Heat and cold stresses phenotypes of *Arabidopsis thaliana* calmodulin mutants: regulation of gamma-aminobutyric acid shunt pathway under temperature stress

Nisreen A. AL-Quraan, Robert D. Locy, Narendra K. Singh

Department of Biological Sciences, Auburn University, Auburn, USA

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 and high temperature for seed germination, susceptibility to low and high temperature induced oxidative damage, and changes in the levels of gamma-aminobutyric acid (GABA) shunt metabolites in response to temperature stress. Exposure of wild type (WT) and *cam* mutant seeds at 4°C showed reduction in germination of *cam5-4* and *cam6-1* seeds. Exposure of *cam* seedlings to 42°C for 2 hr showed reduction in seed germination and survival of seedlings in *cam5-4* and *cam6-1* mutants compared to WT and other *cam* mutants. Oxidative damage by heat and cold stress measured as the level of malonaldehyde (MDA) was detected increased in root and shoot tissues of *cam5-4* and *cam6-1*. Oxidative damage by heat measured as the level of MDA was detected in root and shoot of most *cam* mutants with highest levels in *cam5-4* and *cam6-1*. Level of GABA shunt metabolites in seedlings were gradually increased after 1 hr and 3 hr with maximum level after 6 hr and 12 hr treatments at 4ºC. GABA shunt metabolites in both root and shoot were generally elevated after 30 min and 1 hr treatment at 42°C, and increased substantially after 2 hr at 42°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. Alanine levels showed significant decreases in all *cam* mutants and in WT for 30 and 60 min of heat stress. 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 calmodulin protein (*cam*) in the activation of glutamate decarboxylase (GAD) after exposure to cold, while increased metabolite levels may indicate involvement of other factors like reduction in cytoplasmic pH in cold regulation. Initial general elevation in GABA shunt metabolites after 30 min heat treatment in *cam* mutants suggests regulation of GABA level by *cam*. 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.

Correspondence: Nisreen A. AL-Quraan, Department of Biotechnology and Genetic Engineering, Faculty of science and arts, Jordan University of Science and Technology, Irbid 22110, Jordan. Tel. +962.2.7201000 ext. 23460; Fax: +962.2.7201071.
E-mail: naquraan@just.edu.jo

Key words: Arabidopsis, Calmodulin, *CAM*, CaM, Ca^{+2}, GABA, Cold stress, heat stress, Metabolism, low Temperature, low Temperature.

Received for publication: 3 October 2011.
Revision received: 16 February 2012.
Accepted for publication: 9 March 2012.

International Journal of Plant Biology 2012; 3:e2
doi:10.4081/pb.2012.e2

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.[1-3] Changes in membrane lipid composition and accumulation of sugars had been reported under low temperature acclimation.[4,5] Plants have evolved strategies to prevent and repair such damages that caused by rapid changes in the surrounding temperature. Acquired thermotolerance is one such strategy and involves production of heat shock proteins.[6,7] Heat shock proteins act as molecular chaperons to protect cellular proteins against heat-induced denaturation and to facilitate refolding of heat-damaged proteins.[8] The Hsp100 family proteins are essentials for the acquisition of thermotolerance in Arabidopsis[9-11] and *Zea mays*.[12] Activation of gamma-aminobutyric acid (GABA) shunt pathway in response to various stresses has been shown.[13-16] A rapid increase in GABA level in response to draught in cotton,[17] heat in cultured cowpea cells;[13] heat and induced-oxidative stress in Arabidopsis,[16,18] cold stress and mechanical damage in soybean[19] and in wheat and barely under temperature stress[20] have been reported. GABA is synthesized by a cytosol-localized Ca^{2+}-calmodulin-dependent glutamate decarboxylase (GAD) protein.[21,22] Following irreversible decarboxylation from glutamate, GABA is metabolized to succinate semialdehyde by the GABA shunt pathway which bypasses two steps in the tricarboxylic acid 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,[15,23] are located in the mitochondria. Calcium/calmodulin complex (Ca^{2+}/*cam*) provide a possible level of control for the activation of the GAD enzyme activity[15,23] since this protein is demonstrates a calmodulin binding domain.[24] Such activation could account for the control of the GABA shunt pathway and the concomitant accumulation of GABA during stress. Herein we have used calmodulin T-DNA insertion mutants of *A. thaliana* to examine the role of specific calmodulin protein in tolerance to temperature stress with respect to seed germination, seedlings growth, oxidative damage and GABA shunt metabolite levels in various *cam* mutants under heat and cold treatments.

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, USA, while seeds of all other mutants were obtained from Dr. Janet Braam, Rice University, Texas, USA. 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[25] medium (pH 5.7)

supplemented with 2% (w/v) sucrose, solidified with 1.2% (w/v) agar. Seedlings were grown under continuous illumination (40 µmol m^{-2} s^{-1}) provided by cool white fluorescent lamps at 25ºC.

