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ORIGINAL ARTICLE

Elevated carbon dioxide decreases the adverse effects of higher temperature and drought stress by mitigating oxidative stress and improving water status in *Arabidopsis thaliana*

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Abstract

Main conclusion **This study revealed that elevated carbon dioxide increases *Arabidopsis* tolerance to higher temperature and drought stress by mitigating oxidative stress and improving water status of plants.**

Abstract Few studies have considered multiple aspects of plant responses to key components of global climate change, including higher temperature, elevated carbon dioxide (ECO_2), and drought. Hence, their individual and combinatorial effects on plants need to be investigated in the context of understanding climate change impact on plant growth and development. We investigated the interactive effects of temperature, CO_2, watering regime, and genotype on *Arabidopsis thaliana* (WT and ABA-insensitive mutant, *abi1-1*). Plants were grown in controlled-environment growth chambers under two temperature regimes (22/18 °C and 28/24 °C, 16 h light/8 h dark), two CO_2 concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) for 18 days. Plant growth, anatomical, physiological, molecular, and hormonal responses were determined. Our study provided valuable information about plant responses to the interactive effects of multiple environmental factors. We showed that drought and ECO_2 had larger effects on plants than higher temperatures. ECO_2 alleviated the detrimental effects of temperature and drought by mitigating oxidative stress and plant water status, and this positive effect was consistent across multiple response levels. The WT plants performed better than the *abi1-1* plants; the former had higher rosette diameter, total dry mass, leaf and soil water potential, leaf moisture, proline, ethylene, *trans*-zeatin, isopentyladenine, and *cis*-zeatin riboside than the latter. The water-stressed plants of both genotypes accumulated more abscisic acid (ABA) than the well-watered plants; however, higher temperatures decreased the ability of WT plants to produce ABA in response to drought. We conclude that drought strongly, while higher temperature to a lesser extent, affects *Arabidopsis* seedlings, and ECO_2 reduces the adverse effects of these stressors more efficiently in the WT plants than in the *abi1-1* plants. Findings from this study can be extrapolated to other plant species that share similar characteristics and/or family with *Arabidopsis*.

Keywords ABA-responsive genes · *Abi1-1* mutant · Abiotic stress · Climate change · Phytohormones · Plant growth and development

Abbreviations

ACO_2	Ambient CO_2
Chl	Chlorophyll
CK	Cytokinin
ECO_2	Elevated CO_2
MDA	Malondialdehyde
ROS	Reactive oxygen species

Introduction

Climate change is a serious threat to plant growth and development. The components of climate change include elevated carbon dioxide (ECO_2) concentration and higher temperature, as well as increases in other extreme abiotic stresses, such as water deficit, flooding, and salinity (Meehl

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and Tebaldi 2004). Human activities have rapidly increased the atmospheric CO_2 concentration, and the current global CO_2 concentration of 400 µmol mol^{-1} is expected to surpass 700 µmol mol^{-1} by 2100 (Stocker et al. 2013). Elevated atmospheric CO_2 can increase the global surface temperature by 1.1–6.0 °C due to its heat-trapping potential (Stocker et al. 2013). ECO_2 and higher temperature are expected to affect global precipitation patterns and, in turn, water stress events in soils (Allison et al. 2009). ECO_2 improves growth and biomass of plants through increased photosynthesis and water use efficiency, and decreased stomatal conductance and transpiration (Qaderi et al. 2006; Jones 2013). ECO_2 also increases growth rate of apical meristem (Teng et al. 2006) because of increased production of phytohormones, such as auxins and gibberellins (Yong et al. 2000). High temperature, on the other hand, damages DNA, inhibits CO_2 assimilation, and changes phytohormone concentration, and the balance between reactive oxygen species (ROS) and antioxidants (Jia et al. 2017). Additionally, higher temperature decreases plant biomass by reducing photosynthesis through increased transpiration and stomatal conductance (Jones 2013). It also reduces chlorophyll (Chl) *a*, Chl *b*, and Chl/carotenoid ratio (Cui et al. 2006). Similar to high temperature, water stress changes gene expression and phytohormone levels, declines photosynthates, and induces ROS production and antioxidant activities (Cossu et al. 2014). Water stress decreases net CO_2 assimilation rates by reducing stomatal conductance and Rubisco activity (Reddy et al. 2004). Moreover, it decreases Chl content and fluorescence, plant height, stem diameter, total dry mass, and relative leaf expansion rate and elongation (Kirnak et al. 2001).

Phytohormones, such as abscisic acid (ABA) and ethylene, have a critical role in plant responses to various stress factors (Dodd and Davies 2010). ABA is produced in response to stress factors, such as temperature (Kurepin et al. 2008) and drought (Qaderi et al. 2006), and has an important role in decreasing transpiration during drought conditions by enhancing stomatal closure. Stomatal closure decreases stomatal conductance and, in turn, reduces gas exchange and plant biomass accumulation (Qaderi et al. 2006). Molecular responses of plants to stress factors include interaction among transcription factors and activation of a group of genes (Qu et al. 2013). In *Arabidopsis*, stress responsive genes like *RD29A*, *RD29B*, *RD22*, and varied *LEA* genes are strongly activated by single factors, such as salt and temperature (Hirayama and Shinozaki 2010). Wang et al. (2003) have reported that many of the inducible water-stress genes are activated by ABA. Duan et al. (2013) pointed out the complex interactive effects of ECO_2 and heat stress on plant growth and photosynthesis during water stress. ECO_2 mitigates the effects of environmental stress factors, such as heat and water stress (Naudts et al. 2013; Zinta et al. 2014). Bauweraerts et al. (2013) showed that ECO_2 reduces the negative impacts of heat and water stresses on photosynthetic parameters in loblolly pine (*Pinus taeda* L.) and northern red oak (*Quercus rubra* L.). However, some studies have predicted that future climate change would modify or limit the direct positive effects of ECO_2 on plants. For instance, ECO_2 enhances water status for soil and plants, while heat stress may contradict this effect (Yu et al. 2012), which may be worsened by water stress (Zeppel et al. 2014). Also, Yu et al. (2012) have shown that ECO_2 exacerbates the negative effects of the combined high temperature and water stress on photosynthesis of tall fescue (*Festuca arundinacea*). Variation in the impact of ECO_2 on plant growth and development could come from genetic variation within and among species, tissue type, growth condition (Franks et al. 2013), and experimental design and setting (Tubiello et al. 2007).

Many earlier studies have reported the effects of single factors, such as ECO_2, higher temperature, and water stress on plants (see Qaderi and Reid 2009). Most of these studies have considered the effects of higher temperature and water stress mainly at ambient CO_2 (ACO_2) concentrations (Bhargava and Sawant 2013). Few studies have examined the interactive effects of higher temperature, drought, and ECO_2 on plants (Naudts et al. 2013; Qaderi et al. 2013; Zinta et al. 2014; Oliveira et al. 2016; Roy et al. 2016). Many studies have reported the impacts of ECO_2 on crops, particularly cereals (Gammans et al. 2017), which were grown under both optimal and limiting growth conditions, in addition to studies that have considered weeds, such as *Centaurea nigra* (e.g., Qaderi et al. 2013), and the model plant species, such as *Arabidopsis thaliana* (e.g., Zinta et al. 2014). However, there is still a shortage of knowledge on the effects of CO_2 and other climate change-related factors, such as temperature and watering regime, on weeds and crops other than cereals. Moreover, it will be essential to study the interactive effects of temperature, CO_2, watering regime and other environmental factors on weeds and/or crops and their associated mutants to achieve a complete assessment of plant responses to environmental stress, and to improve plant adaptation to various stress factors. Although earlier studies had documented that both high temperature and drought increase ABA levels in plants (Nilsen and Orcutt 1996), it was found later that higher temperature inhibits the inducing effect of drought stress on the ABA level (Qaderi et al. 2006). Therefore, the use of ABA-insensitive mutant (*abi1-1*) can help to understand how ABA, as a key internal signaling molecule, regulates plant responses to multiple factors, and how its level is affected by temperature. In the present study, we investigated the interactive effects of temperature, CO_2, and watering regime on the WT and its mutant of mouse-ear cress (*Arabidopsis thaliana*) plants grown in pots in controlled-environment growth chambers with the aim (1) to provide a better understanding of plant growth, anatomical, physiological, molecular, and hormonal responses to

the single and combined effects of the three main factors of climate change during vegetative stage, and (2) to determine the effects of temperature on the content of endogenous ABA using *Arabidopsis* (*abi1-1* and its associated WT). On the basis of previous findings, we hypothesized that higher temperature decreases the ability of WT plants to produce ABA in response to drought, whereas ECO_2 increases plant tolerance to stress by mitigating oxidative stress and improving water status of plants, and the mitigating effect is higher in the WT plants than in the *abi1-1* plants.

Materials and methods

Plants and growth conditions

In this study, seeds of two genotypes of *Arabidopsis thaliana* ecotype Landsberg erecta, wild type (WT) and its relative, ABA-insensitive mutant (*abi1-1*; Arabidopsis Biological Resource Center, The Ohio State University, Columbus, OH, USA) were used. First, the seeds were surface sterilized with 95% ethanol and germinated in Petri dishes, containing liquid Murashige and Skoog basal medium (MS; PhytoTechnology Laboratories, Shawnee Mission, KS, USA) in a growth chamber (model ATC26, Conviron, Controlled Environments, Winnipeg, MB, Canada) under control conditions (temperature regime of 22/18 °C, light/dark; photoperiod of 16 h; photosynthetic photon flux density (PPFD) of 300 µmol m^{-2} s^{-1}; and relative humidity (RH) of ~ 65%) for 6 days (four true leaves), essentially as described in Qaderi et al. (2013). For the two *Arabidopsis* genotypes, two seedlings were transplanted to pots (10 cm × 8 cm) containing a mixture of Perlite: Vermiculite: peat moss (1:1:1, by vol.). Then, nine pots containing 18 plants of each genotype were randomly assigned to each experimental treatment (see below). Plants were watered with tap water as needed and fertilized weekly with a slow-release NPK fertilizer (13-14-14 plus micronutrients; Chisso-Asahi Fertilizer Co, Tokyo, Japan). The eight-day-old plants of each genotype were grown under each of the eight experimental treatments, following the experimental design of Qaderi et al. (2013). A split–split–split-plot design was used with four factors (temperature, CO_2, watering regime, and genotype), each with two levels, for a total of 16 treatments, eight for each genotype: (1) lower temperatures (22/18 °C, 16 h light/8 h dark), ambient CO_2 (ACO_2, 400 µmol mol^{-1}), and watering to field capacity (well-watered), considered as control; (2) lower temperatures, ACO_2, and watering at wilting point (water-stressed); (3) lower temperatures, elevated CO_2 (ECO_2, 700 µmol mol^{-1}), and well-watered; (4) lower temperatures, ECO_2, and water-stressed; (5) higher temperatures (28/24 °C, 16 h light/8 h dark), ACO_2, and well-watered; (6) higher temperatures, ACO_2, and water-stressed; (7) higher temperatures, ECO_2, and well-watered; and (8) higher temperatures, ECO_2, and water-stressed. The selected higher temperature and ECO_2 concentration simulate the air temperature and atmospheric CO_2 concentration by the end of this century, based on IPCC predictions (Stocker et al. 2013). Midday leaf water potential ranged from − 1.0 to − 2.0 MPa and soil water potential ranged from − 0.4 to − 1.3, for well-watered and water-stressed plants, respectively. Water potential was measured with a WP4C Dew Point PotentiaMeter (Decagon Devices Inc., Pullman, WA, USA). In the water-stressed plants, a low moisture content was retained in pots during the experimental duration. Pots were rotated within each cabinet twice per week. Two Conviron growth chambers were used, one with lower temperatures and another with higher temperatures. In each chamber, two equal size Plexiglas cabinets of 60 cm depth, 65 cm width, and 50 cm height (GE Polymershapes, Dartmouth, NS, Canada) were placed; one was supplied with ACO_2 and the other with ECO_2 (Air Liquide, Dartmouth, NS, Canada). An electrical fan was used to keep CO_2 circulation constant in each cabinet. The flow of gas from the CO_2 cylinder to the Plexiglas cabinet was regulated by pressure gauge, solenoid valve and flow meter, and regularly monitored by a pSense portable CO_2 meter (CO_2 Meter, Inc., Ormond Beach, FL, USA). Half of the plants in each cabinet was watered to field capacity (determined by the excess water drainage), and the other half at wilting point (determined by the sign of leaf wilting). In each cabinet, PPFD, photoperiod, and RH were similar to the initial growth conditions (Qaderi et al. 2013). The experiments were conducted three times, each time with a different combination of growth chamber and Plexiglas cabinet.

Determination of growth and dry mass

For each treatment, rosette diameter of six of the 18-day-old plants of both genotypes was measured by means of a Digimatic caliper (Mitutoyo Corporation, Kanagawa, Japan). In this study, plants were grown for 18 days and used only in their vegetative stage. At the end of the experiment, from each treatment, three rosettes with average diameter were used to determine leaf number and area, total above (leaves) and belowground (root) dry mass, and leaf moisture content. Leaf (rosette) area was measured with a leaf area meter (Delta-T Devices, Cambridge, UK). For biomass measurement, plant samples were dried for 72 h at 60 °C in a forced-air Fisher Isotemp® Premium oven (model 750F, Fisher, Nepean, ON, Canada) and reweighed, using an analytical balance (model ED224 s, Sartorius, Goetttingen, Germany). For leaf moisture content, for each treatment, three leaves were taken from each of three plants to determine their fresh mass, and then, leaves were dried as described above. Leaf moisture content (%) was calculated using the following

formula: ((LFM − LDM) × 100)/(LFM), where 'LFM' stands for leaf fresh mass and 'LDM' for leaf dry mass.

