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ORIGINAL ARTICLE

Implications of paraquat and hydrogen peroxide-induced oxidative stress treatments on the GABA shunt pathway in *Arabidopsis thaliana* calmodulin mutants

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Abstract *Arabidopsis* mutants with T-DNA insertion in seven calmodulin genes (*CAM*) were used to determine the specific role of CAM in the tolerance of plants to oxidative stress induced by paraquat and hydrogen peroxide (H_2O_2) treatments. *Arabidopsis* calmodulin mutants (*cam*) were screened for seedling growth, seed germination, induced oxidative damage, and levels of γ-aminobutyric acid (GABA) shunt metabolites. Only the *cam*5-4 and *cam*6-1 mutants exhibited an increased sensitivity to paraquat and H_2O_2 during seed germination and seedling growth. In response to treatments with 3 μM paraquat and 1 mM H_2O_2, only the *cam*5-4, *cam*6-1 mutants showed significant changes in malonaldehyde (MDA) levels in root and shoot tissues, with highly increased levels of MDA. In terms of the GABA shunt metabolites, GABA was significantly elevated in root and shoot tissues in response to the paraquat treatments in comparison to alanine and glutamate, while the levels of all shunt metabolites increased in root tissue but not in the shoot tissue following the H_2O_2 treatments. GABA, alanine and glutamate levels were significantly increased in root and shoot of the *cam*1, *cam*4, *cam*5-4, and *cam*6-1 mutants in response to paraquat (0.5, 1 and 3 μM), while they were increased only in the root tissue of the *cam*1, *cam*4, *cam*5-4, and *cam*6-1 mutants in response to H_2O_2 (200 and 500 μM, 1 mM). These data show that the *cam*5-4 and *cam*6-1 mutants were sensitive to the induced oxidative stress treatments in terms of seed germination, seedling growth, and oxidative damage. The accumulation of GABA shunt metabolites as a consequence of the induced oxidative stress treatments (paraquat and H_2O_2 treatments) suggests that the GABA shunt pathway and the accumulation of GABA metabolites may contribute in antioxidant machinery associated with reactive oxygen species and in the acquisition of tolerance in response to induced oxidative stress in *Arabidopsis* seedlings.

Keywords *Arabidopsis* · Calmodulin · Hydrogen peroxide · GABA · Oxidative stress · Paraquat · ROS

Abbreviations

CAM	Calmodulin gene
Ca^{2+}/CaM	Calcium/calmodulin complex
CaM	Calmodulin protein
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
MDA	Malonaldehyde
ROS	Reactive oxygen species
TBARS	Thiobarbiturate reactive substances
UV	Ultra-violet light (UVA or UVB)

Introduction

In plants, oxidative stress leading to production of reactive oxygen species (ROS) may be caused directly through ozone pollution or indirectly as a result of abiotic stresses, such as heat, cold, herbicide applications (e.g., paraquat), drought, or high light intensity (Vranova et al. 2002).

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A common feature among the different ROS is their capacity to cause oxidative damage to proteins, DNA, and lipids (Apel and Hirt 2004). ROS are continuously produced as by-product of various metabolic pathways localized in different cellular compartments (Moller 2001). Under physiological steady state conditions, these molecules are scavenged by different antioxidative defense components that are often confined to specific compartments (Alscher et al. 1997). The equilibrium between production and scavenging of ROS may be disturbed by a number of adverse environmental factors, and such disturbances may result in a rapid rise in the intracellular level of ROS (Prasad et al. 1994). Plants also generate ROS by activating various oxidases and peroxidases that produce ROS in response to certain environmental changes (Bolwell et al. 2002). External conditions that adversely affect the plants can be biotic, imposed by other organisms, or abiotic, arising from an excess or deficit in the physical or chemical environment. The increase in ROS production triggered by either biotic or abiotic stresses is generally attributed to different plant defense mechanisms and/or signaling pathways (Apel and Hirt 2004; Agarwal and Jha 2010). Plants have evolved a number of mechanisms to protect themselves from ROS-mediated damage. Antioxidant enzymes (e.g., ascorbate peroxidase or superoxide dismutase) catalyze such reactions or are involved in the direct processing of ROS (Dröge 2002; Kumutha et al. 2010). Upon being subjected to abiotic stresses, plants induce such ROS scavenging enzymes to decrease the concentration of toxic intracellular ROS levels. The differences in the functions of ROS between biotic and abiotic stresses might rise from the action of hormones and cross-talk between different signaling pathways or from differences in the locations where ROS are produced and/or accumulate during different stresses (Mittler 2002; Mittler et al. 2004). Recent investigations have revealed that ROS, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are central components of the signal transduction cascade involved in plant adaptation to the changing environment (Miller et al. 2007).