Seed germination and thermotolerance assay

Seed sensitivity to heat and cold treatments was performed according to Hong and Vierling[9] with the following modifications: for cold treatment surface sterilized seeds of wild type (WT) and *cam* mutants were suspended in 500 µL of 4ºC sterile distilled water and incubated at 4ºC for 24 hours. For heat treatment surface sterilized seeds of WT and each of the *cam* mutants were suspended in 42ºC 500 µL sterile distilled water and incubated at 42ºC in a closed water bath block for 2 hours. Each tube sample contained 50 seeds. Immediately after treatments seeds were platted on square Petri dishes containing media as described above and allowed to grow vertically under continuous light (40 µmol m^{-2} s^{-1}) at 25ºC 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.

Oxidative damage and thiobarbiturate reactive substances assay

Two set of two week old seedlings of WT and *cam* mutants were exposed to 4ºC for 12 hours in growth chamber and another Two sets of two week old seedlings of WT and *cam* mutants were heat treated at 42ºC for 2 hours. For each treatment, 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 thiobarbiturate reactive substances (TBARS) assay.[26] Three plates with 50 seeds each were used in each replicate of each treatment. Root and shoot tissues after cold and heat treatments were separated and frozen in liquid nitrogen. Tissue (0.50 g) was ground in a 1.5 mL microfuge tube, and 0.5 mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and 0.5 mL 175 mm NaCl in 50 mm Tris-HCl, pH 8 was added to the ground tissues. Tubes were heated to 90ºC 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 MDA was determined as nmol/mgFW from a standard curve of MDA.

Gamma-aminobutyric acid-shunt metabolites extraction and determination

For cold treatments 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 hour, 3 hours, 6 hours and 12 hours. For heat treatments WT and cam seedlings of Arabidopsis grown at 25°C for two week in continuous light were exposed to 42ºC in a growth chamber for 30 min, 1 hour, and 2 hours. Root and shoot tissues were separated and used for metabolite analysis. Three plates with 50 seeds each were used for each treatment. Metabolites from frozen tissues were extracted according to Zhang and Bown[27] with the following modifications; separately harvested root and shoot tissues were ground in 1.5 mL microfuge tubes under LN2 until a fine pow-

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, USA.

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. Germination % for all *cam* mutants under heat (42°C, 2 hours) and cold (4ºC, 24 hours) treatments in *A.thaliana*. Two sets with three replicates of fifty seeds each of the wild type and cam mutants were surface sterilized and exposed to 4ºC for 24 hours and 42ºC for 2 hours, separately. 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. Mutants with (*) represent the significant seed sensitivity (P<0.05).

Mutant	No treatment	Heat (42°C, 2 hr)	Cold treatment (4ºC, 24 hr)
cam1	97	90	89
cam2-1	95	90	86
cam2-2	92	92	87
cam3-2	96	91	92
cam3-3	100	93	90
cam4	97	96	85
cam5-1	98	86	88
cam5-2	95	88	90
cam5-3	94	90	84
cam5-4	95	45*	50*
cam6-1	95	50*	65*
cam7-1	96	85	86
Wild type	100	93	91

der was obtained. To each tube 400 µL 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 15 min, and subsequently centrifuged at full speed for 5 min. Supernatants was removed to new tubes and mixed with 160 µL of 1 m KOH. After 10 min mixing, tubes were centrifuged at full speed for 5 min.The supernatant containing metabolites was transferred to a new tube and used to determine the quantity of specific metabolites.

GABA was measured according to Zhang and Bown[27] with the following modifications. The reaction mixture contained 50 µL of sample extract, 14 µL of 4 mm nicotinamide adenine dinucleotide phosphate, 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 25oC using the microplate reader (BioTek power wave, Max200R, USA). The level GABA nmol/mgFW was determined using an nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) standard curve.

Alanine was measured according to Bergmeyer[28] 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°C using a microplate reader (make and model PowerWave HT, Biotek, USA). The level of alanine as nmol/mgFW was determined using an NADH standard curve. Glutamate was measured according to Bergmeyer[28] 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 0.8 units of glutamate dehydrogenase enzyme suspension (Sigma-Aldrich). The level of glutamate was determined at 340 nm after 60 min incubation using a microplate reader. The level of glutamate was determined as nmol/mgFW using an NADH standard curve. The level of all metabolites was represented as nmol/mg FW fold increase by calculation the metabolite for each point value of each treatment in corresponding with its control according to the following equation: Fold change = value of the metabolites / Control).

Table 3. Oxidative damage for all *cam* mutants under cold (4°C, 12 hours) treatments in *A.thaliana*. The malonaldehyde (MDA) level was determined in root and shoot tissues by the thiobarbiturate reactive substances assay describe in materials and methods. Two set of seedlings were treated at 4°C for 12 hours. 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 (nmol/mg FW). Mutants with (*) represent the significant sensitivity (P<0.05).