Determination of epidermal cell characteristics

From each treatment and each genotype near the upper portion of the plants, full-developed leaves were sampled and used to examine stomatal density, cell density, stomatal index, and cell size of abaxial (lower) epidermis (Yeung 2015). Leaves were decolorized in 50% ethanol for a few days. *Arabidopsis* has small-thin leaves; therefore, leaves were cleared in 4% sodium hydroxide solution and placed in a 60 °C oven for 4 h. After removing the 4% sodium hydroxide solution, the samples were gently rinsed with several changes of distilled water and then were placed into 50% ethanol for 15 min prior to staining. The leaf tissues were stained with safranin solution for about 5 min. The stain was removed by several changes of distilled water. Photomicrographs (with 38.24 mm^2 actual area; at 400 ×) were captured, using an Olympus BX43F compound microscope connected to a DP73 digital camera (Olympus Corporation, Tokyo, Japan). For each genotype, five microscopic fields were randomly examined at the mid areas on each surface of ten leaves from different plants. Epidermal-cell images were later analyzed to determine stomatal density, cell density, stomatal index, and cell size, using the ImageJ software (http://rsb.info.nih.gov/ij/). Stomatal density (stomata mm^{-2}) was calculated as the number of stomata per unit epidermal area (Li et al. 2015). Epidermal cell density (number mm^{-2}) was calculated as the number of epidermal and stomatal cells per unit epidermal area. Stomatal index was estimated, using the formula $(s/(e+s)) \times 100$, where 's' stands for stomata and 'e' for epidermal cells per unit epidermal area (Ceulemans et al. 1995).

Measurement of leaf and soil water potential

Plant water status was assessed by measuring the water potential with a Dew Point PotentiaMeter (model WP4C, Decagon Devices, Pullman, WA, USA). From each treatment, three rosettes and three volumes of soil (~ 2.7 g) were taken at midday and used for measuring water potential (MPa) after calibration with 0.5 mol kg^{-1} of potassium chloride in water (AquaLab, Hoskin Scientific Ltd., Burlington, ON, Canada).

Measurement of photosynthetic pigments

Chlorophyll (Chl) *a*, Chl *b*, carotenoids, total Chl and Chl *a*:*b* ratio were measured according to Hiscox and Israelstam (1979). From each treatment, three leaf samples (~ 50 mg) were harvested from three different plants and incubated at room temperature in 5 ml of dimethylsulfoxide (VWR, Mississauga, ON, Canada) for 24 h in the dark until the pigments were completely bleached. Then, 1 ml of each solution was placed into a cuvette and the absorbance at 664, 648, and 470 nm was measured using a UV/visible spectrophotometer (model Ultraspec 3100 pro, Biochrom Ltd., Cambridge, UK). Pigment content (μg mg^{-1} FM) was calculated based on the absorbance level (Chappelle et al. 1992).

Measurement of proline, lipid peroxidation, and membrane permeability

Proline content was estimated by the method of Bates et al. (1973). From each treatment, three samples of fresh leaves (60 mg) were collected from three different plants and quickly homogenized using a mortar and a pestle in 5 ml of 3% aqueous sulfosalicylic acid. Then, the homogenate was centrifuged at 4000*g* for 10 min, and 2 ml of the filtrate was mixed with 2 ml acid-ninhydrin and 2 ml glacial acetic acid. The mixture was boiled at 100 °C for 30 min, then cooled in ice bath, and extracted with 5 ml of toluene. The absorbance was measured at 520 nm for the aqueous (upper) layer with a UV/visible spectrophotometer, using toluene as a blank. A standard curve was used to determine the proline content on a fresh mass basis (μmol g^{-1} FM).

Lipid peroxidation was determined by measurement of malondialdehyde (MDA) using 2-thiobarbituric acid assay procedure of Guo et al. (2012). From each treatment, three samples of fresh leaves (50 mg) were collected from three different plants and quickly frozen in liquid nitrogen and homogenized, using a mortar and a pestle in a solution composed of 1.5 ml 0.1% trichloroacetic acid and 1.5 ml 0.5% 2-thiobarbituric acid. Then, the homogenate was centrifuged at 4000*g* for 15 min at 4 °C, and the supernatant was boiled for 10 min and cooled on ice. 1 ml of the supernatant was collected and used to measure the absorbance at 532 nm and 600 nm with a UV/visible spectrophotometer. The 0.1% trichloroacetic acid and 0.5% 2-thiobarbituric acid were used as a blank. MDA content (nmol g^{-1} FM) was calculated using the following formula: $[((A532 - A600) \times v) \times 1000]/(\varepsilon \times M)$. In the formula, 'ε' stands for specific extinction coefficient (= 155 mM^{-1} cm^{-1}), 'v' for the volume of extracting medium, 'M' for the leaf fresh mass, and 'A600' and 'A532' for absorbance at 600 and 532 nm wavelengths, respectively.

Membrane permeability was evaluated by measuring the electrolyte leakage using the method of Anjum et al. (2012). From each treatment, three leaf samples (100 mg) were collected from three different plants and rinsed with distilled water and then placed in test tubes containing 15 ml of distilled water and incubated at room temperature for 24 h. The initial conductivity (C1) of the fresh tissue was measured with an HI 98311 DiST® 5 EC/TDS/Temperature Tester (Hanna Instruments Inc., Woonsocket, RI, USA). Samples

were then boiled at 100 °C for 1 h and left to cool down to room temperature. The maximum conductivity of the dead tissue (C2) was measured and the electrolyte leakage was calculated as the percentage ratio of C1–C2.

Measurement of ethylene evolution

Ethylene evolution was measured according to Qaderi et al. (2006) with some modifications. From each treatment, three samples of fresh leaves (~ 200 mg) were collected and incubated under the control condition of our experiment for 20 min in a 3-ml syringe. Then, a 1-ml sample of gas from each syringe was manually injected into a Varian 3900 gas chromatograph equipped with a flame ionization detector (Varian Canada, Mississauga, ON, Canada) and a Carboxen 1006 PLOT capillary column (30 m × 0.53 mm ID; Supelco, Bellefonte, PA, USA). The retention time was ~ 11.5 min. The rate of ethylene evolution was quantified based on leaf fresh mass and standard curve of the gas (pmol g^{-1} FM h^{-1}).

Extraction, purification, and quantification of endogenous abscisic acid and cytokinins

The *Arabidopsis* leaf samples were weighed (approximately 0.1 g FM) and freeze-dried (BenchTop Pro with Omnitronics, VirTis SP Scientific, Warminster, PA, USA). The tissue was suspended in 1 ml of extraction buffer Bieleski#2 (CH_3OH:H_2O:HCOOH [15:4:1, by vol.]), spiked with internal standards (144.7 ng of $^2H_4$ ABA (PBI, Saskatchewan, Canada) and 10 ng of each of the deuterated internal standard cytokinins (CKs) (OlChemim Ltd., Olomouc, Czech Republic; see Noble et al. 2014), and homogenized (ball mill, RetschMM300; 5 min at 25 RPM) at 4 °C with zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, QC, Canada). A modified protocol by Quesnelle and Emery (2007) and Farrow and Emery (2012) was used for the ABA and CKs extraction by robot liquid handler. Hormones were identified and quantified by electrospray ionization, liquid chromatography-tandem mass spectrometry, HPLC-(ESI)-MS/MS (Shimadzu LC-10ADvp HPLC connected to a QTrap 5500 Mass Spectrometer Sciex Applied Biosystem). Positive-ion mode was used for all CKs profiling and negative-ion mode for ABA analyses. A 20-µl sample volume was injected on a Kinetex reversed-phase C18 column (Phenomenex; 3 µm, 50 × 2.1 µm, Torrance, CA, USA). CKs and ABA were eluted with an increasing gradient of 0.08% acetic acid in acetonitrile (B) mixed with 0.08% acetic acid in Milli-Q water (A) at a flow rate of 0.4 ml min^{-1} (CKs) and 0.28 ml min^{-1} (ABA). The initial conditions for CKs were 95% A and 5% B, changing linearly over 8.5 min to 5% A and 95% B for 1.5 min, then returning to initial conditions for 5 min. The initial conditions for ABA were 5% A and 95% B, changing linearly over 3.1 min to 100% A and 0% B for 2 min, and then returning to initial conditions for 5 min. The effluent was introduced into the electrospray source (source block temperature of 700 °C), using conditions specific for each CK/ABA and analysis was obtained by multiple reaction monitoring (MRM) of the protonated intact CK molecule $[M+H]^+$ and the specific product ion.

RNA extraction and RT-PCR

As the *RD22* and *RD29B* are abiotic stress-responsive genes, regulated by ABA signal, we examined the expression pattern of these genes in the 18-day-old seedlings of the two genotypes, using a reverse transcription (RT)-PCR procedure. From WT and *abi1-1* plants, total RNA was isolated, using Ribozol extraction method (AMRESCO; VWR, Mississauga, ON, Canada), according to the manufacturer's instructions. All RNAs were stored at − 80 °C until needed. A 0.2-µg portion of total RNA in a final volume of 20-µl reverse transcription reaction was reverse-transcribed using SuperScript III and RNase H reverse transcriptase (Invitrogen) following manufacturer instructions. From the resulting cDNAs, 2 µl was then used in 50 µl PCR reactions utilizing Taq DNA polymerase with ThermoPol® buffer following manufacturer instructions. PCR amplification for the *RD22* (TAIR ID: AT5G25610) and the *RD29B* (TAIR ID: AT5G52300) was performed with initial denaturation at 94 °C for 3 min followed by 35 cycles of incubations at 94 °C for 45 s, 48 °C for 30 s, and 72 °C for a minute, and a final extension at 72 °C for 10 min. Gene-specific oligonucleotide primers were used to distinguish *RD22* (RD22F, 5′-taggagtcggtaaaggcggt-3′ (forward); and RD22R, 5′-catcggtgcgttcttcttagc-3′ (reverse)) and *RD29B* (RD29BF2, 5′-gaccacaccaaaacccattgag-3′; and RD29BR2, 5′-gcttctccacctttatgcgtg-3′) transcripts by RT-PCR. *EF1alfa* (TAIR ID: AT1G07920) gene was used as a positive internal control for all RT-PCR reactions. The same PCR amplification reaction was set up for the *EF1alfa*, except that the amplification was through 40 cycles. The primers were as follows: EF1alfa-F, 5′-tgaggcacttcccggtgaca-3′; and EF1alfa-R, 5′-gttggcggcacccttagctg-3′. 10 µl of the reaction products was run on a 1 × Tris–Borate-EDTA (TBE) plus 1.6% agarose gel electrophoresis containing Orange G dye and then visualized with a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system (Montreal Biotech, Montreal, QC, Canada).

Data analysis

The effects of temperature, carbon dioxide, and watering regime on growth and biomass, anatomical features, chemical and biochemical properties, physiological parameters, hormonal regulation, and molecular aspects of

Arabidopsis plants (WT and *abi1-1* mutant) were analyzed, using ANOVA for split–split-split-plot design (SAS Institute 2011). For the split–split–split-plot analysis, temperature regime, CO_2 concentration, watering regime, genotype, and growth chamber were treated, respectively, as the main plot, subplot, split-subplot, split–split-subplot and replication. A one-way ANOVA was used to determine differences among treatments, using Scheffé's test at the 5% probability level (SAS Institute 2011). Pearson's correlation coefficient was used to determine relationship between plant parameters (Minitab Inc. 2014). Data are reported as mean ± standard error.

Results

Plant growth

ECO_2 increased rosette diameter and leaf number and leaf area, while water stress and higher temperatures decreased them. The *abi1-1* plants had a smaller rosette diameter and leaf number and area than the WT plants (Table 1, Fig. 1). The three-way interaction among carbon dioxide (C) × watering regime (W) × genotype (G) (Table 2) revealed that rosette diameter and leaf number were highest for the well-watered WT plants at ECO_2, and lowest for the water-stressed *abi1-1* plants at ACO_2 (Fig. 1).

Dry mass accumulation

Higher temperatures and water stress reduced leaf, root, and total biomass, whereas ECO_2 increased them. These parameters were also higher for the WT plants than for the *abi1-1* plants (Table 1, Fig. 2). Differences between CO_2 concentrations were significant for root, leaf, and total biomass (Table 1). However, differences between watering regimes and genotypes were significant for all parameters (Table 1). On the basis of interactions among these factors (Table 2), the well-watered WT plants under lower temperatures at ECO_2 had highest root, leaf, and total biomass, whereas the water-stressed *abi1-1* plants under higher temperatures at ACO_2 had lowest biomass of these parts (Fig. 2).

Light microscopy of epidermal cells

Higher temperatures and ECO_2 decreased, but water stress increased, stomatal density, which was higher in the *abi1-1* plants than in the WT plants (Table 1). On the basis of C × W × G (Table 3), the water-stressed *abi1-1* plants at ACO_2 had highest stomatal density, whereas the well-watered WT plants at ECO_2 had lowest stomatal density (Fig. 3a, b).

