 +/- 1.76; **GFP/UN (untreated with Surflan)**: 3.44 +/- 1.3; **wt/UN**: 2.93 +/- 0.35)**.**

Figure 2.12. The total average fruit weight (g) for CBP expressing and control plants. A. The total average fruit weight of CBP expressing lines 1 and 2 (35S:GFP-CBP-1 and 2) was lower than control, wild type and 35S:GFP lines and the difference was significant ($p<0.05$). However CBP expressing line 4 (35S:GFP-CBP-4) showed the same average fruit weight compared to wild type and 35S:GFP lines but not with sibling control lines. **B.** The average fruit weight of CBP expressing lines was compared to treated control lines with Surflan AS (wild type and 35S:GFP) and untreated control lines from the previous generation (wild type and 35S:GFP). The average fruit weight in CBP expressing lines was lower than that for treated control lines and untreated control lines and the difference was significant ($p<0.05$). Bars represent standard deviation. (n= 13 for sibling lines, n= 15 for wild type and 35S:GFP, n= 9 for CBP-expressing line 1, n= 9 for CBP expressing line 2, n= 9 for CBP expressing line 4). **C.** Fruits from 35S:GFP control line, sibling plant (4-4-6), CBP expressing line 2 (35S:GFP-CBP-2, (plant 2-8-1)) and CBP expressing line 4 (35S:GFP-CBP-4, (plant 4-1-7)) are shown. As shown in the figure, 35S:GFP vector control and sibling plants have higher fruit weight than CBP expressing lines.

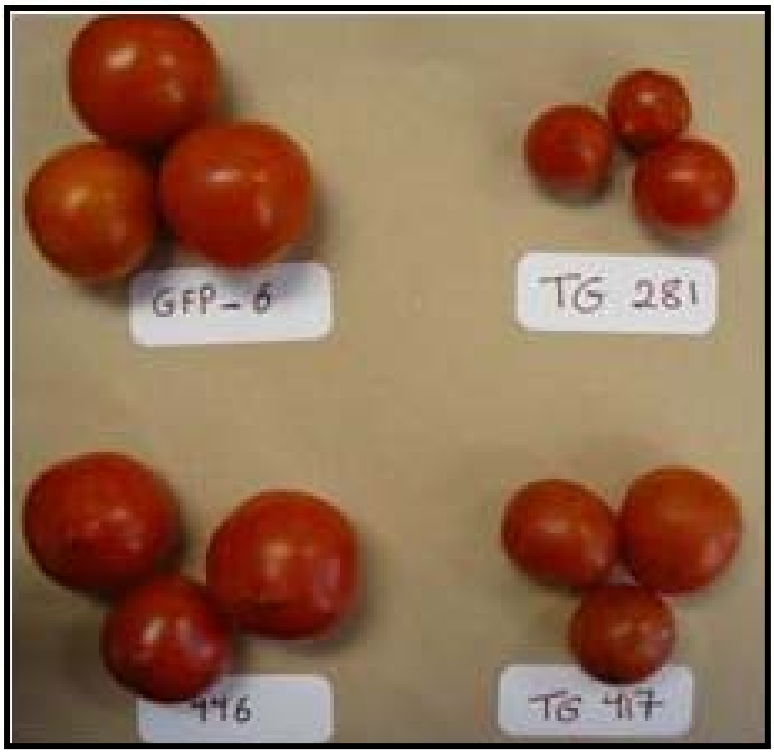
A.


Fruit weight/ripe fruit/plant (gr)
0
1
2
3
4
5
6
7
b
ab
ab
a
a
c
(P=0.007)
(P<0.01)
(P<0.05)
446/415
GFP
WT
TG-4
TG-2
TG-1

B.


Average Fruit weight (g)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
b
c
c
d
b
b
e
e
(P<0.05)
(P<0.05)
TG-4
TG-2
TG-1
446/415
GFP
WT
GFP/UN
WT/UN

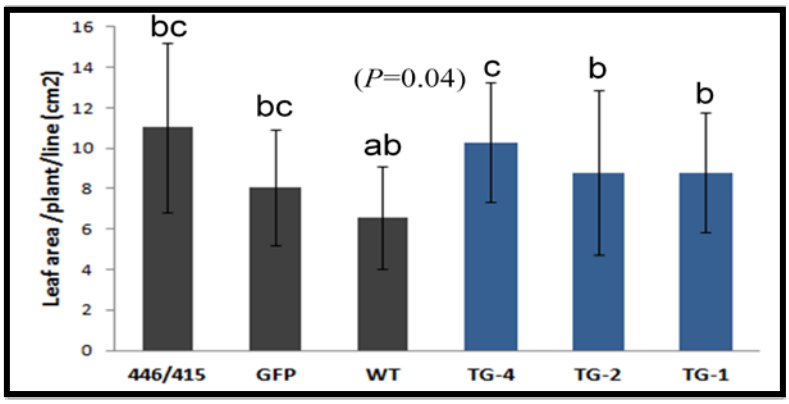
C.



Average Leaf Area of CBP Expressing and Control Lines

The total average leaf area in CBP expressing lines 1 and 2 (35S:GFP-CBP-1 and 2) was approximately the same compared to control lines (wild type, 35S:GFP and siblings), except for CBP expressing line 4 (35S:GFP-CBP-4), which showed a higher average leaf area than control wild type and 35S:GFP lines but not from sibling control lines. No significant difference was found between CBP expressing lines 1 and 2 (35S:GFP-CBP-1, 35S:GFP-CBP-2) and control lines. CBP expressing line 4 (35S:GFP-CBP-4) showed a significant difference compared to wild type line (p=0.04), but not compared to 35S:GFP and sibling control lines. A significant difference was also found between wild type control plants and sibling lines (p=0.02) as shown in **Fig. 2.13.A**. Representative, same-age leaves from CBP expressing lines 1 and 4 (35S:GFP-CBP-1, 35S:GFP-CBP -4) and control lines (35S:GFP and siblings) are shown in **Fig. 2.13.B.** (**TG1**: 8.80 +/- 2.93; **TG-2**: 8.81 +/- 4.06; **TG-4**: 10.30+/- 2.96), (**wt**: 6.56 +/- 2.54; **GFP**: 8.09 +/- 2.87; **sibling**: 11.02 +/- 4.19).

A.



B.

Figure 2.13. Average leaf area of CBP expressing and control tomato lines. A. The average leaf area of CBP expressing lines 1 and 2 (35S:GFP-CBP-1, 2) was approximately the same as control lines. Only CBP expressing line-4 showed a higher average than wild type and the difference was significant (p=0.04). Wild type and sibling lines also showed a significant difference in average leaf area ratio (p=0.02). Bars represent standard deviation. n= 13 for sibling lines, n= 15 for wild type and 35S:GFP, n= 9 for CBP-expressing line1, n= 9 for CBP expressing line 2, n= 9 for CBP expressing line 4. **B.** Example leaves from 35S:GFP plant and sibling control plant (4-4-6), as well as CBP expressing line 4 (35S:GFP-CBP-4, (plant 4-4-1)) and CBP expressing line 1 (35S:GFP-CBP-1, (plant 1-15-1)) are shown. CBP expressing line-4 shows a higher leaf area as 35S:GFP control plant but not from sibling control plant. CBP expressing line-1 shows the same leaf area as the 35S:GFP and sibling control plants.

Average Seed Dry Weight of CBP Expressing and Control Tomato Lines

The total average seed dry weight was measured compared to CBP expressing lines (35S:GFP-CBP-1, 2and 4) and control tomato lines (wild type, sibling and 35S:GFP). The average seed dry weight in CBP expressing plants was higher than that for wild type and 35S:GFP control lines. A significant difference was found between CBP expressing lines and control lines treated with Surflan (p<0.01) as shown in **Fig. 2.14**. (**TG1**: 42.7 +/- 8.43; **TG-2**: 45.8 +/- 9.13; **TG-4**: 41.3+/- 16.05), (**wt**: 18.8 +/- 7.56; **GFP**: 26.0 +/- 7.29; **sibling**: 31.7 +/- 19.70; **GFP/UN (untreated with Surflan)**: 19.8 +/- 9.0; **wt/UN**: 20.6 +/- 6.0)**.**

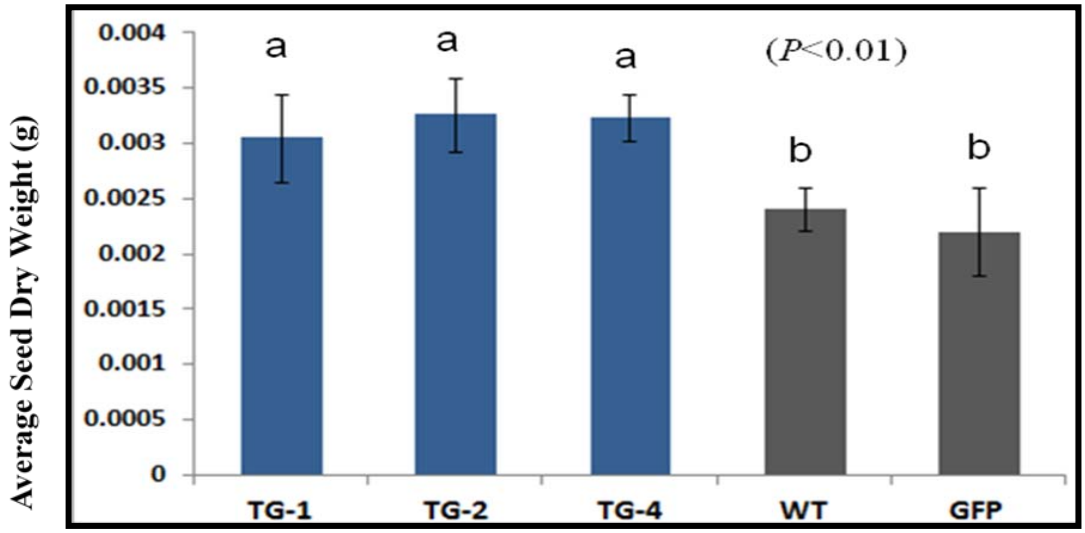


Figure 2.14. The average seed dry weight of CBP expressing and control tomato lines. The average seed dry weight of CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2and 4) was higher than control lines (wild type, sibling and 35S:GFP) and the difference was found to be significant (p<0.01). Bars represent standard deviation. Five plants per each line was used (n=5).

Discussion

In this chapter, the morphological traits of 35S:CBP-GFP expressing tomato plants were characterized. We found that constitutive expression of the C-domain of maize Calreticulin in tomato has distinct effects on the morphology of tomato plants. Specifically, the root and shoot fresh and dry weight of the 35S:GFP-CBP plants compared to the control samples. Also, there was a significant increase in the average number of fruits per plant per line in 35S:GFP-CBP CBP expressing plants compared to control lines. However, the average fruit weight in CBP expressing lines (35S:GFP-CBP) was lower than that for control lines. Furthermore, seed production was also increased in CBP expressing tomato lines (35S:GFP-CBP) compared to wild type and 35S:GFP control lines.

The role of Calreticulin in regulating calcium homeostasis has also been previously highlighted. It has been reported that induction of full length rabbit Calreticulin in HEK-293 cells increased the free calcium concentration within the ER lumen and doubled the rate of ER refilling [17]. Moreover, the increase in calcium storage and release has been reported in plant cells in a study by Persson et al., (2001) which demonstrated that overexpression of Calreticulin in tobacco suspension cells affects the calcium pool in the ER [18].

Jia et. al., (2008) showed that TaCRT-over-expressing tobacco plants (*Nicotiana benthamiana*) exhibited drought resistance compared to control plants under water deficit conditions [19]. In addition, a study by Li et. al., (2008) showed that Calreticulin mutant crt3 in Arabidopsis is involved in plant response to calcium depleted stress and plays an important role in calcium homeostasis and salinity stress [20]. Calreticulin is expressed in plants and shares a similar structure with its homolog protein expressed in animal cells [21]; however

environmental and developmental stimuli affect the expression of Calreticulin in plants. Nevertheless, compared to the animal system, the current knowledge of Calreticulin in plant physiology is limited [21].

We examined how altered expression of C-domain of maize CRT could affect plant's response to stress conditions in tomato plants and if any detectable changes could be observed under non-stress conditions in CBP-expressing lines. Integration of the transgene (C-domain of maize Calreticulin) in CBP expressing tomato plants was confirmed in T_4 and T_5 generation plants **[Fig. 2.1].** Semi-quantitative RT PCR was conducted for selected 35S:GFP-CBP CBP expressing lines to analyze the transcript abundance using maize *CBP* gene specific primers. While no band was observed in wild-type and 35S:GFP vector control plants, RT-PCR showed similar levels of transcript abundance in the leaves of selected 35S:GFP-CBP lines **[Fig. 2.2]**. Moreover, CBP expressing lines were selected to test for protein expression. The total protein from the leaves of independent 35S:GFP-CBP CBP expressing lines (1, 2 and 4), the 35S:GFP vector controls and wild type control tomato plants was extracted and analyzed. No presence of the fusion protein was observed in wild type plants while the GFP antibody showed the GFP expression in 35S:GFP and CBP expressing plants **[Fig. 2.3].**

We assume that because of the higher levels of transgene in CBP expressing plants, protein degradation was observed in selected CBP expressing lines 1and 2. CBP expressing line 4 (35S:GFP-CBP-4) also showed GFP expression while specific GFP antibody was used. The plant 4-4-6 from 35S:GFP-CBP-4 was also negative to the presence of transgene. Semi-quantitative PCR analysis also showed no transcript abundance in 35S:GFP-CBP-4, plant 4-

4-6. Moreover, from the CBP expressing line 4, another plant (4-1-5) was also negative with respect to the integration of the transgene as confirmed by PCR analysis at the DNA and RNA level. Moreover, GFP expression was visible in leaves and roots of transgene expressing tomato lines but not in wild type control plants. In addition, no GFP expression was seen in plant 4-4-6 **[Fig. 2.4].**

Tomato CBP expressing plants (35S:GFP-CBP-1, 2 and 4) were examined to estimate their ploidy level based on the number of the chloroplasts in their guard cells. Ellul et al., (2003) reported that even though in most of the protocols described in the literature using cotyledons as explant source are common, the main technical problem is using methods that produce a high proportion of transgenic plants but lack stable genetic variation [14]. Ellul et al., (2003) reported that based on the analysis of transgenic tomato plants, ploidy levels in transgenic tomato plants depended on the transformation procedure and genotype [14].

Additionally, Joseph et al., (1989) reported that in general, transformed tomato plants had more chloroplasts than plants derived from seeds. Using the chloroplast number as a tool, they determined that 22% of the transgenic tomato plants were polyploid [12].

Furthermore, Sigareva et al., (2004) reported an efficient transformation protocol for tomato plants with no adverse effect on the ploidy level of transgenic tomato plants [15]. The aim of the study was to develop a transformation technique using mannose selection for tomato explants. Interestingly a comparison of the ploidy level of transgenic tomato plants selected on mannose with non-transgenic tomato plants showed no increase in the polyploidy rate of transgenic plants, indicating that the mannose protocol did not contribute to polyploidization [15]. Based on the obtained results in these studies and because *Agrobacterium tumefaciens*-

mediated transformation was used to generate transgenic tomato lines in this project (by Dr. Khodakovskaya) it was important to examine the ploidy level of the transgenic plants.

