Norwegian University of Life Sciences
Faculty of Biosciences
Department of Plant Sciences

Philosophiae Doctor (PhD)
Thesis 2021:65

Growth, hydraulic and transcriptomic responses to high air humidity and the involvement of abscisic acid (ABA)

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To my two boys – anything is possible.

Sheona Innes
Oslo, June 2021

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Abbreviations

ABA	Abscisic acid
ATP	Adenosine triphosphate
ATR-FTIR	Attenuated total reflectance Fourier transform infrared Spectroscopy
Az34	ABA-deficient barley mutant
CO_2	Carbon dioxide
CPD-DNA	Cyclobutane pyrimidine dimers
CPK	calcium-dependent kinases
DEGs	Differentially expressed genes
DW	Dry weight
ET	Ethylene
FDR	False discovery rate
flc	ABA-deficient tomato mutant *flacca*
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
GC	Guard cell
GO	Gene ontology
HPLC	High pressure liquid chromatography
JA	Jasmonic acid
KEGG	Kyoto encyclopaedia of genes and genomes
LAR	Leaf area ratio
LMR	Leaf mass ratio
MAPK	Mitogen activated protein kinase
MoCo	Molybdenum cofactor
NAR	Net assimilation rate

NO	nitrous oxide
NR	Nitrate reductase
RGR	Relative growth rate
RGR_{SHOOT}	Shoot relative growth rate
RH	Relative humidity
RLER	Relative leaf expansion rate
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid
SD	Stomatal density
SDR	Short chain alcohol dehydrogenase/reductase
SLA	Specific leaf area
TD	Trichome density
TW	Turgid weight
UV	Ultraviolet
VPD	Vapour pressure deficit
WPT	Whole plant transpiration

List of papers

Paper I

Elevated air humidity increases UV mediated leaf and DNA damage in pea (*Pisum sativum*) due to reduced flavonoid content and antioxidant power

Sheona N. Innes, Louise E. Arve, Boris Zimmermann, Line Nybakken, Tone I. Melby, Knut Asbjørn Solhaug, Jorunn E. Olsen, Sissel Torre

Photochemical and Photobiological Sciences, 2019,18, p 387-399,

DOI: 10.1039/c8pp00401c

Paper II

Different ABA-deficient mutants show unique morphological and hydraulic responses to high air humidity

Sheona N. Innes, Knut Asbjørn Solhaug, Sissel Torre, Ian C. Dodd

Physiologia Plantarum, 2021, 1-13, DOI: 10.1111/ppl.13417

Paper III

Foliar ABA status mediates transcriptomic responses to evaporative demand solely via altered leaf water status in tomato (*Solanum lycopersicum*)

Sheona N. Innes, Torstein Tengs, Ian C. Dodd, Sissel Torre

(manuscript)

Abstract

The aerial environment, which includes relative air humidity (RH), temperature, vapour pressure deficit (VPD) and CO_2 availability, affects plant growth and development in many ways. The aerial environment determines plant gas exchange and influences plant function from physical trait development down to gene expression. The final regulation of plant gas exchange is through the stomatal pores, which are tightly controlled in order to maximise carbon assimilation while minimising excessive water loss to the atmosphere. Stomatal regulation is complex, and the full extent of signals involved in response to the aerial environment remains equivocal. High RH, or low VPD, plays a significant role in stomatal anatomy, movement and function, yet the sensing and signalling pathways associated with high RH and their specific effects on the regulation of transpiration are yet to be fully understood. Studies have shown that continuous growth in high RH leads to diminished stomatal function and response to stomatal closing stimuli, such as darkness and decreased RH. How this diminished function arises, and strategies to combat it are still under investigation, and the involvement of the phytohormone abscisic acid (ABA) is yet to be fully elucidated.

The aim of this PhD was to investigate the effects of high RH on the growth and water relations of several important crop species: pea, tomato and barley, with a focus on stomatal morphology and function, as well as to analyse the interaction between RH and a potential mitigating factor (UV radiation), and determine the role of ABA in short- and long- term responses to high RH using ABA-deficient mutants. Previous studies have shown that periods of reduced RH, increased temperature, increased proportion of blue

light or spraying with exogenous ABA can alleviate the reduction in stomatal functioning often found in plants grown in high RH. Exposure to UV radiation induces stomatal closure via both ABA-dependent and -independent pathways, thus UV radiation was tested as a potential mitigating factor to alleviate the stomatal effects of growth in high RH in pea plants (paper I). Further studies have indicated species-dependent effects of ABA on plant growth and water relations, thus the effects of growth in high RH of tomato and barley plants and their respective ABA-deficient mutants which have lesions in the same step of ABA biosynthesis were tested (paper II). Finally, the effects of long- and short-term exposure to high RH on WT and ABA-deficient *flacca* (*flc*) tomatoes and their differentially expressed genes (DEGs) were investigated to determine the effect of high RH on gene expression and how this was affected by ABA (paper III).

Pea plants grown in high (90%) RH indicated higher susceptibility to leaf and DNA damage by night-time exposure to 0.15 W m^{-2} UV radiation than plants grown in moderate (60%) RH, which was brought about by a lower total antioxidant power and reduced flavonoid content in these leaves (paper I).