In plants, ROS play two highly contrasting roles, namely, exacerbating damage or signaling the activation of defense mechanisms in response to biotic and abiotic stresses (Neill et al. 2002). These functions necessitate a stringent control of the cellular ROS levels. In plants, catalase scavenges H_2O_2 generated during mitochondrial electron transport, β-oxidation of the fatty acids, and photorespiratory oxidation (Scandalios et al. 1997). Yang and Poovaiah (2002) showed that catalase catalytic activity to regulate H_2O_2 level in *Arabidopsis* is stimulated by the calcium/calmodulin protein complex (Ca^{2+}/CaM) to maintain H_2O_2 homeostasis in plants. ROS generation in cellular compartments, such as the mitochondria or chloroplasts, results in changes in gene expression, indicating that ROS signals are transmitted between the organelles and the nucleus. H_2O_2 treatments induce elevations in Ca^{2+} levels (Rentel and Knight 2004; Hu et al. 2007) and the expression of calmodulin genes (*CAM*) (Desikan et al. 2001). Extracellular CaM stimulates stomatal closure through the activation of heterotrimeric G protein and subsequent promotion of H_2O_2 and intracellular Ca^{2+} elevations (Chen et al. 2004). The activated Ca^{2+}/CaM complex then binds to target proteins and modulates their activities. For example, in the γ-aminobutyric acid (GABA) shunt pathway, Ca^{2+}/CaM binds to the glutamate decarboxylase (GAD) enzyme and induces GABA production and accumulation in response to various abiotic stresses, such as oxidative stress (Shelp et al. 1999; Bouche and Fromm 2004). ROS affect ion transport (Rentel and knight 2004) and the accumulation of compatible solutes and intermediate metabolites, leading to a significant mitigation of ROS stress and ROS-scavenging capacity (Bohnert et al. 1995; Smirnoff and Cumbes 1989; Niyogi 1999; Hong et al. 2000; Cuin and Shabala 2007). It is possible that the interaction and the signaling between all of these components lead to the suppression and regulation of the ROS level and H_2O_2 homeostasis during oxidative stress and environmental factor-induced oxidative stress in plants.

It is vital to investigate such metabolic/signaling interactions and cross-talk between metabolites and signaling components if scientists are to understand the role of oxidative stress, the ROS/antioxidant network, and stress tolerance in plants. In the study reported here, we used CaM T-DNA insertion mutants of *Arabidopsis thaliana* to examine the specific role of CaM in the tolerance of plants to oxidative stress induced by paraquat and H_2O_2 treatments. Tolerance was determined on the basis of seed germination, seedling growth, oxidative damage, and GABA shunt metabolite levels in the various *cam* mutants.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis* (*Arabidopsis thaliana* Ecotype Columbia) and mutant lines with a T-DNA insertion in seven *CAM* genes were used in this study (Table 1). The wild-type, *cam1*, and *cam4* (Ecotype Columbia) seeds were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH, USA; all other *cam* seeds were obtained from the laboratory of Dr. Janet Braam, Rice University, Texas, USA. Homozygous stocks were propagated. Seeds were surface sterilized with 100% bleach (v/v, 6% sodium hypochlorite) for 10 min

Table 1 Mutant lines of *Arabidopsis thaliana* with a T-DNA insertion in calmodulin genes used in this study

Mutant	Insertion location	SALK line no.
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

cam Mutant calmodulin gene, *UTR* untranslated region

All seeds lines were obtained from Dr. Janet Braam, Rice University, TX, except for *cam1* and *cam4* seeds, which were obtained from the Arabidopsis Biological Research Stock Center, Ohio State University, Columbus, OH

followed by five washes with sterile distilled water. The seeds were then sown in petri plates containing sterile 1× MS (Murashige and Skoog 1962) medium (pH 5.7) supplemented with 2% (w/v) sucrose and solidified with 1.2% (w/v) agar. The petri dishes were placed under continuous light (40 μmol m^{-2} s^{-1}) at 25°C for seed germination and seedling growth.

Germination percentage assay

Wild-type seeds and seeds of all *cam* mutants (150 seeds each) were surface sterilized and sown in petri dishes containing 1× MS solid medium supplemented with one of three paraquat concentrations (0.5, 1, 3 μM) or one of three H_2O_2 concentrations (200 and 500 μM, 1 mM), and allowed to grow vertically under continuous light. Emergence of radicles from germinating seeds were scored every 2 days for 2 weeks. Germination percentage was determined by calculating the total number of seeds germinated divided by the total number of seeds plated on the plates.

Root elongation assay

Seven-day-old seedlings grown vertically in petri plates containing 1× MS solid medium under continuous light were transferred to new petri plates containing 1× MS solid medium supplemented with one of three paraquat concentrations (0.5, 1, 3 μM) or one of three different H_2O_2 concentrations (200 and 500 μM, 1 mM). Each plate contained 10–15 seedlings. Three replicate plates were used for each treatment. The increase in primary root length (mm) was measured after 7 days. Immediately after 7 days, root tissues were separated completely from shoot tissues. After collection, the tissues were immediately frozen in liquid nitrogen and used for metabolite extraction and analysis.

Oxidative damage analysis

Seven-day-old seedlings grown vertically in petri plates containing 1× MS solid medium under continuous light were transferred to new petri plates containing 1× MS solid medium supplemented with 3 μM paraquat or with 1 mM H_2O_2. The plates were incubated vertically under continuous light for 7 days. Root tissues were collected separately from shoot tissues and used for the thiobarbituric acid reactive substances (TBARS) assay. Three replicate plates with 15 seedlings on each plate were used for each treatment.

Extraction and determination of the level of GABA shunt metabolites

Metabolites were extracted according to Zhang and Bown (1997) with the following modifications. After transferring 7-day-old seedlings to new petri plates containing 1× MS solid medium supplemented with one of three paraquat concentrations (0.5, 1, 3 μM paraquat) or one of three H_2O_2 concentrations (200 and 500 μM, 1 mM), we separately harvested root and shoot tissues. A 0.25-g sample of each harvested tissue was ground in liquid nitrogen in 1.5-ml microfuge tube until a fine powder was obtained, following which 0.4 ml methanol was added to each tube and the tube contents mixed for 10 min. The liquid from the tubes was removed by vacuum drying, and then 70 mM lanthanum chloride was added to each tube; the tubes were then mixed for 15 min and centrifuged at full speed for 5 min. The supernatant was removed to a new tube and 1 M KOH was added. After 10 min of mixing, the tubes were centrifuged at full speed for 5 min. The supernatant was then transferred to a new tube and used to determine the quantity of the different metabolites.

