Interactive Effects of Main Climate Change Components on Growth and Development of *Arabidopsis Thaliana*

by

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DEDICATION

I am dedicating this thesis to three beloved people who have meant and continue to mean so much to me. My beloved parents, Ibrahim Abo Gamar and Maha Mousa Suleiman, and my beloved wife, Annam Moh'd Said Qaisi.

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Abstract

Higher temperature and elevated carbon dioxide (CO_2) concentration are expected to cause changes in global precipitation patterns and thus increased water stress events in soils. Few studies have reported the interaction between plants and the main three components of climate change (high temperature, elevated CO_2, water stress). In the present study the abscisic acid (ABA)-insensitive mutant (*abi1-1*) and its associated wild-type (WT) of *Arabidopsis thaliana* (Arabidopsis) were used to determine the effects of these three factors on Arabidopsis during vegetative and reproductive stages. Arabidopsis plants were grown 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 – watering to field capacity; and water-stressed – watering at wilting point). I found that the negative effects of higher temperatures and water stress on Arabidopsis plants during their vegetative stage were mitigated by elevated CO_2 by increasing antioxidant activity and plant water status. Also, the WT plants had better performance than the *abi1-1* plants under our experimental conditions, and even under the optimal conditions for plants (lower temperatures, elevated CO_2, and well-watered), suggesting the role of ABA in mitigating the negative effects of stress factors by elevated CO_2 especially in the *abi1-1* genotype. Higher temperatures inhibited the ability of wild-type plants to produce ABA in response to drought. Elevated CO_2 decreased the expression of the two ABA-responsive genes, *RD22* and *RD29B*, without affecting ABA. Higher temperatures and water stress, individually and together, led to decreased growth, yield, and seed quality. Elevated CO_2, in general, did not compensate for the negative effects of higher temperatures and water stress on the measured parameters, such as sound-seed number and seed mass. Elevated CO_2 seems to have more positive effects on the biomass of the reproductive structures (siliques) than on the seed production or biomass. Amino acid contents of Arabidopsis seeds were positively affected by higher temperatures, elevated CO_2 or water stress; with highest effects of higher temperatures. This could compensate for the negative effects of higher temperatures and water stress and no effects of elevated CO_2, respectively, on seed mass, and consequently, on seed quality. Seeds that matured under higher temperatures could have less dormancy and exhibit more germiniability than those that matured under lower temperatures. This study showed that the climate-change related factors would have an effect on the fate of seeds after dispersal. Climate change seems likely to have little effects on the germinability of WT seeds, but can decrease germiniability of *abi1-1* seeds through changing their phenolics and other such compounds. Therefore, this study indicates that there are factors other than ABA that are involved in controlling seed germination in response to climatic factor. Based on these findings, rising CO_2 will have more positive effects on plants at the vegetative stage than at the reproductive stage. In addition, ABA is important to help plants survive under stressful growth conditions, but it seems that under climate change other factors would also affect the plant survival through, for example, changing seed germiability.

Keywords: Abscisic acid, Amino acid, *Arabidopsis thaliana*, Carbon dioxide, Climate change, Gene expression, Genotype, Growth and development, Phenolic compounds, Reproductive yield, Seed germination, Seed production, Seed quality, Temperature, Water stress.

List of Abbreviations and Symbols Used

ABA	Abscisic Acid
ACO_2	Ambient CO_2
ANOVA	Analysis of variance
CA	Cell area
CD	Cell density
CFCs	Chlorofluorocarbons
Chl	Chlorophyll
cisZR	Cis-zeatin riboside
CO_2	Carbon dioxide
CKs	Cytokinins
^oC	Degree Celsius
DHZR	Dihydrozeatin riboside
DW	Dry weight
EC	Electrical conductivity
ECO_2	Elevated CO_2
FM	Fresh mass
G	Genotype
g	Gram
GC-MS	Gas chromatography mass spectrometry
h	Hour
HFC	Hydrofluorocarbons
HT	Higher temperatures
IAA	Indole-3-acetic acid
Ip	Isopentyladenine
IPCC	Intergovernmental Panel on Climate Change
iPR	Isopentenyladenosine riboside
LMC	Leaf moisture content
LT	Lower temperatures
MDA	Malondialdehyde
mg	Milligram
MPa	Water potential Mega Pascal
mm	Millimeter
µl	Microliter
MS	Murashige and Skoog medium
N_2O	Nitrous oxide
RH	Relative humidity
ROS	Reactive oxygen species
s	Second
SD	Stomatal density

SE	Standard error
SI	Stomatal index
T	Temperature
TBA	2-thiobarbituric acid
tZ	TransZeatin
tZR,	Trans-zeatin riboside
UV	Ultraviolet
W	Watering regime
WS	Water-stressed
WT	Wild-type
WUE	Water use efficiency
WW	Well-watered

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CHAPTER 1

Introduction

1.1 Climate Change

The term climate change refers to "any significant change in the measures of climate, such as changes in temperature, precipitation, wind patterns, lasting for an extended period" (Huntingford *et al.,* 2013). Before the Industrial Revolution (1750), anthropogenic actions, such as deforestation and destruction of the natural landscape had limited impact on living organisms that could be regional or in some cases continental (Schroeder & Castello, 2010). However, after the Industrial Revolution, human effects became global because of the anthropogenic actions (Schroeder & Castello, 2010). It is expected that the global population will reach nine billion by 2050, which will require a 70% increase in crop production to meet demand (Bita & Gerats, 2013). Climate change affects plants at the level of molecular function, developmental processes, morphological traits, and physiological parameters (Gray & Brady, 2016). Due to the stressors brought on by climate change, such as temperature increase and reduction in soil moisture, plants must adapt their growth, development, and hormone levels to different stress factors to mitigate negative effects (Moretti *et al.,* 2010).

Greenhouse gases are emitted in significant amounts from different sources, such as the burning of fossil fuels for energy generation, industrial processes, and agricultural practices. These gases trap long-wave infrared radiation around the earth and re-emit it into the atmosphere – a phenomenon called the greenhouse effect (Titus, 1990; Hatfield, 1993). Although the greenhouse effect is necessary to sustain life on earth, an excessive buildup of gases leads to continuously increasing temperature, which significantly changes the ecosystem (Hoegh-Guldberg & Bruno, 2010). Most of the greenhouse gases are natural compounds, such as carbon

dioxide (CO_2), methane (CH_4), water vapour, and nitrous oxide (N_2O) (Montzka *et al.,* 2011). Anthropogenic activities also contribute to the production of these gases. For example, agriculture is responsible for 25% of carbon (mostly from deforestation), 50% of methane, and 75% of N_2O emitted annually (IPCC, 2013). Carbon dioxide enters the atmosphere by fossil fuel combustion and industry, such as cement manufacture (Ebi, 2006), deforestation, land use changes, and burning fossil fuels (Crate & Nuttall, 2016). Natural processes, such as respiration and volcano eruptions (Crate & Nuttall, 2016), also release carbon dioxide. Methane, on the other hand, is emitted by the burning of oil and coal and by seeping of natural gas reservoirs. Livestock, agricultural practices, such as rice production, and decay of organic compounds also play a significant role in the emission of CH_4 (Howarth *et al.,* 2011). Furthermore, through anthropogenic activities, many synthetic fluorinated greenhouse gases, including chlorofluorocarbons (CFCs), perfluorocarbons (PFCs), hydrofluorocarbons (HFCs) and sulfur hexafluoride (SF6) have been emitted to the atmosphere (Li *et al.,* 2014).

King (2013) reported that carbon dioxide emissions in the 21st century would lead to climate change effects on earth on both short and long-time scales that would be irreversible. This irreversibility is reasoned to the great level of inertia or saturation of the oceans and other climate control systems (Solomon *et al.,* 2009), and to the current-slow interaction between climate control system and environmental changes (Caldeira *et al.,* 2003). Ice sheet collapse is a possible irreversible climate change, but it is still highly uncertain, while another important irreversible event to climate change, such as sea level rise and precipitation changes, have been already assured (Solomon *et al.,* 2009). Greenhouse gasses persist in the environment for different time periods; CH_4 persists in the atmosphere up to 12 years (Kirschke *et al.,* 2013), while CO_2 for many hundreds of years or longer (Myhre *et al.,* 2013). Therefore, it seems that reversing the

climate change process and its negative impacts on earth and bringing the earth back to the pre-industrial era appears to be a practically impossible mission. Because of this, there is a serious concern within the academic community about the consequences of the continuous increase in the concentration of CO_2 and other greenhouse gases, and how to reduce their emission.

1.2 Elevated Atmospheric Carbon Dioxide

The carbon dioxide concentration of the earth's atmosphere has varied throughout geologic time. During ice ages, the CO_2 concentration was ~200 µmol mol^{-1}, and during the warmer interglacial periods, it reached to ~280 µmol mol^{-1} (Cook *et al.,* 2013). Since the Industrial Revolution, this concentration increased by more than a third by anthropogenic activities (Crate & Nuttall, 2016). Ice core data since 1700 A.D. and direct atmospheric sampling data since 1958 showed that the carbon dioxide concentration increased to 315 µmol mol^{-1} by 1958 and to about 355 µmol mol^{-1} by 1990 (Keeling *et al.,* 1989). The rate of increase of atmospheric carbon dioxide is about 0.5 percent per year, which means that the change is accelerating. In 2013, CO_2 concentrations exceeded 400 µmol mol^{-1} since measurements began in 1958. Ancient air bubbles trapped in ice cores were taken from a part of Antarctica known as Allan Hills showed that the current CO_2 concentration in the atmosphere is higher than it was at any time in the past 450,000 and 800,000 years (Higgins *et al.,* 2015). This happened because of the failure or delay of many countries to adopt methods to reduce greenhouse gas emissions, which caused a continuous and rapid increase in atmospheric carbon concentration to reach the current concentration (King, 2013). In addition, CO_2 anthropogenic emission without land use change, such as clearing or planting forests, is 30 billion tons/year (Cawley, 2011). This caused the concentration of CO_2 in the atmosphere to increase at ~ 2 µmol mol^{-1}/year, or ~15 billion tons/year (NOAA, 2017).

Therefore, the current CO_2 concentration (406.99 μmol mol^{-1}) is expected to reach 700-1000 μmol mol^{-1} by 2100 (IPCC, 2014). It has been argued that the increase in the atmospheric CO_2 concentration happened because of natural processes, such as changes in ocean carbonate chemistry (Broecker *et al.,* 1999) and natural loss of terrestrial biomass (Indermuhle *et al.,* 1999). However, the isotopic observations confirmed that the increase in atmospheric CO_2 comes from the anthropogenic activities, not from natural processes (Levin & Hesshaimer, 2000). Moreover, the current continuous elevation in CO_2 concentration displays a strong association with fossil fuel burning based on the simple foundation that ~60% of fossil fuel emissions remain in the atmosphere (NOAA, 2017).

The continuous increase in the CO_2 concentration has many negative consequences, such as ocean acidification, decreased atmospheric O_2 and increased earth temperature (Cawley, 2011). Oceans, which are the largest carbon dioxide absorbers, have reached their maximum absorption capacity, and that caused more greenhouse gases accumulation in the atmosphere escorted by continuous changes in the climate (Khatiwala *et al.,* 2009). Additionally, some of the terrestrial and marine species may face extinction because of their mild susceptibility or inability to tolerate these changes in the surrounding environment (McNutt, 2013). In addition, changes in the environment brought by the climate change may also occur to quickly for many species to adapt or to tolerate. Even so, other species that might tolerate the new changes could nonetheless decline because of the destruction or collapse of their original habitats (McNutt, 2013). Accumulation of CO_2 in the atmosphere, because of human activities, increases the retention of heat in the atmosphere, causing global warming leading to a range of damaging impacts in different regions and sectors (Cline, 1991).

1.3 Increased Air Temperature

The "greenhouse effect" is the term used to describe the retention of heat in the Earth's lower atmosphere (troposphere) due to the high concentration of carbon dioxide, methane, and other greenhouse gases (Anenberg *et al.,* 2010). The greenhouse effect itself happens when short-wave infrared radiation, which is not blocked by the greenhouse gases, heats the earth surface, and then the energy is reemitted back through the earth's atmosphere as long-wave, with a longer wavelength. In the wavelengths 5-30 µm lots of this thermal radiation is absorbed by water vapour and CO_2, which in turn radiate it toward the earth surface, therefore heating the atmosphere and earth surface to make it fit to live in (West, 2006). Without this natural greenhouse effect, the average surface temperature of the earth would decrease to -18°C at night, which is about the same as on the moon (Ravishankara *et al.,* 2009). Increased concentration of CO_2 and other greenhouse gases means that heat will take longer time to be emitted to space from the earth lower atmosphere, and thus temperature at the earth's surface is likely to increase. The heat trapped by CO_2 and other greenhouse gases has increased the earth's surface temperature by 0.75°C during the last 100 years (Cawley, 2011). It has been speculated that an increase in the atmospheric CO_2 concentration to 700-1000 µmol mol^{-1} by 2100 can increase the global temperature by 1.1-6.0ºC due to its heat-trapping potential (IPCC, 2013). This change in the global temperature may continue unabated even if the concentrations of greenhouse gases are reduced to zero today. Therefore, temperature of the earth would most likely continue to increase for over thousand years (Solomon *et al.,* 2009). Areas that experience heat stress more readily are those that are quite dry or have limited precipitation throughout the year. These areas, such as the Canadian prairies, are generally in the center of continents and tend to be regions where crops, such as maize, wheat, and canola are grown (Xu *et al.,* 2012). In recent studies, areas such

as the Canadian prairies were listed as regions in which soil moisture is likely to decrease, and where consumptive water use will increase (Liu *et al.,* 2013). In regions of low latitude, it is likely that a modest increase of 1-2ºC would have negative effects on crop yield (DaMatta *et al.,* 2010). This is evident by the 1-1.7% yield loss by every 1°C increase in temperature above 30°C (Lobell *et al.,* 2011), and although cereal yields have recently stabilized, production levels are approximately 25% less than they need to be to meet agricultural demand by 2050 (Mwongera *et al.,* 2014). Additionally, yield reductions of approximately 10% are expected in the future, and will exacerbate food security problems (IPCC, 2013), especially in developing countries where this is already an issue. It has been reported that global warming is harming the environment by causing desertification, melting of snow and ice and consequently sea level rise and increasing the incidence of stronger storms and extreme events (Le Quéré *et al.,* 2012). Moreover, elevated CO_2 concentration and higher temperature together are expected to cause global changes in precipitation patterns and thus increased water stress events in soils (Allison *et al.,* 2009).

1.4 Soil Water Deficit

Scientists have started to talk about the relationship between more intense water-stress events worldwide and climate change (Taub *et al.,* 2008). The main reason is that more greenhouse gases, such as CO_2, methane and water vapour, are released into the atmosphere, causing an increase in the atmosphere temperature, which results in more water evaporating from land and lakes, rivers, and other water bodies (Levin & Hesshaimer, 2000). Warmer temperatures also increase evaporation from soils, which negatively affects plant life by reducing the stomatal conductance and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Cawley, 2011). When rain falls on drought-stricken places, the drier soils are less able to absorb the

water, increasing the occurrence of flooding. With predicted temperature increases and subsequent reduction of precipitation, water stress may occur more frequently in future years (Moretti *et al.,* 2010). As reported, in July 2017, there was a significant variation in the precipitation pattern across the northwestern U.S.A, Brazil, Paraguay, southern Argentina, central and southern Chile, central Asia, and southern Japan. Meanwhile, wetter than normal conditions were notable across the eastern half of the U.S.A, Alaska, northern Argentina, Uruguay, northern parts of Europe, central Russia, northwestern India, northern Japan, and across parts of China (NOAA, 2017). Elevated CO_2, higher temperature, and drought are the main components of climate change that result in physiological, morphological, biochemical, and molecular changes in plants (Salazar-Parra *et al.,* 2012). Therefore, studies on the effects of climate change components on plants are important (Bauweraerts *et al.,* 2013).

1.5 Effects of Atmospheric Carbon Dioxide on Plants

1.5.1 Vegetative Stage

Most studies that were conducted over the last 30 years have shown that plants grown under elevated CO_2 concentrations have significantly more biomass in comparison to those that were grown under ambient CO_2 concentrations. However, this elevation in atmospheric CO_2 concentration has both positive and negative impacts on plants (Qaderi *et al.,* 2006). Elevated CO_2 (e.g. 700 μmol mol^{-1}) increased height, photosynthesis, and biomass of aerial and underground organs of *Chrysolaena obovata* by 40%, 63%, and 47% and 32%, respectively (Oliveira *et al.,* 2010). Elevated CO_2 improves growth and biomass of plants through an increase in photosynthesis rate and water use efficiency (WUE) (Yazaki *et al.,* 2004); Morgan *et al.,* 2011). Moreover, elevated CO_2 decreases transpiration through reducing stomatal conductance

(Long *et al.,* 2004) by decreasing the degree of stomatal opening and stomatal number in order to keep the maximum rates of CO_2 uptake and low rates of transpiration (Taub, 2010). Less oxidative damage (e.g., lipid peroxidation) caused by stress factors under elevated CO_2 has been reported (Zinta *et al.,* 2014). This correlates with a previous report that demonstrated that elevated CO_2 mitigates oxidative stress induced by abiotic factors (Yan *et al.,* 2010). Plants grown under elevated CO_2 concentrations had increased apical meristem growth rate (Teng *et al.,* 2006), because of increased production of phytohormones, such as auxins and gibberellins (Yong *et al.,* 2000). Elevated CO_2 increases leaf photosynthesis by 30%–50% in C_3 plants and 10%–25% in C_4 plants (Tubiello & Ewert, 2002). Moreover, for a wheat (*Triticum aestivum* L.) plant, elevated CO_2 increased total biomass by 8 and 15 % under well-watered and water-stressed conditions, respectively (Tubiello & Ewert, 2002). Increases in above-ground biomass at 550 $\mu mol\ mol^{-1}$ CO_2 for trees are in the range 0–30% with the higher values observed in young trees (Nowak *et al.,* 2004) and little to no response observed in the few experiments conducted in mature natural forests (Norby *et al.,* 2003; Körner *et al.,* 2005). Since plant responses during vegetative stage to elevated CO_2 are not always well correlated with those of reproductive traits (Jablonski, 1997), predicting the responses of plants to future atmospheric CO_2 conditions requires investigation of the effects of CO_2 enrichment throughout a plant's life cycle (Norby *et al*., 2003).

1.5.2 Reproductive Stage

Reproductive characters are key features for predicting the response of plants to atmospheric CO_2. Seed yield can be affected by elevation in CO_2. For example, Kimball *et al.* (2002) reported that yield is increased when plants are grown at elevated CO_2 concentration because of increased

photosynthesis and enhanced vegetative development under optimum growth temperature. Moreover, it has been reported that elevated CO_2 positively affects reproductive characteristics, such as seed size and pollen viability (Prasad *et al.,* 2006). Seed mass is also increased at elevated CO_2 concentrations in canola (*Brassica napus* L.) (Qaderi *et al.,* 2007). With all the previously mentioned positive effects of elevated CO_2 on plant growth and development, some scientists have reported that crop positive response to elevated CO_2 concentration could be lower than previous expectations (Long *et al.,* 2006). In addition, some plant physiologists and modellers see that there is an overestimation for the actual field and farm-level responses to elevated CO_2 in different experimental settings (Tubiello *et al.,* 2015). Flowering time in 40 published studies including both crops and non-crops grown under elevated CO_2 (concentration ranges from 350 to 1000 μmol mol^{-1}) include 28 cases in which flowering time occurred earlier (average 8.6 days), and 12 cases where flowering was delayed (average 5.2 days) (Jagadish *et al.,* 2016). It has also been reported that reproductive features in non-crop and wild species have less response to elevated CO_2 than crop species (Ainsworth & Long, 2005*).* It has been reported that crop plants showed some degree of enhancement in their reproductive features under elevated CO_2, while wild species were evenly distributed between those that were enhanced and those that were inhibited by elevated CO_2 (Jablonski et *al.,* 2002). The allocation of resources in wild species to reproduction was found to decrease 14% at elevated CO_2, which means more carbon allocation to structural, defensive, or other non-reproductive tissues. The increase in yield under elevated CO_2 is dependent on the presence of other important factors, such as nutrients and temperature (Oliveira *et al.,* 2016). Elevated CO_2 concentrations can increase the carbon-to-nitrogen ratio (C/N ratio) (Steinger *et al.,* 2000). This increase in C/N ratio results in a reduction for seed proteins, and consequently a reduction in the proteins essential for embryonic

development in the growing seed (Andalo *et al.,* 1996). At low N, although there was an increase in total crop dry mass in winter wheat (*Triticum aestivum* L. cv. Mercia), elevated CO_2 did not positively affect grain yield and caused a significant decrease under ambient temperature conditions (Mitchell *et al.,* 1993). This can be explained in terms of enhancement of early vegetative growth by elevated CO_2 resulting in a reduction for N available later for grain formation and filling (Mitchell *et al.,* 1993).

1.5.3 Plant Responses to Atmospheric Carbon Dioxide

One of the key factors that affect species response to elevated CO_2 is photosynthetic type. Most plant species (~90%) use a photosynthetic mechanism named C_3 photosynthesis. Other plant species use one of two physiologically different mechanisms called C_4 and crassulacean acid metabolism (CAM) photosynthesis (Taub, 2010). For C_3 plants, the driving force for the growth response to elevated CO_2 is higher leaf CO_2 assimilation rates. C_4 species respond to elevated CO_2 by reducing stomatal conductance, which may result in some indirect improvement of photosynthesis by aiding plants to avoid low water availability under water stress conditions (Leakey, 2009). However, evidence does suggest that C_4 as well as CAM plants are unresponsive or less responsive to elevated CO_2 (Taub, 2010). For example, free-air CO_2 enrichment (FACE) studies on C_4 plants show no increase in yield (Long *et al.*, 2006). Moreover, in FACE experiments, it has been found that the enhancement of photosynthesis by elevated CO_2 in C_4 plants is less than in C_3 plants. C_4 plants have been found to have little or no growth improvement (dry matter production) in some studies (Ainsworth & Long, 2005). There is a very little data available on effects of elevated CO_2 on the concentrations of nitrogen and protein in C_4 plants (Cotrufo *et al.,* 1998).

Carbon dioxide assimilation rates at elevated CO_2 depends on temperature with maximum absolute increases occurring at temperatures that do not cause flower abortion (Conroy *et al.,* 1994). In the absence of temperature increase, many studies have shown that the net effect of doubling of CO_2 was increased yield of rice (Kim *et al.,* 2003). Rice showed 20% decrease in yields in the northwestern India when grown under elevated CO_2 and higher temperature (Lal *et al.,* 1998).

1.6 Effects of High Temperature on Plants

1.6.1 Vegetative Stage

The effect of temperature on plants is a prominent area of study and has particular significance when speaking about agriculture. Heat stress is defined as the increase in temperature above a critical threshold for a period long enough to cause irreversible impairment to plant growth and development (Wahid *et al.,* 2007). Plants experience heat stress when the temperature rises outside the optimal temperature for normal developmental stages to occur (Moretti *et al.,* 2010; Prasch & Sonnewald, 2015). The optimal temperature for plant growth is generally between zero and 40°C (Moretti *et al.,* 2010). Each plant, however, has an optimal range of temperature in which they grow and reproduce most effectively, and produce the most yield (Went, 1953). By increasing the average temperature of the atmosphere, global warming may limit the successful growth and development of individual plant species (Hu *et al.,* 2006; Xu *et al.,* 2012; Craparo *et al.,* 2015). In addition, duration of heat stress most of time caused negative changes in growth and developmental stages of plants (Bita & Gerats, 2013). Dry mass accumulation is considered when assessing the effects of heat stress on plants. Plants facing heat stress show compromised development in the form of shorter and thinner stems, small and thick leaves, and an overall

reduced biomass (Qaderi *et al.,* 2006). There is usually a comparatively more extensive root system in heat-stressed plants (King *et al*., 1997). Overall, dry mass accumulation and shoot to root mass ratio are generally lower in heat-stressed plants than in plants grown in optimal conditions (Qaderi & Reid, 2009*a*).

Higher temperatures cause a reduction in plant biomass by reducing photosynthesis through increasing transpiration and stomatal conductance (Jones, 1992). In addition, higher temperature decreases CO_2 solubility in leaf mesophyll (Jordan & Ogren, 1984). Earlier studies have shown that higher temperatures may lead to a complete loss (Crafts-Brandner & Salvucci, 2000) or reduction in Rubisco catalytic activity by decreasing the enzyme activity of Rubisco activase (Kim & Portis, 2005). The denaturing of Rubisco under high temperatures is also detrimental in relation to reactive oxygen species (ROS). In comparison to C_4 plants, Rubisco in C_3 plants grown under higher temperature tends to fix more O_2 than CO_2 because of the differential effects of temperature on solubility of CO_2 and O_2 and more direct impacts of temperature on Rubisco activity in C_3 plants than in C_4 plants (Brook & Farquhar, 1985). Rubisco is responsible for the control of ROS under lower temperatures. However, during periods of heat stress Rubisco may become compromised, allowing ROS to proliferate and cause damage to the internal membrane and cell structures within the plant, including photosystem II (Qaderi & Reid, 2009*b*).

Moreover, higher temperatures cause structural and functional disruptions of chloroplasts. For example, it induces proton (H^+) leakage through the thylakoid membrane, leading to a loss in the coupling of adenosine triphosphate (ATP) synthesis with electron transport, as well as a reduction in ribulose 1,5 bisphosphate (RuBP) regeneration (Cen & Sage, 2005). It also reduces chlorophyll *a*, chlorophyll *b*, and chlorophyll/carotenoid ratio (Cui *et al.,* 2006). As reported, high temperature damages DNA, inhibits CO_2 assimilation, changes phytohormone

concentration, and the balance between reactive oxygen species (ROS) and antioxidants (Jia *et al.,* 2017). In addition, higher temperature causes injury to the cell membrane and reorganizes microtubules and cytoskeleton, thus it changes the permeability of the membrane and modifies the process of cell differentiation, elongation, and size (Rasheed, 2009). However, regardless of all these negative impacts of heat stress on plants, Fahad *et al.* (2017) reported that the optimum temperature requirements for photosynthesis are expected to rise with elevated concentration of CO_2 in the atmosphere. Studying changes in reproductive features is very important for predicting the response of plants to global warming.

1.6.2 Reproductive Stage

Even though increasing temperature could have some benefits especially for crop production in some cooler regions of the world, it seems that the overall impact on global food security is still negative (Challinor *et al.,* 2014). Rice responses to high temperature depend on the stage of development, with the highest sensitivity noted at the reproductive stage (rang *et al.,* 2011). In Tanzania, Craparo *et al.* (2015) showed that Arabian coffee (*Coffea arabica* L.) yields have severely declined due to temperature increases. As well, mung bean (*Vigna radiata* L.) has been shown to have significant reductions in biomass and yield when exposed to high temperatures (Kaur *et al.,* 2015). Global wheat production has been shown to decrease by 6% for each degree Celsius increase in earth temperature (Asseng *et al.,* 2015). Wheat yield response to temperature is likely to depend on the phenology of the crop and the rate of supply of resources. For instance, high temperature throughout wheat reproductive stage reduces photosynthesis and leaf area, shoot and grain mass in addition to weight and sugar content of kernels (Shah & Paulsen, 2003). High temperature also accelerated wheat and rice development, therefore, grain yield is

decreased since there is less time for radiation interception during the vegetative stage (Conroy *et al.,* 1994). Furthermore, the reductions of wheat yield under higher temperature (Thorne & Wood 1987) is expected to happen because of changes in phenology, which resulted in a decrease in the quantity of photosynthate and nutrients that can be accumulated in grains (Cantero-Martfnez *et al.,* 1995).

It has been reported that the reproductive stage of plants, including flowering and pollen production, is especially sensitive to increased temperature compared to the vegetative stage (Wahid *et al*., 2007). Flowering process in many legumes and cereals has been shown to have a sensitivity to higher temperature (Frank *et al.,* 2009), most probably because of less water and nutrient transport throughout the reproductive stage (Young *et al.,* 2004). Additionally, in many temperate cereal crops, both grain weight and number seem to be negatively affected by higher temperature, with a decrease in grain number directly related to increasing temperature during flowering and grain filling stages (Porter & Semenov, 2005). Increased temperature altered the composition of lipids and proteins within the grain, as well as caused a decrease in several carbohydrates, suggesting an alteration in physiology and nutritional content (Högy *et al.,* 2013). Heat stress during seed development may result in reduced germination and loss of vigor, leading to the reduced emergence and seedling establishment as has been shown for several crop plants (Akman, 2009; Ren *et al.,* 2009). Between 1950 and 2010, the national annual mean surface air temperature for Canada increased by 1.5^{o}C (Vincent *et al.,* 2012), therefore, with such a drastic change in temperature over a short amount of time, plants must use adaptive mechanisms to ensure their survival.

1.6.3 Plant Responses to High Temperature

Responses within the plant to air temperature influence its physiological and morphological characteristics (Qaderi *et al.,* 2012; Xu *et al.,* 2012; Martel & Qaderi, 2016).

Plants mitigate heat stress by bringing physical alterations. The effects of overheating are reduced by rolling or dropping leaves and temporary wilting (Nicotra *et al.,* 2008). These adaptive mechanisms may result in reduced photosynthesis as they aim at blocking out excessive heat as well as incident solar radiation (Hasanuzzaman *et al*., 2013). Arndal *et al.* (2018) found that net root production was higher in ingrowth cores, but fine root number and length in minirhizotrons was lower because of heat stress in a mixed heathland–grassland.

Plants have developed different physiological, biochemical, and molecular mechanisms to respond to increased temperature. Some major ways in which plants adapt for tolerance include production of late embryogenesis abundant proteins, osmoprotectants, antioxidant defense, and factors involved in signaling cascades (Hasanuzzaman *et al*., 2013). Primarily, plants alter their metabolism in order to produce appropriate solutes, which help them manage proteins and cellular structures during heat stress, maintain cell turgidity by osmotic adjustment, and re-establish the redox balance of cells by modifying the antioxidant system and restore cell homeostasis (Janská *et al*., 2010). As the temperature of the Earth rises, water is evaporated more readily from its surface. Due to a decline in soil water availability, many plants are experiencing more water stress than in previous years, and this stress will continue to increase (IPCC, 2013). Research has shown that water stress is one of the most detrimental stressors in relation to plant health (Qaderi *et al.,* 2012).