Tomato and barley substantially differed in their response to growth in high RH, as well as the role of ABA therein (paper II). High RH alleviated somewhat the effects of ABA-deficiency in both species, though the mechanism was most likely through effects on water status in tomato, but not in barley. The differences between the two species may be linked to inherent differences between eudicots (tomato) and monocots (barley), but

may also have arisen as an artefact of nitrate reductase (NR) deficiency in ABA-deficient barley (Az34), but not in *flc*.

Finally, ABA concentration determined transpiration differences in response to RH in WT tomato plants, but this was driven by an ABA-independent mechanism, possibly a hydraulic response, in *flc* plants (paper III). However, the greater response of WT plants suggested that while hydraulics may induce stomatal and transpirational responses to RH, the presence and regulation of ABA has a greater effect. Furthermore, a lack of ABA resulted in the up- and down-regulation of thousands of genes in *flc* compared to WT plants and in response to changing RH, due to changes in water status, rather than ABA-deficiency *per se*. Despite turgor loss in moderate RH, *flc* plants did not undergo osmotic adjustment to regulate water loss, and showed increased ethylene (ET) biosynthesis and signalling compared to WT plants in moderate RH.

In conclusion, this PhD work has shown species and genotypic differences in the effects of high RH on plant growth and hydraulic responses, with responses driven by different pathways in different species.

Sammendrag

Klimafaktorer som relativ luftfuktighet (RF), temperatur, lys og CO_2 påvirker plantevekst og utvikling. Luftklima, og spesielt RF, bestemmer i stor grad gassutvekslingen og dermed transpirasjon hos planter. Reguleringen av gassutveksling skjer gjennom spalteåpningene, som har en viktig funksjon for å maksimere karbonassimilasjon og minimere vanntapet til atmosfæren. Spalteåpningsregulering er kompleks, og mange ulike typer signaler er involvert. Høy RF (>90%) har stor betydning for spalteåpningenes anatomi og morfologi, samt evne til bevegelse og funksjon. Forståelsen av hvordan signaleringen er aktivert under høy RF og dens spesifikke effekt på regulering av transpirasjon er imidlertid mangelfull. Studier har vist at vekst under kontinuerlig høy RH fører til nedsatt spalteåpningsfunksjon og dermed redusert lukkerespons på signaler som normalt fører til lukking, slik som tørkestress og mørke. Hvordan denne nedsatte funksjonsevnen oppstår og hvordan det er mulig å unngå en slik situasjon krever mer kunnskap, spesielt når det kommer til hvilken rolle plantehormonet abscisinsyre (ABA) har i prosessen. ABA er et av de viktigste signalene som styrer åpning og lukking av spalteåpninger.

Målet med dette PhD-arbeidet var å undersøke effektene av høy RF på vekst og vannrelasjoner hos flere viktige matplanter: ert, tomat og bygg. Arbeidet har fokusert på spalteåpningsmorfologi og funksjon, samt å analysere samspillet mellom RF og ultrafiolett (UV) stråling, og fastslå rollen til ABA i både kort- og lang-tids responser på høy RF ved å benytte ABA-manglende mutanter som verktøy. Studier har vist at funksjonen til spalteåpninger utviklet under høy RF kan forbedres ved at de eksponeres for kortere perioder med lavere RF, økt temperatur, økt andel av blått lys

daglig eller sprøytes med ABA. UV-stråling vil normalt indusere lukking av spalteåpninger gjennom både ABA-avhengig og -uavhengige prosesser, og derfor ble det testet om UV-eksponering kunne forbedre funksjonene til spalteåpninger hos ert utviklet under høy RH (artikkel 1). Studier har vist at effektene av ABA på plantevekst og vannrelasjoner er artsspesifikke, og dermed ble effektene av høy RF på tomat og bygg med deres tilsvarende ABA-manglende mutanter, som er mutert ved samme steget i ABA biosyntese undersøkt (artikkel II). I den siste artikkelen ble effektene av både lang- og kort-tids eksponering av høy RF på villtype og ABA-manglende *flacca* (*flc*) tomat og deres differensielt uttrykte gener undersøkt ved hjelp av RNA-sekvensering (artikkel III).

Ert dyrket i høy RF (90%) viste økt følsomhet for skader på blad og DNA som et resultat av UV eksponering (0.15 W m^{-2}) sammenlignet med planter dyrket i moderat (60%) RF. Økt skade oppsto på grunn av lavere total antioksidantkapasitet (FRAP) og redusert innhold av flavonoider (artikkel 1).

Det ble funnet store forskjeller i respons på RF mellom tomat og bygg, og deres ABA-mutanter (artikkel II). Høy RF dempet effekten av ABA mangel hos begge arter, mest sannsynlig gjennom endringer i vannrelasjoner hos tomat, men ikke hos bygg. Forskjellen mellom artenes respons er muligens et resultat av innebygde forskjeller mellom tofrøbladet (tomat) og enfrøbladet (bygg) arter, men kan også henge sammen med at ABA-manglende bygg (Az34) mangler nitrat reduktase (NR) mens *flc* fortsatt har intakt NR