γ-Aminobutyric acid (GABA) γ-Aminobutyric acid level was measured according to Zhang and Bown (1997) with the following modifications. The assay system contained 0.05 ml of sample extract, 4 mM NADP+, 0.5 M K-pyrophosphate buffer (pH 8.6), 2 U/μl GABASE enzyme (Sigma-Aldrich, St. Louis, MO; the GABASE enzyme was suspended in 0.1 M K-pyrophosphate buffer pH 7.2 containing 12.5% glycerol and 5 mM β-marcaptoethanol), and 5 mM α-ketoglutarate. The change in absorbance at 340 nm after the addition of α-ketoglutarate was

recorded after a 90-min incubation at 25°C using a PowerWave microplate reader (Max200R; BioTek, Winooski, VT). The level of GABA [expressed as nmol mg^{-1} fresh weight (FW)] was determined using a NADPH standard curve.

Alanine Alanine level was measured according to Bergmeyer (1983) with the following modifications. The reaction contained 0.05 M Na-carbonate buffer (pH 10), 30 mM β-NAD+, 0.01 ml of sample extract, and 0.3 U/µl alanine dehydrogenase (Sigma-Aldrich) enzyme suspension. The change in absorbance at 340 nm after the addition of alanine dehydrogenase was recorded after a 60-min incubation at 25°C using a microplate reader. The level of alanine (expressed as nmol mg^{-1} FW) was determined using a NADH standard curve.

Glutamate Glutamate level was measured according to Bergmeyer (1983) with the following modifications. The deamination reaction contained 0.1 M Tris-HCl pH 8.3, 7.5 mM β-NAD+, 0.01 ml of sample extract, and 0.8 U/ml glutamate dehydrogenase enzyme suspension (Sigma-Aldrich). The level of glutamate was determined at 340 nm after a 60-min incubation using a microplate reader. The level of glutamate (expressed as nmol mg^{-1} FW) was determined using a NADH standard curve.

TBARS assay

The TBARS assay was performed according to Heath and Packer (1968). Root and shoot tissues from seedlings after separate treatments with 3 µM paraquat and 1 mM H_2O_2 were used for the TBARS assay. Tissues (0.50 g per sample) were frozen in liquid nitrogen and ground to a fine powder in a 1.5-ml microfuge tube, following which 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and 175 mM NaCl in 50 mM Tris–HCl pH 8 were added to the tube. The tubes were heated to 90°C for 25 min and then centrifuged at full speed for 20 min. The supernatant was collected and absorbance measured at 532 nm, with a subtracted reading at 600 nm to account for non-specific turbidity. The level of malonaldehyde (MDA) (expressed as nmol mg^{-1} FW) was determined from a standard curve of MDA.

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

Mutants sensitive to the paraquat and H_2O_2 treatments

In response to the paraquat treatments, the germination of *cam5-4 and cam6-1* seeds was inhibited and significantly ($P < 0.05$) reduced over a 2-week period (Fig. 1). Compared to the wild type, only 55–70% of *cam5-4* and *cam6-1* seeds germinated; in contrast, the other mutants showed 100% or close to 1000% germination at all three paraquat concentrations. More specifically, on MS medium containing 0.5, 1, or 3 µM paraquat, the germination of *cam5-4* and *cam6-1* seeds after 4–8 days reached 75, 70, and 55–60% respectively, while the seeds of the wild type and other *cam* mutants showed complete or close to complete germination (80–97%) after 4 days at the same treatments over the 2-week period record (data not shown).

The same pattern of tolerance/sensitivity of seed germination was observed during the H_2O_2 treatments. In response to H_2O_2 stress, the germination of *cam5-4* and *cam6-1* seeds was inhibited and significantly reduced ($P < 0.05$) (Fig. 2), with a 70–73% germination percentage at 200 µM H_2O_2 after 8 days, a 62–65% germination percentage at 500 µM H_2O_2 after 8 days, and a 53–55% germination percentage at 1 mM H_2O_2 after 8–10 days over the 2-week period. However, the seed of the wild type and other *cam* mutants were able to fully germinate at the three different H_2O_2 concentrations (200 and 500 µM, 1 mM) after 4–6 days over the same study period (data not shown).

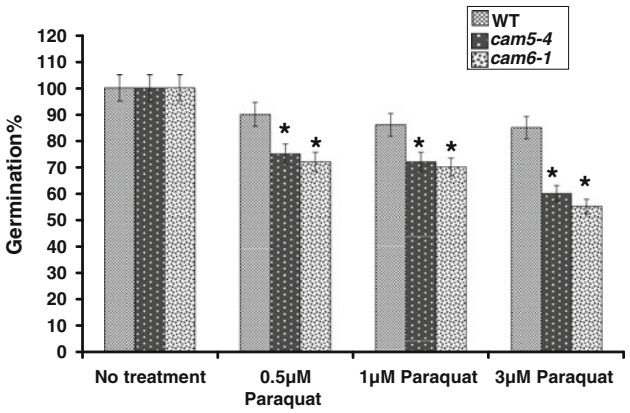

Fig. 1 Seed germination under paraquat stress conditions. A total of 150 seeds of each calmodulin (*cam*) mutant and the wild type (*WT*) were surface sterilized and grown on MS medium containing one of three paraquat concentrations (0.5, 1, 3 µM). Germination was scored every 2 days for 2 weeks. Seed germination percentage after 10 days of culture on paraquat-containing medium was determined. *Error bars* Standard error (SE) over three replicate plates for each mutant. All *cam* mutants' seeds except *cam*5-4 and *cam*6-1 showed no phenotype at all paraquat concentrations. *Asterisk* indicates significance at $P < 0.05$