Higher temperatures increased, but water stress decreased, stomatal index (Table 1), which was significantly affected by the main factors and their interactions (Table 3). These interactions revealed that the well-watered *abi1-1* plants under higher temperatures at ECO_2 had highest stomatal index, whereas the water-stressed *abi1-1* plants under lower temperatures at ECO_2 had lowest stomatal index (Fig. 3c, d).

Higher temperatures and ECO_2 decreased, but water stress increased, cell density, which was higher in the *abi1-1* plants than in the WT plants (Table 1). Interactions of the main factors (Table 3) revealed that the water-stressed WT plants under lower temperatures at ACO_2 had highest cell density, whereas the well-watered WT plants under higher temperatures at ECO_2 had lowest cell density (Figs. 3e, f, 4b, g).

In contrast to cell density, cell area was decreased by water stress, but increased by higher temperatures and ECO_2, and the WT plants had higher cell area than the *abi1-1* plants (Table 1). Interactions among C × W × G (Table 3) revealed that the well-watered WT plants at ECO_2 had highest cell area, whereas the water-stressed *abi1-1* plants at ACO_2 had lowest cell area (Fig. 3g, h).

Soil and leaf water potential and leaf moisture

Higher temperatures and water stress reduced leaf and soil water potential and leaf moisture, whereas ECO_2 increased them (Table 1). Plants from the *abi1-1* genotype had lower leaf and soil water potential and leaf moisture content than the WT plants (Table 1). On the basis of C × W × G (Table 3), all these parameters were highest for the well-watered WT plants at ECO_2, but lowest for the water-stressed *abi1-1* plants at ACO_2 (Fig. 5). On the basis of T × C × G (Table 3), ECO_2 caused highest leaf water potential for the WT plants under lower temperatures, but ACO_2 resulted in lowest leaf water potential for the *abi1-1* plants under higher temperatures. On the basis of interactions among T × W × G (Table 3), leaf water potential was highest for the well-watered WT plants under lower temperatures, but lowest for the water-stressed *abi1-1* plants under higher temperatures. Interactions of the main factors (Table 3) revealed that leaf water potential was highest in the well-watered WT plants under lower temperatures at ECO_2, but lowest in the water-stressed *abi1-1* plants under higher temperatures at ACO_2 (Fig. 5c, d).

Photosynthetic pigments

Higher temperatures increased Chl *a* and carotenoid contents (Table 1). Overall, ECO_2 decreased, whereas water stress increased, Chl *a*, Chl *b*, carotenoids, and total Chl. The *abi1-1* plants had higher carotenoids than the WT plants (Table 1). On the basis of T × C × G (Table 4), the *abi1-1*

Table 1 Effects of temperature, carbon dioxide, watering regime, and genotype on growth, physiological, biochemical, and hormonal parameters of *Arabidopsis thaliana*

Parameter	Temperature		Carbon dioxide		Watering regime		Genotype	
	Lower	Higher	Ambient	Elevated	Well-watered	Water-stressed	Wild type	*abi1-1* mutant
RD (mm)	26.0 ± 2.4a	18.7 ± 1.6b	19.6 ± 1.8b	25.1 ± 2.3a	30.8 ± 1.7a	13.9 ± 0.7a	24.5 ± 2.4a	19.3 ± 1.8b
Leaf number ($plant^{-1}$)	9.4 ± 0.5a	7.2 ± 0.3b	7.3 ± 0.3b	9.3 ± 0.4a	9.7 ± 0.4a	6.9 ± 0.2b	8.9 ± 0.4a	7.6 ± 0.4b
Leaf area (cm^2 $plant^{-1}$)	2.7 ± 0.4a	1.4 ± 0.2b	1.6 ± 0.3b	2.5 ± 0.4a	3.3 ± 0.3a	0.82 ± 0.1b	2.4 ± 0.4a	1.6 ± 0.3b
Root mass (g)	0.06 ± 0.01a	0.02 ± 0.00b	0.03 ± 0.00b	0.06 ± 0.01a	0.07 ± 0.01a	0.01 ± 0.00b	0.06 ± 0.01a	0.03 ± 0.00b
Leaf mass (g)	0.13 ± 0.02a	0.06 ± 0.01b	0.06 ± 0.01b	0.13 ± 0.02a	0.16 ± 0.02a	0.03 ± 0.00b	0.12 ± 0.02a	0.06 ± 0.01b
Total biomass (g)	0.19 ± 0.03a	0.09 ± 0.01b	0.09 ± 0.01b	0.19 ± 0.03a	0.24 ± 0.03a	0.04 ± 0.00b	0.19 ± 0.03a	0.09 ± 0.01b
SD (number mm^{-2})	227.9 ± 10.5a	181.3 ± 12.2b	240.6 ± 10.2a	168.5 ± 9.5b	177.3 ± 11.0b	231.7 ± 11.1a	186.1 ± 10.5b	223.4 ± 12.8a
SI (%)	15.5 ± 0.7b	22.3 ± 0.5a	18.8 ± 0.8a	19.05 ± 1.1a	19.4 ± 0.8a	18.4 ± 1.0b	19.1 ± 0.9a	18.7 ± 0.9a
CD (number mm^{-2})	1574 ± 101a	852 ± 65b	1389 ± 99a	1036 ± 114b	1007 ± 92b	1418 ± 116a	1100 ± 109b	1324 ± 112a
CA (mm^2)	7172 ± 486b	14,909 ± 1609a	8340 ± 629b	13,720 ± 1753a	13,422 ± 1735a	8638 ± 766b	12,499 ± 1716a	9589 ± 976b
LWP	− 1.3 ± 0.08a	− 1.7 ± 0.09b	− 1.7 ± 0.09b	− 1.3 ± 0.08a	− 1.2 ± 0.07a	− 1.8 ± 0.08b	− 1.4 ± 0.08a	− 1.7 ± 0.10b
SWP	− 0.7 ± 0.09a	− 1.06 ± 0.11b	− 1.05 ± 0.12b	− 0.7 ± 0.10a	− 0.4 ± 0.03a	− 1.3 ± 0.03b	− 0.7 ± 0.09a	− 1.00 ± 0.12b
LMC	80.3 ± 2.1a	68.2 ± 3.3b	70.0 ± 3.4b	78.5 ± 2.3a	85.1 ± 1.2a	63.5 ± 2.6b	77.9 ± 2.7a	67.8 ± 3.1b
Chl *a* (μg mg^{-1} FM)	1.38 ± 0.04b	1.47 ± 0.06a	1.55 ± 0.06a	1.31 ± 0.03b	1.28 ± 0.03b	1.58 ± 0.05a	1.43 ± 0.05a	1.37 ± 0.05a
Chl *b* (μg mg^{-1} FM)	0.41 ± 0.01a	0.44 ± 0.01a	0.46 ± 0.02a	0.40 ± 0.01b	0.38 ± 0.01b	0.47 ± 0.01a	0.43 ± 0.01a	0.41 ± 0.01a
Carotenoids (μg mg^{-1} FM)	0.31 ± 0.00b	0.34 ± 0.01a	0.35 ± 0.01a	0.30 ± 0.00b	0.29 ± 0.00b	0.35 ± 0.01a	0.31 ± 0.01b	0.33 ± 0.01a
Total Chl (μg mg^{-1} FM)	1.80 ± 0.06a	1.92 ± 0.08a	2.01 ± 0.08a	1.71 ± 0.04b	1.66 ± 0.04b	2.05 ± 0.07a	1.86 ± 0.07a	1.78 ± 0.07a
Chl *a:b*	3.36 ± 0.04a	3.34 ± 0.06a	3.39 ± 0.06a	3.31 ± 0.04a	3.34 ± 0.04a	3.35 ± 0.06a	3.31 ± 0.02a	3.25 ± 0.07a
Proline (μmol g^{-1} FM)	34.6 ± 3.6a	19.1 ± 1.6b	33.7 ± 3.4a	20.02 ± 2.3b	18.44 ± 1.4b	35.2 ± 3.6a	30.7 ± 3.7a	22.9 ± 2.3b
MDA (mmol g^{-1} FM)	0.08 ± 0.01a	0.02 ± 0.00b	0.07 ± 0.01a	0.03 ± 0.00b	0.02 ± 0.00b	0.07 ± 0.01a	0.04 ± 0.00b	0.05 ± 0.01a
EC (%)	36.8 ± 4.0a	16.9 ± 2.0b	32.8 ± 4.3a	21.0 ± 2.7b	17.3 ± 2.0b	36.4 ± 4.1a	20.0 ± 2.3b	33.8 ± 4.3a
Ethylene (pmol g^{-1} FM h^{-1})	350.7 ± 45.3a	186.3 ± 23.5b	206.9 ± 19.4b	330.1 ± 49.9a	281.8 ± 16.5a	255.2 ± 53.9b	342.7 ± 46.1a	186.5 ± 24.4b
ABA (pmol g^{-1} FM)	117.8 ± 28.5b	263.7 ± 90.2a	255.4 ± 87.2a	126.1 ± 38.1b	52.4 ± 8.3b	326.5 ± 87.7a	120.3 ± 22.7b	261.1 ± 92.1a
Total CKs (pmol g^{-1} FM)	487.5 ± 56.1a	443.9 ± 33.8a	543.4 ± 56.3a	387.9 ± 23.5b	418.7 ± 45.6a	512.7 ± 56.9a	502.8 ± 49.0a	428.6 ± 42.5a
tZ (pmol g^{-1} FM)	3.01 ± 0.70a	2.88 ± 0.50a	3.54 ± 0.76a	2.35 ± 0.35a	3.28 ± 0.62a	2.61 ± 0.59a	4.81 ± 0.59a	1.08 ± 0.30b
iP (pmol g^{-1} FM)	1.94 ± 0.14b	2.26 ± 0.11a	2.19 ± 0.16a	2.01 ± 0.09a	1.91 ± 0.12b	2.28 ± 0.13a	2.24 ± 0.09a	1.95 ± 0.15b
tZR (pmol g^{-1} FM)	6.26 ± 1.04a	4.67 ± 0.70a	6.94 ± 1.09a	3.98 ± 0.48b	5.61 ± 0.91a	5.31 ± 0.89a	6.19 ± 0.99a	4.73 ± 0.77a
cisZR (pmol g^{-1} FM)	11.02 ± 3.66a	3.88 ± 1.33a	7.43 ± 2.57a	7.46 ± 3.17a	7.96 ± 3.56a	6.93 ± 1.89a	11.32 ± 3.78a	3.57 ± 0.84b

Table 1 (continued)

Parameter	Temperature		Carbon dioxide		Watering regime		Genotype	
	Lower	Higher	Ambient	Elevated	Well-watered	Water-stressed	Wild type	*abi1-1* mutant
DHZR (pmol g^{-1} FM)	1.65 ± 0.21b	3.72 ± 0.88a	2.94 ± 0.74a	2.43 ± 0.61a	1.57 ± 0.20b	3.79 ± 0.88a	1.26 ± 0.18b	4.11 ± 0.84a
iPR (pmol g^{-1} FM)	18.50 ± 4.29a	12.15 ± 2.65a	19.03 ± 4.38a	11.62 ± 2.46a	14.81 ± 4.30a	15.84 ± 2.78a	16.33 ± 4.42a	14.32 ± 2.58a

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C. Data are mean ± SE of nine samples from three trials. Means followed by different letters within each parameter and condition are significantly different ($P < 0.05$) according to Scheffé's test. The detected leaf CKs were: free bases—tZ (trans-zeatin) and iP (isopentyladenine); and ribosides—tZR (*trans*-zeatin riboside), cisZR (*cis*-zeatin riboside), DHZR (dihydrozeatin riboside), and iPR (isopentenyladenosine riboside)

CA cell area, *CD* cell density, *Chl* chlorophyll, *EC* electrical conductivity, *LM* leaf moisture, *LWP* leaf water potential, *MDA* malondialdehyde, *RD* rosette diameter, *SD* stomatal density, *SI* stomatal index, *SWP* soil water potential

plants under higher temperatures at ACO_2 had highest Chl *a*, but the WT plants under lower temperatures at ECO_2 had lowest Chl *a*. On the basis of T × W × G (Table 4), the water-stressed *abi1-1* plants under higher temperatures had highest Chl *a*, whereas the well-watered *abi1-1* plants under higher temperatures had lowest Chl *a*. With regards to the interaction of the C × W × G (Table 4), the water-stressed *abi1-1* plants at ACO_2 had highest Chl *a*, whereas the well-watered