We tested tomato transformed plants (T_4 generation) for their polyploidy level. CBP expressing tomato plants (35S:GFP-CBP-1, 2 and 4) showed an increased chloroplast number while no increased chloroplast number was observed in wild type plants. That indicates polyploidy in the CBP expressing tomato lines. Some of the 35S:GFP vector control plants showed an increased number of chloroplasts, but the average range of the chloroplast number was in the range of 4-6, while the average in CBP expressing lines was in the range of 8-10. **[Fig. 2.6].** Because plants 4-4-6 and 4-1-5 were negative with respect to the presence of the transgene but showed increased numbers of chloroplasts, we selected these plants (siblings) to control for ploidy effects rather than CBP-expression in our study.

In order to increase the chloroplast number in control tomato plants, wild type and 35S:GFP control plants were treated with the herbicide Surflan A.S. (40.4% Oryzalin) **[Fig. 2.7].**

Jones et al., (2008) reported a novel method for inducing polyploidy in *Rhododendron* seedlings [13]. Their aim was to develop an effective method for induction of polyploidy and to evaluate the use of treatments of Surflan solution in *Rhododendron* seedlings. They found that semi-solid agar was an effective medium for treatments with Surflan solution (40.4 % Oryzalin) when applied to the apical shoots of the seedlings. While treatments of seedlings resulted in ploidy levels, no visual toxic symptoms were observed. However, the death percentages were random among the seedlings with different ploidy levels, suggesting a sensitivity of seedlings to Surflan [13]. We selected wild type and GFP vector control lines for treatment with Surflan A.S. containing 40% Oryzalin. From the obtained results, we

found that the chloroplast number in the guard cells of treated control plants was increased. These plants were used as ploidy controls for the phenotypic study **[Fig. 2.7].** During the experiment, Surflan solution was suspended in an agar medium and applied on the meristematic region of the tomato seedlings. The meristematic zone is a suitable region for the treatment the central zone of the meristem contains a group of cells that give rise to other cells. However, most seedlings showed sensitivity to Surflan during the treatment. Symptoms such as yellowing cotyledons and growing deficiency were observed during the treatment. The surfaces of the leaves were covered with the agar solution containing Surflan during the treatment which caused burning of the leaves. While this method is a simple and reliable technique to apply and could be a good alternative to increasing the ploidy level (due to their depolymerization effects on microtubules), but adverse side-effects of this process need to be considered.

The most important side effects are due to the fact that the herbicides delay plant growth through the inhibition of the cell division and elongation. Karimiani et al., reported the effect of Surflan on the *in-vitro* growth of *Gerbera jamesonii.* As the concentration of Surflan (Oryzalin) or the exposure period was increased the growth rate of the seedlings were found to decrease [22].

CBP expressing tomato lines (35S:GFP-CBP-1, 2 and 4) as well as control lines (wild type, 35S:GFP and siblings) were evaluated for phenotypic analysis in the phytotron under non-stress growth conditions. Khodakovskaya et al., (unpublished data) have reported that, over-expressed tomato CBP expressing lines (C-domain of maize Calreticulin) showed an increased level of lycopene content compared to wild type and 35S:GFP control plants It was

reported that the lycopene content was 3-fold higher in mature fruits of CBP expressing lines compared to control lines. However, no change was detected in terms of shape, size and the number of fruits per plant by the expression of CBP in tomato plants [11]. Nevertheless, in contrast with T_3 generation, we found differences in the number and the size of the fruits in CBP expressing lines compared to control lines in T_4 and T_5 generation. CBP expressing lines (35S:GFP-CBP-1, 2 and 4) had a more fruits per plant per line but lower average fruit weight compared to control lines **[Fig. 2. 12-13]**. The increased fruit number on tomato plants may lead to a decrease in average fruit weight since a larger number of fruits in plants compete for nutrients among fruits which causes plant stress.

The increase in fruit production was observed in our CBP expressing tomato lines (35S:GFP-CBP) compared to control lines (wild type, 35S:GFP and siblings). We think that this increase might be due to the involvement of calcium in CBP expressing tomato plants through the expression of the C-domain of maize Calreticulin. The effect of calcium on plants has been highlighted in several studies; calcium as a plant nutrient plays an important role, including control of water uptake, increasing nitrogen metabolism and making plant more tolerant to diseases. Another important function of calcium is that calcium is an essential part of the cell wall structure and increases fruit set in plants [23]. Desouky et al., (2009) have shown that spraying olive trees with boron and calcium is a promising treatment to improve fruit set, oil content and oil quality in olives [24]. In a study done by Feagley et al., it was shown that applying soluble calcium with urea improved crop production. It was reported that increased ammonium absorption (caused by calcium) increased the rate of photosynthesis in tomato, squash and cabbage [25].

Siddiq et al., (2009) reported that the growth and yield of tomato (*Lycopersicon enculentum* Mill) have a direct relationship with soil applied calcium carbide and L-methionine (an established precursor of ethylene) [26]. The effect of two precursors, calcium and ethylene was thus investigated in tomato plants [26].

It is thus plausible to suggest that results obtained from tomato CBP expressing plants (ER-GFP-CBP) indicate the potential role of calcium on plant's morphology in terms of fruit production and biomass. While fertilizers are commonly used to increase crops production yield and biomass, the use of reverse CBP expressing techniques (e.g. expression of CBP) may lead to desirable phenotypes in plants.

Moreover, we found that the fruit number between Surflan treated 35S:GFP vector control lines were slightly higher compared to untreated 35S:GFP control lines although the difference was not significant. **[Fig. 2.12].**

In addition, the total average fruit weight in both wild type and 35S:GFP vector control lines treated with Surflan was significantly lower than that in untreated wild type and control 35S:GFP lines. This indicates that the induction of chloroplast number due to the ploidy level could lead to polyploidy leaves and therefore could affect fruit weight in tomato plants. In our study a lower average fruit weight in Surflan-treated control lines was observed. Although the treated control lines showed a decrease in fruit weight compared to untreated control lines, Surflan treated control lines exhibited a higher average fruit weight than CBP expressing lines [**Fig. 2.13].**

In addition, blossom end rot (BER) was observed in some of the fruit tissues of CBP expressing plants. The primary cause of BER is suggested to be due to the calcium deficiency

in the distal fruit tissue [27]. The symptom of BER is a necrotic lesion, presumably as a consequence of cell death and the leakage of solutes into the extracellular space [27]. Some of the CBP expressing lines showed a necrotic lesion at the bottom of the fruit tissue. Over-expressing the C-domain of maize Calreticulin in CBP expressing lines might increase the capacity of calcium storage and release in the ER, however, disruption of calcium delivery to the fruit tissues (which causes local calcium deficiency) may result in weakening of cell walls and ultimately lead to BER symptom development.

Moreover, CBP expressing tomato lines (35S:GFP-CBP-1, 2 and 4) showed significantly higher average fresh and dry root / shoot weight compared to wild type and 35S:GFP vector control lines. No significant difference was found between CBP expressing lines with sibling control lines. The difference between wild type and 35S:GFP vector control lines compared to sibling lines was found to be significant only for root fresh weight.. The total average fresh root length in CBP expressing lines was slightly higher compared to control lines as well. However, only CBP expressing line-2 showed a significant difference with control lines **[Fig. 2.8-11].** We used control sibling lines in our study due to the fact that the transgene was not expressed in siblings but they exhibited an increased chloroplast number. However, based on the morphological analysis, sibling control lines showed a different phenotype compared to wild type and 35S:GFP vector control lines and shared more similarity to CBP expressing line 4 and 1. This indicates that the differences identified between CBP-expressing and control lines are more likely due to the difference in the ploidy level and the expression of the CBP.

The reason for the absence of transgene in sibling lines (offspring of CBP expressing line-4) is not known. Therefore to elucidate the role of Calreticulin in CBP expressing tomato lines it would be prudent to use an alternative control sample. For instance, knockout (crt) tomato plants could be generated to investigate the impact of CRT deficiency. In addition, tomato lines carrying only the CBP gene without GFP coding sequence (ER-targeted CBP protein without GFP) might also be used to investigate the role of Calreticulin in tomato plants.

A low level of calcium has been reported to lead to poor root development, blossom end rot, fruit cracking and leaf necrosis [28]. It has also been reported that an increased concentration of calcium from (10^{-6} to 10^{-2} M $CaCl_2$) increases the size of the roots (length and dry weight) in barley [29]. A change in calcium concentration in plant cells plays an important role in plant development. The role of calcium in cell division and cell growth has been investigated [30]. Wymer et al., (1997) reported the localization of calcium gradient in root hair tips is required for root hair elongation in *Arabidopsis thaliana* [31]. Ivashuta et al., (2005) have developed an RNAi-based screen and identified a *Medicago truncatula* gene (*CDPK1*), which encodes a calcium dependent protein kinase [32]. They reported that the identified gene plays a critical role in root development. Suppression of *CDPK1* gene expression resulted in short roots and short root hairs. CDPKi roots had short cortical cells [32]. Alteration in the root growth and development is an important mechanism because plants adapt to different soil conditions, facilitate water uptake and respond to environmental stimuli through the roots [33]. We found that CBP expressing tomato plants (35S:GFP-CBP) showed increased root development compared to control plants (wild type, 35S:GFP), however, this was probably caused by the difference in ploidy levels of the CBP-expressing

lines, because Surflan-treated CBP expressing (but not CBP-expressing) control plants were not significantly different from the CBP-expressing lines.

Based on the results obtained in this study the average seed dry weight was higher in tomato plants expressing the transgene (C-domain of maize Calreticulin) compared to control wild type and 35S:GFP plants treated with Surflan. Jin et al., (2009) previously reported that by silencing the cell wall invertase inhibitor protein, cell wall invertase activity was increased and this increase in tomato plants led to a delay in leaf senescence, increase seed weight and fruit hexose [34]. Khodakovskaya et al., based on microarray analysis, have reported up-regulation of invertase inhibitor gene in tomato CBP expressing lines (over-expressing the C-domain of maize Calreticulin) compared to wild type control plants [11]. Invertase hydrolyzes sucrose into fructose and glucose; eliminating the inhibitor activity of the cell wall invertase elevates cell wall activity which as a result increases seed weight and fruit sugar level [34]. It has been reported that an increase in seed weight and sugar level in tomato fruits is due to the enhanced apoplasmic sucrose hydrolysis, phloem unloading and hexose accumulation through the silencing of *INVINH1* which resulted in increased invertase activity [34]. The increased invertase activity could enhance sucrose hydrolysis in the apoplasm and facilitate phloem unloading. Increased hexose levels may cause accumulation of dry matter which could lead to an increase in seed weight and size [34].

Accordingly, the observed increased seed size and weight in CBP expressing tomato plants in this study could be caused by an increased expression of the cell wall invertase inhibitor as supported by results obtained from the microarray analysis [11].

In conclusion, the ectopic expression of the ER-targeted maize CBP in tomato plants may lead to increases in Ca^{2+} level in the ER which could have resulted in phenotypic changes such as increased biomass and fruit production. However, to verify that such phenotypic changes in CBP expressing tomato lines are due to the extra calcium store, cytosolic calcium concentration under normal conditions should be measured. Cytosolic calcium changes can be measured using aequorin fluorescence [35]. Genetic manipulation of the processes that lead to increased level of calcium in plant cells have been shown to impact plant growth and plant cell responses against stress conditions as also reported previously [36]. The CBP expressing plants contain higher total calcium compared to wild-type plants, and over-expression of calcium binding protein Calreticulin, e.g. in Arabidopsis, seems to increase plant survival under depleted calcium conditions [10]. Therefore, it appears that the manipulation of Calreticulin or other Ca^{2+}-binding proteins may be one way to engineer more robust plant varieties. Future studies could examine drought on tomato transgenic lines, to verify this hypothesis.

Material and Methods

Previous work; Transformation

In a previous work, CRT C-domain with GFP was cloned and transformed to wild type tomato plants [9]. Using an Eppendorf Pulse system, pBIN2011-GFP binary plasmids (containing GFP sequence) and pBIN2311-GFP-CRT (containing the C-domain of Calreticulin fused with GFP sequence) were electroporated into *Agrobacterium tumefaciens*

strain LBA 4404. Wild type tomato plants (cultivar Micro-Tom) were transformed with *Agrobacterium* using cotyledon explants.

DNA isolation, RNA isolation and PCR analysis

CBP expressing tomato lines T_4 generation were selected and propagated for our study. Total genomic DNA was isolated from leaf tissue using genomic DNA extraction miniprep with CTAB/CHCI3/Isopropanol (adapted from Demeke et al., (2009) [37]). Amplification of the recombinant gene was carried out by PCR (Bio-Rad, CA). Total RNA was isolated using a promega kit (Wisconsin, USA). Synthesis of cDNA was carried out according to the SuperScript III First Strand Synthesis System Kit protocol (Invitrogen, CA). Primer dT16-oligonucleotide was used. One microliter of cDNA was used for the RT-PCR reaction (Bio-Rad, CA). Gene-specific primers for CBP were designed using Primer3 (http://frodo.wi.mit.edu/cgibin/primer3-www.cgi).

PCR amplification was performed with initial denaturation at 94 ^{o}C for 2 min followed by 35 cycles of incubations at 94 ^{o}C for 30 s, 53 ^{o}C for 30 s,72 ^{o}C for 1 min and final extension at 72 ^{o}C for 5 min using *CBP* specific forward 5'-ACAGCATGCCCCTATGATTGACAACC-3' and reverse 5'-ACATGCATGCCGATCTAGAGCTCGTC-3' primers. Actin was used as an internal loading control (ACTINF: 5'-GGATCTTGCTGGTCGTGATT-3', ACTINR: 5'-CTTGTCCATCAGGCAATTCA-3').

GFP Expression Analysis

Leaves and roots of T_4 generation CBP expressing and control constructs were selected and analyzed for GFP fluorescence. Fluorescence images were acquired two weeks after germination using a Leica MZ12 fluorescence dissecting microscope (Leica, Deerfield, IL, USA) using 488 nm excitation laser for GFP.

Protein Extraction and Immunoblot Analysis

Using western blots, CBP expressing and control lines were analyzed for the expression of GFP or the GFP-CBP fusion protein. Total Protein was extracted from the leaves of two months old WT, and CBP expressing 35S:GFP and 35S:GFP-CBP expressing plants. Using 100 mg of leaf tissue from each line tested, the total proteins were extracted using a plant total protein extraction kit (Sigma-Aldrich, St. Louis, MO, USA).

Samples were frozen with liquid Nitrogen and ground well, mixed with a 2x sample buffer adapted from Ausubel et al., (1992) [39]. Protease inhibitor cocktail was added to each of the samples (Sigma-Aldrich, St. Louis, MO, USA). Extracted protein from CBP expressing and control plants was loaded into each lane of a 12% SDS polyacrylamide gel. Proteins were separated by electrophoresis and transferred to a PVDF membrane (Bio-Rad, Bethesda, MD) using Bio-Rad Mini Trans-Blot Assembly for 6 h at 85 V. The membrane was blocked with 5% (w/v) non-fat milk in Tris-buffered saline with 0.2% (v/v) Tween 20 overnight at -4 ^{o}C. Fusion protein was detected using polyclonal anti-GFP antibody raised in goat (Genscript, NJ) (1:50000 dilution in 1X TBST). Goat anti-rabbit antibody (1:500 dilution in 1X TBST) conjugated to horseradish peroxidase was used as the secondary antibody. Signal was

developed using a chemiluminescent substrate, Supersignal West Pico (Pierce Scientific, Rockford, IL).