Mutant	Root			Shoot		
	No treatment	4°C, 12hr	4°C, 12hr-recovery	No treatment	4°C, 12hr	4°C, 12hr-recovery
cam1	0.062	0.07	0.03	0.121	0.07	0.05
cam2-1	0.073	0.11	0.28	0.120	0.10	0.06
cam2-2	0.060	0.70	0.17	0.120	0.10	0.08
cam3-2	0.080	0.05	0.15	0.110	0.06	0.05
cam3-3	0.070	0.07	0.17	0.120	0.07	0.06
cam4	0.087	0.12	0.05	0.107	0.09	0.09
cam5-1	0.072	0.09	0.08	0.108	0.02	0.05
cam5-2	0.070	0.09	0.26	0.120	0.06	0.07
cam5-3	0.074	0.11	0.26	0.105	0.02	0.06
cam5-4	0.086	0.20*	1.23*	0.113	0.15*	0.32*
cam6-1	0.072	0.36*	0.06	0.121	0.14*	0.05
cam7-1	0.088	0.06	0.10	0.108	0.05	0.05
Wild type	0.075	0.139	0.208	0.146	0.100	0.069

Table 4. Oxidative damage for all *cam* mutants under cold (42°C, 2 hr) treatments in *A.thaliana*. The malonaldehyde (MDA) level was determined in root and shoot tissues by the thiobarbiturate reactive substances assay describe in materials and methods. Two set of seedlings were treated at 42°C for 2 hours. 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 (nmol/mg FW). Mutants with (*) represent the significant sensitivity (P<0.05).

Mutant	Root			Shoot		
	No treatment	42°C, 2hr	4°C, 12hr-recovery	No treatment	42°C, 2hr	42°C, 2hr-recovery
cam1	0.062	0.09	0.30	0.121	0.12	0.16
cam2-1	0.073	0.16	0.62	0.120	0.31	0.26
cam2-2	0.060	0.05	0.29	0.120	0.29	0.25
cam3-2	0.080	0.13	0.59	0.110	0.30	0.24
cam3-3	0.070	0.11	0.47	0.120	0.34	0.16
cam4	0.087	0.17	0.13	0.107	0.14	0.23
cam5-1	0.072	0.18	0.36	0.108	0.27	0.24
cam5-2	0.070	0.23	0.31	0.120	0.22	0.45
cam5-3	0.074	0.16	0.45	0.105	0.26	0.39
cam5-4	0.086	0.48*	1.15*	0.113*	0.88*	0.92*
cam6-1	0.072	0.48*	0.75*	0.121*	1.22*	1.16*
cam7-1	0.088	0.18	0.12	0.108	0.38	0.42
WT	0.075	0.094	0.36	0.146	0.203	0.24

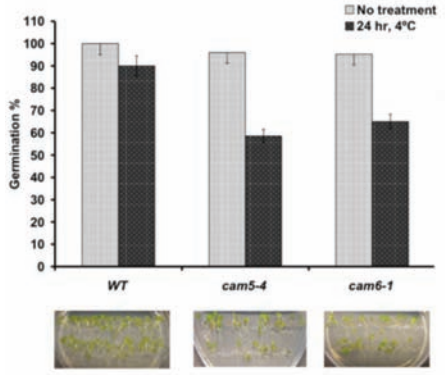

Figure 1. Germination of seeds of wild type, *cam5-4*, and *cam6-1* mutants of *A. thaliana* after exposure to 4°C for 24 hours. Fifty seeds each of the wild type and cam mutants were surface sterilized and exposed to 4°C for 24 hours. Treated seeds were plated on MS salt-agar medium and allowed to germinate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

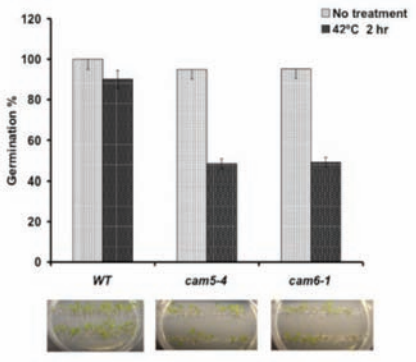

Figure 2. Germination of *A. thaliana* seeds of wild type and T-DNA insertion mutant alleles *cam5-4* and *cam6-1* after exposure to 42°C temperature. Fifty seeds each of genotype were surface sterilized and exposed to 42°C for 2 hours. Heat treated and control seeds were allowed to germinate described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

Table 5. The level of gamma-aminobutyric acid, 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 various cold treatments at 4°C as indicated in material and method. Metabolite levels in mutants were calculated as nmol mg^{-1} FW fold increase in root and shoot tissues separately. Mutants with (*) represent the significant level of changes in metabolite levels ($P<0.05$).

