ROLE OF *ARABIDOPSIS THALIANA* CALMODULIN ISOFORMS

IN TOLERANCE TO ABIOTIC STRESS

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ROLE OF *ARABIDOPSIS THALIANA* CALMODULIN ISOFORMS IN TOLERANCE TO ABIOTIC STRESS

Nisreen AL-Quraan

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VITA

Nisreen AL-Quraan, daughter of Mr. Adnan, AL-Quraan and Mrs. Fayzeh, AL-Quraan was born on June 3, 1976 in Irbid, Jordan. She graduated from AL-Taybeh High School in Irbid, Jordan in 1994. She graduated in 1998 with Bachelor of Science degree from the Department of Biological Sciences, Yarmouk University, Jordan. She joined the graduate program in the Department of Biological sciences, Yarmouk University and received her Master of Science degree in Plant Biochemistry and Molecular biology in 2001. After completion of her MS, she worked as research and teaching assistant for two years in the Department of Biological Sciences, Yarmouk University, Jordan. She was married to Dr. Abdelsalam Talafha, Professor in Large Animal Medicine (Theriogenology) in 2002 and came to the University of Georgia, Athens with her husband. On May, 2004 she joined the Department of Biological Sciences, Auburn University to pursue her PhD degree in Plant Biochemistry and Molecular Biology. She and her husband are gifted with their first child, Morad born in February, 2005.

DISSERTATION ABSTRACT

ROLE OF *ARABIDOPSIS THALIANA* CALMODULIN ISOFORMS

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Calmodulin and Ca^{2+} have been implicated in adaptation of plants to changes in its environment. *Arabidopsis thaliana* (Arabidopsis) genome has nine genetic loci encoding calmodulin protein isoforms. We have used wild type (WT) and T-DNA insertion mutants of seven calmodulin genes in Arabidopsis to determine role of specific isoform of calmodulin in abiotic stress in Arabidopsis.

To understand the role of specific *CAM* genes in heat stress, the steady-state level of mRNA for the nine *CAM* genes in root and shoot tissues of WT seedlings grown at normal growth temperature (25^{o}C) and during heat stress at 42^{o}C for 2 hr was determined using gene specific primers. Normalized steady-state level of all *CAM* RNA in WT and T-DNA insertional mutant lines (*cam*) at normal growth temperature (25^{o}C) and after heat treatment at 42^{o}C was determined. Compared to growth at 25°C, the mRNA levels

of all *CAM* genes were upregulated in both root and shoot after heat treatment with the notable exception of *CAM5* in root and shoot, and *CAM1* in shoot where the mRNA levels were reduced. At 25^{o}C all *cam* mutants showed varying levels of mRNA for corresponding *CAM* genes with the highest levels of *CAM5* mRNA being found in *cam5-1* and *cam5-3*. *CAM5* mRNA was not observed in the *cam5-4* allele which harbors a T-DNA insertion in exon II. Compared to wild type, the level of mRNA for all *CAM* genes varied in each *cam* mutant, but not in a systematic way. During heat stress at 42^{o}C the level of *CAM* gene mRNAs was also variable between insertional mutants, but the level of *CAM1* and *CAM5* mRNA was consistently greater in response to heat stress in both root and shoot. These results suggest differential tissue specific expression of *CAM* genes in root and shoot tissues, and specific regulation of *CAM* mRNA levels by heat. Each of the *CAM* genes appears to contain noncoding regions that play regulatory roles in the interaction between *CAM* genes leading to changes in specific *CAM* gene mRNA levels in Arabidopsis. With respect to expression of specific *CAM* gene only exonic mutation lead to a loss of function of *CAM* genes.

All *cam* mutants were screened for phenotypic alterations in seed germination, survival and treatment induced oxidative damage under a variety of environmental stress. Screening of *cam* mutants demonstrated that *cam5-4* and *cam6-1* accumulate significantly higher level of melonaldehyde and are sensitive to all abiotic factors tested including heat and low temperature exposures, osmotic and salinity stress. Surprisingly, other *cam5* alleles with T-DNA insertions in either 5' UTR or 3'UTR did not show any significant difference from WT in germination, survival and oxidative damage in response to various stresses. This difference in phenotype between *cam5-4* and other

cam5 alleles suggests important role for CaM5 protein in tolerance to abiotic stress in *cam5-4*. T-DNA insertion in *cam6-1* is at the 3'UTR of *CAM6* gene with significant residual level of *CAM6* mRNA. It seems likely that sensitivity of *cam6-1* to all environmental stress tested may be mediated indirectly through a different mechanism.

An initial elevation in the level of GABA shunt metabolites followed by a significant and substantial increase in its level after prolonged exposure to same stress or exposure to increasing level of stress was observed in WT and most *cam* mutants. Root tissues accumulated higher level of metabolites than the shoot tissues in WT and *cam* mutants after exposure to stress, except for GABA level in shoot tissues at higher level of salinity stress. Level of GABA and glutamate changed more significantly than alanine under stress. Different *ca*m mutants showed significant variations in levels of GABA shunt metabolites. These results suggest that collective amounts of CaM proteins may regulate GAD activity in response to abiotic stress rather thanspecific CaM protein under different stress conditions. Steady-state level of *CAM* mRNA in WT and *cam* mutants provides support for some level of compensation in expression *CAM* genes in *Arabidopsis thaliana*.

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I. LITERATURE REVIEW

Introduction

Environmental fluctuations in temperature and water availability limit crop productivity (Bray et al., 2000). Estimated losses of productivity due to biotic and abiotic stresses range from 65% to 87% worldwide (Bray et al., 2000). Organisms respond to the environment by adaptive mechanisms in growth and development involving a complex network for perception and transmission of stress signals to initiate cellular responses by changes in enzyme activity, gene expression, and transport of molecules across membranes (Boyer, 1982; Mittler, 2006; Shelp et al., 2006).

Stress tolerance in plants involves a variety of genetic and molecular adaptive mechanisms (Bohnert et al., 1995) such as osmoregulation and the accumulation of osmoprotectants (McNeil et al., 1999), calcium signaling (Hetherington and Brownlee, 2004), and the accumulation of γ-aminobutyric acid (GABA) (Steward, 1949; Breitkreuz and Shelp, 1995; Mayer et al., 1990; Locy et al., 2000).

The pathway that converts glutamate to succinate via GABA is called the GABA shunt. The GABA shunt pathway in plants is composed of a cytosolic enzyme glutamate decarboxylase (GAD), and mitochondrial enzymes GABA transaminase (GABA-TA) and succinate semialdehyde dehydrogenase (SSADH) (Shelp et al., 1999; Bouché and Fromm, 2004). Rapid accumulation of GABA in response to unfavorable or extreme environments sparked interest in the elucidation of the GABA shunt pathway and its association with mechanisms including pH regulation, TCA cycle fluxes, nitrogen

metabolism, protection against oxidative stress, osmoregulation, and stress signaling (Shelp et al., 1999; Kinnersley and Turano, 2000).

Heat stress and thermotolerance

Plants perceive high temperature in different ways. Adaptation or acclimation to heat occurs differentially over time and across tissue organization. Heat affects a variety of cellular structures and functions (Taiz and Zeiger, 2002). The classical response to heat stress is the production of heat shock proteins (HSPs), which function as molecular chaperons to maintain proper protein folding. Binding of trimeric heat shock factor (HSF) to a heat shock DNA sequence element (HSE) is essential for the transcription of HSP mRNA. As a response to heat stress the synthesis of HSPs enable cells to cope with high levels of cellular protein dentauration during heat stress (Prandl et al., 2001).

Membranes and proteins damaged by heat may produce reactive oxygen species (ROS) during photosynthesis possibly causing acceleration of senescence (Larkindale et al., 2005). The oxygen evolving complex, reaction center, and light harvesting complexes can be disrupted by elevated temperature. The low molecular weight HSPs in chloroplast are involved in PS II thermotolerance (Heckathorn et al., 1998). Phytohormones, such as abscisic acid (ABA), salicylic acid (SA), and ethylene have been linked to heat stress signaling (Cherry and Nielsen, 2004). The role of ABA and SA in the induction of thermotolerance and protection against oxidative damage during heat stress either by prevention or rapid repair of oxidative damage has been demonstrated (Larkindale and Knight, 2002; Larkindale and Huang, 2004; Larkindale et al., 2005).

Ethylene as a potential signaling molecule in tolerance to heat stress and prevention of heat induced oxidative damage without its involvement in HSP induction

during heat stress has been demonstrated (Larkindale and Huang, 2004; Suzuki and Mittler, 2006; Kotak et al., 2007).

The role of calcium signaling in heat stress has been debated for many years. In maize calcium (Ca^{2+}) and calmodulin (CaM) induce HSP70 binding to heat shock elements *in vitro*, suggesting a role for Ca^{2+}/CaM in HSP gene expression through the regulation of HSFs (Sun et al., 2000; Li et al., 2004). Liu et al. (2003) suggested that regulation of DNA-binding activity of HSF is mediated by direct binding of CaM to cytoplasmic HSP70 or by regulation of HSF phosphorylation involving a CaM-dependent kinase activity. Yang and Poovaiah (2000) also demonstrated that the chloroplast chaperonin 10 in Arabidopsis is a Ca^{2+}/CaM-binding protein and suggested that the calcium signaling system can regulate protein folding in chloroplast. Exposure of Arabidopsis to heat results in increased cytoplasmic Ca^{2+} that activates glutamate decarboxylase (GAD) via activation Ca^{2+}/CaM and GABA accumulation in roots, and this effect is reversed by CaM and Ca^{2+} inhibitors (Locy at al., 2000). Calcium channel blocker and CaM inhibitors induce oxidative damage to membranes and enhance reactive oxygen substance (ROS) production while pre-treatment with H_2O_2 can enhance thermotolerance (Larkindale et al., 2005; Larkindale and Knight, 2002; Suzuki and Mittler, 2006).

Low temperature and cold acclimation

Plants exhibit extensive changes in lipid composition, soluble protein content, and simple sugars type and content in response to low temperature. Genetic analysis in plants suggests the involvement of many genes in cold tolerance (Thomashow et al., 1999). Plants can sense cold stress through low temperature induced changes in membrane

fluidity, conformational changes in macromolecules, and metabolite concentration. Pharmacological analyses have shown that plasma membrane rigidification induces *COR* (cold responsive) genes and results in cold acclimation in alfalfa and *Brassica napus* (Orvar et al., 2000; Sangwan et al., 2001). CBF (C-repeat binding factors) regulates the expression of genes involved in phosphoinositide metabolism, transcription of many *Cor* genes, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism, signaling, and a number of additional protective functions. CBFs, also know as dehydration-responsive element-binding proteins (DREBs), signaling is conserved in plants, but varies during cold tolerance versus freezing tolerance (Fowler and Thomashow, 2002; Lee et al., 2005; Maruyama et al., 2004). Central young leaves of Arabidopsis rosettes exhibit a faster and greater freezing tolerance than peripheral mature leaves during cold acclimation (Takagi et al., 2003). Sugar accumulation contributes to this differential freezing tolerance during the early days of cold treatment, but the degree of freezing tolerance in different leaves was not associated with the CBF/DREB1 family proteins or with the COR78/RD29A protein (Kawamura and Uemura, 2003). Proteins induced in the central leaves are associated with membrane repair, protection of the membrane against osmotic stress, enhancement of carbon dioxide fixation, and proteolysis (Kawamura and Uemura, 2003)

Cold-regulated genes in Arabidopsis constitute between 4% and 20 % of the genome (Lee et al., 2005). Expression of CBFs and APETALA2/ETHYLENE RESPONSE FACTOR family transcription factors is induced by cold exposure. The above transcription factors can bind to cis-elements in the promoters of *COR* genes to increase expression. Transgenic plants with ectopic expression of CBF induce expression

of *COR* genes and cold acclimation even at normal growth temperatures (Stockinger et al., 1997; Liu et al., 1998; Chinnusamy et al., 2007).

An increase in the level of ABA during cold exposure has been demonstrated (reference), and pretreatment of plants with ABA can cause the freezing tolerance of plants to increase suggesting a role for ABA in low temperature stress (Gilmour et al., 1991). CIPK3 is a Serine/Therionine kinase associated with calcineurin B-like calcium sensor and regulates ABA responses during seed germination and gene expression in Arabidopsis (Kim et al., 2003). The expression of the *CIPK*3 gene is responsive to ABA and cold (Wasilewsk et al., 2008). These findings suggest a link between low temperature exposure, ABA, and gene expression during stress cold stresses.

The activity and transcription of genes encoding ROS-scavenging enzymes are increased by cold (O`Kane et al., 1996). A number of cold responsive genes such as *RD*29A, *KIN*2, *COR*15A, *COR*47, *DREB*1A, *DREB*2A, and *ERD*10 have been characterized in Arabidopsis (Thomashow, 1999). Some of the *COR* gene products may be involved in ROS scavenging (Lee et al., 2002). The Arabidopsis frostbite1 (*fro*1) mutant displayed reduced expression of cold-responsive genes such as *RD*29A, *KIN*1, *COR*15A, and *COR*47, and constitutively accumulated ROS. The *FOR*1 gene encodes a mitochondrial complex I protein, suggesting that expression of the cold-responsive genes and ROS accumulation might be modulated by disruption of a mitochondrial function (Suzuki and Mittler, 2006). Such studies demonstrate a close link between ROS, ROS signaling, and the cold stress response.

Cytosolic calcium in Arabidopsis seedlings increases significantly at the start of cooling (Larkindale and Knight, 2002). This increase is likely mediated through

activation of calcium channels and phospholipid signaling (Vergnolle et al., 2005; Williams et al., 2005; Chinnusamy et al., 2006; Komatsu et al., 2007). Involvement of CaM in cold induction of *COR* genes and the induction of CaM by low temperature has been shown (Braam and Davis, 1990). Over-expression of *CAM3* in Arabidopsis resulted in decreased expression of cold-induced *COR* genes suggesting a negative regulatory role for CaM in regulation of *COR* expression in plants (Townely and Knight, 2002). Since GAD is a Ca^{2+}/CaM-regulated enzyme (Snedden et al., 1996), Ca^{2+} influx following a temperature drop (Monroy and Dhindsa, 1995) could be responsible for the activation of the GAD enzyme during cold stress.

Drought and non-ionic osmotic stress

Drought induces a number of metabolic pathways in various plants. Changes in the metabolism and accumulation of sugars (such as raffinose, sucrose, and trehalose), sugar alcohols (such as mannitol, and sorbitol), amino acids (such as proline, glutamate, alanine, and GABA), and amines (such as glycine betaine and polyamines) are well documented durning osmotic stresses (Taji et al, 2002; Bartels and Sunkar, 2005). Many of these metabolites are believed to function as compatible osmolytes and antioxidants by protecting cellular functions or maintaining structural components of cells (Umezawa et al., 2006, Seki et al., 2007).

Drought-related water deficit triggers the production of Abscisic acid (ABA), which causes stomatal closure and enhances the expression of a number of specific osmotic ssstress-related pdofsins such as ABA-responsive element binding protein1 (AREB1), dehydration-responsive element binding protein 2A (DREB2A), Receptor-like kinase1(RPK1), SNF1-related protein kinase2C (SRK2C), and guard cell-expressed

calcium-dependent protein kinases 3 and 16 CPK3 and CPK6 (Umezawa et al., 2004; Fujita et al., 2005; Osakabe et al., 2005; Furihata et al., 2006; Mori et al., 2006). Calcineurin B-like (CBL) proteins are calcium sensors that are induced by a number of stresses (Luan et al., 2002). Gene disruption of *CBL*1 in Arabidopsis suggests that CBL1 may be a positive regulator of osmotic stress and a negative regulator of cold responses in plants (Cheong et al., 2003).

The cellular responses to osmotic stress involve water channels (aquaporin), electrolyte transport, the accumulation of osmolytes (Booth and Lewis, 1999), and the protection of proteins and sub-cellular structures (Nuccio et al., 1999; Mager et al., 2000). Upon exposure to osmotic stress, plants exhibit a wide range of response at the molecular, cellular, and whole plant levels (Hasegawa et al., 2000). These include, morphological and developmental changes (such as life cycle, inhibition of shoot growth, and enhancement of root growth), adjustment in ion transport (such as uptake, extrusion, and sequestration of ions), and metabolic changes (such as carbon metabolism, the synthesis of compatible solutes). Some of these responses are triggered by the primary osmotic signals, whereas others may result from secondary stresses/signals caused by the primary signals. These secondary signals can be phytohormones (ABA and ethylene), ROS, and intracellular second messengers (phospholipids). Some of these secondary signals may not be confined to the primary stress sites such as the root, and their ability to move to other parts of the plant contributes to the co-ordination of whole plant responses to stress conditions (Xiong and Zhu, 2002).

To cope with osmotic stress plants accumulate compatible osmolytes to function in osmotic adjustment by lowering the cellular osmotic potential to facilitate water

absorption. Compatible osmolytes are synthesized by virtually all organisms under hyperosmotic stress (Yancey et al., 1982). Common osmolytes include sugars, polyols, quaternary ammonium compounds, and amino acids or their derivatives. These compounds appear to have little interference in cellular function, even at high concentrations (Nuccio et al., 1999; Zhu, 2002).

Many plant species accumulate glycine betaine (GB), which is a quaternary ammonium compound that is abundant mainly in the chloroplast. GB plays a vital role in adjustment and protection of thylakoid membranes and maintains photosynthetic efficiency (Bray et al., 2000). GB is known to accumulate in response to stress in various plant species (Yang et al., 2003), while some plants such as rice (*Oryza sativa*), tobacco (*Nicotiana tabaccum*), and Arabidopsis (*Arabidoposis thaliana*) naturally do not produce GB under stress or non-stress conditions. In these species, transgenic plants overexpressing GB-synthesizing genes exhibited increases in the production of GB and an enhancement in tolerance to salt, osmotic, cold, or high temperature stress (Rhodes and Hansen, 1993; Ashraf and Foolad, 2007). In cultured tobacco cells accumulation of sucrose, GABA, alanine, glutamate, proline, malate, citrate, GB, K^+, NO_3^-, Na^+, and Cl^- increased with decreased external water potential (Handa et al., 1983).

Water deficit in barley roots led to electrolyte leakage from the leaf due to dehydration-induced membrane injury (Kocheva et al., 2004). In yeast and mammalian systems, protein phosphorylation is central to osmotic stress signaling. In *Saccharomyces cerevisiae*, the osmoregulatory pathways begin with either a Src-homology3 (SH3)-domain-containing membrane protein or a two-component histidine

kinase, which activates a mitogen-activated protein kinase (MAPK) cascade and leads to increased osmolyte synthesis and accumulation (Gustin et al., 1998; Zhu, 2001).

Plants also have several MAPKs that are activated by hyperosmotic stress. In alfalfa cells, a 46-kDa MAPK, named Stress-Inducible MAP Kinase (SIMK), is activated by another kinase called SIMKK upon hyperosmotic stress (Munnik et al., 1999, Kiegerl et al., 2000; Cardinale et al., 2002). In tobacco cells, a similar MAPK named SIPK (Salicylic-acid-induced protein kinase) is activated by hyperosmotic stress (Mikolajczyk et al., 2000). This tobacco MAPK is also activated by hypoosmotic stress, salicylic acid, or fungal elicitors (Droillard et al., 2000). Two Arabidopsis kinases, *At*MPK4 and *At*MPK6, are activated by cold, drought, salt, and osmotic stress suggesting a role for these two kinases in osmotic stress (Ichimura et al., 2000). Involvement of a MAPK cascade has been demonstrated in a protoplast transient assay system in response to cold and salt stress (Teige et al. 2004).

A MAPKs signaling cascade is also activated in response to biotic stresses such as fungal elicitors. This provides evidence for the key position of the MAPKs at the crossroads of signaling pathways (Boudsocq and Lauriere, 2005). Transgenic tobacco plants overexpressing the *Nt*C7 receptor-like kinase showed greater tolerance to osmotic stress (Yoshiba et al., 1997). The function of *Nt*C7 was attributed to the regulation of compatible solutes (Yoshiba et al., 1997; Tamura et al., 2003). An Arabidopsis *srf*6 mutant is impaired in the accumulation of cold proteins and fails to acclimate during freezing and drought, suggesting that SRF6 is involved in both osmotic and cold stress signaling (Boyce et al., 2003).

CaM and CaM-binding proteins (CaMBPs) interact with target proteins implicated in osmotic stress responses. Such interactions between CaM/CaMBPs proteins have positive or negative effects on stress tolerance (Snedden and Fromm, 2001; Yang and Poovaiah, 2003). Perruc et al. (2004) identified a stress-responsive CaM-binding protein, *At*CaMBP25, in Arabidopsis that is a nuclear localized, regulatory protein involved in osmotic stress signaling. Reduction in the expression of *At*CaMBP25 protein resulted in enhanced tolerance to salt and osmotic stress, suggesting its role as a negative regulator of salt and osmotic stress tolerance with a less important role in cold tolerance (Perruc et al., 2004).

Salinity and ion toxicity

Nearly three quarters of earth is covered by salt water. Hence, it is not surprising that salt in soil affects a significant proportion of the world's land surface. These salt-affected areas fall broadly into sodic and saline categories. Sodic soils are dominated by excess sodium on exchange sites, a high concentration of carbonate/bicarbonate anions with pH greater than 8.5 and up to 10.8, high sodium absorption ratio, and poor soil structure. Saline soils are generally dominated by sodium ions with chloride and sulphate anions, and a lower pH and sodium absorption ratio, and higher electrical conductivity. Salt-affected soils contain sufficient concentrations of soluble salts to reduce the growth of most plant species. Salt-tolerant plants have evolved to grow on these soils with a wide range of adaptations. However, most agronomically important crops are salt-sensitive (Flowers and Flowers, 2005).

Salinity effects in plants are caused by ionic and osmotic stress. Ionic stress is caused when the amount of free ions disrupt cellular function. The plant deals with

salinity and associated ion toxicity by various detoxification processes reestablishing normal cellular functions (Denby and Gehring, 2005). Salt-tolerant plants differ from salt-sensitive ones by having a low rate of Na^+ and Cl^- transport to leaves. The ability to compartmentalize these ions into vacuoles is critical to preventing their build-up in the cytoplasm, and thus avoiding salt toxicity (Munns, 2002).

A secondary aspect of salt stress is the production of ROS, including superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). ROS may be products of chloroplast and mitochondrial metabolism during stress, and can also be produced by other processes. ROS cause oxidative damage to cellular components including membrane lipids, proteins, and nucleic acids (Haliwell and Gutteridge, 1986). Plants use low molecular mass antioxidants such as ascorbic acid and reduced glutathione and employ a diverse array of enzymes such as superoxide dimutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione S-transferases (GST), and glutathione peroxidases (GPX) to scavenge ROS. Transgenic tobacco plants overexpressing both GST and GPX displayed improved seed germination and seedling growth under stress (Roxas et al., 1997; Apse and Blumwald, 2002).

Salinity leads to ABA accumulation (Xiong and Zhu, 2003). Turgor reduction induced by the hyperosmotic component of salt stress is responsible for the increase in endogenous ABA production, particularly in root (Jia et al., 2002). Accumulation of ABA is mediated by induction of ABA biosynthesis genes and suppression of ABA degradation (Xiong and Zhu, 2003). Changes in the level of ABA regulate stomatal closure and water loss and regulate water balance by inducing genes involved in osmolytes and compatible solute biosynthesis (Hasegawa et al., 2000; Zhu, 2002).

Plant cells accumulate compatible solutes in response to salinity such as essential elemental ions like K^+, simple sugars (mainly fructose and glucose), complex sugars (trehalose, raffinose, and fructans), quaternary aminonium derivatives (glycine betaine, β-alanine betaine, proline betaine), and amino acids (proline, GABA, alanine) (Bohnert and Jensens, 1996; Zhu, 2001). The synthesis of these compatible osmolytes is often achieved by diversion of basic intermediary metabolites into unique biochemical reactions without disturbing intracellular metabolic pathways. These compatible solutes have the capacity to preserve the activity of enzymes with minimal effect on pH or charge balance of the cytosol or lumenal compartments of cellular organelles (Binzel et al., 1987; Bohnert and Jensens, 1996).

Three genetically linked Arabidopsis loci (*SOS*1, *SOS*2 and *SOS*3) are components of a stress-signaling pathway, also called Salt-Overly-Sensitive signal transduction pathway that controls ion homeostasis and salt tolerance (Hasegawa et al., 2000a; Sanders, 2000; Zhu, 2000; Zhu, 2001). Genetic analysis of Na^+/Li^+ sensitivity established that *SOS*1 is epistatic to *SOS*2 and *SOS*3 (Zhu, 2000; Zhu, 2002). These sos mutants also exhibit a K^+ deficient phenotype in media supplemented with 50 μM K^+ and Ca^{2+}. K^+, and the K^+ deficiency of sos2 was suppressed with 100 mM Ca^{2+} (Zhu et al., 1998). The sos1 mutant exhibits hyperosmotic sensitivity unlike sos2 and sos3. Together these results indicate that the SOS signaling pathway regulates Na^+ and K^+ homeostasis, and it is Ca^{2+} activated. SOS3 encodes a Ca^{2+} binding protein with sequence similarity to the regulatory B subunit of calcineurin (protein phosphatase2B) and neuronal Ca^{2+} sensors (Liu and Zhu, 1998). Interaction of SOS3 with the SOS2 kinase (Liu et al., 2000) and SOS2 activation is Ca^{2+} dependent (Halfter et al., 2000).

ACA4 is a novel member of the subfamily of N-terminal CaM-regulated Ca^{2+}-ATPases, and it is localized in the membrane of Arabidopsis vacuoles. ACA4 with vacuolar H^+/Ca^{2+} antiporter CAX1 might adjust cytosolic calcium concentrations by filling vacuolar compartments where calcium is stored (Hirschi et al., 1996; Muir and Sanders, 1997). Overexpression of ACA4 in yeast increased salt tolerance indirectly via the reestablishment of intracellular calcium level (Geisler et al., 2000). The *in planta* function of SOS3 as a salt tolerance determinant is dependent on Ca^{2+} binding and N-myristoylation (Ishitani et al., 2000). The SOS2 protein is a Serine/Therionine kinase (446 amino acids) and has a 267 amino acid N-terminal catalytic domain that is similar in sequence to yeast SNF1 (sucrose non-fermenting) kinase and the mammalian AMPK (AMP-activated protein kinase) (Liu et al., 2000; Zhu, 2000; Zhu, 2002). The kinase activity of SOS2 is essential for its tolerance determinant function.

The C-terminal regulatory domain of SOS2 interacts with the kinase domain to cause auto-inhibition. A 21 amino acids motif in the regulatory domain of SOS2 is the site where SOS3 interacts with the kinase and is the auto-inhibitory domain of the kinase (Guo et al., 2001). Binding of SOS3 to the 21 amino acid motif blocks the SOS2 auto-inhibitory kinase activity. Deletion of the auto-inhibitory domain results in constitutive SOS2 activation, independent of SOS3, and also a threionine (168) to aspartate mutation in the activation loop of the kinase domain constitutively activates SOS2. The plasma membrane localized Na^+/H^+ antiporter SOS1 is controlled by the SOS pathway at the transcriptional and post-transcriptional level (Guo et al., 2001; Zhu, 2002).

The compartementation of Na^+ into vacuoles provides an efficient mechanism to minimize the toxic effect of Na^+ in the cytosol. The transport of Na^+ into vacuoles is

mediated by an Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-pyrophosphatase (H^+-PPase) (Blumwald, 1987). The overexpression of AtHK1, a vacuolar Na^+/H^+ antiporter from Arabidopsis, in Arabidopsis resulted in transgenic plants that were able to grow in high salt concentration (Aspe et al., 1999). Overexpression of Arabidopsis plasma membrane Na^+/H^+ antiporter *SOS*1 improves plant tolerance to salt by limiting Na^+ accumulation (Shi et al., 2003). Disruption of *At*HK1 suppresses the salt sensitive phenotype of sos3-1, indicating that SOS pathway negatively controls this Na^+ influx system in plant cells (Rus et al., 2001). Such studies indicated that the Ca^{+2} and SOS signaling pathways are essential in regulation of ion homeostasis in salt tolerance (Yokoi et al., 2002; Zhu, 2002).

Ultraviolet (UV) irradiation

Heat-induced cellular damage was enhanced by growth in thelight compared to that in the dark (Jeong et al., 2002; Larkindale and Knight, 2002). High light results in increased ROS production potentially damaging to the photosynthetic apparatus (Niyogi, 1999). UV-A, UV-B, and blue light are important factors in perception of the light signal and in the regulation of genes in flavonoid biosynthesis, such as chalcone synthase (CHS), This may involve Ca^{2+} signaling (Preisig and Moreau, 1994). The UV-A and UV-B/ blue light signaling pathways are distinct and involve reversible protein phosphorylation and *de novo* protein synthesis (Christie and Jenkins, 1996).

An Arabidopsis succinate semialdehyde dehydrogenase mutant (*ssadh*) showed a rapid increase in hydrogen peroxide production when subjected to UV irradiation leading to necrosis (Bouché` et al., 2003). Mutants deficient in ascorbate, zeaxanthinin or

glutathione had dramatically reduced survival under high light and heat (Larkindale et al., 2005). Such results suggest that ROS-scavenging enzymes play an important role in preventing photo-oxidative damage under a combination of temperature and high-light stress (Suzuki and Mittler, 2006).

ROS and oxidative stress

ROS (Reactive Oxygen Species) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and nitric oxide (NO•) are critical parts of many physiological processes, and usually exist in the cell in a balance with biochemical antioxidants. Increases in ROS resulting from exposure to environmental oxidants, toxicants, radiation, or stresses disturb cellular oxidation-reduction (redox) balance to a more oxidized state affecting biological functions. This condition is referred to as "oxidative stress" and may be detrimental to the organism and lead to cell death (Scandalios, 2001). There are many potential sources of ROS in plants. ROS are unavoidable by-products of aerobic metabolism (Dat et al., 2000; Mittler, 2002). Other sources of ROS are glycolate oxidase in peroxisomes during photorespiration, NADPH oxidases, amine oxidases, and cell-wall-bound peroxidases. These are highly regulated processes and generate ROS leading to programmed cell death (PCD) (Hammond-Kosack and Jones, 1996; Dat et al., 2000; Grant and Loake, 2000).

Primary sources of ROS production are the Mehler reaction in antenna pigments of chloroplast (Asada and Takahashi, 1987). Production of ROS is enhanced by conditions limiting CO_2 fixation, such as drought, salt, temperature stress, and a combination of these conditions with high light. Limiting CO_2 in C_3 plants activates the photorespiratory pathway resulting in generation of H_2O_2 in peroxisomes by glycolate

oxidase (Mittler, 2002). Over-reduction of the electron transport chain is the main source of $O_2^{\bullet -}$ production under specific stress conditions in mitochondria (Moller, 2001). Additional sources of ROS in plant cells include the detoxifying reactions catalyzed by cytochromes in both the cytoplasm and the endoplasmic reticulum (Urban et al., 1997).

Major ROS-scavenging enzymes of plants include superoxide dimutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PrxR). Together with the antioxidants ascorbic acid and glutathione (Noctor and Foyer, 1998), these enzymes provide cells with highly efficient machinery for detoxifying $O_2^{\bullet -}$ and H_2O_2. The balance between SODs and the different H_2O_2-sacvenging enzymes in cells is considered to be crucial in determining the steady-state level of $O_2^{\bullet -}$ and H_2O_2 (Apel and Hirt, 2004; Mittler et al., 2004). Compatible solutes such as sorbitol, myo-inositol, and proline are effective scavengers of hydroxyl radicals and could protect cells against internally generated hydroxyl radicals (Smirnoff and Cumbes, 1989). Recent studies in Arabidopsis have uncovered some of the key components involved in the ROS signal transduction pathway in plants. It is suggested that plant cells sense ROS via at least three different mechanisms: (i) unidentified receptors proteins; (ii) oxidation-reduction-sensitive transcription factors; and (iii) direct inhibition of phosphatases by ROS (Mittler, 2002; Neill et al., 2002; Vranova et al., 2002; Apel and Hirt, 2004).

H_2O_2 activates several MAPks in Arabidopsis including MPK3, and MPK6 via a MAPK cascade (Kovtun et al., 2000). H_2O_2 also increases expression of the Arabidopsis nucleotide diphosphate (NDP) Kinase2 (Moon et al., 2003). Overexpression of *At*NDPK2 reduced the levels of H_2O_2 and enhanced tolerance to multiple stresses

including cold, salt, and oxidative stress. These studies suggest ROS-induced activation of MAPK appears to be central in mediating cellular responses to multiple stresses (Mitller, 2002; Mittler et al., 2004, Suzuki and Mittler, 2006).