C**hloroplast Count Measurement**

Epidermal peel taken from the leaf of the tomato plants was evaluated. The peel was placed in a drop of Kl/I_2 (potassium iodine) stain on a microscope slide for about 1 min. A cover slip was added. Stained chloroplasts were counted under a bright field microscope with magnification using 40X objective. The chloroplast number was counted from the guard cells by counting 12-15 stomata per leaf. Two leaves were sampled from each plant (thus resulting in 48-60 guard cells being sampled from each plant). A total of 60 plants were tested for this experiment.

Evaluation of Plant's Growth under Controlled Environmental Conditions

Tomato CBP expressing seeds as well as control seeds (wild type, 35S:GFP and sibling lines) were propagated in the mist house for two weeks. Seedlings of each tomato line were transferred to 350 ml pots with growing medium containing 50% of sand and 50% of peat and maintained in the same conditions. All plants were grown in a growth chamber and kept in 9 h light (26 ºC), 750 μmol m^{-2} s^{-1} light intensity, 15 h dark (22 ºC), and 70% humidity conditions. Plants were watered every day and nutrient solution once a day. (http://www.ncsu.edu/phytotron/manual.pdf) [38].

Treatment of Seedlings with Surflan A.S.

Wild type and 35S:GFP vector control seeds were propagated in the mist house for 2 weeks. The control seedlings were transferred to the growth chamber. Surflan A.S. was applied using Agar drops methods on seedlings. Seedlings were treated as the first true leaves started to become apparent. Surflan concentration of 50 μM was suspended in an agar concentration of 5.5 gL^{-1}. The agar + Surflan solution was pipetted onto the meristem forming a drop on the seedling. The treated plants were then placed inside a humidity chamber and maintained at 100% humidity. To obtain 100% humidity, a humidifier was installed in the chamber. The treatment was repeated 3 times 4 days apart. After the treatment the plants were placed in mist to wash off the solution. Plants were tested for the study after reaching the transplant size.

Biomass and Leaf Area Measurements

CBP expressing and control plants were measured for fresh and dry weight and length. The plant's roots were washed off and weighed. Shoot and roots of CBP expressing and control plants were kept at room temperature for one week to dry and then measured. A total of 27 plants from CBP expressing lines 1, 2 and 4; 12 plants from treated control lines and 19 plants from sibling lines were analyzed, according to the described techniques, under normal conditions. For leaf area measurements, leaves from each plant per line were measured using a leaf area meter (Li-Cor Model 3100C), (Nebraska, USA).

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Characterization of Drought Stress Responses of Tomato Plants Expressing the C-domain of *Zea mays* Calreticulin

Introduction

The scarcity of fresh water is a major global concern that impact food supplies, because drought stress causes reduced plant growth and decreases crop yield. Due to the rapidly growing world population, the increasing need for crops and predictions of exacerbated drought conditions under climate change, development of drought-tolerant plants will ultimately benefit mankind and reduce the stress on the planet's diminishing resources. In spite of the importance of tomato as a world food crop and the increase of drought and soil salinization there are not many reports on drought resistance mechanisms in tomato. This project will provide insights about the role of CBP expression on drought tolerance of the economically important tomato, with potential application to other crop species. Understanding the molecular basis of plant adaptation to abiotic stress responses will allow us to generate crop plants with improved tolerance of drought conditions that maintain or increase yield. The insights from these findings will also make it possible to reduce water consumption from irrigation demands.

Calcium plays an important role as second messenger in signal transduction pathways which regulate stress gene expression in plants [1]. Some calcium binding proteins have been shown to act as calcium sensors and detect calcium concentration changes in the cytosol [1]. Cheong et al., (2003) have studied the overexpression of Calcineurin B-like protein (CBL1) in *Arabidopsis* plants under drought and salt stress conditions. CBL1 over-expressing *Arabidopsis* plants showed tolerance under stress conditions, suggesting involvement of

CBL1 in stress response pathways [1]. Moreover, changes in free calcium concentrations in the cytosol in response to drought and salt were detected by studying *Arabidopsis* seedlings [2]. Based on the obtained results, Knight et al., (1997) have reported that, mannitol induced calcium concentration change in the cytosol maybe caused by the release of calcium from the vacuole and this release was occurring through IP_3-dependent calcium channels [2]. In addition, the role of plant calcium dependent protein kinases (CPK10) in *Arabidopsis* has been identified in response to drought stress [3]. It was demonstrated that CPK10, with the interaction of Heat Shock Protein 1 (HSP1) plays main role in stomatal movements under drought stress through the regulation of Abscisic acid (ABA) and calcium [3]. Here we report the functional characterization of maize CBP (calcium binding peptide) in tomato plants under drought stress. Calreticulin (CRT) plays an important role as chaperone protein in endoplasmic reticulum and regulates cellular calcium homeostasis through its calcium binding domains [4]. The expression and localization of Calreticulin depends on tissue and developmental stage. It appears to be present in most plant cells and tissues [5]; however, it has been reported that calreticulin is expressed abundantly in floral tissues and in germinating seeds [5]. Calreticulin appears to reside in the ER. Other studies have also localized CRT to the nuclear envelope in plant cells [6].

The role of Calreticulin in response to salt and drought stress has been highlighted. Jia et al., (2008) have reported the role of wheat Calreticulin in response to drought. TaCRT protein was isolated from wheat (*triticum aestivum* L). TaCRT over-expressing tobacco (*Nicotiana benthamiana*) plants were generated and exhibited enhanced drought resistance to water

deficit by maintaining higher water use efficiency, water retention ability and relative water content [7].

In addition, Calreticulin is also considered to be involved in calcium storage in endoplasmic reticulum in response to stress signal transduction, via its acidic C-domain binding domain (20-50 moles of calcium per mole of protein) [8]. Transgenic *Arabidopsis* plants expressing maize Calreticulin under the control of a heat shock promoter exhibited delayed loss of chlorophyll after induction compared to control lines when transferred to calcium depleted (CD) medium containing 10 mM EGTA. Furthermore, transgenic *Arabidopsis* lines (mGFP5-CBP) transferred to CD medium containing an additional 10 mM calcium (CDC medium) maintained over 90% of their total chlorophyll content under the constitutive expression of 35S promoter, whereas WT and vector control maintained 30-50%. The data suggested that ectopic expression of C-domain of maize Calreticulin in transgenic *Arabidopsis* plants increased calcium stores and under stress conditions this calcium reserved could be used by plants [8]. Moreover, CRT expression was also found to be involved in ABA-induced salt tolerance in potato lines (*Solanum tuberosum*) [4]. Calreticulin expression increased after NaCl stress in early maturing tolerant (EMT) and late maturing tolerant (LMT) potato clones. Calreticulin expression and salt stress tolerance appear to be regulated by the roots through the involvement of Calreticulin in ABA-induced salt tolerance [4].

Enhanced endogenous expression level of Calreticulin mRNA and protein in response to stress conditions such as cold, salt and phytohormones have been reported [9]. Increased Calreticulin mRNA level was observed under NaCl and high temperature stress conditions in *Brassica napus* seedlings [9]. It is possible that the increased Calreticulin expression

modulates the gene expression or could trigger other metabolism pathways in order to adapt plant to the stress environments. In a study by Komatsu et al., (2007) the role of Calreticulin in response to cold has been demonstrated [10]. It was shown that rice Calreticulin is involved in the signaling pathway and can be phosphorylated. The relationship among Calreticulin together with CRTintP (a Calreticulin interacting protein) and CDPK13 (calcium-dependent protein kinase 13) was investigated. Calreticulin and CRTintP1 accumulation was demonstrated in CRTintP1 and Calreticulin transgenic rice respectively. In addition, transgenic rice lines were reported to be more tolerant to cold stress when compared to control lines [10].

It was also reported that a disorder in calcium uptake and transport occurs under drought stress [11]. Calcium channels and membrane disorders (induced by drought) were complemented when treated with calcium in stressed *Vicia faba* plants. It was reported that while reduced fresh weight and dry mass of *Vicia faba* plants was observed under drought stress, supplemented calcium improved this reduction to a large extent [11].

We also wanted to test tomato plants transformed with the GFP-CBP construct under drought stress conditions. The hypothesis was that the constitutive expression of the C-domain of maize Calreticulin (CBP expressing lines) in tomato affects the stress tolerance of tomato plants under drought stress conditions. We measured stomatal conductance, leaf area and chlorophyll content in CBP expressing and control tomato lines. Fresh weight and dry mass of transgenic and control lines were also measured under drought conditions.

The offspring of CBP expressing tomato lines (35S:GFP-CBP) as well as control lines (wild type and sibling, 415 and 446) were selected (T_5 generation). Wild type plants were treated

with Surflan A.S. to increase chloroplast number (as described in Chapter 2). Selected CBP expressing as well as control plants were drought stressed by watering in one, two or three week intervals (every one week watered plants (E1W), every 2 weeks watered plants (E2Ws) and every 3 weeks watered plants (E3Ws) were labeled for simplicity). Selected CBP expressing and control lines were watered every other day and used as a control during the experiment. In general we found that the CBP expressing lines expressing the C-domain of maize Calreticulin responded to drought stress by closing their stomata and reducing their leaf area, photosynthesis and transpiration rate under severe drought stress; however, chlorophyll content was not affected by drought stress in CBP expressing tomato lines. Moreover, CBP expressing lines exhibited reduced biomass and yield under severe drought stress, though; compared to control lines the effects were insignificant. Overall, based on the experimental analysis, it revealed that CBP expressing tomato lines expressing the C-domain of maize Calreticulin not show any significant difference in their response to drought stress compared to control wt and sibling lines.

Results

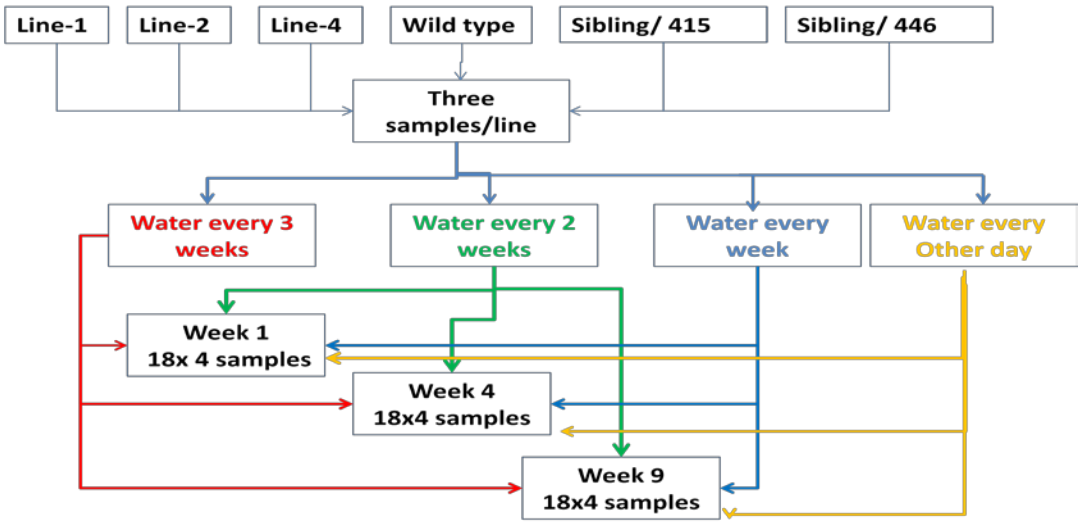
Tomato CBP expressing lines (expressing the C-domain of maize Calreticulin) as well as control plants (wild type and sibling lines, [415-446]) were propagated in the greenhouse under mist condition for 2 weeks. Wild type control tomato plants were treated with Surflan A.S. to increase their ploidy level (as described in Chapter 2). After treatment, control lines, as well as CBP expressing lines were transferred to the larger pots and kept in the chamber to reach the pot capacity before exposure to drought. Drought stress was imposed by watering

plants in a growth chamber for every one week [E1W], every two weeks [E2Ws] and every three weeks [E3Ws]. As a stress control, CBP expressing lines (35S:GFP-CBP) and control (wild type and sibling) tomato plants were watered every other day. The experiment continued for 12 weeks. The developmental stage of the tomatoes was evaluated in the experiment by collecting samples at different periods during the time course of the experiment; week 1 (vegetative stage-14 days after transplanting, [DAT]), week 4 (flowering stage-35 DAT) and week 9 (fruiting stage-65 DAT). Samples were also evaluated on week 12 (79 DAT) for biomass and yield analysis. Four treatments were arranged in a randomized block design with 12 plants per treatment for a total of 288 plants. Random number sequence generator was used to obtain random positions for each of the samples in the treatment blocks. **Table 3.1.** shows the CBP expressing and control tomato lines used in drought experiment.

Table 3.1. CBP expressing and control tomato lines used in drought stress experiment. Drought stress imposed by withholding water for one, two and three weeks for the selected plants. Every other day watered plants were used as control in this study. Four treatments are arranged in a randomized block design (RBD) with 12 plants per treatment for a total of 288 plants. Numbers in cells represent plants for each treatment, i.e. plants 1 to 12 (1-12).

Line Type	Watering Frequency			
	Once a week	Once every 2 weeks	Once every 3 weeks	Every other day (Control)
35S:GFP-CBP–1	1-12	13-24	25-36	217-228
35S:GFP-CBP - 2	37-48	49-60	61-72	229-240
35S:GFP-CBP - 4	73-84	85-96	97-108	241-252
Wild type	109-120	121-132	133-144	253-264
Sibling (4-4-6)	145-156	157-168	169-180	265-276
Sibling (4-1-5)	181-192	193-204	205-216	277-288

Measurements of chlorophyll content, leaf area, root and shoot fresh and dry weight, fruit and seed production and gas exchange parameters such as photosynthesis rate, transpiration rate and stomata conductance were recorded in week 1, week 4 and week 9. A total of 72 (18x4) samples were measured for gas exchange parameters as well as leaf area and chlorophyll measurements as schematically shown in **scheme 3.1.**



Scheme 3.1 Total measurement of CBP expressing and control tomato plants under different water deficit conditions. Three CBP expressing lines (35S:GFP-CBP-1, 2 and 4) as well as control plants (wild type and sibling) were evaluated under drought stress conditions. Four treatments (watered every other day, every week (E1W), every two weeks (E2Ws) and every three weeks (E3Ws)) were included in the experiment for 3 samples from each of the line types (for a total of 18 samples). Thus for each treatment a total of 72 samples (18x4) was evaluated for each selected week.

