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# β-aminobutyric acid mediated drought stress alleviation in maize (*Zea mays* L.)

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**Abstract** The present study highlights the role of β-aminobutyric acid (BABA) in alleviating drought stress effects in maize (*Zea mays* L.). Chemical priming was imposed by pretreating 1-week-old plants with 600 μM BABA prior to applying drought stress. Specific activities of key antioxidant enzymes and metabolites (ascorbate and glutathione) levels of ascorbate-glutathione cycle were studied to unravel the priming-induced modulation of plant defense system. Furthermore, changes in endogenous ABA and JA concentrations as well as mRNA expressions of key genes involved in their respective biosynthesis pathways were monitored in BABA-primed (BABA+) and non-primed (BABA−) leaves of drought-challenged plants to better understand the mechanistic insights into the BABA-induced hormonal regulation of plant response to water-deficit stress. Accelerated stomatal closure, high relative water content, and less membrane damage were observed in BABA-primed leaves under water-deficit condition. Elevated APX and SOD activity in non-primed leaves found to be insufficient to scavenge all $H_2O_2$ and $O_2^{\cdot-}$ resulting in oxidative burst as evident after histochemical staining with NBT and DAB. A higher proline accumulation in non-primed leaves also does not give much protection against drought stress. Increased GR activity supported with the enhanced mRNA and protein expressions might help the BABA-primed plants to maintain a high GSH pool essential for sustaining balanced redox status to counter drought-induced oxidative stress damages. Hormonal analysis suggests that in maize, BABA-potentiated drought tolerance is primarily mediated through JA-dependent pathway by the activation of antioxidant defense systems while ABA biosynthesis pathway also plays an important role in fine-tuning of drought stress response.

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## Introduction

Drought stress negatively affects the performance and productivity of maize (*Zea mays*) throughout the world. Limited water availability affects maize yield to some degree at almost all growth stages; however, the crop is the most susceptible during seedling and reproductive stages (Grant et al. 1989). Water deficit at flowering stage is severe enough to delay silking and cause ear abortion (Bolanos and Edmeades 1996). Prolonged anthesis-to-silking interval ultimately results in a substantial yield reduction in terms of ear and kernel number per plant or even complete crop failure (Cattivelli et al. 2008).

Pre-exposure of mild stress often help the plants to cope with a subsequent harsh environment, a phenomenon known as priming (Macarisin et al. 2009). The primed plants display faster and stronger activation of defense responses that typically get activated after pathogen attack or in response to environmental stress (Beckers et al. 2009; Prime-A-Plant Group et al. 2006). Pretreatment of plants with various natural and synthetic compounds can also trigger the "primed state" condition, where the plant's metabolic investment for the constitutive activation of the defense system is reduced or prevented (Prime-A-Plant Group et al. 2006; Ahn et al. 2007). Although the phenomenon has been known for years, most progress in our understanding of priming has been made only recently. Induced priming has been extensively used by various research groups to enhance the plant's acquired resistance against infections by various pathogens (Zimmerli et al. 2000; Ton and Mauch-Mani 2004; Ton et al. 2005). In contrast, few reports are available on priming induced plant tolerance against abiotic stressors such as heavy metal cadmium (Cao et al. 2009; Hossain et al. 2012a), acid rain (Liu et al. 2011), and salinity (Jakab et al. 2005). Though the effect of β-aminobutyric acid (BABA), a non-protein amino acid in enhancing drought tolerance has been investigated in *Arabidopsis* (Jakab et al. 2005; Macarisin et al. 2009), wheat (Du et al. 2012), and potato (Sos-Hegedus et al. 2014), the potential application of chemical priming in mitigating water stress effects has not so far been explored in other crops including maize.

Drought inevitably reduces $CO_2$ assimilation and increased electron transfer from photosynthetic electron carriers toward $O_2$, triggering an excess generation of toxic reactive oxygen species (ROS) in stressed leaves (Carvalho and Amancio 2002; Asada 2006; Miller et al. 2010). Additionally, roots suffer from periodic or prolonged deprivation of water that interferes with the respiration process at the electron transport level (Lin et al. 2006). Absence of a suitable electron acceptor leads to saturated redox chains, accumulation of NAD(P)H, and a decline in the generation of ATP (Asada 1992; Kennedy et al. 1992). All these events eventually result in oxidative stress damages to cellular components like lipids, proteins, and nucleic acids (Halliwell and Gutteridge 1999). To counter the harmful effects of ROS, plants have developed sophisticated enzymatic antioxidant defense system that includes superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), etc. (Hossain et al. 2006, 2012b). The ROS are mainly comprised of superoxide radicals ($O_2{}^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide ($H_2O_2$), and singlet oxygen ($^1O_2$). Within the plant cell, SOD constitutes the first level of defense against $O_2{}^{\cdot-}$. In order to dismute $O_2{}^{\cdot-}$, it simultaneously produces another reduced oxygen species, hydrogen peroxide ($H_2O_2$), as the reaction product (Fridovich 1986). The $H_2O_2$ is in turn scavenged by

the activity of peroxidase and catalase (Fridovich 1986). Within the ascorbate–glutathione cycle, enhanced activities of DHAR and MDAR result in increased ascorbate reduction. GR, the rate-limiting enzyme also plays a crucial role by maintaining the GSH (reduced glutathione)/GSSG (oxidized glutathione) ratio favorable to ascorbate reduction (Gossett et al. 1996). Apart from the enzymatic defense system, plants also possess low molecular weight antioxidant metabolites (ascorbic acid, reduced glutathione, carotenoids), which readily scavenge $^1O_2$, $O_2{}^{\cdot-}$, and $OH^{\cdot}$, thus, alleviating ROS-induced oxidative stress damages (Foyer and Noctor 2005; Kuzniak and Sklodowska 2001; Smirnoff 2000).

The drought-mediated reduced water availability, which is first perceived by the roots, results in closure of the leaf stomata and the resulting reduction in transpiration, at least in part through the action of the stress hormone abscisic acid (ABA) (Wilkinson and Davies 2002). In plants, ABA is derived from the $C_{40}$ epoxycarotenoid precursors through an oxidative cleavage reaction in plastids (Xiong and Zhu 2003). The first step of ABA biosynthesis pathway involves the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, catalyzed by the enzymes zeaxanthin epoxidase (ZEP). The aldehyde oxidase (AAO) catalyzes the last step in the ABA biosynthesis, converting ABA-aldehyde to ABA. The extent and rate of drought-induced ABA accumulation vary with the severity of drought as well as species and cultivars differing in drought tolerance (Tuberosa et al. 1992; Pekic et al. 1995). Wang et al. (2004) demonstrated that drought tolerance is negatively related to ABA accumulation during short term drought stress in Kentucky bluegrass (*Poa pratensis*). This is in agreement with the findings that drought-tolerant wheat (*Triticum aestivum*) accumulates lower ABA levels, which correlates with a lower ABA biosynthesis and a higher ABA catabolic gene expression (Ji et al. 2011). A low foliar ABA accumulation is often used as a primary selection criterion in breeding programs for improved drought tolerance of major crops including maize (Landi et al. 2001; Wang et al. 2004). In spring wheat, BABA priming is found to enhance the desiccation tolerance by triggering the accumulation of ABA which acts as a non-hydraulic root signal resulting closing of stomata and thus reducing water loss (Du et al. 2012).

Apart from ABA, little is known about the roles of the other hormones in relation to drought stress (Huang et al. 2008). Among them, jasmonate appears to be the most important and has a well-established role in defense responses against biotic stressors (insects and pathogens) and adverse environmental factors such as ozone (Sasaki et al. 2005), wounding (Lorenzo and Solano 2005), and flooding (Arbona and Gomez-Cadenas 2008). The jasmonic acid (JA) was reported to be involved in drought-induced antioxidant responses, including ascorbate metabolism (Ai et al. 2008; Norastehnia and Asghari 2006). A recent study has shown that a burst of JA in

roots of citrus triggers a more progressive ABA accumulation that induces plant defense responses against severe drought stress (de Ollas et al. 2013). The JA biosynthesis pathway involves the subsequent action of both plastidial and peroxisomal enzymes (Poltronieri et al. 2013). It originates from the lipid oxidation pathways in the chloroplast. Phospholipids such as linolenic (18:2) and α-linolenic (18:3) acid liberated from chloroplast membranes through the action of phospholipases (PLDs) act as a substrate for the sequential action of lipoxygenases (LOXs) (Turner et al. 2002). The plastidial 13-LOX produces 13-hydroperoxy-octadecatrienoic acid (13-HPOT), which is then converted into 12-oxo-phytodienoic acid (OPDA) in the plastids via enzymatic reactions catalyzed first by allene oxide synthase (AOS) and subsequently by allene oxide cyclase (AOC) (Schaller 2001). The OPDA is finally reduced/converted to JA through the action of oxo-phytodienoic acid reductase (OPR) followed by three cycles of β-oxidation in the peroxisome (Lyons et al. 2013).