The tolerance/sensitivity of *cam* mutants to paraquat and H_2O_2 stresses was also examined in terms of seedling growth (Figs. 3, 4). As shown in Fig. 3, root growth (mm) of all mutants and the wild type was highly retarded and reduced by as much as 90% on MS medium containing paraquat compared to the non-treated plants cultured under the same conditions. However, the *cam5-4* and *cam6-1*, mutants showed an extra sensitivity ($P < 0.05$) to all paraquat treatments, with significantly reduced root lengths ($P < 0.05$) that were up to >50% shorter than those of wild type and all other mutants lines at all paraquat treatments (Fig. 3).

All three H_2O_2 treatments (200 and 500 μM, 1 mM) affected root elongation in all mutants and the wild type, with a 50–75% reduction in root length after 7 days compared to that of non-treated plants (Fig. 4). However, root length of the *cam5-4* and *cam6-1* mutants was significantly ($P < 0.05$) retarded under all H_2O_2 treatments, with up to a 70% reduction at 200 μM H_2O_2, 80% reduction at 500 μM H_2O_2, and 90% reduction at 1 mM H_2O_2 after 7 days of culture (Fig. 4).

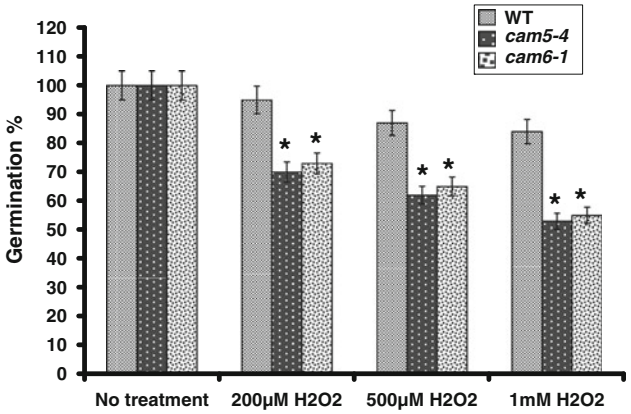

Fig. 2 Seed germination under hydrogen peroxide (H_2O_2) stress conditions. A total of 150 seeds of all *cam* mutants and WT were surface sterilized and grown on MS medium containing one of three H_2O_2 concentrations (200 and 500 μM, 1 mM). Germination was scored every 2 days for 2 weeks. Seed germination percentage after 10 days of culture was scored. *Error bars* SE over three replicate plates for each mutant. All *cam* mutants' seeds except *cam*5-4 and *cam*6-1 showed no phenotype at all H_2O_2 concentrations. *Asterisk* indicates significance at $P < 0.05$

Oxidative damage in response to paraquat- and H_2O_2-induced oxidative stress in *cam* mutants

In all mutants that showed a significant phenotype, there was a higher accumulation of MDA in root tissues than in shoot tissues after 7 days of growth on 3 μM paraquat. As shown in Table 2, *cam5-4* mutants showed a significantly higher ($P < 0.05$) accumulation of MDA in root tissues (404% of wild-type values) than in shoot tissues (196% of wild-type values) in response to the paraquat treatment. Similarly, *cam6-1* mutants accumulated MDA at highly significant level (549% of wild-type values, $P < 0.05$) only in root tissues at the same paraquat treatment (3 μM) (Table 2).

The *cam* mutants that accumulated significant MDA ($P < 0.05$) following exposure to H_2O_2 showed a similar accumulation level and pattern in both root and shoot tissues following a 7-day culture on MS medium supplemented with 1 mM H_2O_2 (Table 3). Among all *cam* mutants that were tested for MDA accumulation, only the