1.7 Effects of Water Stress on Plants

1.7.1 Vegetative Stage

Plants that grown under water stress display an array of visible symptoms. Plant height, stem diameter, total dry mass, and relative leaf expansion rate and elongation are decreased under water stress (Kirnak *et al.,* 2001). El Neomani *et al.* (1990) found that water stress throughout the rapid vegetative stage limits maize (*Zea mays* L.) growth and development. Pace *et al.* (1999) performed an experiment in Texas to examine how cotton (*Gossypium hirsutum* L.) plants respond to a brief water stress followed by a recovery period and they concluded that water stress significantly decreased the height of the plant, the diameter of the stem, number of nodes, and dry weight of cotton plants. Furthermore, water stress reduced the length and fresh/dry weights of alfalfa (*Medicago sativa* L.) shoots. In contrast, root length was reported to be increased in this experiment (Zeid & Shedeed, 2006). Similarly, water stress induced during the vegetative stage of rice resulted in a great reduction in the growth and development of this crop (Manickavelu *et al.,* 2006). In addition, Okcu *et al.* (2005) reported that water stress negatively affects germination and early growth of seedlings. When higher plants grow under severe water stress, cell elongation is inhibited due to interrupted water flow from xylem to surrounding growing cells in the plant (Nonami, 1998). Furthermore, inadequate water delays mitosis and expansion of cells, thus resulting in plants with reduced height and leaf area under water stress conditions (Hussain *et al.,* 2008). When plants are under water stress, mechanisms, such as leaf wilting help to slow loss of water due to transpiration. Water stress causes cells to lose their turgor pressure (Shinozaki & Yamaguchi-Shinozaki, 1997). Leaf wilting occurs when there is a reduction in the amount of water being supplied to the leaf (Cai *et al.,* 2013). The cells within the

leaf and petiole lose their turgor pressure and become limp; this is correlated with stomatal closure and a reduction in leaf water potential (Maherali *et al.,* 2009).

In extreme cases, the plant may undergo leaf abscission or senescence. The plant gradually builds a layer of suberized tissue where the petiole of the leaf meets the stem. As the layer extends across the base of the petiole, water, minerals, and nutrients are blocked from entering the leaf (Sakamoto *et al.,* 2008), and the leaf begins to die.

An increased production of abscisic acid (ABA) under water stress prompts the guard cells to open efflux ion channels and change their ion concentrations to close stomata (Pantin *et al.,* 2013). This narrows the opening between cells and helps to reduce both the transpiration rate and net carbon dioxide assimilation by limiting the amount of gas exchange allowed between the plant and the surrounding environment and retaining water within the plant (Prasch & Sonnewald, 2015).

The water content decreased by nearly 57% of Barbary fig (*Opuntia ficus-indica* L.), when it was grown under water stress (Nerd & Nobel, 1991). The same parameters of relative water content, turgor potential, stomatal conductance, transpiration, and water-use efficiency were reported to decrease in Chinese hibiscus (*Hibiscus rosa-sinensis* cv.) under water stress (Egilla *et al.,* 2005).

Similar to higher temperature, water stress affects plant growth by enhancing the production of the ROS (Reddy *et al.,* 2004). Water stress induces ROS production and antioxidant activities (Cossu *et al.,* 2014). During periods of drought, the efficiency of Rubisco is severely decreased, and an oxidative burst may occur. A burst act as a secondary messenger for stress metabolite and enzyme production, as ROS can severely damage protein and lipid compounds in the plant. Antioxidant enzymes, such as superoxide dismutase, peroxidase, and catalase, can directly target

ROS. They may also be used to strengthen protein and lipid compounds, such as the cellular membrane, and thereby limit the amount of destruction caused by ROS (Kalantar Ahmadi *et al.,* 2015; Prasch & Sonnewald, 2015). Therefore, water stress affects gas exchange, transpiration, protein production and accumulation of metabolites (Ohashi *et al.,* 2006). In addition, it decreases net CO_2 assimilation rates by reducing stomatal conductance and Rubisco activity (Reddy *et al.,* 2004). Water stress has been reported to have less effect on crops during their vegetative stage than reproductive stage (Fereres *et al.,* 2006).

1.7.2 Reproductive Stage

Water stress may negatively affect global crop productivity more than any other stress factor (Boyer & Westgate, 2004). Maize (*Zea mays* L.) plants were grown in buckets under normal conditions and then water stress around pollination showed formation of embryos, but abortion happened, and kernel number was reduced significantly (Zinselmeier *et al.*, 1999). They also observed that all the intermediates in starch synthesis were depleted and the starch contained in the ovary almost disappeared during this abortion. Similarly, Setter *et al.* (2001) assessed the developments of kernel grown under 5-day water stress and shading during the pre-pollination and early post-pollination stages and found that both water stress and light reduction at both stages reduced kernel set especially in apical ear parts. It has been reported that corn seems to be relatively tolerant to water stress during the vegetative stage and during ripening and that, the highest reduction in grain yields is triggered by water stress during the flowering stage (Fereres *et al.,* 2006). In a similar study, rice was found to be more sensitive to water stress, especially during the flowering stage, which causes severe yield losses (Liu *et al.,* 2006). Changes in the physiological processes during the sensitive flowering stage have a negative effect on spikelet

development and fertility under water stress (Liu *et al.,* 2006) and pollen germination (Saini, 1997). Furthermore, reduction in both panicle (O'Toole & Namuco, 1983) and peduncle length (He *et al.,* 2009) were partially responsible for increased sterility under water stress. Nesmith and Ritchie (1992) studied short- and long-term responses of corn to a parenthesis water stress and found 15–25% losses in yield because of the long-term negative effect of water stress. Growing sunflower plants for more than 12 days under water stress during their grain filling and flowering stage was the most damaging, and therefore caused a large reduction in sunflower achene yield (Reddy *et al.,* 2004). In maize, water stress significantly decreased grain yield, which was dependent on the defoliation level because of water stress during early reproductive stage (Monneveux *et al.,* 2006). Water stress decreases seed yield in soybean mostly because of the production of a smaller number of pods and seeds per pod (Specht *et al.,* 2001).

1.7.3 Plant Responses to Water Stress

The response of plants to water stress depends on the duration and severity of the stress (Araus *et al.,* 2002) and the developmental stage (Zhu *et al.,* 2005). Plants alter their physiological response in several ways. Osomoprotection, antioxidation, and ROS scavenging system are among the major physiological responses to tolerate and survive through water stress (Farooq *et al.,* 2009). Osmotic adjustment allows plants to increase the influx of water and thus maintain turgor (Shabala & Lew, 2002). Other than osmotic adjustment, dehydrins also help plant tolerance because they maintain high tissue water potential (Bray, 2002). Another way in which plants tolerate water stress is by ensuring antioxidant defense. This system involves enzymes, such as superoxide dismutase, catalase, and peroxidase, and non-enzymatic resources, such as cysteine, reduced glutathione, and ascorbic acid (Farooq *et al.,* 2009). ROS are formed in plant

cells because of water stress. They are removed by antioxidant enzymes or by the inorganic scavenging molecules. In addition, Kavar *et al.* (2008) reported that various genes are activated in response to the onset of drought at transcriptional level; the products of these genes are important in establishing tolerance to drought. The genetic response may be triggered directly due to drought or because of secondary stress or injury; in either case, the genetic response to drought is a complex phenomenon that takes place as a concerted action of numerous genes (Agarwal *et al.,* 2006). Another major response to drought occurs in the form of special proteins, such as aquaporins. Usually, the synthesis of these proteins is a ubiquitous response that helps to cope with stressful drought conditions by hydration of cellular structures (Wahid *et al.,* 2007) or synthesis of transcription factors required for expression of other stress proteins and genes (Legay *et al.,* 2011). The amino acid proline serves as an indicator for dryness and accumulates under environmental stress (Szabados & Savoure, 2009). Plant hormone signaling has an important role in many physiological and developmental processes, including stress response.

1.8 Plant Hormones

Plants are sessile, thus they must be able to cope with environmental factors and biological environments, such as light, temperature, and humidity levels, and the chemical composition of the soil. Hormones are a group of small organic compounds that occur naturally within living systems. Similar to mammalian systems, these compounds occur in very small concentrations in plant systems and have the ability to influence physiological processes, such as growth and development (Dodd & Davies, 2010). Synthesis of plant hormones can be either localized or generalized, and they can be made in a variety of cells or tissues and then transported to other areas or remain localized (Dodd & Davies, 2010).

Most plant hormones, such as abscisic acid, cytokinin (a purine derivative), gaseous hormone; ethylene, indole acetic acid peptide hormones, and steroid hormones (Fedoroff, 2002) have a critical role in plant responses to various stress factors, such as, temperature and water stress (Dodd & Davies, 2010).

Abscisic acid (ABA) is a terpenoid that was discovered in the 1960s; it is largely involved in plant development and stress responses (Hauser *et al.,* 2011). ABA is produced in response to various stress factors mentioned above, such as temperature (Kurepin *et al.,* 2008) and water stress (Qaderi *et al.,* 2006; Samarah *et al.,* 2009). ABA can be made in most major organs of a plant and travels through the xylem and phloem in order to act in specific locations (Nambara & Marion-Poll, 2005; Hussain *et al.,* 2012). ABA is transferred through the xylem to the shoot, where it causes stomata to close and reduces leaf expansion, thus preventing transpiration from leaf body (Yang *et al.,* 2001a). Its main functions in plants deal with water relations as well as growth and development, and it has been found in every major organ of the shoot and root systems (Hussain *et al.,* 2012). ABA is well known for its use in the regulation of seed dormancy and seedling development, promotion of leaf abscission and prevention of damage caused by ROS (Nambara & Marion-Poll, 2005; Raghavendra *et al.,* 2010; Hauser *et al.,* 2011; Pantin *et al.,* 2013). ABA is synthesized and regulated through both biosynthetic and catabolic pathways within the plant (Raghavendra *et al.,* 2010). Expression, or overexpression, of genes controlling the xanthophyll cycle, 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, carotenoid synthesis, and other ABA response genes have been shown to increase the production of ABA (Raghavendra *et al.,* 2010). Some genes directly linked to ABA synthesis may be triggered by stress, such as drought or high temperatures (Nambara & Marion-Poll, 2005). There have been some debates on the effect of temperature on ABA synthesis. Earlier studies have suggested that

both water stress and elevated temperature promote ABA synthesis, but reduce indole acetic acid (IAA) production (Nilsen & Orcutt, 1996), whereas more recent studies determined that high temperature reduces ABA synthesis (Qaderi *et al.,* 2006, 2012).

Cytokinins (CKs) are a large group of plant hormones, which control different important processes during plant growth and development. CKs are found as bound forms in the tRNA of the majority of living organisms, including plants, but plants also have substantial quantities of free CKs (Haberer & Kieber, 2002). CKs are derived from the nitrogenous base adenine and are categorized according to their side chain to isoprenoid or aromatic compounds (Noble *et al.,* 2014). Isoprenoid CKs are found in four different groups: trans-zeatin, cis-zeatin, dihydrozeatin and isopentenyladenine, in addition to aromatic CKs involving benzylaminopurine and topolins (Noble *et al.,* 2014). CKs stimulate cell division and differentiation processes in vascular plants and shoot and root differentiation (Noble *et al.,* 2014). CKs also affect biomass distribution and delay leaf senescence (Kim *et al.*, 2006). Furthermore, CKs stimulate shoot branching and regulate shoot and root growth in plants (Gajdošová *et al.,* 2011). CKs also regulate the signaling of the availability of inorganic nitrogen to the roots, seed germination, and responses to pathogens (Gajdošová *et al.,* 2011). Enhancement of cell division is the most distinctive response provoked when exogenous CKs applied to higher plants and algae (Noble *et al.,* 2014).

Ethylene is a gaseous-plant hormone that causes fruit ripening and affects plant growth and development in different ways. Ethylene is produced throughout different higher plant tissues from methionine and its production differs according to tissue type, plant species, and the developmental stage (Romagnano, 2008). It has been suggested that ethylene evolution increases by both water stress and elevated CO_2 (Finlayson *et al.,* 1999), but decreases by high temperature (Yu *et al.,* 1980). Aminocyclopropanecarboxylate (ACC) oxidases are the key enzyme involved

in ethylene biosynthesis and were reported to be upregulated in response to heat stress (Nie *et al.,* 2002). Moreover, it has been reported that pretreatment with ethylene enhances the thermotolerance ability of plants (Larkindale & Knight, 2002).

The molecular responses to stress factors include interaction among transcription factors and activation of a group of genes (Qu *et al.,* 2013). In Arabidopsis, stress-responsive genes, such as protein phosphatase 2C (PP2C), *Arabidopsis thaliana* homeobox 7 (ATHB-7), responsive to desiccation 29A (RD29A), responsive to desiccation 29B (RD29B), responsive to desiccation 22 (RD22), and varied late embryogenesis abundant (LEA) genes were strongly activated by single stresses, such as salt and temperature, after 1-12 hours (Hirayama & Shinozaki, 2010). Wang *et al.* (2003) have reported that many of the inducible water stress genes are activated by ABA. The interaction between different environmental factors greatly affects growth and development, as well as physiological and biochemical makeup, of plants.

1.9 Combined Effects of Temperature, CO_2 and Watering Regime on Plants

There have been few studies that investigate plant responses to multiple environmental factors applied in combination. Such studies that show the interaction between different environmental factors are very important because many factors occur in combination and not individually, thus have more effect on plants (Mickelbart *et al.,* 2015). The negative impact of heat and water stresses on plant growth, for example, could be alleviated by elevated CO_2. For example, it has been postulated that plant growth and leaf area increase because of the enhancement in water status when plants grow under elevated CO_2 and moderate water stress conditions (Vu & Allen, 2009). Bauweraerts *et al.* (2013) showed that elevated CO_2 reduced the negative impacts of heat and water stresses on photosynthetic parameters in loblolly pine (*Pinus taeda* L.) and northern

red oak (*Quercus rubra* L.). Similarly, elevated CO_2 also mitigated the negative effects of water and high light stresses on growth rate and photosynthesis in cork oak (*Quercus suber* L.) plants (Pardos *et al.,* 2006). The combined heat and water stresses on Arabidopsis were mitigated by elevated CO_2 at multiple organizational levels (Zinta *et al.,* 2014). In addition, elevated CO_2 mitigated the negative effect of higher temperature and water stress on sugar and amino acid metabolism, but not on fatty acids in Arabidopsis (Zinta *et al.,* 2018). Improvement in tissue turgor and high CO_2 assimilation rate for Cerrado species (*Viguiera discolor* Baker) plants, which were grown under elevated atmospheric CO_2 concentration and water stress, was reported by Oliveira *et al.* (2013). Miranda-Apodaca *et al.* (2018) studied the interactive effect of water stress and elevated CO_2 on two grassland species. They found that the negative effect of water stress on turgor potential in red clover (*Trifolium pratense* L.) was partially alleviated by elevated CO_2 through helping the plant conduct osmotic adjustment and increasing the root to shoot ratio, while in common bent (*Agrostis capillaris* L.) by increasing the hydraulic conductance that caused the plants to have higher leaf relative water content. The above-ground biomass accumulation in grassland ecosystems under multiple stress factors was increased to greater extent under elevated CO_2 than under ambient CO_2 concentration by improving their antioxidant defenses and carbohydrate metabolism (Naudts *et al.,* 2013). However, Arndal *et al.* (2018) found the allocation of carbon (C) to roots was increased in common heather (*Calluna vulgaris* L.) plants in comparison to aboveground biomass under high temperature, elevated CO_2, and water stress. Most climate models predict that the frequency, intensity, and duration of water stress events will decrease with rising CO_2 concentration and increase with increasing temperature (Allison *et al.,* 2009). Nonetheless, Duan *et al.* (2013) pointed to the complex interactive effect of elevated CO_2 and heat stress on plant growth and photosynthesis during

water stress. Paudel *et al.* (2018) showed that the improvement of tree growth grown under elevated CO_2 is much less than originally estimated; this is because the overabundance of carbon can increase wood carbon storage and stomatal decrease in numbers and therefore decreased water-use efficiency. Thus, some studies expected that future climate change would work on modifying or limiting the direct positive effects of elevated CO_2 on different crops and plant species. For example, elevated CO_2 enhances water status for soil and plants, while heat stress may counteract this effect (Yu *et al.,* 2012), which may be worsened more by water stress (Zeppel *et al.,* 2012). This indicates that the interaction between high temperature and water stress could eliminate the positive effect of CO_2 on water status and other physiological parameters. The combination of elevated CO_2 and warming did not modify water stress in loblolly pine (*Pinus taeda* L.) seedlings (Wertin *et al.,* 2012), but it exacerbated water stress in mugga ironbark (*Eucalyptus sideroxylon* L.) seedlings (Zeppel *et al.,* 2012). Similarly, Yu *et al.* (2012) mentioned that elevated CO_2 exacerbated the negative effect of the combined high temperature and water stresses on photosynthesis in tall fescue (*Festuca arundinacea* L.). This means enhanced-down regulation of photosynthesis under combined stress factors. Elevated CO_2 did not mitigate the negative impacts of water stress on the vegetative growth of grape vine (*Vitis vinifera* L.) and white Tempranillo (Kizildeniz *et al.,* 2018). The short-term photosynthetic responses of two oak species, evergreen oak (*Quercus ilex* L.) and Turkey oak (*Quercus cerris* L.), to a higher temperature and elevated CO_2 were studied by Killi *et al.* (2018). The results showed that *Q. ilex* L. is more adaptive than *Q. cerris* L. to the combination of elevated CO_2 and temperature, which suggest that responses of plants to climate change could be species specific. Similar to this, elevated CO_2 exacerbated the negative effects of heat stress on Norway spruce (*Picea abies* L.), but not on Scots pine (*Pinus sylvestris* L.) (Kurepin *et al.,*

2018). It seems that the amelioration effect of CO_2 for high temperature and water stresses also depends on the exposure period. Thus, the improvement of plant growth and development due to of elevated CO_2 concentration may be lessened simultaneously by high temperature and water stresses, particularly in the long term (Albert *et al.,* 2011). It has been reported that elevated CO_2 initially increased the rate of photosynthetic activity, but this response tends to reduce over the long-term exposure, as plants adapt to this new condition (Rajashekar, 2018).

Flowering responses of dominant species in a grassland ecosystem were more robust to high temperature alone than to the interactive effect of high temperature, elevated CO_2, and water stress, but not to water stress or elevated CO_2 alone (Bloor *et al.,* 2010). The grain yield and biomass of wheat in Australia were increased when plants were grown under elevated CO_2 and 2°C warming, regardless of watering regime, but this additive effect disappeared when temperatures increased more than 2°C due to water stress (Oliveira *et al.,* (2013). Therefore, increased temperatures could reduce elevated CO_2 positive impacts indirectly, by increasing the need for water (Xiao *et al.,* 2005). Also, increased temperatures may cause crops growing under elevated CO_2 to have reduced seed number, size, and quality because of water stress (Caldwell *et al.,* 2005).

Earlier studies have shown that crop productivity has changed under combined effects of environmental stress factors (Frenck *et al.,* 2011). For example, the productivity of sugarcane (*Saccharum officinarum* L.) plants was increased by elevated CO_2 through lowering stomatal conductance and transpiration rates (37 and 32%, respectively), and higher water-use efficiency (De Souza *et al.,* 2016). Seed yield is positively affected by elevated CO_2 concentration (Qaderi *et al.,* 2007). It has been reported that the viability and production of pollen decreased under heat stress, thus affecting pod set in chickpea (*Cicer arietinum* L.) (Devasirvatham *et al.,* 2012). In

addition, both water (Frederick *et al.,* 1991) and heat (Prasad *et al.,* 2006) stresses reduce the seed-filling period, thus resulting in small seeds. Higher temperature has caused a reduction in seed set in rice during anthesis, but the combined effects of heat stress and elevated CO_2 concentration did not affect seed set (Madan *et al.,* 2012). Similarly, Williams *et al. (*2007) have reported that the combined effects of elevated CO_2 concentration and heat stress in grass species did not affect seed production. The interactive effects of high temperature with elevated CO_2 and drought on soybean (*Glycine max* L.) seed isoflavone content showed that elevated CO_2 could moderately converse the high temperature and drought effects on the content of *G. max* L. seed isoflavone (Caldwell *et al.,* 2005). The major nutrients in food crops including phosphorus, potassium, calcium, iron, and zinc are known to be suppressed by elevated CO_2 levels (Rajashekar, 2018). Elevated CO_2 increase carbohydrate content accumulation, but decreases nitrogen abundance in plants thus affecting their C-N ratio. Although elevated CO_2 can reduce the contents of major nutrients, it may enhance a certain group of health-promoting phytochemicals in food crops (Rajashekar, 2018).

1.10 Model Plant: *Arabidopsis thaliana*

1.10.1 Botanical Description

Arabidopsis thaliana (mouse-ear cress) is a small annual weed that belongs to the family Brassicaceae. This plant is native to different parts of the world, such as Europe, central Asia, and northwestern Africa, and can be found in many other places in the world (Bos, 2008).

Arabidopsis is selected for this study because of a few characteristics that make it an excellent experimental model. The life cycle of *A. thaliana* is short and under standard growth conditions, it completes its entire life cycle within six to eight weeks (Koornneef & Meinke,

2010). *A. thaliana* reproduces mainly by self-fertilization in contrast to most of the other members belong to the Brassicaceae family that are self-incompatible (Tang *et al.,* 2007). The process of self-pollination helps *A. thaliana* to easily disperse to new areas, gives the plants reproductive assurance, and eliminates dependence on pollinators (Jarne & Charlesworth, 1993). Furthermore, the low growing demand of *A. thaliana*, such as relatively little light (illumination from cool, white fluorescent bulbs is sufficient) and temperatures in the range of 22-26°C make it easily grown in pots in greenhouses or even in Petri dishes in the laboratory (Bos, 2008). The leaves of this plant form a rosette at the base of the plant and their lengths range between 1.5 and 5 cm. The central stem that carries flowers emerges within two-three weeks of germination in a process called bolting. The height of the inflorescence is normally 25 cm. The diameter of the flowers is around 3 mm and organized in a raceme. The fruit is called a silique and each silique usually contains 20-30 seeds, therefore, thousands of seeds can be produced by an individual plant, 10,000–40,000 plant^{-1} (Bos, 2008). Additionally, it is very suitable for genetic research. It has one of the smallest genomes known in plants consisted of five chromosomes containing 25,498 genes coding for proteins belonging to 11,000 families, and the complete genomic sequence is available (Arabidopsis Genome Initiative, 2000), which simplifies designing primers for genotype studies.

In this study, wild-type (WT) *A. thaliana* and its associated ABA-insensitive mutant (*abi1-1*) were used. All are derived from the Landsberg "erecta" ecotype. A number of mutants of *A. thaliana*, ABA-insensitive1 (*ABI1*) to ABA-insensitive5 (*ABI5*), with insensitivity to ABA were all found for seeds capable of germinating when applying exogenous ABA concentrations (3-10 µM) that are inhibitory to the wild-type (Koornneef *et al.,* 1984). The initial characterization of *abi1*, *abi2*, and *abi3* mutants showed that they have a marked reduction in seed dormancy and up

to a 10-fold decrease in sensitivity to exogenous ABA for germination inhibition when compared to wild-type plants (Finkelstein & Somerville, 1990). However, none of the mutants show a complete loss of sensitivity to ABA, which means that they are either leaky mutations or influenced only one of numerous ABA response pathway (Finkelstein & Somerville, 1990).

ABI1 mutant is semidominant mutations, have a reduction in its sensitivity to ABA, and severely decreases the catalytic activity of the *ABI1* type 2C protein phosphatase (PP2C) (Leung *et al.,* 1994). PP2C is an important phosphatase-like protein in eukaryotic organisms that can negatively regulate protein kinase cascade ABA signal system through phosphorylation and carry out vital roles in various cell processes (Fedoroff, 2002).

The ABA regulation of most of the ABA-responsive genes have been shown to be impaired in the *abi1-1* (Hoth *et al.,* 2002). Mutation of *abi1-1* eliminates the ability of the ABA to activate both the plasma membrane outward- and inward- rectifying K^+ channels, however, this ABA sensitivity of both K^+ channels is restored by the protein kinase inhibitors H7 and staurosporine (Armstrong *et al.,* 1995). The *abi1-1* mutations decrease the ability of ABA to induce Ca^{2+} increases in stomata-guard cells but do not affect the ability of Ca^{2+} to induce stomatal closure (Allen *et al.,* 1999). In the *abi1-1*, ABA regulation of about 84.5% and 6.9% of the identified ABA-responsive genes by applying massively parallel signature sequencing (MPSS) was reduced and strongly impaired, respectively; nevertheless, 8.6% of the genes stayed properly regulated (Hoth *et al.,* 2002). Moreover, the existence of a group of genes that were not fully impaired in the *abi1-1*, continued to be appropriately controlled by ABA, proposes the existence of at least two ABA signaling pathways; only one of which is obstructed in *abi1-1* (Hoth *et al.,* 2002).

1.10.2 Lifecycle

The switch from vegetative to reproductive growth represents an important part of the life cycle of *A. thaliana* when the leaf-producing meristems shift to a flower producing meristems (Bos, 2008). *A. thaliana* is an annual weed that flowers only once a year and this makes the timing critical for reproduction to happen and success in *A. thaliana*. Flowering process in *A. thaliana* is regulated by different internal signals, such as the stage of development of the plant and by external (environmental) signals, such as temperature (Martinez-Zapater *et al.,* 1990).

A. thaliana has developed two main flowering strategies (Michaels *et al.,* 2003). First, early flowering or summer annuals strategy, in which the *A. thaliana* plants flower very quickly and can finish its entire lifecycle in 4 to 6 weeks. Second, winter annuals or late flowering strategy, the *A. thaliana* plants need a long time of low temperatures after which they quickly start flowering, a process known as vernalization (Michaels *et al.,* 2003). The summer annual is thought to be an adaptation to hot and dry environments, which allow germination and flowering to happen quickly in spring before environments become excessively hot, and dry. The winter annual is apparently an adaptation to colder environments where it germinates and grows throughout summer and autumn and after that spends winter below the snow as a rosette. When spring starts, the plant moves from vegetative to reproductive growth and yields seeds (Pigliucci, 2002).

1.11 Thesis Objectives

The objectives of this study are (1) to determine how *Arabidopsis thaliana* plants respond to the interactive effects of temperature, CO_2, and watering regime at vegetative and reproductive stages; (2) to investigate how elevated CO_2 might mediate these stress responses; (3) to evaluate

the role of ABA in mitigating the negative effect of stress thus contributing to increased plant health; and (4) to determine the possible interaction between these four factors (temperature, CO_2, watering regime, and genotype).

To test these objectives, it is hypothesized that high temperature and water stress will have negative impacts on WT and *abi 1-1* mutant of *A. thaliana* at various developmental stages (vegetative and reproductive stages), but elevated carbon dioxide will mitigate these negative impacts. Additionally, ABA will help the plants tolerate stress factors and contribute to increased plant health.

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CHAPTER 2

Higher Temperature and Drought Adversely Influence Sensitive Plants, but Tolerant Genotypes May Adapt to these Stress Factors

2.1 Abstract

Few studies have considered multiple aspects of plant responses to key components of global climate change. It is, therefore, critical to assess the single and interactive effects of main components of climate change on plant growth and development. We investigated the combined effects of temperature, carbon dioxide, watering regime, and genotype on *Arabidopsis thaliana* (wild-type and *abi1-1* mutant). 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 carbon dioxide 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. We showed that drought and elevated CO_2 had larger effects on plants than higher temperatures. Elevated CO_2 alleviated the detrimental effects of temperature and drought by increasing antioxidant activity and plant water status, and this positive effect was consistent across multiple response levels. In addition, the wild-type plants had better performance than the *abi1-1* plants. Water-stressed plants of both genotypes accumulated more abscisic acid (ABA) than the well-watered plants, and higher temperatures inhibited the ability of wild-type plants to produce ABA in response to drought. We conclude that drought strongly, but higher temperature to a lesser extent, affects Arabidopsis seedlings, and elevated CO_2 reduces the negative effects of stress factors more in the wild-type plants than in the *abi1-1* plants.

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2.2 Introduction

Climate change is a serious threat to plant growth and development. The components of climate change include elevated carbon dioxide (CO_2) concentration and higher temperature, as well as increases in other extreme abiotic stresses, such as water deficit, flooding, and salinity (Meehl 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^{o}C due to its heat-trapping potential (Stocker et al. 2013). Elevated CO_2 and higher temperature are expected to affect global precipitation patterns and, in turn, water stress events in soils (Allison et al. 2009). Elevated CO_2 improves growth and biomass of plants through increased water use efficiency (Qaderi et al. 2006), photosynthesis rate (Yazaki et al. 2004), and photoassimilation rate (Duan et al. 2014). Elevated CO_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 *a*, chlorophyll *b*, and chlorophyll/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 chlorophyll 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 (Qaderi et al. 2006). Stomatal closure decreases stomatal conductance and, in turn, gas exchange and plant biomass (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, drought responsive genes like *RD29A*, *RD29B*, *RD22*, and varied *LEA* genes are strongly activated by single factors, such as salt and water stress (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 elevated CO_2 and heat stress on plant growth and photosynthesis during water stress. Elevated CO_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 elevated CO_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 elevated CO_2 on plants. For instance, elevated CO_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 elevated CO_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 elevated CO_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 higher temperature (Way and Oren 2010), water stress (Wu et al. 2011), and elevated CO_2 (Hyvönen et al. 2007) on plants. Most of these studies have considered the effects of higher temperature and water stress mainly at ambient CO_2 concentrations (Bhargava and Sawant 2013). Few studies have examined the interactive effects of temperature, drought, and elevated CO_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 elevated CO_2 on crops, particularly cereals (Gammans et al. 2017), which were grown under both optimal and limiting growth conditions, but few studies have considered weeds, such as *Arabidopsis thaliana* (e.g., Zinta et al. 2014) and *Centaurea nigra* (e.g., Qaderi et al. 2013), for such studies. Therefore, 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, effect 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. In the present study, we investigated the interactive effects of temperature, CO_2, and watering regime on wild type and its relative ABA-insensitive mutant (*abi1-1*) 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 concentration of

endogenous ABA by using Arabidopsis (ABA insensitive mutant and its associated wild-type). We hypothesized that elevated CO_2 may initially has a positive effect on *Arabidopsis thaliana*, but the positive effect may decrease by higher temperatures and water stress as a result of metabolic disturbances. In addition, higher temperature inhibits the inducing effect of water stress on ABA.