The aim of the present work is to investigate the role of BABA in alleviating drought stress effects in monocot model plant maize. Antioxidant enzymes activities were studied to unravel the priming-induced modulation of plant defense system. Furthermore, changes in endogenous ABA and JA concentrations as well as expression of key genes involved in their respective biosynthesis pathways were monitored in BABA-primed (BABA+) and non-primed (BABA−) leaves of drought-challenged plants to better understand the mechanistic insights into the BABA-induced hormonal regulation of plant response to water-deficit stress. Antioxidant metabolites of ascorbate–glutathione cycle, membrane damage, RWC, in vivo ROS detection, foliar $H_2O_2$, stomatal closure, and proline accumulation were also studied in detail to get an overview of BABA-mitigated drought stress response. To our best knowledge, this study is the first endeavor to counter drought stress in maize exploiting the priming phenomenon. An understanding of how the BABA ameliorates the impact of water stress will provide valuable information necessary for designing drought-tolerant crops.

## Materials and methods

### Plant material, BABA treatment, and sample collection

Seeds of maize (Zea mays L.) inbred line HKI-161, obtained from the Indian Agricultural Research Institute, New Delhi, were used as plant material for the present investigation. Seeds were first surface sterilized in sodium hypochlorite solution and then allowed to germinate in plastic cups (~200 mL capacity) containing steam-sterilized soil and farmyard manure (FYM) mixture (3:1). Plants (30/treatment/replicate) were maintained at 25 °C under a 16 h photoperiod and 60 %

relative humidity. To induce chemical priming, 7-day-old maize plants were treated with 600 μM β-aminobutyric acid (BABA, Sigma-Aldrich, USA) for a consecutive period of 5 days. Soil drenching with BABA was performed as previously described (Zimmerli et al. 2000 and Jakab et al. 2005). These BABA-primed (BABA+) plants were then subjected to drought stress by withholding water for 9 days. A parallel set of 7-day-old plants were maintained with equal amount of water (15 mL/cup) for the next 5 days and then exposed to drought stress for 9 days. These plants without BABA treatment were considered as non-primed (BABA−) plants. Control set of plants were maintained under same growth condition with regular watering throughout the experimental period. On 9th day of stress, morphological parameters like root length, root weight, shoot length, and shoot weight were recorded. Shoots were randomly harvested and subsequently stored at −80 °C for biochemical and molecular analysis. In total, three independent biological experiments were performed under the same growth conditions. For detection of ROS and cell death, freshly collected leaves and roots were used.

### In vivo detection of ROS

#### Histochemical staining

Foliar hydrogen peroxide ($H_2O_2$) accumulation was detected using 3′3′-diaminobenzidine (DAB) assay (Thordal-Christensen et al. 1997; Iriti et al. 2006). In vivo infiltration of leaves with 5 mM DAB at pH 3.8 forms deep brown polymerization products upon reaction with $H_2O_2$ in the presence of peroxidase (Thordal-Christensen et al. 1997). Superoxide was detected by infiltration of leaves in 6 mM nitroblue tetrazolium (NBT) that produce dark blue insoluble formazan on reaction with superoxides (Fryer et al. 2002). Chlorophylls were removed from the leaves by infiltration with lacto–glycerol–ethanol ($1:1:4 v/v/v$) solution followed by boiling in water bath for 10 min. Images were captured using Olympus CX41 microscope (Olympus, Tokyo, Japan) equipped with digital camera (ProgRes CT3).

#### ROS imaging with CM-H2DCFDA staining

Root tips (~1.0 cm in length) of the control and drought-stressed maize plants (non-primed, BABA primed) were stained with 12.5 μmol/mL CM-H2DCFDA (chloromethyl derivative of 2′,7′-dichlorofluorescin diacetate, Invitrogen, USA) for 10 min followed by washing with distilled $H_2O$. The fluorescence images of the stained roots were observed using a fluorescence microscope (Dewinter, excitation 400–490 nm, emission ≥520 nm).

## Root cell viability assay by Evans blue staining

Root cell viability was determined by Evans blue staining (Tamas et al. 2004). Freshly collected roots were washed thoroughly in ddH$_2$O followed by overnight staining in 0.25 % (w/v) aqueous solution of Evans blue (Sigma, USA) at room temperature. On the next day, the stained roots were washed several times with ddH$_2$O until no further blue color eluted from the roots. For quantitative assessment of staining, stained root tips were excised and immersed in 200 μL N,N-dimethylformamide for 24 h at 4 °C. After the incubation, the absorbance of Evans blue released from the root tips was measured spectroscopically at 600 nm.

## Estimation of relative water content

Relative water content (RWC) of leaf was estimated according to the method of Whetherley (1950). The turgid weight of 1 g of fresh leaf sample was determined by keeping it in water for 4 h. Dry weight (DW) was measured by drying the same sample in a hot air oven (80 °C) until constant weight was achieved. Data was expressed in percentage and calculated with the formula RWC (%)=[(FW−DW)/(turgid weight−DW)]×100.

## Hydrogen peroxide estimation

Foliar hydrogen peroxide content was estimated according to the method of Brennan and Frenkel (1977). One hundred milligrams of frozen leaf tissue was macerated in 4 mL cold acetone and homogenate was filtered through Whatman No. 1 filter paper. Two milliliters of this filtrate was treated with 1 mL of titanium reagent (20 % titanium tetrachloride in concentrated HCl, v/v) and 1 mL of concentrated ammonia solution to precipitate the titanium–hydroperoxide complex. After centrifugation (at 5000×g for 30 min), precipitate was dissolved in 2 N H$_2$SO$_4$ and the absorbance was read at 415 nm. H$_2$O$_2$ content was calculated from a standard curve prepared in the similar way and expressed as micromoles per gram of FW.

## Measurement of malondialdehyde concentration

Malondialdehyde (MDA) concentration was measured following the procedure of Hodges et al. (1999). Frozen leaf tissue was homogenized in 80 % cold ethanol and centrifuged to pellet debris. Different aliquots of the supernatant were mixed either with 20 % trichloroacetic acid or with a mixture of 20 % trichloroacetic acid and 0.5 % thiobarbituric acid. Both mixtures were allowed to react in a water bath at 90 °C for 1 h. After that, samples were cooled down in an ice bath and centrifuged. Absorbance of the supernatant was read at

440, 534, and 600 nm against a blank. MDA concentration was expressed in terms of nanomoles per gram of FW.

## Estimation of free proline content

Proline content was measured spetrophotometrically using the method of Bates et al. (1973). Frozen leaf tissue (100 mg) was homogenized with 5 mL of 3 % sulphosalicylic acid and centrifuged at 5000×g for 10 min. Supernatant was treated with acid-ninhydrin and acetic acid, boiled for 1 h at 100 °C. The reaction was then terminated in an ice bath. Reaction mixture was extracted with 2 mL toluene. Absorbance of chromophore containing toluene was determined at 520 nm. Proline content was expressed as micromoles per gram of FW.

## Antioxidant enzymes assays

Frozen leaf tissue (0.5 g) was homogenized in 1.5 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol, and 2 % (w/v) polyvinyl pyrrolidone (PVP) using chilled mortar and pestle kept in ice bath. The homogenate was centrifuged at 15,000×g at 4 °C for 30 min. Clear supernatant was used for enzymes assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM ascorbate in addition to the other ingredients. All assays were done at 25 °C. Soluble protein content was determined according to Bradford (1976) using BSA as a standard. All spectrophotometric analyses were conducted using an UV/visible Spectrophotometer (Genesis 10S UV–Vis, Thermo Scientific).

## Ascorbate peroxidase

APX (EC 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981). Three milliliters of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H$_2$O$_2$, and 0.1 mL enzyme extract. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm ($\in$=2.8/mM/cm). APX activity was expressed as micromoles of ascorbate oxidized per minute per milligram of protein.

## Superoxide dismutase

Total SOD (tSOD) (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay according to Beyer and Fridovich (1987). In this method, 1 mL of solution containing 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 μM NBT, 0.025 % Triton X-100 was added into small glass tubes followed by 20 μL of sample. Reaction was started by adding 10 μL of riboflavin solution (4.4 mg/100 mL) followed by placing the tubes in an

aluminum foil-lined box having two 20-W fluorescent lamps for 7 min. A parallel control was run where buffer was used instead of sample. After illumination, absorbance of solution was measured at 560 nm. A non-irradiated complete reaction mixture was served as a blank. SOD activity was expressed as units per milligram of protein. One unit of SOD was equal to that amount which causes a 50 % decrease of SOD-inhibitable NBT reduction.