Downstream signaling events associated with ROS sensing involve Ca^{2+} and Ca^{2+}-binding proteins (Kinght and Knight, 2001), the activation of G-protein (Baxter-Burrell et al., 2002), and the activation of phospholipids signaling (Anthony et al., 2004; Rentel et al., 2004). It is possible that the localization of ROS signals in specific cellular sites is similar to that of Ca^{2+} signals in response to external stimuli (Coelho et al., 2002; Mittler et al., 2004). H_2O_2 treatments induce elevation in Ca^{+2} (Rentel and Knight, 2004; Hu et al., 2007) and the expression of genes encoding calmodulin in plants (Desikan et al., 2001). Calmodulin stimulates stomatal closure through activation of heterotrimeric G protein and subsequent production of H_2O_2 and intracellular Ca^{+2} elevations in Arabidopsis (Chen et al., 2004). Yang and Poovaiah (2002) showed that catalase regulates H_2O_2 level in Arabidopsis stimulated by Ca^{+2}/CaM to maintain H_2O_2 homeostasis. Kwon et al. (2007) have suggested that radish catalase is involved in defense against stress induced ROS. These studies indicate that CaM is involved in ROS signaling in plants.

Involvement of Ca^{2+} in H_2O_2 signaling is shown by the expression of Glutathione-S-Transferase (*GST*1) in Arabidopsis. The magnitude of Ca^{2+} response is correlated with the level of *GST*1 expression (Rentel and Knight, 2004). ABA induced an increase in the concentration of cytosolic Ca^{2+} and expression of *CAM*1 and antioxidant genes such as superoxide dismutase4 (*SOD*4), cytosolic ascorbate peroxidase (*cAP*X), and glutathione

reductase (*GR*1). Hu et al. (2007) suggest that Ca^{2+}-CaM is involved in ABA-induced antioxidant defense and ABA signaling in response to H_2O_2 and ROS productions.

Arabidopsis mutants deficient in SSADH enzyme showed inhibition of gamma-hydroxybutyric acid (GHB), ROS accumulations, and improvement in growth (Fait et al. 2005). This result is in agreement with Bouché et al. (2003) showed that high level of ROS is a major cause of *ssadh* aberrant phenotype. Oxidation of polyunsaturated fatty acids, lipid peroxidation, and production of ROS has been used as an index for oxidative damage in plant tissues (Hodges et al., 1999, Rael et al., 2004).

Biotic stress

The resistance of plants to invading pathogens is accomplished by the deployment of a complex array of defense responses. These responses include rapid death of challenged cells leading to the formation of local lesions (hypersensitive response, HR), and non-specific immunity to subsequent infection by a variety of pathogens known as systemic acquired resistance (SAR). Typical rapid response to pathogen attack include ion fluxes, production of ROS, cell wall fortification, synthesis of defense compounds, and changes in gene expression (Dangel and Jones, 2001).

Ca^{2+} influx into the cytosol is among the earliest of these responses and likely serves as a messenger to regulate specific downstream defense pathways. CaM and CaM-like proteins (CaML) are components of plant defense signal transduction. *SCaM*4 and *SCaM*5 in soybean are rapidly induced by elicitor, and this induction is enhanced by using Ca^{2+} ionophores and prevented by Ca^{2+} chelators (Heo et al., 1999; Park et al., 2004). In tomato CaM transcripts increased in response to wounding or the wound signal molecule system and were constitutively high in transgenic plant overexpressing system

(Bergey and Ryan, 1999). Expression profile of Arabidopsis identified CaM-like protein (CaML9) as one of the earliest genes induced after bacterial infection (de-Torres et al., 2003).

Modulation of Calcium (Ca^{+2}) effect via Ca^{+2}-binding proteins

Calcium (Ca^{2+}) is a universal second messenger and acts as a mediator of stimulus responses coupling regulation of diverse cellular functions (Trewavas and Malho, 1998; Knight, 2000; Allen and Schroeder, 2001). Cytosolic Ca^{2+} in plants is rapidly elevated via an increased Ca^{2+} influx in response to stimuli (Reddy, 2001). Transient Ca^{2+} elevations are sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins, which contain so called EF-hand motif(s), a helix-loop-helix structure (Snedden and Fromm, 2001; Yang and Poovaiah, 2003). In Arabidopsis, it is estimated that there are around 250 putative EF-hand proteins (Day et al., 2002). CaM is one of the most conserved Ca^{2+}-binding proteins in eukaryotes. Like animals, plant CaM has four EF-hands embedded within two separate globular regions in the N-and C-terminal regions separated by flexible central helix (Chin and Means, 2000). Once calcium binds to a CaM EF-hand through electrostatic interactions (Hoeflich and Ikura, 2002), t\he Ca^{2+}-CaM complex interacts with GAD to change its conformation to the Ca^{2+}-CaM/GAD active complex, which activates the first step of GABA shunt in plants (Snedden et al., 1995). The first evidence for Ca^{2+}-stimulated GAD activity was obtained by screening a Petunia cDNA expression library with ^{35}S-labeled calmodulin (Baum et al., 1993) where Ca^{2+}-CaM/GAD complex was activated at neutral pH (Snedden et al., 1996). Either Ca^{2+} or H^{+} elevation appears to be sufficient to activate GAD and GABA synthesis (Shelp et al., 1999).

Calmodulin (CaM)

Calmodulins (CaM) are highly conserved, small acidic proteins present in all eukaryotes (Snedden and Fromm, 2001). CaM protein is 149-150 amino acids long with pI 3.92. The CaM prototype is comprised of two globular domains connected with a long flexible helix. Each globular domain contains a pair of intimately linked EF-hands. One EF-hand motif is composed of a specialized helix-loop-helix structure that binds one molecule of Ca^{2+} (Hoeflich and Ikura, 2002).

CaM binds to a short and specific amino acid sequence within target proteins. Conformation of the target protein changes after binding to CaM, resulting in activation of the target protein in response to intracellular Ca^{2+} concentration (McCormack et al., 2005). To facilitate this interaction, the central region of CaM forms a random coil. The globular domains at the N- and C- terminus of the molecule can then wrap around and engulf the target peptide (Bouché` et al., 2005).

In plants there is an extended family of CaM isoforms and CaM-like proteins (CaML). In addition, CaM-like proteins are harboring an EF-hand structure like CaM, but differences in functions such as target specificity, subcellular localization, and Ca^{+2} affinities are known to play an important role in the regulation of physiological processes (Luan et al., 2002; Reddy et al., 2002; McCormack and Braam, 2003; McCormack et al., 2005). CaM and CaML proteins have varied EF-hands, from the three EF-hand in wheat CaM-III (Yang et al., 1996) to the six *Arabidopsis* TCH3 (Sistrunk et al., 1994).

Calmodulin genes (*CAM*) show differential temporal and spatial expression in response to external stimuli such as touch, heat shock, cold, light, and pathogens and to internal stimuli such as hormones. *CAM* expression was elevated in embryo and reduced

in aleurone cells, starchy endosperm, and developed root and shoot tissues of wheat (Yang et al., 1998). In *Pisum staivum* L, *PsCAM*1 and *PsCAM*2 were detected in dry axes and cotyledons with highest accumulation during imbibition and prior to radicle protrusion, while *PsCAM*3 was detected only upon radicle protrusion (Duval et al., 2002). In *Nicotiana plumbaginifolia*, the level of *NpCAM*1 was high in response to cold and high wind, while *NpCAM*2 was not induced by either of the treatments (Van der Luit et al., 1999). Liu et al. (2005) demonstrated increased expression of *AtCAM3* and *AtCAM*7 genes in *Arabidopsis* after heat stress at 37°C. Increased expression of *CAM*3 in *Arabidopsis* seedlings inhibits the expression of *COR* gene in response to cold (Townley and Knight, 2002). In soybean, *SCAM*4 expression was induced within 30 min of pathogen or NaCl exposure (Park et al., 2004).

CaM isoforms and *CAM* genes in Arabidopsis: The genomic database of *Arabidopsis thaliana* shows 9 *CAM* gene loci. The first 7 calmodulins, (CaM1-CaM7), are 149 amino acids long, while CaM8 and CaM9 are predicted to be 151 amino acids long (TAIR web site). *CAM* genes share a high level of sequence identity to vertebrate CaM isoforms (McCorrmack and Braam, 2003). The first seven *CAM* genes in Arabidopsis encode for four CaM protein isoforms. The first isoform is encoded by *CAM1* and *CAM4*, the second isoform is encoded by *CAM2, CAM3* and *CAM5*, the third isoform is encoded by *CAM6,* and *CAM7* encodes the fourth CaM isoform. CaM1 and CaM4 differ from CaM7 by four amino acids; CaM2, CaM3, CaM5 and CaM6 differ from CaM7 by a single amino acid (Kretsinger, 1998). The *Arabidopsis* CaM isoforms are highly similar but minor changes in CaM might contribute to target specificity (Bhattacharya et al., 2004).

CaM genes are differentially expressed in response to numerous external stimuli such as touch, heat shock, cold, light, and pathogens and to internal stimuli like hormones (Townley and Knight, 2002). CaMs are also differentially expressed in different stages as well as in different tissue and cell type (Snedden and Fromm, 2001; Yang and Poovaiah, 2003).

Mechanisms of CaM action: Three CaM-activating mechanisms have been studied in animal systems (Hoeflich and Ikura, 2002). The first activation mechanism involves relieving auto-inhibition. In this mechanism, a CaM binding site is adjacent to or within an auto-inhibitory domain of an enzyme and binding of CaM induces a conformational change that displaces the pseudosubstrate inhibitory domain and allows full enzyme activity (Chin and Means, 2000). The second activation mechanism is active site remodeling. This mechanism was found in anthrax adenylyl cyclase (oedema factor) where four regions of the oedema factor form a surface that recognizes an extended conformation of CaM. Upon CaM binding, a helical domain of the oedema factor undergoes a 30° rotation away from the catalytic core, stabilizing a disordered loop and enzyme activation occurs (Drum et al., 2002). The third activation mechanism is CaM-induced dimerization. Two CaM molecules interact with two K^+ channel domains of the Ca^{2+}-activated potassium channels upon Ca^{2+}-binding. The C-terminal EF-hands mediate tethering to the channel and the N-terminal EF-hands are responsible for Ca^{2+}-induced dimerization leading to channel gating (Schumacher et al., 2001).

In plant systems the best studied CaM system involves the activation of glutamate decarboxylase. The C-terminal CaM-binding domain of glutamate decaroxylase (GAD) binds to a CaM and changes its conformation to GAD/CaM active complex in plant (Yap

et al., 2003). CaM proteins are composed of two pairs of Ca^{2+}-binding sites joined by a linker domain (Chin and Means, 2002). Each binding site is composed of two helices, called the E and F helices, flanking a Ca^{2+}-binding loop called the EF-hand.Conformational changes in CaM affects interactions of CaM with target proteins for modulation of the activity of the target (Snedden and Fromm, 2001; Day et al., 2002; McCormack et al., 2005). The ability of CaM to regulate the target enzyme activity resides in hydrophobic regions that are exposed after conformational change caused by Ca^{2+} binding to CaM (Hoeflich and Ikura, 2002). In addition, electrostatic interactions play a role in CaM-binding, such as interaction between basic helix-loop-helix DNA-binding proteins with CaM (Onions et al., 2000).

CaM is a cytosolic protein. However, it has been found in the nucleus (Van Der Luit et al., 1999), peroxisome (Yang and Poovaiah, 2002), and extracellular matrix (Ma et al., 1999). The necessity of multiple sub-cellular locales for CaM is expected because the CaM-target proteins are present in different subcellular locations. The regulatory activities of CaMs are result from its ability to modulate certain sets of enzymes. CaM has a diverse set of target proteins that are involved in a variety of processes, including ion transport (Arazi et al., 1999; Sze et al., 2000), gene regulation (Yang and Poovaiah, 2002; Bouché` et al., 2002), cytoskeleton organization (Reddy, 2001), disease resistance (Kim et al., 2002), and metabolism and stress tolerance (Reddy, 2001; Snedden and Fromm, 2001). The CaM-binding proteins have been classified into three groups. The first group is plant-specific CaM-binding proteins (e.g., auxin responsive SAURs and pollen-specific MPCBP) (Yang and Poovaiah, 2000; Safadi et al., 2000). The second group is composed of proteins that are similar to animal homologs but have an additional

CaM-binding domain (e.g ,GAD, kinesin, and catalase) (Snedden and Fromm, 2001; Reddy, 2001; Yang and Poovaiah, 2002). The third group is composed of proteins that are similar to the CaM homologs in animals (e.g , CGCG protein, transcription factor, and nuclear proteins) (Bouché` et al., 2002). Many CaM-binding proteins are plant-specific.

Other Calcium-binding Proteins

Calmodulin Like proteins (CML): CaM-like proteins (CML) are characterized by the presence of EF-hand Ca^{2+}-binding motifs. Plants have a divergent and large number of CML proteins. The Arabidopsis genome has circa 50 CML genes. The divergent CML family is likely to have evolved from progenitor CaMs (McCormack and Braam, 2003). Many of the CMLs appear to have undergone significant subfunctionalization. Several CMLs have expression limited to a small subset of organs and have dramatic expression changes in response to a variety of biotic and abiotic stimuli (White and Broadley, 2003). Even the CMLs with closely related paralogs are generally found to display distinct expression characteristics. Thus, the different CML family members might have evolved in ways that enable their continued presence in the Arabidopsis genome to be under selective pressure (McCormack et al., 2005). Determination of the biochemical and physiological functions of this large family of potential Ca^{2+} signal sensors remains an important challenge.

Calcium Dependent Protein Kinase (CDPK): CDPKs form a large subfamily of protein kinases in plants and have been implicated in the control of aspects of plant growth and development (Cheng et al., 2002). The CDPKs have four well characterized conserved motifs: 1) an ATP-binding domain; 2) a catalytic domain; 3) an auto-inhibitor

domain is predicted in the region immediately following the kinase domain; and 4) a number of functional calcium-binding EF-hands in a C-terminal regulatory domain. CDPKs modulate other proteins during calcium signaling under various abiotic stress conditions in plants (Roberts, 1993; Xiong et al., 2002).

Calcinurein B (CBL): CalcineurinB is a Ca^{2+}/calmodulin-dependent serine-therionine phosphatase that plays an important role in transducing Ca^{2+}-dependent signals in a variety of cell types. It is a heterodimer of two subunits: calcineurin B/CBL, the 19-kDa Ca^{2+}-binding and regulatory subunit, and calcineurin A/CAL, ~61-kDa catalytic subunits. Multiple catalytic subunits of calcineurin are derived from at least two structural genes, type one (calcineurin A-alpha) and type two (calcineurin A-beta), each of which can produce additional alternatively spliced transcripts (Sharma and Deswal, 2004). CBL belongs to the family of EF-hand Ca^{2+}-binding proteins. Both CBL and CaM are important for the activation of the phosphatase activity of calcineurin (Kim et al. 2007). Arabidopsis SOS3 is a CBL-like protein involved in the regulation of salt tolerance. SOS3 activates the SOS2 protein kinase, which is a regulator of SOS1 and *At*NHX1, the plasma membrane, and tonoplast Na^+/H^+ antiporter (Wang et al., 2007; Xu et al., 2006; Ma et al., 2005).

Regulation of the GABA shunt

The non-protein amino acid, γ-Aminobutyric acid (GABA), is a four carbon amino acid found in all organisms. GABA is a zwitterion with the amino group on the γ-carbon and exists in an unbound water soluble from in the cell (Shelp et al., 1999). GABA is synthesized from glutamate by glutamate decarboxylase (GAD) and serves as a neurotransmitter inhibitor in the nervous system of vertebrates and some invertebrates

(Sattelle, 1990; Bormann, 2000). GABA is synthesized from glutamate and polyamines in fungi (Kumar and Punekar, 1997). Some of the proposed functions of GABA in fungi include nutritional roles, involvement in conidiation and germination, acidogenesis (Kumar and Punekar, 1997), and as a source of nitrogen and carbon during stress (Solomon and Oliver, 2002).

Glutamate decarboxylase (GAD) (EC 4.1.1.15) catalyzes irreversible decarboxylation of glutamate to GABA in the presence of cofactor pyridoxal phosphate (PLP). GAD is a cytosolic enzyme (Breitkreuz and Shelp, 1995) involved in stress-induced GABA synthesis caused by cytosolic acidosis and the consequent stimulation of GAD as a mechanism to regulate cell pH (Snedden et al., 1992). Five putative genes encoding GAD in *Arabidopsis thaliana* have been identified (Shelp et al., 1999). Two *A. thaliana* genes encoding GAD isoforms, *GAD*1 and *GAD*2 have differential tissue specific expression; *GAD*1 is expressed in root, whereas *GAD*2 is expressed in all tissues (Turano anf Fang, 1998; Zik et al., 1998; Bouché et al., 2004). Arabidopsis GAD1 is a 57.1 KDa protein while GAD2 is a 56.1 kDa protein with 82% identity at the level of amino acids sequence (Zik et al., 1998).

Both GAD1 and GAD2 are calcium/ calmodulin (Ca^{2+}/CaM) regulated enzymes and their calmodulin (CaM)-binding domain differs in their potential phosphorylation sites (Turano and Fang, 1998). GAD2 lacks all four serine and therionine residues present in the CaM-binding domain of GAD1, suggesting differences in regulatory mechanisms (Yevtushenko et al., 2003; Bouché` et al., 2004). In rice (Oryza sativa) two GAD isoforms were identified, *Os*GAD1 and *Os*GAD2, which encode polypeptides of 501 and 500 amino acids, respectively. These proteins share 69% amino acid identity.

*Os*GAD2 is not activated by Ca^{2+}/CaM in vitro (Akama et al., 2001; Yevtushenko et al., 2003). At least four tobacco (*Nicotiana tabacum) GAD* genes are highly conserved and do not have CaM binding domains (McLean et al., 2003).

GAD is a homohexameric enzyme of about 330 kDa with three types of domains, conserved catalytic domain (~250 amino acids), Pyridoxal-5`-phosphate (PLP) binding domain (~10-20 amino acids), and the C-terminal CaM-binding domain (~33-50 amino acids). Plant GAD enzymes with Ca^{2+}-dependent CaM binding domain (Baum et al., 1993) differ from bacterial enzyme in having a C-terminal extension of 33 amino acids within which resides CaM binding domain with in vitro maximal activity at pH 5.8 (Ling et al., 1994; Arazi et al., 1995; Yuan and Vogel, 1998; Zik et al., 2006). GAD activity was found in extracts of various plant species to be modulated by Ca^{2+}/CaM by binding to GAD CaM-binding domain and to change its conformation to GAD/CaM active complex by inducing the enzyme complex dimerization (Yuan and Vogel, 1998; Yap et al., 2003). Plant GAD is specific for L-glutamate, requires PLP, is inhibited by reagents known to react with Sylfhydryl groups, possesses a CaM-binding domain, and exhibits a sharp acidic pH optimum of 5.8 (Baum et al., 1993; Shelp et al., 1999).

GAD enzyme is widely distributed among eukaryotes and prokaryotes, but its function varies in different organisms. In animals, especially in vertebrates, GAD has an important role in the central nervous system where it is responsible for the synthesis of GABA, the major inhibitory neurotransmitter. The majority of vertebrates GAD occur in two isoforms, GAD65 and GAD67, and both are active at neutral pH (Soghomonian and Martin, 1998). In Saccharomyces cerevisiae GAD is Ca^{2+}/CaM dependent enzyme. It

has a C-terminal CaM-binding domain (~40 amino acids) and its expression is required in abatement of oxidative stress (Coleman et al., 2001).

The bacterial GAD is a homohexamer of about 330 kDa, and its maximal activity is at acidic pH of 3.8-4.6. The activation of GAD in bacteria at acidic pH involves association of the hexameric complex with membranes, a process that involves the N-terminus of the enzyme (Capitani et al., 2003). Bacterial GAD has some features similar to those of the plant enzyme: it exhibits an acidic pH optimum (3.8-4.6) (Sukhareva et al., 1994), forms hexamer, and is expressed in response to environmental stresses (Blankenhorn et al., 1999; De Biase et al., 1999; Shelp et al., 2006).

GAD is a cytosolic enzyme and stress-induced GABA synthesis is thought to be the result of cytosol acidosis (Shelp et al., 1992; Shelp et al., 1999). Stress factors such as heat or cold shock, which stimulates GABA level, are also known to increase cytosolic Ca^{2+} levels (Kinght et al., 1991; Breitkreuz and Shelp, 1995), which stimulate the activity of Ca^{2+}-CaM/GAD complex and GABA synthesis. Rapid accumulation of GABA within 1 min without change in pH suggests that either Ca^{2+}or H^{+} is sufficient to stimulate GABA synthesis (Bown and Shelp, 1997; Kinnersley and Turano, 2000; Bouché and Fromm, 2004).

GABA is converted reversibly to succinate semialdehyde (SSA) by two types of GABA-transaminases (GABA-TA) (EC 2.6.1.19) inside the mitochondrial matrix. GABA-TA uses either α-ketoglutarate (GABA-TK) or pyruvate (GABA-TP) as amino group acceptors, producing glutamate or alanine (Shelp et al., 1999; Bouché and Fromm, 2004). In mammals, only GABA-TK is functionally active, while both GABA-TK and GABA-TP are present in tobacco leaf and can be separated from each other using ion

exchange chromatography. GABA-TK and GABA-TP activities exhibit a broad pH optimum from 8 to 10 (Van Cauwenberghe et al., 2002). Knockout mutants of *A. thaliana* in GABA-TA gene have elevated GABA level by 100-fold in flowers suggesting that GABA-TP is the functional enzyme *in vivo* with specific function in pollen tube guidance (Palanivelu et al., 2003).

Succinate semialdehyde dehydrogenase (SSADH) (EC 1.2.1.16) is the third enzyme in the GABA shunt pathway. SSADH irreversibly oxidizes SSA to succinate inside the mitochondria where succinate enters the TCA cycle. Plant SSADH has been localized to the mitochondria (Breitkreuz and Shelp, 1995), while SSADH in yeast is localized in the cytosol (Coleman et al., 2001). SSADH is specific for SSA oxidation and reduction of NAD^+ to produce NADH with optimal pH~9. Both ATP and NADH negatively regulates SSADH activity (Bouché and Fromm, 2004). There is a locus in Arabidopsis genome encoding for SSADH (ssadh1). SSADH1 cDNA encodes a protein of 528 amino acids (56 kDa) with high homology to *E. coli* and human SSADH (Busch and Fromm, 1999). The recombinant Arabidopsis SSADH protein is similar to the multimeric SSADHs of other eukaryotes with the 53 kDa subunit assembled into a native enzymes of 197 kDa implying that the native enz;yme is a homotetramer similar to SSADH from barley and animals (Chambliss and Gibson, 1992; Busch and Fromm, 1999). Disruption of SSADH in Arabidopsis results in necrotic cell death in response to UV-B irradiation or heat stress apparently due to the accumulation of reactive oxygen species (Bouché et al., 2003).

γ-Hydroxybutyrate dehydrogense (GHBDH) is another enzyme responsible for the conversion of SSA to γ-hydroxybutyrate (GHB). In a mammalian brain, GHB is

present in small quantities relative to the GABA level (Maitre, 1997). GHBDH in Arabidopsis is a 289 amino acids polypeptide containing a NADP-binding domain, showing increased activity in response to flooding and oxygen deficiency (Breitkreuz et al, 2003). Oxygen deficiency increases the production of NADPH and reactive oxygen species (ROS) such as superoxides and hydrogen peroxide in plants (Bouché et al., 2003). However, the oxidation of NADPH as well as NADH via the mitochondrial respiratory chain is limited. These findings suggest that GHBDH and SSADH activities in plants are regulated in a complementary fashion by Redox balance and that GHB functions in oxidative stress tolerance (Breitkreuz et al, 2003; Bouché and Fromm, 2004).

GABA serves as a source of carbon and nitrogen in bacteria (Jin et al., 1990). The integrity of GABA synthesis is essential for coping with acidic stress (Cotter et al., 2001). The GAD controls acidification of cytosol by decarboxylation of an acidic glutamate into a neutral GABA via incorporation of H^+ ion (Meng and Bennett, 1992; Cotter et al., 2001). Rapid accumulation of GABA in plants in response to heat, cold, drought, salinity, anoxia, and wounding has been demonstrated (Steward. 1949; Mayer et al., 1990; Breitkreuz and Shelp, 1995; Bown and Shelp, 1997; Shelp et al., 1999; Locy et al., 2000). Also, a possible role of GABA as a signaling molecule in plants has been proposed (Kinnersley and Turano, 2000; Bouché et al., 2003; Shelp et al., 2006).

GABA has been considered as a metabolite that accumulated in response to stress in plants (Mayer et al. 1990; Chung et al., 1992; Shelp et al. 1999). An increase in GABA level in wheat and barley during cold stress is caused by GAD activation and accompanied with induction of the transcription of genes encoding GABA-shunt enzymes (Mazzucotelli et al., 2006). Accumulation of GABA in response to cytosolic

acidification supports the involvement of GABA in pH regulation (Crawford et al., 1994).

In soybean leaves the accumulation of GABA in response to low temperature likely results from activation of glutamate decarboxylase activation or lowering of protoplasmic pH (Wallace et al., 1984). Increases in GABA under hypoxia and no change in its level under drought suggests different mechanisms of GABA accumulations under these two stresses in soybean (Serraj et al., 1998). GABA shunt metabolites accumulated in rice root in response to anoxia, but its level was depleted after treatments with Ca^{2+}-channel blockers and calmodulin antagonists (Reggiani et al., 1988). The accumulation of GABA under hypoxia provides an immediate substrate upon recovery from stress (Wallace et al., 1984; Shelp et al., 1995). GABA may be involved in recycling nitrogen and carbon in plant growth, development, and defense (Micallef and Shelp, 1989), and as a compatible osmolyte (Shelp et al., 1999).

Polyamines may be involved in higher plant responses to salt stress, playing a key role in ion balance, anti-senescence, free radical scavenging, and membrane stabilization (Bouchereau et al., 1999). Xing et al. (2007) proposed that polyamines could perform their function through their oxidative products such as GABA under salinity in soybean. High levels of GABA in soybean root along with increases in diamine oxidase (DAO) activity in response to salt treatment, and reductions in GABA levels after treatment with aminoguanidine (AG, a specific inhibitor of diamine oxidase) during salt stress resulted in the reduction of GABA by 40%. This result suggests a contribution of polyamine oxidation to GABA leavels via DAO (Xing et al., 2007).

GAD1 protein is required for normal oxidative stress tolerance in yeast (Coleman et al. 2001). GAD1 protein increases resistance to H_2O_2 and diamide oxidants, suggesting that GAD and glutamate catabolism via the GABA shunt pathway is required to buffer redox changes in the cell in response to oxidative stress in yeast.

GABA accumulation has been demonstrated in response to mechanical stress and wound healing (Petrivalsky et al., 2007). GABA ingestion in oblique-banded leaf-roller larvae showed reduced growth and survival of larvae suggesting GABA as a first line of plant defense (Ramputh and Bown, 1996; Bown et al., 2006).

Beside its role as a stress metabolite, GABA plays a critical role in the regulation of gene expression, including the expression of the 14-3-3 gene family in plants\(Lancien and Roberts, 2006). Recent studies with Arabidopsis suggest a possible role of GABA as a signal molecule (Bouché and Fromm, 2003; Palanivelu et al. 2003).

Pollen tubes carrying sperm cells navigate through several female tissues during reproductive development in plant (Mascarenhas, 1993). POP2 (pollen-pistil interaction2) protein in Arabidopsis appears to mediate interaction on both male and female sides (Wilhelmi and Preuss, 1996). The homozygous *pop*2 mutant is self-sterile even though the pollen tube can germinate and penetrate the stigma, but its guidance is abnormal (Ma, 2003). Pollen tube guidance was investigated in pop2 mutant (Palanivelu et al. 2003). POP2 encodes a GABA transaminase (GABA-TA) that degrades GABA (Van Cauwenberghe et al. 2002).

Increases in the level of GABA occured along the pollen tube path in wild type, while the *pop*2 mutant showed a disturbed gradient of GABA. This disturbance lead to the inhibition and misguidance of pollen tubes in *pop*2 pistils (Bouché et al. 2003). The

*pop*2 phenotype demonstrates the role of GABA in plant reproduction and development (Bouché et al., 2003; Ma, 2003; Palanivelu et al., 2003; Bouché et al., 2005).

In duckweed (*Lema minor*), promotion of growth by GABA was associated with an increase in mineral content of treated plants in a dose dependent manner (Kinnersley and Lin, 2000), while in transgenic tobacco plants expressing a mutant GAD that lacked an auto-inhibitory CaM-binding domain, higher GABA levels, lower glutamate levels, and less stem elongation resulted (Baum et al., 1996). Since the intracellular Ca^{2+} pools in these plants were unaltered, growth inhibition may result from altered GABA metabolism.

Brassinosteroid (BR) promotes stem elongation and cell divisions (Clouse, 2002). BR plays an important role in coupling environmental factors, especially light, with plant growth and development (Nemhauser and Chory, 2004). Arabidopsis DWARF1 (DWF1) is responsible for an early step in brassinosteroid biosynthesis that converts 24-methylenecholesterol to Campesterol (Choe et al., 1999). DWF1 is a Ca^{2+}/CaM-binding protein and this binding is critical for function. Loss of CaM binding completely abolished the function of DWF1, whereas partial loss of calmodulin binding resulted in partial dwarf phenotype in Arabidopsis (Du and Poovaiah, 2005).

Several abiotic stress pathways share common elements that have potential for cross-talk. Cross talk can also occur between pathways in different organs of the plant when a systemic signal such as hydrogen peroxide moves from a stimulated cell into another tissue to elicit a response (Knight and Knight, 2001). Genetic analysis and biochemical characterization will be required to determine specificity and cross-talk in abiotic stress signaling pathways in plants (Chinnusamy et al., 2004).

The Ca^{2+}/CaM-mediated signal network affects many aspects of plant growth, development, and response to environmental changes (Chinnusamy et al., 2004; Yang and Poovaiah, 2003). GABA accumulation in response to diverse stimuli is associated with various physiological responses and signaling cascades in plants (Bown and Shelp, 1997; Kinnersley and Turano, 2000; Bouché and Fromm, 2004). The GABA shunt and its components (Ca^{2+}/CaM, shunt enzymes, and metabolites) may have both metabolic and signaling function in plants to create a link in the chain of events leading from perception of environmental stimuli to precise physiological responses.

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II. THE REGULATION OF CALMODULIN GENE mRNAs IN WILD TYPE AND CALMODULIN MUTANTS OF *ARABIDOPSIS THALIANA* UNDER HEAT STRESS

ABSTRACT

Calmodulin protein (CaM) regulates the activity of a number of key enzymes and plays important roles in cellular response to environmental changes. The *A. thaliana* genome contains nine calmodulin (*CAM*) genes. To understand the role of specific *CAM* genes in heat stress, the steady-state level of mRNA for the nine *CAM* genes in root and shoot tissues of seedlings grown at normal growth temperature (25^{o}C) and during heat stress at 42^{o}C for 2 hr was compared in T-DNA insertional mutant lines of 7 *CAM* genes and the wild type using gene specific primers and RT-PCR. Compared to growth at 25°C, the mRNA levels of all *CAM* genes were upregulated in both root and shoot after heat treatment with the notable exception of *CAM5* in root and shoot, and *CAM*1 in shoot where the mRNA levels were reduced. At 25^{o}C all *cam* mutants showed varying levels of mRNA for corresponding *CAM* genes with the highest levels of *CAM5* mRNA being found in *cam5-1* and *cam5-3*. *CAM5* mRNA was not observed in the *cam5-4* allele which harbors a T-DNA insertion in exon II. The level of respective *CAM* gene mRNAs were reduced in all *cam* alleles compared to levels in wild type except for increased expression of *CAM5* in roots and shoots of *cam5-1* and *cam5-3*. Compared to wild type, the level of mRNA for all *CAM* genes varied in each *cam* mutant, but not in a systematic

way. In general any nonexonic T-DNA insertion produced a decrease in the mRNA level of the *CAM2* and *CAM3* genes, and the levels of *CAM* gene mRNAs was the same as wild type or lower in the *cam1, cam4, cam5-2,* and *cam6-1* nonexonic mutant alleles. However the level of mRNA for all genes except *CAM2* and *CAM3* genes was upregulated in all *cam2* and *cam3* alleles and in the *cam5-1* and *cam5-3* alleles. During heat stress at 42^{o}C the level of *CAM* gene mRNAs was also variable between insertional mutants, but the level of *CAM1* and *CAM5* mRNA was consistently greater in response to heat stress in both root and shoot. These results suggest differential tissue specific expression of *CAM* genes in root and shoot tissues, and specific regulation of *CAM* mRNA levels by heat. Each of the *CAM* genes appears to contain noncoding regions that play regulatory roles in the interaction between *CAM* genes leading to changes in specific *CAM* gene mRNA levels in Arabidopsis. With respect to *CAM* gene expression only exonic mutations lead to a loss of function of *CAM* genes.

Key words:

Arabidopsis, Calmodulin, *CAM*, CaM, Ca^{+2}, Expression, Heat stress, RT-PCR.