2.3 Materials and methods

2.3.1 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*), were used. The use of *abi1-1* mutant helps us understand that ABA, as a key internal signaling molecule, regulates plant responses to multiple factors. First, the seeds were treated with 95% ethanol and germinated in Petri dishes, containing liquid Murashige and Skoog basal medium (MS) 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 six 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 volume). 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 30 pellets of slow-release NPK fertilizer; each granule contained all 3 of NPK (13-14-14; 0.208g N:0.224g P:0.224g K/30 pellets); 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 (400 μmol mol^{-1}), and watering to field capacity (well-watered), considered as control; (2) lower temperatures, ambient CO_2, and watering at wilting point (water-stressed); (3) lower temperatures, elevated CO_2 (700 μmol mol^{-1}), and well-watered; (4) lower temperatures, elevated CO_2, and water-stressed; (5) higher temperatures (28/24°C, 16 h light/8 h dark), ambient CO_2, and well-watered; (6) higher temperatures, ambient CO_2, and water-stressed; (7) higher temperatures, elevated CO_2, and well-watered; and (8) higher temperatures, elevated CO_2, and water-stressed. 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. Half of the plants in each cabinet was watered to field capacity, and the other half at wilting point. 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 different combinations of growth chamber and Plexiglas cabinets.

Measurements were included a number of growth, anatomical, physiological, molecular, and hormonal responses, as described below.

2.3.2 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 in order to use them only in their vegetative stage. At the conclusion 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 ED224s, 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.

2.3.3 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). Cell area was measured using the ImageJ software (http://rsb.info.nih.gov/ij/) by manually measuring the area of each epidermal cell per picture (all pictures had the same magnification and same size) and then taking the average of cell areas of all cells in the picture.

2.3.4 Measurement of leaf and soil water potential

Water potential was measured with a Dew Point PotentiaMeter (model WP4C, Decagon Devices, Pullman, WA, USA). From each treatment, three rosettes and three volumes (~2.7 g) of soil were used for water potential (MPa) after calibration with 0.05 mol kg^{-1} of potassium chloride in water (AquaLab, Hoskin Scientific Ltd., Burlington, ON, Canada).

2.3.5 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 dimethyl sulfoxide (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 measured for absorbance at 664, 648, and 470 nm, using a UV/visible spectrophotometer (model Ultraspec 3100 pro, Biochrom Ltd., Cambridge, UK). Pigment concentration (µg mg^{-1} FM) was calculated based on absorbance (Chappelle et al. 1992).

2.3.6 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 pistil 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 ºC 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 concentration on a fresh weight 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 pistil 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 in the ice. A 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 according to Sohrabi et al. (2012), using the following formula: $[((A532-A600) \times v) \times 1000] / (\varepsilon \times M)$. In the formula, 'ε' stands for specific extinction coefficient (=155 mM 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 ratio of C1 to C2, and expressed in percentage.

2.3.7 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}).

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

The Arabidopsis young leaf samples were collected from the top part of the rosette, 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, v/v/v]), 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, 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 back 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.

2.3.9 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. Two µL of the resulting cDNAs were 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 94ºC for 3 min followed by 35 cycles of incubations at 94ºC for 45 s, 48ºC for 30 s, and 72ºC for a minute, and a final extension at 72ºC

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′-gaccacaccaaacccattgag-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 were run on a 1 × 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).

2.3.10 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 (wild-type 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, watering regime, genotype, and growth chamber were treated, respectively, as the main plot, subplot, split-subplot, split-split-subplot and replication (see Potvin 2001). A one-way ANOVA was used to determine differences among treatments according to Scheffé's multiple-comparison procedure at the 5% 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.

2.4 Results

2.4.1 Plant growth

ECO_2 increased rosette diameter and leaf number and area, but water stress and higher temperatures decreased them. Plants of *abi1-1* mutant had a smaller rosette diameter and leaf number and area than WT (Table 2.1; Fig. 2.1). The three-way interaction among C × W × G (Table 2.2) revealed that rosette diameter and leaf number were highest for the well-watered WT plants at ECO_2, but lowest for the water-stressed *abi1-1* plants at ACO_2 (Fig. 2.1).

2.4.2 Dry mass accumulation

Higher temperatures and water stress reduced leaf, root and total biomass, but ECO_2 increased them. These parameters were also higher for the WT plants than for the *abi1-1* plants (Table 2.1, Fig. 2.2). Differences between CO_2 concentrations were significant for root, leaf, and total biomass (Table 2.1). However, differences between watering regimes and genotypes were significant for all parameters (Table 2.1). On the basis of interactions among these factors (Table 2.2), 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. 2.2).

Table 2.1 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	14909 ±1609a	8340 ± 629b	13720 ± 1753a	13422 ± 1735a	8638 ± 766b	12499 ± 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} FW)	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} FW)	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} FW)	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} FW)	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 (µmole g^{-1} FW)	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 (mmole g^{-1} FW)	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 (pmole g^{-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

FW h^{-1})								
ABA (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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
DHZR (pmole g^{-1} FW)	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 (pmole g^{-1} FW)	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

Arabidopsis plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 ten days, after eight days of initial growth under 22/18ºC. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters). Means followed by different letters within each parameter and condition are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. CA, cell area; CD, cell density; Chl, chlorophyll; EC, electrical conductivity; LMC, leaf moisture content; LWP, leaf water potential; MDA, malondialdehyde; RD, rosette diameter; SD, stomatal density; SI, stomatal index; SWP, soil water potential. 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).

Table 2.2 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	–	–	–	–	–	–

Arabidopsis plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 ten days, after eight days of initial growth under 22/18ºC. Experiments were conducted three times. Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Fig. 2.1 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 two temperature regimes (22/18ºC and 28/24ºC; 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 multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of nine samples from three different trials (three samples per trial for all measured parameters)

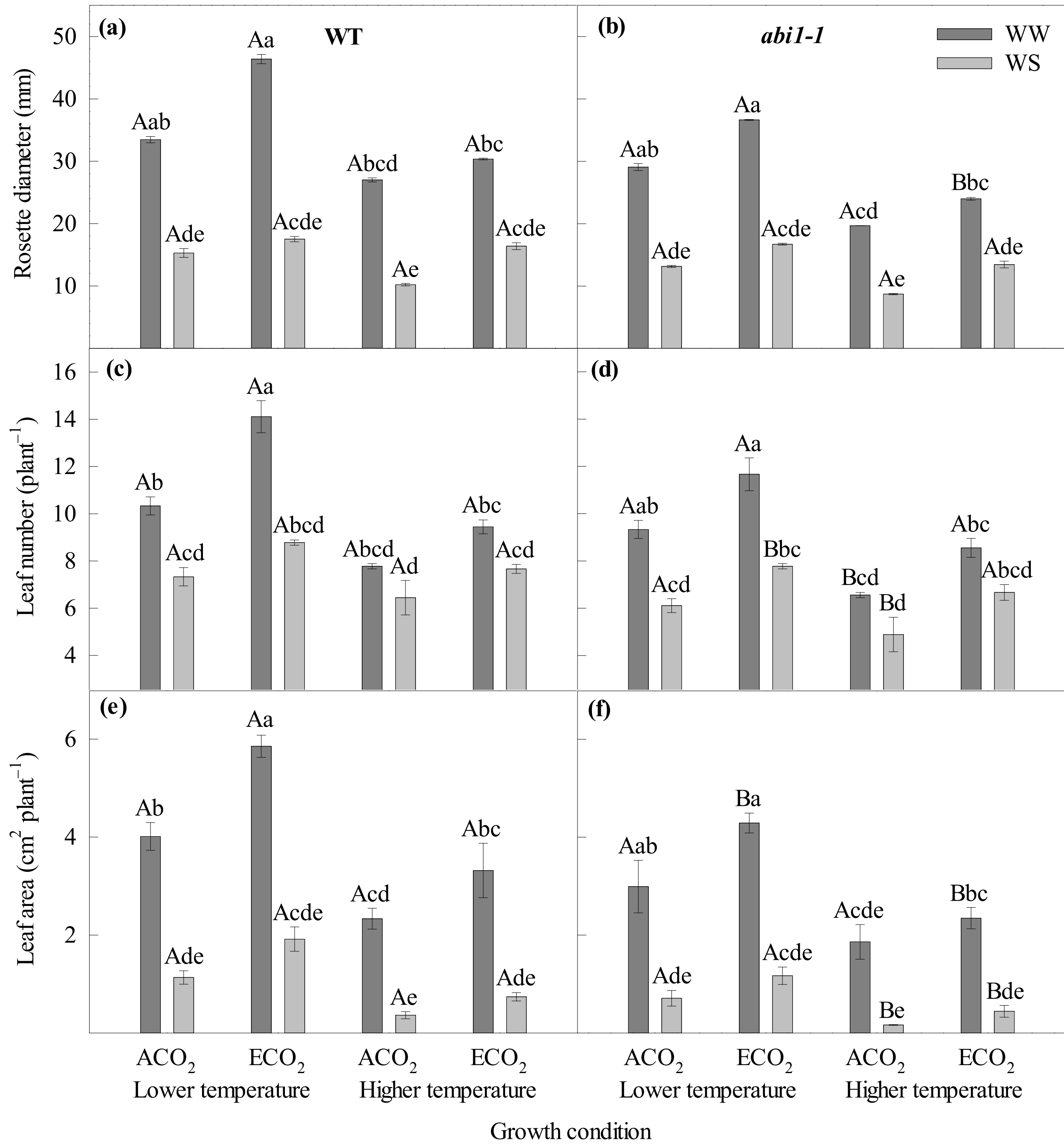
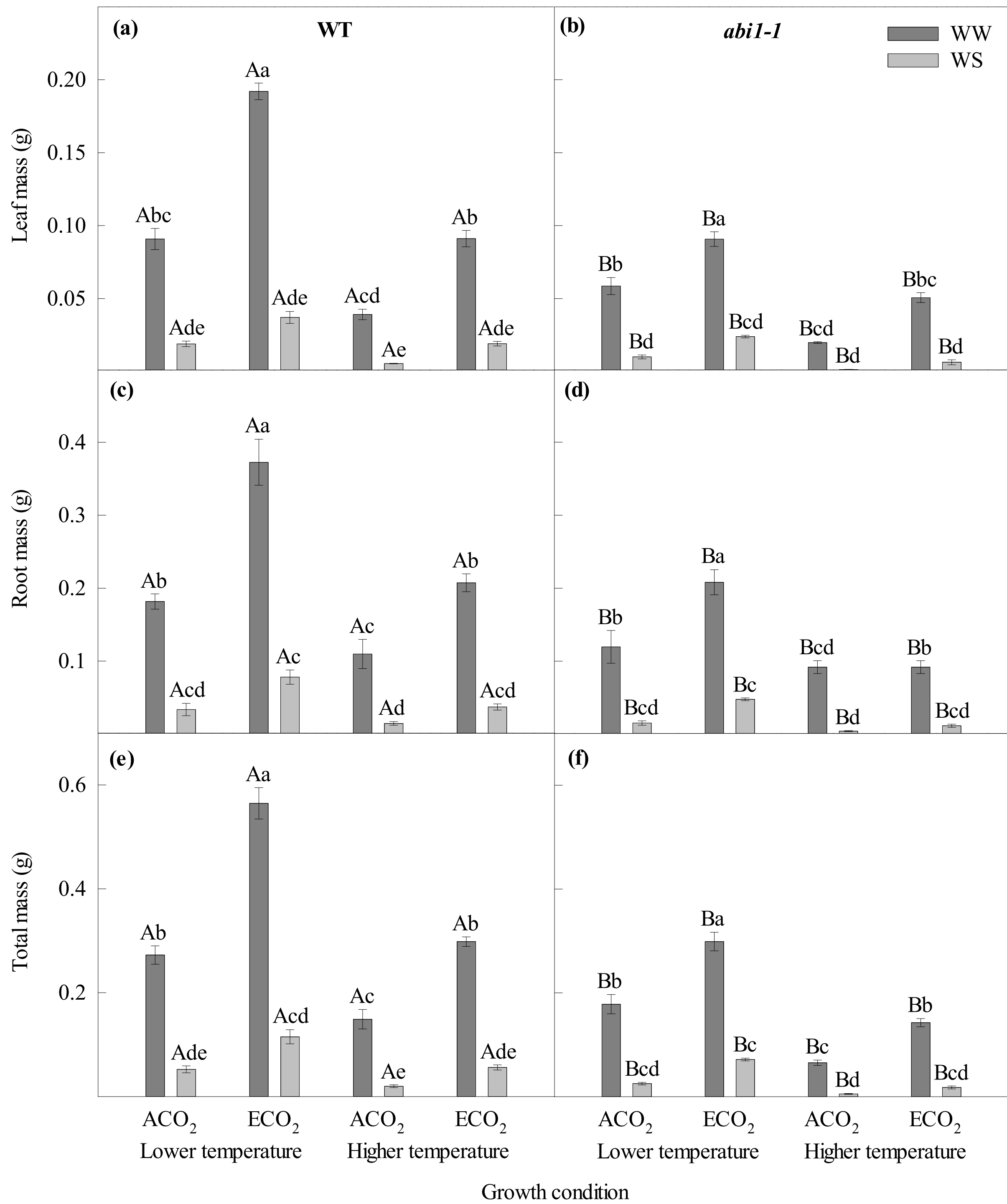

(a)
WT
(b)
abi1-1
WW
WS
Rosette diameter (mm)
Aab
Ade
Aa
Acde
Abcd
Ae
Abc
Acde
Aab
Ade
Aa
Acde
Acd
Ae
Bbc
Ade
(c)
(d)
Leaf number (plant^{-1})
Ab
Acd
Aa
Abcd
Abcd
Ad
Abc
Acd
Aab
Acd
Aa
Bbc
Bcd
Bd
Abc
Abcd
(e)
(f)
Leaf area (cm^2 plant^{-1})
Ab
Ade
Aa
Acde
Acd
Ae
Abc
Ade
Aab
Ade
Ba
Acde
Acde
Be
Bbc
Bde
ACO_2
ECO_2
ACO_2
ECO_2
Lower temperature
Higher temperature
Growth condition

Fig. 2.2 Effects of temperature, carbon dioxide and the watering regime on dry mass accumulation of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Leaf dry mass (**a**–**b**), root dry mass (**c**–**d**), and total dry mass (**e**–**f**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters)



(a)
WT
(b)
abi1-1
WW
WS
Leaf mass (g)
0.20
0.15
0.10
0.05
Abc
Ade
Aa
Ade
Acd
Ae
Ab
Ade
Bb
Bd
Ba
Bcd
Bcd
Bd
Bbc
Bd
(c)
(d)
Root mass (g)
0.4
0.3
0.2
0.1
Ab
Acd
Aa
Ac
Ac
Ad
Ab
Acd
Bb
Bcd
Ba
Bc
Bcd
Bd
Bb
Bcd
(e)
(f)
Total mass (g)
0.6
0.4
0.2
Ab
Ade
Aa
Acd
Ac
Ae
Ab
Ade
Bb
Bcd
Ba
Bc
Bc
Bd
Bb
Bcd
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
Growth condition

2.4.3 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 2.1). Based on C × W × G (Table 2.3), 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. 2.3a–b).

Higher temperatures increased, but water stress decreased, stomatal index (Table 2.1), which was significantly affected by the main factors and their interactions (Table 2.3). 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. 2.3c–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 2.1). Interactions of the main factors (Table 2.3), 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. 2.3e–f, 2.4b, 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 2.1). Interactions among C × W × G (Table 2.3) 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. 2.3g–h).

2.4.4 Soil and leaf water potential and leaf moisture

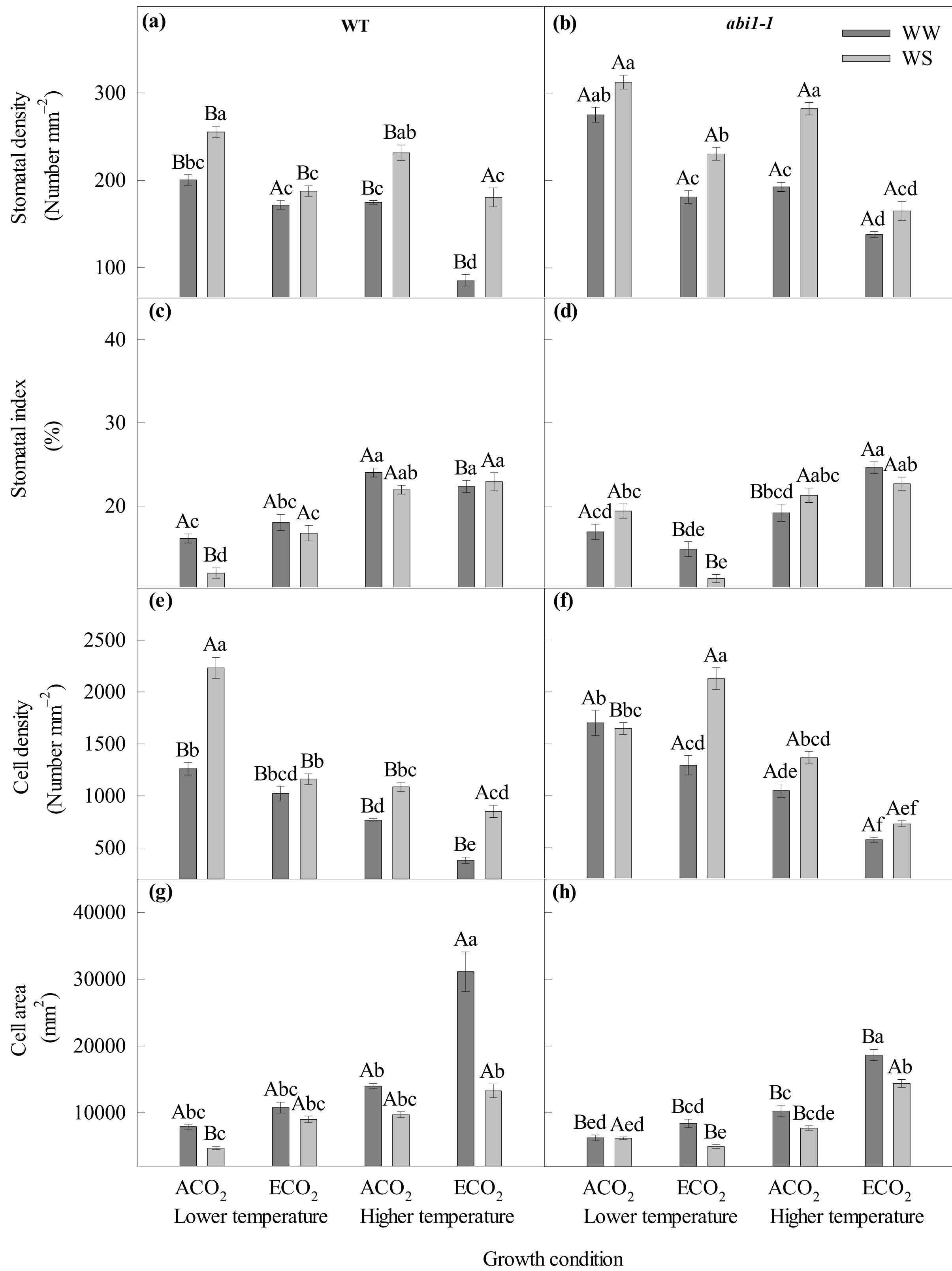
Higher temperatures and water stress reduced leaf and soil water potential, and leaf moisture, whereas ECO_2 increased them (Table 2.1). Plants from the *abi1-1* genotype had lower leaf and soil water potential and leaf moisture content than the WT plants (Table 2.1). Based on C × W × G (Table 2.3), 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. 2.5). On the basis of T × C × G (Table 2.3), 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. Based on the interactions among T × W × G (Table 2.3), 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 2.3) 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. 2.5c–d).

Table 2.3 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*

		Leaf anatomical feature				Leaf and soil water status		
Source	*df*	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 x 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 x W	1	0.99	89.50***	5.64	5.64	0.14	0.07	0.27
C x W	1	84.59***	417.64****	60.00**	39.18**	67.34**	156.42***	141.89***
T x C x 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 x G	1	1.59	33.61***	6.53*	4.37	5.02	13.48**	0.42
C x G	1	116.84****	845.25****	66.23****	31.07***	41.53***	177.00****	67.99****
T x C x G	1	1.99	29.15***	7.08*	3.94	3.02	7.48*	1.02
W x G	1	120.62****	1071.87****	67.28****	31.48***	76.95****	288.31****	69.55****
T x W x G	1	1.66	33.11***	6.64*	4.28	5.03	13.48**	0.49
C x W x G	1	126.41****	2018.16****	66.58****	30.83***	15.98**	90.71****	67.09****
T x C x W x G	1	2.22	23.78**	7.42*	3.67	3.21	8.03*	1.32
Split-split-subplot error	8	–	–	–	–	–	–	–

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. *A. thaliana* plants (wild–type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 eight days of initial growth under 22/18ºC. 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 10 leaves per treatment were analyzed

Fig. 2.3 Effects of temperature, carbon dioxide, and watering regime on the anatomical features of the lower epidermis of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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, 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**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters)



(a)
WT
(b)
abi1-1
WW
WS
Stomatal density (Number mm^{-2})
300
200
100
Bbc
Ba
Ac
Bc
Bc
Bab
Bd
Ac
Aab
Aa
Ac
Ab
Ac
Aa
Ad
Acd
(c)
(d)
Stomatal index (%)
40
30
20
Ac
Bd
Abc
Ac
Aa
Aab
Ba
Aa
Acd
Abc
Bde
Be
Bbcd
Aabc
Aa
Aab
(e)
(f)
Cell density (Number mm^{-2})
2500
2000
1500
1000
500
Bb
Aa
Bbcd
Bb
Bd
Bbc
Be
Acd
Ab
Bbc
Acd
Aa
Ade
Abcd
Af
Aef
(g)
(h)
Cell area (mm^2)
40000
30000
20000
10000
Abc
Bc
Abc
Abc
Ab
Abc
Aa
Ab
Bed
Aed
Bcd
Be
Bc
Bcde
Ba
Ab
ACO$_2$
ECO$_2$
ACO$_2$
ECO$_2$
Lower temperature
Higher temperature
Growth condition

Fig. 2.4 Light photomicrograph of lower epidermis from wild-type and *abi1-1* mutant leaves of *A. thaliana*. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Plants were grown under lower temperatures, ambient CO_2, well-watered (**a–i**), lower temperatures, ambient CO_2, water-stressed (**b–j**), lower temperatures, elevated CO_2, well-watered (**c–k**), lower temperatures, elevated CO_2, water-stressed (**d–l**), higher temperatures, ambient CO_2, well-watered (**e–m**), higher temperatures, ambient CO_2, water-stressed (**f–n**), higher temperatures, elevated CO_2, well-watered (**g–o**), and higher temperatures, elevated CO_2, water-stressed (**h–p**). Scale bar = 500 μm

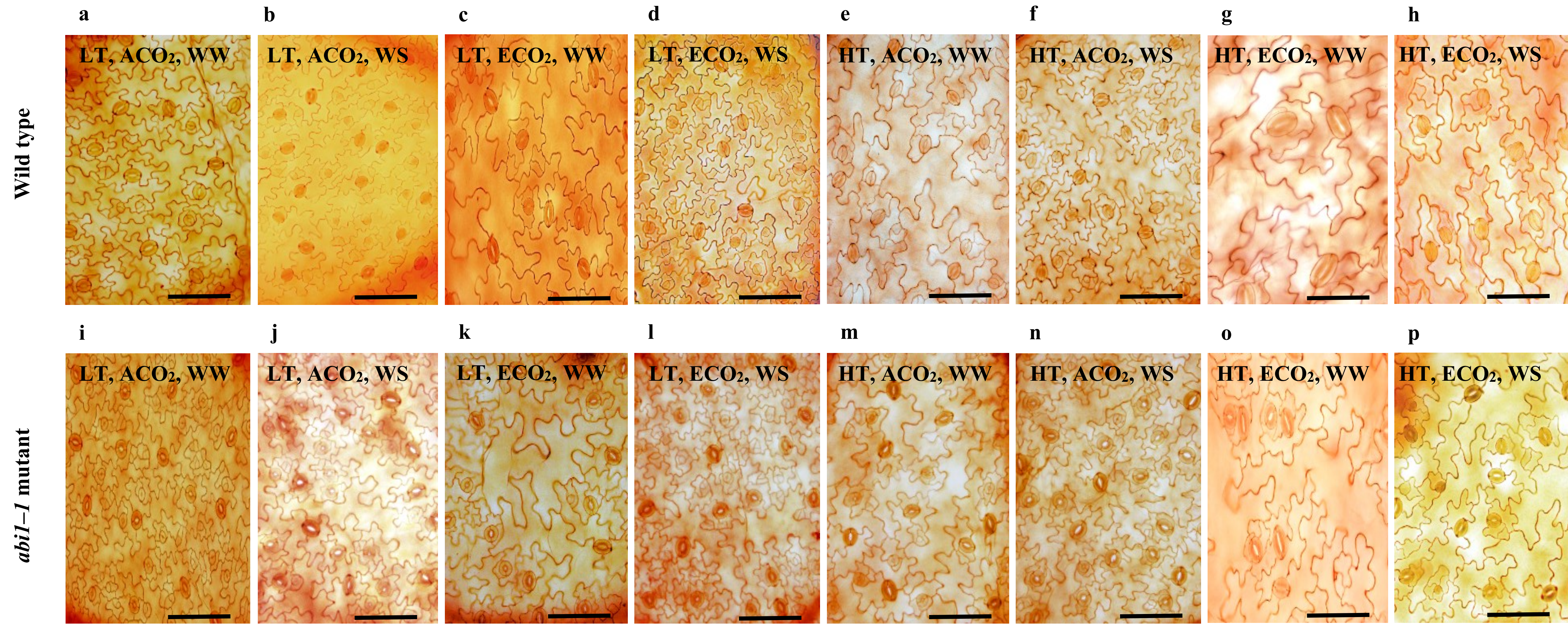
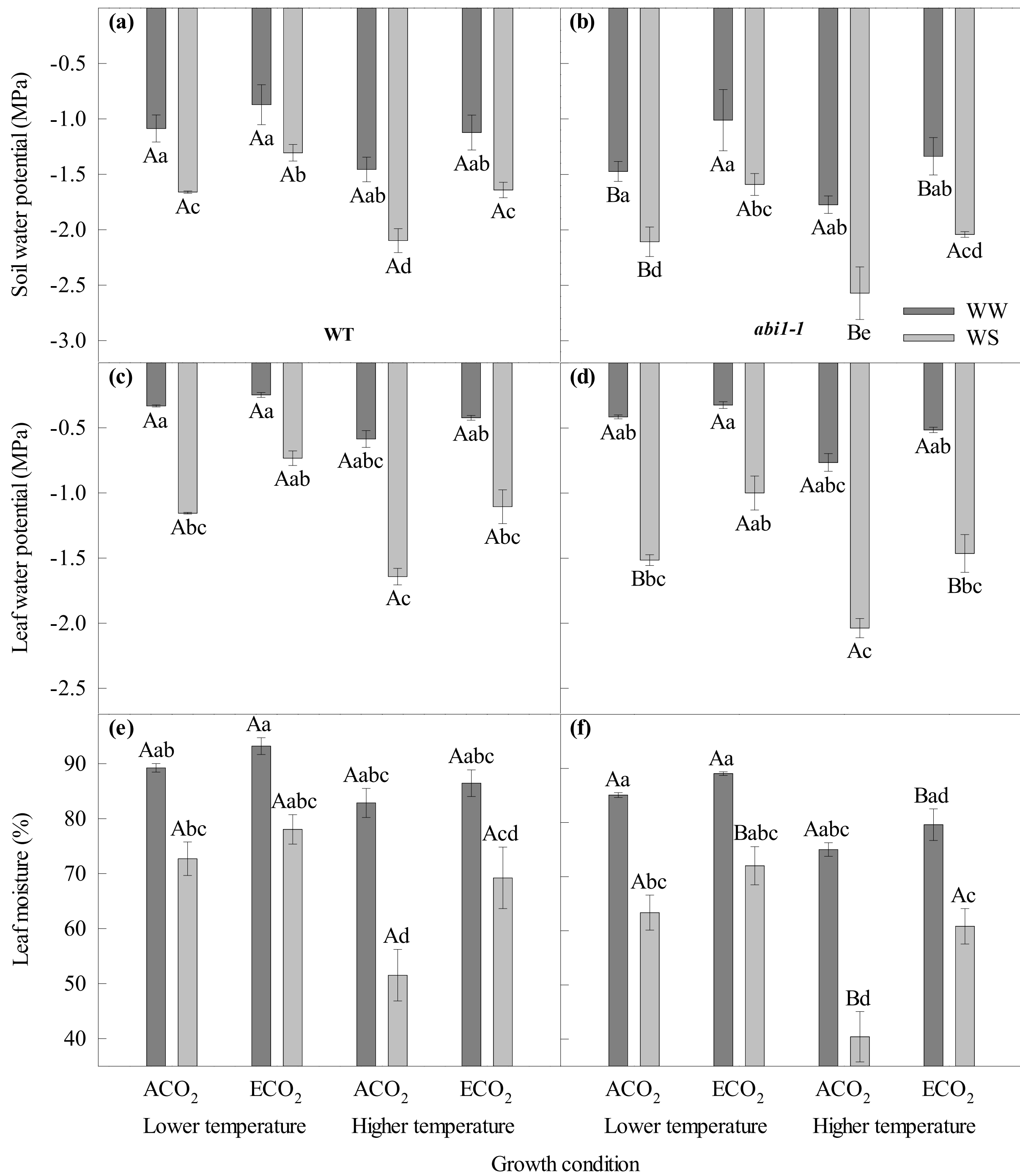

a
LT, ACO2, WW
b
LT, ACO2, WS
c
LT, ECO2, WW
d
LT, ECO2, WS
e
HT, ACO2, WW
f
HT, ACO2, WS
g
HT, ECO2, WW
h
HT, ECO2, WS
Wild type
i
LT, ACO2, WW
j
LT, ACO2, WS
k
LT, ECO2, WW
l
LT, ECO2, WS
m
HT, ACO2, WW
n
HT, ACO2, WS
o
HT, ECO2, WW
p
HT, ECO2, WS
abi1-1 mutant

Fig. 2.5 Effects of temperature, carbon dioxide, and watering regime on water potential and leaf moisture of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 $\mu mol\ mol^{-1}$) 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. Soil water potential (**a**–**b**), leaf water potential (**c**–**d**), and leaf moisture (**e**–**f**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters)


(a)
(b)
(c)
(d)
(e)
(f)
Soil water potential (MPa)
Leaf water potential (MPa)
Leaf moisture (%)
WT
abi1-1
WW
WS
Aa
Ac
Aa
Ab
Aab
Ad
Aab
Ac
Ba
Bd
Aa
Abc
Aab
Be
Bab
Acd
Aa
Abc
Aa
Aab
Aabc
Ac
Aab
Abc
Aab
Bbc
Aa
Aab
Aabc
Ac
Aab
Bbc
Aab
Abc
Aa
Aabc
Aabc
Ad
Aabc
Acd
Aa
Abc
Aa
Babc
Aabc
Bd
Bad
Ac
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
Growth condition

2.4.5 Photosynthetic pigments

Higher temperatures increased Chl *a* and carotenoids (Table 2.1). Overall, ECO_2 decreased, but water stress increased, Chl *a*, Chl *b*, carotenoids and total Chl. The *abi1-1* plants had higher carotenoids than the WT plants (Table 2.1). On the basis of T × C × G (Table 2.4), the *abi1-1* 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*. Based on T × W × G (Table 2.4), 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 interactions of the C × W × G (Table 2.4), the water-stressed *abi1-1* plants at ACO_2 had highest Chl *a*, whereas the well-watered *abi1-1* plants at ECO_2 had lowest Chl *a* (Fig. 2.6a–b). On the basis of the four-way interaction of the main factors (Table 2.4), 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. 2.6c–d). In addition, on the basis of interactions among these factors (Table 2.4), 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. 2.6e–f). Based on C × W × G (Table 2.4), 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. 2.6g–h). Interactions of the main factors (Table 2.4) 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. 2.6i–j).