Det siste manuskriptet i avhandlingen viser at transpirasjonen sannsynligvis er drevet av endringer i mengde ABA hos villtype tomat, men at den er drevet av en ABA-uavhengig mekanisme, muligens hydraulisk respons hos *flc* (artikkel III) i respons på RF. Dessuten, gitt at responsen var sterkere i tomat villtype, ble det konkludert at hydrauliske mekanismer muligens induserer spalteåpnings- og transpirasjons-responser ved endringer i RF, men at tilstedeværelse og regulering av ABA har en sterkere effekt. Mangel på ABA (*flc*) førte til både opp- og ned-regulering av tusenvis av gener sammenlignet med villtype tomat. Den store forskjellen mellom *flc* og villtype oppsto på grunn av endringer i vannstatus, heller enn ABA-mangel *per se*. Til tross for tap av turgor under moderat RF har ikke *flc* planter evne til å gjennomgå osmotiske justeringer for å regulere vanntap, men viste økt biosyntese og signalering av etylen sammenlignet med villtype under moderat RF

Denne avhandlingen viser at effektene av høy RF på plantenes vekst og hydraulisk respons er art-spesifikke, og at responsene er drevet av ulike mekanismer i forskjellige arter. Det er derfor viktig å forstå ulike arters luftfuktighetsresponser for optimal vekst og stresstoleranse.

Introduction

5.1 Background for the study

Plant gas exchange with the atmosphere is one of the most important physiological processes on Earth, utilising atmospheric carbon dioxide (CO_2) and producing life-giving oxygen to nearly all organisms in Earth's biosphere. The colonisation of land by plants was made possible by the acquisition of stomata, which allow plants to both take in CO_2 for carbon assimilation and control transpirational water loss (Fanourakis et al. 2013; Franks & Farquhar 1999). One of the main driving factors of plant transpiration is the difference in vapour pressure deficit (VPD) between leaf tissues and the atmosphere, where a lower atmospheric water potential drives evaporation from the leaf, where the water potential is higher and often assumed to be 100% in intercellular air spaces, via stomatal pores (Fanourakis et al. 2020a). Thus, high relative air humidity (RH), or low VPD, decreases atmospheric evaporative demand and slows transpiration rates (Armstrong & Kirkby 1979; Grange & Hand 1987). High RH is frequently found in natural environments (see Fanourakis et al. 2020a for a review). Indeed, one of the predicted consequences of global climate change is an increase in RH in certain areas, most notably in the northern latitudes, due to an increase in precipitation and cloudiness (IPCC 2013). Yet it is also often found in densely packed controlled environment plant production systems as a result of a trade-off between energy saving and ventilation in high-latitude production during winter (Mortensen 2000; Terfa 2013).

In greenhouse production, the humidity is both determined by and determines the rate of transpiration of the plants, being therefore both an

input and an output in a closed system (Bakker 1991). Humidity control in greenhouse production has previously been overlooked (Mortensen & Gislerød 1999), yet it plays an important role in determining plant transpiration by driving differences in evaporative demand. The rate of transpiration affects several processes in plants, including water balance, evaporative cooling and nutrient and ion uptake (Fanourakis et al. 2020a; Terfa 2013), which in turn affects nearly all aspects of plant growth, development and survival. Energy efficiency is a strong concern in greenhouse production, where costs of heating and ventilation may encompass up to 85% of total operation costs in high latitude systems (Fanourakis et al. 2020a). Strategies such as insulation and temperature integration comprise some of the means employed to make greenhouse production more efficient, yet the control of humidity remains a limiting factor (Körner & Challa 2003). The optimum RH range in greenhouse production of most crops is 60-85%, which keeps transpiration at a manageable rate without the plants getting water stressed, yet is not humid enough for biotic stress (i.e. pathogen infection) to be a problem (Shamshiri et al. 2018). However, the RH in the microclimate within a crown or a dense canopy is usually higher than the surrounding air, adding a further level of difficulty in controlling RH over large scale production (Fanourakis et al. 2020a).

5.2 Transpiration and stomatal functioning in high RH

The rate of total transpiration is determined by two factors: 1) water loss through the cuticle, which may be regulated by factors such as epicuticular wax deposition and polar pore formation in the cuticle (Schreiber et al. 2001), and 2) water loss through stomatal pores, which may be regulated

through stomatal development and aperture control (Fanourakis et al. 2020a). The amount of water lost via the cuticle is often deemed negligible as the stomatal component of transpiration is considerably higher (Rezaei Nejad & Van Meeteren 2005). However, any increase in stomatal resistance (i.e. stomatal closure) increases the fraction of cuticular transpiration to the total transpiration, thereby increasing its importance (Fanourakis et al. 2020a). It has previously been thought that the presence of epicuticular wax was the main barrier to cuticular transpiration, based on the crystalline structure of the waxes (Sánchez et al. 2001). Studies have shown that plants grown in high RH environments, such as *in vitro* culture, have lowered cuticular resistance to transpiration and higher rates of cuticular water loss, attributed to the decreased formation of epicuticular wax in tissue culture-grown plants (Fabbri et al. 1986; Sutter & Langhans 1982). Yet Torre et al. (2003) found no difference in epicuticular wax formation in roses grown in high (90%) RH compared to moderate (60%) RH. In addition to its role in transpiration, the plant cuticle also plays a role in plant-pathogen interactions, as well as abiotic stress resistance, such as protection from ultraviolet (UV) radiation (Macková et al. 2013; Zeisler-Diehl et al. 2018).