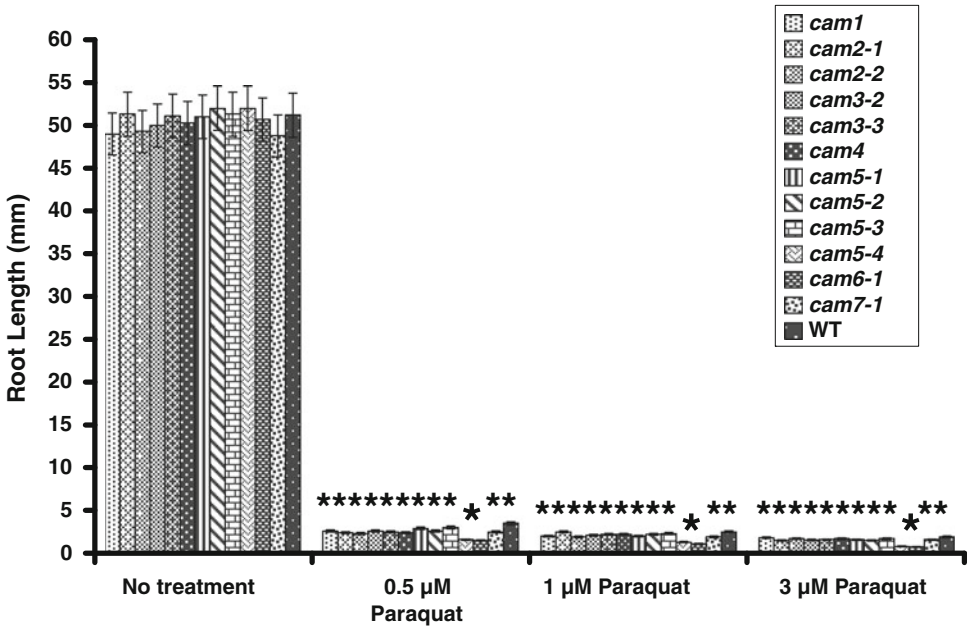

Fig. 3 Root elongation of *cam* mutants and WT seedlings after 7 days of growth on MS medium supplemented with one of three paraquat concentrations (0.5, 1, 3 μM). The results are presented as root length (mm) for each of the *cam* mutants and the WT. *Error bars* SE over three replicate plates for seedlings of each mutant and WT seedlings. All *cam* mutant and WT seedlings showed growth sensitivity to paraquat treatments. *Asterisk* indicates significance at $P < 0.05$

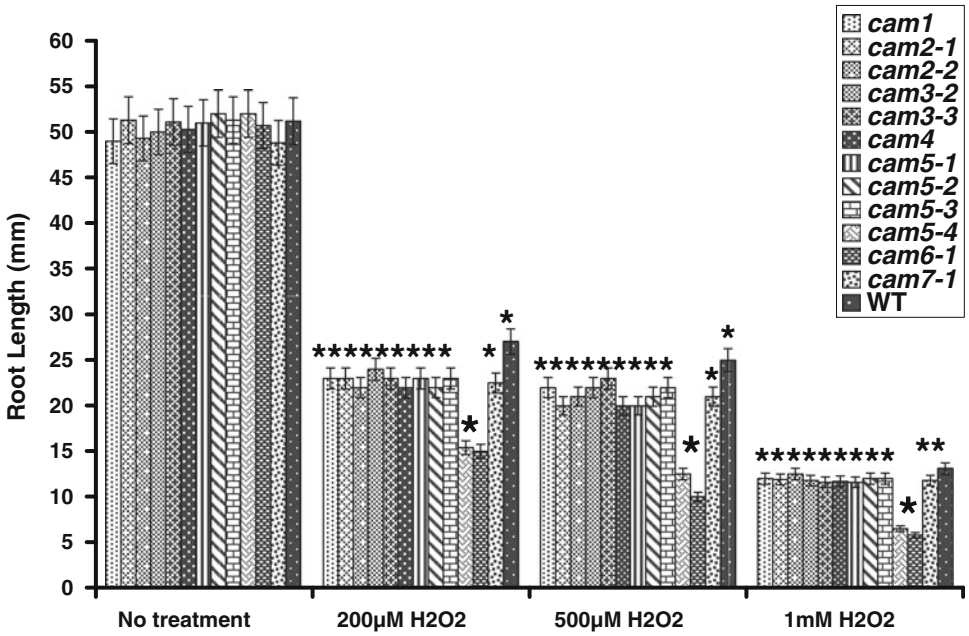

Fig. 4 Root elongation of *cam* mutants and WT seedlings after 7 days of growth on MS medium supplemented with one of three H_2O_2 concentrations (200 and 500 µM, 1 mM). The results are presented as root length (mm) for each of the *cam* mutants and the WT. *Error bars* SE over three replicate plates for seedlings of each mutant and WT seedlings. All *cam* mutants and WT seedlings showed growth sensitivity to H_2O_2 treatments. *Asterisk* indicates significance at $P < 0.05$

Table 2 Oxidative damage in *cam* mutants of *Arabidopsis thaliana* following exposure to a high concentration (3 µM) of paraquat

Mutants	Root		Shoot	
	No treatment	3 µM paraquat	No treatment	3 µM paraquat
cam1	83	107	76	100
cam2-1	80	90	108	100
cam2-2	78	100	108	100
cam3-2	115	110	100	104
cam3-3	80	115	110	109
cam4	116	102	73	98
cam5-1	98	105	105	114
cam5-2	111	110	106	100
cam5-3	83	90	92	100
cam5-4	115	404*	78	196*
cam6-1	96	549*	100	104
cam7-1	117	151*	110	109

Values are given as the percentage of malonaldehyde [MDA; nmol mg^{-1} fresh weight (FW)] relative to the MDA level in wild-type roots and shoots. One-week-old seedlings were exposed to 3 µM paraquat for 7 days under continuous light at 25°C. After 7 days, root and shoot tissues were used for the thiobarbituric acid reactive substances (TBARS) assay and for determination of the MDA level. MDA levels were determined in both root and shoot tissues separately

* Significant oxidative damage ($P < 0.05$)

Table 3 Oxidative damage in *cam* mutants of *A. thaliana* following exposure to a high concentration of hydrogen peroxide (H_2O_2)

Mutants	Root		Shoot	
	No treatment	1 mM H_2O_2	No treatment	1 mM H_2O_2
cam1	83	106	76	102
cam2-1	74	88	103	111
cam2-2	73	90	107	100
cam3-2	75	90	76	90
cam3-3	80	88	100	103
cam4	106	87	73	88
cam5-1	98	100	105	111
cam5-2	91	93	116	100
cam5-3	83	88	92	110
cam5-4	115	176*	78	188*
cam6-1	96	175*	117	183*
cam7-1	107	87	74	86

Values are given as the percentage of MDA (nmol mg^{-1} FW) relative to the MDA level in wild-type roots and shoots. One-week-old seedlings were exposed to 1 mM H_2O_2 for 7 days under continuous light at 25°C. After 7 days, root and shoot tissues were used for the TBARS assay and for determination of the MDA level. MDA levels were determined in both root and shoot tissues separately

* Significant oxidative damage ($P < 0.05$)

cam5-4 and *cam6-1* mutants showed significant ($P < 0.05$) accumulation of MDA in both root and shoot tissues following exposure to H_2O_2, with *cam6-1* accumulating significant levels of MDA in both the roots and shoots that were 175–183% of wild-type values ($P < 0.05$) and *cam5-4* accumulating significant levels of MDA ($P < 0.05$) in the roots and shoots that were 176 and 188%, respectively, of wild-type values (Table 3).