Abbreviations:

CAM: Calmodulin gene

CaM: Calmodulin protein

Ca^{+2}/CaM: Calcium/Calmodulin complex

RT-PCR: Reverse transcriptase- Polymerase Chain Reaction

UTR: 3` or 5` gene Un-Translated Region

INTRODUCTION

Calmodulins (CaM) are highly conserved and ubiquitous transducers of Ca^{+2} signals in all eukaryotes. The regulatory abilities of CaMs are revealed by their ability to modulate the activities of enzymes involved in various physiological responses including growth, gravitropism, phototropism, environmental stress, and biotic defense responses (McCormack and Braam, 2003; Yang and Poovaiah, 2003; McCormack et al. , 2005). CaM harbors no intrinsic enzyme activity of its own, but calmodulin proteins are highly conserved with 4 repeating units called EF-hands. Each EF-hand binds a single Ca^{+2} ion (Strynadka and James, 1989; Crivici and Ikura, 1995). Upon Ca^{+2} binding to the EF-hand domains, CaM alters its structure (Wriggers et al., 1998) by revealing hydrophobic surfaces that serve to interact with target proteins. In this way the Ca^{+2}-dependent regulation of target proteins is mediated.

The genomic database of *A. thaliana* reveals 9 *CAM* gene loci. The first 7 calmodulins proteins, (*CAM1-CAM7*) produce proteins consisting of 149 amino acids each, While CaM8 and CaM9 are predicted to code for proteins that are 151 amino acids each (http://www.arabidopsis.org). Calmodulins are generally considered cytosolic proteins, but they have also been found in the nucleus, mitochondria, chloroplasts, the extracellular matrix, and perixosomes (Jarrett et al. , 1982; Roberts et al. , 1983; Ma et al. , 1999; Van der Luit et al. , 1999; Yang and Poovaiah, 2002). Some *CAM* genes show differential temporal and spatial expression in response to external stimuli such as touch, heat shock, cold, light, pathogens, and hormones (Yang et al., 1998; Duval et al. , 2002; Van der Luit et al. , 1999; Townley and Knight, 2002; Park et al. , 2004).

Exposure to high temperature increases the level of CaM protein in maize seedlings (Gong et al., 1997). Expression of the CaM-related touch (TCH) genes in heat shocked Arabidopsis cells has also been shown (Braam, 1992). In maize, HSP70 binds CaM inside the cytoplasm in a Ca^{+2}-dependent manner (Sun et al, 2000). Ca^{+2}/CaM complexes activate the binding of heat shock factor (HSF) to DNA resulting in the induction of HSP (Li et al. , 2004; Liu et al. , 2007), and increases in the intracellular Ca^{+2} level during heat stress have been shown to result in the induction of *CAM* gene expression and the synthesis of HSP under heat stress in wheat (Fan et al., 2000; Liu et al., 2003). Liu et al. (2005) demonstrated increased expression of *CAM3* and *CAM7* genes in Arabidopsis after heat stress at 37°C.

To elucidate the role of calmodulin isoforms in tolerance to heat stress in plants, we have examined the steady state levels of the nine Arabidopsis *CAM* genes in wild type and in a series of insertion mutations in seven of the 9 *CAM* genes in Arabidopsis seedlings in response to heat stress.

MATERIALS AND METHODS

Plant material and growth conditions

The wild type and mutant lines of Arabidopsis (*Arabidopsis thaliana* Ecotype Columbia) bearing T-DNA insertions in various calmodulin genes (*cam*) used in this study are listed in Table 1. Wild type, *cam1* and *cam4* seeds were obtained from Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH, while seeds of all other mutants were obtained from Dr. Janet Braam, Rice University, Texas. Homozygous seed stocks were propagated for this study. Seeds were surface sterilized with bleach (v/v, 6% sodium hypochlorite) for 10 min followed by five washes

with sterile distilled water. Seeds were plated in Petri dishes on sterile 1X Murashige and Skoog (1962) medium (pH 5. 7) supplemented with 2% (w/v) sucrose, solidified with 1. 2% (w/v) agar, Seedlings were grown under continuous illumination (40μmol m^{-2} s^{-1}) provided by cool white fluorescent lamps at 25 ºC.

Heat treatment

Two-week-old Arabidopsis seedlings of wild type and *cam* mutant lines were grown at 25ºC (control) and at 42ºC for 2 hr (heat treated) (Locy et al. , 2000). Root and shoot tissues of all control and heat treated samples were harvested separately, frozen in liquid nitrogen (LN_2) and used for total RNA extraction. Three replicates of each control or treated sample consisting of 50 seedlings each were collected.

RNA extraction

Total RNA from frozen samples was extracted using the RNeasy Plant Mini kit (Qiagen,) according to manufacturer instructions. Total RNA was suspended in RNase-free water. RNA concentrations were determined be their absorbance A_{260} using GeneQuant*pro*RNA/DNA calculator (New England Biolab), and the integrity of RNA was determined after separation of RNA on a 1% (w/v) agarose gel after electrophoresis and staining with ethidium bromide.

Estimation of steady state levels of mRNA by reverse transcriptase-PCR

Gene specific primers for each of the nine *A. thaliana* calmodulin genes (*CAM*) were designed for RT-PCR analysis of steady state mRNA levels as shown in Table 2. One-step Reverse Transcriptase-PCR (RT-PCR) reaction was performed using primer pairs, SuperScript™ III One-step RT-PCR system with platinum® Taq DNA polymerase

(Invitrogen, Inc.) according to manufacturer's instruction, and 1μg total RNA as determined above. Primers were used at a concentration of 0.2μM in a reaction volume of 15μL.

The annealing temperatures were calculated on the basis of the *Tm* values of each primer pair (Table 2). The following temperature program was used for RT-PCR in a PTC-100 thermocycler (MJ Research): 30 min cDNA synthesis at 50°C, 2 min dentauration at 94C, followed by 40 cycles each of dentauration at 94°C for 1 min, primer annealing for 1 min at 45°C to 55°C (based on the *Tm* of the primers), and extension at 68°C for 1 min. The program ended with a 5 min final extension at 68°C.

A primer pair for the *CBP20* protein (Marín-Vinader et al. 2006) was used as an internal control to normalize RNA concentrations in each reaction tube. RT-PCR amplification products were separated on 1% agarose gels and stained with ethidium bromide. The expression level of each calmodulin (*CAM*) gene in all *cam* mutant lines and the wild type were determined by measuring the fluorescence of RT-PCR amplicon bands using the Gel Documentation and Image Analysis System (Alpha Innotech, CA). The amount of fluorescence in a cDNA amplicon representing specific RNA in each sample was used as a measure of the level of expression. The level of RNA in each tube was normalized with respect to the fluorescence of the *CBP20* internal control. The background fluorescence on agarose gels was subtracted from the fluorescence value of each DNA band. Each determination represents an average of three different biological replicates with standard deviation. Data was represented as Log_2 Fold change in the level of *CAM* gene expression after heat treatment compared to the normal growth (Control).

RESULTS AND DISCUSSION

Comparative steady state levels of *CAM* mRNAs in heat treated Arabidopsis seedlings

The steady state level of mRNA for the 9 *CAM* genes was estimated in root and shoot tissues of wild type Arabidopsis at 25^{o}C (Figure 1A) using RT-PCR. RNA levels of the *CAM* genes in root and shoot tissue was approximately equal for all genes except *CAM2* where the level in shoot was only 60% of the level in root. The levels of individual *CAM* mRNAs in wild type at 25^{o}C were variable. The lowest mRNA level was observed for *CAM7* and *CAM1* in both root and shoot tissues. While the highest mRNA levels were observed for *CAM2* in root, and *CAM3* in root or shoot tissues. All other *CAM* genes showed roughly equivalent mRNA levels.

The change in *CAM* mRNA levels after a 2 hr heat treatment at 42^{o}C was also determined (Figure 1B). All *CAM* genes showed a two to eight-fold increase in expression after heat treatment in root and shoot tissues with the notable exception of *CAM1* in shoot and *CAM5* in both root and shoot (Figure 1B) which decreased in expression by as much as two to over 50-fold. The level of *CAM2*, *CAM3*, *CAM7*, and *CAM8* mRNAs in root was higher than the level of mRNAs for *CAM1*, *CAM4*, *CAM6*, and *CAM9* after heat treatment (Figure 1B). *CAM5* mRNA was not detected in root tissue after heat treatment. The highest levels of *CAM2*, *CAM3*, *CAM8*, and *CAM9* mRNAs were observed in shoot followed by the levels of mRNAs for the *CAM7*, *CAM4* and *CAM6* genes, respectively. The level of *CAM5* mRNA in shoot was greatly reduced under heat as was the level of *CAM1* mRNA (Figure 1B). It can be concluded that heat stress modulates tissue-specific expression of *CAM* genes in Arabidopsis.

Members of the gene family are usually differentially regulated to accomplish specific functions in spatial or temporal manner. Differential expression of *CAM* genes in response to environmental stimuli such as touch, heat shock, cold, light, pathogens and hormones have been shown in plants (Perera and Zielinski, 1992; Botella and Arteca, 1994; Kudla et al. , 1999; Heo et al. , 1999; Yang and Poovaiah; 2000; Yamakawa et al. , 2001; Pandey et al. , 2002; Ali et al. , 2003; Park et al. , 2004; Takabatake et al. , 2007). Accumulation of CaM protein during heat stress has been shown in maize seedlings (Gong et al. , 1997; Sun et al. , 2000). Differential induction of specific *CAM* genes by high temperature was demonstrated in wheat (Fan et al. , 2000; Liu et al. , 2003) and rice (Pheanopas et al. , 2005).

In Arabidopsis all *CAM* genes including the divergent forms, *CAM8* and *CAM9,* are ubiquitously expressed (Zielinski, 2001). Expression level of *CAM3* and *CAM7* mRNA increased in Arabidopsis in response to a 37^{o}C heat treatment (Liu et al. 2005). This observation was supported by the results presented here that showed that at 42°C (still a nonlethal temperature) *CAM3* and *CAM7* were among those *CAM* genes that increased in expression (Figure 1A). The level of *CAM2* and *CAM5* mRNA was reduced in response to heat stress at 37^{o}C (Liu et al. 2005). For *CAM5* this observation was also supported by the observations presented here. However, the *CAM5* expression was measured as total plant tissue expression under heat stress at 37C.

Expression of *CAM* genes in the corresponding *cam* mutants

To potentially elucidate a role of specific *CAM* genes and/or specific isoform(s) of CaM in heat stress, we have used a set of available T-DNA insertion mutants in the first 7 *CAM* genes in Arabidopsis (see Table 1). It should be noted that all *cam* mutants have

T-DNA insertions in either the 5'UTR, 3' UTR or in the single intron of a *CAM* gene, except *cam5-4*, which contains an insertion in the *cam5* exon II.

The level of mRNA observed for each of the *CAM* genes was determined in root and shoot tissues in corresponding *cam* mutants grown at 25^{o}C (Figure 2A). The steady state levels of *CAM* gene mRNAs in corresponding *cam* mutants were variously affected with the highest levels of *CAM5* mRNA being found in root and shoot tissues of *cam5-1* and *cam5-3*. *CAM5* expression was not detected in the *cam5-4* mutant.

The level of mRNA of each of the *CAM* genes in the corresponding *cam* mutants was compared to its expression in wild type, and the increase in mRNA levels is shown in Figure 2B. The level of the *CAM5* gene mRNA in various *cam5* alleles was variable depending upon location of insertion in the *CAM5* gene. In the *cam5-4* allele (bearing the exonII insertion) mRNA was not detected in root or shoot tissues consistent with the *cam5-4* allele being a true knock-out mutation. A higher than wild type level of *CAM5* mRNA in the *cam5-1* allele (bearing an insertion in the 3`UTR) and in the *cam5-3* allele (bearing an insertion in 5`UTR) was seen in both root and shoot tissues, while the *cam5-2* allele (bearing a different 3`UTR insertion) showed a 15-fold reduction in the expression of *CAM5* in root and an 8-fold reduction in shoot tissues. The level of *CAM7* gene mRNA was increased in the shoot of *cam7-1* compared to its level in wild type which also bears a 3`UTR insertion.

The level of mRNA for all other *CAM* genes was reduced in the corresponding *cam* mutants compared to their levels in wild type (Figure 2B). Thus this series of non-exonic T-DNA insertions did not completely eliminate *CAM* expression as anticipated, although insertions in several of the UTRs and introns dramatically reduced

corresponding mRNA levels in some tissues. Only the *cam5-4* exonic insertion allele completely eliminated *CAM5* expression in both root and shoot tissues. Depending upon the location of insertion, the level of the respective *CAM* gene was variable, but the expression of most of the *CAM* genes was reduced in *cam* mutants compared to the respective level in wild type, with the exception of some *cam5* alleles and *cam7-1* allele where mutations produced tissue specific increases in mRNA level.

Alleles of *CAM* genes have pleiotrophic effects on the expression of the other *CAM* genes

It is striking that the steady state level of most *CAM* gene mRNAs in all of the T-DNA insertional alleles of the Arabidopsis *CAM* genes is dramatically lower than the mRNA levels in wild type (Figure 3) except for the *cam2*, *cam3* alleles, and *cam5-1*and *cam5-3* alleles. This particularly applies to the level of *CAM1* and *CAM7* mRNA levels which demonstrate the highest fold increases observed as a result of T-DNA insertion in a *CAM* gene. Additionally, modest increases in the mRNA levels for *CAM4, CAM5,* and *CAM6* are shown in most of the mutant alleles listed above. The levels of *CAM8* and *CAM9* mRNA in various alleles are more variable, particularly in root, but in shoots these *CAM* genes follow the same trend as the other *CAM* genes except for the *cam2-1* and *cam3-3* alleles.

In contrast to the above T-DNA insertion alleles, the *cam1, cam4, cam5-2, cam5-4, cam6-1,* and *cam7-1* insertion alleles all demonstrate lower or no change in the overall expression of all of the *CAM* genes except for slight increases in the level of mRNA for *CAM7* in the *cam4, cam5-2* , and *cam7-1* alleles in shoots and for the *CAM1* gene in the

cam5-3 allele and *cam7-1* allele in roots. The *cam5-3* allele also demonstrates higher levels of mRNA for the *CAM6* and *CAM7* gene in roots.

These observations suggest that there are complex interactions involving either the expression of *CAM* genes or the processing and stability of *CAM* gene mRNAs. It is clear that insertion in any *CAM* gene that was examined in this study lowers the steady state mRNA levels of the *CAM2* and *CAM3* genes. In most of the alleles the levels of *CAM8* and *CAM9* mRNA were similarly affected. While there are a few allele septic effects in root versus shoot, the general pattern of increased mRNA levels of the *CAM1, CAM4, CAM5, CAM6* and *CAM7* genes and decreased mRNA levels in *CAM2, CAM3, CAM8* and *CAM9* in both root and shoot in those alleles which did not lead to lower expression of all *CAM* genes suggests that there is a critical regulatory function for *CAM* genes located in the 3'-UTR of these alleles, since all of the alleles which show some upregulation are insertions in the 3'-UTR while almost all of the other strictly down regulated *CAM* mRNA level alleles involve insertions in regions other than the 3'-UTR. It is also clear that the complete loss of function of the *CAM5* gene (*cam5-4* exonic insertion allele), leads to the down regulation of mRNAs of nearly all of the *CAM* genes in both root and shoot tissues. Other alleles also lead to such strong downregulation of *CAM* gene mRNA levels (*cam1, cam4, cam5-2,* and *cam6-1).* However, none of these alleles demonstrate a strong phenotype at 25 C.

Expression of nine CAM genes in Arabidopsis cam mutants after heat treatment

To better understand how each of the nine *CAM* genes are regulated by heat and whether *CAM* genes interact during heat stress, we determined the steady state mRNA levels for the nine *CAM* genes in root and shoot tissues of all of the cam mutant alleles

after heat treatment and compared this level to the level in plants grown at 25 C. The results are shown in Figure 4 (color plot diagram) as the fold increase in mRNA level at 42 ^{o}C compared to the level in plants grown at 25^{o}C.

In wild type the mRNA levels of all nine CAM genes are upregulated between 1.8 and 8 fold by heat treatment in both root and shoot except for *CAM5* mRNA in roots which is 1.6-fold downregulated, and *CAM1* and *CAM5* mRNAs in shoots which are downregulated 3.1-fold and 52-fold respectively. Although variable from mutant to mutant, it is apparent that none of the mutant alleles examined in this study show an expression pattern during heat-stress that is similar to the wild type. Most notable among the observe differences is the level of *CAM5* mRNA induced by heat stress in all of the *cam2, cam3, cam4,* and *cam5* mutant alleles (except the exon insertion *cam5-4*) where instead of decreasing as in wild type the mRNA level for the *CAM5* gene increases by as much as much as 420-fold. There is no detectable *CAM5* mRNA found in the *cam5-4* allele, but the expression of all other *CAM* genes is downregulated during heat-stress by as much as 2- to 129-fold in roots, and 2- to 325-fold in shoots.

The mutant alleles which are most distinct from this wild type pattern of heat-stress *CAM* gene mRNA levels and which share the down regulation of *CAM* genes with the *cam5-4* allele are: *cam1,* where the mRNAs for all *CAM* genes are significantly downregulated in roots and shoots except that *CAM1* and *CAM5* mRNAs are upregulated in shoots; *cam6-1,* where all *CAM* gene mRNAs are downregulated except *CAM3* mRNA in root and *CAM5* mRNA in shoot; and *CAM7-1*, where all *CAM* gene mRNAs are downregulated except *CAM5* mRNA in shoot which is upregulated by 2-fold.

The expression of nine Arabidopsis *CAM* genes in various *cam* mutants presented here provides evidence for complex interactions between *CAM* gene leading to the regulation of the mRNA levels of other *CAM* loci and the modulation of such interactions by heat. This finding is unexpected as T-DNA insertions in 3-UTRs, 5-UTRs, or introns often lead to loss of gene function. The data presented here elucidate a role of the 3'-UTR in many of the mutant alleles in the regulation of calmodulin mRNAs by heat stress. Additionally, both the expression of *CAM5* during heat stress and the regulation of *CAM5* gene mRNA by other *CAM* genes suggests the importance of this calmodulin gene during heat stress.

Calmodulins are involved in the regulation of many proteins under stress (Ma et al., 2004; Bouche et al. , 2005). Arabidopsis *PP7* (Ser/Ther Phosphatase) interaction with CaM regulates expression of HSP genes during heat stress (Liu et al. , 2007). Functional and regulatory interaction between phyA and phyB in light signaling was demonstrated in Arabidopsis (Casal and Mazella, 1998; Hennig et al., 1999; Neff and Chory, 1999). Because of a central role for CaM in plants under stress, it may be necessary to compensate the amount of CaM in *cam* mutants as an evolutionary necessity for plants to cope with continuously changing environmental cues. Complexity of Ca^{2+} signaling pathways involving competition among CaM binding target proteins for CaM isoforms may be responsible for transcriptional regulation of *CAM* genes.

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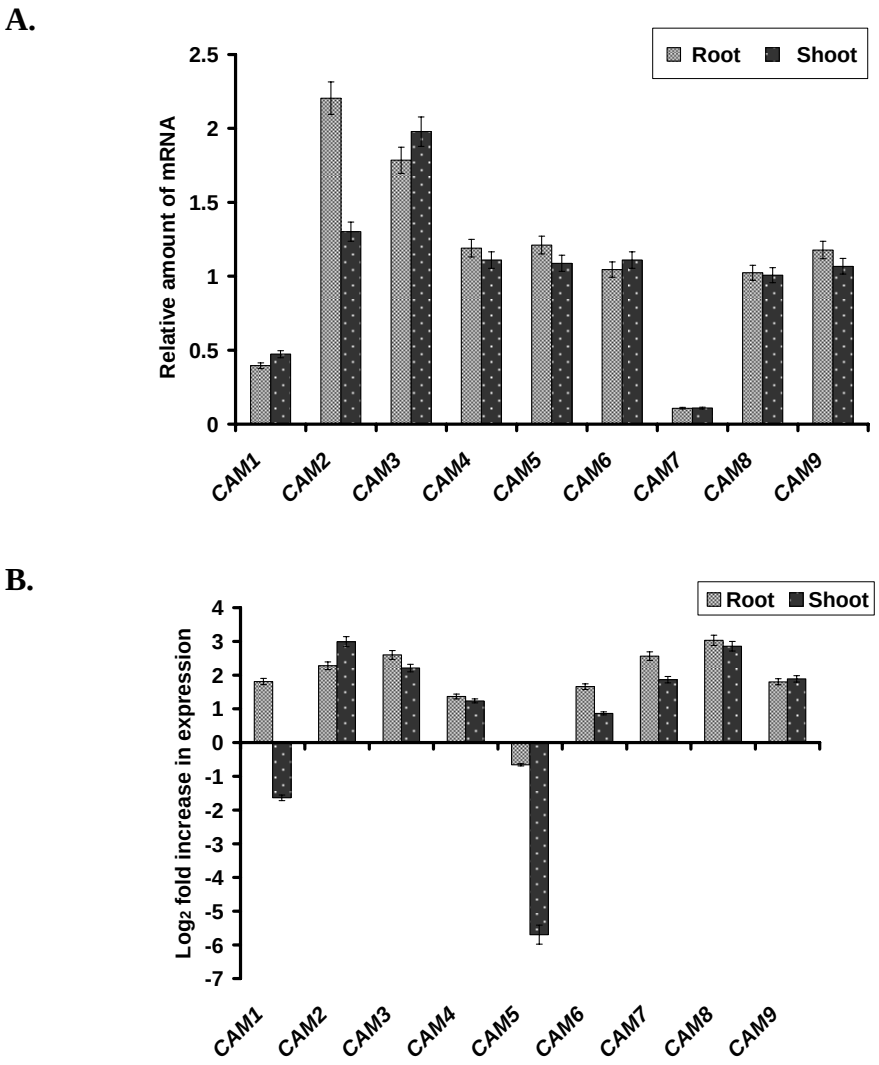

Figure 1. **Estimates of normalized levels of steady state mRNA of nine *CAM* genes in root and shoot tissues of wild type *A. thaliana* grown at 25°C (control) (A), and fold increase in the levels of nine *CAM* genes nRNAs in root and shoot tissues of wild type *A. thaliana* after 2 hr heat treatment at 42°C compared to growth at 25°C (B). Total RNA from root and shoot tissues of 2 week-old seedlings was used to perform RT-PCR according to procedure outlined in Materials and Methods. *CPB20* was used as an internal standard, and all mRNA levels were normalized to the save level of *CPB20* mRNA. The expression level of each *CAM* gene was calculated as outlined in materials and methods. Error bars represent SD of three replicate samples from which RNA was isolated.**

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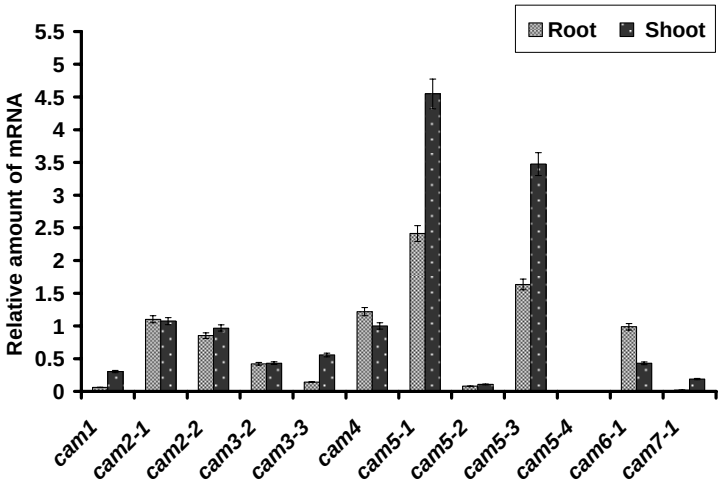

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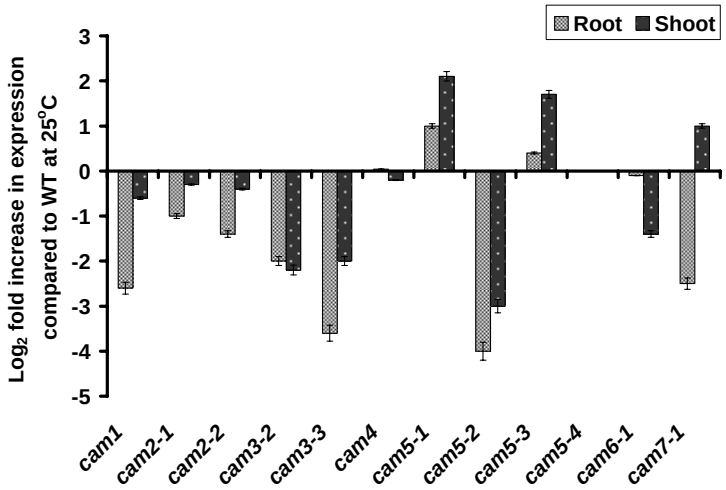

Figure 2. **Extimates of normalized steady state mRNA levels of nine *CAM* genes in root and shoot tissues of the corresponding *cam* mutants of *A. thaliana* at 25°C (A) and the fold increase in the expression of *CAM* genes in root and shoot tissues of corresponding *cam* mutants compared to its expression in wild type at 25°C (B). Total RNA from root and shoot tissues of 2 week-old control and heat-treated seedlings were used to perform RT-PCR using gene specific primers as described in Materials and Mehtods. *CPB20* was used as an internal control.**

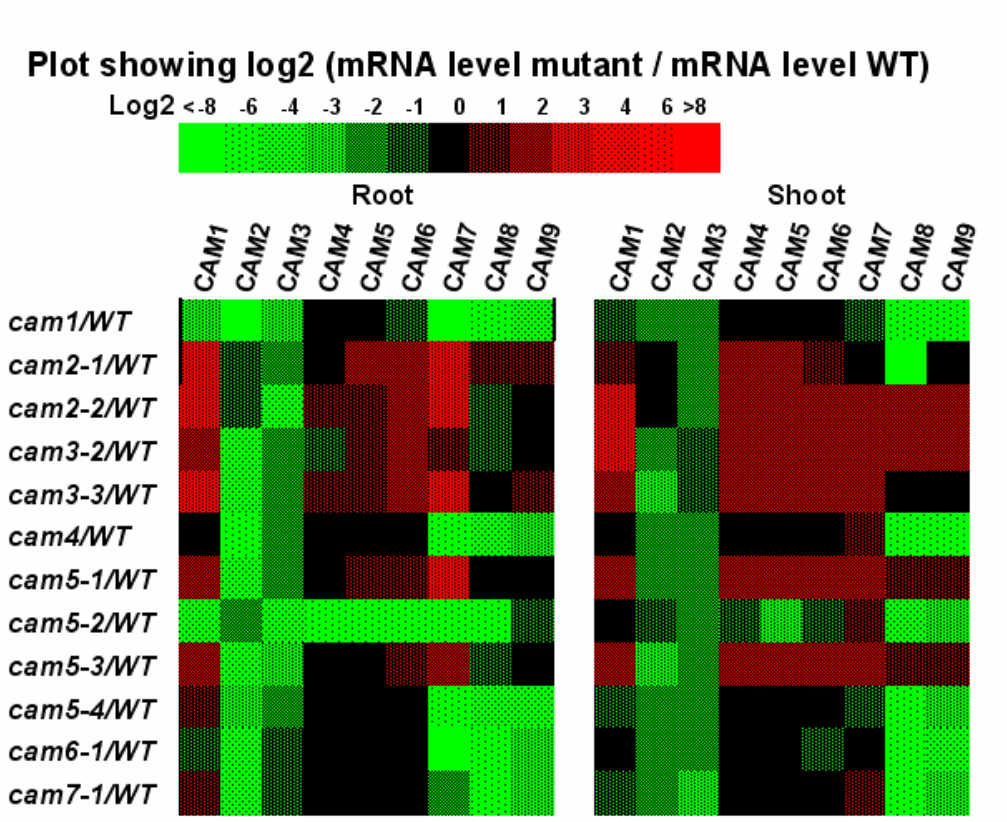

Figure 3. **The fold change of the nine Arabidopsis *CAM* gene mRNAs in root and shoot tissues of each of the *cam* alleles of seven of the *CAM* genes compared to wild type. The color scale show indicates the colors corresponding to a decrease in mRNA level in the mutant relative to wild type (green) or an increase in mRNA level in mutant relative to wild type (red). mRNA levels were measured as described in Materials and methods using *CPB*20 as an internal standard.**

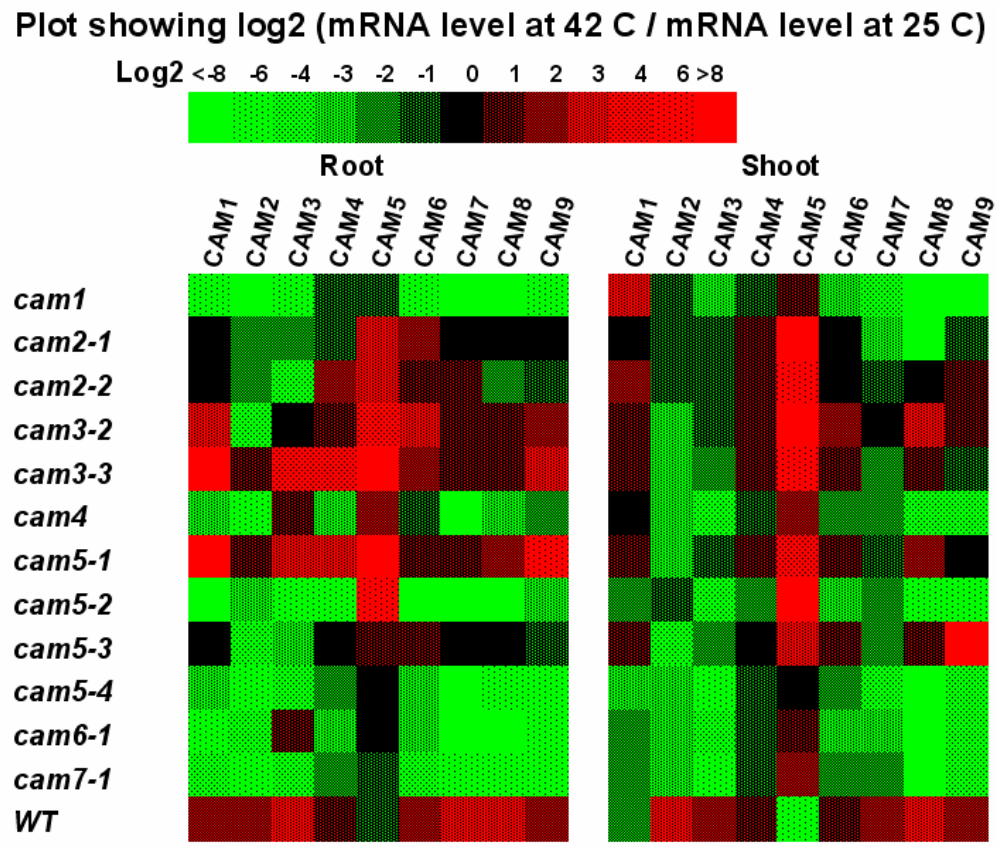

Figure 4 . The fold change in the level of mRNA for the nine Arabidopsis *CAM* gene mRNAs in root and shoot tissues of each of the *cam* alleles of seven of the *CAM* genes and wild type after 2 hr heat treatment at 42ºC compared to growth at 25ºC. The color scale show indicates the colors corresponding to a decrease in mRNA level in the mutant relative to wild type (green) or an increase in mRNA level in mutant relative to wild type (red). mRNA levels were measured as described in Materials and methods using *CPB20* as an internal standard.