2.4.6 Proline, lipid peroxidation, and electrical conductivity

Higher temperatures and ECO_2 decreased, but water stress increased, proline (Table 2.1). WT plants had a higher proline content than the *abi1-1* plants (Table 2.1). The C × W × G interaction (Table 2.4) 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. 2.7a–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 2.1). Based on interactions of the four factors (Table 2.4), 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. 2.7c–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 2.1). On the basis of the C × W × G interaction (Table 2.4), 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. 2.7e–f).

Table 2.4 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*

		Photosynthetic pigment					Chemical stress indicator		
Source	*df*	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 x 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 x W	1	5.94	9.00*	10.71*	4.36	12.73*	0.86	6.81	1.22
C x W	1	27.43**	224.98***	300.09****	5.58	34.36**	11.32*	323.42****	10.21*
T x C x 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 x G	1	7.09*	15.59**	14.98**	5.07	8.28*	1.39	20.77**	1.86
C x G	1	2.92	29.56***	38.12***	14.45**	192.08****	13.05**	79.37****	12.14**
T x C x G	1	5.47*	10.45*	9.85*	4.18	8.67*	1.76	13.40**	2.25
W x G	1	5.50*	121.67****	136.34****	0.08	90.31****	12.84**	221.68****	12.05**
T x W x G	1	7.05*	16.03**	15.47**	4.92	7.34*	1.46	21.32**	1.93
C x W x G	1	30.72***	0.00	0.00	52.42****	332.20****	13.39**	1.57	12.34**
T x C x W x G	1	4.84	11.10*	10.67*	3.30	5.44*	1.97	14.64**	2.48
Split-split-subplot error	8	–	–	–	–	–	–	–	–

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. *A. thaliana* plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 eight days of initial growth under 22/18ºC. Experiments were conducted three times. Chl, chlorophyll; EC, electrical conductivity; MDA, malondialdehyde

Fig. 2.6 Effects of temperature, carbon dioxide and watering regime on photosynthetic pigments of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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, 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**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters)

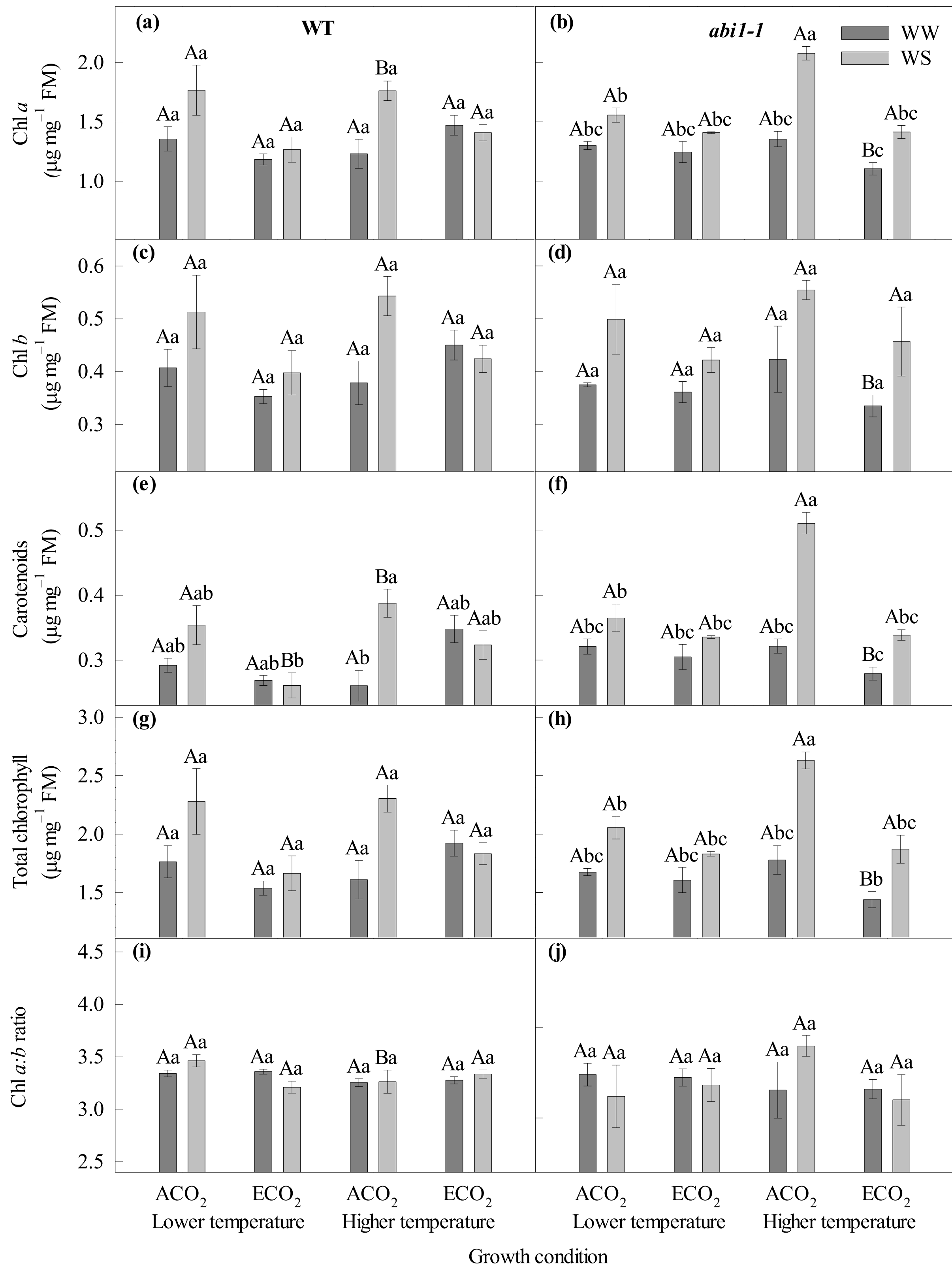
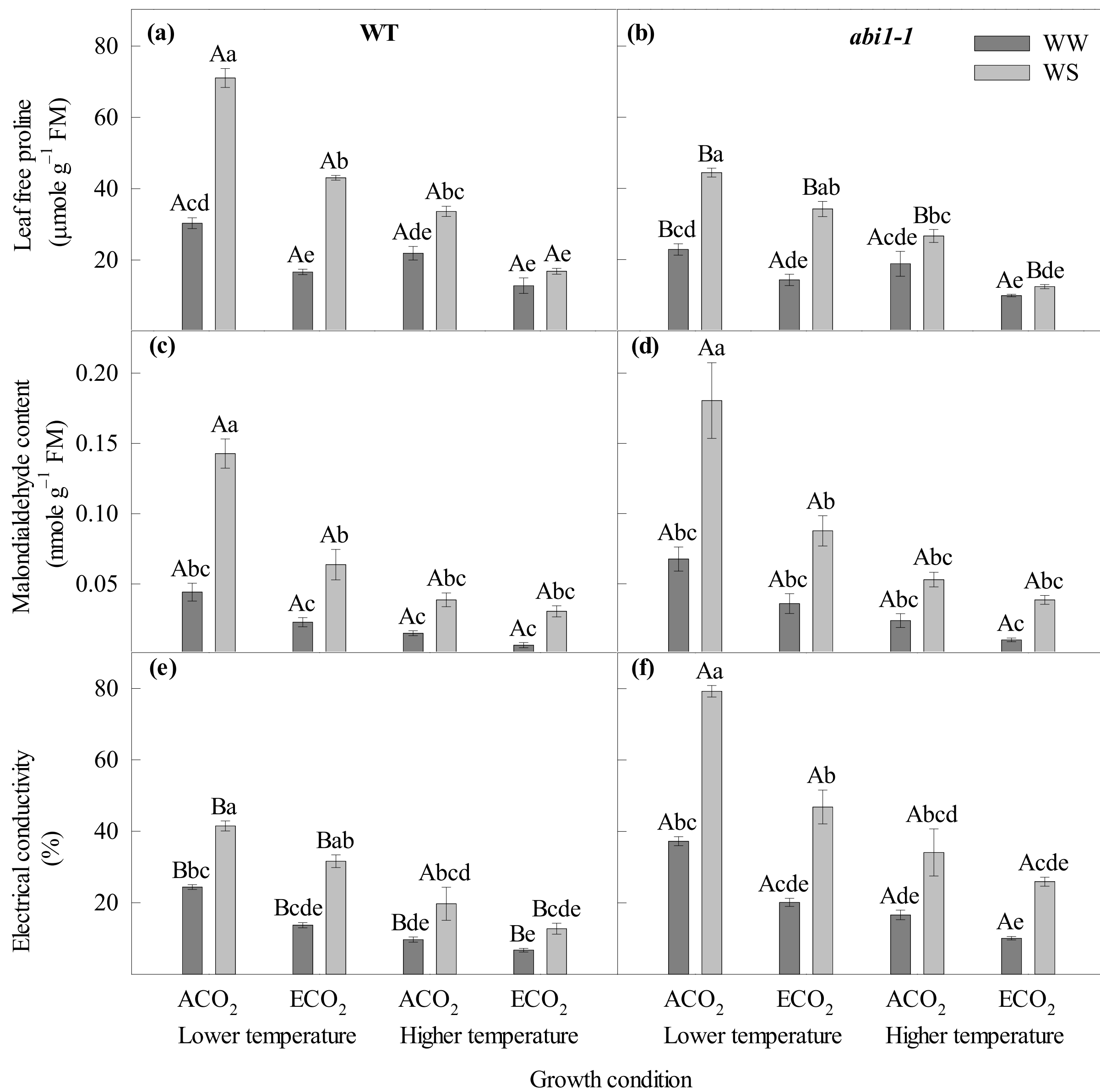

(a)
WT
(b)
abi1-1
WW
WS
Chl a
(μg mg−1 FM)
Chl b
(μg mg−1 FM)
Carotenoids
(μg mg−1 FM)
Total chlorophyll
(μg mg−1 FM)
Chl a:b ratio
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
ACO2
ECO2
Lower temperature
Higher temperature
Growth condition

Fig. 2.7 Effects of temperature, carbon dioxide, and watering regime on leaf free proline, malondialdehyde and electrical conductivity of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Leaf free proline (**a**–**b**), malondialdehyde content (**c**–**d**), and electrical conductivity (**e**–**f**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of in total nine samples from three different trials (three samples per trial for all measured parameters)


(a)
WT
(b)
abi1-1
WW
WS
Leaf free proline (μmole g⁻¹ FM)
80
60
40
20
Aa
Acd
Ab
Ae
Abc
Ade
Ae
Ae
Ba
Bcd
Bab
Ade
Bbc
Acde
Ae
Bde
(c)
(d)
Malondialdehyde content (nmole g⁻¹ FM)
0.20
0.15
0.10
0.05
Aa
Abc
Ab
Ac
Abc
Ac
Abc
Ac
Aa
Abc
Ab
Abc
Abc
Abc
Ac
Abc
(e)
(f)
Electrical conductivity (%)
80
60
40
20
Ba
Bbc
Bab
Bcde
Abcd
Bde
Bcde
Be
Aa
Abc
Ab
Acde
Abcd
Ade
Acde
Ae
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
Growth condition

2.4.7 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 2.1). Based on C × W × G (Table 2.5), 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. 2.8a–b).

2.4.8 Abscisic acid and cytokinins

Higher temperatures and water stress increased ABA, whereas ECO_2 reversed their effects (Table 2.1). Interestingly, the *abi1-1* plants had higher ABA than the WT plants (Table 2.1, Fig. 2.8c–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 *abi1-1* plants (Fig. 2.8c–d).

Total CKs were increased only by ECO_2 (Table 2.1). On the basis of C × W × G interaction (Table 2.5), 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. 2.8e–f).

Detailed analysis of free base CKs revealed the presence of transZeatin and isopentyladenine in both genotypes (Table 2.5). Higher temperatures and water stress significantly increased isopentyladenine. The WT plants had higher transZeatin and isopentyladenine than the *abi1-1* plants (Table 2.1). On the basis of C × G (Table 2.5), ACO_2 resulted in highest transZeatin in the WT plants, but ECO_2 resulted in lowest transZeatin in the *abi1-1* plants. Based on C × W × G (Table 2.5), the well-watered WT plants at ACO_2 had highest transZeatin, whereas the water-stressed WT plants at ECO_2 had lowest transZeatin. The T × C interaction (Table 2.5) revealed that plants under higher temperatures at ACO_2 had highest isopentyladenine, while plants under

lower temperatures at ACO_2 had lowest isopentyladenine. On the basis of C × W × G (Table 2.5), 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).

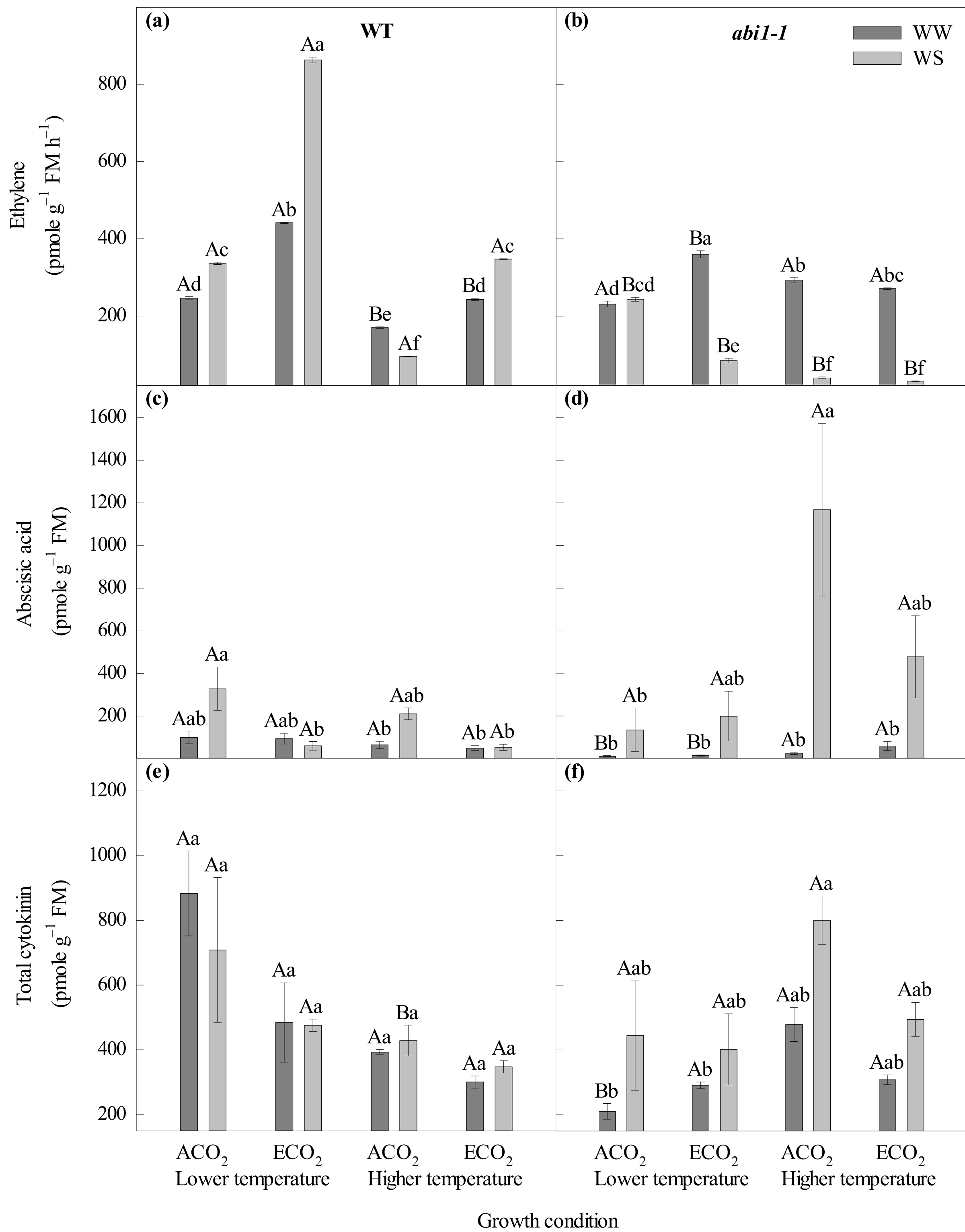
Four major riboside CKs, trans-zeatin, cis-zeatin riboside, dihydrozeatin and isopentenyladenosine riboside, were detected in the WT and *abi1-1* mutant plants. ECO_2 decreased trans-zeatin riboside, which was significantly affected by the main factors and their interactions, except by T × C (Table 2.5). Based on four-way interaction, 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 2.1), but it was not affected by other factors (Table 2.5). Dihydrozeatin riboside was significantly increased by higher temperatures and water stress (Table 2.1). In contrast to cis-zeatin riboside, dihydrozeatin riboside was significantly higher in the *abi1-1* plants than in the WT plants (Table 2.1). Based on C × W × G (Table 2.5), 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).

Table 2.5 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 x 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 x W	1	2.83	0.52	0.02	0.89	4.97	26.38**	0.39	1.37	0.29
C x W	1	50.34**	1.82	40.99**	0.10	1.90	281.27****	1.99	0.27	7.48
T x C x 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 x G	1	2.30	0.65	0.00	1.92	1.46	36.30***	0.85	0.57	0.69
C x G	1	32.29***	2.45	101.50****	9.37*	14.63**	185.99****	3.05	1.94	9.80*
T x C x G	1	3.12	0.48	0.12	1.76	1.05	40.80***	0.94	0.46	0.92
W x G	1	33.49***	2.53	105.57****	1.70	0.81	140.78****	2.51	0.96	9.31*
T x W x G	1	2.46	0.61	0.00	1.84	1.35	36.63***	0.86	0.53	0.73
C x W x G	1	31.41***	2.38	98.44****	22.96**	44.65***	239.47****	3.68	3.27	10.40*
T x C x W x 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	–	–	–	–	–	–	–	–	–

Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. *A. thaliana* plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 eight days of initial growth under 22/18ºC. 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 wild-type 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)

Fig. 2.8 Effects of temperature, carbon dioxide and the watering regime on the leaf hormone concentrations of 18-day-old *A. thaliana* plants. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Ethylene (**a**–**b**), abscisic acid (**c**–**d**), and total cytokinins (**e**–**f**). Different letters above the bars (mean ± SE) denote significant differences within each parameter according to Scheffé's multiple–comparison procedure. Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes. Data are means ± SE of nine samples from three different trials (three samples per trial for all measured parameters)

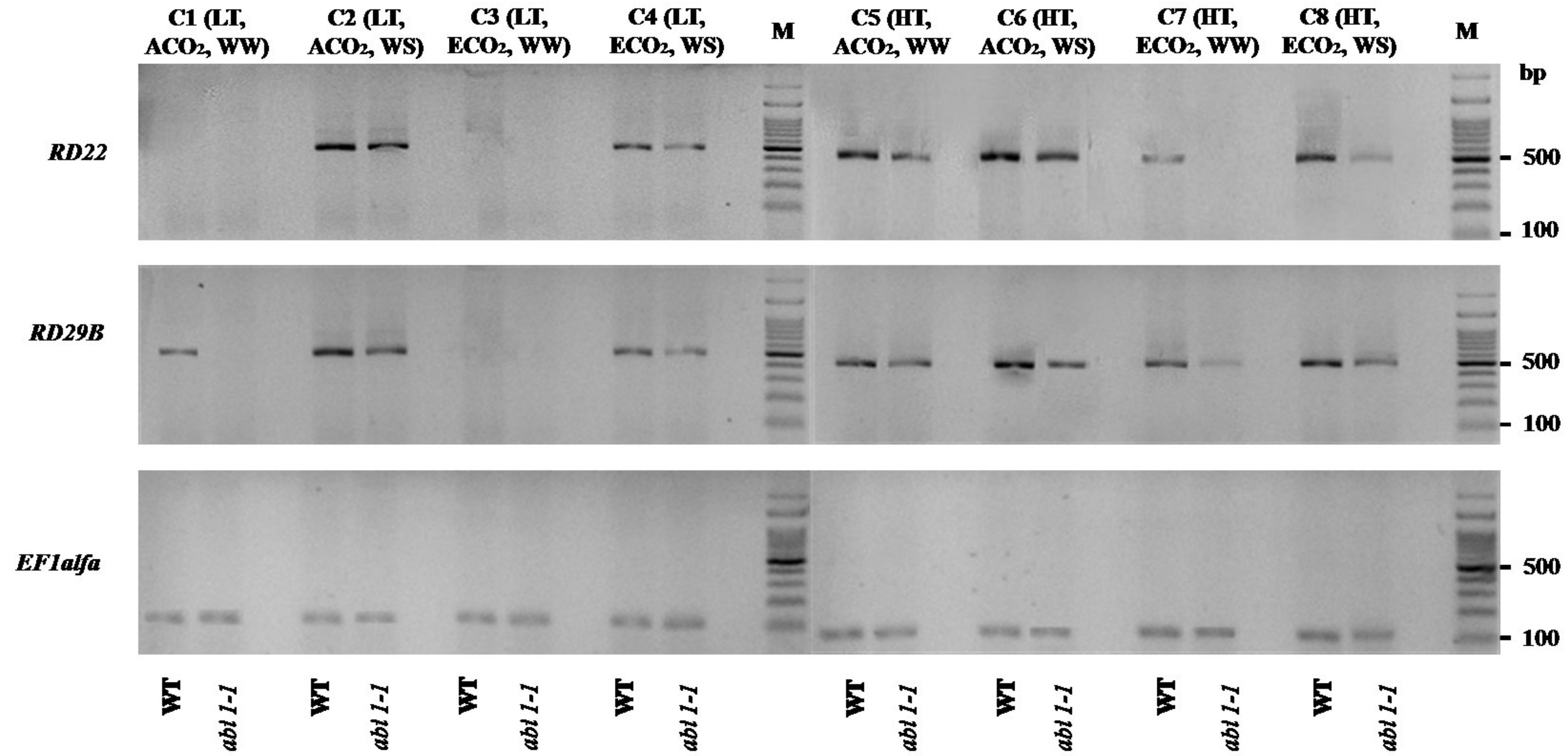


(a)
WT
(b)
abi1-1
WW
WS
Ethylene
(pmole g⁻¹ FM h⁻¹)
800
600
400
200
Aa
Ab
Ac
Ad
Be
Af
Bd
Ac
Ad
Bcd
Ba
Be
Ab
Bf
Abc
Bf
(c)
(d)
Abscisic acid
(pmole g⁻¹ FM)
1600
1400
1200
1000
800
600
400
200
Aa
Aab
Aab
Ab
Ab
Aab
Ab
Ab
Bb
Ab
Bb
Aab
Ab
Aa
Ab
Aab
(e)
(f)
Total cytokinin
(pmole g⁻¹ FM)
1200
1000
800
600
400
200
Aa
Aa
Aa
Aa
Aa
Ba
Aa
Aa
Bb
Aab
Ab
Aab
Aab
Aa
Aab
Aab
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
Growth condition

2.4.9 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. 2.9. 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. 2.9, C7–C8). *RD22* and *RD29B* genes were activated by stress conditions and relatively maintained similar patterns of induction (Fig. 2.9). 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* plants under lower temperatures at ACO_2, or under higher temperatures at ACO_2 (Fig. 2.9).

Fig. 2.9 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. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Expression of the *EF1alfa* gene was used as a constitutive internal control. C1 (control): lower temperatures, ambient CO_2, well-watered, C2: lower temperatures, ambient CO_2, water-stressed, C3: lower temperatures, elevated CO_2, well-watered, C4: lower temperatures, elevated CO_2, water-stressed; C5: higher temperatures, ambient CO_2, well-watered, C6: higher temperatures, ambient CO_2, water-stressed, C7: higher temperatures, elevated CO_2, well-watered, and C8: higher temperatures, elevated CO_2, water-stressed


C1 (LT, ACO2, WW)
C2 (LT, ACO2, WS)
C3 (LT, ECO2, WW)
C4 (LT, ECO2, WS)
M
C5 (HT, ACO2, WW
C6 (HT, ACO2, WS)
C7 (HT, ECO2, WW)
C8 (HT, ECO2, WS)
M
bp
RD22
RD29B
EF1alfa
500
100
500
100
500
100
WT
abl 1-1

2.4.10 Relationship between plant parameters

Pearson's correlation analysis revealed significant ($P < 0.05$) relationships between plant parameters (Table 2.6). For instance, 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 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 ($r = –0.548$).

Table 2.6 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	-

Significance values: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *A. thaliana* plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 eight days of initial growth under 22/18ºC. Ψ, water potential; EC, electrical conductivity

2.5 Discussion

Climate change is inevitable within our lifetime, even if the anthropogenic production of greenhouse gases is stopped today (Stocker et al. 2013). A large number of studies have considered the effects of individual components of climate change on plants (Wu et al. 2011), but very few have assessed the interactive effects of stress factors, such as higher temperature and water stress, with factors, such as elevated CO_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 Chl *a* and ABA, but decreased rosette diameter, leaf number and area, biomass of all plant parts, stomatal and cell density, proline, MDA, electrical conductivity, and ethylene evolution (Table 2.1). Reduced growth that resulted in decreased biomass of all plant parts under higher temperatures coincides with previous results on other plant species (Sacks and Kucharik 2011; 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 average 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). In spite of the higher concentrations of Chl *a* under higher temperatures, our plants had lower growth and total biomass (Tables 2.1, 2.6). It was suggested that heat stress deactivated Rubisco, which would further reduce biomass through reduced photosynthetic capacity (Dutta et al. 2009). It is also possible that higher temperatures caused plants to allocate more photosynthate for metabolic activities, which helped plants cope with stress factors and, therefore, less photosynthate allocated to growth and biomass

for these plants. The reduction of proline level under higher temperatures (Table 2.1) is consistent with earlier studies on Arabidopsis (Rizhsky et al. 2004). Furthermore, *A. thaliana* plants grown under the higher temperature stress have been shown to accumulate sucrose instead of proline (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 2.1, Fig. 2.7e–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 temperatures decreases ethylene evolution (Yu et al. 1980), but increases ABA level (Qaderi et al. 2006), which could have negatively affected growth and biomass of our plants. LeNoble et al. (2004) reported that ABA partially maintains shoot growth by suppressing ethylene evolution. Stomatal conductance, transpiration, and carbon dioxide assimilation are all negatively affected by increased ABA (Reddy et al. 2004).

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 (Table 2.1). 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 (Long et al. 2004). ECO_2 has also been reported to increase leaf size (Qaderi and Reid 2005). Our results support earlier reports, showing reduced stomatal density (Woodward and Kelly 1995; 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 chlorophyll (Table 2.6) 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 less 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 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, we observed a negative correlation between ABA and rosette diameter (Table 2.6). 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. 2.9, C7–C8), without affecting ABA (see fig. 2.8c–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, proline, MDA, electrical conductivity, ABA, isopentyladenine, and dihydrozeatin riboside, but decreased leaf number and area, plant dry mass, cell area, leaf and soil water potential, leaf moisture, and ethylene evolution (Table 2.1). Effects of water stress on growth and biomass are consistent with findings on canola and other species (Rodrıguez et al. 2005; 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 2.6) indicates

that increased transpiration because of high stomatal density 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 (Lambers et al. 2008) 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 our plants. ABA accumulation under water stress is a normal and expected plant reaction, which has been shown to restrict ethylene production or responsiveness, and therefore reduced growth (Sharp et al. 1998). 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 cytokinins isopentyladenine and dihydrozeatin riboside increased in water-stressed plants (Table 2.1). 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. 2.9). Activation of these genes by ABA occurs through a specific group of transcription factors, which bind 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, transZeatin, 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 between genotypes due to genetic make-up 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 concentration than the WT plants (Table 2.1). A positive correlation between ABA and carotenoids (Table 2.6) 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 concentrations of transZeatin, isopentyladenine, and cis-zeatin riboside, but higher dihydrozeatin riboside, than the WT plants (Table 2.1), 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 cytokinin degradation enzymes and this can be the reason why we don't see them decreasing as fast as the other CK fractions do under water stress and higher temperatures.