Changes in GABA shunt metabolites in response to paraquat and H_2O_2 treatments in *cam* mutants

Relative to alanine and glutamate levels, the level of GABA was significantly elevated in root and shoot tissues

Table 4 Level of GABA shunt metabolites (GABA, alanine, and glutamate) in 1-week-old seedlings of all *cam* mutants of *A. thaliana* after culture on MS solid medium supplemented with three different paraquat concentrations (0.5, 1, 3 μM) for 7 days under continuous light at 25°C

Mutants	0.5 μM paraquat						1 μM paraquat						3 μM paraquat					
	Root			Shoot			Root			Shoot			Root			Shoot		
	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu
cam1	242*	100	100	86	120	139	253*	144*	144*	198*	138*	142*	291*	204*	204*	313*	158*	158*
cam2-1	56	78	67	88	86	67	74	89	90	100	100	96	87	100	100	107	106	100
cam2-2	51	45	55	89	88	60	70	89	70	99	100	78	88	100	100	110	110	99
cam3-2	89	75	89	89	89	70	99	89	100	97	100	89	110	100	110	110	110	100
cam3-3	75	36*	50	78	67	50	89	80	80	89	90	64	101	99	100	105	100	100
cam4	227*	136*	136*	204*	141*	141*	253*	144*	144*	239*	150*	151*	264*	180*	180*	327*	158*	160*
cam5-1	55	66	70	90	90	70	76	79	80	100	99	88	89	100	100	114	110	100
cam5-2	77	86	60	89	79	85	88	100	89	100	98	90	100	104	100	116	110	110
cam5-3	76	88	70	89	85	78	100	100	89	100	100	90	110	106	100	110	108*	100
cam5-4	156*	136*	136*	173*	71	71	216*	141*	141*	286*	76	84	269*	156*	153*	493*	82	92
cam6-1	245*	186*	186*	199*	150*	150*	280*	198*	198*	200*	153*	153*	325*	201*	201*	500*	167*	167*
cam7-1	169*	124*	124*	116	112	111	200*	133*	133*	150*	131*	133*	221*	150*	141*	293*	140*	150*

Metabolite levels in mutants were calculated as nmol mg^{-1} FW and are expressed in the table as the percentage of wild-type values in root and shoot tissues separately

GABA γ-Aminobutyric acid, *Ala* alanine, *Glu* glutamate

* Significant level of changes in metabolite levels ($P < 0.05$)

in response to the paraquat treatments (Table 4). In the *cam1* and *cam4* mutants, GABA was significantly accumulated ($P < 0.05$) in both the roots (136–291% of wild-type values) and shoots (204–327% of wild-type values) in comparison with alanine and glutamate levels (139–180% of wild-type values) at all paraquat treatments. The *cam5-4* mutant significantly accumulated ($P < 0.05$) glutamate and alanine only in root tissues (136–156% of wild-type values) at all paraquat treatments, while GABA was significantly accumulated to a high level in both root (156–269% of wild-type values) and shoot (173–493% of wild-type values) tissues following exposure to 0.5, 1, and 3 μM paraquat (Table 4). The *cam6-1* mutant had a significantly increased level of GABA ($P < 0.05$) in both the roots (245–325% of wild-type values) and shoots (1199–500% of wild-type values), while the alanine and glutamate levels were elevated up to 200 and 167% of the wild-type values in the roots and shoots, respectively, at all three different paraquat concentrations. Similarly, the *cam7-1* mutant accumulated a higher level of GABA than of alanine and glutamate in both the root and shoot tissues at the three paraquat concentrations ($P < 0.05$; GABA: 150–293% of wild-type values; alanine and glutamate: 124–150% of wild-type values), as shown in Table 4. Following exposure to H_2O_2, the levels of all shunt metabolites were increased only in root tissue, with no significant accumulations or changes in GABA, alanine, and glutamate in the shoot tissues at all H_2O_2 treatments. In the *cam1* and *cam4* mutants, the accumulation of GABA (138–280% of wild-type values), alanine, and glutamate (150–171% of wild-type values) was significantly ($P < 0.05$) increased in root tissues at all H_2O_2 treatments, as shown in Table 5. For the *cam5* mutants, only the *cam5-4* mutant significantly accumulated GABA (138–379% of wild-type values, $P < 0.05$) at 500 μM and 1 mM H_2O_2, and alanine and glutamate (131% of wild-type values, $P < 0.05$) at 1 mM H_2O_2 in root tissues. On the other hand, no significant phenotype in *cam5-1*, *cam5-2*, and *cam5-3* mutants was detected at all H_2O_2 treatments. As shown in Table 5, the *cam6-1* mutant significantly accumulated more GABA (203–324% of wild-type values, $P < 0.05$) than alanine and glutamate (133–161% of wild-type values) in root tissues at 500 μM and 1 mM H_2O_2, while the *cam7-1* mutant significantly accumulated GABA (133–183% of wild-type values, $P < 0.05$) in root tissues, with no observalbe changes in alanine and glutamate levels (Table 5).