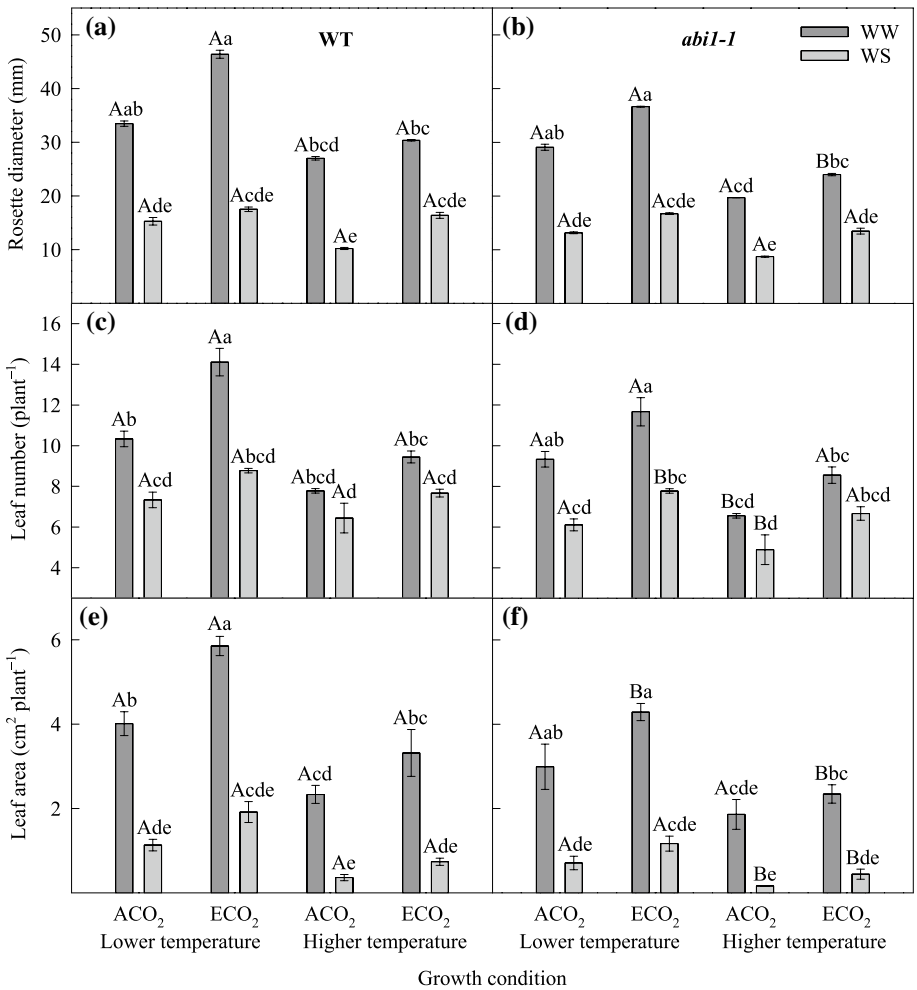

Fig. 1 Effects of temperature, carbon dioxide, and the watering regime on plant growth characteristics of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers. The WT (**a**, **c**, **e**) and *abi1-1* (**b**, **d**, **f**) genotypes were used in this study. Rosette diameter (**a**, **b**), leaf number (**c**, **d**), and leaf area (**e**, **f**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's test. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes

Table 2 Summary of split–split-split-plot ANOVA (*F* value) for effects of temperature, carbon dioxide, watering regime, and genotype on growth and dry mass of *Arabidopsis thaliana*

Source	*df*	Plant growth			Dry mass		
		Rosette diameter	Leaf number	Leaf area	Root	Leaf	Total
Temperature (*T*)	1	0.18	0.02	0.00	7.82	9.34	11.02
Main plot error	2	–	–	–	–	–	–
Carbon dioxide (C)	1	10.94	374.00**	0.45	22.36*	18.62*	15.62
T×C	1	0.26	0.06	0.06	1.79	1.46	1.19
Subplot error	2	–	–	–	–	–	–
Watering regime (W)	1	12.60*	52.38**	0.03	187.90***	168.13***	145.29***
T×W	1	0.16	0.02	0.03	7.82*	6.81	5.66
C×W	1	11.65*	40.92**	0.33	350.85****	318.29****	278.93****
T×C×W	1	0.19	0.00	0.04	8.42*	7.34	6.11
Split-subplot error	4	–	–	–	–	–	–
Genotype (G)	1	9.81*	96.72****	0.37	380.80****	323.32****	268.59****
T×G	1	0.34	2.99	1.33	17.74**	16.39**	14.87**
C×G	1	10.67*	146.69****	0.70	72.91****	59.23****	47.02***
T×C×G	1	0.51	4.52	1.22	11.34**	10.63*	9.79*
W×G	1	10.38*	122.02****	0.01	201.33****	168.71****	138.30****
T×W×G	1	0.36	2.91	1.30	18.28**	16.81**	15.17**
C×W×G	1	11.06*	175.24****	2.39	2.08	0.28	0.00
T×C×W×G	1	0.60	4.52	1.20	12.54**	11.56**	10.47*
Split-split-subplot error	8	–	–	–	–	–	–

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C. Experiments were conducted three times

Significance values: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$

abi1-1 plants at ECO_2 had lowest Chl *a* (Fig. 6a, b). On the basis of the four-way interaction of the main factors (Table 4), the water-stressed *abi1-1* plants under higher temperatures at ACO_2 had highest Chl *b*, whereas the well-watered WT plants under lower temperatures at ECO_2 had lowest Chl *b* (Fig. 6c, d). Also, on the basis of interactions among these factors (Table 4), the water-stressed *abi1-1* plants under higher temperatures at ACO_2 had highest carotenoids, whereas the well-watered WT plants under higher temperatures at ACO_2 had lowest carotenoids (Fig. 6e, f). On the basis of C×W×G (Table 4), total Chl was highest in the water-stressed *abi1-1* plants at ACO_2, but lowest in the well-watered WT plants at ACO_2 (Fig. 6g, h). Interactions of the main factors (Table 4) revealed that the water-stressed *abi1-1* plants under higher temperatures at ACO_2 had highest Chl *a:b* ratio, whereas the water-stressed *abi1-1* plants under higher temperatures at ECO_2 had lowest Chl *a:b* (Fig. 6i, j).

Proline, lipid peroxidation, and electrical conductivity

Higher temperatures and ECO_2 decreased, but water stress increased, proline level (Table 1). WT plants had a higher proline content than the *abi1-1* plants (Table 1). The C×W×G interaction (Table 4) indicated that proline content was highest in the water-stressed WT plants at ACO_2, but lowest in the well-watered *abi1-1* plants at ECO_2 (Fig. 7a, b).

Higher temperatures and ECO_2 decreased, but water stress increased, MDA content, which was higher in the *abi1-1* plants than in the WT plants (Table 1). On the basis of four-way interaction (Table 4), MDA was highest in the water-stressed *abi1-1* plants under lower temperatures at ACO_2, but lowest in the well-watered WT plants under higher temperatures at ECO_2 (Fig. 7c, d).

Higher temperatures and ECO_2 decreased, but water stress increased, electrical conductivity, which was similar to the effects on MDA. The *abi1-1* plants had higher electrical conductivity than the WT plants (Table 1). On the basis of C×W×G interaction (Table 4), the water-stressed *abi1-1* plants at ACO_2 had highest electrical conductivity, whereas the well-watered WT plants at ECO_2 had lowest electrical conductivity (Fig. 7e, f).

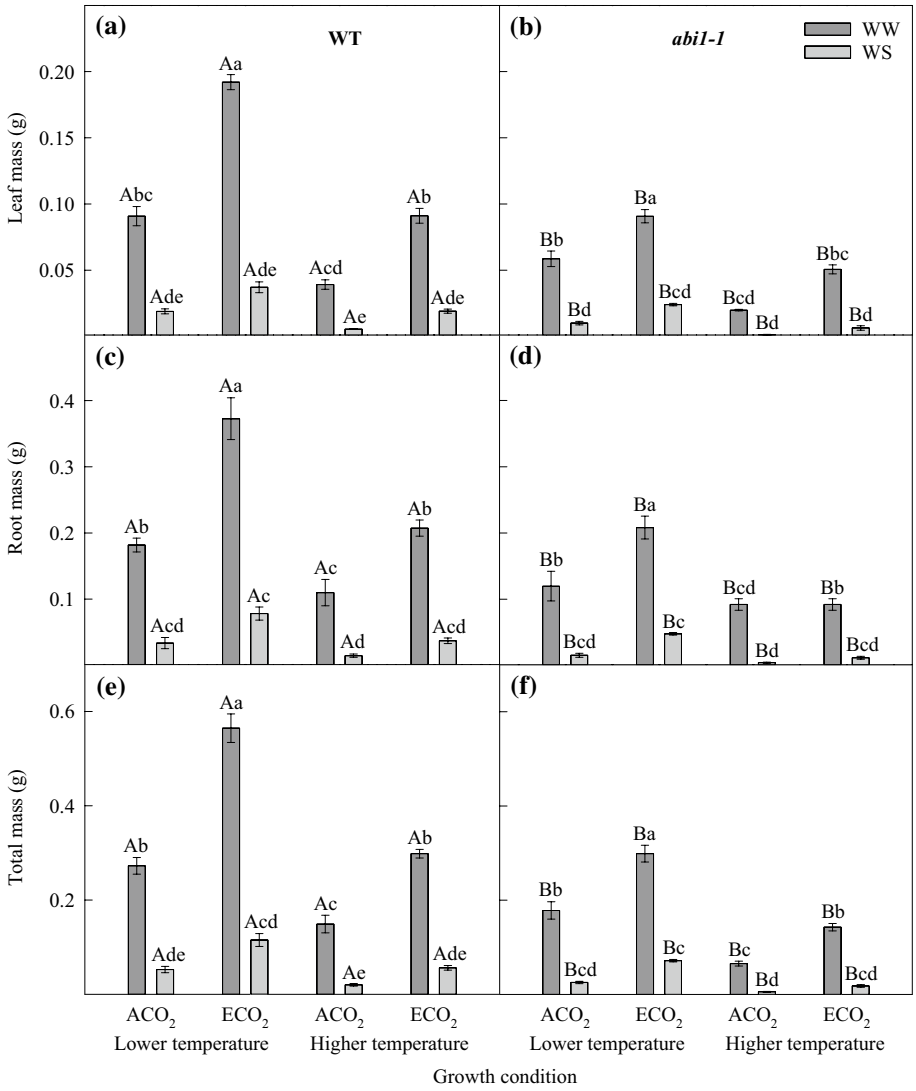

Fig. 2 Effects of temperature, carbon dioxide, and watering regime on dry mass accumulation of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**) and *abi1-1* (**b**, **d**, **f**) genotypes were used in this study. Leaf dry mass (**a**, **b**), root dry mass (**c**, **d**), and total dry mass (**e**, **f**). Other details as in Fig. 1

Ethylene evolution

ECO_2 increased, but higher temperatures and water stress decreased, ethylene evolution, which was higher from the WT plants than from the *abi1-1* plants (Table 1). On the basis of C × W × G (Table 5), the water-stressed WT plants at ECO_2 had highest ethylene evolution, whereas the water-stressed *abi1-1* plants at ECO_2 had lowest ethylene evolution (Fig. 8a, b).

Abscisic acid and cytokinins

Overall, higher temperatures and water stress increased ABA content, whereas ECO_2 reversed their effects (Table 1). Interestingly, the *abi1-1* plants had higher ABA than the WT plants (Table 1, Fig. 8c, d). Importantly, higher temperature inhibited the inducing effect of water stress on ABA content in the WT plants regardless of the watering regime, but not in the *abi1-1* plants (Fig. 8a, b).

Total CKs were increased only by ECO_2 (Table 1). On the basis of C × W × G interaction (Table 5), the well-watered WT plants at ACO_2 had highest total CKs, whereas the well-watered *abi1-1* plants at ECO_2 had lowest total CKs (Fig. 8e, f).

Detailed analysis of free base CKs revealed the presence of trans-zeatin and isopentyladenine in both genotypes (Table 5). Higher temperatures and water stress significantly increased isopentyladenine. The WT plants had higher *trans*-zeatin and isopentyladenine than the *abi1-1* plants (Table 1). On the basis of C × G (Table 5), ACO_2 resulted in highest *trans*-zeatin in the WT plants, but ECO_2 resulted in lowest *trans*-zeatin in the *abi1-1* plants. On the basis of C × W × G (Table 5), the well-watered WT plants at ACO_2 had highest *trans*-zeatin, whereas the water-stressed WT plants at ECO_2 had lowest *trans*-zeatin. The T × C interaction (Table 5)

Table 3 Summary of split–split-split-plot ANOVA (*F* value) for effects of temperature, carbon dioxide, watering regime, and genotype on leaf anatomical features and water status of *Arabidopsis thaliana*

Source	*df*	Leaf anatomical feature				Leaf and soil water status		
		Stomatal density	Stomatal index	Cell density	Cell area	Soil water potential	Leaf water potential	Leaf moisture
Temperature (*T*)	1	1.03	159.11**	5.77	5.40	3.36	12.01	0.39
Main plot error	2	–	–	–	–	–	–	–
Carbon dioxide (C)	1	114.65**	765.35**	65.29*	33.31*	15.69	40.77*	80.58*
T×C	1	1.54	38.24*	6.54	4.45	0.14	0.19	0.58
Subplot error	2	–	–	–	–	–	–	–
Watering regime (W)	1	91.00***	1252.95****	60.89**	39.36**	44.32**	111.56***	146.17***
T×W	1	0.99	89.50***	5.64	5.64	0.14	0.07	0.27
C×W	1	84.59***	417.64****	60.00**	39.18**	67.34**	156.42***	141.89***
T×C×W	1	1.05	85.47***	5.74	5.52	0.21	0.18	0.38
Split-subplot error	4	–	–	–	–	–	–	–
Genotype (G)	1	110.60****	282.45****	66.51****	31.53***	119.32****	413.25****	69.00****
T×G	1	1.59	33.61***	6.53*	4.37	5.02	13.48**	0.42
C×G	1	116.84****	845.25****	66.23****	31.07***	41.53***	177.00****	67.99****
T×C×G	1	1.99	29.15***	7.08*	3.94	3.02	7.48*	1.02
W×G	1	120.62****	1071.87****	67.28****	31.48***	76.95****	288.31****	69.55****
T×W×G	1	1.66	33.11***	6.64*	4.28	5.03	13.48**	0.49
C×W×G	1	126.41****	2018.16****	66.58****	30.83***	15.98**	90.71****	67.09****
T×C×W×G	1	2.22	23.78**	7.42*	3.67	3.21	8.03*	1.32
Split-split-subplot error	8	–	–	–	–	–	–	–

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C. Experiments were conducted three times for the leaf and soil water potential and the moisture content. For the anatomical features, five microscopic fields on each surface of ten leaves per treatment were analyzed

Significance values: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$

revealed that plants under higher temperatures at ACO_2 had highest isopentyladenine level, while plants under lower temperatures at ACO_2 had lowest isopentyladenine. On the basis of C×W×G (Table 5), the well-watered WT plants at ACO_2 had highest isopentyladenine (2.58 ± 0.23), whereas the well-watered *abi1-1* plants at ACO_2 had lowest isopentyladenine (1.42 ± 0.55).