Table 1. **Mutant lines with T-DNA insertion in calmodulin genes of *A. thaliana* used in this study. All seeds lines were obtained from Dr. Janet Braam, Rice University, except *cam1* and *cam4* seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus.**

Mutant	Insertion Location	SALK Line #
cam1	3`UTR	SALK_107507
cam2-1	Intron	SALK_066990
cam2-2	Intron	SALK_089283
cam3-2	5`UTR	SALK_075669
cam3-3	3`UTR	SALK_042391
cam4	5`UTR	SALK_149142
cam5-1	3`UTR	SALK_007371
cam5-2	3`UTR	SALK_073480
cam5-3	5`UTR	SALK_138758
cam5-4	Exon II	SALK_027181
cam6-1	3`UTR	SALK_071609
cam7-1	3`UTR	SALK_074336

Table 2. **Nucleotide sequence of primers for *CAM* genes and CBP20 gene used as an internal standard for RT-PCR amplification.**

CAM gene	Gene locus	Primer sequences
CAM1	At5g37780	F: 5`AAAGAGAGCGACTCTGAAT 3`
		R: 5`AAAGCAACAAAATGTGGGTA3`
CAM2	At2g41110	F: 5`AGCTGATCTTCAGGACTTGA 3`
		R: 5`TCAGACCCTATTGGCATAAA 3`
CAM3	At3g56800	F: 5`TTTCCAGCAGAGACACTTTT 3`
		R: 5`CGCAAAAACATTCGCGTCTT 3`
CAM4	At1g66410	F: 5`CTTCTCTGAAACGAAAAACC 3`
		R: 5`CCGAAATCTAAACAGCCATT 3`
CAM5	At2g27030	F: 5`ACACACACCAACGTTGATT 3`
		R: 5`CCTGGTACTTCACATAATAT 3`
CAM6	At5g21274	F: 5`GAGAGGCAAATAATATATTC 3`
		R: 5`AGGGCATTGCTTTAAGATAA 3`
CAM7	At3g43810	F: 5`AAAGGAGAAGGAACATTCAC 3`
		R: 5`AAAGCAAATGATGAGTGCAA 3`
CAM8	At4g14640	F: 5`CAGAGATTTAAGCACTTCAAACC 3`
		R: 5CACGAAGTAGTAACAACTAACAAGG3`
CAM9	At3g51920	F: 5`CATGAGTATTGACCTTGACCG 3`
		R: 5`CGACCGAAAAAAATCATTG 3`
CBP20	At5g44200	F: 5`ATGGCTTCTTTGTTCAAGGAGC 3’
		R: 5`TTAAGATCTTCTCTTCCGATCATC3’

III. SENSITIVITY OF CALMODULIN MUTANTS OF *ARABIDOPSIS THALIANA* TO HIGH TEMRERATURE EXPOSURE

ABSTRACT

Arabidopsis mutants with T-DNA insertion in seven calmodulin genes (*CAM*) were used to determine the role of specific *CAM* genes in tolerance of plants to high temperature. Arabidopsis calmodulin mutant alleles (*cam*) were screened for survival, seed germination, heat-induced oxidative damage, and GABA shunt metabolite levels. Exposure of *cam* seedlings to 42^{o}C for 2 hr showed reduction in seed germination and survival of seedlings in *cam5-4* and *cam6-1* mutants compared to wild type (WT). Oxidative damage by heat measured as the level of malonaldehyde (MDA) was detected in root and shoot of most *cam* mutants with highest levels in *cam5-4* and *cam6-1*. The MDA level was reduced slightly in the root and shoot of *cam1*, the root of *cam2-2,* and the shoot of *cam4*. GABA shunt metabolites in both root and shoot were generally elevated after 30 min and 1 hr treatment at 42^{o}C, and increased substantially after 2 hr at 42^{o}C comparing to the control (no treatment). GABA and glutamate levels were increased significantly more than alanine in root and shoot tissues of all *cam* mutants and wild type compared to the control. After 30 min, the level of GABA and glutamate increased 0.2-1.5-fold and 0.2-2.5-fold respectively in all *cam* mutants and in wild type root and shoot tissues compared to the control. The shoot of *cam1* and *cam7-1* mutants

were exceptions to this trend. After 1 hr, the level of GABA and glutamate increased 0.7-3-fold and 1-4.3-fold respectively in all *cam* mutants and wild type root and shoot tissues. The root of *cam5-4* was an exception to this trend. After 2 hr at 42^{o}C, GABA levels increased in all *cam* mutants and WT up to 5-fold in the root *cam7-1* and in the shoot of *cam2-2*, *cam5-1*, *cam5-2*, and *cam5-4*. The glutamate level increased 3-11-fold in all *cam* mutants and WT with the highest levels observed in root of *cam5-4* and *cam6-1* (11 fold) and the shoot of *cam1* (3 fold). Alanine levels showed significant decreases in all *cam* mutants and in WT for 30 and 60 min of heat stress. Initial general elevation in GABA shunt metabolites after 30 min heat treatment in *cam* mutants suggests regulation of GABA level by CaM. A significant increase in GABA levels in *cam* mutants after 2 hr of heat treatment was observed. These data suggest that regulation by factors other than CaM is likely, and that this factor may relate to the regulation of GAD by intracellular pH and/or metabolite partitioning under heat stress.

Key words:

Arabidopsis, Calmodulin, *CAM*, CaM, Ca^{+2}, GABA, GAD, Heat stress, Metabolism, Thermotolerance

Abbreviations:

CAM: Calmodulin gene

CaM: Calmodulin protein

Ca^{+2}/CaM: Calcium/Calmodulin complex

GAD: Glutamate Decarboxylse

GABA: γ-Aminobutyric Acid

MDA: Malonaledehyde

NAD^{+}: Nicotinamide adenine dinucleotide

$NADP^{+}$: Nicotinamide adenine dinucleotide phosphate

TBARS: Thiobarbiturate reactive substances

INTRODUCTION

Plants have evolved strategies to prevent and repair damage caused by rapid changes in the surrounding temperature. Acquired thermotolerance is one such strategy and involves production of heat shock proteins (Vierling, 1991; Larkindale et al., 2005). Heat shock proteins act as molecular chaperons to protect cellular proteins against heat-induced dentauration and to facilitate refolding of heat-damaged proteins (Boston et al., 1996). The Hsp100 family proteins are essentials for the acquisition of thermotolerance in Arabidopsis (Hong and Vierling, 2000, 2001; Hong et al., 2003) and *Zea mays* (Nieto-Sotelo et al., 1999).

Activation of GABA shunt pathway in response to various stresses has been shown (Mayer et al., 1990; Bown and Shelp, 1997; Shelp et al., 1999; Locy et al., 2000). A rapid increase in GABA level in response heat in cultured cowpea cells (Mayer et al., 1990) and Arabidopsis (Locy et al., 2000), have been reported.

GABA is synthesized by a cytosol-localized Ca^{2+}-calmnodulin-dependent glutamate decarboxylase (GAD) protein (Baum et al., 1993; Snedden et al., 1996). Following irreversible decarboxylation from glutamate, GABA is metabolized to succinate semialdehyde bypassing two steps in the TCA cycle. This pathway is referred to as the GABA shunt. In plants, the last 2 steps of the GABA shunt take place in mitochondria. These steps are catalyzed by a GABA-transaminase using either α-ketoglutarate or pyruvate as amino group acceptor and succinate semialdehyde dehydrogenase (Shelp et al., 1999, Bouche and Fromm, 2004).

Ca^{2+}/CaM provides a possible level of control for the activation of the GAD enzyme activity (Shelp et al., 1997; Bouche and Fromm, 2004) since this protein is

demonstrates a calmodulin binding domain (Zik et al., 1998). Such activation could account for the control of the GABA shunt pathway and the concomitant accumulation of GABA during stress. Possible roles of the GABA shunt in processes such as carbon-nitrogen balance, cytosolic pH regulation, protection from damage by reactive oxygen, defense against insects, and plant growth and development have been suggested (Shelp et al., 1997; Kinnersley and Turano, 2000; Bouche and Fromm, 2004). A proline transporter (*At*ProT2 and *Le*ProT1) is able to recognize GABA as a substrate (Schwacke et al., 1999; Grallath et al., 2005), and the presence of an Arabidopsis high affinity GABA transporter (*At*GAT1) (Meyer et al., 2006) may play a role in intercellular or intracellular GABA transport.

We have used calmodulin T-DNA insertion mutants (*cam*) of Arabidopsis to examine the role of specific *CAM* genes in high temperature tolerance with respect to survival, germination, oxidative damage, and changes in the levels of GABA shunt metabolites.

MATERIALS AND METHODS

Plant material and growth conditions

The wild type and mutant lines of Arabidopsis (*Arabidopsis thaliana* Ecotype Columbia) bearing T-DNA insertions in various calmodulin genes (*cam*) used in this study are listed in Table 1. Wild type, *cam1* and *cam4* seeds were obtained from Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH, while seeds of all other mutants were obtained from Dr. Janet Braam, Rice University, Texas. Homozygous seed stocks were propagated for this study. Seeds were surface

sterilized with bleach (v/v, 6% sodium hypochlorite) for 10 min followed by five washes with sterile distilled water. Seeds were plated in Petri dishes on sterile 1X Murashige and Skoog (1962) medium (pH 5. 7) supplemented with 2% (w/v) sucrose, solidified with 1. 2% (w/v) agar, Seedlings were grown under continuous illumination (40µmol m^{-2} s^{-1}) provided by cool white fluorescent lamps at 25 ºC.

Seed germination

Surface sterilized seeds of WT and each of the *cam* mutants were suspended in 500 µL sterile distilled water and incubated at 42ºC in a water bath block for 2 hr. Each tube sample contained 50 seeds. Immediately after heat treatment seeds were plated on square Petri dishes containing media as described above and allowed to grow vertically under continuous light (40µmol m^{-2} s^{-1}) at 25 ºC for 7 days. Emergence of radicle from germinating seeds was recorded and compared to control without heat treatment. Percent germination of each *cam* mutant was compared to WT. The average of three replicate plates was used for each treatment.

Seedling survival

Two week old seedlings of WT and *cam* mutants grown under continuous light (40µmol m^{-2} s^{-1}) at 25 ºC were incubated at 42ºC 30 min, 1 hr, and 2 hr in a growth chamber. Heat treated seedlings were transferred to growth chambers for 5 days at 25^{o}C under continuous light before scoring for the survival. Green and growing seedlings were scored as surviving. An average of 3 replicates with fifty seeds on each plate were used.

Oxidative damage

Two sets of two week old seedlings of WT and *cam* mutants were heat treated at 42ºC for 2 hr. One set was used immediately, while the second set of seedlings was

placed under continuous light at 25°C for a 2 day recovery period. The level of malonaldehyce (MDA) in root and shoot tissues of seedlings exposed to heat and after recovery was determined using the TBARS assay (Heath and Packer, 1968). Three plates with 50 seeds each were used in each replicate of each treatment.

Root and shoot tissues after heat treatment were separated and frozen in liquid nitrogen. Tissue (0.50g) was ground in a 1.5mL microfuge tube, and 0.5mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and 0.5mL 175 mM NaCl in 50 mM Tris-HCl, pH 8 was added to the ground tissues. Tubes were heated to 90°C for 25 min. The supernatant was collected after the samples were centrifuged for 20 min at full speed. The absorbance of the supernatant was measured at 532 nm. The level of malonaldehyde (MDA) was determined as nmol/mgFW from a standard curve of MDA.

GABA-shunt metabolites

WT and *cam* seedlings of Arabidopsis grown at 25°C for two week in continuous light were exposed to 42°C in a growth chamber for 30 min, 1 hr, and 2 hr. Root and shoot tissues were separated and used for extraction for analysis of metabolites. Three plates with 50 seedlings on each plate were used for each sample.

Metabolites were extracted according to Zhang and Bown (1997) with the following modifications. Separately harvested root and shoot tissues were ground in 1.5 mL microfuge tubes under LN_2 until a fine powder was obtained. To each tube 400µL methanol was added, and the samples were mixed for 10 min. Liquid from samples were removed by vacuum drying. Five hundred µL of 70 mM lanthanum chloride was added to each tube: the tubes were mixed for 15min, and subsequently centrifuged at full speed for 5min. Supernatants was removed to new tubes and mixed with 160µL of 1 M KOH.

After 10min mixing, tubes were centrifuged at full speed for 5min. The supernatant containing metabolites was transferred to a new tube and used to determine the quantity of specific metabolites.

GABA (γ-aminobutyric acid) was measured according to Zhang and Bown (1997) with the following modifications. The reaction mixture contained 50µL of sample extract, 14 µL of 4 mM $NADP^+$, 19 µL of 0.5 M potassium pyrophosphate, pH (8.6), 10 µL of (2 u/µL) GABASE enzyme (GABASE enzyme was suspended in 0.1 M potassium pyrophosphate, pH 7.2 containing 12.5% Glycerol and 5 mM β-marcaptoethanol), and 10 µL of α-ketoglutarate. The change in absorbance at 340 nm after addition of α-ketoglutarate was recorded after 90 min incubation at 25^{o}C using the microplate reader (BioTek power wave, Max200R, USA). The level GABA nmol/mgFW was determined using an NADPH standard curve.

Alanine was measured according to Bergmeyer (1983) with the following modifications: the reaction contained 180 µL of 0.05 M Na-carbonate buffer pH (10), 7 µL of 30 mM β-NAD^+, 10 µL of sample extract, and 0.3 units of alanine dehydrogenase (Sigma-Aldrich) enzyme suspension. Changes in absorbance at 340 nm after addition of alanine dehydrogenase was recorded after 60 min incubation at 25^{o}C using a microplate reader (make and model). The level of alanine as nmol/mgFW was determined using an NADH standard curve.

Glutamate was measured according to Bergmeyer (1983) with the following modifications: the deamination reaction contained 180 µL of 0.1 M Tris-HCl, pH 8.3, 8 µL of 7.5 mM β-NAD^+, 10 µL of sample extract, and 0.8u nits of glutamate dehydrogenase enzyme suspension (Sigma-Aldrich). The level of glutamate was

determined at 340nm after 60 min incubation using a microplate reader. The level of glutamate was determined as nmol/mgFW using an NADH standard curve.

Data Analysis

Each data point is expressed as a mean ± standard deviation of the three independent experiments. The values were compared and analyzed by two-way ANOVA (analysis of variance) using LSD multiple comparison tests on the means. Where differences are reported, they are at the 95% confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Sensitivity of *cam* seeds and seedlings to heat exposure

Except for *cam5-4* and *cam6-1*, seeds of the other Arabidopsis *cam* mutants exposed to 42°C for 2 hr germinated normally (data not shown). Germination of *cam5-4* and *cam6-1* seeds was inhibited by 45% and 50% after exposure to 42°C for 2 hr, respectively (Figure 1).

Survival of 2 hr heat treated seedlings after 5 days of recovery at 25°C showed a significant reduction in seedlings survival of *cam5-4* and *cam6-1* (Figure 2). Survival of *cam5-4* seedlings after 2 hr of growth at 42°C was reduced by 40% while *cam6-1* seedlings showed a reduction in survival by 30% after 2 hr at 42°C. Seedling survival of all other *cam* mutants was comparable to wild type seedlings (data not shown).

The significant ($P<0.05$) sensitivity of *cam6-1* and *cam5-4* alleles may be explained by the fact that the protein products of the *CAM5* and *CAM6* genes may be involved in the protection of plants from heat stress involved in protective pathways that contribute to germination and survival of plants at high temperatures.

The nature of the *cam5-4* mutant allele was investigated by examining the mRNA level of all of the *CAM* genes produced in this mutant, and it was observed that the *cam5-4* exonic insertion mutant fails to produce detectable levels of *CAM5* mRNA and produces either the same or reduced levels of the other *CAM* gene mRNAs (see Chapter II, Figure 4). Although the level of *CAM6* mRNAs in the *cam6-1* mutant is reduced it is not eliminated, but the levels of other *CAM* gene mRNAs are either equal to wild type levels or reduced comparable to those in *cam*5-4 (see chapter II, Figure 4). However, the *CAM* gene mRNA levels observed in *cam1* and *cam4* alleles are equally low overall, and the results do not allow an explanation of the seed germination phenotype of *cam6-1* on the basis of *CAM* gene expression alone (see Chapter II, Figure 4).

The sensitivity of the *cam5-4* mutant to heat treatment may due to the absence of CaM5 protein in this mutant, but since the mutation in the *cam6-1* allele did not eliminate *CAM6* gene expression the heat stress sensitivity of the *cam6-1* mutant (and possibly the *cam5*-4 allele) may be due to pleiotrophic effects of one or both of these mutations rather than a direct effect on the expression of these genes.

Oxidative damage by high temperature in *cam* mutants

Malonaldehyde (MDA) is a secondary end product of the oxidation of polyunsaturated fatty acids and is a useful index of general lipid peroxidation and ROS production *in situ* (Hodges et al., 1999, Rael et al., 2004). WT and *cam* mutants were analyzed for the accumulation of malonaldehyde (MDA) as nmol/mgFW. The level of MDA was determined in root and shoot tissues immediately after heat treatment and after recovery for 2 days at 25^{o}C under continuous light. Seedlings of *cam5-4* and *cam6-1* demonstraed dramatically higher levels of MDA in both root and shoot, and the level of

MDA remained significantly higher after 2 days of recovery (Figure 3). Root tissues of *cam2-1*, *cam3-2*, and *cam3-3* demonstrated elevated levels of MDA after heat treatment and recovery compared to wild type, while roots of *cam4* and *cam7-1*showed reduced MDA production after the recovery period. The accumulation of MDA in roots of *cam1* and *cam2-2* was appreciably lower than wild type after the stress period, but was equivalent to wild type after recovery. Shoot tissues of *cam5-2*, *cam5-3* and *cam7-1* had higher MDA after heat treatment and recovery. While *cam2-1*, *cam2-2*, *cam3-2*, *cam3-3*, and *cam5-1* shoots showed lower MDA after recovery. Shoots of *cam1* and *cam4* had reduced MDA compared to wild type after heat treatment and recovery.

The production of ROS and the oxidative damage phenotype of *cam5-4* and *cam6-1* as a result of heat treatment are consistent with the germination and survival sensitivity phenotype of the alleles examined. These results possibly delineate ROS production as a part of the pleiotrophic mechanism involved in reduced thermotolerance of *cam5-4* and *cam6-1*. Photo-peroxidation damage and ROS production have been observed after a wide range of stresses in other system, and thus this finding is consistent with these previously presented results (Foyer et al., 1994; Harndahl et al., 1998; Lu and Zhang, 1999). Under heat stress oxidative damage has been observed in creeping bentgrass (*Agrostis stolonifera*) (Larkindale and Huang, 2004), and Arabidopsis seedlings (Larkindale and Knight, 2002; Larkindale et al., 2005; Larkindale and Vierlang, 2008). Heat induced oxidative damage increased in Arabidopsis seedlings pretreated with Ca^{+2}/CaM inhibitors (Larkindale et al., 2002), and higher CaM proteins levels have been linked to lower levels of heat-induced membrane damage in maize (Gong et al., 1997). The oxidative damage (high MDA level) in the *cam5-4* is directly consistent with these

observations provided that the *CAM5* gene product is the only CAM required for thermal protection. However, the results obtained here with the *cam6-1* mutant suggest a more pleotrophic explanation of the results presented here.

Levels of GABA shunt metabolites in response to high temperature in *cam* mutants

The levels of GABA shunt metabolites (L-glutamate, GABA, and L-alanine) in wild type and 11 alleles of seven of the nine Arabidopsis *CAM* genes were determined after 0 min, 30 min, 1 hr, and 2 hr at 42°C. GABA shunt metabolites in both root and shoot were generally elevated after 30 min and 1 hr of heat treatment at 42°C, and increased substantially after 2 hr at 42°C. Furthermore, GABA and glutamate levels were increased significantly more than alanine in root and shoot tissues of wild type and all *cam* mutants.

After 30 min, the level of GABA (0.2-1.5 fold increase) and glutamate (0.2-2.5 fold increase) were significantly increased in all *cam* mutants and in wild type root and shoot tissues compared to the control except in the shoot of *cam1* and *cam7-1* mutants (Figure 4B). After 1 hr, level of GABA (0.7-3 fold increase) and glutamate (1-4.3 fold increase) were significantly increased in all *cam* mutants and wild type root and shoot tissues compared to the control except in root of *cam5-4* (Figure 4C). After 2 hr at 42°C, the GABA level increased in all *cam* mutants, and WT increased up to 5-fold in root *cam7-1* and shoot of *cam2-2*, *cam5-1*, *cam5-2*, and *cam5-4* compared to the control. Glutamate levels increased in all *cam* mutants and WT (3-11 fold increase) with highest level in root of *cam5-4* and *cam6-1* (11-fold) and shoot of *cam1* (3-fold) by comparison to the glutamate level in the untreated sample (figure 4D).

Alanine level did not increase in WT or any of the *cam* mutants during heat treatment (Figure 4). Our results showed that CaM may involve the regulation and partitioning of GABA shunt metabolites in root and shoot tissue under heat treatments.

GABA accumulation under various abiotic stresses is well documented and studied in various plants system (Hanower and Brazozowska, 1975; Wallace et al., 1984; Mayer et al., 1990; Locy et al., 2000; Mazzucotelli et al., 2006; Kaplan, 2007). The data presented here are consistent with the regulation of GABA shunt pathway by calcium and calmodulin by several possible regulatory mechanisms such as: regulation of GAD activity to produce GABA in response to heat stress in the cytosol, regulation of the translocation of GABA across the mitochondrial membrane for conversion into alanine or glutamate, regulation of the level and the pattern of GABA, alanine and glutamate accumulation in root and shoot tissues, and involvement of the translocation of metabolites between the root and shoot under heat stress.

The GABA shunt and Ca^{+2}/CaM signaling through CaM isoforms may function in multiple pathways acting together to allow plant survival at high temperature. The prevention and repair or membrane and protein damage by ROS extends the ability to tolerate oxidative damage caused by heat stress. The exact balance between these components needed for survival depends both on the plant growth stage and on the duration and severity of heat stress in Arabidopsis seedlings.

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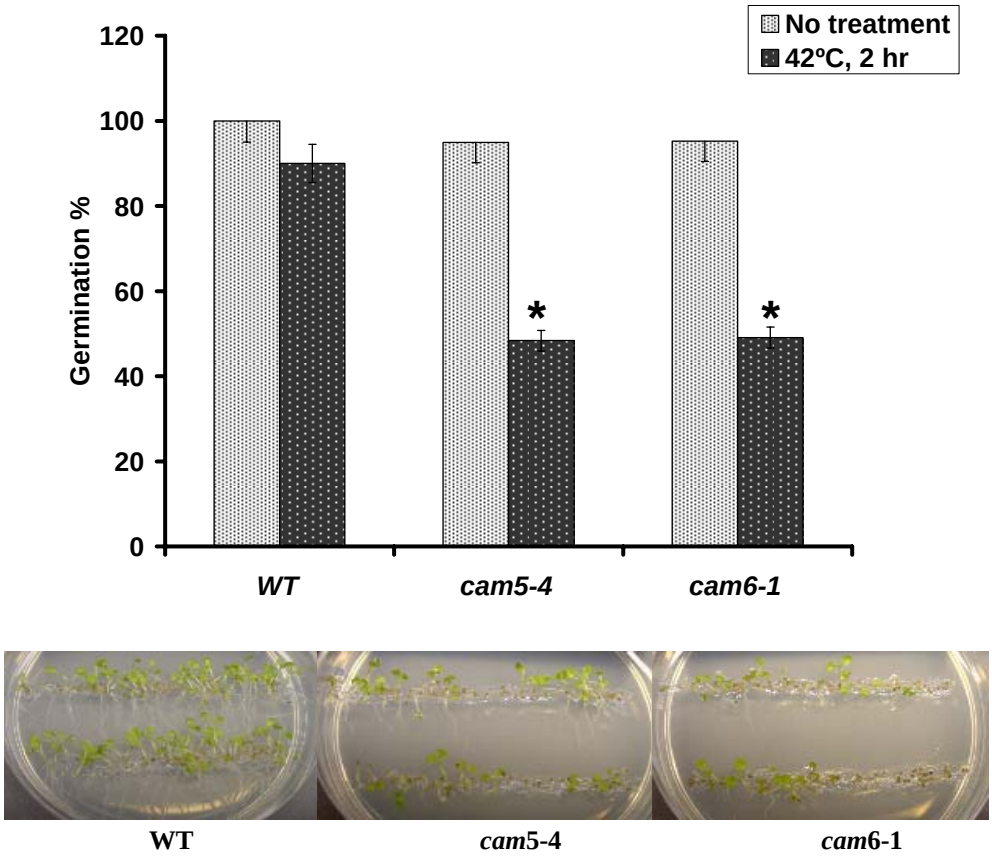


Figure 1: **Germination of *A. thaliana* seeds of wild type and T-DNA insertion mutant alleles *cam5-4* and *cam6-1* after exposure to 42ºC temperature. Fifty seeds each of genotype were surface sterilized and exposed to 42ºC for 2 hr. Heat treated and control seeds were allowed to germinate described in Materials and Methods. The number of germinating seeds was scored. Error bars represent the SD over three replicate plates.**

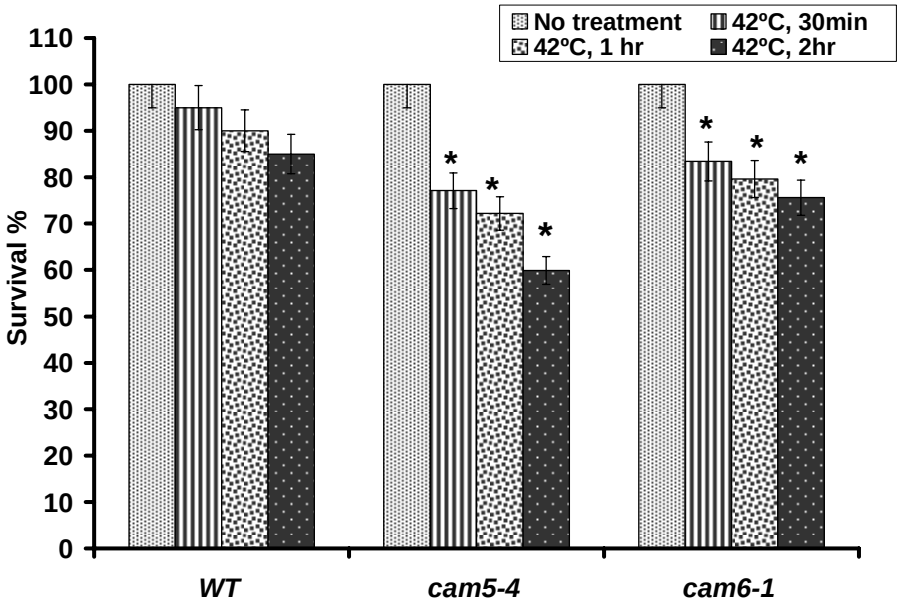


Figure 2: **Survival of *A. thaliana* wild type and T-DNA insertion mutant alleles *cam5-4* and *cam6-1* seedlings exposed to 42ºC heat. Two week old seedlings of each genotype were grown as described in Materials and Methods and exposed to 42°C for 30 min, 1 hr, and 2 hr. After heat treatment, plates containing seedlings were transferred to a growth chamber at 25°C with continuous light for 5 days. Percent survival of was determined. Error bars represent the SD over three replicate plates. Each plate contained 50 seedlings.**

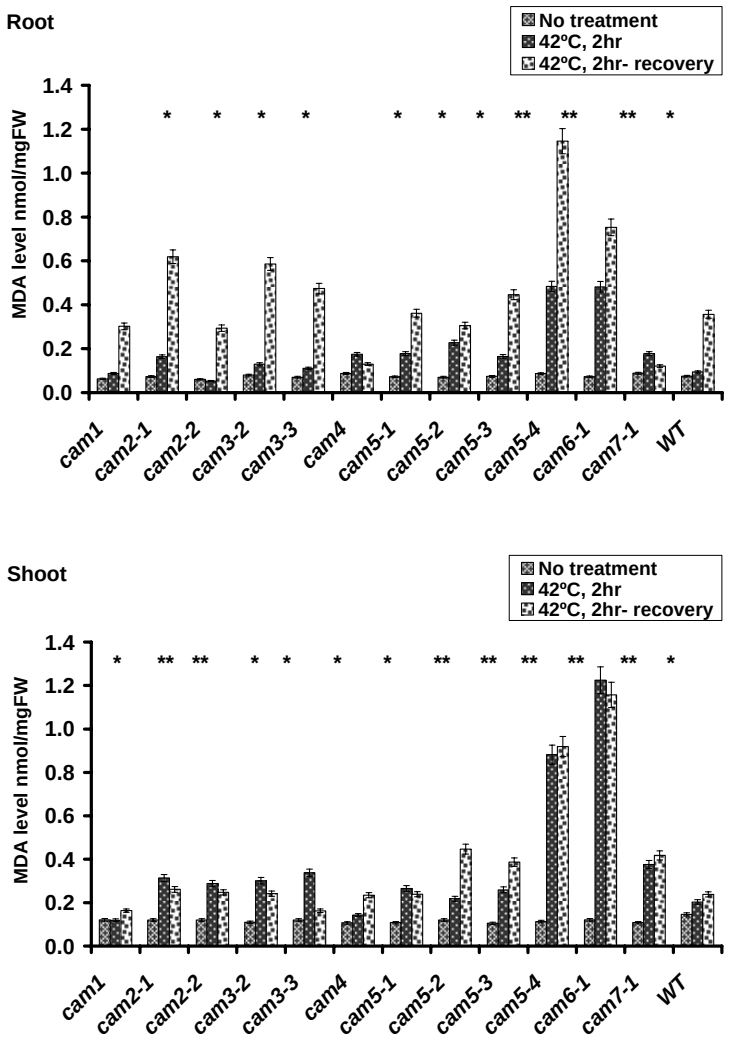
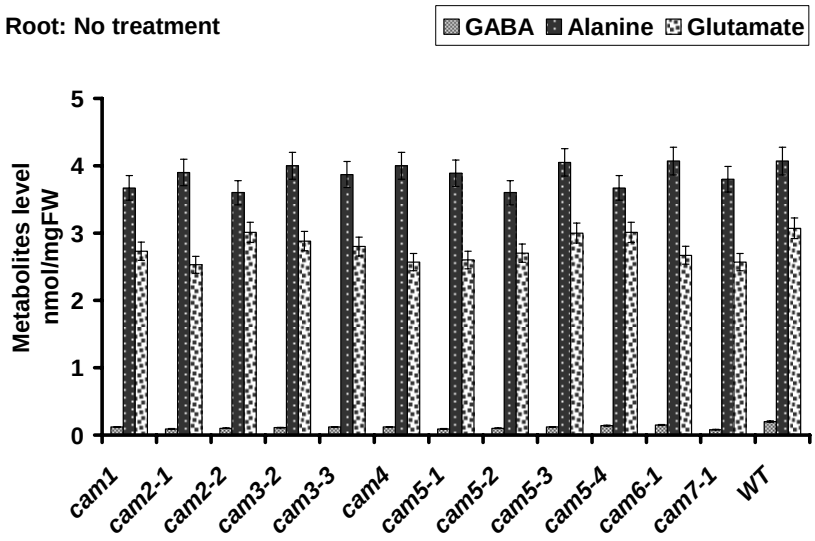
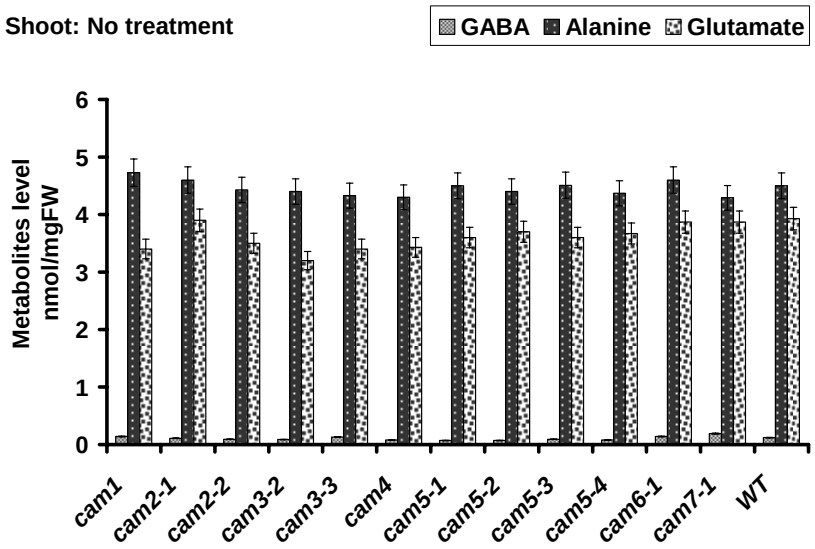


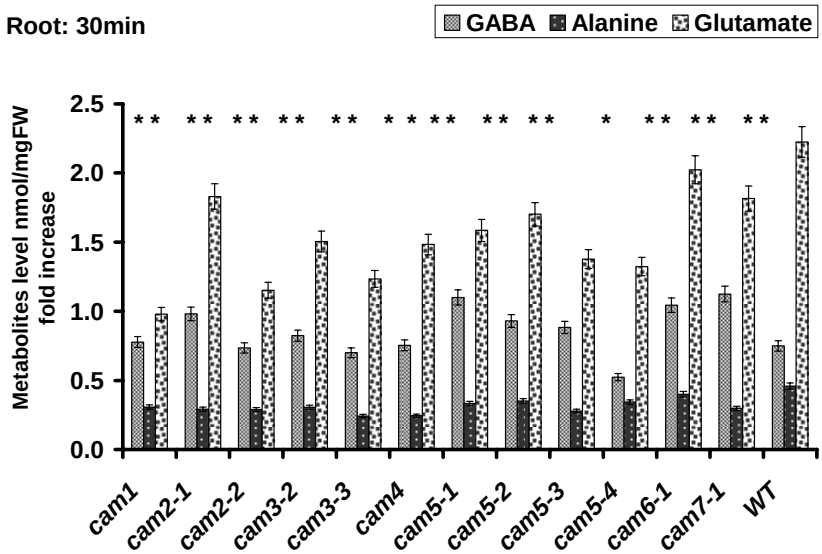
Figure 3: Oxidative damage by high temperature exposure in *cam* mutants of *A. thaliana*. The MDA level was determined in root and shoot tissues by the TBARS assay describe in materials and methods. Two set of seedlings were treated at 42ºC for 2 hr. One set was used immediately for measurement of MDA level, while the other set was allowed to recover for 2 days under continuous light at 25°C before determination of MDA level. Percent of MDA level in *cam* mutants and wild type in root and shoot tissues were determined. Error bars represent the SD over three replicate plates with 50 seedlings on each plate. Mutants with (*) represent the significant oxidative damage (P <0.05).