5.2.1 Stomatal morphology and anatomy

Steady-state transpiration via the stomata is largely based on stomatal anatomy, which is determined during stomatal development, and is thereby affected by the environmental conditions in which a plant is grown. This occurs via the determination of stomatal density (SD; the number of stomata per leaf area), patterning and size, for which stomatal pore length is often used as a proxy (Fanourakis et al. 2020a). On a molecular level, SD and patterning is determined during development by the regulation of three

transcription factors: SPEECHLESS (SPCH), MUTE and FAMA, which in turn are regulated by a peptide signalling pathway which acts through a mitogen activated protein kinase (MAPK) cascade. These are all influenced by plant hormones including brassinosteroids and abscisic acid (ABA), as well as environmental factors such as CO_2 availability and RH (Zoulias et al. 2018). There are also several proteases involved in the stomatal development process which selectively cleave proteins, suggesting that proteolysis is an important aspect of stomatal development (Fanourakis et al. 2020b). However, while the molecular components have been identified, the processes involved in environmental regulation of stomatal development remain equivocal (Fanourakis et al. 2020b; Zoulias et al. 2018).

A safety-efficiency trade-off was proposed to determine stomatal size and density, where species with a higher stomatal conductance under high water availability show a greater sensitivity to dehydration as a closing signal due to having a higher water potential at the point when stomatal conductance reaches 50% under drought (Henry et al. 2019). Thus, species with small, high density stomata have faster response times to water loss and are more stress tolerant, while those with fewer, larger stomata have higher potential growth, yet respond slower to water loss (Henry et al. 2019; Lawson & Blatt 2014). Plants grown in high RH show species-dependent effects on SD (see Fanourakis et al. 2020a for review), but have been found to have universally larger stomata (Aliniaeifard et al. 2014; Bakker 1991; Rezaei Nejad & Van Meeteren 2005; Torre et al. 2003), which would perturb the safety-efficiency balance, and the larger stomata may explain some of the reduced functionality in response to water loss. Increased stomatal size in high RH

has been attributed to lower levels of ABA found in plants grown in high RH (Giday et al. 2014), and this will be discussed further below.

5.2.2 Stomatal aperture control by guard cells

Transient adjustment of stomatal apertures, i.e. in response to environmental conditions and changes thereof, has been thoroughly investigated, and the opening and closing of stomata is a tightly regulated process (Fanourakis et al. 2016; Fanourakis et al. 2020a; Merilo et al. 2018; Tossi et al. 2014). The stomatal pore is surrounded by two guard cells (GCs), and is opened or closed through regulation of GC turgor. This is controlled through the influx/efflux of osmotically active ions and solutes, which determines cellular osmotic potential, thereby affecting water potential and water flux into and out of the cells (Fig. 1A). During stomatal opening, hyperpolarisation of the plasma membrane by H^+-ATPases causes H^+ ion extrusion and activates inward-rectifying K^+ channels. At the same time, H^+/Cl^- symporters transport H^+ and Cl^- ions into the GCs. A similar electrochemical gradient across the tonoplast is driven by V-type ATPases, causing influx of H^+ and Cl^- into the vacuole, while H^+-driven antiporters transport K^+ ions into the vacuole (Matrosova 2015). This results in increased osmotic potential and decreased water potential, driving an influx of water into the GC.

In stomatal closure, K^+ extrusion from the vacuole depolarises the tonoplast, driving Cl^- efflux through anion channels. Subsequent activation of plasma membrane anion channels drives anion efflux from the GCs, depolarising the plasma membrane and driving activation of K^+ outward-rectifying channels and K^+ efflux (Matrosova 2015). GC ion channel and membrane

transport regulation is highly complex, with a large number of genes involved in maintaining cellular ion homeostasis (see Fig. 1B), especially in response to environmental conditions (Saito & Uozumi 2019). Ca^{2+} plays a significant signalling role in GC movement, especially stomatal closure (see Fig. 1B). Ca^{2+} initiates signalling cascades via calcium-dependent kinases (CPKs), inducing activation of the SLOW ANION CHANNEL-ASSOCIATED1/HOMOLOGUES (SLAC1/SLAH) anion efflux channels and GUARD CELL OUTWARD-RECTIFYING K^+ (GORK) channels, driving anion and K^+ efflux from the cytoplasm to increase water potential and reduce GC turgor (Förster et al. 2019; Saito & Uozumi 2019).