Effect of Drought on Stomatal Conductance, Leaf Area and Chlorophyll Content

Stomatal conductance (g_s) measures the maximum rate of passage of either carbon dioxide, or water vapor through the stomata. Humidity, hydration status of the plant and also light

intensity affect stomata conductance [12]. Stomatal conductance declines under mild drought stress and under severe drought stress stomatal conductance declines even more, because the photosynthetic machinery becomes damaged [12]. The plant's adaptive response to water deficit is to produce smaller leaves and increase the amount of leaf waxes to reduce water loss [13]. Also, under drought stress conditions reduced leaf area in plants leads to reduced plant transpiration and cell expansion [13].

However, stomatal closure and leaf growth inhibition has been reported as earliest responses to drought, protecting the plants from water loss [14]. These parameters were measured in this study and results are described below.

Results from stomatal conductance measurements **[Table 3.2]** indicate that in general, CBP expressing lines (35S:GFP-CBP) have the approximately same maximum passage rate of water vapor or carbon dioxide through the stomata compared to control lines (wild type and sibling lines) throughout the experiment. A decline in stomatal conductance in both CBP expressing lines and control plants was seen for E3Ws watered plants on flowering stage and for E2Ws watered plants on fruiting stage (compared to well watered treatment), presumably due to closure of stomata to avoid water loss, though the difference between samples was not significant. Because measurements could not be obtained from three weeks withheld watered samples in week 9 of the experiment (fruit stage), due to severe stress, averages for that period do not include week 9 results and are thus inconclusive. **Fig. 3.1.** shows the stomatal conductance measurement for CBP expressing and control lines during the experiment.

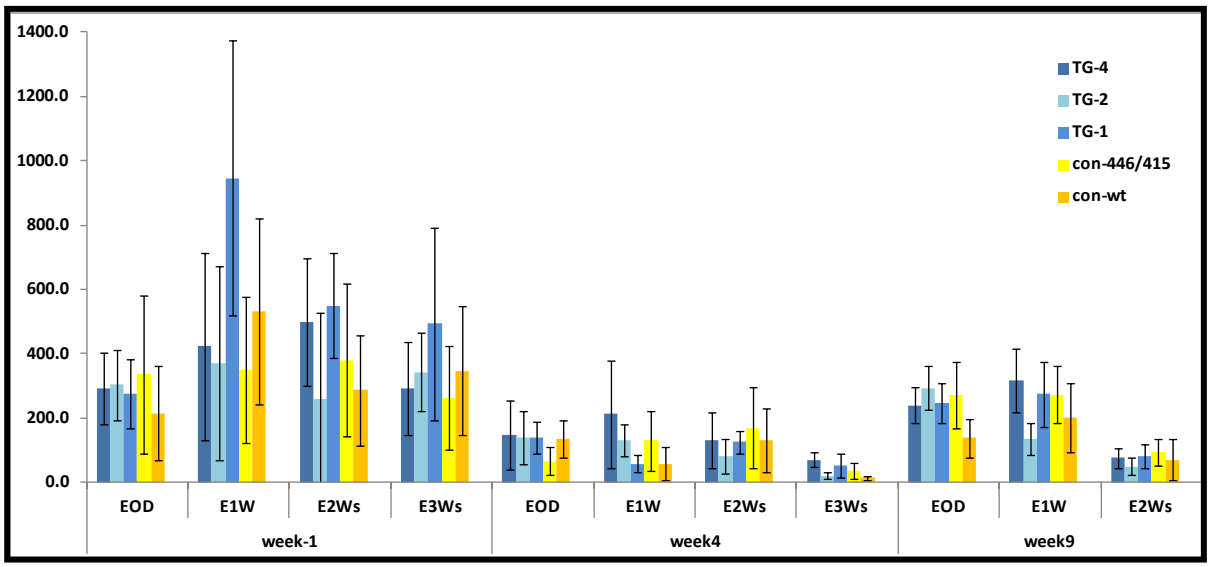


Figure. 3.1. Stomatal conductance of CBP expressing and control lines. Based on the results obtained from the average of two leaves per plant of CBP expressing (35S:GFP-CBP) and control (wild type and sibling) lines, no significant decline was observed in the stomatal conductance rate at the vegetative stage (week 1) on each watering frequency category**.**
A decline in stomatal conductance was observed for E3Ws watered plants on flowering stage (week 4). At fruiting stage (week 9) for E2Ws watered plants, the differnece between the lines were not significant, except in flowering stage (week 4), E3Ws watered CBP expressing line 2 (35S:GFP-CBP-2) and wild type lines showed lower rate of stomatal conductance compared to CBP expresssing lines (35S:GFP-CBP-1 and 4) and sibling lines and the difference was found significant (p<0.05). Error bars represent standard deviations. (n= 9 for CBP expressing lines; n= 3 for wt and n= 6 for sibling line). EOD: every other day waterd plants; E1W: every one week watered plants; E2Ws: every two weeks watered plants; E3Ws: every three weeks waterd plants.

Moreover, the difference of leaf area between the line types (CBP expressing lines vs. control) under drought stress was found not to be significant **[Table 3.2].** A reduction of average leaf area was observed for CBP expressing and control lines which had water withheld for two and three weeks (E2Ws, E3Ws) on fruiting stage (week 9) compared to well watered treatment lines, however, no significant difference was found in any watering frequency category between CBP expressing and control tomato lines. On flowering stage

(week 4) no decrease of leaf area was observed for E2Ws and E3Ws watered CBP expressing and control sibling tomato plants, except E3Ws watered wild type lines showed increased average leaf area. Based on the data from vegetative stage (week 1), E2Ws watered CBP expressing line 4 (35S:GFP-CBP-4) showed higher average leaf area compared to control wild type lines and the difference was significantly presented (p=0.05). **Fig. 3.2.** shows the average leaf area of CBP expressing and control tomato lines under drought stress conditions.

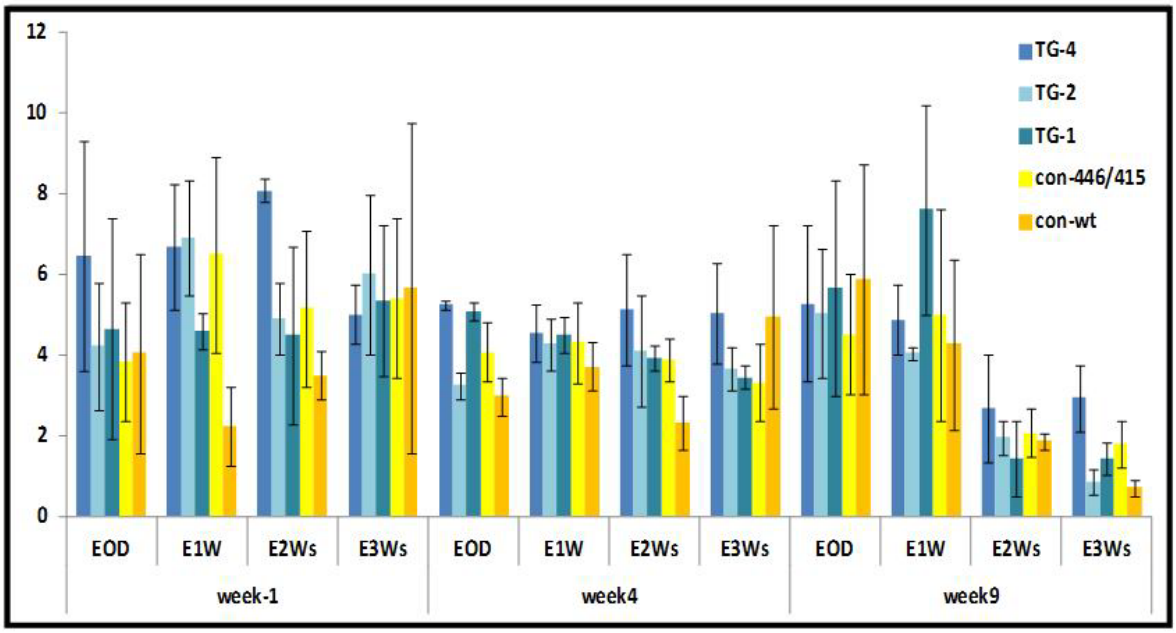


Figure. 3.2. Total average leaf area of tomato CBP expressing and control lines under water deficit conditions. Based on the measurements of leaf areas, CBP expressing lines (35S:GFP-CBP) showed the same average leaf area compared to control lines (wild type and siblings) at the vegetative stage (week 1). An exception is that at flowering stage (week 4), E2Ws watered CBP expressing line 4 (35S:GFP-CBP-4) showed significant higher average leaf area compared to control wild type line (p=0.05). At fruiting stage, E2Ws and E3Ws CBP expressing and control lines showed reduced leaf area compared with well watered lines, however the difference among the stressed lines were not significant. Bars represent standard deviation. (n= 9 for CBP expressing lines; n= 3 for wt and n= 6 for sibling line). EOD: every other day waterd plants; E1W: every one week watered plants; E2Ws: every two weeks watered plants; E3Ws: every three weeks waterd plants.

Chlorophyll content was also measured under drought stress for CBP expressing lines (35S:GFP-CBP-1, 2 and 4) and control lines (wild type and sibling) **[Table 3.2].** Chlorophyll content was not affected by drought stress in any of the tomato lines (CBP expressing line vs. control lines).

The data revealed that at flowering stage treatment (week 4), there was a significant higher chlorophyll content on E3Ws watered) CBP expressing line 4 (35S:GFP-CBP-4) compared to control wild type and sibling lines ($p<0.05$). At fruiting stage all E3Ws watered lines showed reduced chlorophyll content; except CBP-expressing (35S:GFP-CBP-1) line, which showed higher chlorophyll content compared to other plants, though the difference was not significant. **Fig. 3.3**. shows the average of two leaves per plant per line for chlorophyll content measurement at different developmental stages of CBP-expressing and control tomato lines.

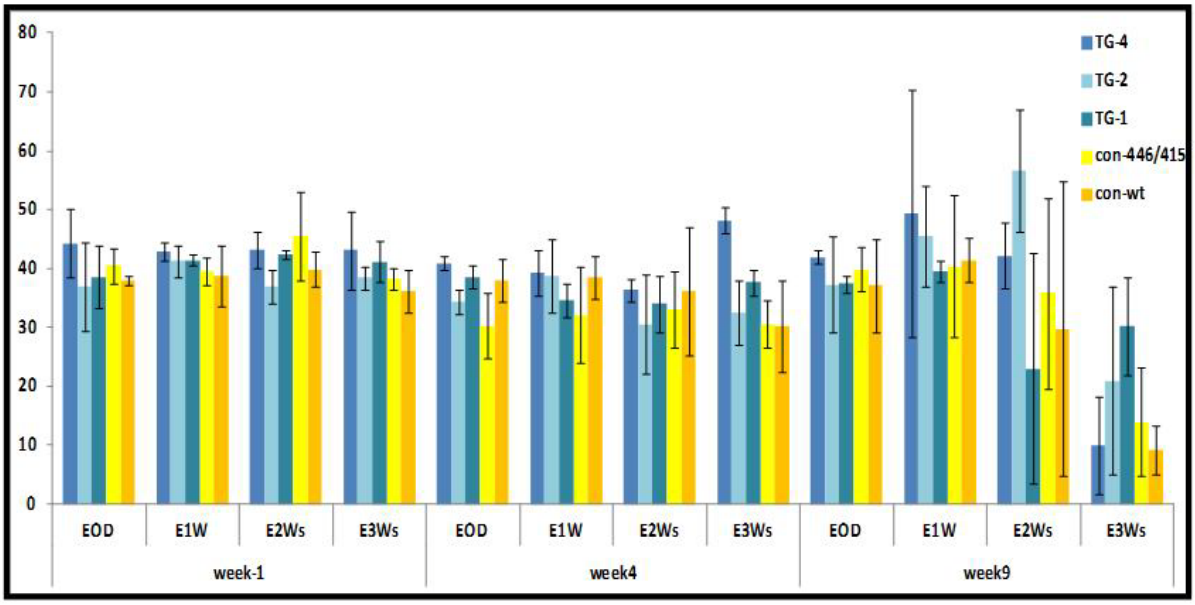


Figure. 3.3. Total average of chlorophyll content of CBP expressing lines vs. control lines exposed to drought stress. During the vegetative (week 1), flowering (week 4) and fruiting (week 9) stages the chlorophyll content of stressed plants remained fairly constant. However, chlorophyll content in CBP expressing lines and control lines after the imposition of drought stress at the fruiting stage for E3WS watered plants began to diminish compared to well water treatment. However, no significant difference was found between the lines. (n= 9 for CBP expressing lines; n= 3 for wt and n= 6 for sibling line). EOD: every other day watered plants; E1W: every one week watered plants; E2Ws: every two weeks watered plants; E3Ws: every three weeks waterd plants.

Table 3.2. Average stomatal conductance, leaf area and chlorophyll content of CBP expressing lines vs. control wild type and sibling lines at week 1, 4 and 9 of the experiment. TG:CBP expressing lines. Con: Control lines. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Stomatal Conductance								
Line type	**TG/ EOD**	**Con/ EOD**	**TG/ EW**	**Con/ EW**	**TG/ E2Ws**	**Con/ E2Ws**	**TG/ E3Ws**	**Con/ E3Ws**
Average/W1	290.44	265.85	592.00	415.71	424.81	354.29	380.88	290.78
SD	103.72	196.23	422.61	257.21	244.01	217.77	219.29	173.65
Average/W4	142.61	104.68	134.03	106.14	112.97	156.39	52.26	29.67
SD	78.83	61.16	115.55	87.70	63.34	116.77	31.97	23.27
Average/W9	262.75	197.81	241.94	249.44	68.64	90.77		
SD	63.55	106.52	117.62	96.74	58.35	41.12		
Leaf Area								
Line type	**TG/ EOD**	**Con/ EOD**	**TG/ EW**	**Con/ EW**	**TG/ E2Ws**	**Con/ E2Ws**	**TG/ E3Ws**	**Con/ E3Ws**
Average/W1	5.11	3.95	6.06	4.90	5.98	4.60	5.50	5.49
SD	2.35	1.97	1.54	2.91	1.99	1.80	1.47	2.58
Average/W4	4.53	3.47	4.44	4.09	4.45	3.37	4.25	3.79
SD	0.99	0.80	0.54	0.88	1.19	0.94	0.93	1.46
Average/W9	5.32	5.19	5.50	4.75	2.01	2.04	2.04	1.58
SD	1.85	2.22	2.12	2.36	1.00	0.53	1.16	0.70
Chlorophyll Content								
Line type	**TG/ EOD**	**Con/ EOD**	**TG/ EW**	**Con/ EW**	**TG/ E2Ws**	**Con/ E2Ws**	**TG/ E3Ws**	**Con/ E3Ws**
Average/W1	40.98	39.12	41.83	39.28	40.80	43.61	40.93	37.62
SD	6.37	2.25	1.74	3.17	3.60	6.72	4.36	2.56
Average/W4	37.90	34.57	37.49	34.26	33.61	34.08	39.44	30.39
SD	3.40	5.85	4.50	7.50	5.55	7.63	7.58	5.02
Average/W9	38.88	38.38	44.84	40.78	40.64	33.83	20.42	12.40
SD	4.15	6.21	12.14	9.74	18.55	18.15	13.14	7.88

Photosynthesis and transpiration rates were measured at flowering stage of stressed and unstressed tomato plants under water deficit conditions **[Table 3.3].**