Four major riboside CKs, *trans*-zeatin, *cis*-zeatin riboside, dihydrozeatin, and isopentenyladenosine riboside, were detected in the WT and *abi1-1* mutant plants. ECO_2 decreased *trans*-zeatin riboside, which was significantly affected by the main factors and their interactions, except by T×C (Table 5). On the basis of four-way interaction, the level of *trans*-zeatin riboside was highest in the well-watered WT plants under lower temperatures at ACO_2 (13.09 ± 5.52), but lowest in the well-watered *abi1-1* plants under higher temperatures at ECO_2 (1.95 ± 1.07). *cis*-zeatin riboside was significantly lower in the *abi1-1* plants than in the WT plants (Table 1), but it was not affected by other factors (Table 5). Dihydrozeatin riboside was significantly increased by higher temperatures and water stress (Table 1). In contrast to *cis*-zeatin riboside, dihydrozeatin riboside was significantly higher in the *abi1-1* plants than in the WT plants (Table 1). On the basis of C×W×G (Table 5), the isopentenyladenosine riboside was highest in the water-stressed *abi1-1* plants at ACO_2 (28.27 ± 11.43), but lowest in the well-watered *abi1-1* plants at ECO_2 (8.43 ± 1.24).

Gene expression pattern of *RD22* and *RD29B*

Expression of the ABA-responsive genes, *RD22* and *RD 29B*, in the WT and *abi1-1* plants is shown in Fig. 9. In the control treatment, *RD22* was not expressed in WT or in *abi1-1*, while *RD29B* was only expressed in WT. These genes were not expressed in the well-watered plants of both genotypes under lower temperatures at ECO_2. Expression of these genes was increased by higher temperatures and water stress, but decreased by ECO_2 (see Fig. 9, C7–C8). *RD22* and *RD29B* genes were activated by stress conditions and relatively maintained similar patterns of induction (Fig. 9). However, stress-mediated induction of these genes was higher in the WT plants than in the *abi1-1* plants. In particular, the induction of these genes was much stronger in the water-stressed plants of WT than that of the *abi1-1*

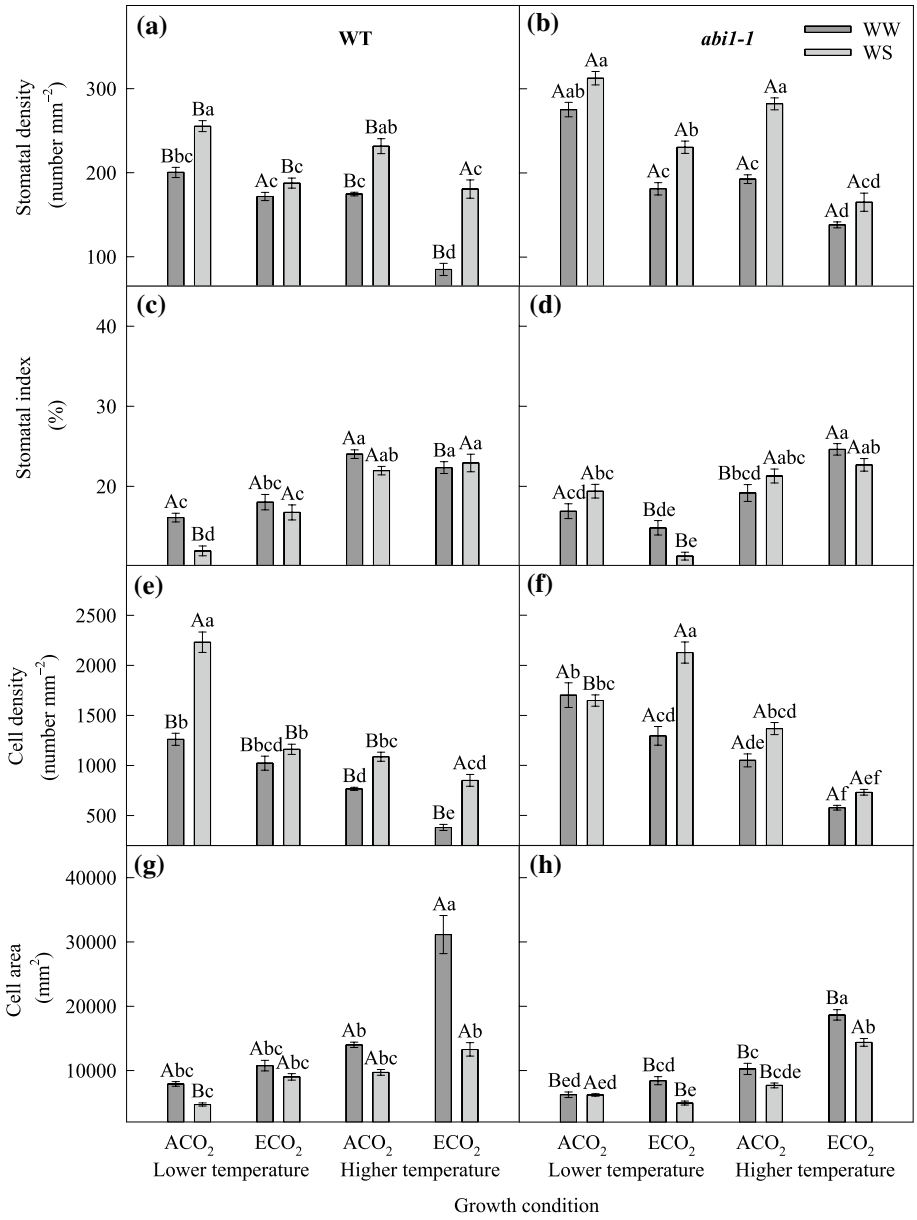

Fig. 3 Effects of temperature, carbon dioxide, and watering regime on the anatomical features of the lower epidermis of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**, **g**) and *abi1-1* (**b**, **d**, **f**, **h**) genotypes were used in this study. Stomatal density (**a**, **b**), stomatal index (**c**, **d**), cell density (**e**, **f**), and cell area (**g**, **h**). Other details as in Fig. 1

plants under lower temperatures at ACO_2, or under higher temperatures at ACO_2 (Fig. 9).

Relationship between plant parameters

Pearson's correlation analysis revealed significant ($P<0.05$) relationships between plant parameters (Table 6). Rosette diameter was positively correlated with leaf area ($r=0.936$), root mass ($r=0.908$), leaf mass ($r=0.905$), total mass ($r=0.913$), and leaf moisture content ($r=0.791$), but negatively correlated with electric conductivity ($r=-0.409$). Leaf area was positively correlated with root mass ($r=0.920$), leaf mass ($r=0.940$), and total mass ($r=0.940$), but negatively correlated with electrical conductivity ($r=-0.367$). Root mass was positively correlated with leaf mass ($r=0.964$) and total mass ($r=0.984$), but negatively correlated with Chl *a* ($r=-0.462$), Chl *b* ($r=-0.448$), total Chl ($r=-0.469$), and electrical conductivity ($r=-0.370$). Leaf mass was positively correlated with total mass ($r=0.996$), but negatively correlated with Chl *a* ($r=-0.446$), Chl *b* ($r=-0.447$), total Chl ($r=-0.457$), and electrical conductivity ($r=-0.401$). A negative correlation was found between total mass and Chl *a* ($r=-0.455$), Chl *b* ($r=-0.451$), and total Chl ($r=-0.465$). Leaf mass area was positively correlated with leaf area ($r=0.490$), root mass ($r=0.609$), and leaf mass ($r=0.677$). Cell area had positive correlation with stomatal index ($r=0.640$), but negative correlation with stomatal density ($r=-0.824$), malondialdehyde (MDA, $r=-0.618$), and electrical conductivity ($r=-0.603$). Correlations were positive between soil water

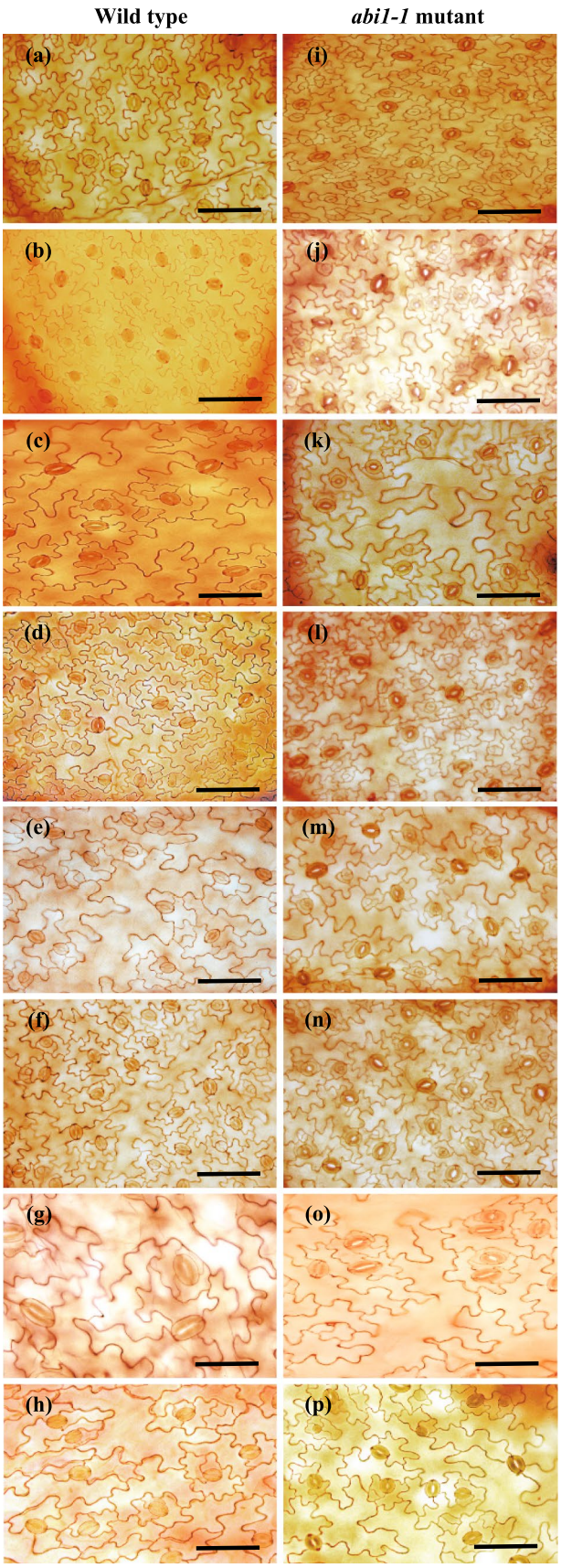

Fig. 4 Light photomicrograph of lower epidermis from WT and *abi1-1* mutant leaves of *A. thaliana*. Growth conditions: lower temperatures, ACO_2, well-watered (**a–i**), lower temperatures, ACO_2, water-stressed (**b–j**), lower temperatures, ECO_2, well-watered (**c–k**), lower temperatures, ECO_2, water-stressed (**d–l**), higher temperatures, ACO_2, well-watered (**e–m**), higher temperatures, ACO_2, water-stressed (**f–n**), higher temperatures, ECO_2, well-watered (**g–o**), and higher temperatures, ECO_2, water-stressed (**h–p**). Scale bar = 500 µm. Other details as in Fig. 1

potential and leaf number (r = 0.794) and leaf water potential (r = 0.860). Leaf moisture content was positively correlated with leaf area (r = 0.814), root mass (r = 0.727), leaf mass (r = 0.735), and total mass (r = 0.738). MDA was positively correlated with electrical conductivity (r = 0.898), but negatively correlated with total mass (r = − 0.367). ABA was positively correlated with Chl *a* (r = 0.682), Chl *b* (r = 0.598), carotenoids (r = 0.740), and total Chl (r = 0.669), but negatively correlated with rosette diameter (r = − 0.593) and leaf area (r = − 0.548).