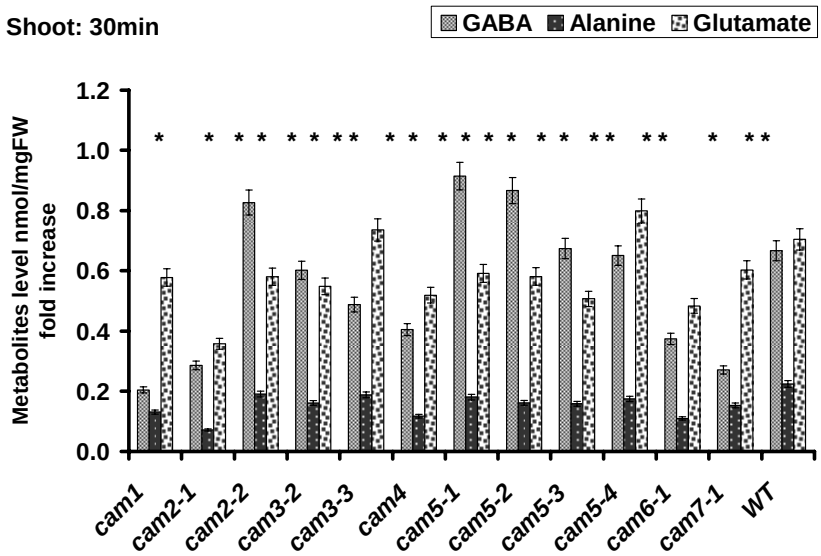
A.


Root: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

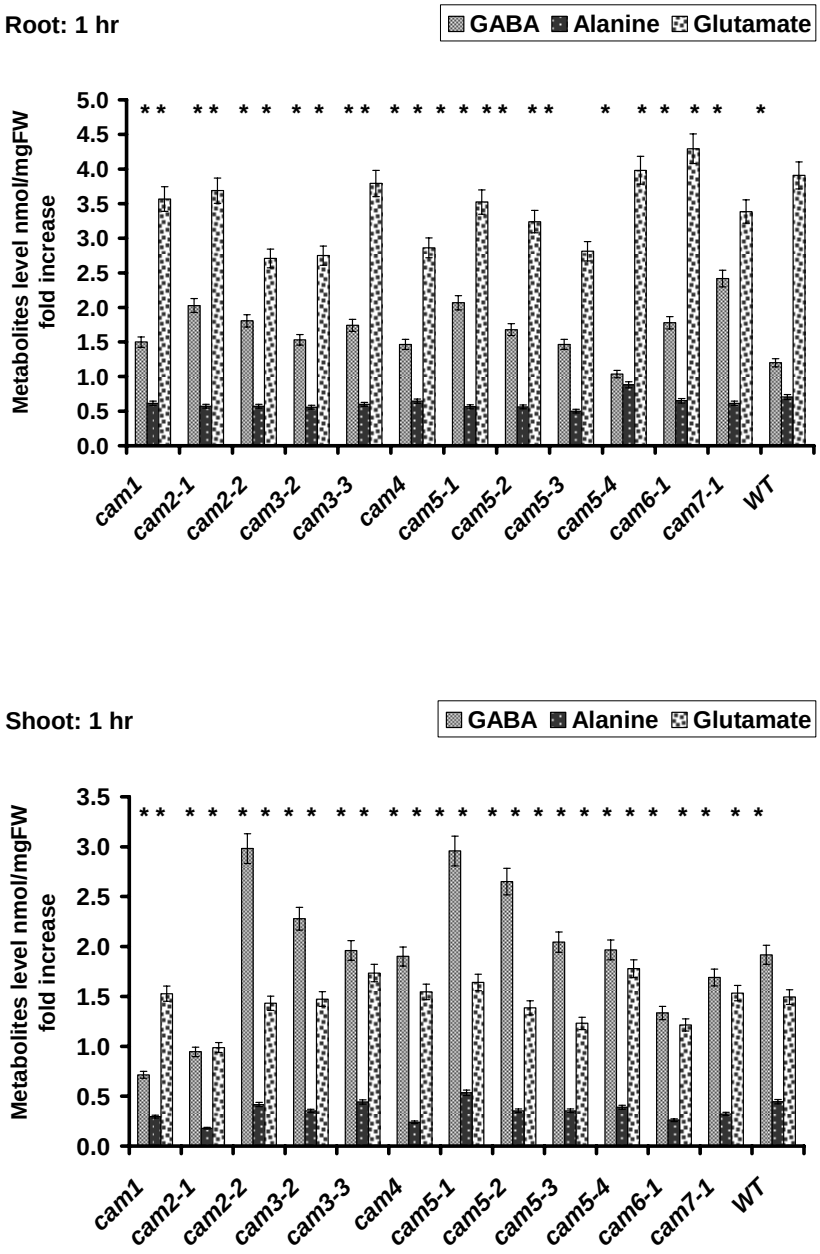

Shoot: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

B.

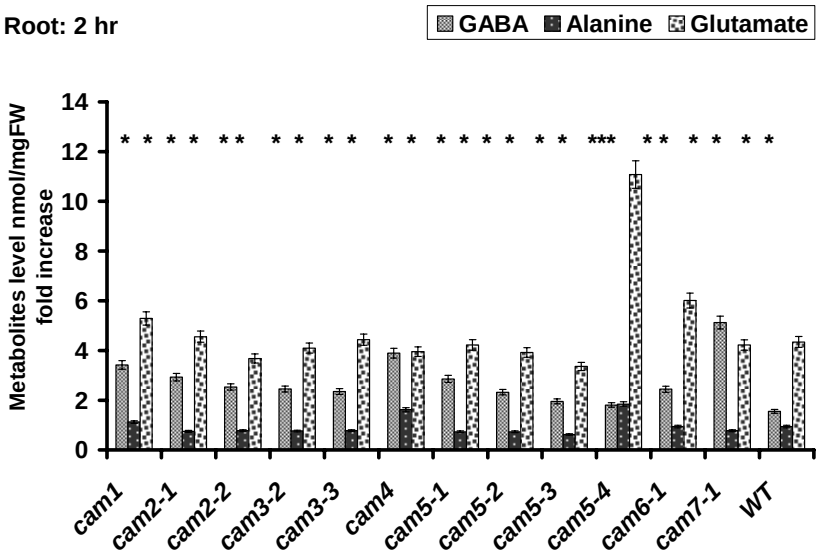

Root: 30min
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
2.5
2.0
1.5
1.0
0.5
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT


Shoot: 30min
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
1.2
1.0
0.8
0.6
0.4
0.2
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

C.


Root: 1 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT
Shoot: 1 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

D.


Root: 2 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0
2
4
6
8
10
12
14
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

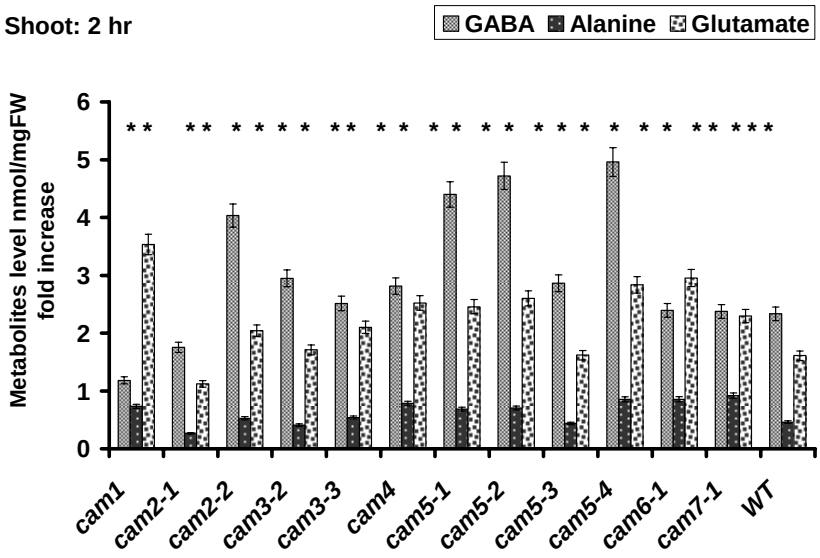

Shoot: 2 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

Figure 4: **The levels of GABA, alanine and glutamate in two-week-old seedlings of *cam* mutants of *A. thaliana* after exposure to heat at 42^{o}C for 30 min, 1 hr and 2 hr. Root and shoot tissues were harvested separately and frozen in liquid nitrogen for determination of the level of metabolites as described in materials and methods. Metabolite levels in wild type and *cam* mutants were calculated and expressed as nmol/mgFW fold increase in root and shoot tissues. A, No treatment B, 30 min. C, 1 hr. D, 2 hr. Error bars represent SD over three replicate plates. Each sample contained 50 seedlings. Mutants with (*) represent the significant level of changes in metabolite levels (P <0.05).**

Table 1. **Mutant lines with T-DNA insertion in calmodulin genes of *A. thaliana* used in this study. All seeds lines were obtained from Dr.Janet Braam, Rice University, except *cam1* and *cam4* seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus.**

Mutant	Insertion Location	SALK Line #
cam1	3`UTR	SALK_107507
cam2-1	Intron	SALK_066990
cam2-2	Intron	SALK_089283
cam3-2	5`UTR	SALK_075669
cam3-3	3`UTR	SALK_042391
cam4	5`UTR	SALK_149142
cam5-1	3`UTR	SALK_007371
cam5-2	3`UTR	SALK_073480
cam5-3	5`UTR	SALK_138758
cam5-4	Exon II	SALK_027181
cam6-1	3`UTR	SALK_071609
cam7-1	3`UTR	SALK_074336

IV. SENSITIVITY OF CALMODULIN MUTANTS OF *ARABIDOPSIS THALIANA* TO LOW TEMPERATURE EXPOSURE

ABSTRACT

Plants have evolved mechanisms to cope with changes in surrounding temperatures. T-DNA insertions in seven calmodulin genes of *Arabidopsis thaliana* were used to investigate the role of specific calmodulin isoforms in tolerance of plants to low temperature for survival, seed germination, susceptibility to low temperature induced oxidative damage, and changes in the levels of GABA shunt metabolites. Exposure of wild type (WT) and *cam* mutant seeds at 4°C showed reduction in germination of *cam5-4* and *cam6-1* seeds. Survival of WT and *cam* seedlings was not affected by exposure to low temperature. After treatment at 4°C, MDA levels increased in root tissues of *cam2-2*, *cam2-3*, *cam4*, *cam5-4* and *cam6-1*, and in shoot tissues of *cam5-4* and *cam6-1*. Level of GABA shunt metabolites in seedlings were gradually increased after 1 hr and 3 hr treatments with maximum level after 6 hr and 12 hr. After 1 hr, the GABA shunt metabolite levels increased up to 1-fold compared to the control (no treatment) in root and shoot tissues of all *cam* mutants and the wild type. After 3 hr treatment at 4°C, GABA level increased 0.65-2.5-fold while alanine and glutamate shoed more modest increases. In response to 6 hr incubation at 4°C, GABA and glutamate increased 1.5-5-fold and alanine increased only 1-fold in root tissues of all *cam* mutants and the wild type compared to the control. In shoot tissues, alanine and glutamate increased at the same

level (0.5-fold) while GABA increased up to 2-fold in all mutants with maximum levels in shoot of *cam2-2*, *cam3-2*, *cam3-3*, *cam4*, *cam5-1*, *cam5-2* and *cam5-4* mutants. After 12 hr treatment at 4°C, GABA and glutamate increased 2-7-fold, and alanine levels increased 0.2-0.5-fold in root tissues of all *cam* mutants and the wild type. Similarly in shoot tissues, GABA and glutamate increased 0.5-3.5-fold, and alanine increased 0.2-0.5-fold in shoots of all the mutants and the wild type compared to the control. GABA levels increased 0.5-1.5-fold in shoots of *cam4*, *cam5-1*, *cam5-2*, and *cam5-4* mutants. Glutamate level increased (1-3.5-fold) in shoots of *cam1*, *cam2-2*, *cam3-2*, *cam3-3*, *cam4*, *cam5-1*, *cam5-2*, *cam5-3*, *cam5-4*, *cam6-1* and *cam7-1* mutants and the wild type. Sensitivity of *cam5-4* and *cam6-1* to low temperatures suggests a role of the *CAM5* and *CAM6* genes in seed germination and protection against cold induced oxidative damage. Increases in the level of GABA shunt metabolites in response to cold treatment after initial reduction in some *cam* mutants suggests a role for CaM in the activation of GAD after exposure to cold, while increased metabolite levels may indicate involvement of other factors like reduction in cytoplasmic pH in cold regulation.

Key words:

Arabidopsis, Calmodulin, *CAM*, CaM, Ca^{+2}, GABA, Cold stress, Metabolism, low Temperature

Abbreviations:

CAM: Calmodulin gene

CaM: Calmodulin protein

Ca^{+2}/CaM: Calcium/Calmodulin complex

GAD: Glutamate Decarboxylase

GABA: γ-Aminobutyric Acid

MDA: Malonaledehyde

NAD^{+}: Nicotinamide adenine dinucleotide

$NADP^{+}$: Nicotinamide adenine dinucleotide phosphate

TBARS: Thiobarbiturate reactive substances

INTRODUCTION

Plants are exposed to either rapid or gradual changes in surrounding temperature. Adaptation to low temperature is associated with many physiological and metabolic processes, which require changes at molecular and biochemical levels, and rearrangement of cell metabolism (Kaye and Guy, 1995). During cold acclimation changes in gene expression occur, especially increased expression of cold-responsive genes (*COR*). A great number of *COR* genes have been characterized from a variety of plant species, but only some of them code for proteins whose biochemical function is known, or can be predicted based on sequence similarity (Guy et al., 1985; Jung et al., 2003). Other factors that contribute to low temperature acclimation or tolerance such as changes in membrane lipid composition and accumulation of sugars do not necessarily rely on changes in gene expression and may involve post-translational modulation of enzymes (Cossins et al., 2002, Stitt and Hurry, 2002).

Activation of GABA shunt pathway in response to various stresses has been shown (Mayer et al., 1989; Bown and Shelp, 1997; Shelp et al., 1999; Locy et al., 2000). A rapid increase in GABA level in response to draught in cotton (Hanower and Brzozowska, 1975), heat in cultured cowpea cells (Mayer et al., 1990) and Arabidopsis (Locy et al., 2000), cold stress and mechanical damage in soybean (Wallace et al., 1984) and in wheat and barely (Mazzucotelli et al., 2006) have been reported.

GABA is synthesized by a cytosol-localized Ca^{2+}-calmnodulin-dependent glutamate decarboxylase (GAD) protein (Baum et al., 1993; Snedden et al., 1996). Following irreversible decarboxylation from glutamate, GABA is metabolized to succinate semialdehyde by the GABA shunt pathway which bypasses two steps in the

TCA cycle. The last two enzymes of the GABA shunt, a GABA-transaminase using either α-ketoglutarate or pyruvate as amino group acceptor and succinate semialdehyde dehydrogenase (Shelp et al., 1999, Bouche and Fromm, 2004), are located in the mitochondria. Ca^{2+}/CaM-dependent modulation of GAD provides activation of GAD enzyme activity, and thus GABA shunt pathway, leading to GABA accumulation during stress.

We have used calmodulin T-DNA insertion mutants of *A. thaliana* to examine the role of specific calmodulin protein in tolerance of Arabidopsis to low temperature with respect to survival of seedlings, seed germination, and oxidative damage and GABA shunt metabolite.

MATERIALS AND METHODS

Plant material

Wild type (WT) (*Arabidopsis thaliana* Ecotype Columbia) and T-DNA insertions in seven calmodulin genes (*cam*) (also in *Arabidopsis thaliana* Ecotype Columbia) used in this study are listed in Table 1. Seeds were obtained from Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH, and from Dr. Janet Braam, Rice University, TX. Homozygous stocks were propagated for use in these stduies.

Seeds were surface sterilized with 100% bleach (v/v, 6% sodium hypochlorite) for 10 min followed by five washes with sterile distilled water. Seeds were plated on sterile 1XMS (Murashige and Skoog, 1962) medium (pH 5.7) on Petri dishes supplemented with 2% (w/v) sucrose and solidified with 1.2% (w/v) agar. Petri dishes were placed under continuous light (40μmol m^{-2} s^{-1}) at 25 °C for seed germination and growth of seedlings.

Seed germination

Surface sterilized seeds of WT and *cam* mutants were suspended in 500 µL of sterile distilled water and incubated at 4°C for 24 hr. Treated seeds were plated on 1X MS Petri dish plates. Plates were vertically placed under continuous light for 7 days at 25°C. Radicle emergence was recorded after 7 days and compared to control seeds without treatment and WT. For each line average of three plates each with 50 seeds were used.

Seedling survival

Two week old seedlings of WT and *cam* mutants were incubated at 4°C for 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr. Seedlings were allowed to recover under continuous light for 5 days at 25°C. Healthy seedlings with green leaves were scored as surviving. For each mutant and WT the average of three plates each with 50 seeds was determined.

Oxidative damage

Two set of two week old seedlings of WT and *cam* mutants were exposed to 4°C for 12 hr in growth chamber. Seedlings from one set were used immediately after treatment for determination of malonaldehyde (MDA) level using the TBARS assay in root and shoot tissues (Heath and Packer, 1968). The second set of seedlings was allowed to recover under continuous light for 2 days at 25°C before determination of MDA levels. Three plates with 50 seedlings each were used for each sample. Root and shoot tissues were separated and frozen in liquid nitrogen before extraction and determination of MDA level.

GABA-shunt metabolites

Two weeks old seedlings of WT and *cam* mutants grown under continuous light on 1X MS agar plates at 25°C were transferred to growth chamber at 4°C for 1 hr, 3 hr, 6 hr and 12 hr. Root and shoot tissues were separated and used for metabolite analysis. Three plates with 50 seeds each were used for each sample. Metabolites from frozen tissues were extracted according to Zhang and Bown (1997). Alanine and glutamate levels were determined according to Bergmeyer (1983) and the level of GABA was determined according to Zhang and Bown (1997) at 25°C using a microplate reader.

Data Analysis

Each data point is expressed as means ±SD of the three independent experiments. The values were compared and analyzed by two-way ANOVA (analysis of variance) using LSD multiple comparison tests on the means. Where differences are reported, they are at the 95% confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Sensitivity of *cam* Mutants to cold treatment

Seeds of the *cam6-1* and *cam5-4* alleles exposed to low temperature at 4°C for 24 hr showed 65% and 50% reduction in germination respectively, while seeds of all other *cam* mutants and WT showed germination rates between 85-93% (Figure 1). Exposure of two-week-old seedlings of WT and cam mutants to 4°C for 24 hr showed no significant between WT and any of the *cam* mutants.

The significant ($P<0.05$) sensitivity of *cam6-1* and *cam5-4* alleles to low temperature germination suggests that the *CAM5* and *CAM6* genes may be directly or indirectly involve in cold stress tolerance in Arabidopsis.

The nature of the *cam5-4* mutant allele was investigated by examining the mRNA level of all of the *CAM* genes produced in this mutant, and it was observed that the *cam5-4* exonic insertion mutant fails to produce detectable levels of *CAM5* mRNA and produces either the same or reduced levels of the other *CAM* gene mRNAs (see Chapter II, Figure 4). Although the level of *CAM6* mRNAs in the *cam6-1* mutant is reduced it is not eliminated, but the levels of other *CAM* gene mRNAs are either equal to wild type levels or reduced comparable to those in *cam5-4* (see chapter II, Figure 4). However, the *CAM* gene mRNA levels observed in *cam1* and *cam4* alleles are equally low overall, and the results do not allow an explanation of the seed germination phenotype of *cam6-1* on the basis of *CAM* gene expression alone (see Chapter II, Figure 4). Since *CAM5* is not expressed in the *cam5-4* the results presented here are consistent with *CAM5* having a direct role in cold tolerance, while *CAM6* acts pleiotrophically rather than directly in thermotolerance.

Oxidative damage by low temperature in *cam* mutants

All *cam* mutants and the wild type were assayed for the accumulation of thiobarbituric acid reactive substances (TBARS) by measuring the accumulation of malonaldehyde (MDA) in root and shoot under cold treatment at 4°C for 12 hrs and after recovery for 2 days under continuous light. In response to cold treatment MDA level accumulated significantly in many *cam* mutants both immediately and after recovery in root and shoot tissues (Figure 2).

A significant MDA accumulation was not observed in the *cam1* mutant while in *cam2* alleles only *cam2-1* accumulated MDA in root tissue up to 0.4 fold after recovery and the *cam2-2* allele accumulated MDA 1.5-fold, ($p<0.05$) only in root compared to wild type. The *cam3-3* allele accumulated MDA 3.5-4-fold ($P<0.05$) in root after immediate assay compared to wild type, and the *cam4* mutant showed 4–fold ($P<0.05$) in roots immediately and showed a 0.4-fold increase in the recovery assay. The *cam5-1*, *cam5-2,* and *cam5-3* mutants accumulated MDA up to 0.5-fold in root after the recovery assay while the *cam5-4* allele showed a significant 4 to 5 fold after recovery in both root and shoot when compared to wild type. For *cam6-1* MDA accumulated 1.5-fold in both root and shoot tissues but not when assayed after recovery. The *cam7-1* allele had no phenotype in either the root or shoot (Figure 2).

The production of ROS by the *cam5-4* allele during cold treatment was consistent with the germination phenotype of this mutant. However, the production of TBARS reactive substances does not produce a reasonable explanation of the increased cold sensitivity of *cam5-4* allele since the accumulation of MDA was not greater in tissues of *cam5-4* than in other *cam* mutants which were not more thermo-sensitive. The MDA accumulation that we observed in *cam* mutants and wild type especially in root tissues might be modulated by disruption of a mitochondrial function and changes in membrane fluidity as a result of cold treatment (Suzuki and Mittler, 2006).

Level of GABA shunt metabolites in *cam* mutants after cold treatment

Since GABA shunt pathway has been reported to be activated following cold stress in plant systems (Wallace et al., 1984; Mazzucotelli et al, 2006), the levels of

glutamate, alanine, and GABA in roots and shoots during cold treatment at 4°C was determined (figure 3).

The levels of the three amino acids gradually increased after 1 hr of cold treatment with a maximum level reached after 6 hr and 12 hr of treatment. After 1 hr, GABA shunt metabolite levels increased up to 1-fold compared to the control (no treatment) in root and shoot tissues of all *cam* mutants and the wild type. In root tissue, glutamate levels increased (0.8-1-fold) while GABA and alanine levels showed a smaller increase (0.2-0.6-fold). The maximum increase occurred in root of *cam2-1*, *cam3-3*, *cam4*, *cam5-1*, *cam5-2*, *cam6-1* and the WT (Figure 3B). In shoot tissue, GABA levels increased (0.7-fold increase) to a greater extent than alanine and glutamate (0.2-0.5-fold increase) with higher levels in shoot of *cam2-2*, *cam4*, *cam5-1*, *cam5-2* and *cam5-4* mutants (Figure 3B).

After a 3 hr cold treatment at 4°C, GABA levels increased 0.65-2.5-fold while alanine and glutamate increased less (0.5-1.5-fold) in all mutants and the wild type and in both root and shoot tissue compared with the control. GABA increased in root tissue of *cam1*, *cam2-1*, *cam5-1* and *cam7-1* and in shoot tissue of *cam2-2*, *cam3-2*, *cam4*, *cam5-1*, *cam5-2*, and *cam5-4* mutants. Alanine and glutamate increased in all *cam* mutants and the wild type greatest increases in roots of *cam3-3*, *cam4*, *cam5-1*, *cam5-2* and *cam6-1* and shoots of *cam2-2*, *cam3-2*, *cam4*, *cam5-1*, *cam5-2* and *cam5-4* mutants (Figure 3C).

After 6 hr incubation at 4°C, GABA and glutamate were increased 1.5-5-fold while the alanine level only increased 1-fold in root tissues of all *cam* mutants and the wild type compared to the cold untreated control. GABA levels increased 2-3-fold in roots of *cam1*, *cam2-1*, *cam4*, *cam5-1*, and *cam7-1* mutants. Glutamate levels increased

3-5-fold in roots of the *cam1*, *cam3-3*, *cam4*, *cam5-1*, *cam6-1* and *cam7-1* mutants (Figure 3D). In shoot tissues, alanine and glutamate increased 0.5-fold while GABA increased up to 2-fold in all mutants with maximum increases in shoots of the *cam2-2*, *cam3-2*, *cam3-3*, *cam4*, *cam5-1*, *cam5-2* and *cam5-4* mutants as shown in Figure 3D.

After 12 hr of cold treatment at 4°C, GABA and glutamate accumulated 2-7-fold while alanine accumulated to a lower level of 0.2-0.5-fold increase in root tissues of all *cam* mutants and the wild type compared to the cold untreated control. GABA levels increased 2-7-fold in roots of *cam1*, *cam2-1*, *cam4*, *cam5-1*, *cam5-4* and *cam7-1* mutants. Glutamate levels increased 3-7-fold in roots of *cam3-3*, *cam5-1*, *cam5-2*, *cam6-1* and *cam7-1* mutants (Figure 3E). Similarly in shoot tissues, GABA and glutamate increased 0.5-3.5-fold while alanine increased 0.2-0.5-fold in shoots of all the mutants and the wild type compared to the control. GABA levels increased 0.5-1.5-fold in shoots of *cam4*, *cam5-1*, *cam5-2*, and *cam5-4* mutants. Glutamate levels increased 1-3.5-fold in shoots of *cam1*, *cam2-2*, *cam3-2*, *cam3-3*, *cam4*, *cam5-1*, *cam5-2*, *cam5-3*, *cam5-4*, *cam6-1* and *cam7-1* mutants and the wild type as shown in Figure 3E.

During cold treatment almost all of the mutants showed a major changes in the levels of glutamate, alanine, and GABA in both root and shoot tissues (Figure 3). The accumulation of GABA in response to low temperature may result from an altered intracellular compartementation of glutamate, alteration glutamate decarboxylase activity, or from the intracellular damage and lowering of protoplasmic pH that might lead to the activation of glutamate decarboxylase.

CaM functions as a ubiquitous Ca^{+2}-binding protein that may be involved in GABA shunt pathway regulation by various possible mechanisms such as: regulation of

GAD activity in response to cytosol calcium accumulation, regulation of the translocation of GABA across the mitochondrial membrane, regulation of the level and the pattern of GABA, alanine and glutamate pools, and regulating translocation of GABA, alanine and/or glutamate between roots and shoots.

Mazzucotelli et al (2006) in their study for characterization of GABA shunt metabolites and GABA shunt genes during cold acclimation and freezing in wheat and barely found that GABA shunt metabolite accumulation and shunt gene expression were activated in response to low temperature, while GABA accumulation, glutamate availability, and GAD activity were correlated to the severity of the stress. Since GABA shunt may play a protective role in a pH-stat mechanism counteracting cytosol acidification due to membrane leakage as a result of cold stress, CaM signaling through activation of functional GAD (H^+-consuming properties) may be involved in regulating the accumulation rate of GABA and subsequently alanine and glutamate during cold stress.

Increased levels of GABA shunt metabolites in response to cold treatment after an initial reduction in some *cam* mutants suggests that activation of GAD does occur after exposure to cold stress in response to the intracellular damage and low cytoplasmic pH. Since GAD is a Ca^{2+}/CaM-regulated enzyme; the Ca^{2+} influx that occurs following a temperature drop could be responsible for the activation of the CaM/GAD complex and induction of GABA shunt pathway in response to cold stress.

In Conclusion the data presented here suggest an important role of the GABA shunt and CaM signaling in *Arabidopsis* seedlings under cold stress and highlights the

role of CaM and CaM-mediated signaling in adaptation and tolerance to temperature stress.

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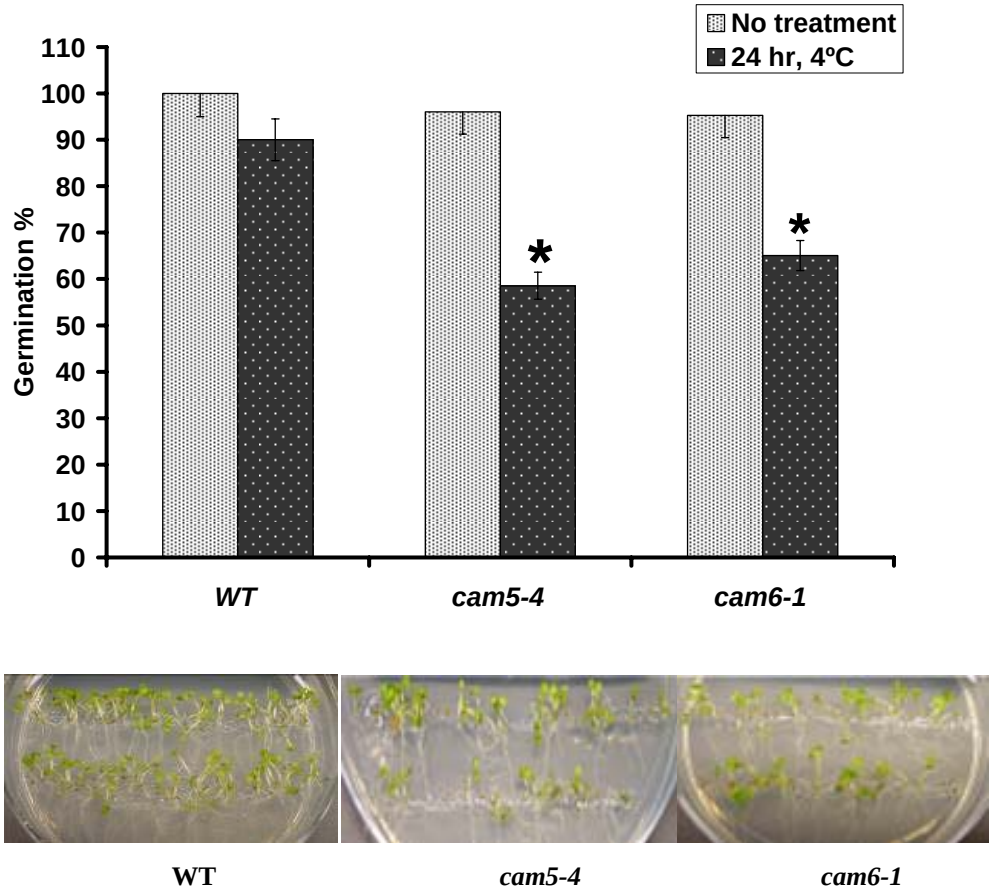
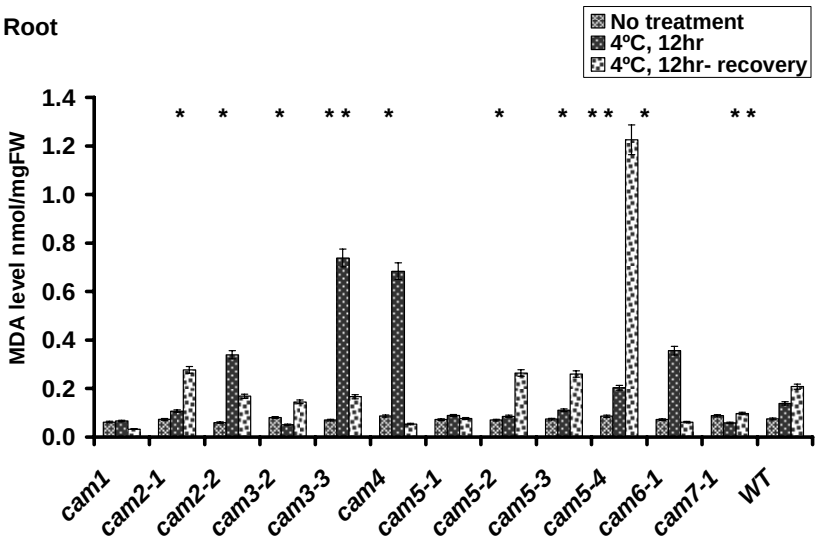


Figure 1. **Germination of seeds of wild type, *cam5-4*, and *cam6-1* mutants of *A. thaliana* after exposure to 4ºC for 24 hr. Fifty seeds each of the WT and *cam* mutants were surface sterilized and exposed to 4ºC for 24 hr. Treated seeds were plated on MS salt-agar medium and allowed to germinate at 25ºC under continuous light for seven days. The percent of germinating seeds were scored. Error bars represent SD over three replicate plates. Except for *cam5-4* and *cam6-1*, all cam mutants showed germination of seeds comparable to wild type.**


Root
No treatment
4ºC, 12hr
4ºC, 12hr- recovery
MDA level nmol/mgFW
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