Table 3.3. **Average photosynthesis and transpiration rate of transgene expressing lines and control lines under drought stress.** EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

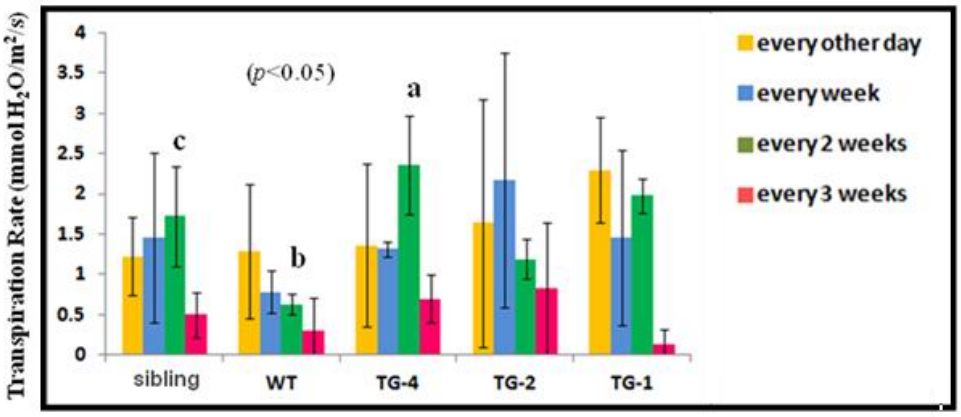
Photosynthesis rate	**Average**				**SD**			
Samples	**EOD**	**EW**	**E2Ws**	**E3Ws**	**EOD**	**EW**	**E2Ws**	**E3Ws**
Sibling	0.005	0.006	0.007	0.002	0.002	0.003	0.002	0.002
WT	0.009	0.004	0.004	0.001	0.005	0.003	0.001	0.003
TG-4	0.007	0.007	0.010	0.003	0.005	0.001	0.001	0.000
TG-2	0.005	0.009	0.006	0.004	0.004	0.003	0.001	0.005
TG-1	0.007	0.006	0.008	0.000	0.002	0.004	0.001	0.001
Transpiration rate	**Average**				**SD**			
Sibling	1.226	1.457	1.726	0.497	0.48	1.06	0.62	0.28
WT	1.291	0.781	0.629	0.307	0.84	0.27	0.13	0.40
TG-4	1.357	1.314	2.360	0.699	1.01	0.09	0.61	0.31
TG-2	1.641	2.168	1.189	0.819	1.54	1.58	0.25	0.83
TG-1	2.298	1.452	1.979	0.130	0.66	1.09	0.21	0.18

In general, photosynthesis and transpiration rates decreased in all severely stressed CBP expressing lines and control lines when they were exposed to drought (every three weeks watered plants). Because one of the first responses of plants under drought stress is stomatal closure, the decrease in photosynthesis rate in drought stressed plants can be attributed to reduction in CO_2 uptake **[Fig. 3.4.A]**. The effect of drought stress on transpiration was similar to that on photosynthesis **[Fig. 3.4.B]**. While well watered plants had higher transpiration rates, CBP-expressing and control lines exposed to drought stress showed lower transpiration rates (every three weeks watered plants [E3Ws]). Results obtained from transpiration rate measurements indicated no significant difference between E3Ws watered

CBP expressing lines (35S:GFP-CBP) and control (wild type and sibling) lines, however, for E2Ws watered lines, CBP expressing line 35S:GFP-CBP-4 showed significantly higher transpiration rate compared to control lines (wild type, sibling) (p<0.05). Moreover, E2Ws watered wild type and sibling line differed significantly (p=0.01) as shown in **Fig. 3.4 A.**

Data obtained from photosynthesis rate measurements revealed that, when water was withheld for three weeks, CBP expressing line and control lines showed declines in the photosynthesis rate, but the difference was not significant. However, watering every two weeks (E2Ws), the CBP expressing lines (35S:GFP-CBP-1, 2 and 4) had a significantly higher photosynthesis rate compared to wild type control lines (p<0.05). Sibling and wild type lines also showed significant differences when water was withheld for two weeks (p=0.01) as shown in **Fig. 3.4 B**.

A.



B.

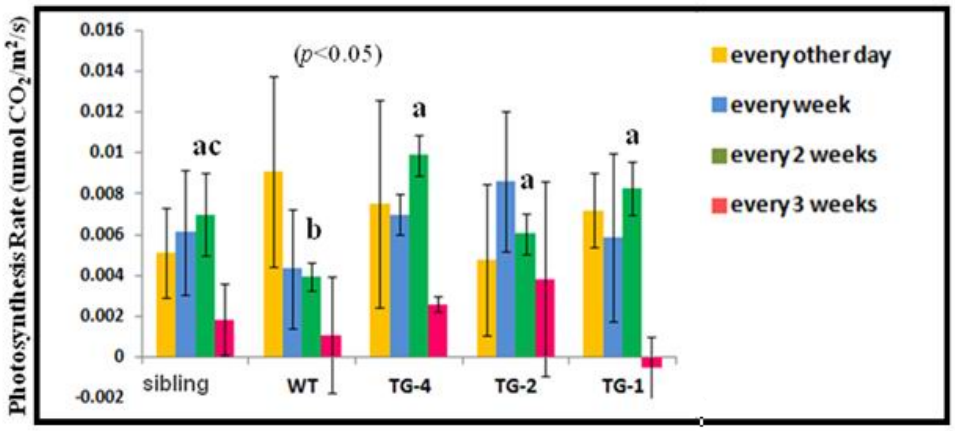


Figure. 3.4. Photosynthesis and transpiration rate of CBP expressing lines vs. control lines under drought stress conditions. The effect of drought stress on photosynthesis and transpiration rates was evaluated in CBP expressing and control tomato lines. Compared to unstressed lines, photosynthesis and transpiration rates of stressed CBP expressing line and control lines diminish when water was withheld for three weeks (E3Ws). Significant difference was found in transpiration rate between E2Ws watered CBP expressing line 4 (35S:GFP-CBP-4) and wild type and sibling lines **(A)**. For photosynthesis rates, CBP expressing lines 35S:GFP-CBP-1, 2 and 4 and wild type control line differed significantly for E2Ws watered treatment **(B).** (n= 9 for CBP expressing lines; n= 3 for wt and n= 6 for sibling line).

Biomass and Yield Analysis under Drought Conditions

CBP-expressing tomato lines 35S:GFP-CBP-1, 35S:GFP-CBP-2 and 35S:GFP-CBP-4 (T_5 generation) were planted in the greenhouse for this drought tolerance experiment. Control tomato plants for the drought experiment include wild type and sibling lines. Control wild type plants were treated with Surflan A.S. before exposing to drought. When all the tomato plants reached the pot capacity the experiment was started. Six weeks old tomato plants were exposed to drought by watering plants for every one (E1W), two (E2Ws) and three (E3Ws) weeks. Every other day watered plants were used as controls in the experiment. Total dry weights of CBP expressing and control plants, as well as fruit and seed number and weight were assessed at the end of the experiment (week 12). The data were analyzed using t-test.

Analysis of Dry Root and Shoot Weight and Length under Drought

Total average dry weights of CBP expressing lines (35S:GFP-CBP-1, 2 and 4) and control lines for every watering category was measured **[Table 3.4].** In general, there was no effect on biomass by watering frequency between line types (CBP expressing line vs. control) under drought conditions. The effect of drought on total dry weight for CBP expressing line and control lines were the same. Both CBP expressing lines and control lines showed decline in total dry weight when water was withheld for two or three weeks. Based on the t-test analysis, no significant difference was found among all the lines. Significant differences were found only between CBP expressing line 1 (35S:GFP-CBP-1) and sibling plants under every week watering frequency (p=0.02). **Fig. 3.5.** shows the total average dry weight of CBP expressing lines and control lines under water deficit conditions.

Table 3.4. **Total average dry weight (g) of transgene expressing lines and control lines under drought stress.** EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	2.9	1.8	0.9	1.0
SD	1.2	0.5	0.3	0.4
Wt	1.9	2.3	1.1	1.0
SD	1.7	1.0	1.0	0.5
TG-4	3.5	2.5	1.3	1.3
SD	1.2	0.8	0.8	0.5
TG-2	3.8	2.6	0.8	0.9
SD	1.2	0.9	0.9	0.1
TG-1	2.7	2.9	1.0	1.1
SD	0.6	0.9	0.9	0.2

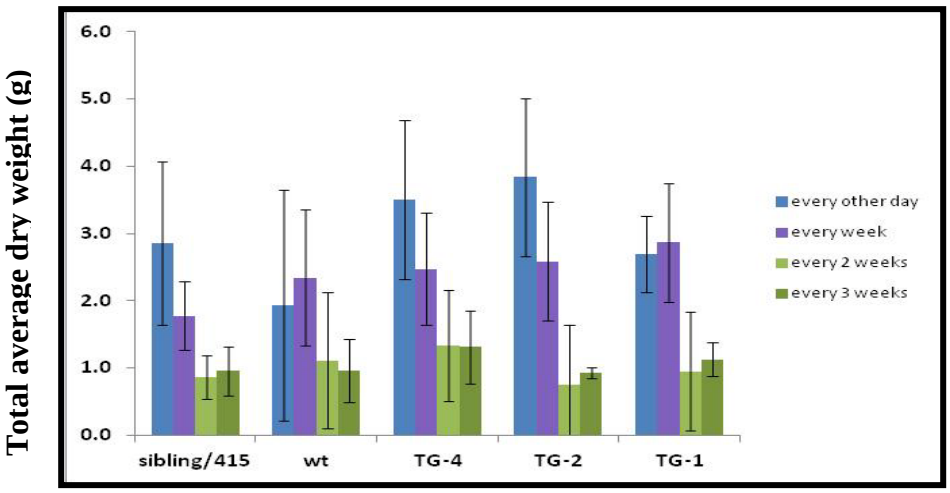


Figure. 3.5. Total average dry weight of CBP expressing lines and control lines. A decline of total dry weight in all the lines were observed between every 2 and 3 weeks withold water plants (E2Ws, E3Ws) compared to control every other day watered plants. No significant difference was found between CBP expressing line and control lines in each category of watering frequency. Error bars indicate SD (n= 10 for each of the line type).

A decline of shoot and root dry weight was observed in both CBP expressing lines (35S:GFP-CBP) and control wild type and sibling lines when watered plants for E2Ws and E3Ws compared to every other day watered control plants **[Table 3.5].** However, average dry shoot weight of CBP expressing lines were the same as control lines (wild type and sibling) for each watering frequency. T-test results indicate that there is no significant difference of average shoot dry weight between CBP expressing lines and control lines for each category of watering frequency watered plants. The only significant difference was found between CBP expressing line 1 (35S:GFP-CBP-1) and sibling plants for every week watering frequency (E1W) (p=0.01).

Table 3.5. Total average shoot dry weight (g) of transgene expressing lines vs. control lines. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	1.916	1.345	0.761	0.777
SD	0.757	0.420	0.230	0.270
Wt	1.900	1.654	0.901	0.817
SD	0.137	0.800	0.800	0.280
TG-4	2.580	1.872	0.887	0.920
SD	0.740	0.800	0.800	0.230
TG-2	1.990	1.840	0.709	0.772
SD	0.425	0.630	0.630	0.140
TG-1	1.753	2.059	0.787	0.910
SD	0.324	0.540	0.540	0.250

Moreover, CBP expressing lines showed approximately the same average dry root weight as control lines **[Table 3.6].** Only CBP expressing line 1 and 2 (35S:GFP-CBP-1 and 35S:GFP-CBP-2) showed higher average dry root weight than sibling plants for every week watering frequency (E1W) and the difference was found significant (p=0.04). **Fig. 3.6**. shows the average shoot and root dry weight in CBP expressing lines and control lines under drought stress conditions.

Table 3.6. Total average root dry weight (g) of transgene expressing lines vs. control lines. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	0.936	0.424	0.126	0.110
SD	0.516	0.170	0.090	0.100
Wt	2.040	0.776	0.177	0.153
SD	0.905	0.500	0.500	0.210
TG-4	0.923	0.604	0.346	0.382
SD	0.682	0.220	0.220	0.290
TG-2	1.835	0.738	0.112	0.080
SD	1.110	0.270	0.270	0.040
TG-1	0.950	0.811	0.162	0.182
SD	0.201	0.400	0.400	0.150

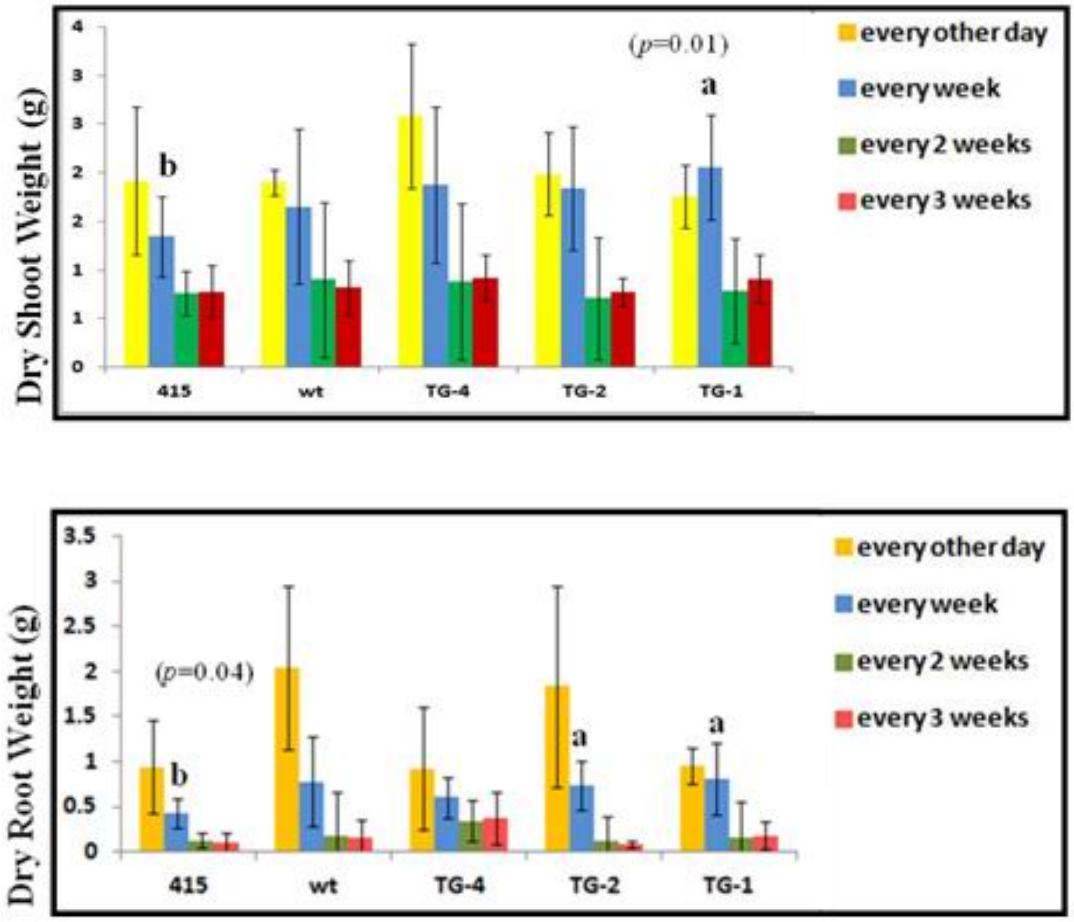


Figure. 3.6. Total average shoot and root dry weight of CBP expressing lines and control lines under drought stress conditions. A decline of total shoot and root dry weight of all the lines were bserved when every other day watered plants were compared to two and three weeks watered plants (E2Ws and E3Ws). However no significant difference was found between CBP expressing lines and contol lines (wt and sibling 415). Error bars represent SD. (n= 10 for each of the line type).