	1 hr , 4°C						3 hr , 4°C					
	Root			Shoot			Root			Shoot		
Mutant	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu
cam1	0.12	0.23	0.3	0.11	0.21	0.19	0.93	0.11	0.21	0.14	0.24	0.12
cam2-1	0.13	0.21	0.23	0.14	0.13	0.17	0.97	0.14	0.13	0.19	0.20	0.13
cam2-2	0.21	0.33	0.24	0.38	0.29	0.20	0.99	0.38	0.29	0.39	0.20	0.21
cam3-2	0.19	0.30	0.18	0.28	0.21	0.18	0.99	0.28	0.21	0.40	0.23	0.19
cam3-3	0.13	0.40	0.22	0.14	0.20	0.19	0.98	0.14	0.20	0.18	0.25	0.13
cam4	0.13	0.17	0.36	0.42	0.23	0.10	0.96	0.42	0.23	0.42	0.23	0.13
cam5-1	0.15	0.36	0.22	0.43	0.25	0.19	1.00	0.43	0.25	0.36	0.25	0.15
cam5-2	0.12	0.28	0.36	0.43	0.23	0.18	1.01	0.43	0.23	0.45	0.23	0.12
cam5-3	0.22	0.35	0.26	0.30	0.22	0.20	0.88	0.30	0.22	0.33	0.22	0.22
cam5-4	0.33*	0.44*	0.36*	0.70*	0.26*	0.25*	1.07*	0.58*	0.52*	0.73*	0.29*	0.32*
cam6-1	0.34*	0.41*	0.65*	0.26*	0.22*	0.11*	1.20*	0.61*	1.17*	0.29*	0.24*	0.22*
cam7-1	0.14	0.38	0.37	0.16	0.24	0.14	1.03	0.48	0.78	0.21	0.21	0.18
WT	0.20	0.51	0.67	0.25	0.25	0.18	0.65	0.59	0.84	0.25	0.26	0.21
	6 hr , 4°C						12 hr , 4°C					
	Root			Shoot			Root			Shoot		
Mutant	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu
cam1	0.47	0.28	0.73	0.31	0.23	0.21	0.67	0.58	1.23	0.55	0.14	0.56
cam2-1	0.26	0.23	0.27	0.36	0.21	0.13	0.53	0.36	1.30	0.27	0.19	0.36
cam2-2	0.53	0.44	0.53	0.84	0.33	0.29	1.11	0.58	1.23	0.53	0.39	0.84
cam3-2	0.60	0.48	0.41	0.91	0.30	0.21	1.28	0.55	1.24	0.41	0.40	0.91
cam3-3	0.50	0.40	0.54	0.59	0.22	0.20	0.81	0.74	1.30	0.54	0.18	0.59
cam4	0.39	0.34	0.78	0.95	0.17	0.23	1.67	0.52	0.96	0.36	0.42	0.95
cam5-1	0.66	0.58	0.68	0.80	0.36	0.25	1.83	0.59	1.05	0.58	0.36	0.80
cam5-2	0.54	0.48	0.71	0.64	0.28	0.23	1.83	0.58	0.98	0.55	0.45	0.64
cam5-3	0.36	0.30	0.44	0.59	0.35	0.22	0.77	0.51	0.78	0.44	0.33	0.59
cam5-4	1.69*	0.71*	1.41*	1.78*	0.40*	0.50*	4.54*	1.69*	1.87*	2.38*	0.59*	2.82*
cam6-1	1.98*	0.69*	5.22*	0.59*	0.28*	0.36*	2.80*	1.40*	6.70*	0.88*	0.42*	1.91*
cam7-1	0.96	0.65	1.06	0.37	0.23	0.29	1.02	1.03	1.20	0.34	0.32	0.88
WT	1.00	0.62	1.85	0.42	0.28	0.31	1.05	1.15	2.07	0.50	0.38	1.54

Table 6. The level of gamma-aminobutyric acid, 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 various heat treatments at 42°C as indicated in material and method. Metabolite levels in mutants were calculated as nmol mg^{-1} FW fold increase in root and shoot tissues separately. Mutants with (*) represent the significant level of changes in metabolite levels (P<0.05).