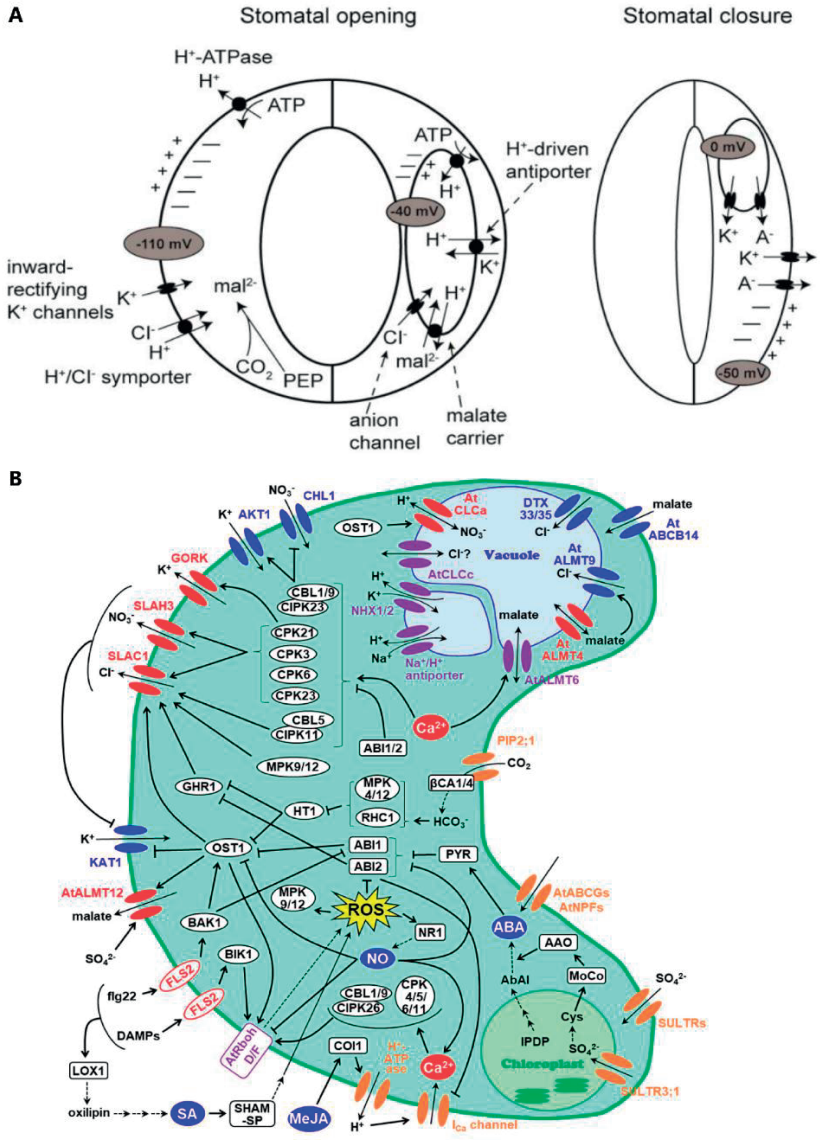


Fig. 1. A) Guard cell (GC) opening and closure is driven by changes in turgor, brought about by the influx and efflux of ions due to changes in membrane channel polarisation. Figure adapted from Matrosova (2015). B) GC ion channels are regulated by a large number of genes and signalling cascades. Figure adapted from Saito and Uozumi (2019).

5.2.3 Hormonal control of stomatal aperture

Hormonal regulation of stomatal movement is mainly driven by ABA, yet is also controlled by other hormones, such as jasmonic acid (JA, as methyl jasmonate (MeJA), in GCs), salicylic acid (SA) (Liu et al. 2003; Munemasa et al. 2015; Saito & Uozumi 2019), strigolactones (SL) (Lv et al. 2018), ethylene, auxin, cytokinin and brassinosteroids (Dodd 2003a). ABA, JA, SA and SL induce stomatal closure by activating SLAC1 ion channels via increases in cytosolic [Ca^{2+}], though through different mechanisms and pathways (Fig. 1B, for a highly detailed description, see Saito & Uozumi 2019). ABA is generally deemed the most important hormone involved in stomatal movement, and it induces stomatal closure via a signalling cascade initiated by its binding to PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) receptors. These further bind to protein phosphatase 2Cs (PP2Cs), which allows phosphorylation of sucrose non-fermenting 1-related subfamily2 (SnRK2) protein kinases. These then activate SLAC1/SLAH ion channels, driving stomatal closure (Fig. 2A) (Munemasa et al. 2015). Stomatal ion channels may also be activated by hormone-independent pathways (Fig. 2B-C). For example, increased [CO_2] acts in an ABA-independent manner, activating ion channels downstream of OST1 to initiate stomatal closure (Fig. 2B) (Hsu et al. 2018), and UV-B radiation, given at doses resembling those in nature, initiates a signalling cascade that is ABA-independent, yet activates NR, which induce nitrous oxide (NO) production and stomatal closure (Fig. 2C) (Tossi et al. 2014).