The average shoot and root length of CBP expressing tomato lines under drought was measured **[Table 3.7-3.8].** It has been reported that water deficit increases root growth and also stimulates the elongation rate in plants [15]. Based on the results, water deficit did not enhance primary root length in CBP expressing lines (35S:GFP-CBP). The CBP expressing

lines (35S:GFP-CBP-1, 2 and 4), showed a decline in root length when watered for E2Ws and E3Ws compared to well watered control plants. However, the decline of root length in wild type lines was more severe and was significant compared to trangenic CBP expressing lines. While a decline of root length was observed for E2Ws and E3Ws watered tomato lines (CBP expressing and control tomato plants) compared to every other day watered plants, no significant decline was found in average shoot length in both CBP expressing lines and control lines when watered for E2Ws and E3Ws. **Fig. 3.7.** shows total average shoot and root length in CBP expressing lines and control tomato lines under water deficit conditions.

Table 3.7. Total average shoot length (inch) of transgene expressing lines vs. control lines. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	6.500	5.308	4.813	4.792
SD	1.620	0.880	1.060	1.010
Wt	5.500	5.050	4.063	4.318
SD	1.323	1.320	1.320	0.840
TG-4	5.767	5.400	4.833	4.556
SD	0.252	0.890	0.890	0.880
TG-2	6.000	5.950	4.833	4.800
SD	0.577	0.740	0.740	0.670
TG-1	5.167	5.357	4.278	4.714
SD	0.764	0.800	0.800	0.990

Table 3.8. Total average root length (inch) of transgene expressing lines vs. control lines. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	6.700	5.042	2.732	2.538
SD	1.037	1.210	1.150	1.130
Wt	7.250	4.460	3.571	1.929
SD	2.475	1.200	1.200	1.510
TG-4	6.667	6.280	3.600	2.917
SD	1.893	2.130	2.130	0.740
TG-2	7.125	6.250	2.857	2.067
SD	1.436	0.990	0.990	0.400
TG-1	6.500	5.643	2.944	2.417
SD	1.323	1.270	1.270	1.160

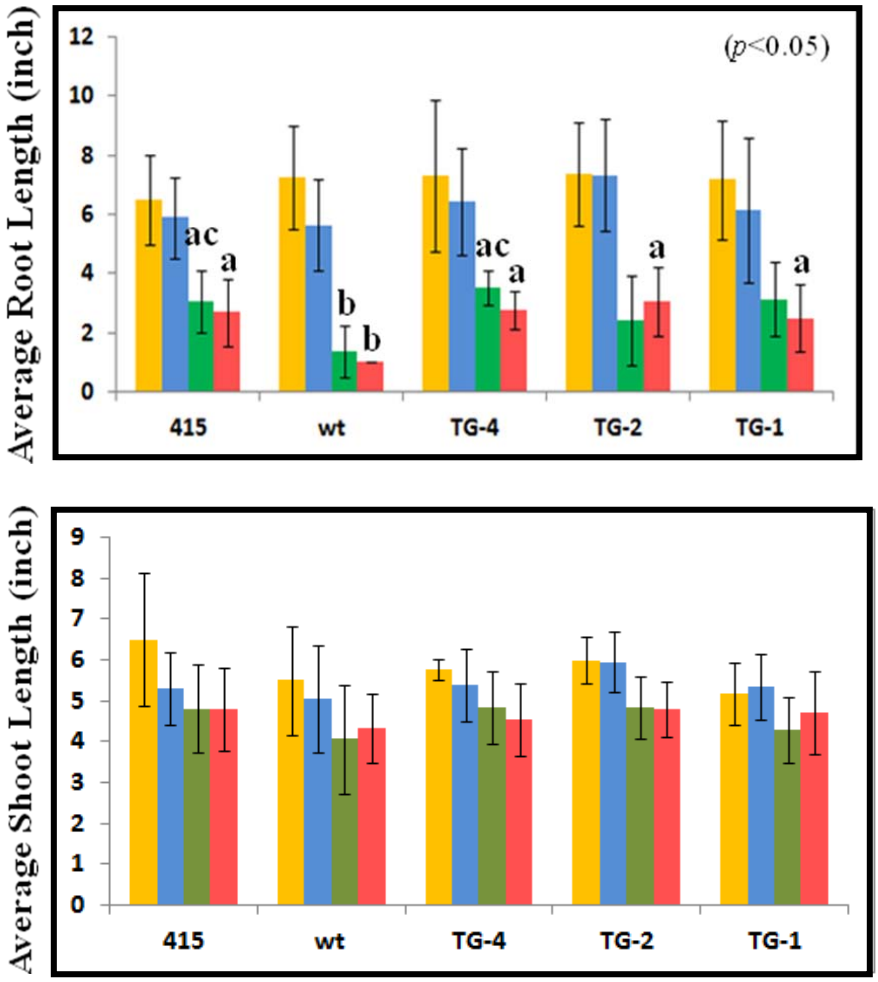


Figure. 3.7. Total average shoot and root lengths in CBP expressing lines and control tomato lines under drought stress. Total average of shoot and root lengths were analyzed in 4 months old CBP expressing lines (35S:GFP-CBP) and control wild type, sibling lines under water deficit conditinos. Compared to well watered plants, both trangenic and control lines showed declined average root length when plants watered every two and three weeks (E2Ws and E3Ws). A significant difference was observed in every three week withhold watered CBP expressing lines compared to wild type, but not to sibling controls. No significant decline was observed in the average shoot length of plants watered E2Ws and E3Ws neither in CBP expressing lines nor control lines. Error bars represent SD. (n= 10 for each of the line type).

Analysis of Fruit and Seed Weight and Number

The effects of the drought treatments on fruit production and seed yield were assessed. Imposition of water stress caused a significant reduction in fruit and seed weight and number of CBP expressing lines (35S:GFP-CBP) and control wild type and sibling tomato lines. Based on the analysis of average fruit weight, CBP expressing lines and control lines did not differ significantly when plants watered for E2Ws and E3Ws. However every week watered (E1W) CBP expressing line 4 (35S:GFP-CBP-4) had significantly higher average fruit weight compared to wild type plants (p=0.01) **[Table 3.9]**. Moreover, every other day and every week watered CBP expressing line 4 (35S:GFP-CBP-4) had significantly higher average fruit weight than CBP expressing line 2 and 1 (35S:GFP-CBP-2 and 35S:GFP-CBP-1) (p<0.05) as shown in **Fig. 3.8.**

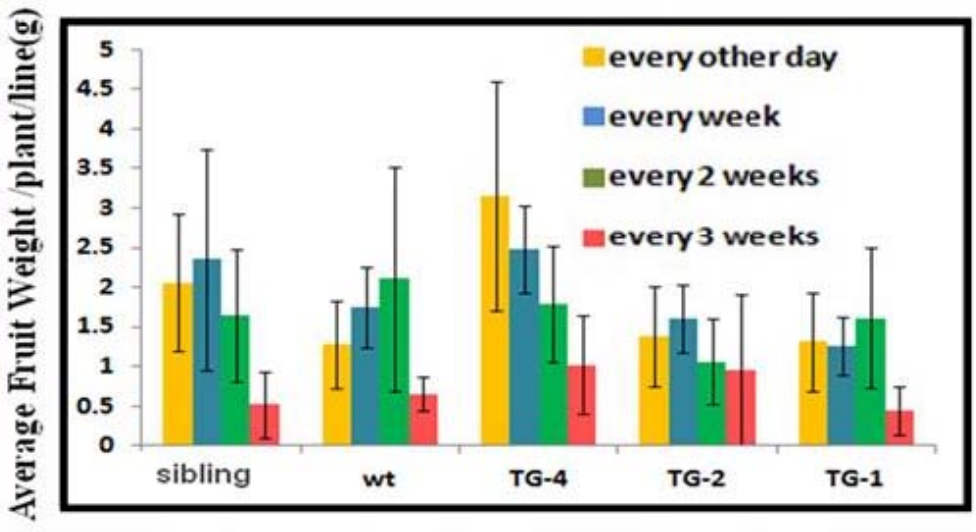


Figure. 3.8. Total average fruit weight of CBP expressing lines and control tomato lines under drought stress. Total average of fruit weight of CBP expressing line (35S:GFP-CBP) and control wild type and sibling lines were analyzed under drought stress conditions. No significnat difference was found for E2Ws and E3Ws watered lines. The only difference was observed in every week withold watered CBP expressing line1 (35S:GFP-CBP-1) compared to sibling lines (p=0.01) and CBP expressing line 4 (35S:GFP-CBP-4) compared to wild type lines (p=0.01). Moreover, every other day and every week withhold CBP expressing line 4 (35S:GFP-CBP-4) also showed higher average fruit weight than CBP expressing line 1 and 2 (35S:GFP-CBP-1 and 35S:GFP-CBP-2) lines and the difference was found significnat (p<0.05). Error bars represent standard deviation. (n= 10 for each of the line type).

Table 3.9. Total average fruit weight (g) of CBP expressing lines vs. control tomato lines under drought stress conditions. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	2.061	2.352	1.646	0.514
SD	0.870	1.400	0.840	0.410
Wt	1.284	1.753	2.104	0.652
SD	0.550	0.510	1.420	0.220
TG-4	3.147	2.480	1.784	1.016
SD	1.450	0.550	0.730	0.620
TG-2	1.371	1.609	1.058	0.943
SD	0.630	0.430	0.540	0.970
TG-1	1.309	1.255	1.613	0.445
SD	0.620	0.360	0.880	0.310

Data presented show the average fruit number per plant for CBP expressing lines 35S:GFP-CBP-1, 2 and 4 as well as control lines (wild type and sibling plants) based on observations from weeks 7-10 and 12 of the experiment (fruiting stage). Data from this period suggest that in general the difference in the average number of fruits per plant per line among CBP expressing and control plants is not statistically significant. E2Ws and E3Ws watered CBP expressing lines and control lines showed the same lower average fruit number compared to well watered plants on weeks 7, 10 and 12. Moreover, no significant difference was observed at week 7 and 10 among all the watering frequency categories between CBP expressing lines and control lines, whereas based on the analysis of week 12 of fruiting, every week watered CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) had a significantly higher average fruit number than sibling lines (p=0.05). In addition, E2Ws watered sibling lines had a significantly higher average fruit number than CBP expressing line 4 (35S:GFP-CBP-4)

(p=0.009). Every three weeks watered (E3Ws) CBP expressing line 4 (35S:GFP-CBP-4) had a higher average fruit number than sibling lines and the difference was significant (p=0.001) as shown in **Fig. 3.9.**

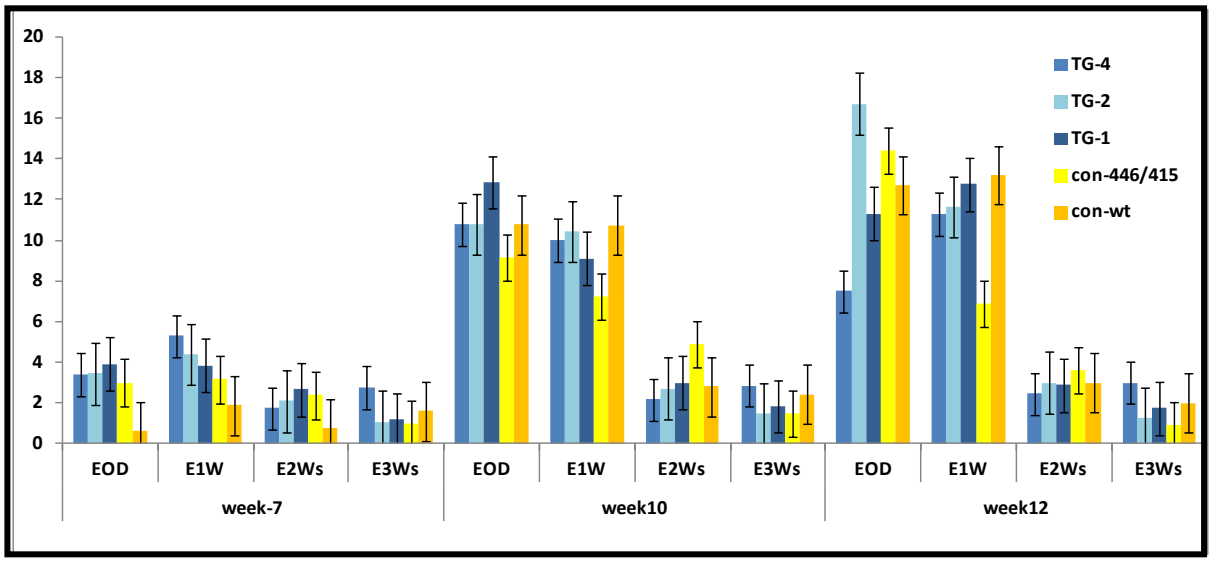


Figure. 3.9. Total average fruit number of CBP expressing lines and control lines under water deficit. A decline of average fruit number was observed in all the lines when plants watered for two and three weeks (E2Ws and E3Ws) compared to every other day watered plants**.** However, on week 12, E2Ws watered sibling lines showed higher average fruit number than CBP expressing line 4 (35S:GFP-CBP-4) and E3Ws watered CBP expressing line 4 (35S:GFP-CBP-4) showed higher average fruit number than sibling lines and the difference was found significant. Error bars represent standard deviation. (n= 10 for each of the line type). EOD: every other day waterd plants; E1W: every one week watered plants; E2Ws: every two weeks watered plants; E3Ws: every three weeks waterd plants.

The total average seed numbers and weights were analyzed for CBP expressing lines (35S:GFP-CBP-1, 2 and 4) as well as control tomato plants (wild type and sibling) under drought stress conditions **[Table 3.10-11].** The total average of seed numbers for E2Ws watered CBP expressing line 1 and 2 (35S:GFP-CBP-2 and 35S:GFP-CBP-1)were lower than those from control sibling and wild type lines and the difference was found significant (p=0.03). A significant difference was also found between every week watered CBP expressing line 1 and 4 (35S:GFP-CBP-1, 35S:GFP-CBP-4) and wild type control lines, in which wild type lines had a significantly higher seed number (p<0.05). However, no significant difference was found for E3Ws watered CBP expressing lines and control lines.