### Glutathione reductase

GR (EC 1.6.4.2) activity was determined by monitoring the glutathione dependant oxidation of NADPH, as described by Carlberg and Mannervik (1985). In a cuvette, 0.75 mL 0.2 M potassium phosphate buffer (pH 7) containing 2 mM EDTA, 75 μL NADPH (2 mM), and 75 μL oxidized glutathione (20 mM) were mixed. Reaction was initiated by adding 0.1 mL enzyme extract to the cuvette and the decrease in absorbance at 340 nm was monitored for 2 min. GR activity was calculated using the extinction coefficient for NADPH of 6.2/mM/cm and expressed as micromoles of NADPH oxidized per minute per milligram of protein.

### Dehydroascorbate reductase

DHAR (EC 1.8.5.1) enzyme activity was measured according to the method of Nakano and Asada (1981). The complete reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.2 mM DHA, and 0.1 mM EDTA in a final volume of 1 mL. Reaction was initiated by adding suitable aliquot of enzyme extract and increase in absorbance was recorded at each 30 s interval for 3 min at 265 nm. Enzyme activity was expressed as micromoles of ascorbate formed per minute per milligram of protein.

### Monodehydroascorbate reductase

MDAR (EC 1.6.5.4) enzyme activity was measured as described by Miyake and Asada (1992). Monodehydroascorbate was generated by ascorbate oxidase using a reaction mixture (1 mL) containing 50 mM HEPES-KOH buffer, pH 7.6, 0.1 mM NADPH, 2.5 mM ascorbate, ascorbate oxidase (0.14 U), and suitable aliquot of enzyme extract. MDAR activity was expressed as micromoles of NADPH oxidized per minute per milligram of protein.

### Estimation of foliar ascorbate and glutathione contents

Ascorbate content was determined according to Law et al. (1983). The assay is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by ascorbate in acidic solution. The $Fe^{2+}$ forms a red chelate with bipyridyl absorbing at 525 nm. DHA was calculated by subtracting AsA from total ascorbate.

The DTNB–GSSG reductase recycling procedure of Anderson (1985) was used for the determination of both total (GSH + GSSG) and GSSG levels. GSH content was calculated by subtracting GSSG content from the total glutathione content.

### RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was extracted from 100 mg of frozen leaf tissue of the control and drought-stressed maize plants (non-primed, BABA primed) using RNA isolation method as described by Ghawana et al. (2011). Total RNA (2 μg) was pretreated with DNase I (Invitrogen, USA) to remove any contaminating DNA followed by first-strand cDNA synthesis using Superscript III (Invitrogen, USA) according to the manufacturer's instructions. To check the cDNA synthesis, PCR was performed using *26S rRNA* forward (5′ CACAATGATAGGAAGAGCCGAC 3′) and reverse (5′ CAAGGGAACGGGCTTGGCAGAATC 3′) primers as described previously (Singh et al. 2004).

All the maize gene sequences used for primer designing were downloaded from NCBI database (www.ncbi.nlm.nih.gov). The primers for quantitative real-time PCR (qRT-PCR) analysis were designed using the primer 3 v.0.4.0 software (http://gmdd.shgmo.org/primer3). Gene amplification and gel electrophoresis was performed to confirm that the primers amplified only a single product of expected size (data not shown). Each qRT-PCR reaction was performed with three biological replicates and three technical replicates. The reaction was performed in 10 μL reaction mixture containing diluted cDNA samples as template, 2× SYBR® Green Master Mix (Applied Biosystems, USA) and 200 nM each of forward and reverse gene specific primers. The reactions were performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using the following program: initial denaturation at 94 °C for 10 min, followed by 40 cycles of amplification (94 °C for 30 s, 54–60 °C for 30 s, and 72 °C for 30 min) and final melt curve analysis was performed. Transcript level of all the genes was normalized with an internal reference, *glyceraldehydes 3-phosphate dehydrogenate* (*GAPDH*) gene from maize. The relative expression ratio of each gene was calculated using comparative Ct value method as described previously by Livak and Schmittgen (2001). Data represented here are relative quantitation (RQ) values of gene expression. Expression is shown after normalization to *GAPDH*. Values were calculated using $\Delta\Delta C_T$ method, and the error bars represented $RQ_{MIN}$ and $RQ_{MAX}$. Data are representative of three biological replicate experiments. All the primers used in this study are listed in Supplementary Table 1.

## HPLC analysis of abscisic acid

An Agilent Technologies 1200 series high-performance liquid chromatograph (HPLC) coupled with a UV–Vis variable detector was utilized for the quantification of abscisic acid (Guinn et al. 1986; Tang et al. 2011). ZORBAX SB-C$_{18}$ ($250 \times 4.6$ mm; 5-$\mu$m particle size) reverse phase column was employed for the chromatographic separation of the compound. The mobile phase composition was 70 % acetonitrile (ACN) + 30 % water (H$_2$O), and the chromatographic separation was performed at a flow rate of 1 mL/min at wave length of 254 nm. The injection volume was 20 $\mu$L. Foliar ABA concentration was determined by comparing HPLC profile with that of standard. Under the above condition, the retention time was 4.2 min. The ABA concentration was expressed as nanograms per gram of FW.

## LC–MS/MS analysis of jasmonic acid

Jasmonic acid was quantified in liquid chromatography coupled with tandem mass spectrometry. The mobile phase composition was (A) water, 5 mM ammonium acetate and 0.1 % acetic acid and (B) methanol, 5 mM ammonium acetate and 0.1 % acetic acid. The HPLC separation was performed on a Alliance 2695 separation module liquid chromatography (Waters, Milford, MA, USA) equipped with a quaternary solvent delivery system on a reversed phase Symmetry C$_{18}$ (5 $\mu$m; $2.1 \times 100$ mm) column (Waters, USA) with a total flow rate of 0.3 mL/min. The injection volume was 20 $\mu$L. The estimation of jasmonic acid under LC–MS/MS was performed in negative ion mode by multireaction monitoring (MRM) with mass transition from parent ion ($m/z$ 209.02) to daughter ion ($m/z$ 58.32), ($m/z$ 80.40), and ($m/z$ 108.83).

## Western blot analysis

A portion (200 mg) of leaf was ground to slurry in 800 $\mu$L of lysis buffer using chilled mortar and pestle at 4 °C. The lysis buffer consists of 50 mM sodium-phosphate buffer (pH 7.0), glycerol, sodium dodecyl sulfate, 2-mercaptoethanol, PMSF, EDTA, and plant-specific broad-spectrum protease inhibitor cocktail (Genetix, India). The homogenate was centrifuged at $12,000 \times g$ for 20 min at 4 °C. The final clear supernatant was used for electrophoresis. Proteins were separated on 12 % SDS–PAGE and transferred onto a polyvinylidene difluoride membrane using a semidry transfer blotter (Atto, Japan). The blotted membrane was blocked with 2 % skimmed milk (Amnesco, USA). The membrane was subsequently incubated with a 1:2500 dilution of anti-actin antibody (Agrisera, Sweden) for 1 h at room temperature. Anti-actin antibody (Agrisera, Sweden) was used as the loading control. Goat anti-rabbit IgG conjugated with alkaline phosphatase (Abcam, USA) served as the secondary antibody. After 1 h incubation,

with the secondary antibody, bands were observed by using NBT/BCIP-chromogenic reagent (Genetix, India). Following the same procedure, anti-glutathione reductase (Agrisera, Sweden) 1:5000 dilution was used. Goat anti-rabbit IgG conjugated with alkaline phosphatase (Abcam, USA) was used as secondary antibody for visualization of GR.

## Statistical analysis

The results are presented as mean values±standard errors. Statistical significance between mean values was assessed using analysis of variance and a conventional Duncan's multiple range test (DMRT), using SPSS-10 statistical software (SPSS Inc., Chicago, IL, USA). A probability of $p < 0.05$ was considered significant.

## Results

### BABA protects maize against drought stress

To evaluate the protective role of BABA against drought, seedling growth was compared among the BABA-primed and non-primed maize plants under drought stress (Fig. 1a). As compared to the control, significant decreases in shoot and root biomass, as well as root length were recorded in drought-stressed seedlings (Table 1). Nevertheless, the magnitudes of the decreases were more severe in non-primed seedlings exposed to drought than the BABA-primed plants. With the progress of drought stress, rolling of leaf blades followed by leaf tip drying were evident. The highest percentage of leaves with dried tips (68.5 %) was recorded in non-primed seedlings, while the leaf damage was significantly lower in BABA-primed plants (57.3 %). Better performance of BABA-pretreated plants under stress strongly indicates BABA-potentiated drought stress alleviation in maize.