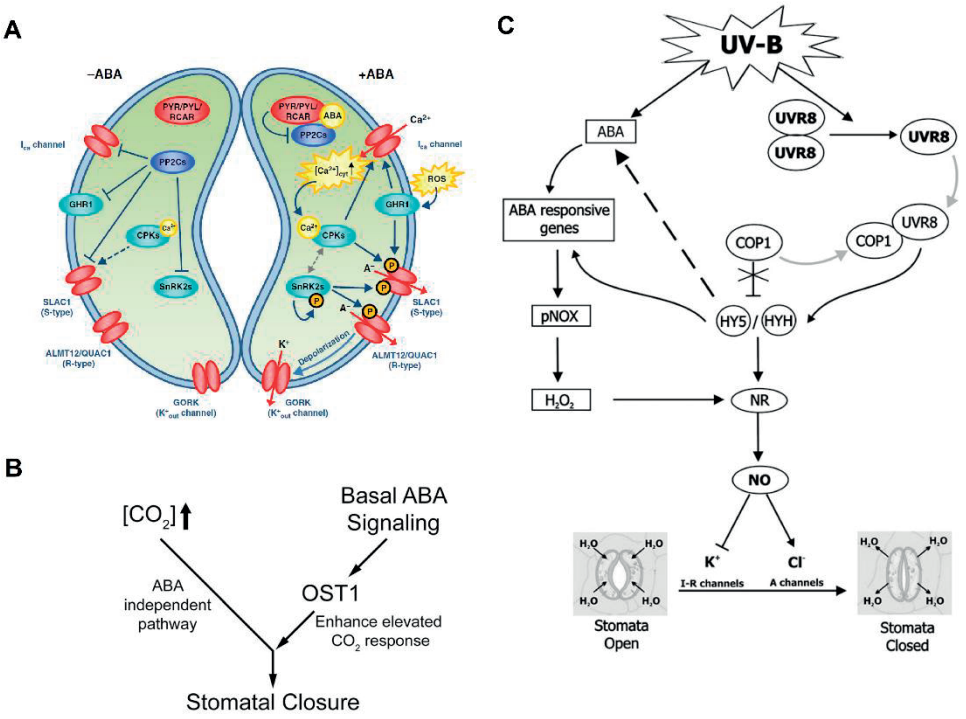


Fig. 2. ABA-dependent and -independent pathways involved in stomatal closure. A) ABA associates with PYR/PYL/RCAR receptors, which bind to PP2Cs. This allows phosphorylation of SnRK2s and stimulation of CPKs, which activate SLAC1 ion channels and facilitate stomatal closure. Figure adapted from Munemasa et al. (2015). B) Increased [CO_2] facilitates stomatal closure via a pathway acting downstream of OST1, figure adapted from Hsu et al. (2018). C) UV-B radiation acts via both ABA-dependent and -independent pathways. In the ABA-independent pathway UV-B initiates a signalling cascade via UVR8, a UV-specific receptor, which binds to COP1, allowing activation of HY5/HYH transcription factors, which then initiate NR and nitrous oxide NO production to induce stomatal closure. Figure adapted from Tossi et al. (2014).

Stomata of plants grown in high RH have been shown to have reduced functionality, in that closing stimuli, (e.g. darkness and desiccation) do not elicit stomatal closure to the same extent as in plants grown in more moderate RH regimes (Arve et al. 2013; Fanourakis et al. 2011; Mortensen 2000; Rezaei Nejad & Van Meeteren 2005; Torre & Fjeld 2001; Torre et al. 2003). Reduced stomatal functioning has been found in several species developed under conditions of low evaporative demand, including roses (Arve et al. 2013; Fanourakis et al. 2011; Torre & Fjeld 2001; Torre et al. 2003), *Tradescantia virginiana* (Rezaei Nejad & Van Meeteren 2005), several tree species (Aasamaa & Sõber 2011; Fordham et al. 2001; Lihavainen et al. 2016; Oksanen et al. 2019), micropropagated *Delphinium* plants (Santamaria et al. 1993), *Vicia faba* (Aliniaeifard et al. 2014; Arve et al. 2014), *Arabidopsis thaliana* (Aliniaeifard & van Meeteren 2014) and tomato (Arve & Torre 2015). Specific treatments to stimulate stomatal closure during growth have been investigated for their potential to combat diminished stomatal functioning in high RH, with results varying with the stimulus (Fanourakis et al. 2016). For instance, partial root zone drying, which transiently promotes root to shoot ABA signalling (Dodd et al. 2015) had no effect on stomatal aperture in high RH (Fanourakis et al. 2016), while root water deficit improved stomatal functionality of high RH plants to the same level as plants grown in moderate RH (Giday et al. 2014). Moreover, while decreasing RH for a period of time every day during growth only slightly improved stomatal functionality in high RH (Arve et al. 2017), increasing the proportion of blue light in the spectrum enhanced stomatal functionality significantly (Terfa et al. 2020). Further investigation into the mechanisms underlying stomatal response to growth under high RH may provide insight for the development of mitigating strategies in

production systems. Thus, one of the aims of this PhD work was to investigate such mechanisms and the role of ABA down to the transcriptomic level (paper III).

Exposure to UV radiation in low doses elicits a range of chemical and photomorphogenic responses in plants (Robson et al. 2015), including biosynthesis of antioxidants including flavonoids (Yin & Ulm 2017), alterations in plant water relations through effects on stomatal movement (Jansen & Van Den Noort 2000) and changes in plant architecture (Robson et al. 2015). Increased stomatal closure is a frequently reported, though not universal, consequence of exposure to UV radiation, most often given as UV-B radiation (He et al. 2005; Tossi et al. 2014). Thus, one of the aims of this study was to investigate the possibility of mitigating diminished stomatal functioning in high RH by promoting stomatal closure using UV radiation (paper I). This was performed in a factorial experiment using pea plants grown in moderate or high RH, either experiencing exposure to UV radiation or not. It was hypothesised that, given the low dose, the UV radiation would promote stomatal closure in high RH and thereby combat diminished stomatal functioning, while enhancing plant antioxidant concentrations and possibly controlling plant architecture to create more compact plants (paper I).