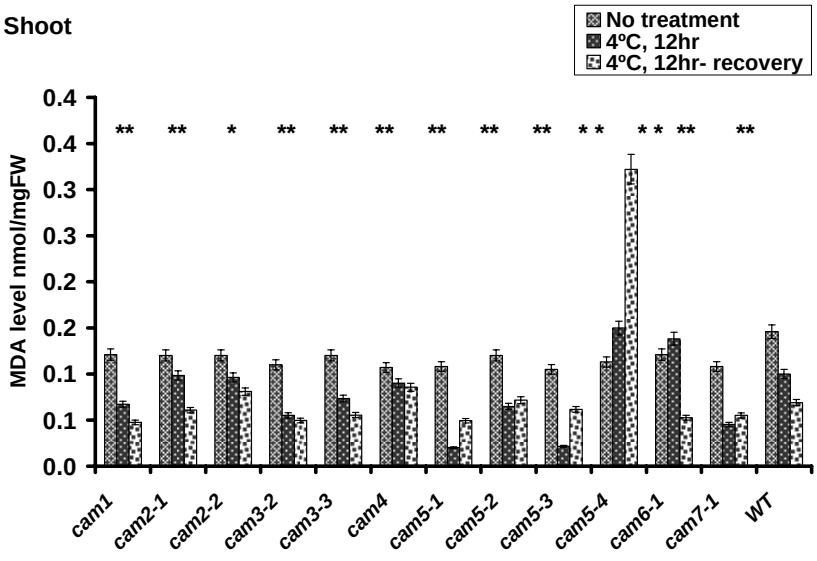
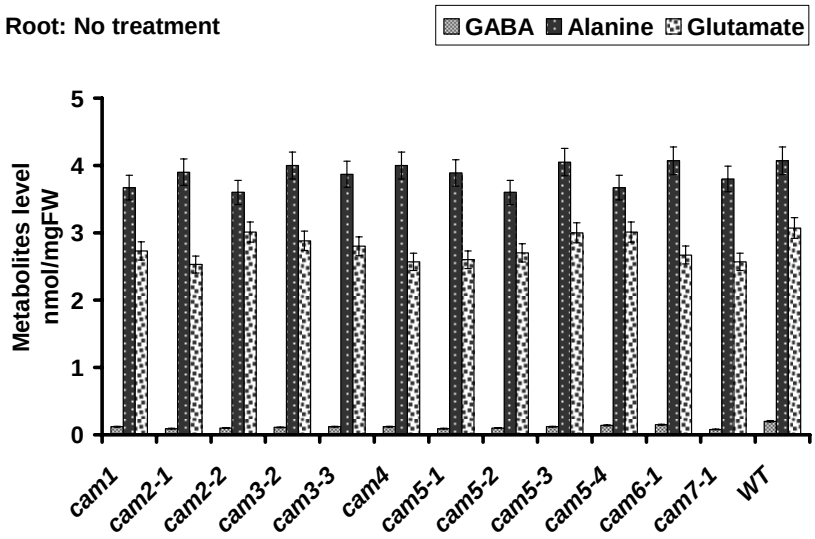
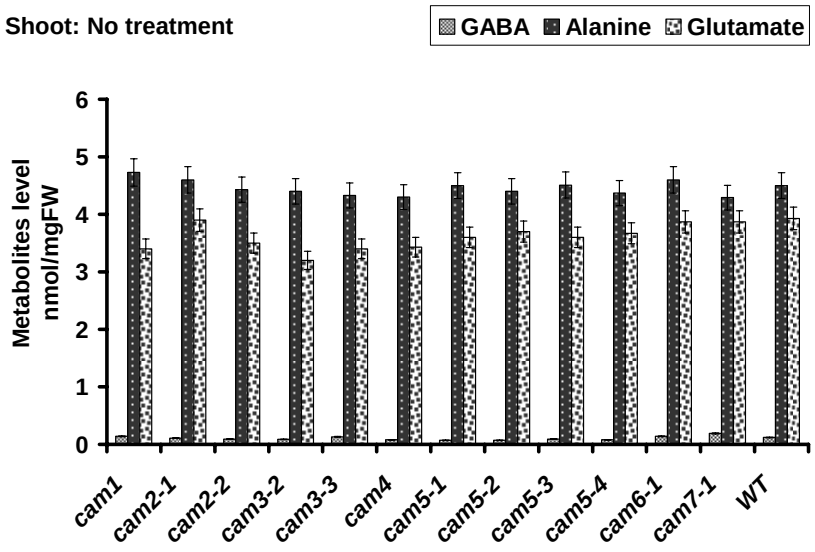

Shoot
No treatment
4ºC, 12hr
4ºC, 12hr- recovery
MDA level nmol/mgFW
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

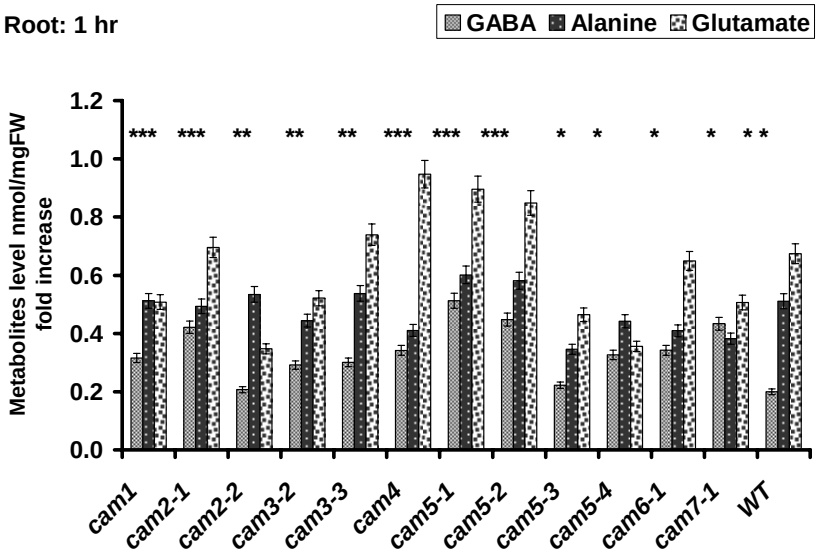
Figure 2. **Oxidative damage in *cam* mutants of *A. thaliana* after exposure to low temperature at 4°C. MDA level was determined in root and shoot tissues by the TBARS assay describe in materials and methods. Two sets of seedlings were treated at 4°C for 12 hr. One set was used immediately for measurement of MDA level while the other set was allowed to recover for 2 days under continuous light at 25°C before determination of MDA level. Percent of MDA level in *cam* mutants and the wild type were determined. Error bars represent the SD over three replicate plates with 50 seedlings on each plate. Mutants with (*) represent the significant oxidative damage (P <0.05).**

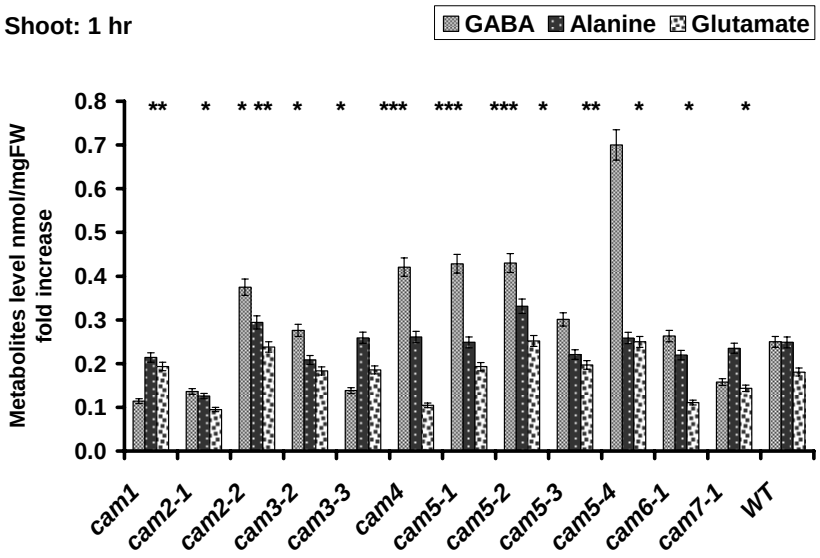
A.


Root: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

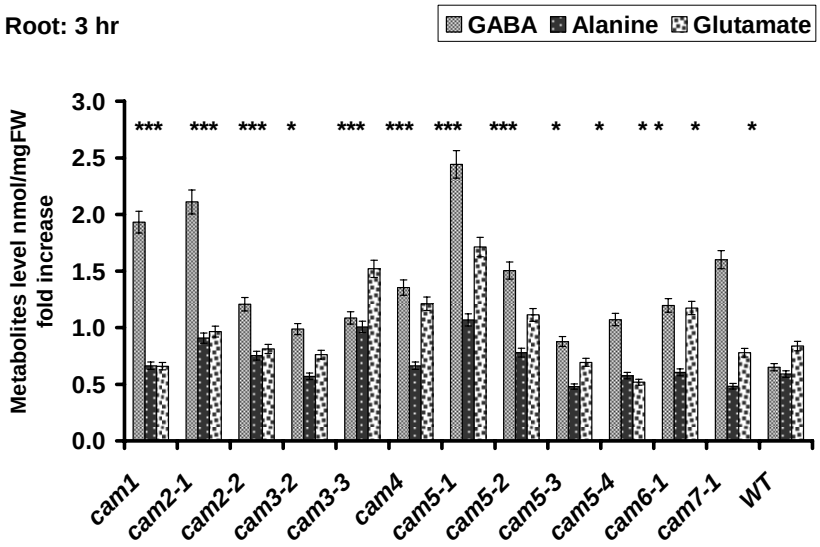

Shoot: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

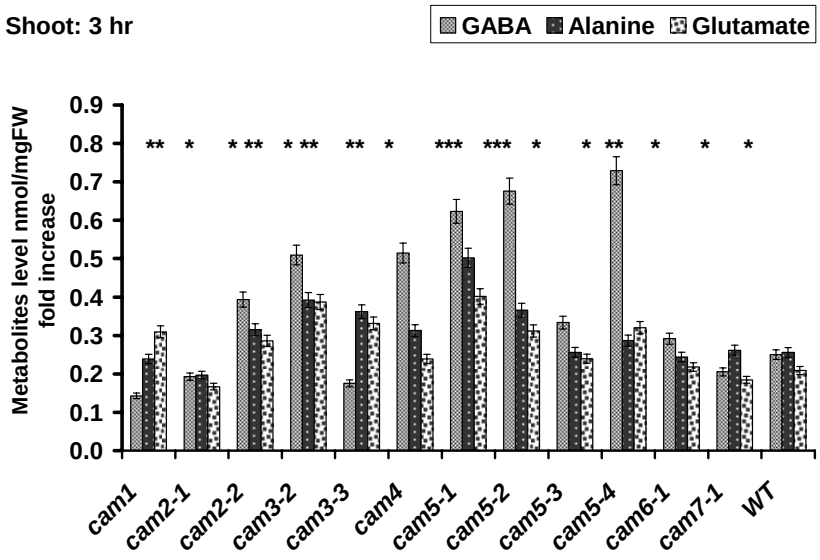
B.


Root: 1 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*** *** ** ** ** *** *** *** * * * * * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

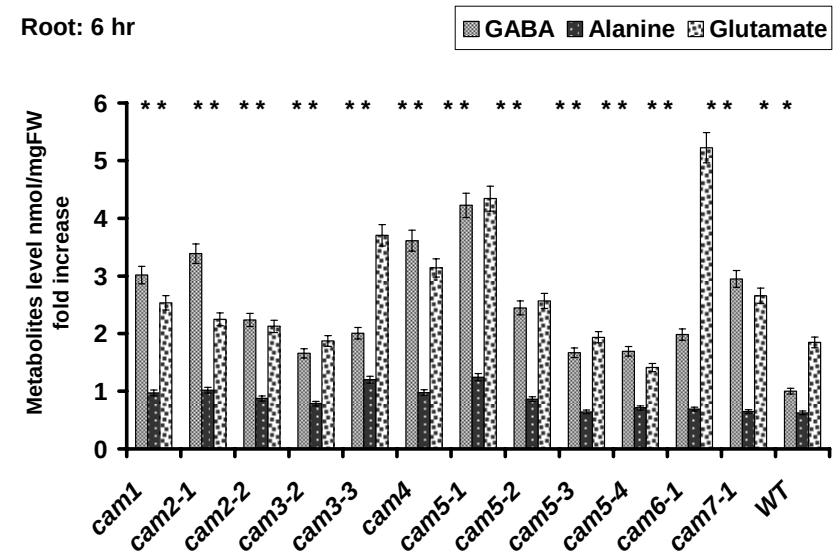

Shoot: 1 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
** * * ** * * *** *** *** * ** * * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

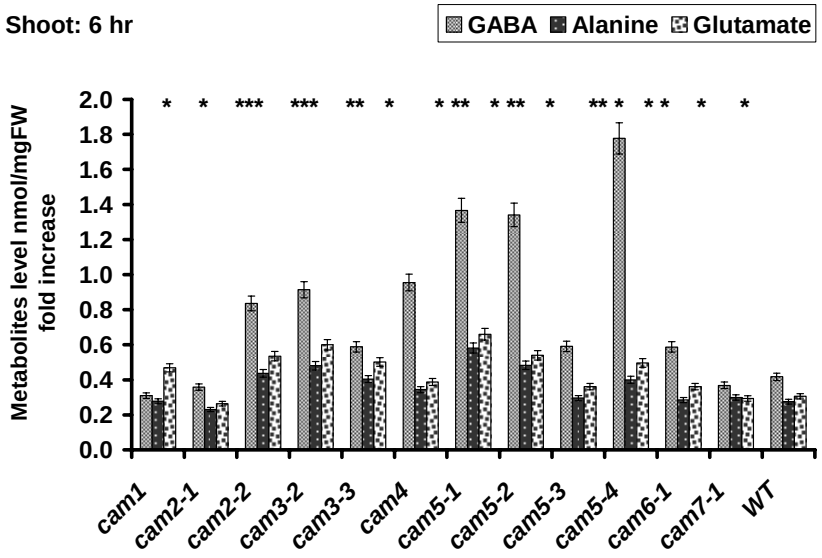
C.


Root: 3 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW fold increase
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*** *** *** * *** *** *** *** * * * * * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

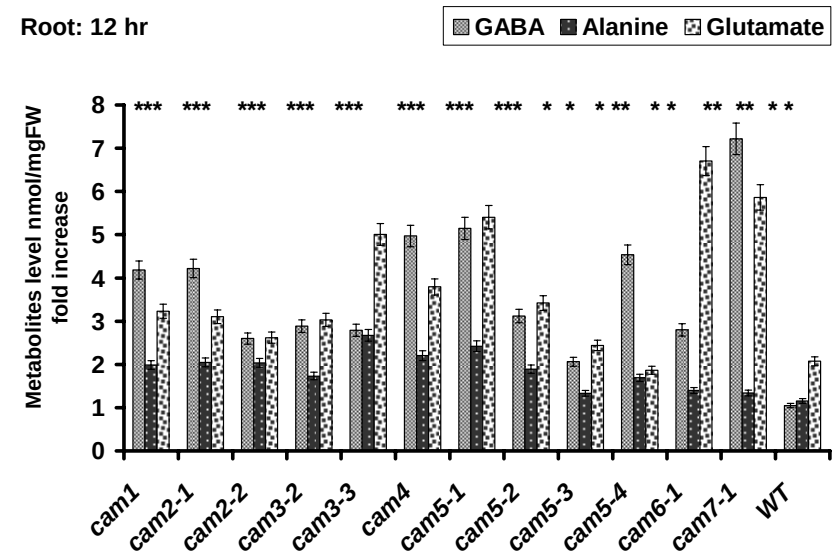

Shoot: 3 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW fold increase
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
** * * ** * ** ** * *** *** * * ** * * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

D.


Root: 6 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT


Shoot: 6 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

E.


Root: 12 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0
1
2
3
4
5
6
7
8
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

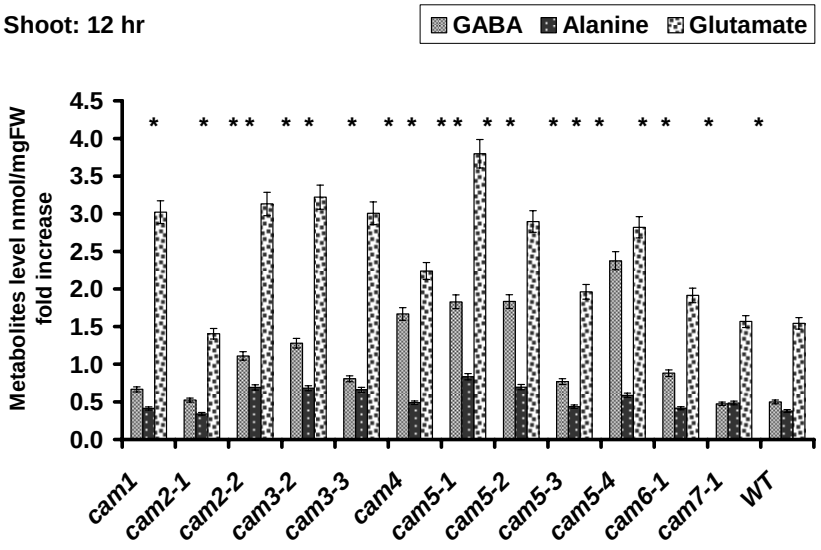

Shoot: 12 hr
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

Figure 3. **The level of GABA, alanine and glutamate in root and shoot tissues of two week old seedlings of wild type and *cam* mutants of *A. thaliana* was determined after exposure to cold as indicated. Seedlings were treated for 0 hr, 1 hr, 3 hr, 6 hr and 12 hr at 4ºC. Root and shoot tissues were harvested separately and frozen in liquid nitrogen for determination of metabolite levels as described in materials and methods. Metabolite levels were determined and expressed as nmol/mgFW fold increase. A, No treatment (0 hr). B, 1 hr. C, 3 hr. D, 6 hr. E, 12 hr. Error bars represent SD over three replicate plates, each containing 50 seedlings each of WT and *cam* mutants.**

Table 1. **Mutant lines with T-DNA insertion in calmodulin genes of *A. thaliana* used in this study. All seeds lines were obtained from Dr.Janet Braam, Rice University, except *cam1* and *cam4* seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus.**

Mutant	Insertion Location	SALK Line #
cam1	3`UTR	SALK_107507
cam2-1	Intron	SALK_066990
cam2-2	Intron	SALK_089283
cam3-2	5`UTR	SALK_075669
cam3-3	3`UTR	SALK_042391
cam4	5`UTR	SALK_149142
cam5-1	3`UTR	SALK_007371
cam5-2	3`UTR	SALK_073480
cam5-3	5`UTR	SALK_138758
cam5-4	Exon II	SALK_027181
cam6-1	3`UTR	SALK_071609
cam7-1	3`UTR	SALK_074336

V. SENSITIVITY OF CALMODULIN MUTANTS OF *ARABIDOPSIS THALIANA* TO SALT AND OSMOTIC STRESS

ABSTRACT

Plants accumulate compatible solutes in response to low water potential caused by ionic and nonionic osmotic stress. T-DNA insertion mutants in seven calmodulin genes of *Arabidopsis thaliana* (Arabidopsis) were used to determine the roles of specific *CAM* genes in tolerance of plants to salinity and osmotic stress induced by NaCl and mannitol. Canges were measured in seed germination, growth, stress induced oxidative damage, and changes in the levels of GABA shunt metabolites. Seed germination and seedling growth was affected by ionic and nonionic stresses in *cam5-4* and *cam6-1* mutants. Oxidative damage measured as the level of malonaldehyde (MDA) was significantly higher in root and shoot of *cam4* and *cam6-1* in response to salinity stress. Exposure to nonionic osmotic stress by mannitol increased the MDA level in root of *cam* mutants except *cam1*, *cam2-1*, *cam2-2*, and *cam7-1* and shoot of *cam5-4* and *cam6-1*. All metabolites levels in both root and shoot were generally increased in response to all salt (NaCl) treatments compared to the control. In response to 75 and 100mM NaCl treatments; GABA, alanine and glutamate level were increased significantly in root tissue of *cam1*, *cam5-4*, *cam7-1*, and shoot tissue of *cam1* and *cam5-4* mutants. All *cam* mutants except the wild type accumulated alanine and glutamate to a greater extent than

GABA in root and shoot tissues at 150mM NaCl treatment. In General, all the shunt metabolites level were accumulated in root more than shoot in response to non-ionic osmotic stress. GABA, alanine, and glutamate levels was significantly increased in root and shoot tissues of *cam1*, *cam4*, *cam5-4*, *cam6-1* and *cam7-1* mutants in response to treatments of seedlings to all mannitol concentrations. Our results show that *cam5-4* and *cam6-1* mutants are sensitive to salt and osmotic stress for seed germination, seedling growth and oxidative damage. High accumulation of GABA, alanine and glutamate under salt and osmotic stress suggest that GABA shunt metabolites may function in osmoregulation and signaling in response to salinity and non-ionic osmotic stress, and may serve as an osmoprotectants in response to low water availability. These results suggest GABA shunt metabolites are differentially used for ionic and non-ionic stress in Arabidopsis.

Key words:

Arabidopsis, Calmodulin, *CAM*, CaM, Ca^{+2}, GABA, NaCl, Mannitol, Salt stress, Osmotic stress, Metabolism

Abbreviations:

CAM: Calmodulin gene

CaM: Calmodulin protein

Ca^{+2}/CaM: Calcium/Calmodulin complex

GAD: Glutamate Decarboxylse

GABA: γ-Aminobutyric Acid

MDA: Malonaledehyde

NAD^{+}: Nicotinamide adenine dinucleotide

$NADP^{+}$: Nicotinamide adenine dinucleotide phosphate

TBARS: Thiobarbiturate reactive substances

INTRODUCTION

Membrane disorganization, reactive oxygen species (ROS) production, metabolic toxicity, and inhibition of photosynthesis are some of the consequences of salt stress on the plants (Yeo, 1998). Plants respond to salt stress by changing in gene expression, metabolic activity, and ion and water transport to minimize stress damage and to reestablish ion and water homeostasis (Zhu, 2001; Shi et al., 2002; Zhu, 2002).

Upon exposure to osmotic stress, plants exhibit a wide range of responses at the molecular, cellular, and whole plant levels (Xiong and Zhu, 2002). Altered water status most likely brings about initial growth reduction and induces the inhibition of cell division and accelerates cell death (Hasegawa et al., 2000).

To withstand osmotic stress, certain plants have evolved a high capacity to synthesize and accumulate non-toxic solutes (osmoprotectants or compatible solutes), predominantly in the cytoplasm, as part of an overall mechanism to reduce intracellular osmotic potenital and thereby maintain both Turgor and drive an osmotic gradient for water uptake (LeRudulier et al., 1984; Smirnoff, 1998). Being non-toxic, osmoprotectants can accumulate to osmotically significant levels without disturbing metabolic pathways inside the cell. Osmoprotectants can protect enzymes and membranes inside the cell against damage from high salt concentration and oxidative damage (Nuccio et al., 1999; Ashraf and Foolad, 2007).

One of the earliest responses to osmotic and salinity stress in plant cells is a transient increases in cytosolic free Ca^{+2} (Knight, 2000). Experimental evidence implicates Ca^{+2} function in salt and osmotic adaptation (Knight and Knight, 2001). Ca^{+2} as a second messenger in abiotic stress (Rudd and Franklin-Tong, 2001; Sanders et al.,

2002) is required for the expression of some stress-induced genes (Knight et al., 1996) and activation of a stress-associated gene promoters in plants (Sheen, 1996). Since Ca^{+2} transduces the stress signal by interacting with protein sensors, Ca^{+2} sensors such as CaM, CaM-dependent protein kinases (CDPK), and Calcineurin B-like proteins (CBLs) are prime candidates that link Ca^{+2} signal to downstream responses during salt and osmotic stress in plants (Zhu, 2002; Cheong et al., 2003; Perruc et al., 2004).

CaM is known to couple Ca^{+2} signals to changes in the activity of downstream target proteins via direct interaction between the Ca^{+2}/CaM complex and target molecules (Bouche et al., 2005). Glutamate decarboxylase (GAD) is one of the target proteins that can be activated by Ca^{+2}/CaM complex (Arazi et al., 1995; Snedden et al., 1996; Yuan and Vogel., 1998; Zik et al., 2006). In response to various abiotic stresses GAD decarboxylates glutamate to produce GABA which subsequently is transported into the mitochondrial matrix and degraded via succinic semialdehyde to succinate, a pathway that is called the GABA shunt (Shelp et al., 1999; Kinneresly and Turano, 2000). During salt and osmotic stress, GABA has been suggested to be involved in osmoregulation, regulation of cytosolic pH, and the regulation of detoxification of reactive oxygen radicals (Shelp et al., 1999; Bouche and Fromm, 2004; Xing et al., 2007).

GABA might also act as a compatible solute and be involved in intracellular signaling during salt and osmotic stress. However, the specific role of GABA in signaling and osmoregulation is not well understood (Bouche et al., 2003). Therefore, the characterization of the role of CaM in GABA shunt metabolite accumulation is crucial to understanding the possible roles of the GABA shunt activation in *Arabidopsis* seedlings under the osmotic and salt treatments.

T-DNA insertion mutants in the calmodulin genes of *A. thaliana* have been used to examine the roles of specific calmodulin genes in tolerance to high salt and osmotic treatments with respect to seed germination, seedlings growth, oxidative damage and GABA shunt metabolite levels.

MATERIALS AND METHODS

Plant material

Wild type (WT) (*Arabidopsis thaliana* Ecotype Columbia) and T-DNA insertions in seven calmodulin genes (*cam*) (also in *Arabidopsis thaliana* Ecotype Columbia) used in this study are listed in Table 1. Seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH and from Dr. Janet Braam, Rice University, Texas. Homozygous stocks were propagated for use in these studies.

Seeds were surface sterilized with 100% bleach (v/v, 6% sodium hypochlorite) for 10 min followed by five washes with sterile distilled water. Seeds were plated on sterile 1XMS (Murashige and Skoog, 1962) medium (pH 5.7) on Petri dishes supplemented with 2% (w/v) sucrose and solidified with 1.2% (w/v) agar. Petri dishes were placed under continuous light (40μmol m^{-2} s^{-1}) at 25 ºC for seed germination and growth of seedlings.

Seed germination

150 surface sterilized seeds of WT and each of *cam* mutants were plated on 1X MS medium supplemented with 0, 75, 100, and 150 mM NaCl and allowed to grow under continuous light at 25^{o}C. A similar set of seeds were plated on 1X MS Petri dish plates supplemented with 0, 200, 300, and 400 mM mannitol and allowed to grow under continuous light at 25^{o}C. Emergences of radicle from germinating seeds were scored

after 10 days. The effect of salt and mannitol on seed germination was calculated for WT and *cam* mutants.

Root elongation

Seven-day-old seedlings of WT and *cam* mutants grown on 1X MS medium under continuous light were transferred onto 1X MS medium supplemented with 0, 75, 100, or 150 mM NaCl, and grown under continuous light for seven days before measurement of increase in root lengths in response to varying salt concentrations. For determination of effect of osmotic stress, seven-day-old seedlings were transferred onto new 1X MS medium supplemented with 0, 200, 300, or 400 mM mannitol and seedlings were allowed to grow for next seven days in continuous light before measurement of the increase in the length of primary root in response to varying concentrations of mannitol. Each plate contained 15 seedlings. Three replicate plates were used for each treatment. The increase in primary root length (mm) was measured after 7 days of growth.

Oxidative damage

Seven-day-old seedlings grown on 1X MS medium under continuous light were transferred onto 1X MS plates supplemented with 0, 150 mM NaCl and 400 mM mannitol. Plates were incubated under continuous light at 25^{o}C for the next seven days. Root and shoot tissues were separated for determination of malonaldehyde (MDA) level by the TBARS assay (Heath and Packer, 1968). Three replicate plates with 15 seedlings on each plate were used.

GABA shunt metabolites

Seven-day-old WT and *cam* seedlings grown under continuous light at 25^{o}C were transferred to 1X MS medium supplemented with 0, 75, 100 and 150mM NaCl, and 0, 200, 300 and 400mM mannitol, respectively and grown under continuous light for seven days. The levels of L-glutamate, GABA, and L-alanine in seedlings grown on control and supplemented medium were determined in root and shoot tissues. Three plates with 15 seedlings on each plate were used for each sample. Metabolites from each sample were extracted according to Zhang and Bown (1997). Glutamate and alanine levels were measured according to Bergmeyer (1983) and GABA level was measured according to Zhang and Bown (1997) using microplate reader.

Data Analysis

Each data point is given as a mean ±SD of the three independent experiments. The values were compared and analyzed by two-way ANOVA (analysis of variance) using LSD multiple comparison tests on the means. Where differences are reported, they are at the 95% confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Sensitivity of *cam* mutants to salt and osmotic stress

To test the sensitivity/tolerance of *cam* mutants to salt and osmotic stress, all the mutants and wild type plants were grown on media containing various concentrations of NaCl (0, 75, 100, 150mM) or Mannitol (0, 200, 300, 400mM). The germination rate and the growth as increase in root length were monitored. As shown in Figure (1A) the germination of *cam6-1* and *cam5-4* was inhibited and significantly ($P<0.05$) as a results

of salt toxicity while there was no significant difference between wild type and the other *cam* mutants. On 75mM, 100mM and 150mM NaCl the germination of *cam5-4* and *cam6-1* seeds reached 75%, 65% and 60% germination respectively after 8-10 days (Figure 1A), while wild type and the other *cam* mutant seeds germinated normally (85-95%) after 4 days on the same treatments (data not shown). The same pattern of germination tolerance/sensitivity was observed under mannitol treatments (Figure 1B). Seed germination was inhibited and significantly reduced ($P<0.05$) in *cam5-4* and *cam6-1* seeds in response to osmotic stress. Consequently; *cam5-4* and *cam6-1* seeds germinated 75% at 200mM Mannitol, 60-65% at 300mM Mannitol, and 56-60% at 400mM Mannitol after 10 days. However, wild type and the other *cam* mutants were able to fully germinate at 200 mM Mannitol after 4 days, 300 mM Mannitol after 6 days, and 300 mM Mannitol after 8 days over the same period.

The tolerance/sensitivity of *cam* mutants to salt and osmotic stresses was also examined at the seedling stage (Figure 2). To this end, 7 days-old seedlings grown on 1X MS medium under continuous light were transferred to media containing 0, 75, 100, or 150 mM NaCl or 0, 200, 300, or 400mM mannitol. Root elongation (mm) was measured after 7additional days. As shown in Figure 2; root growth of all of the mutants and the wild type was retarded on NaCl or mannitol-containing media compared to control media without salt or mannitol treatments. However, most of the mutants showed no significant differences in seed germination assay compared to wild type on 75mM and 100 mM NaCl or 200 mM and 300 mM mannitol after 7 days, while at 75, 100, and 150 mM NaCl or 200, 300, and 400 mM mannitol treatments the root length was significantly ($P<0.05$) retarded and reduced for all *cam* mutants compared to wild type (Figure 2). Only, *cam6-*

1 and *cam5-4* seedlings showed greater sensitivity to NaCl treatments and a significant ($P<0.05$) reduction in root length at 75, 100 and 150 mM NaCl (Figure 2A) or 200, 300 and 400 mM mannitol (Figure 2B) in comparison with all the other mutants and the wild type. Furthermore *cam6-1* and *cam5-4* seedlings showed a highly significant (*,$P<0.05$) sensitivity to 150mM NaCl or 400mM Mannitol demonstrating a reduction of more than 50% in root growth after 7 days.

Taken together, these data are consistent with 2 mutants, *cam5-4* and *cam6-1,* demonstrating greater sensitivity to salt and osmotic stress at both the germination and seedling stages than wild type or any of the other *CAM* mutations studied here. This suggests that CaM5 and CaM6 may be directly or indirectly involved in tolerance and signaling pathways during early development and seedlings growth leading to tolerance to salt and osmotic stress treatments. We investigated the nature of the *cam5-4* mutant allele by looking at the mRNA levels in the wild type and the mutants, and we find that the *cam5-4* mutant does not show any steady state level of *CAM5* mRNA and the levels of all other *CAM* genes are reduced (refer to Chapter II) while the *cam6-1* mutation demonstrates a reduced level of mRNA of *CAM6* in shoot up to 50% and overall reduced levels of all other *CAM* genes as well (refer to Chapter II). The absence of the *CAM5* mRNA in the *cam5-4* mutant may indicate the loss of a critical CaM protein that directly functions in salt and osmotic stress tolerance possibly in signaling pathways or GABA synthesis during seeds germination and seedling growth. However, the incomplete loss of mRNA for CAM6 in the *cam6-1* mutant (refer to Chapter II) despite the fact that this mutant demonstrates a germination and seedling growth phenotype on media containing salt and mannitol could means that this phenotype results not from the loss of CaM6

protein but is related to pleiotrophic effects of the lowered level of CaM6 protein or other proteins including the products of other CaM genes. The exact nature of these effects has not been elucidated at this time.

In Arabidopsis, Ca^{2+}-binding protein CML24 -also known as TCH2 and shares 40% identity with CaM proteins was strongly suggested to positively regulate ABA inhibition of germination and seedling development and negatively regulates ion sequestering under high salt treatment, which implicates its possible role in ion homeostasis (Braam, 1992; Delk et al., 2005). In other studies, the transcription of *CAM* genes was induced when cultured cells from tomato or bean (*Vigna radiate*) are exposed to salinity (Botella and Arteca, 1994; Delumeau et al., 2000). On the other hand, transgenic tobacco plants expressing bovine *CAM* germinate faster on media containing high levels of NaCl comparing to the control (Olsson et al., 2004). Conversely, Arabidopsis CaM binding protein of 25kDa (AtCaMBP25) overexpression exhibits an increased sensitivity to both ionic (NaCl) and non-ionic (Mannitol) osmotic stress during seed germination and seedling growth (Perruc et al., 2004).