**Fig. 1** Comparative growth performances of BABA-primed (BABA+) and non-primed (BABA−) maize plants under drought stress along with control (**a**). *Arrows* clearly indicate that drought-induced leaf drying is more severe in non-primed plants as compared to BABA-primed plants. Fluorescence imaging of the control (**b**), non-primed (**c**), and BABA-primed (**d**) roots visualized by CM-H2DCFDA staining using a Dewinter fluorescence microscope, with a B filter (excitation 400–490 nm, emission ≥520 nm). Roots stained with Evans blue specify nonviable cells (**e**). Absorbance of Evans blue released from the stained roots indicating higher loss of cell viability in non-primed roots (**f**). Control, non-primed, and BABA-primed roots are shown from left to right, respectively. NBT staining for in vivo detection of superoxide accumulation (**g**). *Dark blue spots* (formazan) on the drought-stressed leaves indicate superoxides deposits. Leaves of the control, non-primed, and BABA-primed plants are arranged from left to right, respectively. Enlarged microscopic view of dark blue spots (*arrow marks*) showing superoxides accumulation (**h**). Foliar H$_2$O$_2$ deposits analyzed by DAB assay (**i**). Enlarged microscopic view of a portion of stressed leaf showing dark brown spot (*arrow marks*) indicative of H$_2$O$_2$ deposits (**j**)
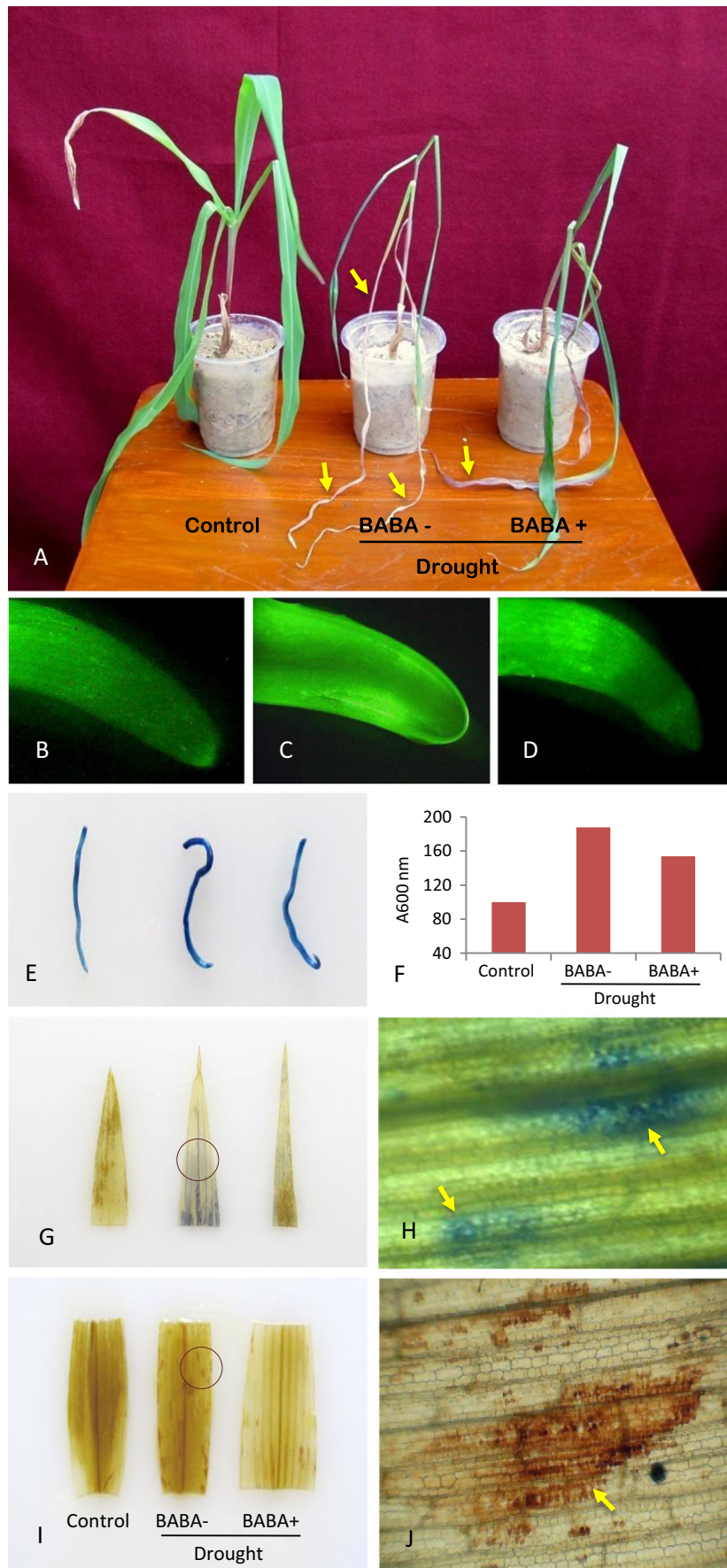
**Table 1** Comparative growth performance of BABA-primed and non-primed maize (*Zea mays* inbred line HKI-161) seedlings under drought stress

| Treatment | | Leaves with dried tips (%) | Shoot length (cm) | Shoot weight (g) | Root length (cm) | Root weight (g) |
|---|---|---|---|---|---|---|
| Control | | 3.11a±1.29 | 38.35a±1.41 | 6.02c±0.11 | 31.09c±0.80 | 4.66c±0.17 |
| Drought | BABA− | 68.50c±3.08 | 35.09a±1.53 | 1.92a±0.09 | 21.20a±1.11 | 1.89a±0.12 |
| Drought | BABA+ | 57.38b±3.24 | 37.96a±1.87 | 3.20b±0.13 | 24.98b±0.77 | 2.35b±0.14 |

Data are expressed as mean±S.E. ($n$=30). Means followed by a common letter are not significantly different at the 5 % level by Duncan's multiple range test (DMRT)

## BABA-pretreated plants experience less oxidative stress on exposure to drought

Histochemical staining with NBT and DAB are very sensitive techniques for in vivo detection of superoxides and $H_2O_2$ in the cells, respectively. Presence of dark blue spots (formazan) on the drought-stressed leaves clearly indicate the location of stress induced superoxides accumulation (Fig. 1g, h). Intensity and severity of dark blue spots were remarkably higher in leaves of non-primed plants exposed to drought stress than the BABA-primed leaves (Fig. 1g). Interestingly, no such clear spots were detected in control leaves. Similarly, staining of stressed leaf blades with DAB showed deep brown spots indicative of $H_2O_2$ deposits (Fig. 1i, j). Maximum spots were observed in leaves of non-primed plants. In contrast, only few spots were evident on the BABA-primed leaves (Fig. 1i). Moreover, drought-induced elevation in ROS generation in roots was visualized by CM-H2DCFDA staining (Fig. 1b–d). CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation by $H_2O_2$ or other hydroperoxides yields a fluorescent adduct that is trapped inside the cell. In comparison with the control, high emissions of green fluorescence by drought stressed roots were observed. Interestingly, the entire root tips of non-primed plants exposed to drought showed green fluorescence, while the emission by BABA-primed roots was comparatively lower (Fig. 1 c, d), indicating low ROS generation under drought stress.

## BABA protects root cell death

Evans blue staining has been the most widely used technique for determining cell viability. It is basically a dye unable to cross the intact membranes and thus used to assess the cells integrity (Gaff and Okongo-Ogola 1971). Figure 1e shows the control and drought-exposed non-primed and BABA-primed roots (from extreme left to right, respectively) stained with Evans blue. The uptake of Evans blue by drought-stressed roots of non-primed plants was comparatively higher than the control and the BABA-primed roots (Fig. 1f), indicating a higher loss of cell viability.

## BABA helps to maintain a high relative water content under drought

Measurement of relative water content (RWC) in leaf tissue is commonly used to assess the water status of the plant. Foliar RWC content was drastically declined in both non-primed and BABA-primed plants under drought treatment (Fig. 2a). Interestingly, the magnitude of decrease was more severe (~3.7-fold) in leaves of non-primed plants as compared to BABA-primed plants (~2-fold decrease over the control).

## BABA does not induce proline accumulation

Increased proline accumulation has been considered as a general selection criterion for drought-tolerant crops (Van Rensburg et al. 1993; Silvente et al. 2012). Significant impact of drought stress in enhancing endogenous free proline level was noticed in maize leaves. After 9 days of stress, leaves of non-primed plants accumulated a very high level (~57-fold) of proline as compared to the control (Fig. 2b). In contrast, only 13.8-fold increase in proline content was observed in BABA-primed plants.