In the current study, we found a pattern of responses to the interactive effects of temperature, CO_2, watering regime, and genotype. 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 and leaf biomass were decreased by higher temperatures and water stress, individually and together, but the negative effects of these two factors was less on 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, shows 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 times 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. 2.8c), 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, irrespective 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's ability to close stomata and prevent transpiration and, in turn, plant biomass (Qaderi et al. 2006). On the other hand, increased accumulation of ABA in the *abi1-1* mutant under the combination of higher temperatures and water stress (Fig. 2.8d) might be because this genotype does not sense the presence of this stress hormone. Our results 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 had less stress and therefore less expression of these two genes. ABA-responsive genes encode different

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

2.6 Conclusion

Water stress and ECO_2 have larger effects, negative and positive, respectively, on plants than do higher temperatures; plants are also influenced by interactions of these factors. ECO_2 reduces stress effects by increasing antioxidant activity and plant water status, 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. Results indicate that the positive effects of ECO_2 could come from increased photosynthesis and antioxidant activity. 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. Interaction of watering regime with higher temperatures negatively affects the ability of WT plants to accumulate ABA and this negatively affects their performance under stress conditions as ABA is involved in plant tolerance to environmental stress.

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CHAPTER 3

SINGLE AND INTERACTIVE EFFECTS OF TEMPERATURE, CARBON DIOXIDE AND WATERING REGIME ON PLANT GROWTH AND REPRODUCTIVE YIELD OF TWO GENOTYPES OF *ARABIDOPSIS THALIANA*

3.1 Abstract

Premise of research. Few studies have investigated the combined effects of temperature, carbon dioxide (CO_2), and watering regime on plants during vegetative stage, but the effects of these factors on plants during reproductive stage have received little attention and deserve further studies.

Methodology. Plants of *Arabidopsis thaliana* (wild-type and *abi1-1*, abscisic acid (ABA)-insensitive mutant) were grown 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 45 days. Plant growth (stem height and diameter), reproductive yield (silique number, mass, length, and width; sound and aborted seed number; sound and aborted seed mass together), and seed amino acids were measured.

Pivotal results. Higher temperatures and water stress decreased plant growth and yield, and only water stress increased aborted seed number. Elevated CO_2 increased silique width and mass, but decreased stem height and aborted seed number. With the exception of aborted seed number, all measured growth and reproductive yield parameters were higher for the WT plants than for the *abi1-1* plants. Stem diameter, and silique width and mass were lowest in the water-stressed *abi1-1* plants grown under higher temperatures at elevated CO_2. Under higher temperatures, elevated CO_2 and water stress, the *abi1-1* plants produced seeds with lower amino acids than the

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WT plants. Most amino acids were increased by higher temperatures and elevated CO_2. Seeds of the *abi1-1* mutant had higher leucine and valine than seeds of the WT plants.

Conclusions. Temperature had more effects on seed quality than elevated CO_2 or water stress. Elevated CO_2 partially mitigated the negative effects of stresses on some parameters in both genotypes, with a larger extent in the WT plants than in the *abi1-1* mutant, indicating the role of ABA in the process.

Keywords: Arabidopsis thaliana, carbon dioxide, climate change, seed quality, temperature, water stress.

3.2 Introduction

Elevated atmospheric carbon dioxide (CO_2), higher air temperature, and drought are the main components of climate change (Qaderi et al. 2006; Stocker et al. 2013). With the massive influx of CO_2 as a result of anthropogenic emissions, its total concentration in the Earth's atmosphere is expected to surpass 700 μmol mol^{-1} by the end of this century, increasing air temperature by 1-6°C, depending on the climate model (Stocker et al. 2013). Elevated CO_2 has been reported to increase photosynthesis and, in turn, plant height and biomass of aerial and underground organs (Oliveira et al. 2010), although effects on seed production are less understood. Seed development is one of the sensitive stages of plant life cycle (Linkies et al. 2010). It has been reported that elevated CO_2 positively affects reproductive characteristics, such as seed size, seed set, yield index and pollen viability (Prasad et al. 2006). Elevated CO_2 has been shown to mitigate the negative effects of environmental stress, such as higher temperature and drought, during plant vegetative stage (Qaderi et al. 2006). However, the ameliorative effects of elevated CO_2 on plants exposed to high temperatures or drought stress during reproductive stage is not well understood. The mitigative effects of CO_2 can be decreased simultaneously by higher temperature and drought in the long run (Albert et al. 2011).

Temperature is the most important factor affecting the production and subsequent germinability of seeds (Hedhly et al. 2009). DaMatta et al. (2010) stated that, in regions of low latitude, a modest increase of 1-2°C adversely affects crop yield. Devasirvatham et al. (2012) have shown that, in chickpea (*Cicer arietinum* L.), heat stress decreased pollen viability and production and, in turn, pod set. Higher temperature can indirectly reduce the positive effects of elevated CO_2 by increasing the need for water (Qaderi and Reid 2009).

Drought is considered as one of the most damaging stress to plants, and global warming has indicated its increased occurrence in many parts of the world (Qaderi et al. 2012). Drought causes plants to adjust physiological processes in order to adapt to moderate stress levels; damage to, or loss of, plant parts may occur during extreme drought levels (Sangtarash et al. 2009). In addition, drought reduces the seed-filling period, thus resulting in seeds with smaller size (Frederick et al. 1991).

It has been widely documented that seed quality is affected by environmental factors, particularly during seed filling when seeds accumulate chemicals (Carrera et al. 2011). Carbon-to-nitrogen ratio (C/N ratio) has been reported to increase under elevated CO_2 concentrations (Steinger et al. 2000). This alteration in C/N ratio results in decreased amount of seed protein, and consequently a reduction in the amino acids essential for embryonic development in the growing seeds (Andalo et al. 1996). Earlier studies have shown the relationship of amino acid accumulation to temperature (Piper and Boote 1999) and availability of water (Carrera et al. 2009), but results have been inconsistent and mostly originated from single factor studies.

To date, most studies of plant responses to the main components of climate change have focused on the effects of individual factors on phenology, photosynthesis, water relation, organ formation, dry matter accumulation, metabolism of carbon and nitrogen, and grain yield (e.g., Teng et al. 2006; Jagadish et al. 2007; Ahmadi et al. 2015). Few studies have considered the combined effects of temperature, CO_2, and watering regime on plants (Roy et al. 2016; Zinta et al. 2018), and very few studies on seed quality (Sanhewe et al. 1996; Madan et al. 2012). However, none of these studies have used mutant-based approach to examine the interactive effects of these factors on seed quality. We hypothesized that higher temperature and water stress decrease plant growth, reproductive yield and seed quality of *Arabidopsis* despite the mitigative

effects of elevated CO_2. Our objectives were to study the effects of these three factors, as individuals and in combination, on two genotypes of *Arabidopsis thaliana* (wild type and abscisic acid (ABA)-insensitive mutant, *abi1-1*) to asses changes in plant growth and reproductive yield.

3.3 Materials and Methods

3.3.1 *Plant Material and Growth Conditions*

In this study, two genotypes of *A. thaliana* ecotype Landsberg erecta (wild-type and *abi1-1* mutant) were used. The *abi1-1* mutant was used to evaluate the ABA involvement in plant responses to the interactive effects of temperature, CO_2, and watering regime during their growth and reproduction. *A. thaliana* seeds were imbibed for 15 min in distilled water, then treated with 95% ethanol for 5 min and germinated in Petri dishes containing liquid Murashige and Skoog basal medium (MS). Petri dishes were placed in a controlled-environment growth chamber (model ATC26, Conviron, Controlled Environments Ltd., Winnipeg, MB, Canada) under 22°/18°C (16h light/8h dark) and photosynthetic photon flux density (PPFD) of 300 photons m^{-2} s^{-1}, and monitored for six days. At the four-leaf stage, the seedlings were transplanted to pots containing a mixture of Perlite, Vermiculite, and peat moss (1:1:1, by volume). Plants were watered with tap water and were supplemented with 30 pellets of slow-release NPK fertilizer; each granule contained all 3 of NPK, (13-14-14; 0.208g N:0.224g P:0.224g K/30 pellets); Chisso-Asahi Fertilizer Co, Tokyo, Japan) every week, the seedlings were grown under the above-mentioned conditions for two more days. After that, eight-day-old seedlings were randomly assigned to lower (22°C light/18°C dark) or higher (28°C light/24°C dark) temperature regimes. A split-split-split-plot design was used with four factors: temperature, CO_2, watering

regime, and genotype to have eight experimental conditions as following: (1) lower temperatures (22/18°C, 16 h light/ 8 h dark), ambient CO_2 (400 μmol mol^{-1}), and well-watered (watering to field capacity), considered as control; (2) lower temperatures, ambient CO_2, and water-stressed (watering to wilting point); (3) lower temperatures, elevated CO_2 (700 μmol mol^{-1}), and well-watered (4) lower temperatures, elevated CO_2, and water-stressed; (5) higher temperatures (28/24°C, 16 h light/8 h dark), ambient CO_2, and well-watered; (6) higher temperatures, ambient CO_2, and water-stressed; (7) higher temperatures, elevated CO_2, and well-watered; and (8) higher temperatures, elevated CO_2, and water-stressed. Plants were grown under their respective treatment for 45 days in one of the two growth chambers (model ATC26, Conviron, Controlled Environments Ltd., Winnipeg, MB, Canada). In each chamber, two equal size Plexiglas cabinets (60 cm × 65 cm × 50 cm; GE Polymershapes, Dartmouth, NS, Canada) were placed; one with ambient CO_2 and another with elevated CO_2 (Air Liquide, Dartmouth, NS, Canada). An electrical fan was used to keep CO_2 circulation in each cabinet constant. Half of the plants in each cabinet were well-watered, and the other half were water-stressed. In each cabinet, PPFD, photoperiod, and RH were similar to the initial growth conditions (Qaderi et al. 2013). In each of the three trials, a different combination of chamber-cabinet was used for each treatment (with five plants), and trays were rotated within the cabinets twice per week, to minimize positional effects. Experiments were conducted three times, and each time for six weeks.

3.3.2 *Growth Characteristics*

Stem height was measured for five plants with average height of both genotypes using a ruler. Stem diameter was also measured for the same plants using a Digimatic caliper (Mitutoyo Corp. Kanagawa, Japan) placed at the midway point between soil and the top end of the stem.

3.3.3 *Reproductive Yield Characteristics*

For each treatment, five six-week-old wild-type (WT) and *abi 1-1* plants were used to have a mean value for the following traits: number of siliques per plant, silique width and length, seed number and category (sound or aborted) per silique, 200-seed mass, and a 100-silique mass. For each of the five plants, the total number of siliques was counted. Then, one fully mature silique was randomly harvested from the middle of the main inflorescence of each of the five plants and their length and width were measured. From these siliques seeds were counted, categorized as sound and aborted, and randomly counting 200 seeds and their mass was determined with an analytical balance (model ED224s, Sartorius, Goettingen, Germany). A 100 empty siliques were randomly selected from each of the five plants and dried at 60°C for 24 h in a forced air Fisher Isotemp® Premium oven (model 750F, Fisher Scientific, Nepean, ON, Canada) to determine silique dry mass, using an analytical balance.

3.3.4 *Seed Surface Structure*

The surface of mature seeds that were collected from plants grown under eight treatments were examined with a scanning electron microscope (SEM; Zeiss 1455VP, Jena, Germany), using smart SEM version 5.05 Zeiss software. To view the fine structures with the SEM, three seeds from three different plants were coated with gold-palladium, using a gold coating system (Leica EM ACE200, Wetzlar, Germany).

3.3.5 *Seed Amino Acid Profiling*

From each treatment, dry seeds were ground into a fine powder with a mortar and pestle. Then, 5 mg of ground seeds were weighed, using an analytical balance, and transferred into 4

mL vials. After that, 2.1 mL of methanol:water:chloroform (4:1:2, v/v/v) and 40 μL of triacontane in chloroform (1 mg mL^{-1}) were added to each vial. Next, the samples were vortexed (mini vortexer MV 1, VWR Scientific Products, Mississauga, Ontario, Canada) and incubated at 50°C for one hour at 117 rpm, using a shaker (MAXQ 4450, Thermo Scientific, Waltham, Massachusetts, USA). After samples were cooled to room temperature, 0.9 mL of distilled water and 20 μL of ribitol in water (2 mg mL^{-1}) were added to each vial. The samples were again vortexed before being incubated at 50°C for one hour at 117 rpm. Then, the samples were transferred to Eppendorf tubes and centrifuged (Heraeus Fresco 17 Centrifuge, Thermo Scientific, Waltham, Massachusetts, USA) for 30 min at 4°C at 2900 × *g*. The upper aqueous polar layer was collected and transferred to 2 mL glass vials, while the lower chloroform non-polar layer was discarded. The polar layer was evaporated by a freeze-dryer (VirTis BenchTop Pro with Omnitronics™, SP Scientific, Gardiner, NY). After the sample was dried, 50 μL of methoxyamine-HCl (20 mg mL^{-1} in pyridine) was added to the vial. The sample was then vortexed for 45 sec and incubated at 50°C in a shaker at 117 rpm for one hour. Then, 50 μL of MSTFA + 1% TMCS (Thermo Scientific, Bellemonte, PA, USA) was added. Finally, the sample was vortexed for 45 sec and incubated at 50°C in a shaker at 117 rpm for one hour. Following incubation, each of the samples was transferred to a vial for analysis. Samples were injected (1 μL) into the GC-MS (model 7820A, Agilent Technologies, Santa Clara, CA, USA), equipped with a capillary column (HP-5, 30 m × 0.25 mm ID; Agilent Technologies, Santa Clara, CA, USA). High purity helium gas (at a flow rate of 1 mL min^{-1}) was used as a carrier gas. The temperature program was a five-min delay at 70°C, followed by the oven temperature increasing to 310°C at 5°C min^{-1}. Then, the temperature was held at 310°C for 6 min, before cooling to 70°C and held for five min, giving an overall analysis time of 64 min per sample.

3.3.6 *Data Analysis*

The effects of temperature, carbon dioxide, watering regime, and their interactions were determined on growth, reproductive yield and seed amino acids of two Arabidopsis genotypes (wild-type and *abi1-1* mutant), using analysis of variance (ANOVA) for split-split-split-plot design (SAS Institute 2011). For this analysis, temperature regime was treated as the main plot, CO_2 as the subplot, watering regime as the split-subplot, and genotype as the split-split-subplot and growth chambers (trials) as replication. A one-way ANOVA was used to determine differences between/among experimental treatments, according to Scheffé's multiple-comparison procedure at the 5% confidence level (SAS Institute 2011). Pearson's correlation coefficient was used to determine relationships between parameters at the 5% confidence level (Minitab Inc 2014). Data are reported as mean ± standard error.

3.4 Results

3.4.1 *Plant Growth*

Plants that were grown under higher temperatures, elevated CO_2 or water stress were shorter, respectively, than plants that were grown under lower temperatures, ambient CO_2 or watering to field capacity (Table 3.1). The WT plants were taller than the *abi1-1* plants (Table 3.1). Carbon dioxide, watering regime, genotype, the two-way interactions between carbon dioxide (C) × watering regime (W), C × genotype (G), and W × G, and the three-way interaction among C × W × G significantly affected stem height (Table 3.2). Based on three-way interaction among C × W × G, (Table 3.2), the well-watered WT plants at ambient CO_2 had tallest stems, whereas the water-stressed *abi1-1* plants at ambient CO_2 had shortest stems (Table 3.2; Fig. 3.1*A–B*).

Higher temperatures and water stress decreased stem diameter (Table 3.1). The WT plants had thicker stems than the *abi1-1* plants (Table 3.1). The stem diameter was significantly affected by watering regime, genotype, the two-way interactions of C × W, T × G, C × G, and W ×G, the three-way interactions of T × C × G and T × W × G, and the four-way interaction (Table 3.2). On the basis of four-way interaction, the well-watered WT plants grown under lower temperatures at elevated CO_2 had the thickest stems, whereas the water-stressed *abi1-1* plants grown under higher temperatures at elevated CO_2 had the thinnest stems (Table 3.2; Fig. 3.1*C*–*D*).

3.4.2 *Reproductive Yield*

Plants grown under higher temperatures or water stress had significantly fewer and shorter siliques than plants grown under lower temperatures or watering to field capacity (Table 3.1). The number and length of siliques were higher for the WT plants than for the *abi1-1* plants (Table 3.1). Watering regime, genotype, and the two-way interactions of C × W were all significant for silique number, but not for silique length (Table 3.2). On the basis of interaction among C × W × G (Table 3.2), the well-watered WT plants at elevated CO_2 produced the highest number of siliques, whereas the water-stressed *abi1-1* plants at ambient CO_2 produced the lowest number of siliques (Fig. 3.2*A*–*B*). Based on four-way interaction, the well-watered WT plants at elevated CO_2 had longest siliques, whereas the water-stressed *abi1-1* plants at elevated CO_2 had shortest siliques (Fig. 3.2*E*–*F*).

Elevated CO_2 increased, but higher temperatures and water stress decreased, silique mass and width, which were higher for the WT plants than for the *abi1-1* plants (Table 3.1). Overall, the mass of siliques was significantly affected by CO_2 and the two-way interaction between T × W,

and the three-way interaction among T × C × W (Table 3.2). Watering regime and genotype significantly affected the mass and width of siliques (Table 3.2). The two-way interactions between C × W, T × G, C × G, and W × G, and the three-way interactions among T × C × G and T × W × G, and the four-way interaction significantly affected the mass and width of siliques (Table 3.2). Based on four-way interaction (Table 3.2), the well-watered WT plants under lower temperatures at elevated CO_2 produced siliques with highest mass and width, whereas the water-stressed *abi1-1* plants under higher temperatures at elevated CO_2 produced siliques with lowest mass and width (Table 3.2; Fig. 3.2*C–D*, *G–H*).

Plants grown under higher temperatures or water stress had significantly smaller seeds and fewer sound seeds than plants grown under lower temperatures or watering to field capacity (Table 3.1). The WT plants produced more sound seeds, which were significantly heavier, than the *abi1-1* plants (Table 3.1). Seed mass and sound seed number were significantly affected only by the interaction among C × W × G (Table 3.2). Based on this interaction (Table 3.2), seed mass and sound seed number were highest for the well-watered WT plants at elevated CO_2, but seed mass was lowest for the water-stressed *abi1-1* plants at ambient CO_2 (Fig. 3.3*E–F*), whereas sound seed number was lowest for the water-stressed *abi1-1* plants at elevated CO_2 (Fig. 3.3*A–B*).

Water stress increased the number of aborted seeds, whereas elevated CO_2 decreased it (Table 3.1). Plants of the *abi1-1* genotype produced more aborted seeds than the WT plants (Table 3.1). Overall, the number of aborted seeds was significantly affected by CO_2, watering regime, genotype, the two-way interactions of C × G and C × W, and the three-way interaction of C × W × G (Table 3.2). With regards to the C × W × G (Table 3.2), the water-stressed *abi1-1*

plants at ambient CO_2 produced the highest number of aborted seeds, whereas the well-watered WT plants at elevated CO_2 produced the lowest number of aborted seeds (Fig. 3.3*C*–*D*).

Table 3.1

Effects of Temperature, Carbon Dioxide, Watering Regime, and Genotype on Growth and Reproductive Yield of *Arabidopsis thaliana*

Parameter	Temperature		Carbon dioxide		Watering regime		Genotype	
	Lower	Higher	Ambient	Elevated	Well-watered	Water-stressed	Wild-type	*abi1-1* mutant
Stem height (cm)	32.05 ± 1.46a	24.64 ± 1.59b	29.15 ± 1.50a	27.54 ± 1.88b	34.03 ± 1.42a	22.66 ± 1.02b	30.47 ± 1.69a	26.22 ± 1.62b
Stem diameter (mm)	0.92 ± 0.06a	0.58 ± 0.04b	0.73 ± 0.04a	0.77 ± 0.08a	0.92 ± 0.06a	0.58 ± 0.04b	0.81 ± 0.06a	0.69 ± 0.05b
Silique number (plant^{-1})	317.05 ± 31.53a	148.11 ± 20.74b	229.11 ± 28.75a	236.05 ± 34.89a	346.43 ± 26.95a	118.73 ± 13.84b	252.29 ± 32.39a	212.87 ± 31.01b
Silique mass (g siliques^{-100})	0.07± 0.01a	0.04 ± 0.00b	0.04 ± 0.00b	0.05 ± 0.01a	0.07 ± 0.01a	0.03 ± 0.00b	0.06 ± 0.01a	0.04 ± 0.00b
Silique length (mm)	6.29 ± 0.52a	3.43 ± 0.29b	4.69 ± 0.41a	5.03 ± 0.60a	6.51 ± 0.48a	3.21 ± 0.24b	5.38 ± 0.52a	4.34 ± 0.48b
Silique width (mm)	0.64 ± 0.05a	0.39 ± 0.03b	0.49 ± 0.03b	0.55 ± 0.06a	0.67 ± 0.04a	0.37 ± 0.02b	0.56 ± 0.05a	0.47 ± 0.05b
Sound seed no. (plant^{-1})	32.19 ± 3.68a	10.49 ± 1.64b	20.15 ± 2.56a	22.53 ± 4.45a	31.75 ± 3.82a	10.93 ± 1.58b	24.38 ± 4.08a	18.30 ± 3.01b
Aborted seed no. (plant^{-1})	4.88 ± 0.55a	5.17 ± 0.85a	6.33 ± 0.81a	3.72 ± 0.47b	3.18 ± 0.35b	6.87 ± 0.77a	3.57 ± 0.47b	6.48 ± 0.79a
Seed mass (mg seeds^{-200})	2.02 ± 0.14a	1.55 ± 0.10b	1.77 ± 0.12a	1.81 ± 0.15a	2.31 ± 0.09a	1.26 ± 0.06b	1.91 ± 0.14a	1.67 ± 0.13b

Note. Arabidopsis plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC. Data are means ± SE of 15 samples from three different trials (five samples per trial for all measured parameters, except for seed mass in which 10 samples of 200 seeds were used per trial). Means followed by different letters within each parameter and condition are significantly different ($P < 0.05$; Scheffé's multiple-comparison procedure).

Table 3.2

Summary of Split-Split-Split-Plot ANOVA (*F* Values) for Effects of Temperature, Carbon dioxide, Watering Regime, Genotype, and Their Interactions on Plant Growth and Reproductive Yield of *Arabidopsis thaliana*

		Plant growth		Reproductive yield						
Source	df	Stem height	Stem diameter	Silique number	Silique mass	Silique length	Silique width	Sound seed number	Aborted seed number	Seed mass
Temperature (T)	1	0.32	12.25	0.97	7.18	0.51	12.74	1.23	0.10	3.13
Main plot error	2	–	–	–	–	–	–	–	–	–
Carbon dioxide (C)	1	37.67*	2.55	7.77	23.32*	6.84	5.88	5.19	37.54*	0.82
T × C	1	0.46	0.32	1.08	1.92	0.55	0.60	1.30	0.00	0.02
Subplot error	2	–	–	–	–	–	–	–	–	–
Watering regime (W)	1	53.08**	43.53**	8.54*	189.50***	6.87	71.84**	5.41	11.35*	0.04
T × W	1	0.25	2.08	0.93	8.10*	0.47	3.28	1.18	0.09	0.33
C × W	1	49.91**	112.17***	8.40*	354.33****	3.96	160.65***	4.98	7.51	4.24
T × C × W	1	0.32	2.20	0.98	8.71*	0.51	3.49	1.22	0.06	0.30
Split-subplot error	4	–	–	–	–	–	–	–	–	–
Genotype (G)	1	33.52***	99.92****	7.71*	393.82****	3.08	141.48****	4.67	11.49**	3.81

T × G	1	0.54	13.66**	0.98	18.16**	2.11	13.08**	1.46	0.57	3.02
C × G	1	35.27***	5.40*	7.40*	75.06****	8.17*	14.33**	5.13	23.74**	1.84
T × C × G	1	0.98	10.28*	1.22	11.59**	2.28	9.34*	1.66	0.78	2.63
W × G	1	34.91***	39.04***	7.67*	207.94****	5.38*	63.08****	4.97	17.27**	0.10
T × W × G	1	0.59	13.57**	1.03	18.74**	2.11	13.10**	1.50	0.53	2.97
C × W × G	1	35.98***	0.00	7.20*	1.96	11.61**	0.00	5.35*	31.49***	8.76*
T × C × W × G	1	1.18	10.12*	1.36	12.85**	2.35	9.42*	1.78	0.67	2.50
Split-split-subplot error	8	–	–	–	–	–	–	–	–	–

Note. Arabidopsis plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC. Experiments were conducted three times. Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Fig. 3.1 Effects of temperature, carbon dioxide and watering regime on plant growth characteristics of 45-day-old plants of *Arabidopsis thaliana*. (*A*–*B*) stem height; and (*C*–*D*) stem diameter. (*A*, *C*) wild-type (WT); and (*B*, *D*) *abi1-1* mutant. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Dark-grey bars represent well-watered plants and light-grey bars water-stressed plants. Data are means ± SE of 15 samples from three different trials (five samples per trial for all measured parameters). Different letters above the bars (mean ± SE) denote significant differences within each parameter ($P < 0.05$; according to Scheffé's multiple-comparison procedure). Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes.