	30 min, 42°C						1 hr , 42°C						2 hr , 42°C					
	Root			Shoot			Root			Shoot			Root			Shoot		
Mutant	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu
cam1	0.23	0.31	0.21	0.14	0.13	0.58	0.73	0.61	2.57	0.71	0.31	1.53	1.42	1.12	5.29	1.18	0.66	0.53
cam2-1	0.21	0.29	0.13	0.19	0.07	0.36	0.27	0.57	2.69	0.95	0.29	0.99	0.93	0.75	4.55	1.75	0.27	1.12
cam2-2	0.33	0.29	0.29	0.39	0.12	0.58	0.53	0.57	1.71	1.98	0.29	1.09	0.53	0.78	3.68	1.06	0.53	1.04
cam3-2	0.30	0.31	0.21	0.40	0.14	0.55	0.41	0.56	1.75	1.28	0.31	1.07	0.45	0.76	4.09	1.95	0.41	1.71
cam3-3	0.40	0.24	0.20	0.18	0.15	0.47	0.54	0.60	2.79	0.96	0.24	1.37	0.35	0.77	4.44	1.51	0.54	1.10
cam4	0.17	0.24	0.23	0.42	0.12	0.52	0.78	0.65	1.86	1.05	0.24	1.35	1.89	1.45	3.95	1.81	0.67	1.52
cam5-1	0.36	0.33	0.25	0.36	0.13	0.59	0.68	0.56	2.52	0.96	0.33	1.40	0.86	0.73	4.22	1.40	0.68	1.46
cam5-2	0.28	0.35	0.23	0.45	0.12	0.58	0.71	0.56	2.24	0.65	0.35	1.39	0.33	0.73	3.92	1.73	0.58	1.60
cam5-3	0.35	0.28	0.22	0.33	0.14	0.51	0.44	0.50	1.81	1.04	0.28	1.23	0.96	0.62	3.36	2.86	0.44	1.62
cam5-4	0.52*	0.34*	1.32*	0.65*	0.17*	0.80*	1.04*	0.88*	3.98*	1.97*	0.39*	1.78*	1.81*	1.84*	11.08*	4.96*	0.86*	2.83*
cam6-1	1.04*	0.40*	2.02*	0.37*	0.11*	0.48*	1.78*	0.65*	4.29*	1.33*	0.26*	1.21*	2.44*	0.94*	6.01*	2.39*	0.86*	2.95*
cam7-1	0.66	0.30	1.82	0.27	0.10	0.50	0.92	0.61	3.39	1.06	0.22	1.30	5.13	0.77	4.22	1.37	0.62	1.30
WT	0.75	0.46	2.22	0.67	0.22	0.70	1.20	0.71	3.91	1.92	0.44	1.49	1.55	0.94	4.34	2.33	0.46	1.61

Data analysis

Each data point was expressed as the mean ± standard deviation (SD) of three independent experiments. The values were compared and analyzed by two-way analysis of variance (ANOVA) using least significant difference (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 and heat treatments

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% under all treatments (Figure 1). Except for *cam5-4* and *cam6-1*, seeds of the other Arabidopsis cam mutants exposed to 42°C for 2 hours germinated normally (Table 2). Germination of *cam5-4* and *cam6-1* seeds was significantly (P<0.05) inhibited by 45% and 50% after exposure to 42°C for 2 hours, respectively (Figure 2). The sensitivity of *cam6-1* and *cam5-4* alleles to low and high temperature germination may be explained by the fact that the protein products of the *CAM5* and *CAM6* genes may be involved in protective pathways that contribute to germination and survival of plants at low and high temperatures.

The nature of the *cam5-4* mutant allele was investigated by examining the mRNA level of the entire *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.[29] Although the level of *CAM6* mRNAs in the *cam6-1* mutant is reduced but not eliminated and the levels of other *CAM* gene mRNAs are either equal to wild type levels or reduced comparable to those in *cam5-4*. 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.[29] 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 low and high 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 MDA in root and shoot under cold treatment at 4ºC for 12 hours, heat treatment at 42ºC for 2 hours, and after recovery for 2 days under continuous light. In response to cold treatment MDA level accumulated in many *cam* mutants both immediately and after recovery in root and shoot tissues (Table 3). The *cam5-4* allele showed a high level of MDA (4 to 5 fold increase, P<0.5) after recovery in both root and shoot when compared to wild type while *cam6-1* accumulated 1.5-fold increase, (P<0.05) in MDA in both root and shoot tissues but not when assayed after recovery (Figure 3). In Response to heat treatment seedlings of *cam5-4* and *cam6-1* demonstrated dramatically higher levels of MDA in both root and shoot, and the level of MDA remained higher after 2 days of recovery (P<0.05) (Table 4, Figure 4). 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 thermosensitive. 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. 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. We observed the same sensitivity phenotype of both *cam5-4* and *cam6-1* mutants in response to paraquat and H_2O_2 treatments during seed germination and seedling growth, and oxidative damage.[18] 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.[30-32] Under heat stress oxidative damage has been observed in creeping bentgrass (*Agrostis stolonifera*),[33] and Arabidopsis seedlings.[7,34,35] Heat induced oxidative damage increased in Arabidopsis seedlings pretreated with Ca^{+2}/CaM inhibitors,[34] and higher CaM proteins levels have been linked to lower levels of heat-induced membrane damage in maize.[36] 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 in our study.

Level of gamma-aminobutyric acid shunt metabolites in *cam* mutants after cold treatment

Since GABA shunt pathway has been reported to be activated following cold stress in plant systems,[19,20] the levels of glutamate, alanine, and GABA in roots and shoots during cold treatment at 4ºC was determined (Figure 5). The levels of the three amino acids gradually increased after 1 hr of cold treatment with a maximum level reached after 6 hours and 12 hours of treatment (Table 5). After 1 hour, GABA shunt metabolite levels increased up to 0.5-1-fold compared to the control (no treatment) (Figure 5A) in root and shoot tissues of all *cam* mutants and the wild type (Table 5). The maximum increase occurred in root of *cam5-4*, *cam6-1* and the WT (Figure 5B). 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 *cam5-4* mutant (Figure 5C). After a 3 hours cold treatment at 4ºC, GABA levels increased 0.65-2.5-fold