Total averages of seed weights of CBP expressing lines and control lines exposed to drought were also analyzed **[Table 3.10].** No significant difference was found among the lines in any category of watering frequency, however, only every three weeks watered (E3Ws) CBP expressing lines 4 and 2 (35S:GFP-CBP-4 and 35S:GFP-CBP-2) showed higher average seed weight compared to sibling lines and the difference was found significant (p<0.05). as shown in **Fig. 3.10.**

Table 3.10. Total average seed weight (g) of CBP expressing lines vs. control tomato lines under drought stress conditions. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	0.015	0.014	0.009	0.003
SD	0.004	0.004	0.007	0.001
Wt	0.014	0.013	0.008	0.022
SD	0.008	0.003	0.011	0.033
TG-4	0.012	0.014	0.009	0.010
SD	0.006	0.002	0.001	0.005
TG-2	0.015	0.010	0.008	0.009
SD	0.005	0.002	0.004	0.003
TG-1	0.012	0.010	0.008	0.005
SD	0.007	0.002	0.001	0.005

Table 3.11. Total average seed number of CBP expressing lines vs. control tomato lines under drought stress conditions. EOD: every other day watered plants; EW: every week watered plants; E2Ws: every two weeks watered plants and E3Ws: every three weeks watered plants. SD: Standard Deviation.

Samples	EOD	EW	E2Ws	E3Ws
Sibling	7.66	4.25	5.29	3.33
SD	8.37	1.83	3.46	2.36
wt	10.48	9.44	9.08	15.00
SD	7.28	3.02	5.66	7.94
TG-4	6.85	4.38	7.17	6.40
SD	3.80	0.59	2.84	3.27
TG-2	4.24	3.70	1.50	6.50
SD	1.40	3.25	0.71	3.91
TG-1	9.49	2.91	1.63	3.08
SD	0.85	1.69	0.18	3.42

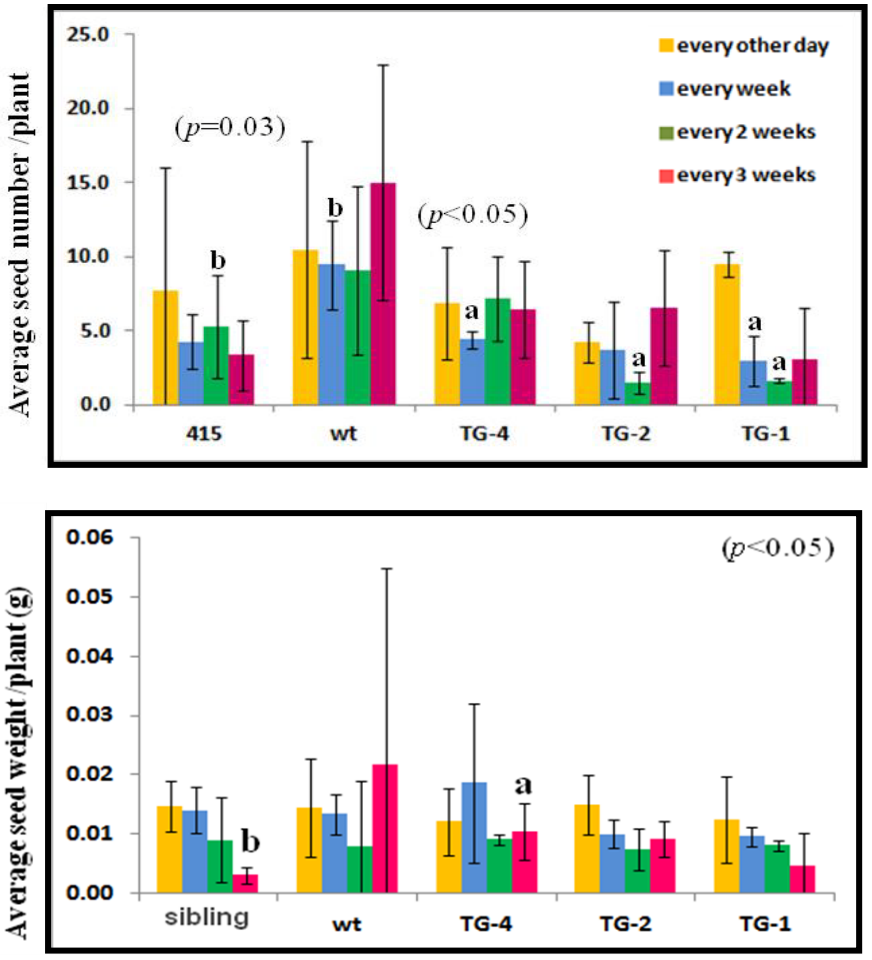


Figure. 3.10. Total average seed weight and number of CBP expressing lines and control tomato lines under drought stress conditions. CBP expressing line and control tomato plants were evaluated based on their seed numbers and seed weights per fruit per plant, after exposure to drought stress condtions. A decline of seed number was observed in evey two weeks watered (E2Ws) CBP expressing line 1 and 2 (35S:GFP-CBP-2 and 35S:GFP-CBP-1), whereas every three weeks watered (E3Ws) CBP expressing line 2 and 1 (35S:GFP-CBP-2 and 35S:GFP-CBP-1) showed higher average seed number. A reduction of average seed weight was observed in stressed CBP expressing line and control lines, however, the only significant difference was found between CBP expressing line 4 and 2 (35S:GFP-CBP-4, 35S:GFP-CBP-2) and control sibling lines when plants watered for E3Ws. Error bars represents standard deviation. (n= 10 for each of the line type).

CBP expressing lines and control plants were evaluated based on their ability to survive under drought stress conditions. As shown in **Fig. 3.11**. almost 40% of the every two weeks watered (E2Ws) CBP expressing line 2 and 4 (35S:GFP-CBP-2 and 35S:GFP-CBP-4) survived under water deficit, whereas, every two weeks watered (E2Ws) wild type and sibling control lines as well as CBP expressing line 1 (35S:GFP-CBP-1) showed near 80% and 60% survival rates respectively. Interestingly, neither of the every three weeeks watered (E3Ws) CBP expressing lines 4 and 1(35S:GFP-CBP-4 and 35S:GFP-CBP-1), nor the wild type and sibling control lines survived under severe drought stress conditions, however, only CBP expressing line 2 (35S:GFP-CBP-2) showed 10% survival rate when exposed to drought stress as shown in **Fig. 3.11**.

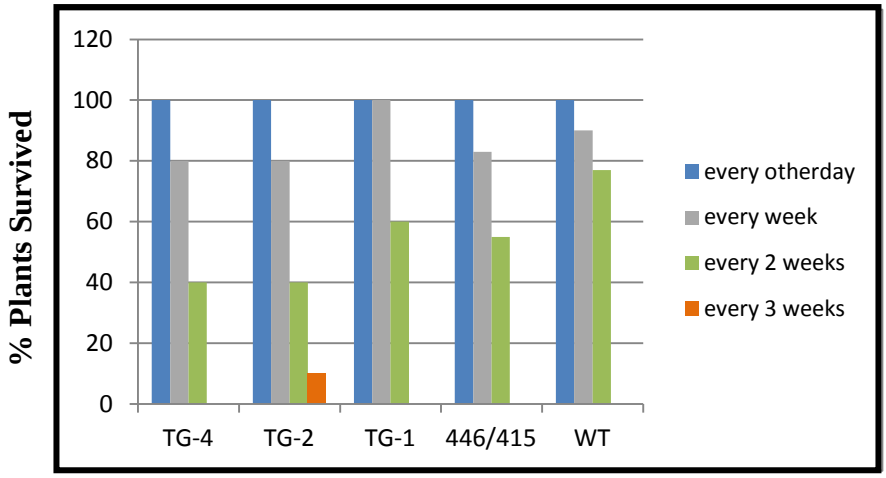


Figure. 3.11. Percentage of CBP expressing lines and control tomato plants surviving under water deficit conditions. Based on the survival rate of the CBP expressing lines and control lines under different water frequency, only CBP expressing line 2 (35S:GFP-CBP-2), showed higher survival rate when watered for three weeks (E3Ws), none of the plants from other lines survived under severe stress conditions. (n=20 for each line type).

Discussion

Drought causes reduced plant growth and decreases crop yield [16]. Under drought stress water availability for plants decreases which leads to a decline in water potential and as a result the photosynthesis rate declines [16]. In the other words, to avoid low water potential plant tissues balance water uptake and loss to avoid dehydration [17]. Based on the avoidance/tolerance model, stomatal closure and accumulation of compatible solutes are among the first responses that enhance plants' resistance to drought [18]. Another response of plant cells under drought stress is cell wall hardening to avoid loss of turgor; this mechanism prevents cell expansion [19]. We investigated whether expression of the C-domain of maize Calreticulin (CBP) in tomato plants would affect the plants response to drought stress and whether CBP expressing tomato lines show differences in the resistance to water stress compared to control lines by measuring the pertinent physiological and morphological parameters during the course of the experiment. Three CBP expressing lines, 35S:GFP-CBP-1, 2 and 4 as well as two control lines, wild type and sibling plants were evaluated under drought stress. In addition, various stages of plant development were evaluated during the experiment, namely the vegetative, flowering and fruiting stage. Observations of leaf area, chlorophyll content, biomass, yield and gas exchange parameters such as stomatal conductance, photosynthesis and transpiration rates were recorded on the selected developmental stage. Biomass and yield analysis were conducted at the end of the drought-stress period. CBP expressing and control tomato plants were exposed to drought by watering plants every one week (E1W), every two weeks (E2Ws) and every three weeks (E3Ws). As controls, all lines were watered every other day.

We found that drought had a significant effect on drought-stressed CBP expressing lines and control lines compared to unstressed plants; however, the difference between the lines was insignificant in most of the measured parameters during the experiment. Based on the obtained results, a decline in the stomatal conductance in both CBP expressing and control plants was observed after two and three weeks of simulated drought though the difference between lines was not significant. Stomatal closure has been reported in many studies as the earliest response to drought [20]. Moreover, the dominant limiting factor to photosynthesis has been reported to be the stomatal closure under mild drought [21]. The decrease in Rubisco activity has been also reported at lower stomatal conductance, (g <100 mM H_2O m^{-2} s^{-1}), whereas permanent photo-inhibition occurs at very low values (g <50 mM H_2O $m^{-2}s^{-1}$) [22]. Results from stomatal conductance measurement indicate that both CBP expressing lines (35S:GFP-CBP) and control wild type and sibling lines showed a reduced stomatal conductance (g_s) at two and three weeks of simulated drought, (g <100 mM H_2O $m^{-2}s^{-1}$); however, except for CBP expressing line 2 (35S:GFP-CBP-2) and wild type control which showed significantly lower stomatal conductance, the difference between the other line types was not significant as shown in **Fig. 3.1.** The reduction in stomatal conductance leads to reduced transpiration rates [23]. Based on the obtained data from the flowering stage, a decline in photosynthesis and transpiration rate was found for CBP expressing lines and control tomato plants that were subjected to water withholding for three weeks, as shown in **Fig. 3.4.** This indicates that drought imposed in CBP expressing lines and control tomato lines affected their transpiration rate in which, it will allow these plants to conserve water during drought, but the difference was not significant between the line types.

However, drought stressed (E2Ws) CBP expressing lines (35S:GFP-CBP-1, 2 and 4) showed a statistically higher photosynthesis rate compared to that in wild type ($p<0.05$) but not with sibling lines. Interestingly, while CBP expressing line 4(35S:GFP-CBP-4) showed the same reduction in stomata conductance compared to wild type plants at the same flowering stage and under the same imposed drought conditions, the leaf area, the photosynthesis and transpiration rate of CBP expressing line 4 (35S:GFP-CBP-4) were found to be significantly higher than those in wild type lines but not significant difference with sibling lines. This could indicate that CBP expressing lines, as well as sibling lines (with showing ploidy level, discussed in chapter 2) might have a high capacity allowing high CO_2 assimilations, since the polyploidy plants tend to have larger leaf area and as a result, higher photosynthesis rate even with partial stomatal closure. The data also could suggests that while stomata closes in response to drought and a net decrease in photosynthesis, stomata conductance is not solely controlled by soil water availability but is also regulated by many internal and external factors such as secondary messengers or epidermal cell turgor [24]. Moreover, although expression of the C-domain of maize Calreticulin in tomato plants (35S:GFP-CBP-1, 2 and 4) may have resulted in closure of stomata but a normal photosynthetic activity was maintained.

Schroeder et al., (2001) reported that calcium oscillations may encode information required for processing closure signals through the induction of abscisic acid (ABA) via a study of *Arabidopsis* guard cells [25].

The chlorophyll content was not affected by drought stress on both CBP expressing lines and control lines at vegetative, flowering and fruiting stages as shown in **Fig. 3.3.** The chlorophyll content of stressed plants remained fairly constant during the experiment, only three weeks watered (E3Ws) CBP expressing and control lines at the fruit stage showed a slight decline in chlorophyll content. However, the difference among the lines was not significant. The only significant difference was found between every three weeks watered (E3Ws) CBP expressing line 4 (35S:GFP-CBP-4) and control lines at flowering stage. It seems, in general CBP expressing lines showed the same effect as control lines in terms of maintaining chlorophyll content under severe water deficit, except in CBP expressing line 4 (35S:GFP-CBP-4) at flowering stage. Jiang et al., (2001) studied the effect of calcium on antioxidant activities and water relations associated with heat tolerance in cool-season grasses [26]. The grasses were treated with $CaCl_2$ (10 mM) and exposed to heat stress (35/30 ^{o}C). In the control species heat stress reduced grass quality, relative water content, and chlorophyll content, whereas calcium treatment increased all three factors in the treated grasses under heat stress.

It has been suggested that external calcium may interfere with the cellular calcium and therefore affect osmotic adjustment of cells under stress conditions [26]. The role of calcium in the regulation of plant responses to stress could be also due to its involvement in signal transduction [27], or gene expression [28] under stress conditions. However, for the CBP expressing line-4 that experienced induced drought (every three weeks watered E3Ws) an increase in the chlorophyll content was observed only at the flowering stage and not in fruiting stage and since only one of the lines compared to other CBP expressing lines (line 1

and 2) showed an effect thus the effect of expressed calcium binding domain of maize CRT in CBP expressing lines is not conclusive.

The effect of water stress on biomass yield was also evaluated. Drought stress significantly reduced biomass in CBP expressing lines (35S:GFP-CBP) and control wild type and sibling lines. Zeid et al., (2006) reported the reduction of shoot and root fresh and dry weights in alfalfa in response to water deficit induced by polyethylene glycol. However, the root length was increased [29].

Total biomass of the drought-stressed tomato plants declined when watered for two and three weeks (E2Ws and E3Ws), while, total biomass of unstressed plants (every other day watered) remained constant. No significant difference was observed between the lines (CBP expressing lines vs. control).

While shoot length was not significantly affected by drought stress in both CBP expressing and control plants, root length showed a decline in stressed plants compared to well-irrigated plants. Comparing stressed CBP expressing lines and control lines, every three weeks watered (E3Ws) CBP expressing lines showed significantly longer roots compared to wild type plants but not to sibling lines **[Fig. 3.7].** This again could indicate the effect of ploidy on CBP expressing and sibling lines in which root length in stressed lines tend to decline compare to well watered lines but compared with stressed wild type plants polyploidy lines had a significant longer average root length in order to obtain more water from the soil.