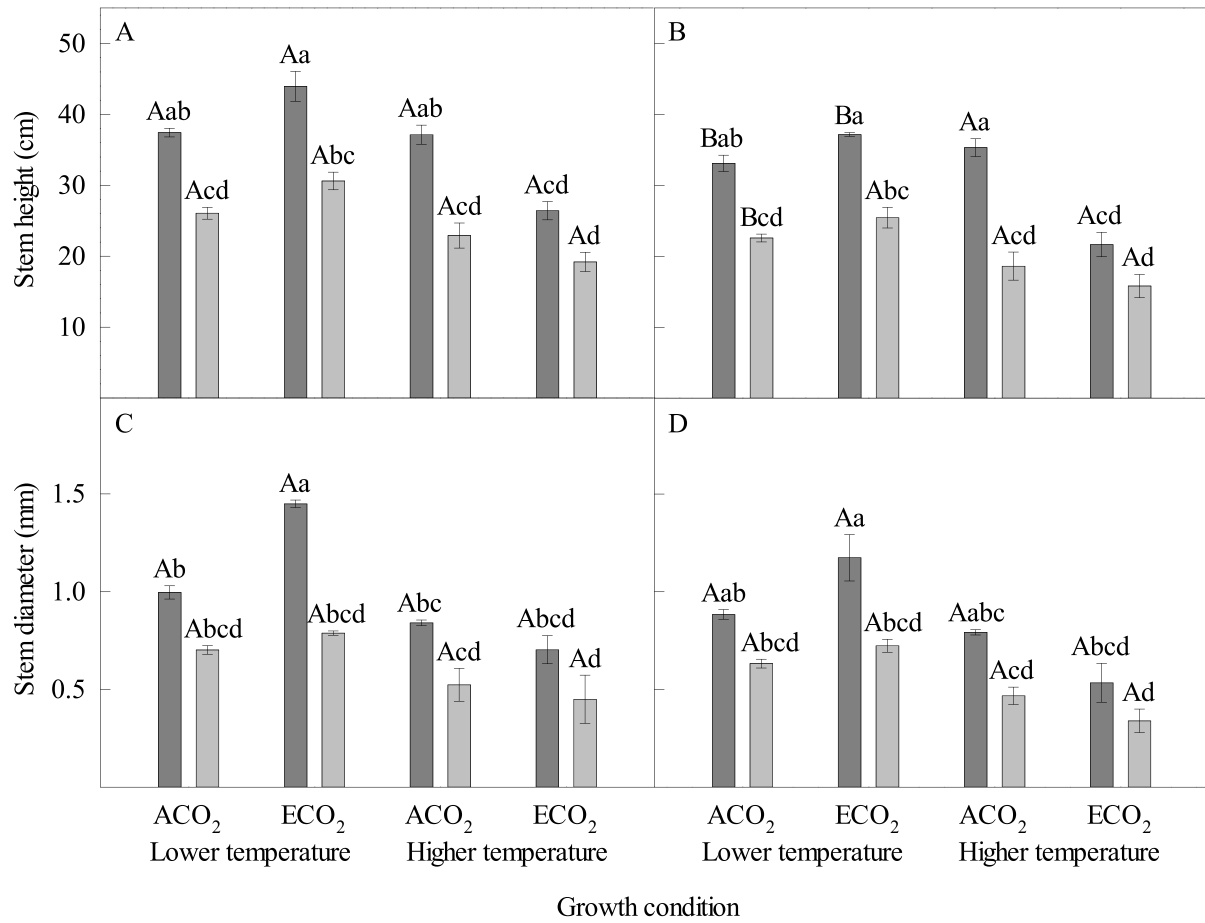
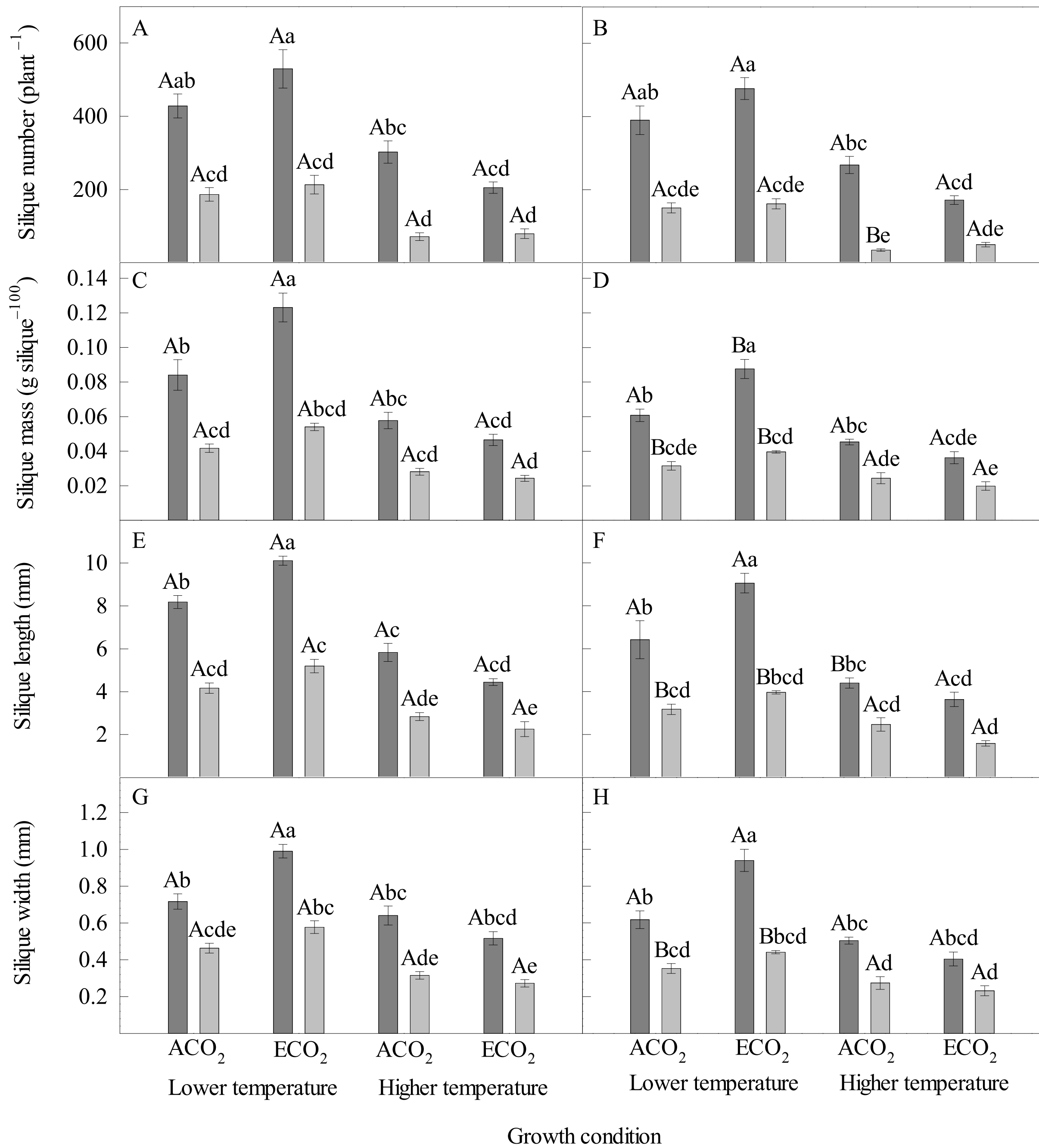
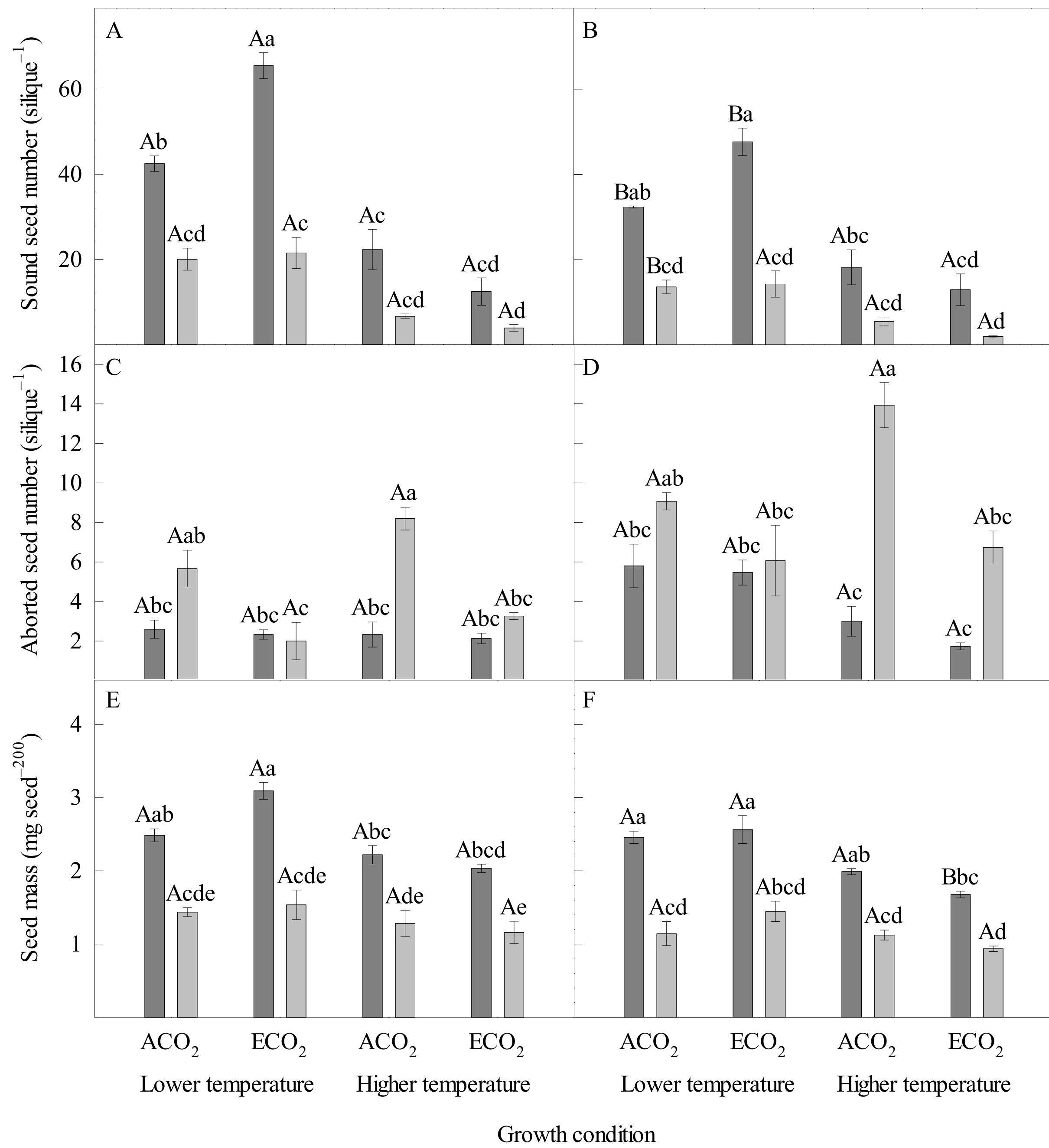

A
B
C
D
Stem height (cm)
Stem diameter (mm)
50
40
30
20
10
1.5
1.0
0.5
Aab
Acd
Aa
Abc
Aab
Acd
Acd
Ad
Bab
Bcd
Ba
Abc
Aa
Acd
Acd
Ad
Ab
Abcd
Aa
Abcd
Abc
Acd
Abcd
Ad
Aab
Abcd
Aa
Abcd
Aabc
Acd
Abcd
Ad
ACO_2
ECO_2
ACO_2
ECO_2
Lower temperature
Higher temperature
ACO_2
ECO_2
ACO_2
ECO_2
Lower temperature
Higher temperature
Growth condition

Fig. 3.2 Effects of temperature, carbon dioxide and watering regime on silique characteristics of 45-day-old plants of *A. thaliana*. (*A–B*) silique number; (*C–D*) silique mass; (*E–F*) silique length; and (*G–H*) silique width. (*A*, *C*, *E*, *G*) WT; and (*B*, *D*, *F*, *H*) *abi1-1*. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. Dark-grey bars represent well-watered plants and light-grey bars water-stressed plants. Data are means ± SE of 15 samples from three different trials (three samples per trial for all measured parameters). Different letters above the bars (mean ± SE) denote significant differences within each parameter ($P < 0.05$; according to Scheffé's multiple-comparison procedure). Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes.



A
B
C
D
E
F
G
H
Silique number (plant^{-1})
Silique mass (g silique^{-100})
Silique length (mm)
Silique width (mm)
Aab
Acd
Aa
Acd
Abc
Ad
Acd
Ad
Aab
Acde
Aa
Acde
Abc
Be
Acd
Ade
Ab
Acd
Aa
Abcd
Abc
Acd
Acd
Ad
Ab
Bcde
Ba
Bcd
Abc
Ade
Acde
Ae
Ab
Acd
Aa
Ac
Ac
Ade
Acd
Ae
Ab
Bcd
Aa
Bbcd
Bbc
Acd
Acd
Ad
Ab
Acde
Aa
Abc
Abc
Ade
Abcd
Ae
Ab
Bcd
Aa
Bbcd
Abc
Ad
Abcd
Ad
ACO_2
ECO_2
Lower temperature
Higher temperature
Growth condition

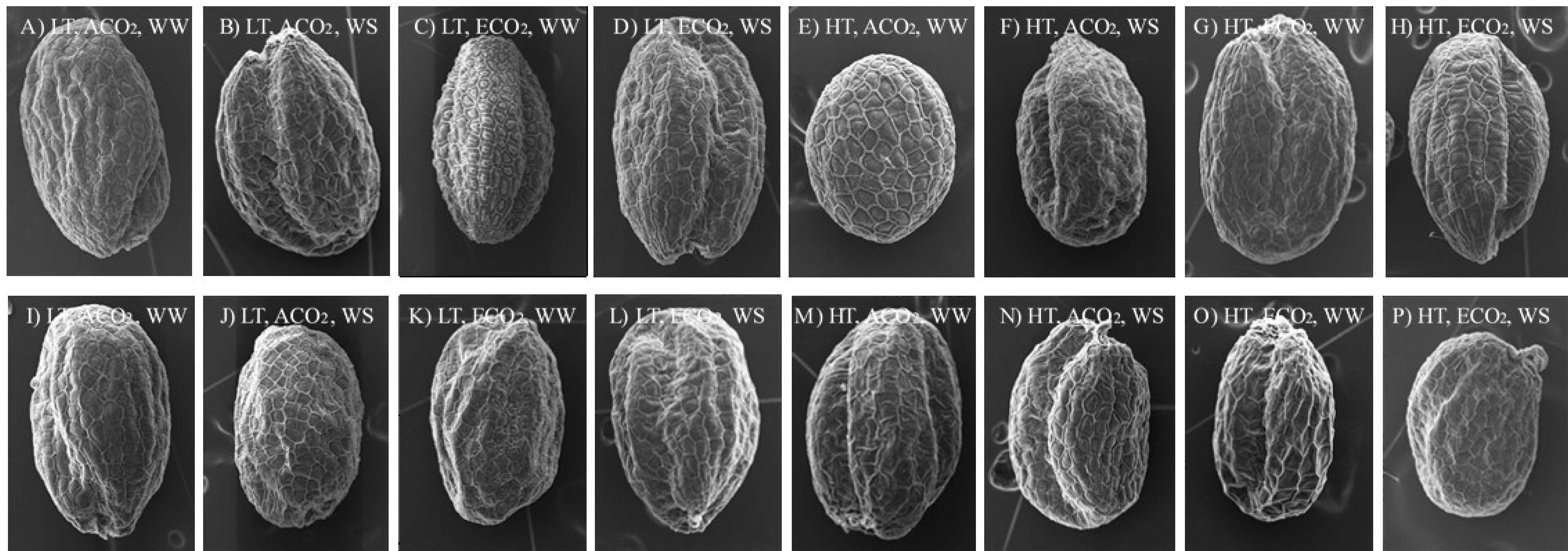
Fig. 3.3 Effects of temperature, carbon dioxide and watering regime on seed characteristics of 45-day-old plants of *A. thaliana*. (*A–B*) sound seed number; (*C–D*) aborted seed number; and (*E–F*) seed mass. (*A*, *C*, *E*) WT, (*B*, *D*, *F*) *abi1-1*. Plants were grown under two temperature regimes (22/18°C and 28/24°C; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 $\mu mol\ mol^{-1}$) and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers. Dark-grey bars represent well-watered plants and light-grey bars water-stressed plants. Data are means ± SE of 15 samples from three different trials (three samples per trial for all measured parameters). Different letters above the bars (mean ± SE) denote significant differences within each parameter ($P < 0.05$; according to Scheffé's multiple-comparison procedure). Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes


A
B
C
D
E
F
Sound seed number (silique^{-1})
Aborted seed number (silique^{-1})
Seed mass (mg seed^{-200})
Ab
Acd
Aa
Ac
Ac
Acd
Acd
Ad
Bab
Bcd
Ba
Acd
Abc
Acd
Acd
Ad
Abc
Aab
Abc
Ac
Abc
Aa
Abc
Abc
Abc
Aab
Abc
Abc
Ac
Aa
Ac
Abc
Aab
Acde
Aa
Acde
Abc
Ade
Abcd
Ae
Aa
Acd
Aa
Abcd
Aab
Acd
Bbc
Ad
ACO$_2$
ECO$_2$
Lower temperature
Higher temperature
Growth condition

3.4.3 *Seed Surface Structure*

The surface structure of the WT and *abi1-1* mutant seeds were examined with a scanning electron microscope. Under control condition (lower temperatures, elevated CO_2, and watering to field capacity) the epidermal cells of the WT and *abi1-1* mutant seed coats displayed the typical properties of Arabidopsis seed coat (Haughn et al. 2012), which has a hexagonal pattern with a volcano-shaped secondary cell wall at their center, known as the columella (Fig. 3.4*A*, *I*). In both genotypes, the seed coat of seeds that matured under higher temperatures had larger epidermal cells with larger columellae (Fig. 3.4*E–H*, *M–P*) than the seeds that matured under lower temperatures (Fig. 3.4*A–D*, *I–L*). Also, the grooves were narrow and deep for the seeds that matured under higher temperatures (Fig. 3.4*F*, *N*) and wide and shallow for seeds that matured under lower temperatures (Fig. 3.4B, J). The epidermal cells and columellae of seed coat of seeds that matured at elevated CO_2 appeared slightly larger and had more observable columellae (Fig. 3.4*A–B*, *I–J*) than the seeds taht matured at ambient CO_2 (Fig. 3.4*C–D*, *K–L*). However, there were slight differences in groove size and depth of seeds that matured at different CO_2 concentrations. Seeds from the water-stressed plants exhibited smaller epidermal cells and columellae, which were obvious (see Fig. 3.4*E–F*, *M–N*). Epidermal cells of *abi1-1* seed coat appeared slightly smaller than those of the WT seeds. However, in both genotypes, the columellae are easily recognized on the surface of seeds (Fig. 3.4*A–H*, *I–P*).

Fig. 3.4 Scanning electron microscopy of wild-type and *abi1-1* mutant seeds of *Arabidopsis thaliana*. The surface morphology of mature dry seeds of *A. thaliana.* (*A–H*) WT; and (*I–P*) *abi1-1* mutant. Seeds matured under the following conditions: (*A*, *I*) lower temperatures, ambient CO_2, well-watered; (*B*, *J*) lower temperatures, ambient CO_2, water-stressed; (*C*, *K*) lower temperatures, elevated CO_2, well-watered; (*D*, *L*) lower temperatures, elevated CO_2, water-stressed; (*E*, *M*) higher temperatures, ambient CO_2, well-watered; (*F*, *N*) higher temperatures, ambient CO_2, water-stressed; (*G*, *O*) higher temperatures, elevated CO_2, well-watered; and (*H*, *P*) higher temperatures, elevated CO_2, water-stressed. Magnification: *A*, *F*, *G*, *N* (×501); *C* (×400); and others (×503).


A) LT, ACO2, WW
B) LT, ACO2, WS
C) LT, ECO2, WW
D) LT, ECO2, WS
E) HT, ACO2, WW
F) HT, ACO2, WS
G) HT, ECO2, WW
H) HT, ECO2, WS
I) LT, ACO2, WW
J) LT, ACO2, WS
K) LT, ECO2, WW
L) LT, ECO2, WS
M) HT, ACO2, WW
N) HT, ACO2, WS
O) HT, ECO2, WW
P) HT, ECO2, WS

3.4.4 *Seed Amino Acid Profiling*

Amino acid profiling has led to the identification of eight amino acids in seeds of the two Arabidopsis genotypes, based on both internal and external standards. Maturation temperature increased seed amino acids, except valine (Table 3.3). Elevated CO_2 increased seed amino acids, except aspartic acid (Table 3.3). Water stress had no effect on any of the amino acids (Table 3.3). Leucine and valine were higher in the *abi1-1* seeds than in the WT seeds (Table 3.3). Interactions between and among factors were significant for the majority of amino acids, and the response of each amino acid differed to the interactive effects of T × C × W (Table 3.4). Seeds that matured on the well-watered plants that were grown under higher temperatures at elevated CO_2 had highest leucine, lysine, serine, and threonine (Fig. 3.5*E–L*); seeds that matured on the water-stressed plants that were grown under higher temperatures at elevated CO_2 had highest glycine (Fig. 3.5*C–D*) and tyrosine (Fig. 3.5*M–N*); seeds that matured on the well-watered plants that were grown under lower temperatures at elevated CO_2 had lowest glycine (Fig. 3.5*C–D*), serine (Fig. 3.5*I–J*), and threonine (Fig. 3.5*K–L*); seeds that matured on the water-stressed plants that were grown under lower temperatures at elevated CO_2 had lowest leucine (Fig. 3.5*E–F*) and tyrosine (Fig. 3.5*M–N*); and seeds that matured on the water-stressed plants that were grown under lower temperatures at ambient CO_2 had lowest lysine (Fig. 3.5*G–H*). On the basis of interaction among T × C × G (Table 3.4), in the WT plants, seeds that matured under higher temperatures at elevated CO_2 had highest serine, whereas seeds that matured under lower temperatures at elevated CO_2 had lowest serine (Fig. 3.5*I–J*).

Each amino acid responded differently to the interactive effects of C × W × G (Table 3.4). Seeds that matured on the water-stressed WT plants that were grown at elevated CO_2 had highest aspartic acid, glycine, lysine, serine, and threonine (Fig. 3.5*A–D*, *J–L*); seeds that matured on the

well-watered *abi1-1* plants grown at elevated CO_2 had highest leucine and valine (Fig. 3.5*E–F*, *O–P*); seeds that matured on the well-watered WT plants grown at elevated CO_2 had highest tyrosine (Fig. 3.5*M–N*); seeds that matured on the well-watered WT plants that were grown at elevated CO_2 had lowest aspartic acid (Fig. 3.5*A–B*); seeds that matured on the water-stressed WT plants at ambient CO_2 had lowest glycine, leucine, and valine (Fig. 3.5*C–F*, *O–P*); seeds that matured on the well-watered WT plants at ambient CO_2 had lowest lysine, serine, and tyrosine (Fig. 3.5*G–J*, *M–N*); and seeds that matured on the water-stressed *abi1-1* plants at elevated CO_2 had lowest threonine (Fig. 3.5*K–L*).

Table 3.3

Effects of Temperature, Carbon Dioxide, Watering Regime, and Genotype on Seed Amino Acids of *Arabidopsis thaliana*

	Temperature		Carbon dioxide		Watering regime		Genotype	
Parameter	Lower	Higher	Ambient	Elevated	Well-watered	Water-stressed	Wild-type	*abi1-1* mutant
Aspartic acid	7.27 ± 0.21b	7.99 ± 0.23a	7.83 ± 0.21a	7.44 ± 0.25a	7.44 ± 0.26a	7.83 ± 0.19a	7.50 ± 0.24a	7.76 ± 0.21a
Glycine	2.63 ± 0.28b	4.25 ± 0.69a	2.65 ± 0.24b	4.23 ± 0.71a	3.41 ± 0.46a	3.47 ± 0.64a	3.61 ± 0.30a	3.27 ± 0.44a
Leucine	2.26 ± 0.06b	2.58 ± 0.11a	2.22 ± 0.05b	2.62 ± 0.10a	2.52 ± 0.10a	2.32 ± 0.08b	2.35 ± 0.06b	2.48 ± 0.09a
Lysine	6.48 ± 0.07b	6.98 ± 0.17a	6.43 ± 0.07b	7.02 ± 0.16a	6.77 ± 0.13a	6.69 ± 0.15a	6.70 ± 0.07a	6.76 ± 0.12a
Serine	4.77 ± 0.27b	7.83 ± 0.89a	4.96 ± 0.23b	7.63 ± 0.93a	6.10 ± 0.63a	6.45 ± 0.82a	6.31 ± 0.28a	6.28 ± 0.55a
Threonine	4.76 ± 0.27b	4.83 ± 0.89a	4.96 ± 0.23b	7.63 ± 0.93a	6.10 ± 0.63a	6.49 ± 0.82a	6.31 ± 0.28a	6.28 ± 0.55a
Tyrosine	5.41 ± 0.15b	5.83 ± 0.12a	5.35 ± 0.08b	5.90 ± 0.16a	5.62 ± 0.14a	5.63 ± 0.14a	5.63 ± 0.16a	5.62 ± 0.12a
Valine	5.62 ± 0.25a	5.93 ± 0.17a	5.45 ± 0.08b	6.09 ± 0.28a	5.94 ± 0.26a	5.60 ± 0.16a	5.54 ± 0.09b	5.99 ± 0.25a

Note. Mature seeds were collected from Arabidopsis plants (wild-type and *abi1-1* mutant) that were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC, and their amino acids (μmol g^{-1} FW) were measured. Data are means ± SE of four seed samples from four different plants. Means followed by different letters within each parameter and factor are significantly different ($P < 0.05$; according to Scheffé's multiple-comparison procedure).

Table 3.4

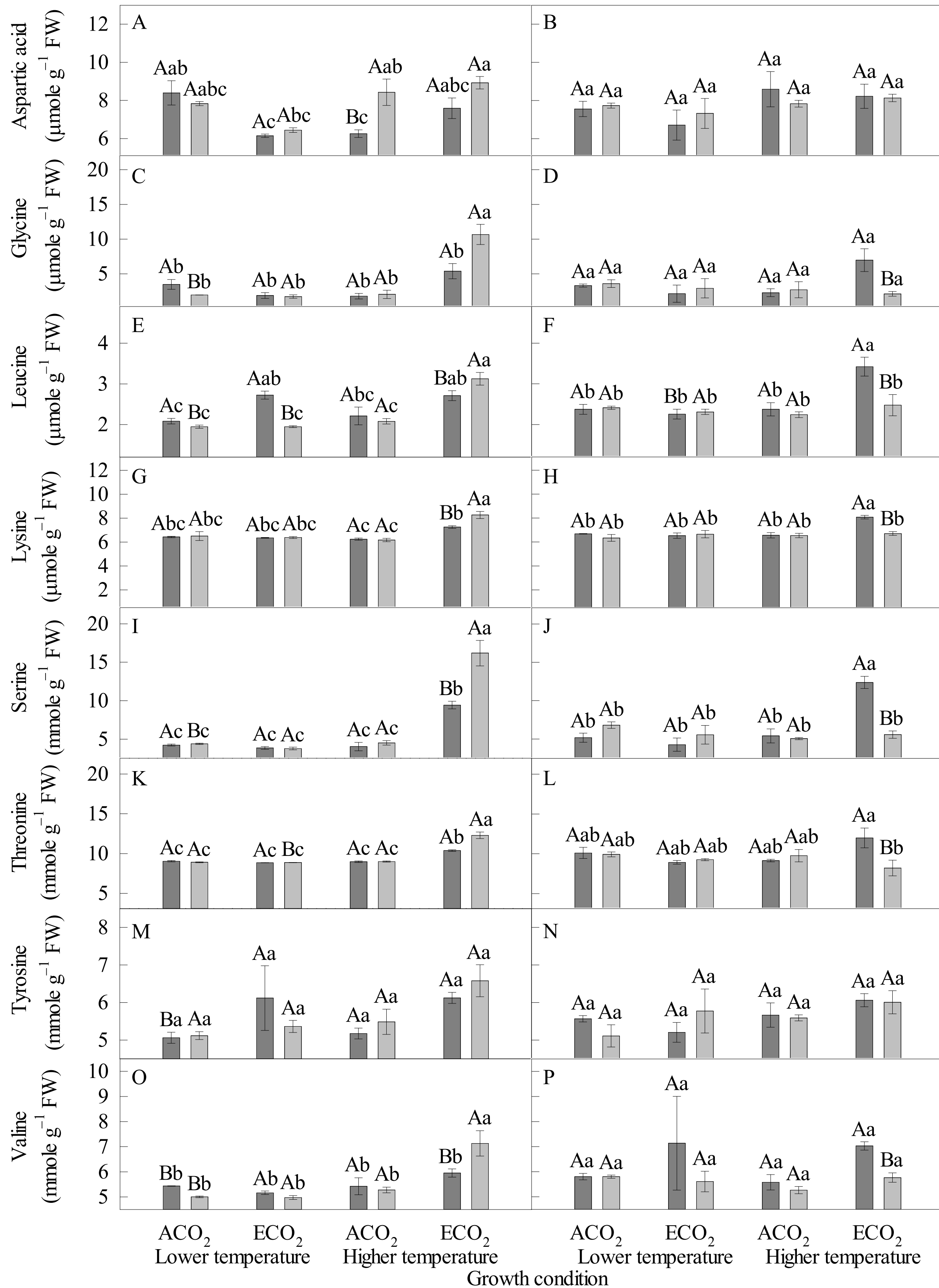
Summary of Split-Split-Split-Plot ANOVA (*F* Values) for Effects of Temperature, Carbon Dioxide, Watering Regime, Genotype, and Their Interactions on Seed Amino Acids of *Arabidopsis thaliana*

Source	df	Aspartic acid	Glycine	Leucine	Lysine	Serine	Threonine	Tyrosine	Valine
Temperature (T)	1	1.77	15.08	13.66	5.88	14.64	6.80	4.18	6.14
Main plot error	2	–	–	–	–	–	–	–	–
Carbon dioxide (C)	1	1535.01****	900.91**	40.50*	1203.1****	543.23****	1543.21****	1012****	235.50**
T × C	1	3.256****	151.87**	3.66	25.31****	78.39****	12.34****	9.32****	1.04
Subplot error	2	–	–	–	–	–	–	–	–
Watering regime (W)	1	127.79**	25.68**	0.05	504.27****	120.94***	1222.84****	175.29***	183.36***
T × W	1	3.41	41.83**	14.50*	17.79*	34.06**	22.29**	11.35*	5.78
C × W	1	81.76***	0.67	34.04**	297.02****	65.91**	903.01****	81.50***	92.73***
T × C × W	1	2.95	40.78**	14.33*	15.71*	32.54**	18.77*	10.34*	5.05
Split-subplot error	4	–	–	–	–	–	–	–	–
Genotype (G)	1	354.28****	0.00	27.24***	333.29****	131.32****	1209.48****	169.83****	55.10****
T × G	1	0.36	4.55	2.02	0.69	16.99**	0.00	1.19	1.41
C × G	1	538.12****	29.36***	0.54	553.15****	237.01****	1578.44****	364.66****	107.88****
T × C × G	1	1.00	3.28	1.77	1.37	10.83*	0.00	1.58	1.93

W × G	1	1104.65****	266.18***	82.51****	1249.08****	581.28****	2689.46****	1040.18****	284.87****
T × W × G	1	0.00	3.83	0.80	0.00	15.50**	0.00	0.00	0.45
C × W × G	1	1396.78****	460.98****	188.12****	1627.75****	776.37****	3186.85****	1448.45****	388.51****
T × C × W × G	1	0.00	0.00	0.00	0.00	0.21	0.00	0.00	0.28
Split-split-subplot error	8	–	–	–	–	–	–	–	–

Note. Mature seeds were collected from Arabidopsis plants that were grown under lower (22/18°C) or higher (28/24°C) temperature regimes at either ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) CO_2 concentrations, and two watering regimes in controlled-environment growth chambers for 45 days, after eight days of initial germination and seedling growth under 22/18°C. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Fig. 3.5 Effects of temperature, carbon dioxide and the watering regime on seed amino acids of 45-day-old plants of *A. thaliana*. (*A*–*B*) aspartic acid; (*C*–*D*) glycine; (*E*–*F*) leucine; (*G*–*H*) lysine; (*I*–*J*) serine; (*K*–*L*) threonine; (*M*–*N*) tyrosine; and (*O*–*P*) valine. (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*) WT; and (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*) *abi1-1* mutant. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 $\mu mol\ mol^{-1}$) and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers. Dark-grey bars represent well-watered plants and light-grey bars water-stressed plants. Data are means ± SE of four seed samples from four different plants. Different letters above the bars (mean ± SE) denote significant differences within each parameter ($P < 0.05$; according to Scheffé's multiple-comparison procedure). Uppercase letters represent differences between genotypes, whereas lowercase letters represent differences within genotypes


Aspartic acid (µmole g−1 FW)
Glycine (µmole g−1 FW)
Leucine (µmole g−1 FW)
Lysine (µmole g−1 FW)
Serine (mmole g−1 FW)
Threonine (mmole g−1 FW)
Tyrosine (mmole g−1 FW)
Valine (mmole g−1 FW)
ACO2 ECO2 ACO2 ECO2
Lower temperature Higher temperature
Growth condition

3.4.5 *Relationship Between Plant Parameters*

Pearson's correlation analysis revealed many significant (P < 0.05) relationships between plant parameters. For instance, stem height was negatively correlated with aspartic acid (r = –0.550, P = 0.027) and serine (r = –0.531, P = 0.034). Seed mass and sound seed number were positively correlated with silique length (r = 0.901, P = 0.000; r = 0.948, P = 0.000), silique mass (r = 0.880, P = 0.000; r = 0.938, P = 0.000), silique width (r = 0.880, P = 0.000; r = 0.925, P = 0.000), stem diameter (r = 0.854, P = 0.000; r = 0.893, P = 0.000), and stem height (r = 0.877, P = 0.000; r = 0.854, P = 0.000), but were negatively correlated with aspartic acid (r = –0.574, P = 0.020; r = –0.532, P = 0.034). Number of aborted seeds was negatively correlated with stem height (r = –0.309, P = 0.033). Silique length was positively correlated with silique mass (r = 0.950, P = 0.000), and silique width (r = 0.975, P = 0.000). Silique mass was positively correlated with silique width (r = 0.938, P = 0.000).