## BABA reduces $H_2O_2$ and lipid membrane peroxide levels

On exposure to drought, hydrogen peroxide ($H_2O_2$) level was significantly increased in both non-primed and BABA-primed plants (Fig. 2c). Notably, the magnitude of the increase in foliar $H_2O_2$ level was more in non-primed plants (~2.7-fold), while only 1.3-fold increase was evident in BABA-primed plants. Additionally, drought caused a significant increase in MDA level, a general marker of oxidative damage to lipid membranes. As compared to BABA-primed plants, leaves of non-primed plants accumulated significantly a higher level of MDA, indicating higher membrane damages under drought stress (Fig. 2d). Comparatively, a high $H_2O_2$ level in non-primed leaves might be responsible for inducing such a high membrane damage.

## BABA pretreatment enhances antioxidant capacity

Under drought stress, activities of antioxidant enzymes were differentially modulated in BABA-primed and non-primed

**Fig. 2** Effect of drought stress on foliar relative water content (**a**), endogenous free proline concentration (**b**), $H_2O_2$ level (**c**), and lipid peroxidation (MDA concentration) (**d**) in BABA-primed and non-primed maize plants. Data are expressed as mean value±S.E. (*n*=6). *Vertical bars* indicate standard errors. Mean values in columns with different letters are significantly different at the 5 % level according to Duncan's multiple range test
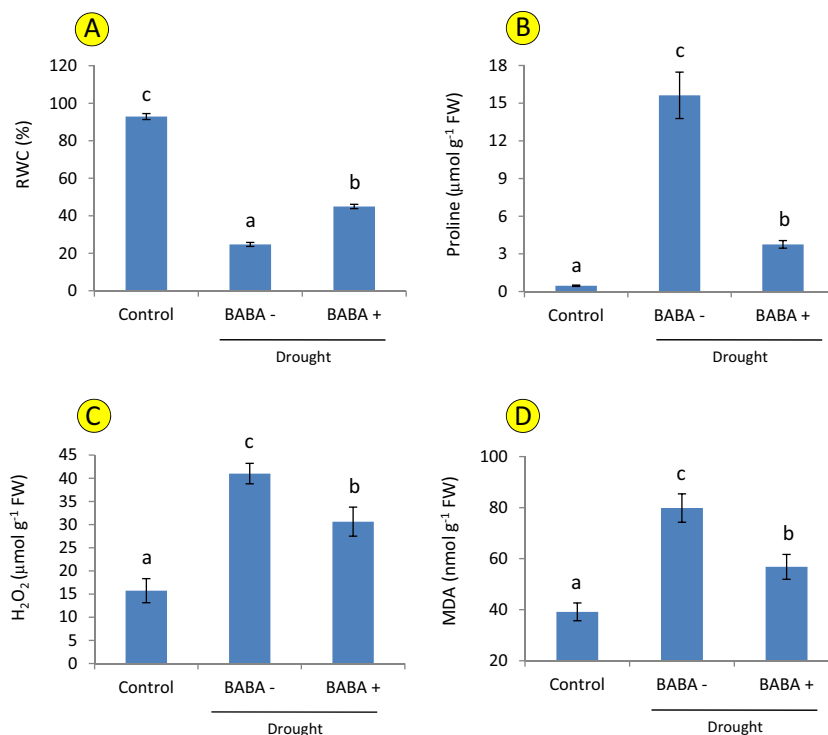
plants. Among the five studied antioxidant enzymes of ascorbate-glutathione cycle, APX activity was significantly increased (~1.8-fold over the control) in leaves of both BABA-primed and non-primed plants exposed to drought (Fig. 3a). Similar trend was also observed in case of tSOD (Fig. 3b). Nevertheless, a magnitude of the increase was more in non-primed leaves (2.5-fold) than the BABA-primed plants (1.7-fold). A completely reverse trend was observed for MDAR and DHAR (Fig. 3 c, d). On exposure to water-deficit stress, both BABA-primed and non-primed leaves exhibited a significant decline (~1.7-fold as compared to the control) in MDAR activity (Fig. 3c). Interestingly, for DHAR, the magnitude of the decline was more severe (~3.6-fold) in non-primed leaves than the BABA-primed plants (~1.6-fold) (Fig. 3d). Like APX and SOD, increased GR activity was evident in leaves of both BABA-primed and non-primed plants under drought (Fig. 3e). Notably, a magnitude of the increase was higher in leaves of BABA-primed plants (~1.8-fold) than the non-primed leaves (~1.2-fold).

Apart from antioxidant enzyme activities, drought also caused marked changes in antioxidant pool. Leaves of both primed and non-primed plants showed significant increase (~3.7-fold over the control) in reduced ascorbate (AsA) level (Table 2). Similar increasing trend for dehydroascorbate (DHA) content was recorded in drought-stressed leaves irrespective of pretreatment. However, non-primed leaves accumulated significantly a higher level of DHA (3.5-fold) as compared to the BABA-primed plants (2.2-fold). As compared to control leaves, no significant variation in AsA/DHA ratio was

observed under drought stress irrespective of treatment (Table 2). Like ascorbate, leaves of both primed and non-primed plants accumulated significantly a high level of reduced glutathione (GSH) in response to drought (Table 3). Interestingly, a magnitude of the increase was significantly more in BABA priming (~3.8-fold) than the non-primed leaves (~2-fold). A similar increasing trend was also recorded in total glutathione (tGSH) content (Table 3). The drought-induced high accumulation of GSH was also reflected in overall GSH/GSSG ratio. Notably, as compared to non-primed plants, BABA-primed leaves maintained a very high GSH/GSSG ratio (~4.4-fold over the control) essential for sustaining balanced redox status to counter drought-induced oxidative stress damages.

### BABA potentiates drought-responsive gene expression

Based on the physiological and biochemical studies, a relative expression analysis of nine genes was conducted in the control and drought stressed plants (non-primed, BABA primed) (Fig. 4). These genes were randomly selected from antioxidant defense mechanism, ABA, and JA biosynthesis pathways operative during drought stress. The genes selected for expression analysis are *ascorbate peroxidase* (*APX*), *manganese superoxide dismutase* (*MnSOD*), *glutathione reductase* (*GR*), *zeaxanthin epoxidase* (*ZEP*), *aldehyde oxidase* (*AAO*), *phospholipase D* (*PLD*), *lipoxygenase* (*LOX*), *allene oxide synthase* (*AOS*), and *allene oxide cyclase* (*AOC*) (Fig. 4). Real-

**Fig. 3** Drought induced changes in foliar APX (**a**), tSOD (**b**), MDAR (**c**), DHAR (**d**), and GR (**e**) activities in BABA-primed (BABA+) and non-primed (BABA−) maize plants. Data are expressed as mean value±S.E. (*n*=6). *Vertical bars* indicate standard errors. Mean values in columns with different letters are significantly different at the 5 % level according to Duncan's multiple range test
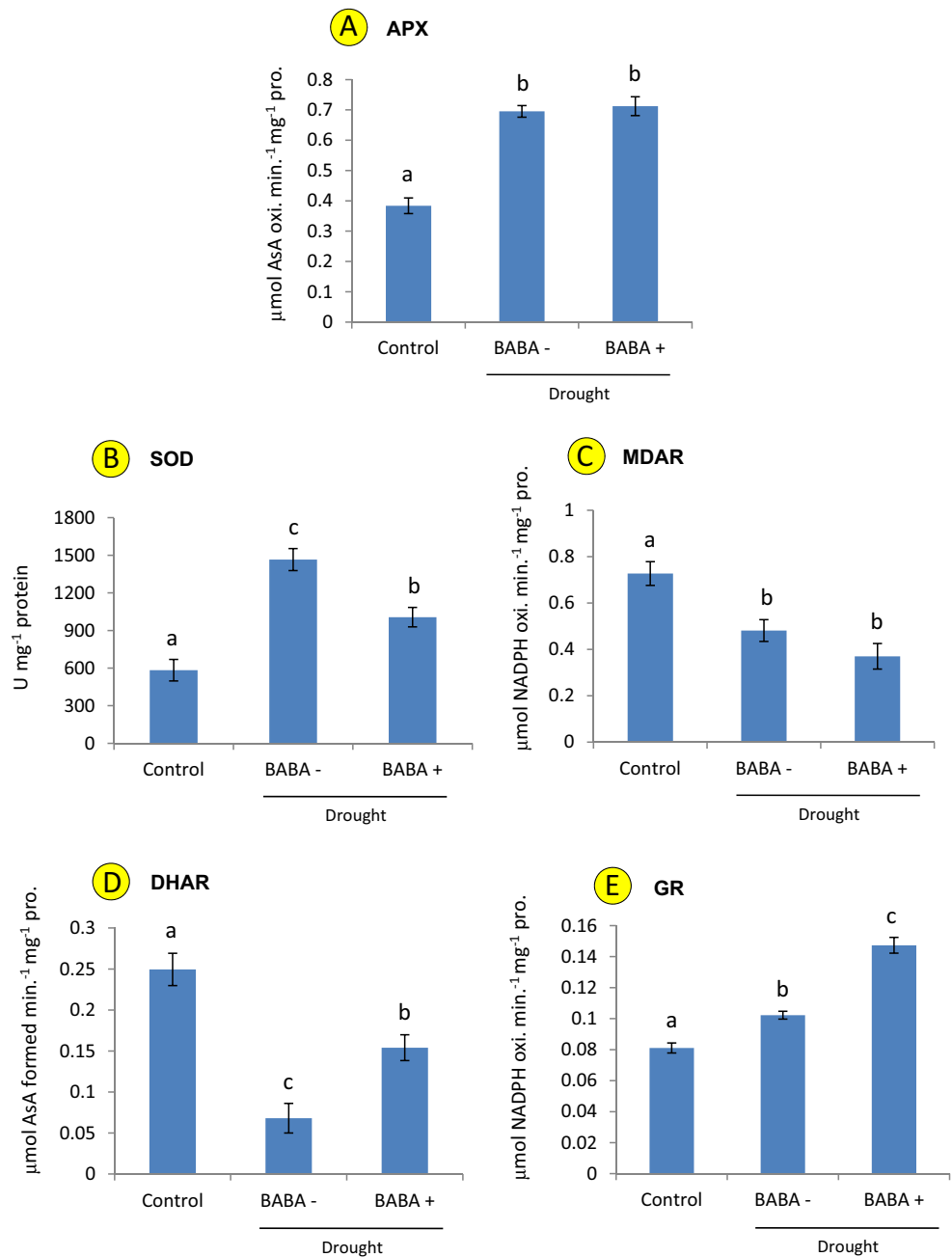
time RT-PCR analysis showed that the expression pattern of the studied genes involved in antioxidant defense mechanism largely corroborates with biochemical analysis (Figs. 3 and 4a–c). Interestingly, the expression of *APX* was upregulated (~2.7-fold over the control) in drought-stressed plants (non-primed, BABA primed) (Fig. 4a). The expression of *MnSOD* was also upregulated in drought-stressed plants; however, the level of expression was more in non-primed leaves (5.0-fold) than the BABA-primed plants (3.6-fold) (Fig. 4b). Notably, the transcriptional abundance of *GR* is in consistency with the enzyme activity trend (Figs. 3e and 4c). Increased mRNA expression of *GR* was recorded following