In agreement with the data presented here CaM might be a regulatory component during seed germination and plant growth during osmotic and salinity stress signaling in Arabidopsis.

Oxidative damage in *cam* mutants by salt and osmotic stress

In order to test the effects of high salt and osmotic stress treatments on oxidative damage and ROS levels in Arabidopsis seedlings. One-week-old seedlings of all *cam* mutants and the wild type were subject to 150 mM NaCl or 400 mM mannitol for 7 days.

After 7 days, the MDA levels (nm/mgFW) were determined in both root and shoot separately using the TBARS assay as shown in Figure 3 and 4. The MDA levels were significantly reduced in root and shoot tissues of most *cam* mutants under high salt and osmotic treatments. The *cam1* mutant accumulated 0.4-fold more MDA ($P<0.05$) only in shoot tissues at 150 mM NaCl, and the *cam5-4* mutant accumulated 1.2-fold higher levels of MDA ($P<0.05$) only in shoot under the same treatment. The *cam6-1* allele accumulated 1.6-fold and 0.4-fold higher MDA levels in both root and shoot respectively under NaCl treatment (Figure 3).

As a Result of high Mannitol treatment (Figure 4): *cam3-2* and *cam3-3* accumulated 0.35-fold more MDA in root at 400 mM mannitol. The *cam4* mutant accumulated 0.5-fold more MDA in root ($P<0.05$), while the *cam6-1* mutant showed a similar phenotype in root (0.85-fold increase) and in shoot (0.3-fold increase) at 400 mM mannitol treatment. All *cam5* alleles showed significant accumulation of MDA in root (1-fold, $P<0.05$) at 400 mM mannitol, while *cam5-4* is the only *cam* allele that accumulated higher level MDA in the shoot (0.6-fold) under the same mannitol treatment after 7 days incubation (Figure 4).

Taken together, these data allow the inference that CaM may be involved in oxidative damage protection during salt and osmotic stress. CaM might indirectly be involved in scavenging hydroxyl radicals and superoxides ions as a second response under salt and osmotic stress. The distinctive phenotype of *cam5-4* and *cam6-1* implicates the possible interactions between different CaM isoforms in signaling and acquired tolerance to oxidative damage in response to high salinity and hyperosmolarity.

Catalase enzyme, which is mainly localized in peroxisomes and glyoxysomes, was found to be activated by Ca^{+2}/CaM to down regulate hydrogen peroxide (H_2O_2) and maintain H_2O_2 homeostasis levels in plants (Yang and Pooviah, 2002). Transgenic plants with reduced catalase activity showed increased sensitivity to salt stress (Willekens et al., 1997). Furthermore, it is strongly indicating that CaM and CaM-signaling are involved in regulating the ROS levels and ROS signaling under salt and osmotic stress in Arabidopsis.

GABA shunt metabolites in *cam* mutants in response to salt and osmotic stress

To investigate changes in the level of GABA, it's substrate glutamate, and alanine, the product of GABA degradation under salt stress levels of these compounds were determined in root and shoot separately of all *cam* mutants and the wild type after seedlings were grown on 0, 75, 100, or 150 mM NaCl for 7 days.

Generally, glutamate, GABA, and alanine all accumulated in root and shoot in all *cam* mutants and wild type compared to the control in response to all salt (NaCl) treatments (Figure 5). In response to 75mM NaCl treatment, GABA increased 0.5-3.5-fold, alanine increased 1-2.4-fold, and glutamate increased 1.5-4.5-fold increased in root tissues of all *cam* mutants and the wild type with a 6.5-fold increase of alanine and an 8.7-fold increase glutamate in roots of the *cam5-4* mutant (Figure 5B). In shoot tissues under the same treatment, alanine and glutamate increased 1-2.5-fold in all *cam* mutants and wild type while the GABA level increased 0.1-1-fold when compared to the control (Figure 5B).

At 100 mM NaCl exposure; glutamate, GABA, and alanine increased in root tissues. GABA, alanine and glutamate level increased up to 8-fold in root tissues of all

mutants and the wild type compared to the control with maximum accumulation in roots of *cam1*, *cam5-4*, and *cam7-1* mutants to 10-, 11-, and 12-fold increase respectively as shown in Figure 5C. On the other hand, in shoot tissues GABA showed higher accumulation levels (5-33-fold increase) than alanine and glutamate (1-4-fold increase) in all *cam* mutants but not in wild type. The maximum GABA, alanine, and glutamate levels were observed in shoot of *cam1* and *cam5-4* mutants (Figure 5C).

After 7 days of seedlings exposure to 150 mM NaCl, GABA accumulated ($P<0.05$) to greater levels than alanine and glutamate in roots and shoots of all *cam* mutants compared to the control except in shoot tissues of wild type (Figure 5D). In root tissues, GABA accumulated up to 25-fold while alanine and glutamate levels reached 14-fold in roots of the *cam1*, *cam2-1*, *cam5-4*, and *cam7-1* mutants. Furthermore, all *cam* mutants showed increases up to 55-fold in GABA levels. This was higher than the levels of alanine and glutamate that were observed (1-3-fold) especially in shoots of *cam1* and *cam5-4* mutants as shown in Figure 5D. Wild type shoot tissues did not accumulate glutamate, GABA, or alanine under 150 mM NaCl treatment (Figure 5D).

To investigate the GABA shunt and its metabolites levels under osmotic stress; GABA, alanine, and glutamate levels were determined in roots and shoots separately for all *cam* mutants and the wild type after 7 days on media containing 0, 200, 300, or 400 mM mannitol.

Over all glutamate, GABA, and alanine increased to a greater extent in roots of all *cam* mutants and the wild type than in shoot tissues compared to the unstressed control under all mannitol treatments (Figure 6). In response to 200 mM mannitol; GABA increased 3.5-18-fold while alanine and glutamate increased 2-4-fold in all *cam* mutants

and the wild type root tissues. Significant accumulation of GABA (17 fold), alanine (17 fold) and glutamate (28 fold) were observed in roots of *cam1*, *cam4* and *cam5-4* mutants (Figure 6B). In shoot tissues, glutamate, GABA, and alanine accumulated in all *cam* mutants and the wild type compared to the control where GABA, alanine, and glutamate levels reached up to 1.7-fold in shoots of the *cam1*, *cam3-2*, and *cam5-4* mutants (Figure 6B).

At 300 mM mannitol treatment; GABA, alanine and glutamate increased up to 25-30-fold in roots of all *cam* mutants and the wild type compared to the control as shown in Figure 6C. GABA increased 8-30-fold; alanine increased 4-22-fold; and glutamate increased 4.5-32-fold. The maximum levels of all metabolites were reached in roots of the *cam1*, *cam4*, *cam6-1*, and *cam7-1* mutants. In shoot tissues, glutamate, GABA, and alanine increased 1-5-fold of all *cam* mutants and the wild type especially in shoots of *cam1*, *cam3-2*, *cam5-1*, *cam5-2*, *cam5-4*, *cam6-1* and *cam7-1* mutants as shown in Figure 6C.

In response to 400 mM mannitol treatment for 7 days glutamate (7-37 fold increase), GABA (12-52 fold increase), and alanine (5-23 fold increase) accumulated significantly in all mutants and the wild type root tissues compared to the control and to the other mannitol treatments (Figure 6D). The maximum accumulation occurred in root tissues of *cam1*, *cam4*, *cam5-4*, *cam6-1* and *cam7-1* mutants. On the other hand, GABA increased 11-fold, alanine increased 1.5-4.5-fold, and glutamate increased 1.5-6-fold in shoot tissues under the same treatment. GABA level reached maximum levels in shoots of the *cam5-1* and *cam5-2* mutants while alanine and glutamate reached maximum levels

only in the shoots of *cam*6-1 mutants compared to the control under 400 mM mannitol treatment as shown in Figure 6D.

GABA shunt pathway has been suggested to be involved in osmoregulation and ion homeostasis under salt and osmotic stress (Shelp et al., 1999; Bouche and Fromm, 2004; Xing et al., 2007). GABA, and the other shunt metabolite levels vary between genotypes, and with salt and osmotic stresses in root versus shoot tissues. High accumulation of GABA under salt stress suggested that GABA might be involved in osmoregulation and signaling in response to salinity. High accumulation of GABA, alanine and glutamate under osmotic stress especially in root tissues suggested that GABA, alanine and glutamate may serve as an osmoprotectants in response to high osmolarity and low water availability under mannitol treatments. High accumulation of GABA shunt metabolites in *cam5-4* mutant's tissues under salt stress suggests that the CaM5 isoform might play a role in metabolite accumulation and partitioning under salt but not necessarily under osmotic stress.

Beside its involvement in osmoregulation; GABA may be involved in ion balance and homeostasis. Kinnersley and Lin (2000) found that promotion of *lemna minor* growth by GABA application was associated with an increase in mineral contents of treated plants. On the other hand, higher GABA accumulation in the roots of soybean (*Glycine max* L.) plants under salt stress was found to be the result of polyamine degradation (PA) to maintain ion balance and enhance salt tolerance (Xing et al., 2007).

Arabidopsis CaM-regulated Ca^{+2}-ATPase (ACA4), is a calmodulin regulated calcium pump that is located on the vacuole membrane. This protein conferred protection against high NaCl and mannitol treatments in yeast (Geisler et al., 2000).

Since *cam*5-4 mutants showed a significant elevation in GABA shunt metabolites under salt stress, it is possible that CaM5 might be involved in Ca^{+2} influx/outflow between the cytosol and other cellular organelles via regulating the membrane localized Ca^{+2} pump, which in turn regulates the GABA shunt in response to salt stress.

In conclusion our data strongly indicating that GABA shunt, CaM, and Ca^{+2}/CaM signaling might be a major components in osmoregulation and adaptation signaling under both ionic and non-ionic osmotic stress in *Arabidopsis*. Further studies, combining metabolic and genetic approaches, will increase the knowledge of the complex interaction between the GABA shunt and CaM in osmoregulation, cross-tolerance, and signaling in plants under salt and osmotic stress.

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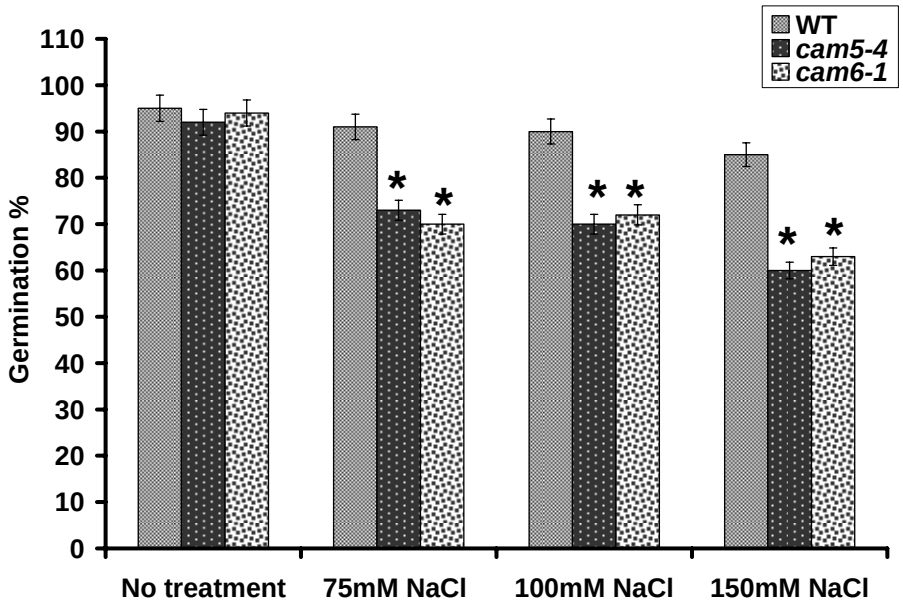
A.


WT
cam5-4
cam6-1
Germination %
110
100
90
80
70
60
50
40
30
20
10
0
No treatment
75mM NaCl
100mM NaCl
150mM NaCl

B.

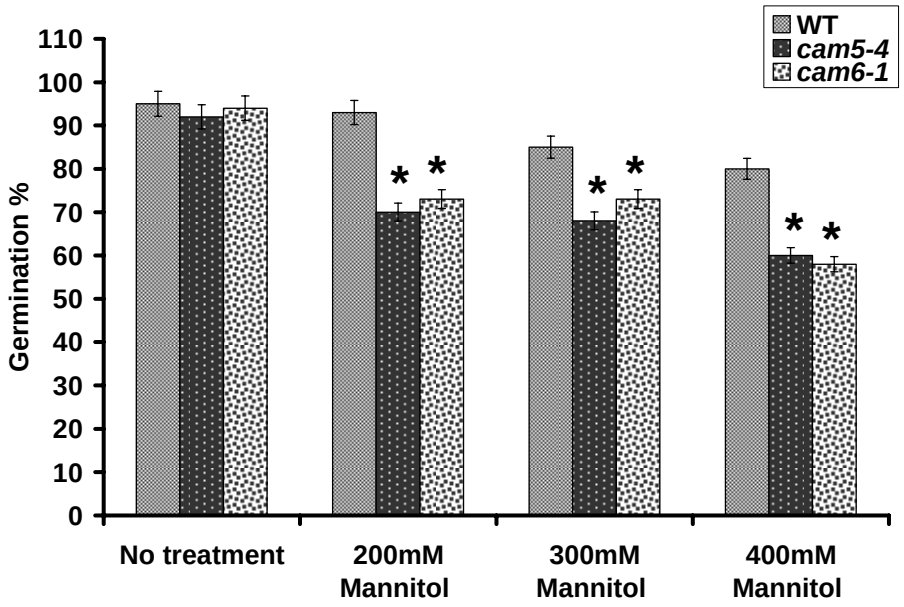
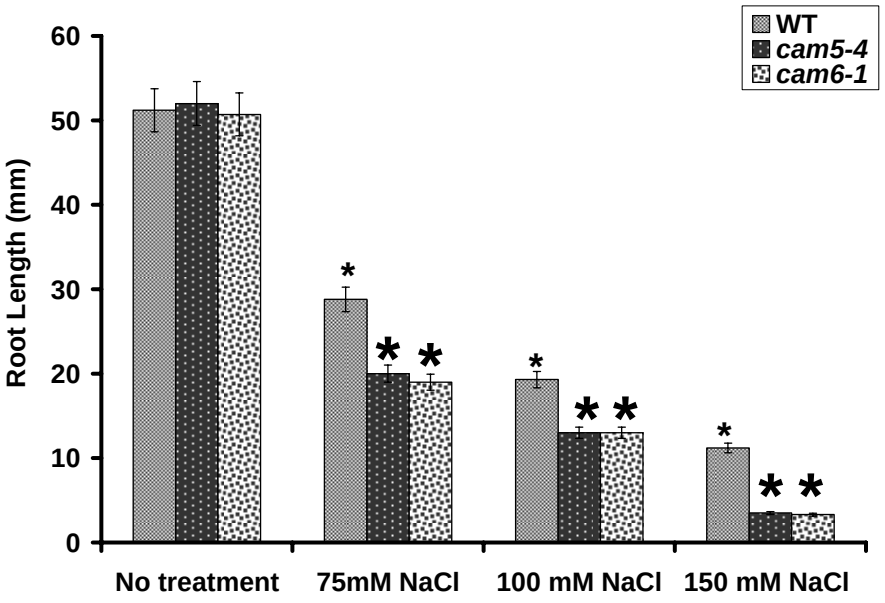

WT
cam5-4
cam6-1
Germination %
110
100
90
80
70
60
50
40
30
20
10
0
No treatment
200mM Mannitol
300mM Mannitol
400mM Mannitol

Figure 1: **Germination of WT and *cam* mutant seeds on various concentrations of NaCl (A) and Mannitol (B). 150 WT and each of the *cam* mutants seeds were surface sterilized and grown on medium containing 0, 75, 100, or 150 mM NaCl, or 0, 200, 300, or 400 mM Mannitol. Germinating seeds were scored after 10 days. Results are presented as percent of germination. Error bars represent the SD over three replicate plates for each of the mutants. All *cam* mutants were similar to WT under all concentrations of NaCl and Mannitol except *cam*5-4 and *cam*6-1.**

A.


WT
cam5-4
cam6-1
Root Length (mm)
60
50
40
30
20
10
0
No treatment
75mM NaCl
100 mM NaCl
150 mM NaCl

B.

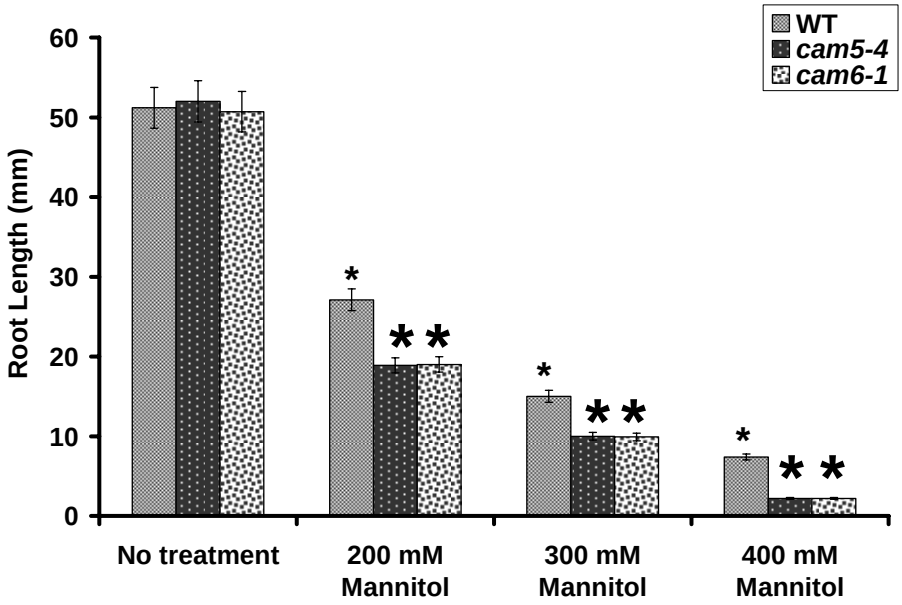
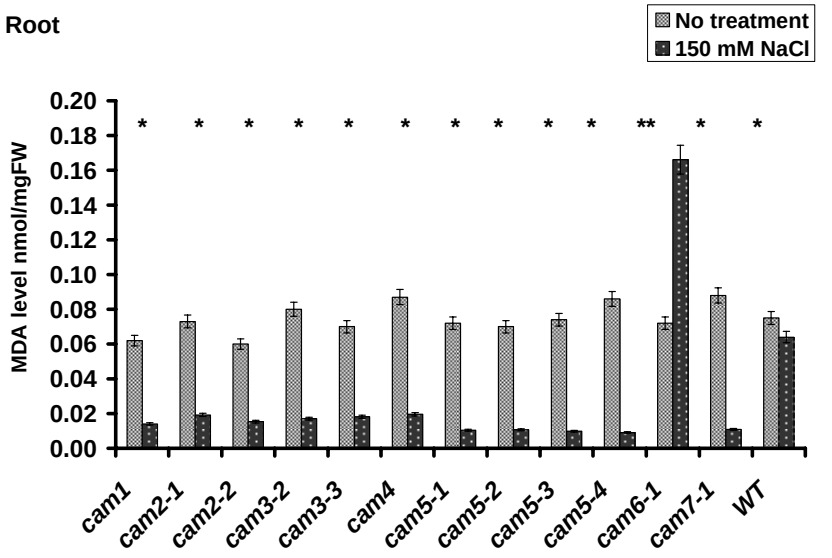

WT
cam5-4
cam6-1
Root Length (mm)
60
50
40
30
20
10
0
No treatment
200 mM Mannitol
300 mM Mannitol
400 mM Mannitol

Figure 2. **Root elongation of *cam* mutants and WT seedlings after 7 days of growth on agar plates supplemented with 0, 75, 100, and 150 mM NaCl (A) and with 0, 200, 300, 400 mM Mannitol (B). Results are presented as root length (mm) for WT and *cam* mutants. Error bars represent the SD over three replicate plates. WT and *cam* seedlings had similar sensitivity to different concentrations of NaCl and Mannitol treatments except for *cam*5-4 and *cam*6-1.**


Root
No treatment
150 mM NaCl
MDA level nmol/mgFW
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
* * * * * * * * * * ** * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

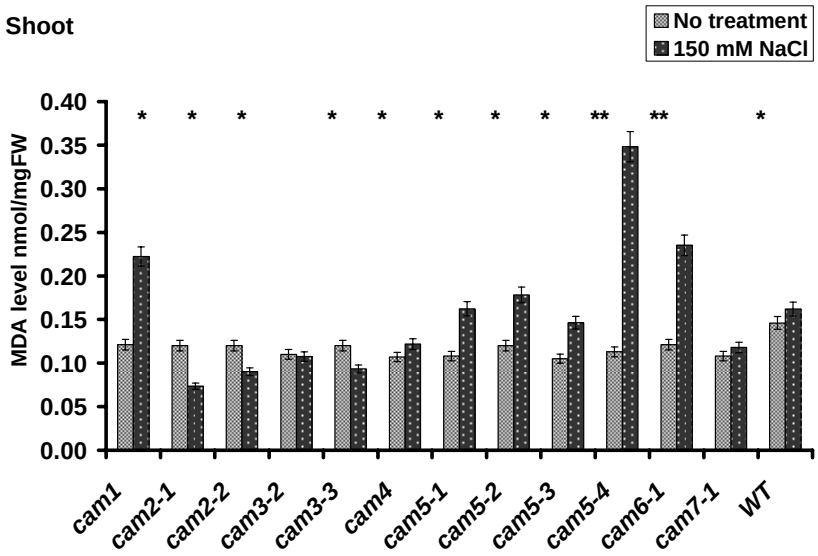
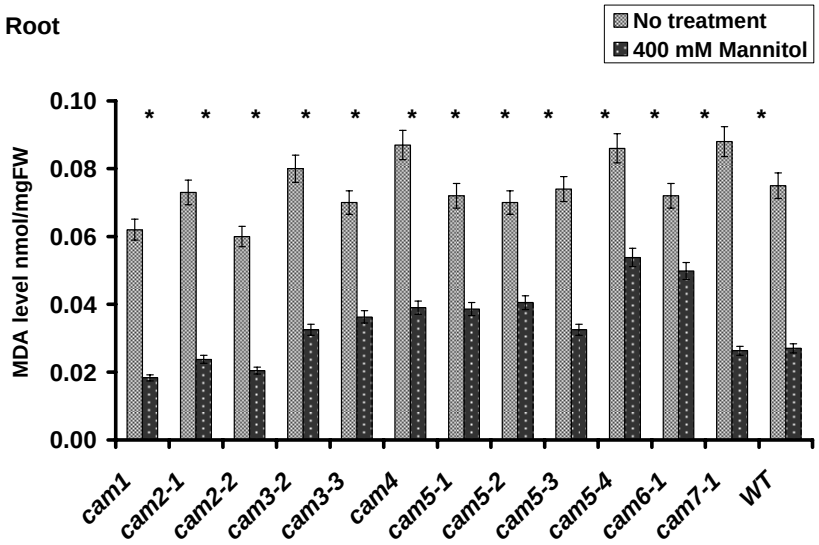

Shoot
No treatment
150 mM NaCl
MDA level nmol/mgFW
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
* * * * * * * * ** ** *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

Figure 3. **Oxidative damage in seedlings of *cam* mutants and wild type *A. thaliana* after exposure to 150 mM NaCl. MDA level was determined in root and shoot tissues by the TBARS assay described in materials and methods. Two sets of one week old seedlings were subjected to two 150 mM NaCl for 7 days under continuous light at 25ºC. After 7 days root and shoot tissues were used for MDA level measurement. Percent of MDA level in *cam* mutants and the wild type in root and shoot tissues were determined. Error bars represent the SD over three replicate plates. Mutants with (*) represent the significant oxidative damage (P <0.05).**


Root
No treatment
400 mM Mannitol
MDA level nmol/mgFW
0.10
0.08
0.06
0.04
0.02
0.00
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

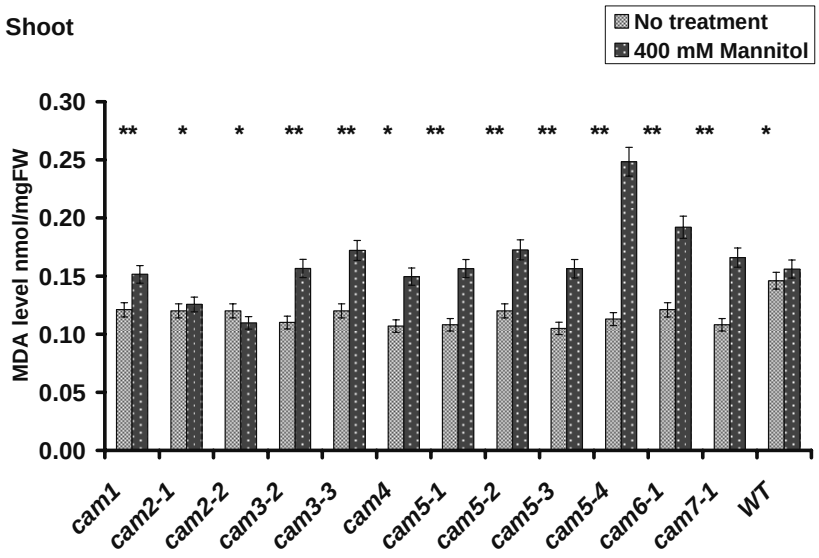
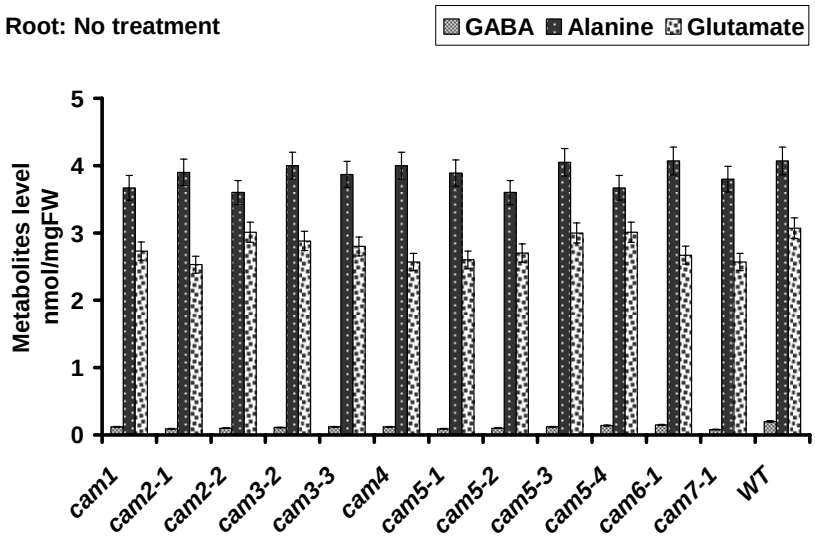
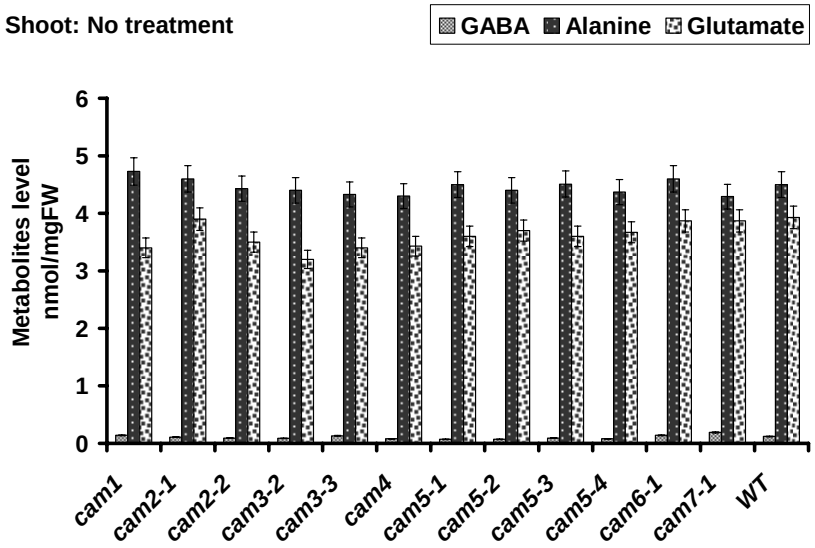

Shoot
No treatment
400 mM Mannitol
MDA level nmol/mgFW
0.30
0.25
0.20
0.15
0.10
0.05
0.00
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

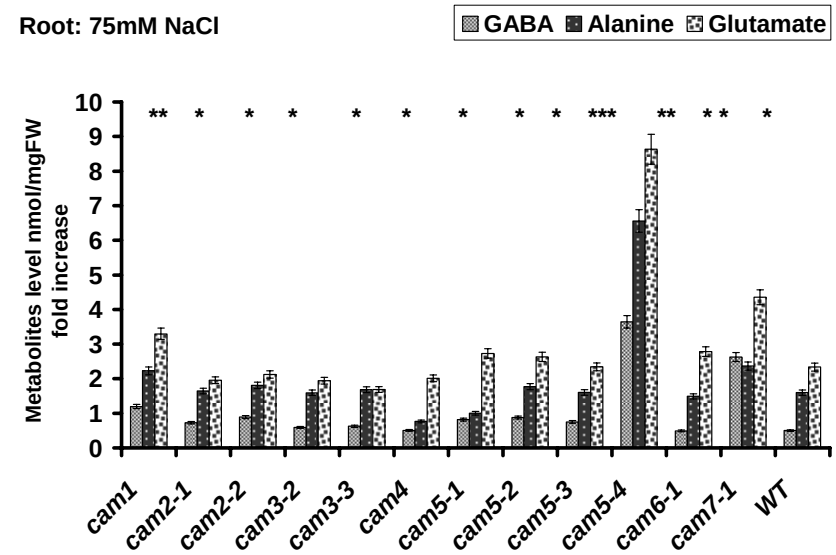
Figure 4. **Oxidative damage in *cam* mutants and wild type *A. thaliana* caused after exposure to 400 mM Mannitol. MDA level was determined in root and shoot tissues by the TBARS assay described in materials and methods. Two sets of one week old seedlings were subjected to 400 mM Mannitol for 7 days under continuous light at 25ºC. After 7 days root and shoot tissues were used for MDA level measurement. Percent of MDA level in *cam* mutants and the wild type in root and shoot tissues were determined. Error bars represent the SD over three replicate plates. Mutants with (*) represent the significant oxidative damage (P <0.05).**

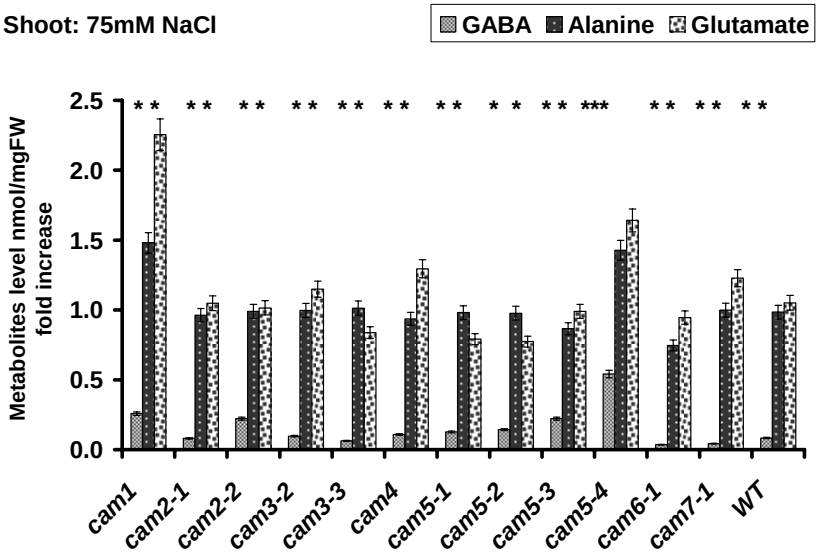
A.