Discussion

In general, our results indicate the sensitivity of *Arabidopsis* mutants to induced oxidative stress during seed germination and plant growth; more specifically, they implicate the possible role of CaM and CaM-mediating signaling during plant development under different oxidative stress-inducing factors. The strong phenotype

Table 5 Level of GABA shunt metabolites (GABA, alanine, and glutamate) in 1-week-old seedlings of *cam* mutants of *A. thaliana* after culture on MS solid medium supplemented with three different H_2O_2 concentrations (200 and 500 μM, 1 mM) for 7 days under continuous light at 25°C

Mutants	200 μM H_2O_2						500 μM H_2O_2						1 mM H_2O_2					
	Root			Shoot			Root			Shoot			Root			Shoot		
	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu	GABA	Ala	Glu
cam1	138*	99	99	44	55	63	144*	164*	150*	73	66	66	230*	170*	164*	100	72	72
cam2-1	88	67	56	45	37*	56	100	89	70	58	47	69	110	107	100	65	60	79
cam2-2	76	56	67	75	60	70	88	80	89	78	78	89	100	107	108	93	86	100
cam3-2	90	70	56	70	70	65	98	100	89	78	80	79	106	108	98	100	82	81
cam3-3	89	88	69	60	56	60	100	100	75	77	69	76	111	110	97	100	79	99
cam4	138*	99	99	47	52	52	250*	157*	157*	56	58	58	281*	171*	171*	71	63	63
cam5-1	89	60	79	90	81	79	101	79	89	100	90	98	111	107	104	115	100	110
cam5-2	70	57	67	75	71	63	100	79	89	89	89	70	111	99	104	110	97	109
cam5-3	80	70	68	79	79	78	86	79	99	100	90	96	101	101	108	106	100	108
cam5-4	79	98	81	73	52	62	138*	105	101	77	57	65	379*	131*	135*	90	68	71
cam6-1	108	102	101	67	48	57	203*	133*	133*	71	51	58	324*	161*	161*	100	57	65
cam7-1	90	99	99	63	72	72	133*	104	104	71	75	74	183*	112	110	80	90	100

Metabolite levels in mutants were calculated as nmol mg^{-1} FW and are expressed in the table as the percentage of wild-type values in root and shoot tissues separately

* Significant level of changes in metabolite levels ($P < 0.05$)

sensitivity of *cam5-4* and *cam6-1* to the paraquat and H_2O_2 treatments as well as to other abiotic stresses that we have previously investigated (AL-Quraan et al. 2010) revealed that CaM5 and CaM6 are involved directly in seed germination and plant growth processes, especially in the signaling pathways and acquisition of tolerance in response to abiotic stresses. Desikan et al. (2001) showed that the *CAM* gene is strongly induced by H_2O_2. Along the same line, based on the results of their cDNA microarray analysis, Weber et al. (2004) showed that CAM2 gene expression increased up to twofold in response to oxidative stress induced by treatment of *Arabidopsis* seedlings with MDA as the factor inducing oxidative damage. Thus, a significant amount of cross-talk occurs between ROS and CaM, and both these signaling molecules mediate cross-tolerance to a variety of stresses. Seed germination in lettuce (*Lactuca sativa* L.) and pigweed (*Amaranthus albus* L.) were promoted by inhibition of the catalase enzyme, where metabolically derived H_2O_2 was found to serve as the oxidant in the pentose pathway of glucose use in dormant seeds prior to water imbibition (Hendricks and Taylorson 1975). Similarly, in *Helianthus annuus* L., seed dormancy-breaking mechanisms through ROS production and oxidation of a specific set of embryo proteins have been found to be enhanced by paraquat treatment (Oracz et al. 2007). In soybean (*Glycine max*) embryonic axes, the level of a fat-soluble anti-oxidant compound (tocopherol [alpha]T) increased during 24-h seed imbibition in the presence of 4 mM paraquat as a cellular adjustment to oxidative stress (Simontacchi et al. 1993), while Rogozhin et al. (2000) found that antioxidant and peroxidase enzyme activity increased significantly in ultraviolet (UV)-treated wheat (*Triticum aestivum* L.) seeds during germination as a compensatory mechanism to inhibit free radicals formation following UV radiation treatment of the wheat seeds. Taken together, these results reveal that ROS production and protein oxidation is a novel mechanism involved in seed dormancy alleviation during seed germination. The ROS scavenging mechanisms that were activated during the UV, paraquat, and H_2O_2 treatments resulted in disturbances to and/or retardation of such mechanisms, which in turn led to a reduction in seed germination during induced-oxidative stress. The extra sensitivity of *cam5-4* and *cam6-1* mutants may be due to the nature of those mutants [exon II and 3′untranslated region (UTR) T-DNA insertion]. Based on the results of our previous study (Al-Quraan et al. 2010), the complete loss of function of the *CAM5* gene (*cam5-4* exonic insertion allele) leads to the downregulation of mRNAs of nearly all *CAM* genes in both root and shoot tissues. Such alterations in gene regulation and possible *CAM/CAM* interaction suggest the important role(s) of CaM5 and CaM6 in ROS and ROS signaling mechanisms during seed germination and plant growth tolerance in response to abiotic stress in *Arabidopsis* seedlings.