drought exposure. The BABA-primed leaves showed strong upregulation (3.9-fold over the control) in *GR* as compared to non-primed plants (2.8-fold) (Fig. 4c). The mRNA expressions of genes involved in ABA biosynthesis pathway namely *ZEP* and *AAO* showed an upregulation under drought stress (Fig. 4d, e). Interestingly, out of the four studied JA biosynthesis pathway-related genes, *PLD*, *LOX*, and *AOS* exhibited significant increases in their transcriptional abundances over the non-primed leaves (Fig. 4f–h). In contrast, an almost equal increase (~2-fold over the control) in *AOC* expression was noticed in both BABA-primed and non-primed plants (Fig. 4i).

**Table 2** Changes in foliar ascorbate contents of BABA-primed and non-primed maize (*Zea mays* inbred line HKI-161) seedlings under drought stress

| Treatment | | tAsA (μmol/g FW) | AsA (μmol/g FW) | DHA (μmol/g FW) | AsA/DHA |
|---|---|---|---|---|---|
| Control | | 1.58a±0.27 | 0.81a±0.05 | 0.77a±0.21 | 1.59a±0.38 |
| Drought | BABA− | 5.97b±0.61 | 3.27b±0.45 | 2.70c±0.17 | 1.18a±0.11 |
| Drought | BABA+ | 4.52b±0.73 | 2.76b±0.47 | 1.75b±0.31 | 1.64a±0.25 |

Data are expressed as mean±S.E. (*n*=6). Means followed by a common letter are not significantly different at the 5 % level by Duncan's multiple range test (DMRT)

## BABA primes JA synthesis

To find out the role of stress hormones in fine-tuning of BABA-mitigated drought stress response, leaves of the control and drought stressed (BABA-primed and non-primed) maize plants were sampled for ABA and JA estimation. Under drought condition, a rapid increase in endogenous ABA level was recorded (Fig. 5a). However, the non-primed (BABA−) leaves showed a higher accumulation of ABA (811.8 ng/g FW) as compared to BABA-primed plants (616.1 ng/g FW). Notably, a different pattern was recorded for endogenous JA profile. The BABA-primed leaves accumulated a high JA (405.6 ng/g FW) after the imposition of drought stress, while only 221 ng/g FW JA concentration was recorded in non-primed leaves (Fig. 5b).

## BABA potentiates GR expression

Analysis of foliar GR protein expression by western blotting was performed to validate the GR mRNA expression data. Similar to transcriptional abundance, a marked increase in GR protein expression was recorded on exposure to drought stress (Fig. 6). Nevertheless, as compared to the control, a magnitude of the increase was more in BABA-primed leaves than the non-primed plants. A maximum GR expression as recorded in BABA-pretreated stressed leaves exactly corroborate with the mRNA expression and biochemical analysis (Figs. 3e, 4c, and 6).

## Discussion

Accumulation of osmolytes such as proline is an inevitable physiological response of plants experiencing severe water stress. Proline not only acts as cytoplasmic osmoticum but also serves as a reservoir of carbon and nitrogen sources for post-stress growth (Fukutaku and Yamada 1984). Moreover, a role of proline in ROS detoxification and stabilization of cell membranes has been proposed (Hamilton and Heckathorn 2001). BABA treatment reported to alter the amino acids balance in *Arabidopsis* (Singh et al. 2010). Analysis of free amino acids content of *Arabidopsis* pre-exposed with BABA revealed a higher accumulation of proline than the water-treated control plants. However, we did not find such BABA-induced enhanced accumulation of endogenous free proline level before the imposition of drought stress (Supplementary Fig. 1). After the 9-day-long water-deficit period, accumulation of endogenous free proline in leaves was much higher in non-primed plants than the BABA-primed plants. However, accumulation of such a high level of proline does not give much protection to the non-primed plants to combat water stress as evident from their growth performance. Furthermore, the protective function of proline in ROS detoxification seems to be negligible, or at least, not effective as indicated by the higher $H_2O_2$ level as well as CM-H2DCFDA staining (Figs. 1b–d and 2c). Earlier, we also observed that excess accumulation of proline could not confer protection against flooding (Arbona et al. 2008) and salinity (Hossain et al. 2007). Previous physiological studies on plant stress responses have established a positive correlation between proline accumulation level and stress pressure (Claussen 2005; Arbona et al. 2008). The present data indicates that the stress pressure exerted by withholding soil watering for 9 days on non-primed plants was higher than the BABA-pretreated plants. In other words, BABA pre-exposure alleviates the drought stress effects that may lead to less accumulation of proline. Our findings are consistent with the proposal of Ibarra-Caballero (1988) to consider the enhanced proline level simply as a stress effect, rather than an indication of drought stress resistance.

**Table 3** Changes in foliar glutathione contents of BABA-primed and non-primed maize (*Zea mays* inbred line HKI-161) seedlings under drought stress

| Treatment | | tGSH (nmol/g FW) | GSH (nmol/g FW) | GSSG (nmol/g FW) | GSH/GSSG |
|---|---|---|---|---|---|
| Control | | 93.85a±2.59 | 34.04a±4.03 | 59.80a±2.49 | 0.58a±0.09 |
| Drought | BABA− | 122.40b±1.51 | 71.39b±1.53 | 51.01b±0.86 | 1.40b±0.04 |
| Drought | BABA+ | 181.14c±1.65 | 130.48c±1.89 | 50.66b±0.81 | 2.58c±0.06 |