Root: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

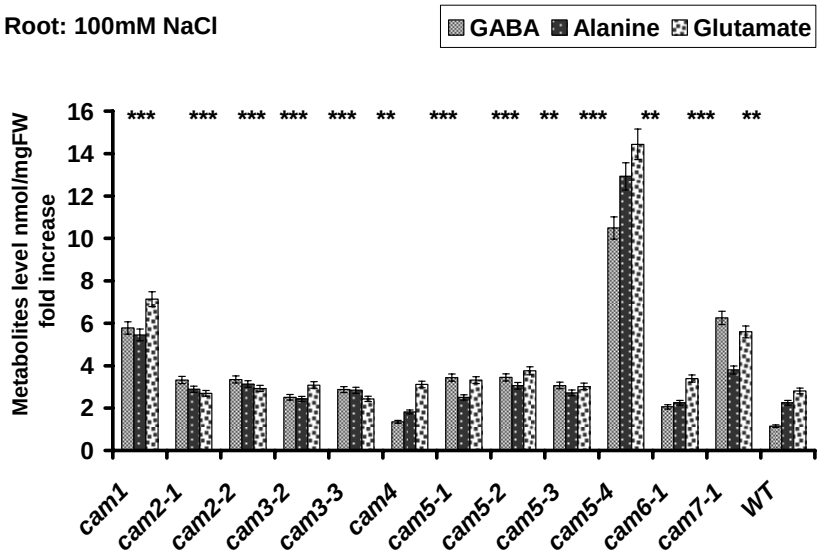

Shoot: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

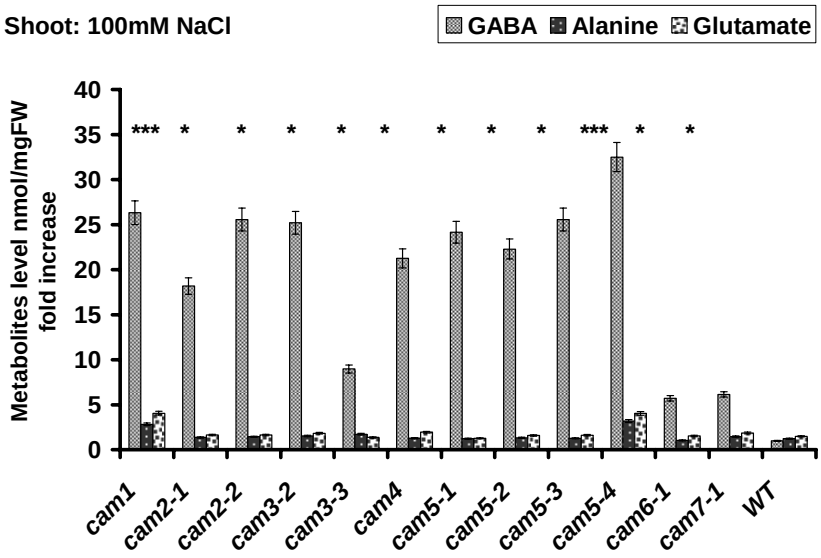
B.


Root: 75mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0
1
2
3
4
5
6
7
8
9
10
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

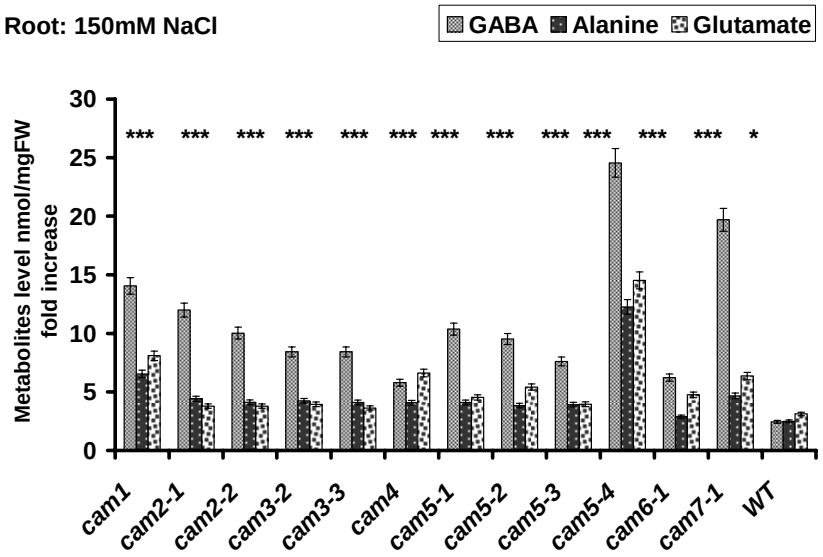

Shoot: 75mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
0.0
0.5
1.0
1.5
2.0
2.5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

C.


Root: 100mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
16
14
12
10
8
6
4
2
0
***
***
***
***
***
**
***
***
**
***
**
***
**
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT


Shoot: 100mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
40
35
30
25
20
15
10
5
0
***
*
*
*
*
*
*
*
*
***
*
*
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

D.


Root: 150mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
30
25
20
15
10
5
0
*** *** *** *** *** *** *** *** *** *** *** *** *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

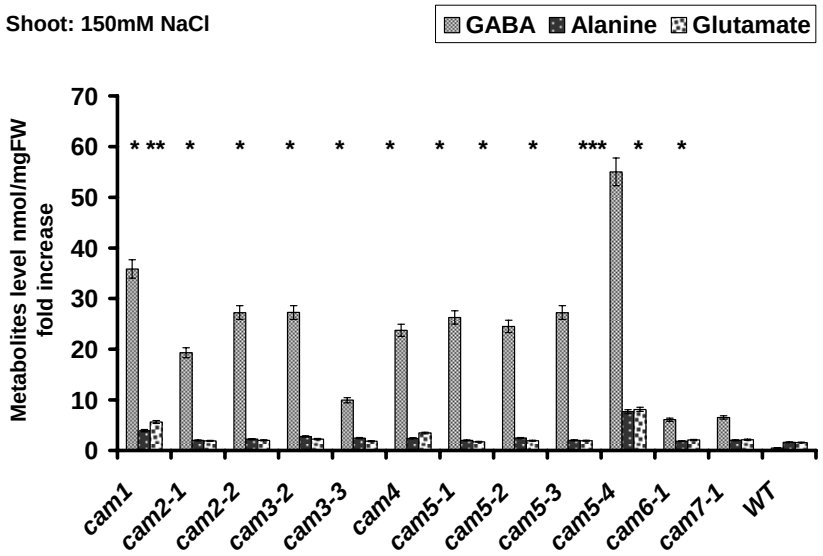
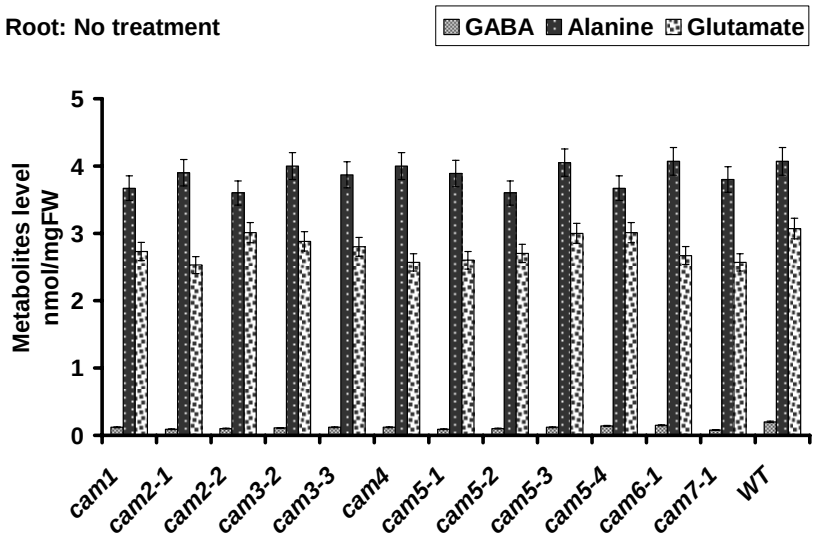
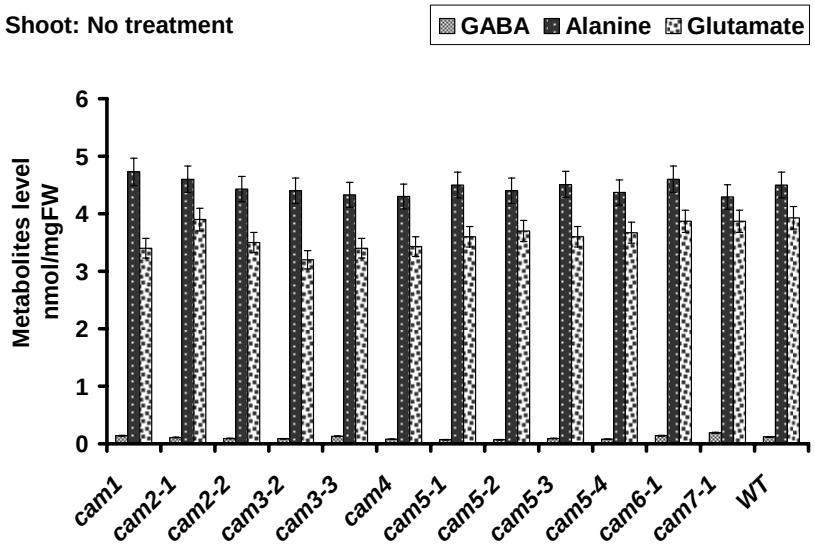

Shoot: 150mM NaCl
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
70
60
50
40
30
20
10
0
* ** * * * * * * * * *** * *
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

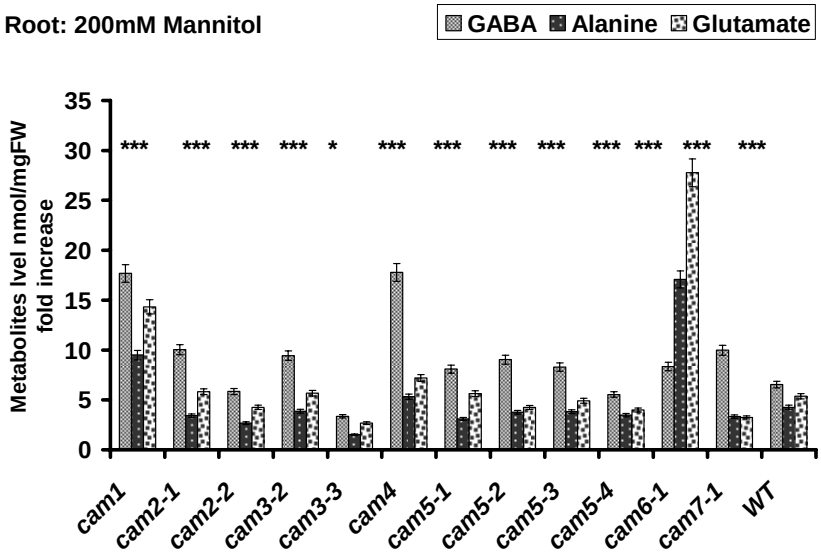
Figure 5. **GABA shunt metabolites in *cam* mutants of *A. thaliana* after exposure to 0, 75, 100, and 150mM NaCl were grown under continuous light at 25ºC for seven days. Root and shoot tissues were harvested and frozen in liquid nitrogen for determination of the level of metabolites as described in materials and methods. Metabolite levels in mutants were calculated as nmol/mgFW and are presented as fold increase in root and shoot tissues of all *cam* mutants and wild type compared to the 0 NaCl control treatment. A, No treatment. B, 75mM NaCl. C, 100mM NaCl. D, 150mM NaCl. Error bars represent the SD over three replicate plates each with 50 seedlings each. Mutants with (*) represent the significant level of changes in metabolite levels (P <0.05).**

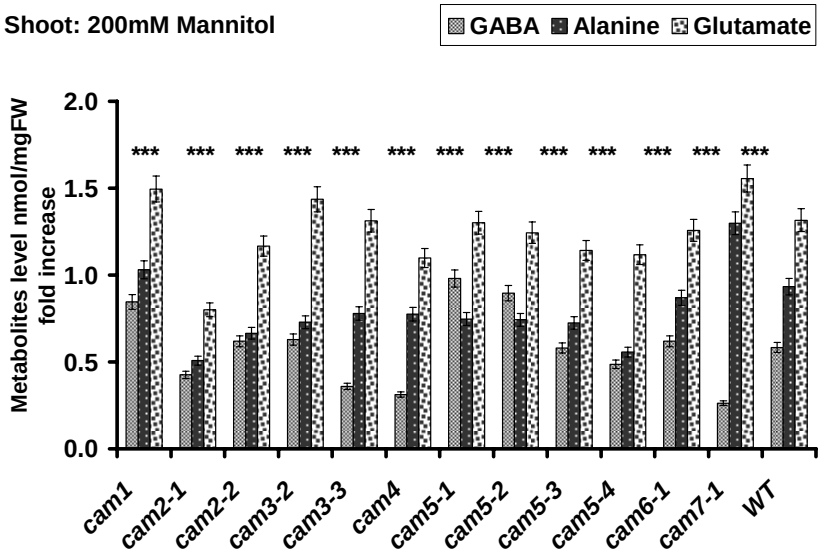
A.


Root: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

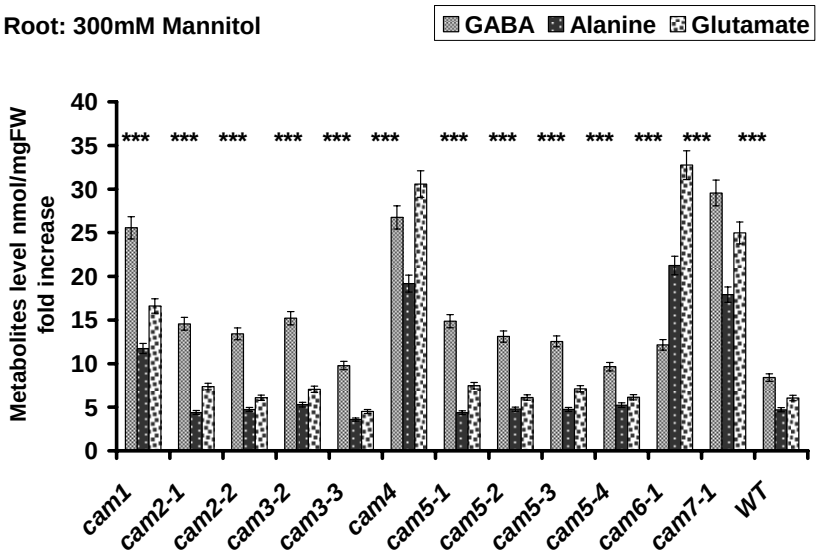

Shoot: No treatment
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
0
1
2
3
4
5
6
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

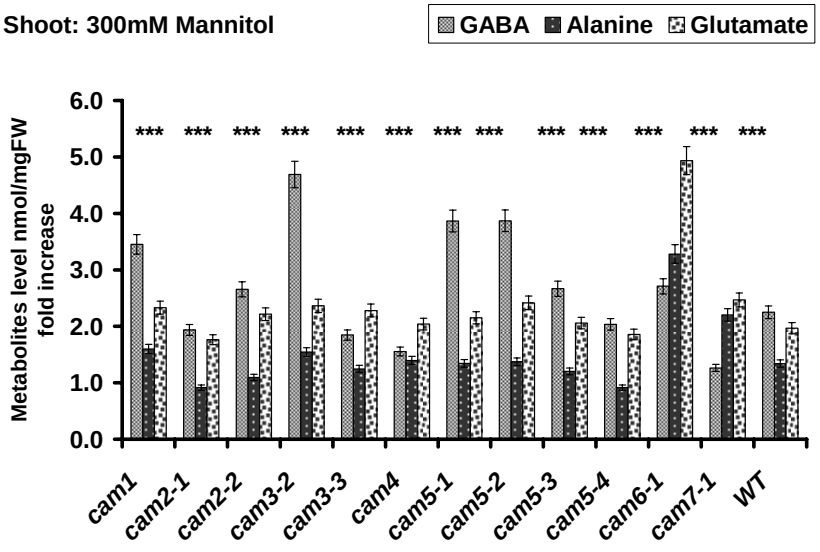
B.


Root: 200mM Mannitol
GABA
Alanine
Glutamate
Metabolites lvel nmol/mgFW
fold increase
35
30
25
20
15
10
5
0
***
***
***
***
*
***
***
***
***
***
***
***
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

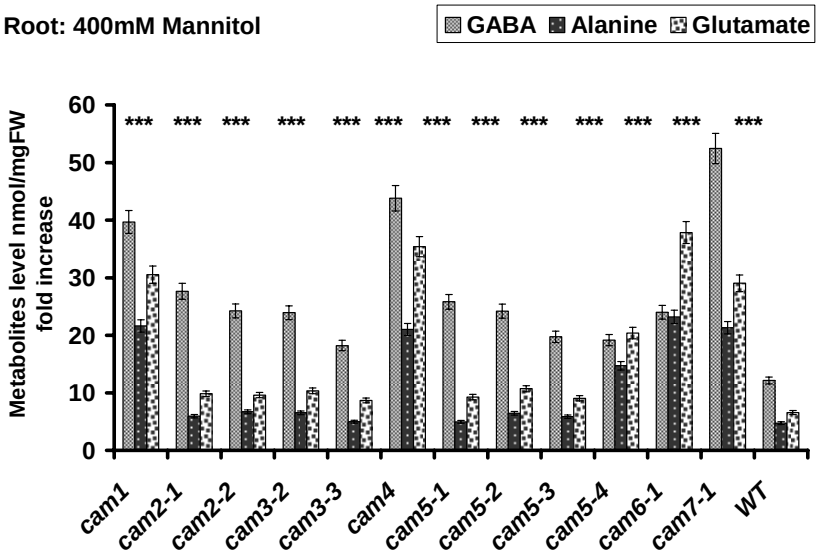

Shoot: 200mM Mannitol
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
***
***
***
***
***
***
***
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

C.


Root: 300mM Mannitol
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
40
35
30
25
20
15
10
5
0
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT


Shoot: 300mM Mannitol
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
6.0
5.0
4.0
3.0
2.0
1.0
0.0
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

D.


Root: 400mM Mannitol
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
60
50
40
30
20
10
0
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

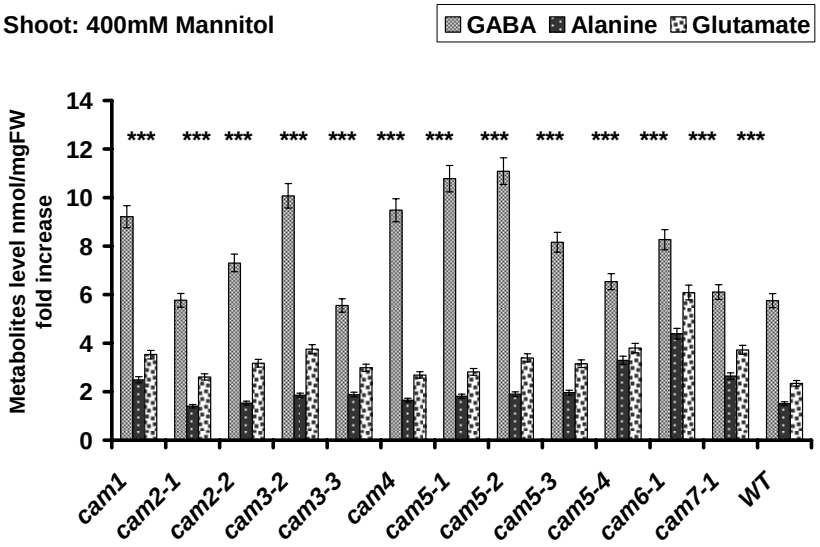

Shoot: 400mM Mannitol
GABA
Alanine
Glutamate
Metabolites level nmol/mgFW
fold increase
14
12
10
8
6
4
2
0
***
cam1
cam2-1
cam2-2
cam3-2
cam3-3
cam4
cam5-1
cam5-2
cam5-3
cam5-4
cam6-1
cam7-1
WT

Figure 6. **GABA shunt metabolites in *cam* mutants and wild type *A. thaliana* seedlings after exposure to 0, 200, 300 and 400mM mannitol grown under continuous light at 25ºC for seven days. Root and shoot tissues were harvested and frozen in liquid nitrogen for determination of the level of metabolites as described in materials and methods. Metabolite levels in mutants were calculated and expressed as nmol/mgFW or fold increase relative to the 0 mM mannitol control in root and shoot tissues of all *cam* mutants and wild type. A, No treatment. B, 200mM Mannitol. C, 300mM Mannitol. D, 400mM Mannitol. Error bars represent the SD over three replicate plates each with 50 one week old seedlings transferred after growth on normal medium. Mutants with (*) represent the significant level of changes in metabolite levels (P <0.05).**

Table 1. **Mutant lines with T-DNA insertion in calmodulin genes of *A. thaliana* used in this study. All seeds lines were obtained from Dr.Janet Braam, Rice University, except *cam1* and *cam4* seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus.**

Mutant	Insertion Location	SALK Line #
cam1	3`UTR	SALK_107507
cam2-1	Intron	SALK_066990
cam2-2	Intron	SALK_089283
cam3-2	5`UTR	SALK_075669
cam3-3	3`UTR	SALK_042391
cam4	5`UTR	SALK_149142
cam5-1	3`UTR	SALK_007371
cam5-2	3`UTR	SALK_073480
cam5-3	5`UTR	SALK_138758
cam5-4	Exon II	SALK_027181
cam6-1	3`UTR	SALK_071609
cam7-1	3`UTR	SALK_074336

APPENDIX I

Summary of the normalized expression in root and shoot tissues of *A. thaliana* wild type at 25ºC normal growth and 42ºC heat treatment for 2 hr.

	Root		Shoot	
	Control	Heat Treated	Control	Heat Treated
Gene	(25ºC)	(42ºC)	(25ºC)	(42ºC)
CAM1	0.3946	1.3834	0.473	0.152
CAM2	2.205	10.6899	1.301	10.3431
CAM3	1.7842	10.8173	1.9786	9.1667
CAM4	1.19	3.0732	1.11	2.6178
CAM5	1.2107	0.7667	1.0873	0.021
CAM6	1.0456	3.3122	1.11	2.0277
CAM7	0.1077	0.6376	0.1083	0.3944
CAM8	1.0233	8.3813	1.0072	7.3041
CAM9	1.1772	4.1119	1.0676	3.9521

APPENDIX II

Summary of Log_2 fold increase in the expression level of nine *CAM* genes in root and tissues of *cam* mutants after 2 hr treatment at 42ºC compared to normal growth at 25ºC. Numbers in parenthesis represent Log_2 fold change. Change that is less than 1 fold was not considered as a significant change.

	Root		Shoot	
Genes	**Increase**	**Decrease**	**Increase**	**Decrease**
CAM1	*cam1* (5) *cam4* (3) *cam5-2* (8) *cam5-4* (3) *cam6-1*(5) *cam7-1*(5) WT (2)	*cam3-2* (3) *cam3-3* (8) *cam5-1* (11)	*cam5-2* (2) *cam5-4* (3) *cam6-1* (3) *cam7-1* (2.4)	*cam1* (3) *cam2-2* (2) *cam3-2* (1) *cam3-3(1)* *cam5-1* (1.2) *cam5-3* (1) WT (2)
CAM2	*cam1*(13) *cam2-1*(2) *cam2-2* (2) *cam3-2* (4) *cam4* (6) *cam5-2* (3) *cam5-3* (4) *cam5-4* (5) *cam6-1* (4) *cam7-1* (6) WT (3)	*cam3-3* (1) *cam5-1* (1)	*cam1* (1) *cam2-1* (1) *cam2-2* (1) *cam3-2* (3) *cam3-3* (3) *cam4* (3) *cam5-1* (3) *cam5-2* (1) *cam5-3* (4) *cam5-4* (3) *cam6-1* (3) *cam7-1* (3) WT (2.3)	None
CAM3	*cam1* (6) *cam2-1* (2) *cam2-2* (4) *cam3-2* (1)	*cam3-3* (4) *cam4* (1) *cam5-1* (3) *cam6-1* (1)	*cam1* (3) *cam2-1* (1) *cam2-2* (1) *cam3-2* (1)	None

	cam5-2 (7) *cam5-3* (3) *cam5-4* (4) *cam7-1* (4) WT (3)	None	*cam3-3* (2) *cam4* (4) *cam5-1* (2) *cam5-2* (4) *cam5-3* (2) *cam5-4* (5) *cam6-1* (4) *cam7-1* (6) WT (2)	
CAM4	*cam1* (1) *cam2-1* (2) *cam4* (3) *cam5-2* (6) *cam5-4* (2) *cam6-1* (3) *cam7-1* (2) WT (2)	*cam2-2* (2) *cam3-2* (1) *cam3-3* (4) *cam5-1* (3)	*cam1* (1) *cam4* (1) *cam5-2* (2) *cam5-4* (1) *cam6-1* (1) *cam7-1* (2) WT (1)	*cam2-1*(1) *cam2-2* (1) *cam3-2*(1) *cam3-3* (1) *cam5-1* (1)
CAM5	*cam1* (1) *cam7-1* (1)	*cam2-1* (3) *cam2-2* (3) *cam3-2* (4) *cam3-3* (9) *cam4* (2) *cam5-1* (8) *cam5-2* (6) *cam5-3* (1) WT (1)	None	*cam1* (1) *cam2-1* (7) *cam2-2* (5) *cam3-2* (7) *cam3-3* (6) *cam4* (2) *cam5-1* (4) *cam5-2* (7) *cam5-3* (3) *cam6-1* (1) *cam7-1* (2) WT (2)
CAM6	*cam1* (5) *cam4* (1) *cam5-2* (8) *cam5-4* (3)	*cam2-1* (3) *cam2-2* (2) *cam3-2* (3) *cam3-3* (2)	*cam1* (3) *cam3-2* (1) *cam4* (2) *cam5-2* (3)	*cam3-3* (2) *cam5-1*(1) *cam5-3* (1)

	cam6-1 (3) *cam7-1* (4) WT (2)	*cam5-1* (1) *cam5-3* (1)	*cam5-4* (2) *cam6-1* (3) *cam7-1* (2) WT (1)	
CAM7	*cam1* (13) *cam4* (10) *cam5-2* (8) *cam5-4* (10) *cam6-1* (10) *cam7-1* (7) WT (3)	*cam2-2* (1) *cam3-2* (1.4) *cam3-3* (1) *cam5-1*(1)	*cam1* (4) *cam2-1* (3) *cam2-2* (1.4) *cam3-3* (2) *cam4* (3) *cam5-1* (1) *cam5-2* (2) *cam5-3* (2) *cam5-4* (4) *cam6-1* (3) *cam7-1* (2) WT (2)	None
CAM8	*cam1*(9) *cam2-2* (2) *cam4* (3) *cam5-2* (8) *cam5-4* (7) *cam6-1* (8) *cam7-1* (6) WT (3)	*cam3-2* (1) *cam3-3* (1) *cam5-1* (2)	*cam1* (9.4) *cam2-1* (11) *cam4* (4.4) *cam5-2* (5.4) *cam5-4* (8) *cam6-1* (9) *cam7-1* (9) WT (3)	*cam3-2* (3) *cam3-3* (1) *cam5-1* (2) *cam5-3* (1)
CAM9	*cam1* (7) *cam2-2* (1) *cam4* (2) *cam5-2* (3) *cam5-3* (1.2) *cam5-4* (6) *cam6-1* (5) *cam7-1* (6) WT (2)	*cam3-2* (2) *cam3-3* (3) *cam5-1* (5)	*cam1* (8) *cam2-1* (1.1) *cam3-3* (1) *cam4* (5) *cam5-2* (5) *cam5-4* (4) *cam6-1* (5) *cam7-1* (5) WT (2)	*cam2-2* (1) *cam3-2* (1) *cam5-3* (8)

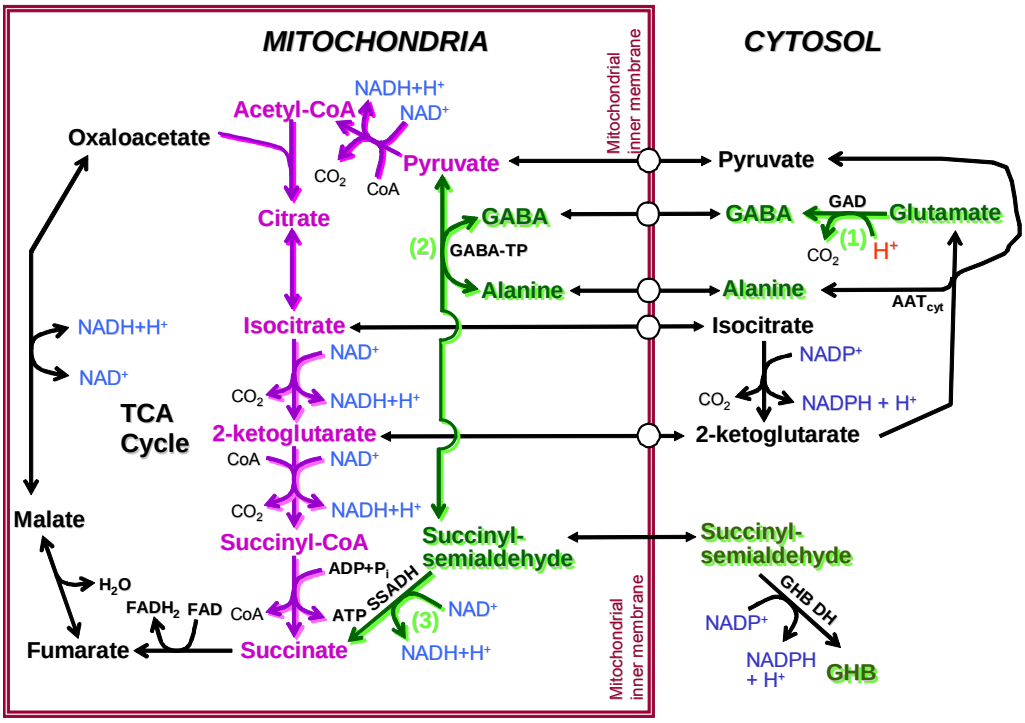
APPENDIX III

Summary of the normalized expression in root and shoot tissues of *A. thaliana cam* mutants with the corresponding primer at 25ºC normal growth and 42ºC heat treatment for 2 hr.

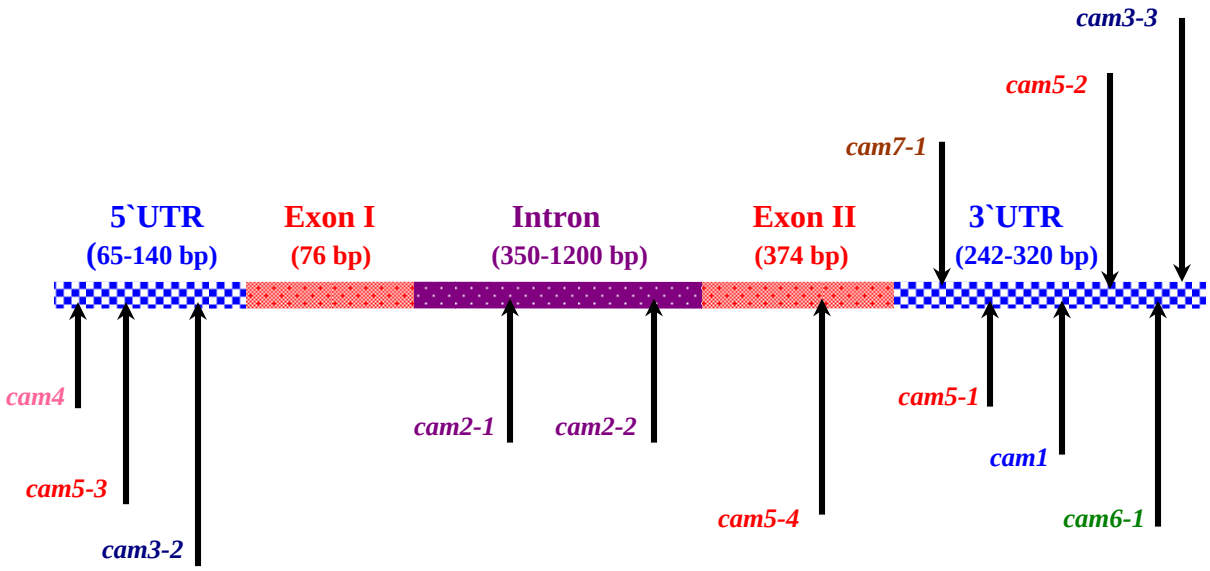
	Root		Shoot	
	Control	Heat Treated	Control	Heat Treated
Mutant	(25ºC)	(42ºC)	(25ºC)	(42ºC)
cam1	0.0631	1.8384	0.3043	0.033
cam2-1	1.1035	4.0001	1.0756	2.4115
cam2-2	0.8534	3.7809	0.9692	2.1136
cam3-2	0.4221	0.7786	0.4327	2.1283
cam3-3	0.143	0.0333	0.5586	2.3187
cam4	1.22	11.3113	1	2.3462
cam5-1	2.4133	0.0113	4.5469	0.2498
cam5-2	0.0807	0.001	0.1076	0.001
cam5-3	1.6362	0.7901	3.4744	0.3276
cam5-4	0	0	0	0
cam6-1	0.9875	7.4035	0.43	4.259
cam7-1	0.0193	1.8125	0.19	0.5849

APPENDIX IV. GABA SHUNT METABOLIC PATHWAY IN PLANT AND ITS RELATION WITH OTHER METABOLIC PATHWAYS

● The major metabolites and enzymes that are specifically associated with the shunt pathway are indicated by green color. (**Copyright©: Dr.Robert D.Locy**)



APPENDIX V. T-DNA INSERTION DIAGHRAMS ON ARABIDOPSIS THALIANA *CAM* GENE OF CAM MUTANTS USED IN THIS STUDY



- **5`UTR: ~ 65-140bp**
- **Exon I: ~ 76bp**
- **Intron: ~ 350-1200bp**
- **Exon II: ~ 374bp**
- **3`UTR: ~ 242-320bp**