As a result of the paraquat and H_2O_2 treatments in our study, the *cam5-4* and *cam6-1* mutants showed a strong phenotype based on the higher MDA levels in root tissues

than shoot tissues under the same paraquat treatment (Table 2), while a distinct phenotype of MDA accumulation in root and shoot tissues due to the H_2O_2 treatments was observed (Table 3). In *Arabidopsis*, Yang and Poovaiah (2002) proved that *Arabidopsis* catalase isoform (AtCat3) has a CaM-binding domain in which CaM binds and activates the catalase enzyme in a Ca^{2+}-dependent manner. On the other hand, the three catalase genes from radish (*Rhaphanus staivus* L.), which show the greatest homology to those of *Arabidopsis*, were found to be differentially expressed in response to white light, xenobiotics, and UV light stress (Kwon et al. 2007). Additionally, catalase enzyme activity extracted from tobacco leaves was stimulated up to twofold by Ca^{2+}/CaM, but not by Ca^{2+} or CaM alone (Yang and Poovaiah 2002). These results indicate that Ca^{2+}/CaM is involved in ROS scavenging and H_2O_2 level regulation in plants through stimulation of the catalytic activity of the catalase enzyme, thereby highlighting the significant role of Ca^{2+}/CaM-mediated signaling in the regulation of ROS level and H_2O_2 homeostasis in response to oxidative stress and environmental changes in plants. In a manner similar to that for abiotic stress, plant defense responses to pathogen attack, biotic stress and antioxidants are involved in both oxidative burst and ROS generation, such as H_2O_2 and superoxides, via the Ca^{2+}/CaM-mediating pathways (Bouche et al. 2005; Du and Poovaiah 2005). Harding et al. (1997) showed that transgenic tobacco cells expressing foreign CaM exhibited a stronger active oxygen burst in response to elicitor and antioxidant treatments as a defense response when compared with the normal control cells challenged with the same stimuli. Given these earlier results, our results suggest that CaM might play an important role in activating ROS production and scavenging hydroxyl radicals and superoxide ions that were activated as a result of oxidative stress damage and cellular impairment. The distinctive phenotype of some of the *cam* mutants, especially *cam5-4* and *cam6-1*, implies that there are interactions and signaling between different CaM isoforms in terms of signaling and acquiring tolerance in mitigating the damaging effects of oxidative stress.

In response to paraquat treatments; GABA shunt metabolites were accumulated to a significant level ($P < 0.05$) in root and shoot tissues at the same treatments (Table 4). Interestingly, challenging the *cam* mutants with H_2O_2 stress (all three treatments) resulted in the accumulation of GABA, alanine, and glutamate metabolites in root tissues at significantly high levels, with no significant elevation in metabolite levels in the shoot tissues (Table 5). Our data strongly suggest the significant elevation phenotype of GABA compound over the other shunt metabolites (alanine and glutamate) in response to induced oxidative stress in most *cam* mutants compared to the non-treated plants. The high accumulation of GABA under induced oxidative stress suggests the crucial role of the GABA shunt pathway in maintaining the redox equilibrium and preventing the accumulation of ROS and cell death during periods of oxidative damage challenges inside the cell. GABA elevation in response to ROS-generating factors indicates the essential role that GABA may play as a contributor to the intracellular pool of antioxidants and to redox buffer changes and signaling inside the cell in response to oxidative stress (Shelp et al. 1999; Bouche and Fromm 2004). Taking into consideration that our measurement of GABA, alanine, and glutamate during the paraquat and H_2O_2 treatments was for the total accumulation in the whole cell (with all of its compartments and organelles), it is possible that GABA and the shunt amino acids accumulated and pooled in different organelles inside the cell in response to stress, thereby acting as compatible solutes and antioxidants via CaM regulation and signaling. Many studies have reported the involvement of the GABA shunt pathway in protection against oxidative stress. In yeast (*Saccharomyces cerevisiae*), Coleman et al. (2001) proved that overexpression of the *GAD1* gene increases resistance to H_2O_2 and amide oxidant treatments. The loss of function of the glutamate decarboxylase gene (*GAD1*) or the succinate dehydrogenase gene (*UGA5*) in yeast reduces the oxidative stress tolerance in yeast cells and increases the level of ROS production. Since the GAD enzyme in yeast is also activated by the Ca^{+2}/CaM complex, there is a high likelihood that Ca^{+2}/CaM regulation of the GABA shunt pathway is involved in redox balance and signaling inside the cell. In support of this possibility, Bouche et al. (2003) showed that the *ssadh* mutant in *Arabidopsis* is sensitive to UV and heat stress and that loss of the function of succinate semialdehyde enzyme (SSADH) causes a rapid increase in the level of H_2O_2, necrotic lesions, hypersensitivity to environmental stresses, and cell death in *ssadh* mutant. The results of these studies indicate the strong relationship and the intriguing link between the GABA shunt Ca^{2+}/CaM-GAD regulation, ROS production and signaling, and plant growth in response to abiotic stresses and induced-oxidative factors in *Arabidopsis* seedlings and various plant species.

Our data strongly imply that the GABA shunt pathway and GABA accumulation might be involved in the antioxidant machinery associated with ROS scavenging, H_2O_2 homeostasis, maintenance of the cellular redox balance, and acquisition of tolerance signaling in response to induced oxidative stress in *Arabidopsis*. Hence, further investigations are needed to address the significant role of GABA shunt and Ca^{+2}/CaM signaling and mediating mechanisms interaction and cross-talk in different cellular compartments and organelles in plants.

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