3.5 Discussion

3.5.1 *Effects Of Temperature*

This study revealed that higher temperatures decreased stem height and diameter (Table 3.1; Fig. 3.1). In previous studies, it has been shown that, under higher temperatures, plants have shorter stems and smaller leaves, although they usually maintain leaf number (Gliessman 1998; Qaderi et al. 2012). It has been well documented that heat stress deactivated Rubisco, which would further reduce biomass through reduced plant ability to perform photosynthesis (Dutta et al. 2009). Abiotic stress factors affect plants during both vegetative and reproductive stages, and higher temperature has greater effects on plant reproduction than other stress factors (Bac-Molenaar et al. 2015). However, in the current study, components of reproductive yield were negatively affected by higher temperatures (Table 3.1; Figs. 3.2 and 3.4). This agrees with a previous study on the effects of temperature on *A. thaliana* ecotypes Bur and Cvi (Huang et al. 2014). Seed mass is usually used as an indicator of seed size (Hampton et al. 2013). Consistent with this, seeds matured under higher temperatures had a smaller mass and, consequently, smaller size than those matured under lower temperatures (Table 3.1; Figs. 3.3–3.4). The positive correlations between silique length and seed mass, and silique length and sound seed number support an earlier study, which reported that shorter siliques produce fewer and lighter seeds than longer siliques (Lebowitz 1989). Higher temperatures decrease yield by reducing seed size and increasing the number of aborted flowers (Bueckert et al. 2015). Also, it decreases seed mass by accelerating the seed growth rate and reducing duration of seed filling (Young et al. 2004), and thus, decreasing dry matter accumulation (Gibson and Paulsen 1999). On the other hand, seed mass was found not to change, or sometimes to increase, under higher temperatures (Peltonen-Sainio et al. 2011). In our study, higher temperatures increased the amount of seven out of eight

measured amino acids, with the exception of valine (Table 3.3). Increased amino acid contents in seeds that matured under higher temperatures could have compensated for the production of smaller siliques with fewer and lighter seeds. An earlier study showed that higher temperatures decrease seed quality (Shinohara et al. 2006). Moreover, the amount of most amino acids was found to decrease in response to temperature stress in potato (Hancock et al. 2014), but was largely unaffected in soybean (Wolf et al. 1982).

3.5.2 *Effects Of Carbon Dioxide*

Elevated CO_2 decreased plant height and the number of aborted seeds, increased silique width and mass, but had no effect on seed mass or number of sound seeds (Table 3.1). It seems likely that plants at elevated CO_2 allocated more resource to silique width and mass than that to stem, resulting in shorter plants under this factor. This is reflected in the negative correlation between stem height and each of the amino acids aspartic acid and serine, which might have supplied by silique. Moreover, higher growth at elevated CO_2 will not be achieved unless the plant has a way to utilize it, but if there is sink limited capacity, a down regulation of photosynthesis will occur (Körner 2000; Kirschbaum 2011). Elevated CO_2 is expected to increase seed mass by increasing plant assimilate availability (Jablonski et al. 2002), and many responses to elevated CO_2 are species-dependent (Hikosaka et al. 2011). However, Kinugasa et al. (2003) reported that elevated CO_2 did not affect production of seeds, whereas capsule mass increased by 86% in Canada cocklebur (*Xanthium canadense* L.). All measured amino acids, except aspartic acid and valine, increased in the seeds matured at elevated CO_2 (Table 3.3). Increased seed mass at elevated CO_2 may result in increased seed C/N ratio in non-legumes (Hampton et al. 2013). However, no such effect was observed on seed mass and amino acid

content in this study (Tables 3.1 and 3.3), which is in agreement with a previous study on rice (*Oryza sativa* L.; Wang et al. 2011). This was likely due to the beneficial effects of fertilizer on the nutrient uptake of plants during our experiment, indicating that the effects of elevated CO_2 on the amino acid content of seeds depends on nutrient availability (Marshall et al. 2010). Also, increase in the amino acid content of seeds at elevated CO_2 could be mainly because of increased nitrogen acquisition (Hikosaka et al. 2011). Therefore, the current study did not concur with an earlier study that reported decreased quality of seeds that matured at elevated CO_2 by increasing the C/N ratio (Jablonski et al. 2002). However, this scenario could be different for wild plants that are already living under limited resources of carbon and nitrogen (Kontopoulou et al. 2014). Vicente et al. (2016) found a higher amino acid contents in seeds of durum wheat (*Triticum turgidum* subsp. *durum*) matured at ambient CO_2 in comparison to elevated CO_2.

3.5.3 *Effects Of Watering Regime*

Significant reduction in components of growth and reproductive yield was found in water-stressed plants (Table 3.1). This could be related to negative effects of water stress on plant metabolic processes, such as photosynthesis and protein synthesis (Ohashi et al. 2006). Water stress significantly decreased leucine, but did not affect the rest of the measured amino acids (Table 3.3). This is inconsistent with earlier studies, particularly in regard to leucine, which has been shown to increase under stress conditions, such as drought (Delauney and Verma 1993). Leucine has been shown to increase in the siliques of *A. thaliana* during water stress, as it is acted as osmoprotectant (Nambara et al. 1998).

3.5.4 *Effects Of Genotype*

This study showed that the WT plants were more resilient to stress factors than the *abi1-1* mutant. The WT plants were taller with thicker stems and had higher reproductive yield than the *abi1-1* mutant (Table 3.1). This is because of the ABA signaling impairment in the guard cells of the *abi1-1* mutant (Pei et al. 1997), causing their stomata to remain open, and thus, increasing transpiration and decreasing growth and development. Seeds of the *abi1-1* mutant had significantly more leucine and valine than seeds of the WT plants (Table 3.3). Amino acids, such as leucine, isoleucine and valine, act as osmoprotectants against abiotic stresses, such as higher temperature and water stress (Obata and Fernie 2012). Therefore, it is clear that ABA signaling is important for plant responses and protection against stress factors.

3.5.5 *Interactive Effects Of Temperature, Carbon Dioxide, Watering Regime, And Genotype*

In this study, for the first time, we investigated the combined effects of temperature, CO_2, watering regime, and genotype on seed quality, and showed that the well-watered WT plants grown at elevated CO_2 were tallest and had highest silique number and heaviest seeds (Figs. 3.1*A*, 3.2*A* and 3.3*E*). In contrast, the water-stressed *abi1-1* plants grown at ambient CO_2 were shortest and had lowest silique number and lightest seeds (Figs. 3.1*B*, 3.2*B* and 3.3*F*). These results are expected as elevated CO_2 provides more building material for plant growth. Variations in growth and seed mass between genotypes are due to impairment of the ABA signaling in the guard cells of the *abi1-1* plants. Watering plants to the field capacity at elevated CO_2 helped them prevent stem shortening, induced by elevated CO_2 alone. Amino acids exhibited differential responses to the T × C × W interaction (Table 3.4; Fig. 3.5), but in general, temperature was the main factor that affected seed amino acids. Stem diameter, and silique mass

and width were highest in the well-watered WT plants grown under lower temperatures at elevated CO_2, but lowest in the water-stressed *abi1-1* plants grown under higher temperatures at elevated CO_2 (Figs. 3.1*C–D* and 3.2*C–D*, *G–H*). It appears that elevated CO_2 did not mitigate the negative effects of water stress and higher temperatures on the *abi1-1* plants, suggesting the importance of the ABA signaling in CO_2-induced stomatal responses. In agreement with this, an earlier study reported the role of ABA and the ABA receptors in enhancing Arabidopsis guard cell to decrease stomatal opening at elevated CO_2 (Chater et al. 2015). Merilo et al. (2013) stated that the response of guard cells to elevated CO_2 was partially disrupted in *abi1-1*. In our study, increased accumulation of all amino acids in the WT seeds matured on water-stressed plants grown under higher temperatures at elevated CO_2 (Fig. 3.5) could be associated with the CO_2-induced improvement in heat and drought tolerance, although the specific mechanisms are not clear. On the other hand, the reduction of amino acid contents, especially those playing an osmoprotectant role (e.g., glycine) in the *abi1-1* seeds matured under the same conditions, could imply that the ABA-mediated regulation of amino acid synthesis in reproductive tissues is required. It has been shown that, in *A. thaliana*, the levels of amino acids during stress responses can be regulated in both an ABA-dependent and an ABA-independent manner (Nambara et al. 1998). Moreover, the same study showed that, under water stress, WT plants accumulated more amino acids than the ABA-deficient mutant (*aba2-2*) plants.

It can be concluded that the predicted higher temperatures and water stress, individually and together, will lead to decreased growth, yield, and seed quality in both genotypes. Elevated CO_2, in general, did not compensate for the negative effects of stress factors on the measured parameters. This study showed that long-term exposure to elevated CO_2 seems to have more positive effect on the biomass of the reproductive structures (siliques), but not on seed mass or

seed production. Amino acid contents of Arabidopsis seeds were positively affected by higher temperatures, elevated CO_2 or water stress; with highest effects by temperature. This could compensate for the negative effects of higher temperatures and water stress, and no effects of elevated CO_2, respectively, on seed mass, and consequently, on seed quality. The responses of measured parameters to elevated CO_2 were found to be genotype dependent, suggesting the importance of ABA in plant protection against stress conditions and improvement of seed quality.

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CHAPTER 4

Interactive effects of Temperature, Carbon Dioxide and Watering Regime on Seed Germinability of Two Genotypes of *Arabidopsis thaliana*

4.1 Abstract

We examined the combined effects of temperature, carbon dioxide (CO_2), and watering regime during seed maturation on subsequent germinability and total phenolics of *Arabidopsis thaliana* (wild-type (WT) and *abi1-1* mutant) seeds. Mature seeds were collected from plants that were grown under lower (22º/18ºC, 16 h light/ 8 h dark) or higher (28º/24ºC, 16 h light/ 8 h dark) temperatures, at ambient (400 µmol mol^{-1}) or elevated (700 µmol mol^{-1}) CO_2 concentration, and well-watered or water-stressed. Germinated and non-germinated (viable, rotten, and empty) seed percentages, germination rate, and total phenolics were determined for both genotypes. Higher maturation temperatures increased seed germination percentage, but decreased germination rate, percentage of rotten and non-germinated viable seeds, and total phenolics. Elevated CO_2 increased seed total phenolics. Water stress decreased the percentage of non-germinated viable seeds. None of the two latter factors affected other measured parameters. Seeds of the *abi1-1* mutant had higher total phenolics. The fate of seeds was mostly affected by higher temperatures and water stress. Also, seeds of the *abi1-1* mutant had higher germination rate, empty seed percentage, and total phenolics than seeds of the WT genotype. Germination percentage was highest for the WT seeds that matured on the water-stressed plants that were grown under higher temperatures at ambient CO_2. It can be concluded that higher temperatures have the greatest effects on seed germinability and other parameters, and elevated CO_2 did not alleviate the negative effects of higher temperatures on seed viability.

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4.2 Introduction

There is undeniable evidence that the Earth's climate has changed in the last century, to an extent that cannot be attributed to normal climate cycles (Wheeler and von Braun, 2013). As a result of anthropogenic emissions, the current CO_2 level (407 μmol mol^{-1}) may surpass 700 μmol mol^{-1} by the end of the century (Stocker *et al.,* 2013). Additionally, warming of 0.65-1.06°C has already occurred between the periods of 1880 to 2012, with the last 30 years being the warmest in nearly a millennium. From 2016 to 2035 alone, climate models predicted a further temperature increase of 0.3-0.7°C, and by 2100 it is expected that temperatures will rise at least another 1.5°C, and as much as 6.4°C (Stocker *et al.,* 2013). Other effects of climate change include increased incidences of water stress and modifications to ecosystem suitability for native species (Stocker *et al.,* 2013). Climate change has been reported to have a large effect on plant recruitment, and especially on the regeneration process. Temperature and water status of soil do not only affect the distribution of plants, but they also control the initiation and breaking of seed dormancy and radical emergence during germination (Walck *et al.,* 2011). Moreover, the chemical properties of seeds and their ability to germinate have been reported to be affected by environmental factors either during their development on the parent plant, or after dispersal in the soil (Gutterman, 2000). In general, seed dormancy can be classified to physical, physiological, morphophysiological, in addition to other categories (Baskin and Baskin, 1998). Physiological dormancy is the most common type of seed dormancy in plants, and it is controlled by the presence of internal germination inhibitors, such as phenolics (Baskin and Baskin, 1998), which can also be present in the testa of seeds (Debeaujon *et al.,* 2000). As shown, seeds of mouse-ear cress (*Arabidopsis thaliana* L.) has a non-deep physiological dormancy, which means that embryo released from surrounding structures grows normally and that dormancy is broken by

stratification or after-ripening (Baskin and Baskin, 2004). In Arabidopsis, seed dormancy has been investigated for many years, with more focus on the genetic and physiological levels (Holdsworth *et al.,* 2008). These studies have shown that the phytohormone abscisic acid (ABA) plays a central role in inducing seed dormancy in this species (Bewley and Black, 1994). However, ABA deficient and insensitive mutants of Arabidopsis have reduced dormancy (Nambara *et al.,* 1994). Several environmental factors are known to affect seed germination. Among them, temperature is the main factor affecting seed germination either by altering the parent plant metabolism and/or changing seed chemical composition (Fitter and Hay, 2012). It has been shown that lower temperatures increase dormancy levels in mature seeds of Arabidopsis (Kendall *et al.,* 2011; Piskurewicz *et al.,* 2016). Higher temperature inhibits the ability of plants to accumulate chemicals, which have an important role in inducing seed dormancy (Dornbos and McDonald, 1986). However, earlier reports have shown that higher temperatures could enhance seed germination (Fenner, 1991; Baskin and Baskin, 1998). Elevated CO_2 has been reported to decrease (Farnsworth and Bazzaz, 1995; Andalo *et al.,* 1996), to increase (Edwards *et al.,* 2001) or to have no effect (Way *et al.,* 2010), on seed germination. The responses of plants to elevated CO_2 are species dependent (Farnsworth and Bazzaz, 1995) and variation among genotypes also exists (Andalo *et al.,* 1996). In their meta-analytical study, considering 79 crop and wild species, Jablonski *et al.* (2002) reported a 14% decrease in seed nitrogen content in response to elevated CO_2, particularly in the non-leguminous species. This could lead to decreased viability of seeds (Andalo *et al.,* 1996). However, higher content of seed nitrogen positively increases germination rate (Hara and Toriyama, 1998), but not germination *per se* (Hampton *et al.,* 2013). However, high concentrations of nitrogen in the seeds can also induce dormancy (Peterson and Bazzaz, 1978; Goudey *et al.,* 1987, 1988; Luzuriaga *et*

al., 2006). Water availability and temperature are the most important factors in determining seed germination (Baskin and Baskin, 1998). Water stress has a negative impact on seed germination as well as on the early seedling growth stages (Toscano *et al.,* 2017). Arabidopsis seed germination was negatively affected by water stress (Auge *et al.,* 2015). However, it has been reported that water stress during seed maturation on the parent plant causes the seeds to transit from the state of development to the state of germination (Kermode *et al.,* 1986). This transition happens because of alterations in protein content (Lalonde and Bewley, 1986) and messenger RNA (Bewley *et al.,* 1989). Germination processes determine the distribution and composition of plant communities (Harper, 1978); however, fewer studies have been conducted on this topic compared to that of plant growth and development in relation to elevated CO_2. Also, several studies have examined the interactive effects of two or more climate change main drivers on seed germination (Alexander and Wulff, 1985; Qaderi *et al.,* 2008; Gurvich *et al.,* 2017), but many more multi-factor research is necessary. The objectives of this study were: (1) to determine germination responses of wild type (WT) and its relative abscisic acid-insensitive mutant (*abi1-1*) of *A. thaliana* seeds to temperature, CO_2, and watering regime, as individual factors and in combination; (2) to examine the effects of these factors on the fate of seeds that are tested for germination; and (3) to evaluate changes in seed total phenolics from WT and *abi1-1* plants and examine its relevance to seed germinability. It was hypothesized that exposure of plants to higher temperature, elevated CO_2 and water stress would decrease subsequent seed germination pattern and increase number of non-germinated seeds through increasing total phenolic content in Arabidopsis, and that seed germinability response would be related to genotype.

4.3 Materials and methods

4.3.1 *Plant material and growth conditions*

Seeds of the WT and *abi1-1* mutant of *A. thaliana* ecotype Landsberg erecta were treated with 95% ethanol for 5 min and germinated in Petri dishes containing liquid Murashige and Skoog basal medium (MS) for 6 days. Seedlings were then potted in a mixture of peat moss, Perlite and Vermiculite (1:1:1, by volume), and fertilized weekly with 30 pellets of slow-release NPK fertilizer; each granule contained all 3 of NPK, (13-14-14; 0.208g N:0.224g P:0.224g K/30 pellets); Chisso-Asahi Fertilizer Co, Tokyo, Japan). Pots were transferred to a growth chamber (model ATC26, Conviron, Controlled Environments, Winnipeg, MB, Canada), set to a temperature regime of 22/18°C on a 16 h photoperiod. The photosynthetic photon flux density (PPFD) was 300 µmol photon m^{-2} s^{-1}. Two seedlings were transplanted to pots and were left to acclimate for two days, and then nine pots containing 18 plants of each genotype were randomly assigned to each experimental treatment: (1) lower temperatures (22/18°C, 16 h light/ 8 h dark), ambient CO_2 (400 µmol mol^{-1}), and watering to field capacity (well-watered) as control; (2) lower temperatures, ambient CO_2, and watering at wilting point (water-stressed); (3) lower temperatures, elevated CO_2 (700 µmol mol^{-1}), and well-watered; (4) lower temperatures, elevated CO_2, and water-stressed; (5) higher temperatures (28/24°C, 16 h light/8 h dark), ambient CO_2, and well-watered; (6) higher temperatures, ambient CO_2, and water-stressed; (7) higher temperatures, elevated CO_2, and well-watered; (8) higher temperatures, elevated CO_2, and water-stressed. One growth chamber was used for each of lower and higher temperature regimes. In each growth chamber, two equal size Plexiglas cabinets (60 cm × 65 cm × 50 cm, GE Polymershapes, Dartmouth, NS, Canada) were placed; one supplied with ambient CO_2 and the other with elevated CO_2 (see above). Plants were grown inside these cabinets for 45 days. Half of

the plants were watered to field capacity (well-watered) and the other half at wilting point (water-stressed), inside the cabinets, pots were rotated weekly to reduce positional effects. Midday leaf water potential ranged from –1.0 to –2.0 MPa and soil water potential ranged from –0.4 to –1.3, for the well-watered and water-stressed plants, respectively. Water potential was measured with a WP4C Dew Point PotentiaMeter (Decagon Devices Inc., Pullman, WA, USA). The experiment was conducted three times and each time the chambers and cabinets were reversed.

4.3.2 *Germination tests*

Seeds from the dry-brown siliques were used for germination experiments. From each treatment and genotype (three plants), 50 seeds were sown in triplicate on blue germination filter paper (Anchor Paper 37 Co., St. Paul, MN), initially moistened with 10 mL of distilled water in 100 × 15 mm Petri dishes. Dishes were not sealed with parafilm; therefore, 2-3 mL of distilled water was added daily when needed to maintain the filter paper moist. Seeds were incubated in a growth chamber under the control conditions essentially as described above. Germination was scored daily, and germinated seeds with 2 mm or longer radicle were counted and removed. Petri dishes were placed randomly in the growth chamber at the beginning of the experiment and replaced in different random patterns after each daily count. At the conclusion of the experiment (58 days), intact seeds were subjected to viability test according to Qaderi *et al.* (2002).

4.3.3 *Total phenolic content*

Phenolic compounds were extracted according to Abdelhady *et al.* (2011) with some modifications. From each treatment, six samples of dry seeds (0.5 g) were collected and

homogenized using a mortar and a pistil in 2.5 mL 80% methanol. Then, the homogenate was incubated for two hours at room temperature and centrifuged at 4000 × *g* for 20 min at 4 ºC. The supernatant was collected and stored at 4 ºC for further analysis. Total phenolic content was estimated by the method of Waterhouse (2002) by using 2N Folin-Ciocalteu reagent and Gallic acid in methanol (concentration range: 0 to 500 mg L^{-1}) as a standard. A 10 µL of each sample was mixed with a 790 µL of water, 50 µL of the 2N Folin-Ciocalteu's reagent, and 150 µL of 20% (w/v) sodium carbonate and incubated at room temperature for two hours in the dark. The absorbance was measured at 760 nm for the solution with a UV/visible spectrophotometer (model Ultraspec 3100 *pro*, Biochrom Ltd., Cambridge, UK). The concentration of phenolics was determined (mg L^{-1}) from the standard curve; then the total phenolic content in samples was expressed in relations of Gallic acid equivalent (GAE) by the following equation: $P = (C \times V) / M$. In the equation, P stands for total phenolic content (mg GAE g^{-1} FM), C for the concentration of Gallic acid calculated from the calibration curve (mg L^{-1}), V for the volume of extracting medium (mL^{-1}), and M for the leaf fresh mass (g^{-1}).

4.3.4 *Data analysis*

The effects of temperature, CO_2, watering regime, genotype, and their interactions were determined on seed germination pattern, fate of seeds that were set for germination, and total phenolic content of the WT and its relative ABA-insensitive mutant (*abi1-1*) of *A. thaliana* using a four-way analysis of variance (ANOVA). Differences among treatments were determined by a one-way ANOVA, using Scheffé's multiple-comparison procedure at the 5% confidence level (SAS Institute, 2011). The following equation was used to calculate the coefficients of germination rate for each replicate: $N/\sum n_i d_i$, where N stands for final germination percentage, n_i

for the number of germinated seeds on the particular day on which a count was made, and d_i stands for the number of days from the start of the experiment (Alm *et al.,* 1993). All values of coefficients of germination rate were between 0 (no germination) and 1 (fastest germination rate), and multiplied by 100 to facilitate interpretation. Several-single Pearson's correlation coefficients at the 5% confidence interval were used to determine relationships between parameters (Minitab Inc., 2014).

4.4 Results

4.4.1 *Germination percentage*

Seeds that matured under higher temperatures had higher percentage of germination than the seeds matured under lower temperatures (Table 4.1). The germination percentage in the *abi1-1* mutant seeds was 90.56 % and in the WT seeds was 89.53 %. Although not significant, seeds of the *abi1-1* mutant had a higher germination percentage than those of the WT genotype (Table 4.1). Overall, subsequent seed germination was significantly affected by the plant growth temperature, the two-way interactions between temperature (T) × watering regime (W), (T) × genotype (G), and W × G, the three-way interaction among carbon dioxide (C) × W × G, and the four-way interaction (Table 4.2). Based on the four-way interaction, the WT seeds that matured on the water-stressed plants that were grown under higher temperatures at ambient CO_2 had the highest germination percentage, whereas the WT seeds that matured on the water-stressed plants that were grown under lower temperatures at elevated CO_2 had the lowest germination percentage ($P < 0.05$; Figs. 4.1A-B, 4.2A-B).

4.4.2 *Coefficient of germination rate*

Seeds that matured under lower temperatures had a higher germination rate than seeds that matured under higher temperatures (Table 4.1). Also, seeds that matured on the *abi1-1* mutant plants had a higher germination rate than the seeds that matured on the WT genotype plants. Germination rate was affected significantly by temperature, genotype, and the two-way interactions between T × W, T × G, and W × G (Table 4.2). The T × W interaction showed that seeds that matured on the well-watered plants that were grown under higher temperatures had the fastest rate, while seeds that matured on the well-watered plants that were grown under lower temperatures had the slowest rate. With regards to the T × G interaction, seeds that matured on the WT plants that were grown under higher temperatures had the fastest rate, and seeds that matured on the *abi1-1* plants that were grown under lower temperatures had the slowest rate. The interaction between W × G revealed that seeds that matured on the water-stressed WT plants had the fastest rate, and seeds that matured on the water-stressed *abi1-1* plants had the slowest rate ($P < 0.05$; Fig. 4.1C-D).

4.4.3 *Fate of non-germinated seeds*

A viability test at the end of this experiment showed that the percentage of non-germinated viable seeds was significantly higher for seeds that matured on plants that were grown under lower temperatures than for seeds that matured on plants that were grown under higher temperatures (Table 4.1). Similarly, seeds that matured on the well-watered plants had higher percentage of non-germinated viable seeds than seeds that matured on the water-stressed plants (Table 4.1). Overall, the percentage of non-germinated viable seeds was significantly affected by temperature, watering regime, the two-way interactions between T × W, T × G, and the four-way

interaction (Table 4.2). Based on the four-way interaction, the *abi1-1* seeds that matured on the well-watered plants that were grown under higher temperatures at elevated CO_2 had the highest percentage of non-germinated viable seeds, whereas the WT seeds that matured on the water-stressed plants that were grown under higher temperatures at ambient CO_2 had the lowest percentage of non-germinated viable seeds ($P < 0.05$; Fig. 4.2A-B).

Germination test revealed that seeds that matured under higher temperatures were less rotten than seeds that matured under lower temperatures (Table 4.1). The percentage of rotten seeds was significantly affected by temperature, the interactions between T × W, T × G, W × G, the three-way interaction among C × W × G, and the four-way interaction (Table 4.2). Based on these interactions, the WT seeds that matured on the water-stressed plants that were grown under lower temperatures at ambient CO_2 had the highest percentage of rotten seeds, whereas the WT seeds that matured on the water-stressed plants that were grown under higher temperatures at ambient CO_2 had the lowest percentage of rotten seeds ($P < 0.05$; Fig. 4.2A-B).

Seeds that matured on the *abi1-1* plants were emptier than seeds that matured on the WT plants (Table 4.1). Genotype and the interactions between T × C, T × W, T × G, and W × G significantly affected the percentage of empty seeds. With respect to the interaction between T × C, seeds that matured on plants that were grown under lower temperatures at ambient CO_2 had highest number of empty seeds, while seeds that matured on plants that were grown under higher temperatures at ambient CO_2 had lowest number of empty seeds. The T × W interaction showed that the water-stressed plants that were grown under lower temperatures produced the highest number of empty seeds, whereas the water-stressed plants that were grown under higher temperatures produced the lowest number of empty seeds. The interaction between T × G revealed that the *abi1-1* seeds that matured on plants that were grown under higher temperatures

had the highest percentage of empty seeds, whereas the WT seeds that matured on plants that were grown under the same temperatures had the lowest percentage of empty seeds. As per the W × G interaction, seeds that matured on the water-stressed *abi1-1* plants had the highest number of empty seeds, whereas seeds that matured on the well-watered WT plants had the lowest number of empty seeds ($P < 0.05$; Fig. 4.2A- B).

4.4.4 ***Total phenolic content***

Seeds that matured under lower temperatures had higher total phenolic content than seeds that matured under higher temperatures (Table 4.1). Elevated CO_2 increased total phenolic content, which was higher in the *abi1-1* seeds than in the WT seeds (Table 4.1). Total phenolic content was significantly affected by temperature, CO_2, genotype, the two-way interaction between T × W, T × G, W × G, the three-way interaction among C × W × G, and the four-way interaction (Table 4.2). The highest total phenolic content occurred in seeds that matured on the water-stressed WT plants that were grown under lower temperatures at elevated CO_2, but the lowest total phenolic content occurred in seeds that matured on the water-stressed WT plants that were grown under higher temperatures at ambient CO_2 ($P < 0.05$; Fig. 4.1E-F).

Table 4.1 Effects of temperature, carbon dioxide, watering regime, and genotype on the germinated and non-germinated seeds, and total phenolic content of *Arabidopsis thaliana*. Mature seeds were collected from *A. thaliana* plants (wild-type and *abi1-1* mutant) that were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC. Seeds were germinated under a temperature regime of 22/18ºC (16 h light/8 h dark), light intensity of 300 µmol m^{-2} s^{-1}, and relative humidity of ~65% for 58 days. Data are means ± SE of nine samples of 50 seeds from three different trials for all measured parameters, except for total phenolic content (means ± SE of six samples from two different trials; three samples per trial). Means followed by different letters within each parameter and condition are significantly different ($P < 0.05$) according to Scheffé's multiple-comparison procedure. For coefficients of germination rate, all means have been multiplied by 100 to facilitate data interpretation.