Discussion

Climate change is inevitable within our lifetime, even if the production of greenhouse gases is stopped today (Stocker et al. 2013). Many studies have considered the effects of individual components of climate change on plants (Qaderi and Reid 2009), but very few have assessed the interactive effects of stress factors, such as higher temperature and water stress, with factors, such as ECO_2, which may alleviate stress on plants (e.g., Zinta et al. 2014). It is, therefore, important to investigate the interactive effects of climate change components on plants, since a comprehensive study considering many aspects of plant responses to multiple factors is needed.

Higher temperatures increased stomatal index, cell area, Chl *a*, carotenoids, ABA, isopentyladenine, and dihydrozeatin riboside, but decreased rosette diameter, leaf number and area, biomass of all plant parts, stomatal and cell density, leaf and soil water potential, leaf moisture content, proline, MDA, electrical conductivity, and ethylene evolution (Table 1). Reduced growth that resulted in decreased biomass of all plant parts under higher temperatures coincides with previous results on other plant species (Qaderi et al. 2015). Decreased stomatal density under higher temperatures may have been an adaptive mechanism to reduce transpiration via stomata and, in turn, might have caused biomass reduction. The size of the individual epidermal cells was significantly larger under higher temperatures, which negatively affects the ability of the epidermal cell to perform mitosis (Qaderi et al. 2013). Despite the higher Chl *a* content under higher temperatures, our plants had lower growth and total biomass (Tables 1, 6). It was suggested that heat stress

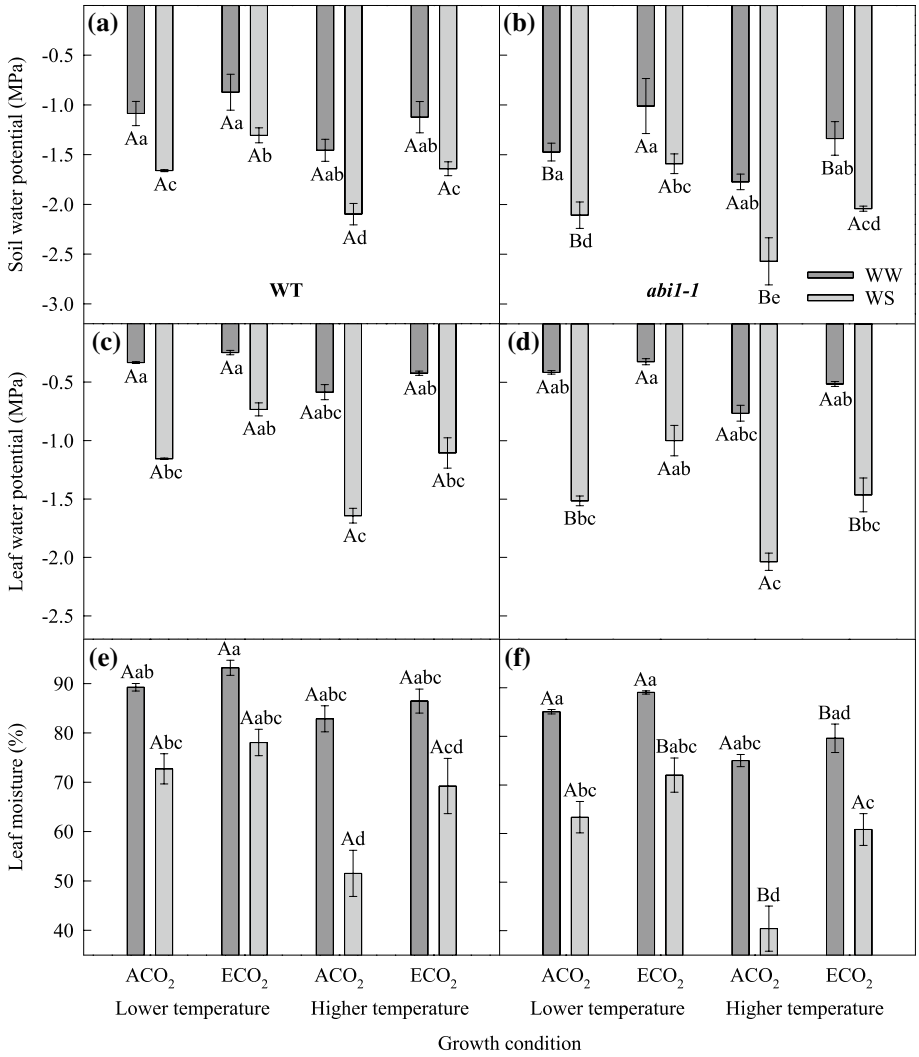

Fig. 5 Effects of temperature, carbon dioxide, and watering regime on water potential and leaf moisture of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**) and *abi1-1* (**b**, **d**, **f**) genotypes were used in this study. Soil water potential (**a**, **b**), leaf water potential (**c**, **d**), and leaf moisture (**e**, **f**). Other details as in Fig. 1

deactivated Rubisco, which would further reduce biomass through reduced photosynthetic capacity (Dutta et al. 2009). The reduction of proline level under higher temperatures (Table 1) is consistent with earlier studies on *Arabidopsis* (Rizhsky et al. 2004). Earlier studies have shown decreased MDA level in the heat-stressed *Arabidopsis* plants (Weber et al. 2004). Changes in electrical conductivity (Table 1, Fig. 7e, f) in the same manner to MDA, in all treatments, suggest that plants grown under higher temperatures acclimated to protect oxidative damage of cell membranes (Zinta et al. 2014). As shown, higher temperature decreases ethylene evolution (Yu et al. 1980), but increases ABA level (Qaderi et al. 2006), which could have negatively affected growth and biomass of plants in this study.

ECO_2 increased rosette diameter, leaf number and area, plant dry mass, cell area, leaf and soil water potential, leaf moisture, and ethylene evolution, but decreased stomatal and cell density, photosynthetic pigments, proline, MDA, electrical conductivity, ABA, total CKs, and *trans*-zeatin riboside (Table 1). It has been well documented that ECO_2 improves growth and biomass of plants through increased leaf photosynthesis rate and water use efficiency, and decreased transpiration (Jones 2013). ECO_2 has also been reported to increase leaf size (Qaderi and Reid 2005). Our results support earlier reports, showing reduced stomatal density (Sekiya and Yano 2008) and cell density (Bray and Reid 2002), and increased epidermal cell area (Bray and Reid 2002) by ECO_2. Reduction of pigments under ECO_2 is in agreement with earlier studies on bull pine (*Pinus ponderosa*; Houpis et al. 1988). Also, the negative correlation of total biomass and total Chl (Table 6) suggests that ECO_2 enables the reallocation of extra nitrogen and other important materials away from the photosynthetic apparatus to other growth-limiting processes, such as antioxidant defense. In this study, ECO_2 decreased proline content, which has been shown to occur in *Betonica officinalis* (Erhardt and Rusterholz 1997). Plants at ECO_2 had less MDA and consequently lower electrical conductivity, as ECO_2 mitigates oxidative stress induced by abiotic factors (Yan et al. 2010). As shown, ECO_2 increases ethylene evolution by enhancing

Table 4 Summary of split–split-split-plot ANOVA (*F* value) for effects of temperature, carbon dioxide, watering regime, and genotype on photosynthetic pigments and chemical stress indicators of *Arabidopsis thaliana*

Source	*df*	Photosynthetic pigment					Chemical stress indicator		
		Chl *a*	Chl *b*	Carotenoids	Total Chl	Chl *a*:*b* ratio	Proline	MDA	EC
Temperature (*T*)	1	2.57	4.65	5.39	2.12	3.97	0.87	5.06	1.22
Main plot error	2	–	–	–	–		–	–	–
Carbon dioxide (C)	1	4.82	14.56	18.07	114.56**	620.30**	13.40	25.83*	12.37
T×C	1	13.43	2.96	2.89	34.51*	13.27	1.26	1.96	1.74
Subplot error	2	–	–	–	–		–	–	–
Watering regime (W)	1	3.23	102.93***	144.46***	0.21	110.18***	12.11*	172.34***	10.82*
T×W	1	5.94	9.00*	10.71*	4.36	12.73*	0.86	6.81	1.22
C×W	1	27.43**	224.98***	300.09****	5.58	34.36**	11.32*	323.42****	10.21*
T×C×W	1	5.76	9.35*	11.20*	4.12	11.07*	0.93	7.36	1.29
Split-subplot error	4	–	–	–	–		–	–	–
Genotype (G)	1	39.59***	267.13****	285.32****	10.47*	25.85**	12.26**	421.3****	11.61**
T×G	1	7.09*	15.59**	14.98**	5.07	8.28*	1.39	20.77**	1.86
C×G	1	2.92	29.56***	38.12***	14.45**	192.08****	13.05**	79.37****	12.14**
T×C×G	1	5.47*	10.45*	9.85*	4.18	8.67*	1.76	13.40**	2.25
W×G	1	5.50*	121.67****	136.34****	0.08	90.31****	12.84**	221.68****	12.05**
T×W×G	1	7.05*	16.03**	15.47**	4.92	7.34*	1.46	21.32**	1.93
C×W×G	1	30.72***	0.00	0.00	52.42****	332.20****	13.39**	1.57	12.34**
T×C×W×G	1	4.84	11.10*	10.67*	3.30	5.44*	1.97	14.64**	2.48
Split-split-subplot error	8	–	–	–	–	–	–	–	–

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C. Experiments were conducted three times

Chl chlorophyll, *EC* electrical conductivity, *MDA* malondialdehyde

Significance values: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$

the production of the enzyme that converts 1-amino-cyclopropane-l-carboxylate (ACC) precursor to ethylene, and by activating this enzyme as well (Sisler and Wood 1988). In this study, a negative correlation between ABA and rosette diameter was observed (Table 6). ABA reduction under ECO_2 is interesting because it could mean that ECO_2 helps the *Arabidopsis* plants to accumulate more biomass by inhibiting the growth-inhibiting factor, ABA. Importantly, ECO_2 decreased the expression of the two ABA-responsive genes, *RD22* and *RD29B* (see Fig. 9, C7–C8), without affecting ABA (see Fig. 8c, d); this indicates that there is another mechanism by which ECO_2 affects the level of expression of the two genes. Decreased number of induced or repressed genes in plants grown under stress factors with ECO_2 has previously been reported (Gillespie et al. 2011).

Water stress increased stomatal and cell density, total Chl, proline, MDA, electrical conductivity, ABA, isopentyladenine, and dihydrozeatin riboside, but decreased leaf number and area, plant dry mass, stomatal index, cell area, leaf and soil water potential, leaf moisture, and ethylene evolution (Table 1). Effects of water stress on growth and biomass are consistent with findings on canola and other species (Qaderi et al. 2006). As shown, increased stomatal density due to water stress has previously been reported (Heckenberger et al. 1998). Limin et al. (2007) reported that increased stomatal density has a positive effect on water use efficiency. However, the observed negative correlation between biomass and stomatal density (Table 6) indicates that increased transpiration might have negatively affected the plant ability to efficiently perform photosynthesis. Despite having higher photosynthetic pigments, water-stressed plants had lower growth and biomass. Reduced water availability triggers the closure of stomata, negatively affecting gas exchange (Jones 2013) and, in turn, plant growth. Water stress increased proline, which has been proposed to protect antioxidant enzymes and plasma membranes (Hare and Cress 1997) by stabilizing different antioxidant enzymes, such as superoxide dismutase (Miller et al. 2010). Since water stress increases ROS and its associated membrane injury (Zhu et al. 2007), it might have led to increased MDA and electrical conductivity in tested plants. As shown, ABA accumulation under water stress prevents excess ethylene production and, in turn, maintains growth of shoot and root (Sharp