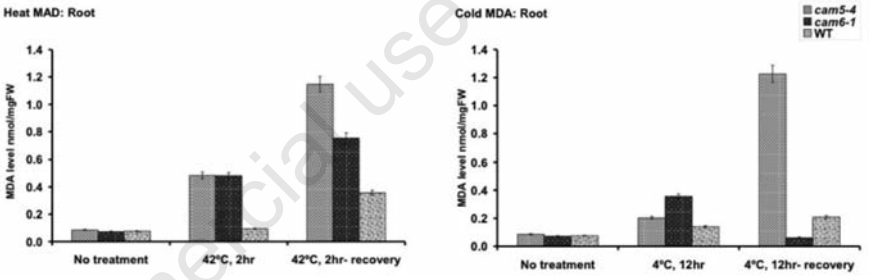

Figure 3. Oxidative damage in *cam* mutants of *A. thaliana* after exposure to low temperature at 4°C. Malonaldehyde (MDA) level was determined in root and shoot tissues by the thiobarbiturate reactive substances (TBARS) assay describe in materials and methods. Two sets of seedlings were treated at 4ºC 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 25oC before determination of MDA level. Error bars represent the standard deviation over three replicate plates with 50 seedlings on each plate.

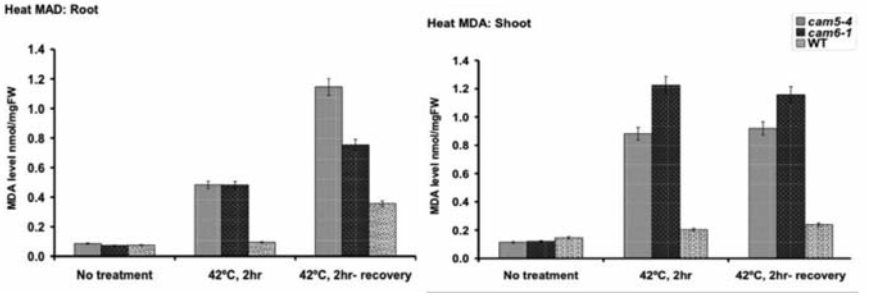

Figure 4. Oxidative damage by high temperature exposure in cam mutants of *A. thaliana*. The malonaldehyde (MDA) level was determined in root and shoot tissues by the thiobarbiturate reactive substances (TBARS) assay describe in materials and methods. Two set of seedlings were treated at 42ºC for 2 hours. 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 (nmol/mg FW). Error bars represent the standard deviation over three replicate plates with 50 seedlings on each plate.

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 *cam5-4* mutants. Alanine and glutamate increased in all *cam* mutants and the wild type greatest increases in roots of *cam5-4* and *cam6-1*. After 6 hours incubation at 4ºC, 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. Glutamate levels increased 3-5-fold in roots of *cam6-1* mutant (Figure 5B). 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 *cam5-4* mutants as shown in Figure 5. After 12 hours of cold treatment at 4ºC, 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 *cam5-4* mutant. Glutamate levels increased 3-7-fold in roots of *cam6-1* (Figure 5B). 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 *cam5-4* mutants. Glutamate levels increased 1-3.5-fold in shoots of *cam5-4* and *cam6-1* mutants and the wild type as shown in Figure 5C.

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 5). The accumulation of GABA in response to low temperature may result from an altered intracellular compartmentation 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.*[20] 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

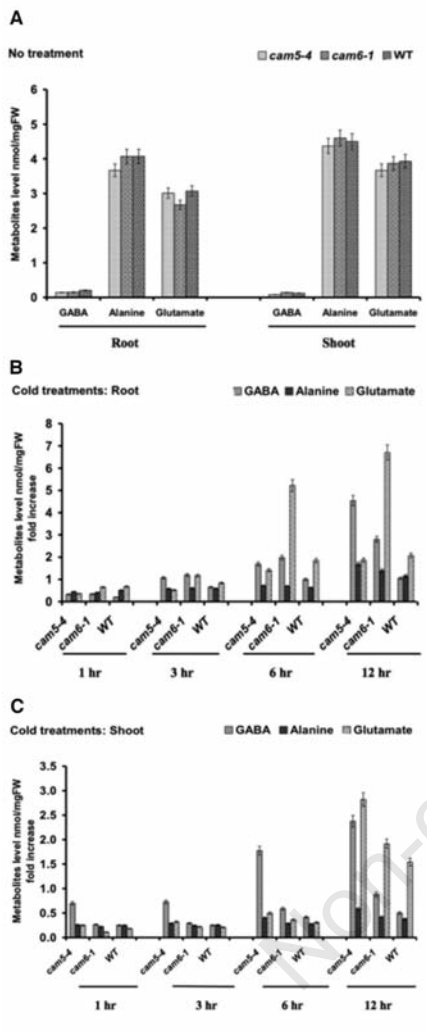

Figure 5. The level of gamma-aminobutyric acid, alanine and glutamate in root and shoot tissues of two week old seedlings of wild type and cam mutants of *A. thaliana* was determined after exposure to cold as indicated. Seedlings were treated for 0 hour, 1 hours, 3 hours, 6 hours and 12 hours at 4ºC. 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; B) Root; C) Shoot. Error bars represent standard deviation over three replicate plates, each containing 50 seedlings each of wild type and *cam* mutants.