Data are expressed as mean±S.E. (*n*=6). Means followed by a common letter are not significantly different at the 5 % level by Duncan's multiple range test (DMRT)
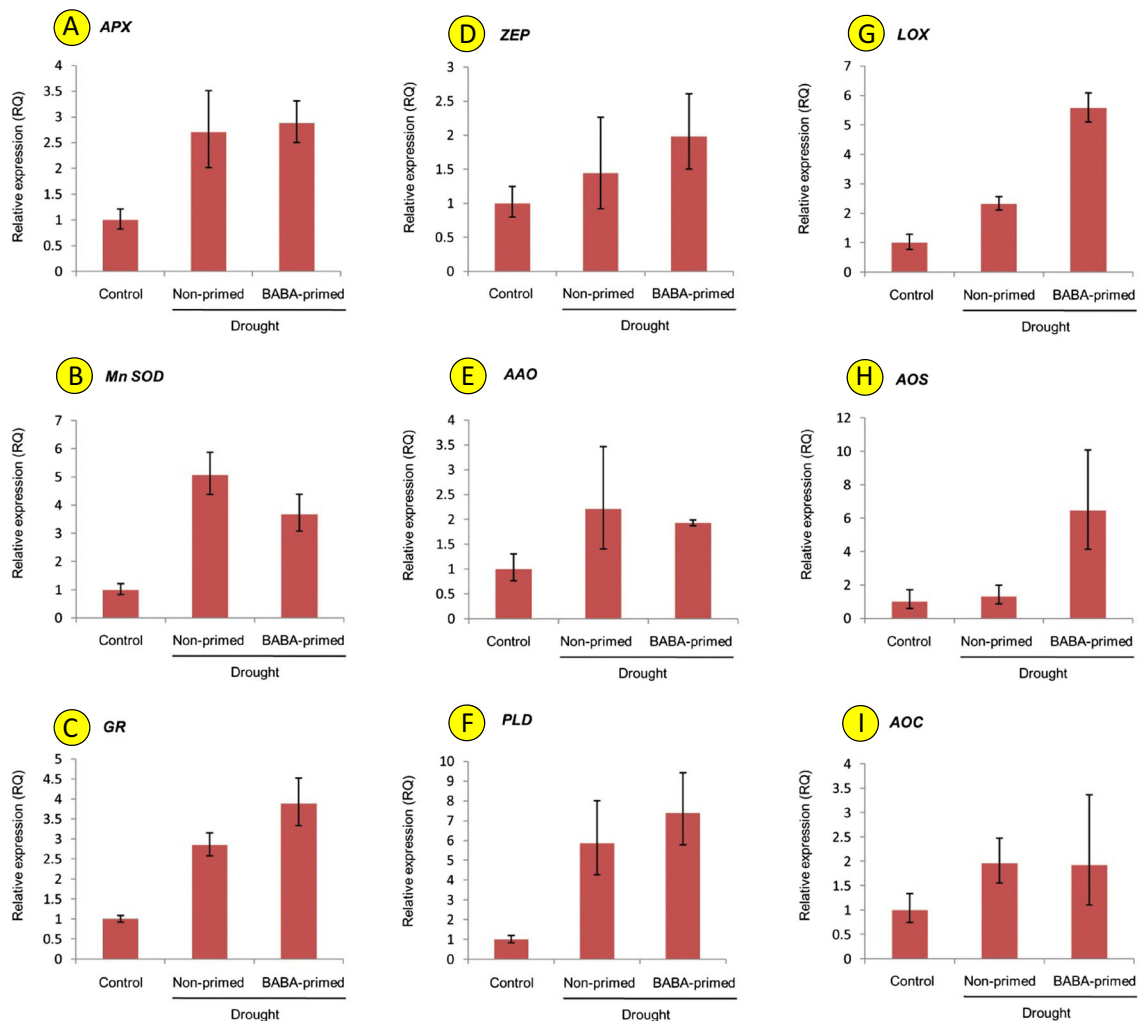
**Fig. 4** qRT-PCR analysis of relative expression of **a** *APX*, **b** *MnSOD*, **c** *GR*, **d** *ZEP*, **e** *AAO*, **f** *PLD*, **g** *LOX*, **h** *AOS*, and **i** *AOC* genes in leaves of the control, non-primed, and BABA-primed plants under drought stress. Data represented here are relative quantitation (RQ) values of gene expression. Expression is shown after normalization to *GAPDH*. Values were calculated using $\Delta\Delta C_T$ method, and the *error bars* represented $RQ_{MIN}$ and $RQ_{MAX}$. Data are representative of three biological replicate experiments

Drought stress is usually characterized by the dehydration of tissues and its impact could be better evaluated by measuring the RWC which indicates the water status of the cells. In the present experiment, a drastic decline in RWC was observed on exposure to water-deficit stress. Notably, as compared to BABA-treated plants, the decrease was more severe in leaves of non-primed plants (Fig. 2a). A higher percentage of leaves with dried tips as observed in non-primed plants also implied that the physiological effects of dehydration stress was more in non-primed leaves than the BABA-treated plants. Moreover, adverse effects of drought stress on shoot and root growth were more evident in non-primed plants as compared to BABA-primed plants. A higher uptake of Evans blue by roots of non-primed drought-stressed plants indicates a higher cell death over the primed roots (Fig. 1f).
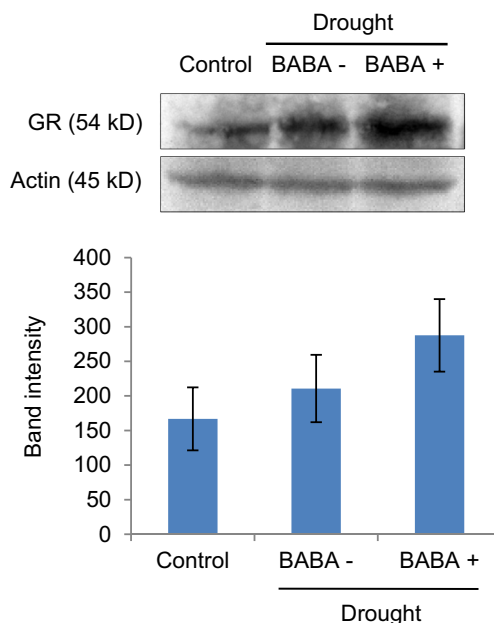
Closure of stomata is an early response of plants to soil drying. Among the phytohormones, ABA is the best known stress hormone that triggers stomatal closure. Moreover, other hormones, such as JA, brassinosteroids, ethylene, and cytokinins also play an essential role in stomatal response to stresses (Daszkowska-Golec and Szarejko 2013). Previous studies have shown that BABA pretreatment triggers ABA accumulation, resulting in stomatal closure thus enhancing drought tolerance (Jakab et al. 2005; Du et al. 2012). In this study, accelerated stomatal closure (Supplementary Fig. 2) together with enhanced accumulation of JA and ABA in BABA-primed drought-stressed leaves suggests that JA interacts with ABA in order to close the stomata. The positive role of JA in regulation of stomatal closure has been documented in earlier studies (Suhita et al. 2003; Munemasa et al. 2007). This BABA-mediated partial closing of stomata might be responsible for maintaining such a high relative water content under water-deficit condition (Fig. 2a).
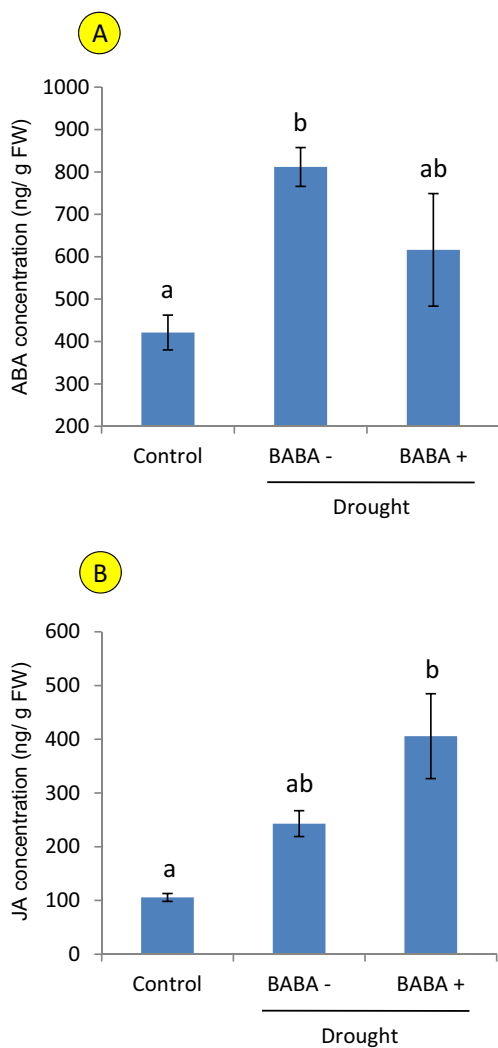
Fig. 5 Drought-induced modulation in ABA (a) and JA (b) accumulation in leaves of BABA-primed (BABA+) and non-primed (BABA−) maize plants. Data are expressed as mean value±S.E. (n=3). *Vertical bars* indicate standard errors. Mean values in columns with different letters are significantly different at the 5 % level according to Duncan's multiple range test



Fig. 6 Change in protein expression level of GR in leaves of BABA-primed and non-primed plants on exposure to drought stress