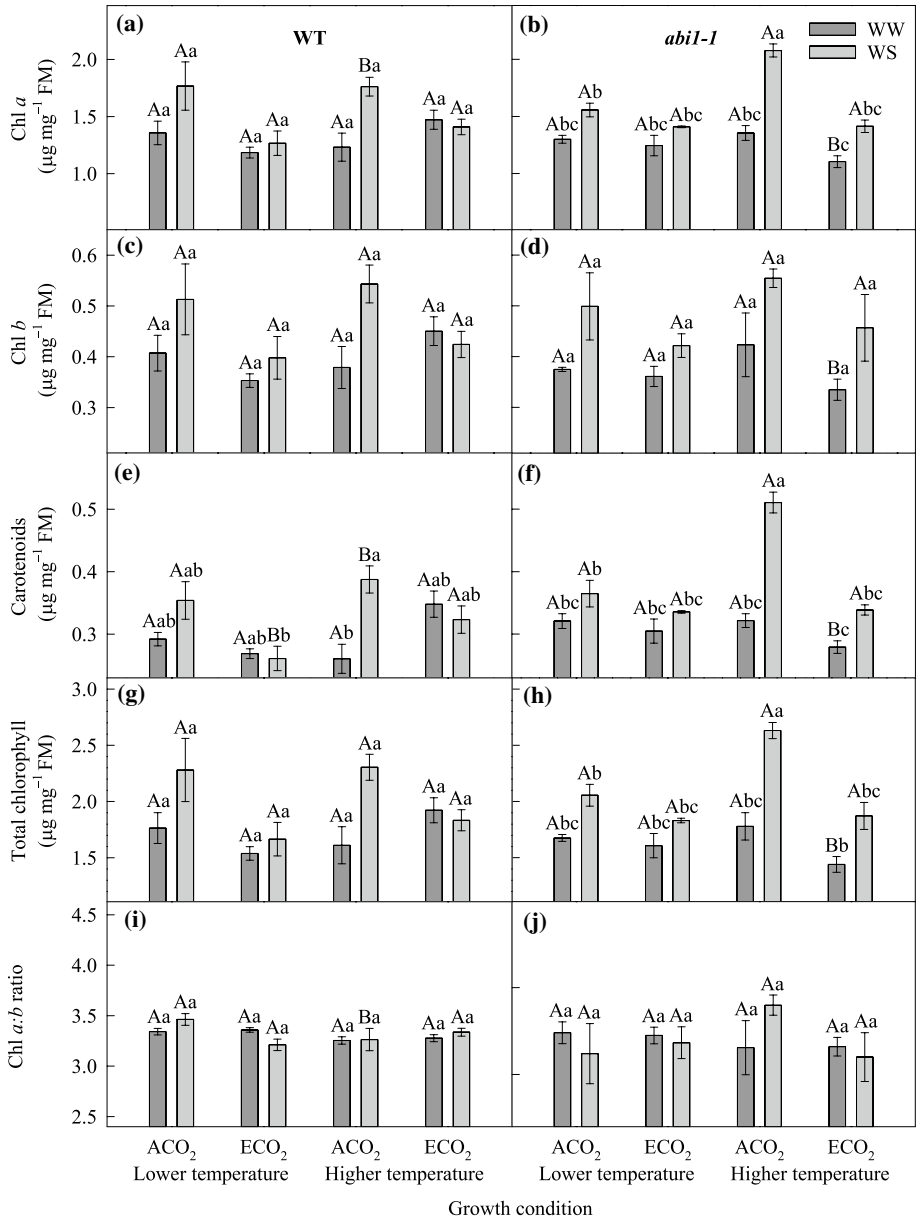

Fig. 6 Effects of temperature, carbon dioxide, and watering regime on photosynthetic pigments of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**, **g**, **i**) and *abi1-1* (**b**, **d**, **f**, **h**, **j**) genotypes were used in this study. Chlorophyll *a* (**a**, **b**), chlorophyll *b* (**c**, **d**), carotenoids (**e**, **f**), total chlorophyll (**g**, **h**), and chlorophyll *a:b* ratio (**i**, **j**). Other details as in Fig. 1

and LeNoble 2002). However, ethylene has been found to be involved in regulating ROS accumulation induced by water stress (Cui et al. 2015). Carotenoids work as non-enzymatic plant antioxidants (Reddy et al. 2004), which could have responded to higher temperatures and water stress as their levels increased in plants grown under these conditions. The CKs isopentyladenine and dihydrozeatin riboside increased in water-stressed plants (Table 1). Higher concentrations of the dihydrozeatin riboside (De Meutter et al. 2003) and isopentyladenine (Piñero et al. 2014) were also detected in the water-stressed and salt-stressed plants, respectively. As expected, the two genes—*RD22* and *RD29B*—were up regulated under water stress because of the high level of ABA under this condition (Fig. 9). Activation of these genes by ABA occurs through a specific group of transcription factors, which binds to specific *cis*-regulatory elements located in their promoters (Lenka et al. 2009).

In this study, the WT plants performed better than the *abi1-1* plants under the experimental conditions. The WT plants had a higher rosette diameter, leaf number and area, total dry mass, cell area, leaf and soil water potential, leaf moisture, proline, ethylene, *trans*-zeatin, isopentyladenine, and *cis*-zeatin riboside, but lower stomatal and cell density, MDA, electrical conductivity, ABA, and dihydrozeatin riboside than the *abi1-1* plants. These results suggest differences

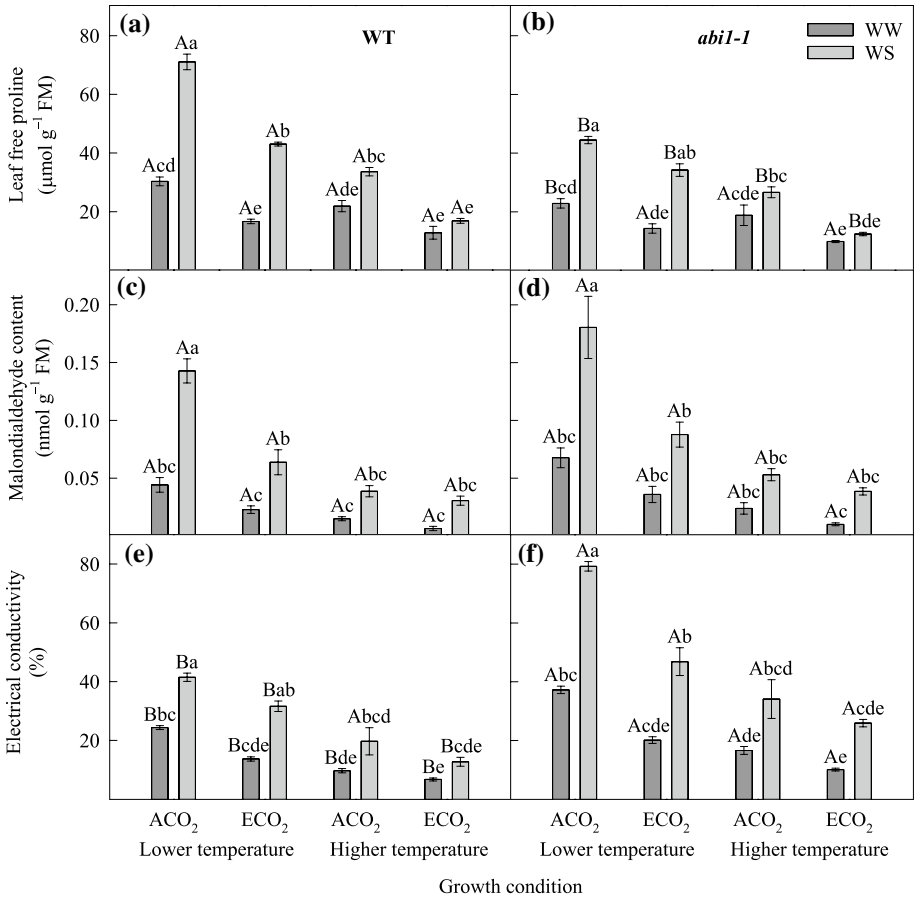

Fig. 7 Effects of temperature, carbon dioxide, and watering regime on leaf free proline, malondialdehyde, and electrical conductivity of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**) and *abi1-1* (**b**, **d**, **f**) genotypes were used in this study. Leaf free proline (**a**, **b**), malondialdehyde content (**c**, **d**), and electrical conductivity (**e**, **f**). Other details as in Fig. 1

between genotypes due to genetic background and varied levels of tolerance and sensitivity to stress factors. ABA-signalling mutants in the protein phosphatases 2C, such as *abi1-1*, show diminished ability to close stomata and, in turn, to tolerate heat (Larkindale and Knight 2002) and water stress (Christmann et al. 2007) by increasing transpiration and decreasing their growth and development. The *abi1-1* plants also had significantly higher carotenoid content than the WT plants (Table 1). A positive correlation between ABA and carotenoids (Table 6) supports an earlier study, which reported that ABA is synthesized from phytoene, a carotenoid produced from pyruvate and glyceraldehydes-3-phosphate (Cutler and Krochko 1999). Interestingly, the *abi1-1* plants had significantly lower contents of *trans*-zeatin, isopentyladenine, and *cis*-zeatin riboside, but higher dihydrozeatin riboside, than the WT plants (Table 1), and this might be an adaptive mechanism to enhance their ability to tolerate stress. Also, dihydrozeatin forms of CKs, such as dihydrozeatin riboside, are more resistant to the CK degradation enzymes and this can be the reason why they were not decreasing as fast as the other CK fractions did under water stress and higher temperatures.

In the current study, a pattern of responses to the interactive effects of temperature, CO_2, watering regime, and genotype was found. For example, root biomass was largest in the well-watered plants under lower temperatures, but smallest in the water-stressed plants under higher temperatures. Rosette diameter, leaf number, and dry mass increased at ECO_2 regardless of the watering regime. Increased plant growth and biomass at ECO_2 indicates that ECO_2 provides more building material for plant growth. Plant biomass was lowest in the *abi1-1* plants under higher temperatures, but highest in the WT plants under lower temperatures. Also, the water-stressed *abi1-1* plants had lowest growth and biomass, whereas the well-watered WT plants had highest biomass. It is not surprising to see these variations in growth and biomass between genotypes, as the outcome is due to inability of the *abi1-1* plants to close stomata under stress conditions. Moreover, reduced rosette diameter, leaf number, and higher stomatal density in the *abi1-1* plants caused them to have the lowest biomass and growth rate. The well-watered plants grown under lower temperatures at ECO_2 had highest root biomass, whereas the water-stressed plants grown under higher temperatures at ACO_2 had lowest root biomass. Root

Table 5 Summary of split–split-split-plot ANOVA (*F* value) for effects of temperature, carbon dioxide, watering regime, and genotype on leaf hormones of *Arabidopsis thaliana*

Source	*df*	Ethylene	ABA	Total CKs	tZ	iP	tZR	cisZR	DHZR	iPR
Temperature (*T*)	1	2.95	0.51	0.01	1.73	2.54	497.89**	0.36	1.22	0.30
Main plot error	2	–	–	–	–	–	–	–	–	–
Carbon dioxide (C)	1	36.44*	2.28	76.08*	4.21	1394.23***	110.79**	3.21	2.35	10.09
T×C	1	2.81	0.53	0.00	0.20	546.82**	8.89	0.54	1.90	0.53
Subplot error	2	–	–	–	–	–	–	–	–	–
Watering regime (W)	1	51.19**	1.85	41.57**	1.74	0.83	403.76****	2.58	0.77	8.29*
T×W	1	2.83	0.52	0.02	0.89	4.97	26.38**	0.39	1.37	0.29
C×W	1	50.34**	1.82	40.99**	0.10	1.90	281.27****	1.99	0.27	7.48
T×C×W	1	3.07	0.49	0.00	0.81	4.72	28.66**	0.41	1.32	0.33
Split-subplot error	4	–	–	–	–	–	–	–	–	–
Genotype (G)	1	33.67***	2.55	106.42****	0.20	4.00	98.93****	1.96	0.31	8.58*
T×G	1	2.30	0.65	0.00	1.92	1.46	36.30***	0.85	0.57	0.69
C×G	1	32.29***	2.45	101.50****	9.37*	14.63**	185.99****	3.05	1.94	9.80*
T×C×G	1	3.12	0.48	0.12	1.76	1.05	40.80***	0.94	0.46	0.92
W×G	1	33.49***	2.53	105.57****	1.70	0.81	140.78****	2.51	0.96	9.31*
T×W×G	1	2.46	0.61	0.00	1.84	1.35	36.63***	0.86	0.53	0.73
C×W×G	1	31.41***	2.38	98.44****	22.96**	44.65***	239.47****	3.68	3.27	10.40*
T×C×W×G	1	3.61	0.38	0.15	1.49	0.20	42.87***	0.99	0.31	1.03
Split–split–subplot error	8	–	–	–	–	–	–	–	–	–

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C. For ABA and CKs, three replications for each treatment and for ethylene nine replications for each treatment of three trials were analyzed. The CKs detected in the leaves of the WT and *abi1-1* mutant plants were: free bases—tZ (*trans*-zeatin) and iP (isopentyladenine); and ribosides—tZR (*trans*-zeatin riboside), cisZR (*cis*-zeatin riboside), DHZR (dihydrozeatin riboside), and iPR (isopentenyladenosine riboside)

Significance values: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$

and leaf biomass accumulation was decreased by higher temperatures and water stress, individually and together, but the negative effects of these two factors were lower in plants at ECO_2. The well-watered WT plants under lower temperatures at ECO_2 had 144.1, 93.9, and 106.5 times higher root, leaf, and total dry mass, respectively, than the water-stressed *abi1-1* plants grown under higher temperatures at ACO_2. Also, these interactions revealed that leaf water potential was highest in the well-watered WT plants under lower temperatures at ECO_2, but lowest in the water-stressed *abi1-1* plants under higher temperatures at ACO_2, with about three times difference between the two genotypes. Increased carotenoids under higher temperatures and water stress, individually and together, revealed increased antioxidants. Since higher temperatures and water stress increase the level of ROS in leaves, increased carotenoids likely protect plants against oxidative damage through xanthophyll cycle (Jones 2013). It seems, therefore, that ECO_2 mitigated some of the negative effects of water stress and higher temperatures on plants. The effects of combined heat and water stress on *Arabidopsis* were mitigated by ECO_2 at multiple organizational levels (Zinta et al. 2014). Lipid peroxidation was about 28-fold higher in the water-stressed *abi1-1* plants under lower temperatures at ACO_2 than in the well-watered WT plants under higher temperatures at ECO_2. Alteration in lipid composition has been shown to provide protection for plasma membrane against different stress factors (Burgos et al. 2011). It has been reported that the total antioxidant capacity was considerably improved by the interaction of heat and water stress, and to a higher degree at ECO_2 (Zinta et al. 2014).