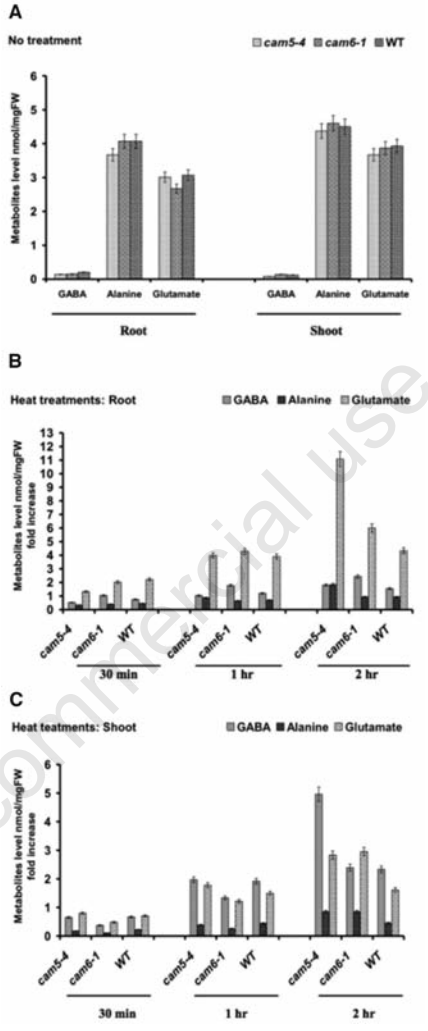

Figure 6. The levels of gamma-aminobutyric acid, alanine and glutamate in two-week-old seedlings of cam mutants of *A. thaliana* after exposure to heat at 42°C for 30 min, 1 hour and 2 hours. Root and shoot tissues were harvested separately and frozen in liquid nitrogen for determination of the level 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) Root; C) Shoot. Error bars represent standard deviation over three replicate plates. Each sample contained 50 seedlings.

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.

Levels of gamma-aminobutyric acid 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 *cam* mutants of Arabidopsis CAM genes were determined after 0 min, 30 min, 1 hour, and 2 hour at 42ºC. GABA shunt metabolites in both root and shoot were generally elevated after 30 min and 1 hour of heat treatment at 42°C, and increased substantially after 2 hour at 42°C. Furthermore, GABA and glutamate levels were increased more than alanine in root and shoot tissues of wild type and all *cam* mutants (Table 6). After 1 hour, level of GABA (0.7-3 fold increase) and glutamate (1-4.3 fold increase) were increased in all *cam* mutants and wild type root and shoot tissues compared to the control except in root of *cam5-4* (Figure 6). After 2 hr at 42°C, the GABA level increased in all *cam* mutants, and WT increased up to 5-fold in root *cam5-4* compared to the control. Alanine level did not increase in WT or any of the *cam* mutants during heat treatment. Glutamate levels increased in all *cam* mutants and WT (3-11 fold increase) with highest level in root of *cam5-4* and *cam6-1* (6-11-fold) by comparison to the glutamate level in the untreated sample (Figure 6B). 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.[16,17,19,20,37,38] Previously we reported that GABA, alanine and glutamate levels were significantly increased in root and shoot of the *cam5-4*, and *cam6-1* mutants in response to paraquat (0.5, 1 and 3 μM), while they were increased only in the root tissue of the *cam5-4*, and *cam6-1* mutants in response to H_2O_2 (200 and 500 l m, 1 mm).[18] 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.

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.