CBP expressing lines 1 and 2 (35S:GFP-CBP-1 and 35S:GFP-CBP-2) also showed significantly higher average dry root weight than sibling plants for every week watering frequency (p=0.04). However, since this was only shown in every week watered lines but not

in severely stressed lines (E2Ws and E3Ws) the effect of CBP (C-domain of maize CRT) in tomato lines is not conclusive **[Fig. 3.6].**

In general, stressed CBP expressing lines and control lines showed a decline in both shoot and root weight compared to those in unstressed lines. The shoot length was unaffected by drought, while the root length was declined in all lines. This could indicate that plants retain their shoot lengths to enhance their access to the light and improve photosynthesis. Because the rate of photosynthesis was reduced under water deficit, due to the closure of stomata, a reduction in shoot weight was observed. The reduced shoot weight under drought could also be due to the fact that plants tend to lose their leaves under stress to enhance the hydraulic pressure in the plant, although the loss of leaves could affect the overall biomass and yield.

Drought stress also caused reduced fruit size and number in tomato CBP expressing lines (35S:GFP-CBP) as well as in wild type and sibling control lines. Reduced fruit size and number under water deficit has been reported on high bush blueberry (*Vaccinum corymbosum L.*) [30]. Overall, average fruit weight in CBP expressing line 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) and control sibling and wild type lines was reduced under severe drought stress compared to well watered plants.

Furthermore, the average fruit number obtained from fruiting stage (week 12) revealed that under very mild stress (every week watered E1W) CBP expressing lines 1, 2 and 4 (35S:GFP-CBP-1, 2 and 4) had a significantly higher average fruit number than sibling control lines. However under severe stress (every three weeks watered E3Ws) only CBP expressing line 4 (35S:GFP-CBP-4) showed a significantly higher average fruit number compared to that in sibling lines (p=0.01). Whereas, every two weeks watered sibling lines

(E2Ws) showed a higher average fruit number than that in CBP expressing line 4 (35S:GFP-CBP-4). Fruit growth depends on long-distance transport of sugars and water. Water is transported through xylem and phloem, whereas sugars are only imported from the phloem [31]. Because fruits have to compete for water with the rest of the plant, xylem influx is sensitive to the changes of water potential in the whole plant [31]. The data suggests that although the average fruit number showed a decline under severe drought, CBP expressing lines and control lines showed different average fruit number on selected weeks of fruiting depending on the stress level. However, a significant effect of CBP-expression on fruit number and size in drought stressed plants could not be determined.

Seed weight of tomato plants was also affected by imposed stress treatments. It is well known that under drought stress seed yield is reduced [32]. It has been reported that under stress conditions seed filling period shortens, which results in reduced final seed size [32]. Seed yield in tomato lines was minimized through the influence of severe water stress. Heatherly et al., (1993) reported that during seed production of soybean the yield and quality of seeds under drought stress was reduced, suggesting that reproductive growth is an important and sensitive stage to water stress [33]. The average seed number in sibling and wild type control lines showed no significant difference among every category of watering frequency whereas, stressed CBP expressing lines (every two weeks watered E2Ws) CBP expressing line 1 and 2 (35S:GFP-CBP-1 and 2) showed a significant reduction in seed number compared to well watered CBP expressing plants. When comparing the stressed line types (CBP expressing line vs. control), every two weeks watered (E2Ws) CBP expressing line 1 (35S:GFP-CBP-1 and 35S:GFP-CBP-2) had a significantly lower seed number than

control sibling lines (p=0.03) suggesting that CBP expressing line 1 (35S:GFP-CBP-1) was more sensitive during seed production under drought stress conditions.

In summary, in response to drought plants can exhibit drought escape or drought resistance mechanisms [34]. Plant responses to further resistance are classified into drought avoidance and drought tolerance. Drought escape is the ability of plants to complete their life cycle before severe stress takes place, in other words, plants shorten their life cycle or growing season to allow for earlier reproduction and to avoid the period of stress [34].

However, the plants' ability to improve water uptake under drought stress or reduce water loss is considered drought avoidance [35]. Improved root traits, reducing water loss by closing of stomata (reduced stomatal conductance), or reduced evaporation surface (by reducing leaf area), are the mechanisms that confer drought avoidance [36]. Drought tolerance is the ability of plants to resist water deficit under low tissue water potential [37]. Maintaining cell turgor or reducing evaporative water loss through the accumulation of compatible solutes is the mechanism of drought tolerance [37]. Drought avoidance/tolerance mechanism is schematically shown in **Fig. 3.12**.

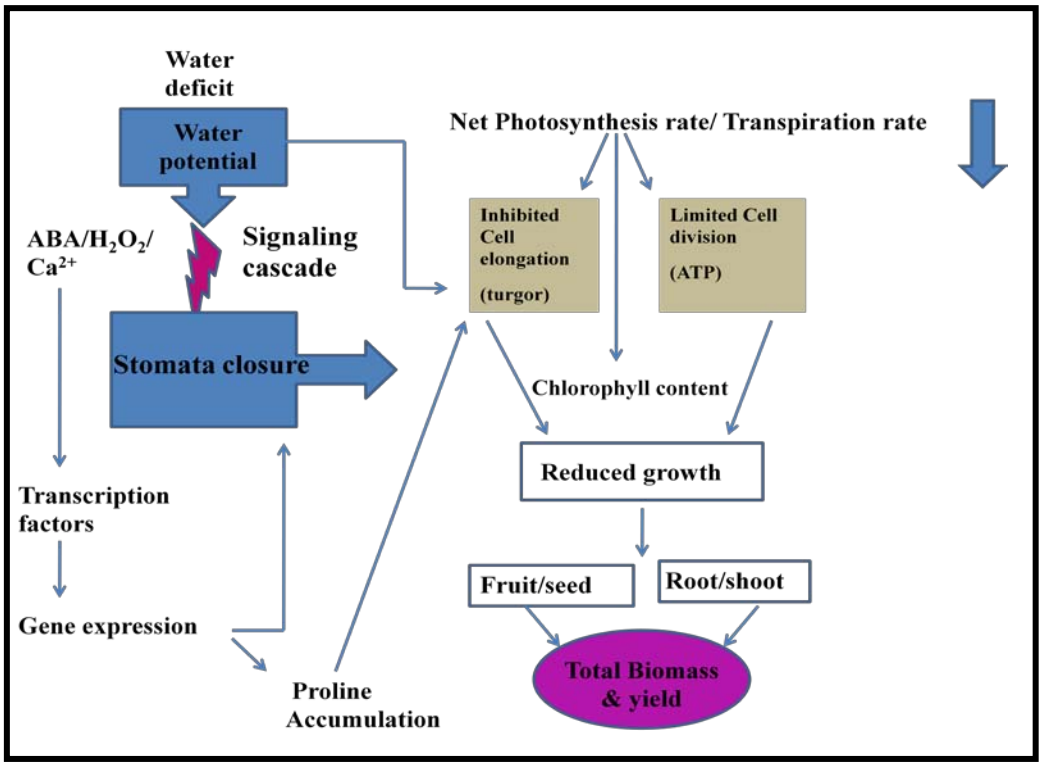


Figure 3.12. Drought avoidance/tolerance mechanism model. Moderate (nonlethal) water stress can be defined as a situation in which reduced water availability leads to the inhibition of plant growth. Water deficit causes reduced water potential in plants' tissues and cells. The stomatal closure and the resulting CO_2 deficit in the chloroplasts is the main cause of decreased photosynthesis under mild and moderate stresses, however, impaired ATP is also a likely explanation for decreased photosynthesis under water stress. The role of ABA, Ca^{2+} and H_2O_2 in signaling pathway in regulation of stomata closure has been well studied. Moreover, reductions of photosynthetic and transpiration rates ultimately cause reduced growth by limiting cell division and inhibiting cell elongation under drought stress. Reduced growth affects biomass and yield by reducing fruit and seed number and weight and dry and fresh weight in plants.

The results obtained in this experiment reveal that CBP expressing tomato lines, expressing the C-domain of maize Calreticulin show no significant difference in the response mechanisms to drought stress compared to the control lines. While drought escape is observed as a response, reduced stomata conductance, photosynthetic and transpiration in

CBP expressing tomato lines also indicate drought avoidance mechanisms. However, the CBP-expressing plants do not seem to show increased drought tolerance, because no increase in their biomass and yield was observed compared to those in control wild type and sibling lines.

In animals, the multi-functional protein Calreticulin has been widely studied pointing to more than 40 cellular functions [37]. However, studies of plant Calreticulin have not fully addressed the particular or specific role of the Calreticulin in response to abiotic stress conditions. We found that constitutive expression of the C-domain of maize Calreticulin in some of the tomato plants leads to some phenotypical and morphological changes in CBP expressing line tomato lines under normal conditions, such as increased fruit production, and seed weight. A number of studies have reported the role of Calreticulin in response to drought [7].

To investigate whether C-domain of maize CRT is specifically involved in plants response to drought stress we evaluated CBP expressing tomato plants under drought stress. CBP expressing tomato lines (35S:GFP-CBP 1, 2 and 4) showed avoidance mechanisims such as reducing their stomatal conductance in order to reduce water loss and exhibited reduced leaf area to reduce evaporation surface under severe drought stress conditions. These responses however were not significantly different when compared to the control lines, indicating that CBP-expression did not cause any increase in avoidance responses. However, it was also shown that water deficit reduced tomato yield and biomass. Although the timing, duration, severity and stage of development undoubtedly had pivotal roles in determining how plant

responded to water deficit, in general, CBP expressing lines did not show significant differences with wild type and sibling control lines.

Calreticulin has also been described as a potential regulator of calcium homeostasis and chaperon activity. However, the molecular mechanism underlying maize CRT role in plant drought resistance still remains unknown. Expression of maize CRT in *Arabidopsis* lines has been shown to lead to increased total calcium content of up to 25%, higher levels of chlorophyll and increased seed yield compared to wild type and 35S:GFP control lines [6]. It was also reported that CBP expressing *Arabidopsis* lines showed increased root growth and better survival under intermittent drought. Further investigation revealed the increased expression of two stress tolerance genes (*DREB1A* and *RD29A*) mediated by the up-regulation of *CIPK*6 (Calcineurin B-like interacting protein kinase) in those CBP-expressing *Arabidopsis* lines [6].

Further studies are needed to test the expression level of drought tolerant stress genes, (*DREB1A* and *RD29A*) to determine whether manipulation of maize calreticulin in CBP expressing tomato plants can lead to increased expression level of drought responsible genes. Moreover, the expression level of CIPK gene family could be analyzed in tomato CBP expressing lines (35S:GFP-CBP) under drought stress conditions. In addition, cytosolic calcium concentration under drought stress conditions should be measured to investigate if Ca^{2+} is increased in the CBP expressing tomato lines and in response to drought stress. A study of tobacco plants expressing the wheat Calreticulin revealed increased tolerance to drought [7] indicating the role of Calreticulin's C-domain (regulation of calcium homeostasis) as well as N- and P-domains (chaperon activity) to tolerating water deficit. In

the tomato plants examined in this study only the C-domain of maize Calreticulin was over-expressed. CBP expressing tomato lines over-expressing various maize CRT domains could be generated for comparison with the C-domain over-expressed lines to assess their role to tolerating stress conditions.

In conclusion, although maize Calreticulin may enable plants to enhance their resistance to drought at some point, results obtained from biomass and yield analysis in this study revealed that in general the difference between the CBP expressing lines and control tomato lines was not significant. Further work is required to facilitate the understanding of Calreticulin in plants against water deficit.

Material and Methods

Growth Condition and Drought Treatment

A randomized block design with three replications was used for this experiment. To realize the drought treatments, plants were subjected to one of the following four irrigation regimes: Control; a well irrigated treatment (no drought stress), drought stress: imposed during the vegetative, flowering and fruiting stage by withholding irrigation for one, two and three weeks. Individual pots were 48 rows. Plant distance within a row was 10 cm. Plants were rotated during the experiment to obtain an even condition for all the individual pots.

Plants were watered with about 100 ml tap water. Tomato plants expressing the C-domain of maize Calreticulin as well as control plants (wild type, GFP and sibling plants) were

propagated in the greenhouse under mist for 2 weeks. Wild type plants were treated with Surflan A.S. (as described in chapter 2) to maintain the increase in ploidy levels as determined by guard cell chloroplast number. After 6 weeks in the mist house, specimen were planted in 4 in^2 (10 cm^2) pots containing the same amount of Fafard 4P (Fafard, Anderson, S.C.) soilless substrate, which includes 0.6 lb/yd^3 N, 0.066 lb/yd^3 P, and 0.332 lb/yd^3 K (0.36, 0.09, and 0.20 $kg \cdot m^{-3}$, respectively) and transferred to the growth chamber (TM, 28 ^oC and RH, 40%). The fertilizer 14-14-14 was added to the plants during transfer to pots. The granular fertilizer sprinkled once at the surface of soil and watered on daily bases. Twelve independent plants from each sample group examined to obtain the physiological parameters. Physiological parameters were measured three times during the course of the experiment (week 1, 4 and 9). Plants (1-1-2, 1-1-3, 1-15-1) from CBP expressing line 35S:GFP-CBP-1; (2-1-3, 2-4-5, 2-2-2) from CBP expressing line 35S:GFP-CBP- 2; and plants (4-4-1, 4-4-5 and 4-4-3) from CBP expressing line 35S:GFP-CBP-4 marked and selected for harvesting leaves.

Yield and Biomass

At the end of the crop cycle, the effects of the drought treatments on seed yield and fruit production were assessed. Fruit and seed weight and number per plant per line were evaluated. CBP expressing and control plants were also harvested for biomass measurements (fresh, dry weight and length). Shoot and roots of CBP expressing and control plants were kept at room temperature for one week to dry and then measured. For biomass analysis, 80 individual pots for control lines, 120 individual pots for CBP expressing lines were analyzed;

for seed weight and number, 80 individual plants from control lines and 120 individual plants from CBP expressing lines were analyzed according to the described techniques, under drought stress conditions.

Stomata Conductance, Leaf Area, Chlorophyll Content and Photosynthesis Rate Measurements

For stomata conductance measurements, two leaves per each plant per each line were analyzed using handheld Delta-T AP4 porometer (Delta-T Devices, UK). Measurements were done between 12:00 and 3:00 h at temperature of 28°C ± 0.5and humidity of 40%.

Leaf Chamber Fluorometer, Li6400-40 (LI-COR Biosciences, Nebraska, USA) was used for measuring photosynthesis and transpiration rate. One leaf per each plant per each line was evaluated for leaf area measurements; leaf from each plant per line was measured using a leaf area meter Li-Cor Model 3100C, (LI-COR Biosciences, Nebraska, USA). Chlorophyll content was measured using Spad meter (Spectrum Technologies, Inc. Plainfield, IL). Two leaves per each plant per each line were evaluated. All the assessments were performed three times during the experimental period, at 14 days (vegetative stage) and 65 days (flowering) and 79 days (fruiting).

Statistical Analysis

Means were compared using Tukey's Studentized Range (HSD) Test at $p = 0.05$. All calculations for stomatal conductance, leaf area and chlorophyll content were performed with the help of the SAS software. For biomass and yield analysis, a Student's *t* test was used to

determine the significance of differences between treatments by using excel software (Microsoft office excel version 7).

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