	Temperature		Carbon dioxide		Watering regime		Genotype	
Parameter	Lower	Higher	Ambient	Elevated	Well-watered	Water-stressed	Wild-type	*abi1-1* mutant
Germinated (%)	87.78 ± 1.51b	92.31 ± 1.31a	90.31 ± 1.38a	89.78 ± 1.59a	89.89 ± 1.05a	90.19 ± 1.82a	89.53 ± 1.71a	90.56 ± 1.21a
Germination rate	43.10 ± 3.22a	35.19 ± 2.76b	37.28 ± 2.69a	41.01 ± 3.44a	37.81 ± 2.66a	40.48 ± 3.48a	31.95 ± 1.72b	46.34 ± 3.45a
Non-germinated (%)								
Viable	2.44 ± 0.27a	1.58 ± 0.39b	1.86 ± 0.38a	2.17 ± 0.32a	2.47 ± 0.37a	1.56 ± 0.31b	2.03 ± 0.33a	2.00 ± 0.37a
Rotten	8.31 ± 1.26a	4.97 ± 0.77b	6.47 ± 0.89a	6.81 ± 1.27a	6.44 ± 0.65a	6.83 ± 1.41a	7.50 ± 1.37a	5.78 ± 0.69a
Empty	1.47 ± 0.32a	1.14 ± 0.32a	1.36 ± 0.35a	1.25 ± 0.29a	1.19 ± 0.31a	1.42 ± 0.34a	0.94 ± 0.33b	1.67 ± 0.30a
Total phenolics (mg GAE g^{-1} FM)	3.29 ± 0.37a	2.25 ± 0.39b	2.46 ± 0.38b	3.08 ± 0.41a	2.75 ± 0.36a	2.80 ± 0.45a	2.59 ± 0.46b	2.96 ± 0.28a

Table 4.2 Analysis of variance (*F* value) for effects of temperature, carbon dioxide, watering regime, and genotype on germination pattern, non-germinated seeds (viable, rotten and empty), and total phenolic content in seeds of *Arabidopsis thaliana*. Arabidopsis (wild–type and *abi1–1* mutant) plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC. Experiments were conducted three times. Mature seeds were collected and germinated under a temperature regime of 22/18ºC (16 h light/8 h dark), light intensity of 300 µmol m^{-2} s^{-1}, and relative humidity of ~65% for 58 days. Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

		Germination pattern		Non-germinated (%)			
Source	*df*	Germination (%)	Germination rate	Viable	Rotten	Empty	Total phenolics (mg GAE g^{-1} FM)
Temperature (T)	1	25.09****	8.08**	5.46*	15.52***	0.90	44.19****
Carbon dioxide (C)	1	0.34	1.79	0.69	0.16	0.10	15.79**
Watering regime (W)	1	0.11	0.92	6.19*	0.21	0.40	0.10
Genotype (G)	1	1.29	26.71****	0.01	4.14	4.22*	5.50*
T × C	1	2.46	1.68	1.28	0.11	4.23*	0.02
T × W	1	111.09****	15.42***	13.64***	60.02****	21.03****	104.73****
T × G	1	43.65****	7.99**	14.78***	17.66***	8.10**	137.80****
C × W	1	0.08	2.60	0.05	0.07	1.22	0.03
C × G	1	0.00	1.78	0.46	0.02	0.10	3.12
W × G	1	16.70***	11.19**	1.28	8.73**	4.90*	42.39****
T × C × W	1	0.16	0.51	0.28	0.21	0.40	0.99
T × C × G	1	0.16	0.02	0.01	0.04	0.23	0.01
C × W × G	1	6.51*	1.52	0.46	6.22*	0.03	11.82**
T × C × W × G	2	4.66*	1.28	3.57*	5.14*	0.36	11.74***

Fig. 4.1 Effects of temperature, carbon dioxide and watering regime on germination pattern and total phenolic content of *Arabidopsis thaliana* seeds. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 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. (A,C,E) WT, (B,D,F) *abi1-1*. (A–B) germination percentage; (C-D) coefficients of germination rate; and (E–F) total phenolic content. Dark-grey bars represent well-watered plants and light-grey bars water-stressed plants. Different letters above the bars (mean ± SE) denote significant differences ($P < 0.05$) within each parameter according to Scheffé's multiple-comparison procedure. Lowercase letters represent differences within genotypes, whereas uppercase letters represent differences between genotypes. Data are means ± SE of nine samples of 50 seeds from three different trials for germination % and coefficient of germination rate, but for total phenolic content means ± SE of six samples from two different trials; three samples per trial.

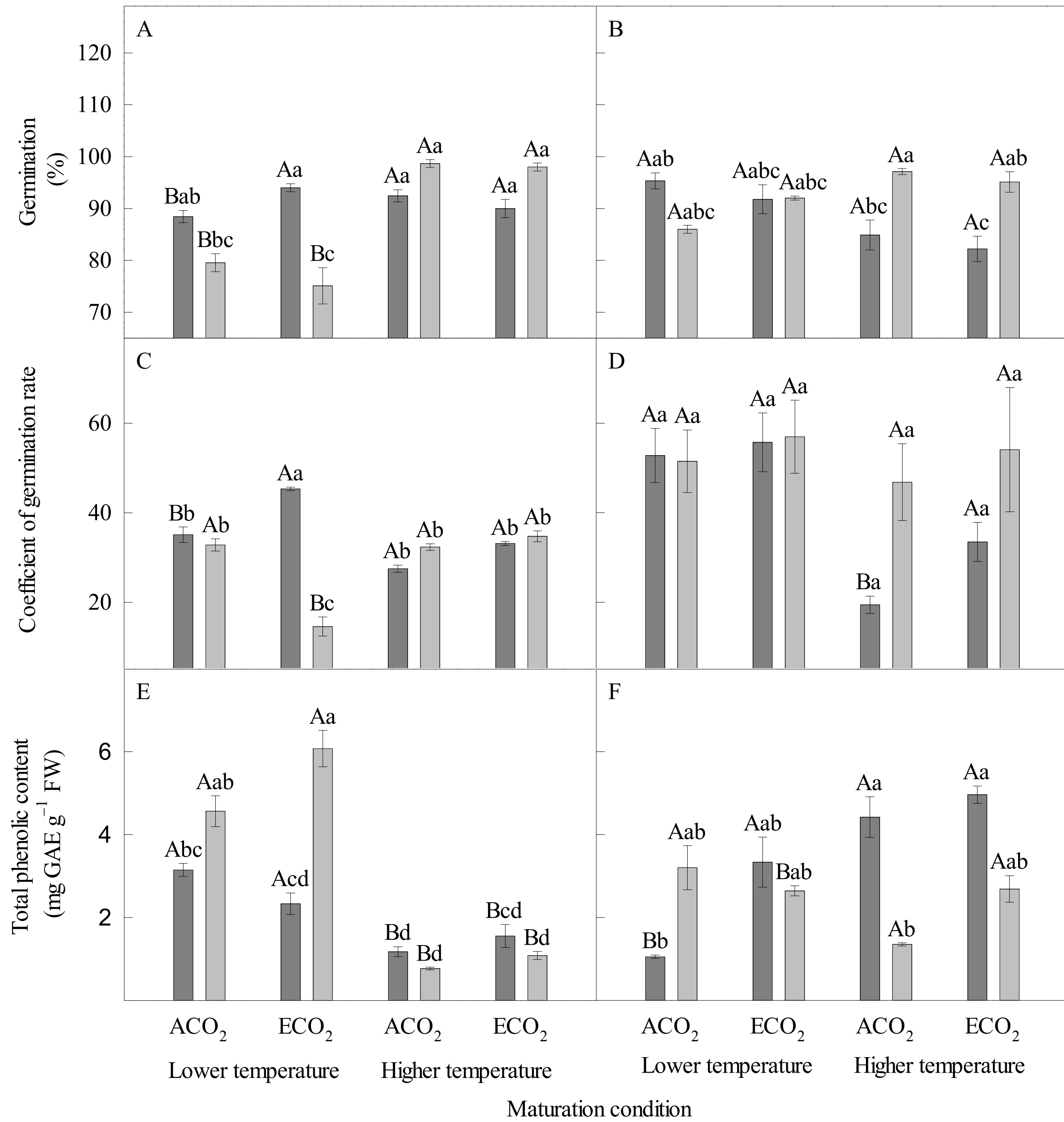
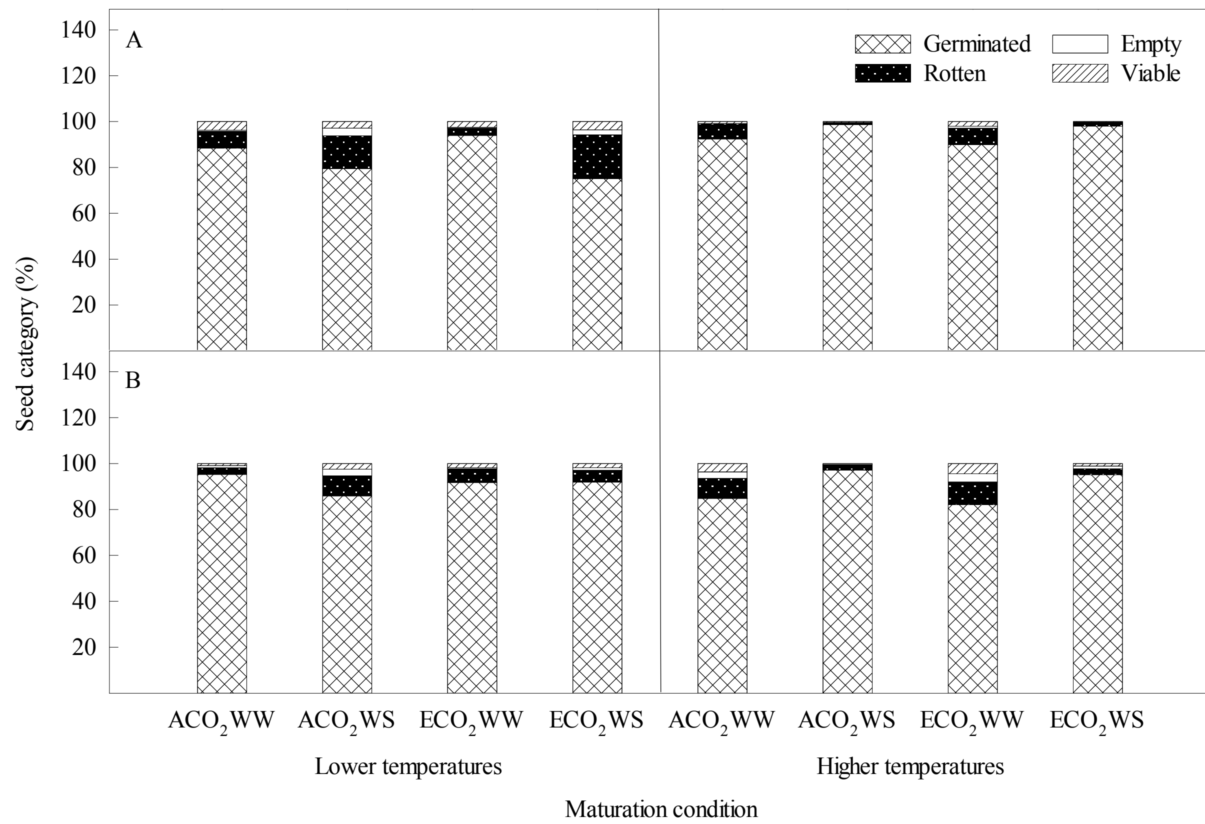

A
B
C
D
E
F
Germination (%)
Coefficient of germination rate
Total phenolic content (mg GAE g^{-1} FW)
120
110
100
90
80
70
60
40
20
6
4
2
Bab
Bbc
Aa
Bc
Aa
Aa
Aa
Aa
Aab
Aabc
Aabc
Aabc
Abc
Aa
Ac
Aab
Bb
Ab
Aa
Bc
Ab
Ab
Ab
Ab
Aa
Aa
Aa
Aa
Ba
Aa
Aa
Aa
Abc
Aab
Acd
Aa
Bd
Bd
Bcd
Bd
Bb
Aab
Aab
Bab
Aa
Ab
Aa
Aab
ACO2
ECO2
ACO2
ECO2
Lower temperature
Higher temperature
Maturation condition

Fig. 4.2 Effects of temperature, carbon dioxide and watering regime on the fate of non-germinated seeds of *Arabidopsis thaliana*. Plants were grown under two temperature regimes (22/18ºC and 28/24ºC; 16 h light/8 h dark), two carbon dioxide concentrations (400 and 700 $\mu mol\ mol^{-1}$), and two watering regimes (well-watered and water-stressed) in controlled-environment growth chambers. (A) WT, (B) *abi1-1*. ACO_2, ambient CO_2; ECO_2, elevated CO_2; WW, well-watered; WS, water-stressed.


A
B
Germinated
Empty
Rotten
Viable
140
120
100
80
60
40
20
Seed category (%)
ACO2WW
ACO2WS
ECO2WW
ECO2WS
Lower temperatures
Higher temperatures
Maturation condition

4.4.5 *Relationship between seed categories*

Pearson's correlation analysis showed numerous significant and interesting relationships between seed germination pattern, fate of none-germinated seeds and total phenolic content (Table 4.3). For example, germination percentage was negatively correlated with non-germinated seeds (viable, rotten, and empty); similarly, germination percentage was negatively correlated with total phenolic content. Germination rate had a significant positive relationship with germination percentage, but a negative relationship with rotten seeds. The percentage of non-germinated viable seeds was positively correlated with the percentage of rotten and empty seeds and total phenolic content (Table 4.3).

Table 4.3 Pearson's correlation coefficients between germination percentage, coefficients of germination rate, non-germinated seeds (viable, rotten, and empty), and total phenolic content in seeds of *Arabidopsis thaliana*. Arabidopsis plants (wild-type and *abi1-1* mutant) were grown under two temperature regimes (22/18ºC and 28/24ºC; 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 45 days, after eight days of initial growth under 22/18ºC. Experiments were conducted three times (n=9), except for the total phenolics (two times, n=6). Seeds were collected and germinated under a temperature regime of 22/18ºC (16 h light/8 h dark), light intensity of 300 µmol m^{-2} s^{-1}, and relative humidity of ~65% for 58 days. Significance values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Parameter	Germination percent	Germination rate	Non-germinated viable	Rotten	Empty	Total phenolics
Germination percent	-					
Germination rate	0.370*	-				
Non-germinated viable	-0.629***	-0.110	-			
Rotten	-0.928***	-0.398*	0.372*	-		
Empty	-0.643***	-0.204	0.393*	0.407*	-	
Total phenolics	-0.851***	-0.290	0.716***	0.712***	0.589***	-

4.5 Discussion

4.5.1 *Effects of temperature*

This study showed that maturation temperatures had the highest effects on subsequent seed germinability and total phenolic content of the resulted seeds. In the current study, higher maturation temperatures were followed by more complete and faster germination of Arabidopsis seeds (Table 4.1; Fig. 4.1A-D). The positive effect of higher temperatures on germination percentage and rate comes in agreement with the results of earlier studies on seeds from other plant species (Qaderi *et al.,* 2006; Pérez-Sánchez *et al.,* 2011). As shown, higher temperatures increased the germination percentage of Scotch thistle (*Onopordum acanthium* L.) cypselas either by making the cypsela coats thinner (Qaderi *et al.,* 2003), or by lowering phenolic compounds and surface wax in cypselas (Qaderi *et al.,* 2006). In the current study, it is likely that higher temperatures have led to increased germination percentage by decreasing the germination-inhibiting factor - the phenolic compounds (Tables 4.1, 4.3; Fig. 4.1E-F).

A significant reduction in the percentage of non-germinated viable seeds was found in seeds that matured under higher temperatures (Table 4.1). In addition, negative correlation between germinated seeds and non-germinated viable seeds revealed that the germinability of seeds was increased by higher temperatures, as expected (Table 4.3). The thinner outer covering of seeds that usually forms under higher temperatures may also enhances the leaching of germination inhibitors from the embryo (Porter and Wareing, 1974) and, thus, decreases the number of non-germinated viable seeds that matured under higher temperatures. In the current study, seeds that matured under higher temperatures had decreased number of rotten seeds at the end of the germination test (Table 4.1). Because seeds that matured under lower temperatures took longer time to germinate, possibly prolonged soaking of seeds increased the number of rotten seeds,

which were followed by seed mortality. The negative relationship between germinated and rotten seeds may also explain the lower germination percentage of seeds that matured under lower temperatures, as more rotten seeds were found from this temperature regime upon viability test (Tables 4.1, 4.3).

4.5.2 *Effects of carbon dioxide*

Elevated CO_2 during seed maturation significantly increased total phenolic content (Table 4.1), but did not affect seed germination percentage or rate. Way *et al*. (2010) also found that neither germination percentage nor germination rate of loblolly pine (*Pinus taeda* L.) seeds were affected by CO_2 treatment. In the current study, the increased total phenolic content of seeds that matured at elevated CO_2 (Table 4.1) is in agreement with the result of Karowe and Grubb (2011) who found higher concentration of phenolic compounds in the oilseed rape (*Brassica rapa* L.) plants grown at elevated CO_2.

4.5.3 *Effects of watering regime*

Seeds that matured on the water-stressed plants had decreased percentage of non-germinated viable seeds (Table 4.1). Unfavorable maturation conditions for seeds could interrupt their development and result in light and shriveled seeds (DeLouche, 1980). Water stress has been reported to increase secondary dormancy in Arabidopsis (Auge *et al.,* 2015). Moreover, water stress has been reported to decrease seed germination and/or viability and to increase seed dormancy (Hawkes, 2004).

4.5.4 *Effects of genotype*

The current study showed that seeds that matured on the WT plants had a lower germination rate, lower percentage of empty seeds, and decreased total phenolic content than the seeds that matured on the *abi1-1* mutant plants (Table 4.1). Non-significant difference in seed germination percentage between WT and *abi1-1* is unusual. The Arabidopsis *abi1-1* phosphatase mutation, which reduced abscisic acid-induced dormancy in seeds (Koornneef *et al.,* 1984) made the *abi1-1* mutant seeds quicker and more germinable and therefore their germination rate was higher (Fig. 4.1C-D). However, the higher percentage of empty seeds and phenolic content of the *abi1-1* mutant seeds (Table 4.1; Figs. 4.1E-F, 4.2A-B), caused them to have similar germination percentage to that of WT seeds. The use of ABA-insensitive mutant seeds showed that under climate change, the ABA content of mature embryo will not be the only factor that can reduce or prevent seed germination**.** Also, increased empty seed number produced by the *abi1-1* plants is related to the stronger effect of stress factors on the *abi1-1* mutant than on the WT genotype. This is mainly because of the ABA signaling impairment in the guard cells of the *abi1–1* mutant (Pei *et al.,* 1997), causing their stomata to remain open and, consequently, increasing transpiration and decreasing photosynthesis. Decreased accumulation of phenolics in the WT seeds could mean that these seeds were less affected by temperature and water stress than the *abi1-1* seeds, as stressed plants usually accumulate more phenolic compounds (Harborne and Williams, 2000).

4.5.5 *Interactive effects of temperature, carbon dioxide, watering regime, and genotype*

Germination percentage was highest for seeds that matured on the water-stressed plants under higher temperatures, but lowest for seeds that matured on the water-stressed plants under lower

temperatures. This result did not concur with the study of Gurvich *et al.* (2017) who found that low water potentials and high temperatures were negatively affected seed germination in cactus (*Echinopsis candicans* L.). Seeds that matured on the water-stressed WT plants that were grown under higher temperatures at ambient CO_2 had 1.3 times higher germination percentage than the seeds that matured on the well-watered WT plants that were grown under lower temperatures at elevated CO_2 (Figs. 4.1A-B, 4.2A-B). Higher maturation temperatures and water stress, individually and together, increased germination percentage, but seeds that matured at elevated CO_2 had decreased germination percentage. Germination percentage has been shown to decrease in Arabidopsis at elevated CO_2 (Andalo *et al.,* 1996). Elevated CO_2 has been shown to increase the C/N ratio and that can cause a reduction in seed protein content, which can be used to provide amino acids necessary for embryo growth during germination (Andalo *et al.,* 1996). Seeds that matured on the well-watered *abi1-1* plants that were grown under higher temperatures at elevated CO_2 had 10 times higher non-germinated viable seeds than the seeds that matured on the water-stressed WT plants that were grown under higher temperatures at ambient CO_2 ($P < 0.05$; Fig. 4.2A-B). It is not surprising to see that seeds matured on the *abi1-1* plants were not more germinable than those of WT plants; the outcome may be due to the accumulation of more phenolic compounds in those seeds. Also, the non-significant positive effect of elevated CO_2 on seed viability is in agreement with the study of Prasad *et al*. (2003) who showed that elevated CO_2 did not counteract the negative effects of high temperatures on seed viability. Earlier studies have shown that *abi1-1* mutation lead to a total (Webb and Hetherington, 1997) or 50 % (Leymarie *et al.,* 1998) suppression of CO_2, sensing in stomata of the *abi1-1* mutant. The seeds from the water-stressed WT plants that were grown under lower temperatures at elevated CO_2 had the highest total phenolic content; this could explain the lowest germination percentage and

the highest germination rate for seeds that matured under this condition in comparison to other conditions (Fig. 4.1A-F).

4.6 ***Conclusion***

Among the main climate change components discussed in the current study, maturation temperature has more effects on subsequent seed germination, germination rate, rotten seeds, and total phenolic content than do CO_2 and watering regime. Seeds that matured under higher temperatures could have less dormancy and exhibit more germiniability than those that matured under lower temperatures. The negative effects of higher temperatures on the non-germinated viable seeds that matured on the *abi1-1* plants was not mitigated by the elevated CO_2. It is, therefore, most likely that higher temperature and water stress will have a strong impact on plant performance, especially on the sensitive plant cultivars, and this could have implications on the survival of sensitive plants under future climates. In addition, this study showed that the effects of the climate-change related factors on the germination rate, germinability, etc., could have implications for seed fate (e.g. germinatin and new plant or seed death in the environment). Based on the results of this study, climate change might have little effects on the germinability of WT seeds, such as weed, but could decrease seeds of ABA-insensitive mutants (e.g., *abi1-1*) through changing their phenolics and other such compounds. This study indicates that there are factors other than ABA that are involved in controlling seed germination in response to climatic factors. Further studies are required to fully understand seed germination patterns under multiple climate change factors.

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CHAPTER 5

General Conclusions

5.1 Conclusions

In general, environmental stress factors negatively affect growth and developmental processes of plants. These factors can affect plants by altering their phenological, morphological, physiological, and biochemical characteristics. Studying the way that plants interact with climate change as well as the various environmental biotic and abiotic stress factors is complex. This complexity comes from the genetic variation within and among species, tissue type, growth conditions and the experimental designs. In the future, the main factors, which are expected to affect plant growth and development will be temperature, CO_2, and water availability. Due to the increased presence of greenhouse gases like carbon dioxide, methane, and nitrous oxide in the atmosphere, temperature of the Earth is rising, and is likely to increase between 1-4°C by the end of this century (Triacca *et al.,* 2014).

While studies examining individual environmental stress factors are abundant, few examine multiple factors and their interactions. Since stress factors occur in combination in natural environments, it is important to understand how plants respond to multiple stresses simultaneously. Crop responses to multiple factors are often greater than observed with individual factors and combining stress factors can lead to antagonistic responses. Increased temperatures and decreased water availability have been shown to reduce plant growth and the quantity and quality of plant yield (Mekonnen *et al.,* 2016). In most cases, elevated CO_2 alleviated the negative impacts of heat and water stress on plant growth and development, but in few cases, the CO_2 effect was not obvious (Gammans *et al.,* 2017). Therefore, this issue requires further investigation to understand the mechanism by which ECO_2 alleviates the negative effects

of different stress factors. This study provides an analysis of the effects of co-occurring environmental stress factors, temperature, CO_2 and watering regime, on plant growth and development.

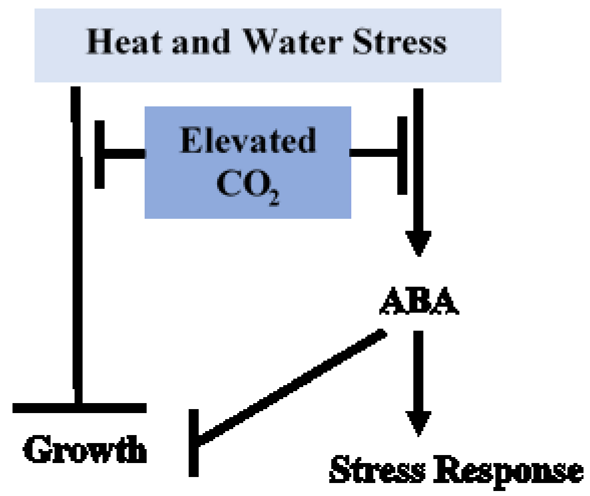
The main work that is described in this thesis is concerned with investigating how plant respond to the combined effects of the three main components of climate change (higher temperature, ECO_2, and water stress) at their vegetative and reproductive stages. We aimed to determine if elevated CO_2 would be able to ameliorate the negative effects of heat and water stresses on plants. This analysis is important given that some reports showed that the improvement of plant growth and development due to elevated CO_2 concentration may be lessened simultaneously by high temperature and water stresses (Albert *et al.,* 2011).

In the first research chapter (Chapter 2), our main purpose was 1) to evaluate to the combined effects of the three factors on *Arabidopsis thaliana* growth at the vegetative stage and 2) determine the role of the hormones such as ABA in plant response to all three factors combined. The results showed that heat and water stresses, alone or combined, inhibited the growth of wild-type *Arabidopsis thaliana* and, importantly, elevated CO_2 alleviated the negative effects of these stresses on growth (Fig. 5.1). For example, compared to the non-stressed condition, biomass decreased by 48% under heat stress, 23% under water stress, and 75% under combined heat and water stresses (Fig. 2.2). However, the observed decrease in biomass was lower when elevated CO_2 was included in the analysis. In the presence of elevated CO_2, biomass decreased by 23.9% under heat stress, 7.3% under water stress, and 26% under heat and water stresses combined (Fig. 2.2). This trend was also observed when other parameters, such as rosette diameter, leaf area, and leaf number, were measured (Fig. 2.1). The positive effects of elevated CO_2 on growth of stressed plants may be due to the observed lower levels of ABA (Fig. 2.8c), which is known to

inhibit growth under unfavourable environmental conditions. The lower ABA levels correlated with a decrease in the expression of ABA-responsive genes such as *RD22* (Fig. 2.9). To gather further insight into the effects of elevated CO_2 on ABA-mediated stress response the growth of the ABA insensitive mutant *abi1-1* was also assessed. Compared to the wild type plants, the *abi1-1* plants were smaller (e.g. lower biomass) and produced higher levels of ABA under heat and water stresses (Fig. 2.1, 2.2 and 2.8d). However, similar to wild type, elevated CO_2 reduced the impact of stress on growth including lowering ABA levels in *abi1-1* plants. Overall these results demonstrate a positive role for CO_2 in promoting tolerance to environmental stresses, such as heat and drought.

The positive effect of elevated CO_2 on stressed plants at their vegetative stage led us to address questions in Chapters 3 and 4 which investigated the impact of the gas on stressed plants at the reproductive stage. In Chapter 3, the study focused on examining the effects of temperature, CO_2, watering regime, and genotype on plant quality and quantity (plant growth, reproductive yield components, and seed amino acids), while the focus in Chapter 4 was on studying the effect of these factors on seed germinability. We found that in contrast to the vegetative stage (Chapter 2) in which we found that elevated CO_2 had positive effects on plants, plant quality and quantity and seed germinability were most affected by higher temperatures. In addition, the results showed that the predicted environmental changes will lead to loss of seed quality, particularly seed mass (Chapter 3), and possibly decreased germination in both genotypes, but more in seeds of *abi1-1* mutants (Chapter 4). However, while seed mass will also change, this does not necessarily imply any negative effect on seed amino acid as they increased in seeds matured under higher temperature, elevated CO_2, and water stress (Tables 3.1 and 3.3).

Fig. 5.1 A proposed model depicting the interaction of the main components of climate change and effects on growth in *Arabidopsis thaliana*. Flowchart representing the proposed model for the role of elevated CO_2 in alleviating the negative effect of higher temperature and water stress on growth and ABA production in *Arabidopsis thaliana*. Elevated CO_2 reduced the impact of stress factors on growth by lowering ABA levels.


Heat and Water Stress
Elevated CO2
ABA
Growth
Stress Response

This result supported our findings in Chapter 4 on germination where we found that seeds that matured under higher temperatures were more germinable than seeds matured under lower temperatures. Moreover, exploring response differences between the WT and the *abi1-1* mutant in terms of their ability to acquire and retain good seed quality (Chapter 3) and germinability (Chapter 4) under stressful environments showed that elevated CO_2 was, in general, less able to mitigate the negative effects of higher temperatures and water stress in the WT and *abi1-1* plants. This result was obvious when a reduction happened in all amino acid contents in *abi1-1* seeds matured on heat-stressed plants at elevated CO_2 with water stress (Fig. 3.5). This is attributed to insensitivity of *abi1-1* plants to ABA, which might have alleviated elevated CO_2 to decrease the negative effects of higher temperatures and water stress on seed quality by increasing photosynthesis through closing the stomata, and thus, decreased transpiration and stomatal conductance. Importantly, elevated CO_2 also prompted the accumulation of more phenolic compounds in *abi1-1* seeds causing them to be less germinable (Table 4.1; Fig. 4.1). This result indicates that there are factors other than ABA that are involved in controlling seed germination in response to climatic factors.

Based on these findings, climate change will bring conditions that may not be suitable for some plants. Crop plants grown in the centre of continents, such as wheat, barley and canola, may experience greater climate shifts than plants grown in coastal regions (Xu *et al.,* 2012). In the absence of agricultural practices alleviating climactic stressors, plant survival is limited to internal adaptive mechanisms (Martel & Qaderi 2016). As the global population continues to rise, food security may be placed into jeopardy if crop plants are unable to maintain productivity due to high stress levels; the economy will also suffer if the amount or the quality of exportable crops is reduced.

This research provides valuable information for advancing our understanding of plant interactions with the main components of climate change. Also, this thesis provides evidence that ECO_2 will have a more positive effect on plants at the vegetative stage than at the reproductive stage. This is because higher growth at elevated CO_2 will not be achieved unless the plant has a way to utilize it, but if there is sink limited capacity, which usually happens after long periods of growing plants under elevated CO_2, a down regulation of photosynthesis will occur (Körner 2000; Kirschbaum 2011). In addition, it showed the importance of ABA to help plants survive under stressful growth condition, but it seems that under climate change other factor would also affect the plant survival through, for example, changing seed germiability.

Therefore, to minimize the risk of reductions in plant growth and seed quality we will therefore have to consider intensifying research in this area particularly with greater number of FACE experiments in addition to chamber and greenhouses studies, in order to generate the best possible understanding of plant responses to elevated CO_2 and to improve their performance. May it will be also helpful to change sowing date so that seed filling occurs at lower temperatures. Finally, adoption of different genetic approaches to developed genotypes suitable to cope with abiotic stresses at both vegetative and reproductive stages is needed for crops to grow better under future climatic conditions.

Further studies are required to focus on the following points to determine:

1. The environmental factor among these factors that have the highest effects on plant growth and development.

2. How plant responses to the interactive effects of temperature, elevated CO_2, and water stress and other abiotic factors, such as increased nitrogen deposition.

3. How the interactions of watering regime with higher temperatures negatively affects the ability of WT plants to accumulate ABA.

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