In many cases the reduced stomatal functioning response has been attributed to a decrease in foliar ABA levels in these conditions (Giday et al. 2013a; Rezaei Nejad & van Meeteren 2006; Rezaei Nejad & van Meeteren 2008), due to increased hydroxylation of ABA to phaseic acid by ABA 8'-hydroxylase in *Arabidopsis thaliana* (Okamoto et al. 2009) or decreased

deconjugation from ABA-glucose ester (ABA-GE) to active ABA by β-glucosidase in *Rosa hybrida* (Arve et al. 2013). Previously, it was assumed that foliar [ABA] was controlled by root ABA biosynthesis and xylem transport, but grafting studies with ABA-deficient rootstocks determined shoot ABA biosynthesis to be sufficient for maintaining a WT phenotype (Dodd et al. 2009; Jones et al. 1987). While foliar [ABA] seems to be important, further studies have indicated the importance of ABA receptors and signalling molecules, most notably OST1, in stomatal response to different RH levels (Gonzalez-Guzman et al. 2012; Merilo et al. 2013; Merilo et al. 2018). However, while ABA concentration and signalling seems to play a role, the exact mechanism(s) by which reduced stomatal functioning is brought about in response to high RH are yet to be elucidated. Therefore, one of the aims of this study was to investigate the role of ABA in transpiration and stomatal responses to high RH in different species (papers II and III). This was performed in paper II by testing the morphological and hydraulic responses of two important crop species, tomato (*Solanum lycopersicum*) and barley (*Hordeum vulgare*), along with their constituent ABA-deficient mutants, *flacca* (*flc*) and Az34, which are mutated in the same locus (see Table 1), to growth in high RH.

5.3 Role of RH and ABA on growth and morphology in different species

Growth in high RH not only affects stomatal traits and water relations, it has also been shown to affect aspects of whole plant growth and morphology, and the most commonly reported effects include increasing biomass, leaf area and the number of leaves of several species (Grange & Hand 1987; Hoffman et al. 1971; Hovenden et al. 2012; Innes et al. 2018b; Leuschner 2002; Lihavainen et al. 2016; Mortensen & Gislerød 1990;

Mortensen 2000; Oksanen et al. 2019). Some variation in morphological responses to high RH have been reported, which were determined as dependent on species and soil salinity (Mortensen & Gislerød 1990; Mortensen 2000). Additionally, differences have been reported in the response of leaf thickness and leaf anatomy to high RH, which may be attributed to differences in the mechanism by which leaf expansion occurred – i.e. epidermal cell expansion or cell division (Hovenden et al. 2012; Leuschner 2002; Torre et al. 2003), though increased leaf water status resulting from growth in low evaporative demand plays a role (Leuschner 2002; Lihavainen et al. 2016; Mortensen 2000). Transpiration determines the xylem sap flux through the plant, and while this may increase leaf water status, it may also decrease both nutrient and ion uptake from the rhizosphere (Bakker 1991; Fanourakis et al. 2020a) and mass flow of nutrients in the soil to the rhizosphere (Cramer et al. 2009). Thus, decreased transpiration rates have been shown to induce varying nutrient deficiencies (N, K, P and Ca) in different plant species (del Amor & Marcelis 2005; Gislerød et al. 1987; Gislerød & Mortensen 1990; Holder & Cockshull 1990; Lihavainen et al. 2016). Leuschner (2002) and Oksanen et al. (2019) reported nutrient dilution in temperate woodland herbs and northern forest trees grown in high RH, respectively, and decreased leaf area has been attributed to low leaf [Ca^{2+}] in several species grown in high RH (Mortensen 2000; Oksanen et al. 2019). Lihavainen et al. (2016) found decreased foliar N content in silver birch grown in high RH, which resulted in disturbance of carbon and nutrient homeostasis. However, as indicated by Lihavainen et al. (2016), these are results of short-term experiments, and long-term measurements would indicate whether the nutrient deficiencies indicated

here represent a temporary imbalance in nutrients due to high RH or a more severe deficiency which would affect growth and development.

The effects of high RH on leaf morphology and anatomy are seemingly species dependent. For instance, Leuschner (2002) found larger leaf area in five of six woodland herb species, thicker leaves in two of four species, and significantly higher fractional air space in transverse sections in four of four species grown in high relative to moderate RH. Leaves of *Nothofagus cunninghamii* showed a similar increase in leaf area and thickness, and decreased specific leaf area (SLA) when grown in high RH (Hovenden et al. 2012). Yet roses showed a decrease in leaf thickness, but a similar increase in intercellular air space and decreased vascular density when grown in high relative to moderate RH (Torre et al. 2003). Leuschner (2002) attributed the increase in leaf area to an increase in epidermal cell expansion, while Hovenden et al. (2012) determined a significant increase in epidermal cell division to be the cause. Torre et al. (2003) attributed their results to a reduction in both epidermal thickness and the size of palisade and spongy mesophyll cells. Increased intercellular air spaces in high RH may result from the epidermis expanding at a faster rate than the palisade and spongy mesophyll cells (Waldron & Terry 1987). Thus, not only are the effects of high RH species-dependent, but the mechanisms by which RH affects morphology are seemingly interspecific too.