The cell membrane is one of the primary sites of injury caused by drought stress (Zhang and Kirkham 1996). Large changes in the physical properties of membrane lipids viz. peroxidation during desiccation appear to contribute the loss of membrane's selective permeability. Determination of MDA level as an estimation of oxidative damage to lipid membranes is a widely accepted methodology (Hossain et al. 2006; Arbona et al. 2008). Hydroxyl radical (OH⁻) is the most reactive of all ROS capable of abstracting hydrogen atom from a methylene (-CH$_2$-) group present in polyunsaturated fatty acid (PUFA) side chains of membrane lipids and initiating lipid peroxidation (Barber and Thomas 1978). The OH⁻ is in turn generated from $H_2O_2$ as a result of one electron reduction by Haber–Weiss or Fenton reactions by using metal catalyst (Halliwell and Gutteridge 1999). The impact of stress-

induced membrane damage can be minimized by the elevated action of $H_2O_2$ scavenging enzymes, e.g., APX, CAT that limits the formation of OH⁻ (Gutteridge 1982; Hossain et al. 2006). Here, in response to drought, a significant increase in foliar $H_2O_2$ level was recorded in both primed and non-primed maize plants. Nevertheless, the magnitude of increase was more in non-primed plants than the BABA-priming (Fig. 2c). In vivo detection of $H_2O_2$ through DAB staining further supports a high accumulation of $H_2O_2$ in leaves of non-primed plants (Fig. 1i, j). The prime function of the enzyme peroxidase is to detoxify $H_2O_2$ produced in excess under oxidative stress. Among them, APX is instrumental in regulating $H_2O_2$ level by using ascorbate as reducing agent to catalyze the conversion of hydrogen peroxide to water. During drought period, both BABA-primed and non-primed leaves showed significant increases in APX activity (Fig. 3a). This enhanced activity is also supported by the observed up-regulation of *APX* gene expression irrespective of priming treatments (Fig. 4a). The enhanced APX activity is in agreement with recent proteomic findings on the organ-specific proteomic analysis of drought-stressed soybean (Mohammadi et al. 2012). Authors documented a significant higher *APX* abundance in leaves and hypocotyls of PEG-treated and drought-stressed seedlings. Drought-induced enhanced *APX* expression was also reported in tobacco (Faize et al. 2011), poplar (Yang et al. 2010), creeping bentgrass (Xu and Huang 2010), *Arabidopsis* (Koussevitzky et al. 2008), and pea (Mittler and Zilinskas 1994). Here, instead of elevated APX activity, the presence of comparatively higher level of $H_2O_2$ in non-primed leaves implies a very high rate of $H_2O_2$ production under drought stress. The observed increase in the *APX*

expression seem to be insufficient to give full protection to the non-primed drought-stressed plants in scavenging this deadly ROS, resulting in high membrane damage as compared to BABA-primed plants.

Within plant cell, SOD acts as the first line of defense against oxidative stress as its activity directly modulates the amount of $O_2^{.-}$ and $H_2O_2$, the two important Haber–Weiss reaction substrates (Bowler et al. 1992). On exposure to drought, the non-primed plants showed comparatively a high tSOD activity over the BABA-primed leaves (Fig. 3b). This enhanced trend is consistent with the qRT-PCR expression data for *MnSOD* gene (Fig. 4b). The significantly enhanced tSOD activity in non-primed leaves might not be sufficient enough to scavenge all $O_2^{.-}$, as evident after the NBT staining. Presence of large number of dark blue and deep brown spots as indicative of $O_2^{.-}$ and $H_2O_2$ on the non-primed leaves strongly indicates severe oxidative burst under prolonged drought period (Fig. 1g–j).

Precise metabolic tuning of ascorbate–glutathione cycle allows the cell to maintain favorable GSH/GSSG ratio necessary for cellular redox regulation (Gossett et al. 1996). The GR activity could effectively recycle GSH at the expense of NADPH. Notably, under drought stress, the BABA-primed leaves exhibited a higher GR activity (~1.8-fold over the control) as compared to the non-primed leaves (~1.2-fold). The GR mRNA and protein expression patterns also validate this enhanced GR activity (Figs. 3e, 4c, and 6). Upregulation of *GR* expression has also been reported to occur in other crops in response to drought as well as during recovery period (Torres-Franklin et al. 2008; Ratnayaka et al. 2003). This enhanced GR activity might be the prime reason of a significantly high GSH content as well as a high GSH/GSSG ratio in leaves of BABA-primed plants (Table 3).

Within the ascorbate–glutathione cycle, APX readily dismutes $H_2O_2$ using ascorbate as the electron donor with a concomitant production of another toxic free radical— monodehydroascorbate (MDHA)—that may either disproportionate spontaneously into dehydroascorbate (DHA) and reduced ascorbate (AsA) or be enzymatically converted into DHA by the enzyme MDAR (Hossain et al. 2009). Under drought stress, a significant decline in MDAR activity was noticed in both primed and non-primed leaves (Fig. 3c). A similar trend was also observed in DHAR activity in drought stressed plants. However, the magnitude of the decline was less in BABA-primed leaves. These decreased MDAR and DHAR activities together with the lack of positive correlation between DHAR activity and AsA and DHA levels suggest either an increase in de novo synthesis of AsA or, alternatively, non-enzymatic disproportionation of MDHA might be responsible for such a high accumulation of AsA and DHA.

Plants have the potential to acquire drought tolerance by sensing water stress and activating appropriate resistance mechanisms (Macarisin et al. 2009). The ROS signaling

pathways are interlinked with hormonal networks that eventually regulate plant's stress responses (Pastor et al. 2013). Phytohormones like ABA and JA are the key players in sensing and signaling various environmental stresses including drought (de Ollas et al. 2013). In addition of conferring abiotic stress, BABA has been shown to be effective against pathogens by potentiating both salicylic acid (SA) and abscisic acid (ABA)-dependent defense mechanisms in different plants (Zimmerli et al. 2001; Ton and Mauch-Mani 2004). At moderate water stress levels, soil drenching with BABA was found to enhance ABA accumulation resulting in the closing of stomata and reducing water use, thus increasing the desiccation tolerance in wheat (Du et al. 2012). In separate study, BABA-primed plants not only accumulated callose and lignin and provided protection from pathogen attack but also had increased tolerance to dehydration (Hamiduzzaman 2005). Therefore, cross-talk might exist in the defensive mechanisms operative during pathogen attack and drought stress. Expression analysis performed for different genes involved in ABA and JA biosynthesis pathways showed upregulation in both non-primed and BABA-primed plants during drought stress (Fig. 4d–g, i). Notably, higher expressions of *ZEP*, *PLD*, *LOX*, and *AOS* genes were recorded in BABA-primed leaves as compared to non-primed plants during drought stress (Fig. 4d, f–h). Previously, Zimmerli et al. (2000) reported that *PR-1* gene expression was faster in BABA-treated plants than that in the non-treated control upon infection with pathogens, although BABA did not induce the expression of the *PR-1* gene directly. Also, characterization of *Arabidopsis* mutant *ibs3* revealed that mutant is affected in the regulation of the *ABA1* gene encoding the ABA biosynthesis enzyme ZEP in BABA-induced protection against salt and pathogen (Ton et al. 2005). Ton and Mauch-Mani (2004) also demonstrated that BABA-induced resistance against necrotrophic fungi was regulated by ABA-dependent signaling pathway. PLD, LOX, and AOS are the key enzymes in JA biosynthesis pathway. JA and its derivatives have been proposed to be a signal transducer of defense reactions in plants. Hamiduzzaman et al. (2005) demonstrated that an enhanced level of JA was correlated with primed callose deposition in grapevine against pathogen *Plasmopara*. These observations clearly showed that BABA-mediated response is most likely based on the activation of ABA and JA dependent signaling mechanisms during biotic and abiotic stresses. Our data also showed that expression of genes involved in ABA and JA pathways largely corroborate with the quantified endogenous levels of ABA and JA in the control and drought-stressed plants (Figs. 4e–h and 5). Notably, the BABA-primed (BABA+) leaves accumulated high endogenous JA after the imposition of drought stress, while a relatively low JA level was recorded in non-primed (BABA−) plants (Fig. 5b). Therefore, these data suggest key involvement of both JA and ABA in BABA-potentiated defense response to drought in maize.

## Conclusions

In summary, from our findings, the following conclusions could be drawn: (a) BABA-treatment prior to drought alleviates the stress impact at a significant level; (b) non-primed plants might have experienced drought stress in a more severe way, as evident from a high MDA level and a low RWC; (c) elevated APX and SOD activity in non-primed leaves was found to be insufficient to scavenge all $H_2O_2$ and $O_2^{\cdot-}$ resulting in oxidative burst; (d) a higher proline accumulation in non-primed leaves does not give much protection against drought stress; (e) BABA promotes stomatal closure which might help the primed plants to maintain a high RWC under water-deficit state; (f) enhanced mRNA and protein expression with concomitant increased GR activity in BABA-primed plants might be the determining factor in maintaining a high GSH pool essential for sustaining balanced redox status to counter drought-induced oxidative stress damages; (g) hormonal analysis supported with transcript abundance suggests that in maize, BABA-potentiated drought tolerance is primarily mediated through JA-dependent pathway by the activation of antioxidant defense systems while ABA biosynthesis pathway also plays an important role in fine-tuning of drought stress response.

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