References

1. Kaye C, Guy CL. Prespectives of plant cold tolerance: physiology and molecular responses. Sci Prog 1995;78:271-99.
2. Guy CL, Niemi KJ, Brambl R. Altered gene expression during cold acclimation of spinach. Proc Natl Acad Sci USA 1985;82: 3673-7.
3. Jung SH, Lee JY, Lee DH. Use of SAGE technology to reveal changes in gene expression in Arabidopsis leaves undergoing cold stress. Plant Mol Biol 2003;52:553-67.
4. Cossins AR, Murray PA, Gracey AY, et al. The role of desaturases in cold-induced lipid restructuring. Biochem Society Transactions 2002;30:1082-6.
5. Stitt M, Hurry V. A plant for all seasons: alterations in photosynthetic crabon metabolism during cold acclimation in Arabidopsis. Curr Opin Plant Biol 2002;5:199-206.
6. Vierling E. The role of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 1991;42:579-620.
7. Larkindale J, Hall JD, Knight MR, Vierling E. Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol 2005;138:882-97.
8. Boston RS, Viitanen PV, Vierling E. Molecular chaperonens and protein folding in plants. Plant Mol Biol 1996;32:191-222.
9. Hong SW, Vierling E. Mutants of Arabidopsis thaliana Defective in the Acquisition of tolerance to high temperature stress. Proc Natl Acad Sci USA 2000;97:4392-7.
10. Hong SW, Vierling E. Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J 2001;27:25-35.
11. Hong SW, Lee U, Vierling E. Arabidopsis hot mutants define multiple functions required for acclimation to high temperatures. Plant Physiol 2003;132:757-67.
12. Nieto-Sotelo J, Kannan KB, Martinez LM, Segal C. Characterization of maize heat shock protein 101 gene, HSP101, encoding a ClpB/Hsp100 protein homologue. Gene 1999;230:187-95.
13. Mayer RR, Cherry JH, Rhodes D. Effects of heat shock on amino acid metabolism of cowpea cells. Phytochemistry 1990;94: 796-810.
14. Bown AW, Shelp BJ. The metabolism and functions of γ-aminobutyric acid. Plant Physiol 1997;115:1-5.
15. Shelp BJ, Bown AW, Maclean MD. Metabolism and function of gamma-aminobutyric acid. Trend Plant Sci 1999; 4:446-42.
16. Locy RD, Wu SJ, Bisnette J, et al. The regulation of GABA accumulation by heat stress in arabidopsis. In: Cherry JH, Locy RD, Rychter A. Plant tolerance to abiotic stresses in agriculture: role of genetic engeneering. Dordrech, Netherland: Kluwer Academics; 2000. pp 39-52.
17. Hanower P, Brzozowska J. Influence d'un choc osmotique sur la composition des feuilles de cotonnier en acides amines libres. Phytochemistry 1975;14:1691-4.
18. AL-Quraan NA, Locy RD, Singh NK. Implications of paraquat and hydrogen peroxide-induced Oxidative stress treatments on the GABA shunt pathway in Arabidopsis thaliana calmodulin mutants. Plant Biotech Rep 2011;5:225-34.
19. Wallace W, Secor J, Schrader LE. Rapid accumulation of γ-amino butyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. Plant Physiol 1984;75:170-5.
20. Mazzucotelli E, Tartari A, Cattivelli L, Forlani G. Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barely and wheat. J Exp Bot 2006;57: 3755-66.
21. Baum G, Chen Y, Arazi T, et al. A plant glutamate decarboxylase containing a calmodulin binding domain: cloning, sequence, and functional analysis. J Biol Chem 1993;268:19610-7.
22. Snedden WA, Koutsia N, Baum G, Fromm H. Activation of a recombinant Petunia glutamate decarboxylase by calcium/calmodulin or by a monoclonal antibody which recognizes the calmod-

ulin binding domain. J Biol Chem 1996; 271:4148-53.
23. Bouche N, Fromm H. GABA in Plants: Just a metabolite? Trend Plant Sci 2004; 9:110-5.
24. Zik M, Arazi T, Snedden WA, Fromm H. Two isoforms of glutamate decarboxylase in arabidopsis are regulated by calcium/calmodulin and differ in organ distribution. Plant Mol Biol 1998;37:967-75.
25. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15:472-97.
26. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. Arch Biochem Biophys 1968;125:189-98.
27. Zhang G, Bown AW. The rapid determination of gamma-aminobutyric acid. Phytochemistry 1997;44:1007-9.
28. Bergmeyer HU. Methods of enzymatic analysis. 2nd Edition. Volume I. Weinheim: Verlag Chemie; 1983. p 427.
29. AL-Quraan NA, Locy RD, Singh NK. Expression of calmodulin genes in wild type and calmodulin mutants of Arabidopsis thaliana under heat stress. Plant Physiol Biochem 2010;48:697-702.
30. Foyer CH, Descourvieres P, Kunert KJ. Protection against Oxygen Radicals: an Important Defense Mecahnisms Studies in Transgenic Plants. Plant Cell Environm 1994;17:507-23.
31. Harndahl U, Hall R, Osteryoung K, et al. The chloroplastic small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. Cell Stress Chaperon 1998;4:129-38.
32. Lu C, Zhang J. Effects of water stress on PSII photochemistry and its thermostability in wheat plants. J Exp Bot 1999;50:1199-06.
33. Larkindale J, Huang B. Thermotolerance and antioxidant systems in agrostis stolonifera: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. J Plant Physiol 2004; 161:405-13.
34. Larkindale J, Knight MR. Protection against heat stress-induced oxidative damage in arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol 2002;128:682-95.
35. Larkindale J, Vierlang E. Core genome responses involved in acclimation to high temperature. Plant Physiol 2008; 146:748-61.
36. Gong M, Chen SN, Song YQ, Li ZG. Effects of calcium on intrinsic heat tolerance in relation to antioxidant systems in maize seedlings. Aust J Plant Physiol 1997;24:371-9.
37. Kaplan F, Kopka J, Haskell DW, et al. Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiol 2004;136:4159-68.
38. Kaplan F, Kopka J, Sung DY, et al. Transcripts and metabolite profiling during cold acclimation of arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant J 2007;50:967-81.