In the WT *Arabidopsis* plants (Fig. 8c), our result supports a previous study using canola (*Brassica napus*), which showed that water stress increased ABA, but higher temperatures inhibited the synthesis of ABA, regardless of the watering regime (Qaderi et al. 2006). The inhibition of ABA production in response to the combination of water stress and higher temperatures could be of considerable importance as it could have negative effects on the plant ability to close stomata and prevent transpiration and, in turn, plant biomass (Qaderi et al. 2006). On the

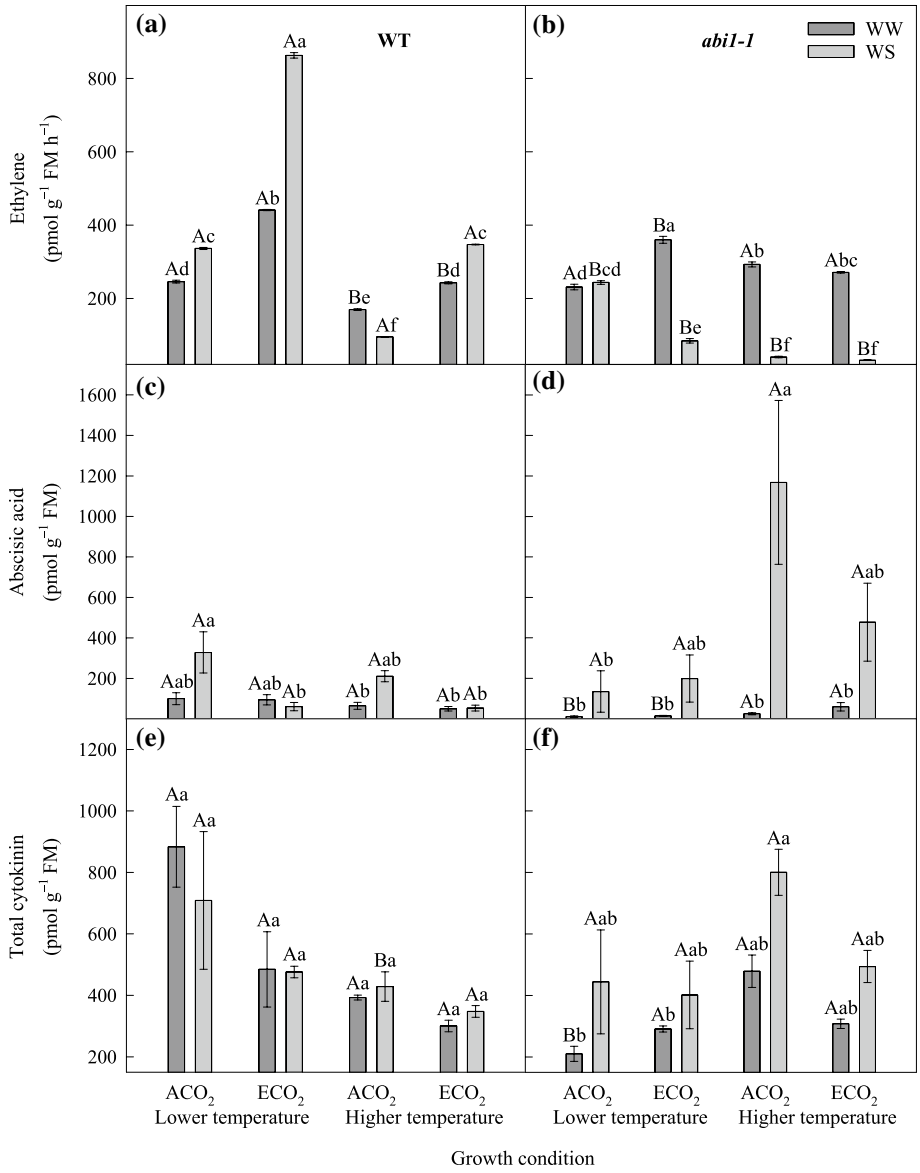

Fig. 8 Effects of temperature, carbon dioxide, and the watering regime on the leaf hormone contents of 18-day-old *A. thaliana* plants. The WT (**a**, **c**, **e**) and *abi1-1* (**b**, **d**, **f**) genotypes were used in this study. Ethylene (**a**, **b**), abscisic acid (**c**, **d**), and total cytokinins (**e**, **f**). Other details as in Fig. 1

other hand, increased accumulation of ABA in the *abi1-1* mutant under the combination of higher temperatures and water stress (Fig. 8d) might be because this genotype does not sense the presence of this stress hormone. The results of this work are consistent with an earlier report in which the *abi1-1* plants accumulated more ABA than the WT plants (Verslues and Bray 2005). Therefore, the use of *abi1-1* mutant confirmed the ability of higher temperatures to block the drought-induced increase of ABA under the combination of these two factors. Overall, ECO_2 could protect plants against oxidative damage, either through enhancing defense mechanisms (Zinta et al. 2014) or decreasing photorespiration (Foyer and Noctor 2009). Our results revealed differences in the expression of the two genes between plants exposed to higher temperatures and water stress at ACO_2 or at ECO_2. This stress-reducing effect of ECO_2 was clearly observed for growth, biomass, and stress indicator parameters, as shown by decreased expression of the two genes; this could mean that plants grown at ECO_2 experienced lower stress level and, therefore, lower expression of these two genes. ABA-responsive genes encode different compounds, such as defensive

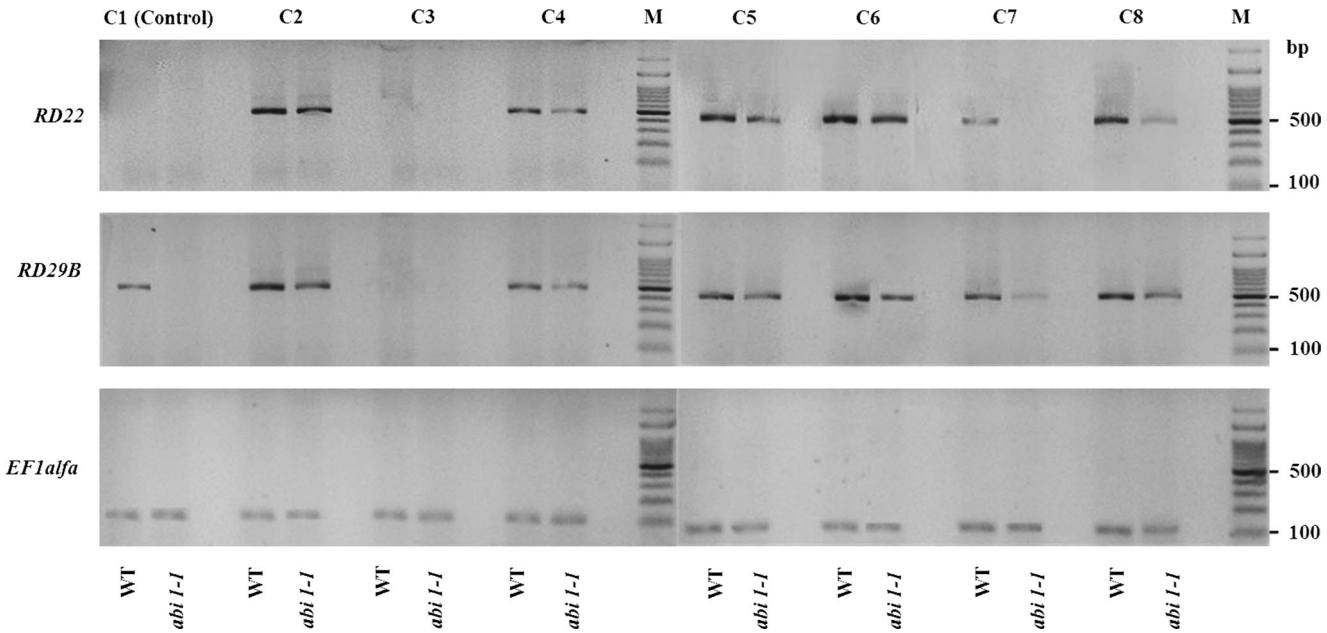

Fig. 9 Effects of temperature, carbon dioxide, and watering regime on the expression of the ABA-responsive genes (*RD22* and *RD29B*) of 18-day-old *A. thaliana* plants. Expression of the *EF1alfa* gene was used as a constitutive internal control. C1 (control): lower temperatures, ACO_2, well-watered, C2: lower temperatures, ACO_2, water-stressed, C3: lower temperatures, ECO_2, well-watered, C4: lower temperatures, ECO_2, water-stressed; C5: higher temperatures, ACO_2, well-watered, C6: higher temperatures, ACO_2, water-stressed, C7: higher temperatures, ECO_2, well-watered, and C8: higher temperatures, ECO_2, water-stressed. Other details as in Fig. 1

proteins, enzymes that help in osmolyte production, and transcription factors for regulation of other gene expression (Bray 2002).

Conclusions

In summary, water stress and ECO_2 have larger effects, negative and positive, respectively, on plants than do higher temperatures. Plants are also affected by the interactions of these factors; interactions between higher temperature and drought can be synergetic in some cases, but antagonistic in others. Overall, both higher temperatures and drought stress increase isopentyladenine, dihydrozeatin riboside and ABA, but decrease leaf number, leaf area, plant dry mass, leaf and soil water potential, leaf moisture, and ethylene evolution. On the other hand, higher temperatures increase stomatal index and cell area, but decrease stomatal and cell density, proline, MDA and electrical conductivity, whereas drought stress has the opposite effects on these parameters. ECO_2 has antagonistic interaction with higher temperature and drought on plant growth and development. ECO_2 reduces stress effects by mitigating oxidative stress and improving water status of plants and helps plants withstand the supra-optimal temperature and limited water conditions, and this reducing effect was consistent across plant parameters. Nevertheless, the negative effects of higher temperatures and water stress enhance adaptive responses in *Arabidopsis*. In this study, differences between the two genotypes in response to multiple factors indicate that higher temperatures and water stress will have strong effects on plant performance, especially on the *abi1-1* plants. Higher temperatures have antagonistic effects on drought regarding the ability of WT plants to accumulate ABA. This negatively affects plant performance under water stress conditions as ABA is involved in stomatal closure in response to drought. Genetic engineering can be used to develop plant cultivars with increased ABA production to withstand such stress conditions. Findings from this study can be extrapolated to other plant

Table 6 Pearson's correlation coefficient between morphological, anatomical, physiological, and hormonal parameters of *Arabidopsis thaliana*

	Parameter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Rosette diameter	–															
2	Leaf number	− 0.261	–														
3	Leaf area	0.936***	− 0.225	–													
4	Root mass	0.908***	− 0.192	0.920***	–												
5	Leaf mass	0.905***	− 0.215	0.940***	0.964***	–											
6	Total biomass	0.913***	− 0.209	0.940***	0.984***	0.996***	–										
7	Stomatal density	− 0.420	0.369	− 0.370	− 0.430	− 0.475	− 0.464	–									
8	Cell area	0.158	− 0.414	0.109	0.202	0.282	0.259	− 0.824***	–								
9	Leaf Ψ	− 0.168	0.859***	− 0.136	− 0.069	− 0.122	− 0.112	0.374	− 0.365	–							
10	Soil Ψ	− 0.058	0.794***	− 0.040	0.047	0.051	0.053	0.190	− 0.187	0.860***	–						
11	Leaf moisture	0.791***	− 0.149	0.814***	0.727***	0.735***	0.738***	− 0.476	0.226	− 0.139	− 0.015	–					
12	Carotenoids	− 0.564***	0.088	− 0.502***	− 0.454**	− 0.441**	− 0.449**	0.583*	− 0.239	0.069	0.021	− 0.713***	–				
13	Total chlorophyll	− 0.606***	0.361*	− 0.531***	− 0.469**	− 0.457**	− 0.465**	0.597*	− 0.323	0.293*	0.314*	− 0.645***	0.886***	–			
14	Malondialdehyde	− 0.375**	0.459**	− 0.347*	− 0.344*	− 0.374**	− 0.367*	0.793***	− 0.618*	0.457**	0.256	− 0.261	0.235	0.334*	–		
15	EC	− 0.409**	0.263	− 0.367*	− 0.370*	− 0.401**	− 0.394**	0.777***	− 0.603*	0.309*	0.122	− 0.298*	0.334*	0.352*	0.898***	–	
16	Abscisic acid	− 0.593*	0.107	− 0.548*	− 0.385	− 0.427	− 0.417	0.387	− 0.189	0.058	− 0.099	− 0.729**	0.740**	0.669**	0.107	0.142	–

A. thaliana plants (WT and *abi1-1* mutant) were grown under two temperature regimes (22/18 °C and 28/24 °C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 µmol mol^{-1}), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers for 10 days, after 8 days of initial growth under 22/18 °C

Ψ water potential, *EC* electrical conductivity

Significance values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

species with similar characteristics, such as canola; both *Arabidopsis* and canola belong to the family Brassicaceae.

Author contribution statement MMQ planned and designed the research. MIAG performed the research. SLS helped with molecular experiments, ECY with anatomical work, and AK and RJNE with hormone analysis. MIAG and MMQ analyzed the data and wrote the manuscript in collaboration with other co-authors. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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