Transpiration rates affect leaf expansion through competition between availability of water for evaporative demand vs. growth due to the importance of epidermal water status in controlling leaf expansion (Waldron & Terry 1987). This means that the reduction of steady-state transpiration rate that occurs in high RH may stimulate leaf expansion by

enhancing leaf water status (Leuschner 2002). The transpiration-leaf water status link may furthermore account for the characteristically smaller leaves of ABA-deficient mutants (Sharp et al. 2000), which generally show considerably higher transpiration rates and lower leaf water contents than their WT counterparts (Bradford 1983; Sagi et al. 2002; Tal 1966; Walker-Simmons et al. 1989). However, ABA-deficiency can also inhibit shoot growth by non-hydraulic mechanisms (Bradford 1983; Mulholland et al. 1996a; Mulholland et al. 1996b), such as increased ethylene (ET) production (Dodd et al. 2009; LeNoble et al. 2004; Sharp et al. 2000), yet this result is not universal (Dodd 2003b; Tardieu et al. 2010). Previous studies that have looked at the effects of ABA and RH have generally focused on attenuating the negative effects of ABA-deficiency on water status by growing the ABA-deficient plants in high RH, yet have failed to consider the effects of high RH *per se* or a possible ABA-RH interaction (Mäkelä et al. 2003; Mulholland et al. 1996a; Okamoto et al. 2009; Sharp et al. 2000; Yaaran et al. 2019). Thus, the experiments performed here to test the effects of high RH and ABA were factorial, to separate the effects of these two factors (papers II and III). The experiments were furthermore performed on a eudicot and a monocot (paper II), to thus investigate possible differences in effect between the two clades. One of the fundamental differences between monocots and eudicots is that the transpiration and leaf expansion zones are spatially separate in monocots, while in eudicots this is not the case (Radin 1983). This means that the effects of high RH and ABA may differ between tomato and barley due to fundamental differences in morphology.

Formal growth analyses, that investigate relative growth rate (RGR, see equation 1) as well as its underlying components (Poorter 2002) have been performed to some extent on ABA-deficient mutants, yet to the best of our knowledge have not been used to investigate the effects of high RH. Studies on the ABA-deficient tomato mutant *sitiens* indicated that decreased RGR in the mutant was the result of lower SLA, while NAR and LMR were unaffected (Mäkelä et al. 2003; Nagel et al. 1994), yet in *flc* the same decrease in RGR was attributed to decreased NAR, while SLA was unaffected (Coleman & Schneider 1996). ABA-deficient barley plants had a higher SLA than WT, yet RGR was not measured in the study (Mulholland et al. 1996a). Given the differences in growth conditions and water status among the extant studies, the details of the effects of ABA and high RH on plant growth in different species remain incomplete. Thus, a formal growth analysis with a factorial experimental design was performed to investigate the separate and combined effects of high RH and ABA-deficiency (paper II), and hypothesised that high RH would promote growth and water status of ABA-deficient mutants.

1) RGR = NAR * (SLA * LMR)

Where, RGR = relative growth rate,
NAR = net assimilation rate, the rate of mass increase per unit leaf area,
SLA = specific leaf area, the ratio of leaf area to total mass,
LMR = leaf mass ratio, the ratio of leaf mass to total plant mass and
LAR = leaf area ratio = SLA * LMR

5.4 The role of high RH and ABA in abiotic and biotic defence responses

Reduced stomatal function and disturbed water relations after growth in high RH leaves plants more susceptible to both abiotic and biotic stress, such as drought, salt, heat and pathogen infection (Fanourakis et al. 2020a). Higher rates of water loss upon experiencing stress facilitates wilting and plant death, as plants are unable to control water loss and the available resources become rapidly depleted (Aliniaeifard & van Meeteren 2013; Torre et al. 2003). Furthermore, effects of high RH on the plant cuticle (see section 5.2) have the potential to increase plant susceptibility to both abiotic stressors, such as UV radiation, and biotic stress such as pathogen infection.

ABA is known as the stress response hormone, and, as indicated above, plays a significant role in both plant growth and stomatal movement regulation. Several abiotic stress conditions, such as drought and salt stress (Zuo et al. 2019), cause upregulation of ABA biosynthesis, which induces stress-related genes and stomatal closure (Lee & Luan 2012). This, together with its synergistic/antagonistic relationship with ET and ET-JA signalling pathways (Anderson et al. 2004) indicate ABA as a mediator at the crossroads between abiotic and biotic stress defence responses (Lee & Luan 2012). High RH is a condition under which plant disease outbreaks often occur in natural systems (Panchal et al. 2016), as open stomata are more susceptible to microbial entry and pathogen infection (Melotto et al. 2006). The reported decline in [ABA] in plants grown in high RH, as well as the potential reduction in stomatal function (see previous sections) leaves plants grown in high RH both more susceptible to pathogen infection (Fanourakis et al. 2020a). ET and JA have a synergistic relationship and their combined

signalling pathway is important for resistance to necrotrophic pathogens (Anderson et al. 2004). Yet ABA has an antagonistic relationship with the ET-JA signalling pathway, such that the presence of ABA may partially attenuate somewhat plant tolerance to such pathogens, with ABA-deficient mutants more resistant to some pathogens (Audenaert et al. 2002; Zhang & Sonnewald 2017).

The relationship between ABA and ET is more complex than that, however. For instance, ET inhibited ABA-induced stomatal closure during drought stress (Tanaka et al. 2005), and inhibit shoot growth in conditions of low [ABA] (LeNoble et al. 2004; Sharp & LeNoble 2002) in *Arabidopsis.* Yet recent studies have shown that the antagonism between the two hormones strongly depends on their endogenous concentrations, where low concentrations of one will activate biosynthesis of the other, despite mutual antagonism when concentrations of both are high (Müller 2021). Not only are ABA-ET relations important in biotic stress responses, both ABA and ET are involved in abiotic stress responses, and the importance of ABA-ET interaction in stomatal movement regulation plays a role in both biotic and abiotic stress responses (Müller 2021). While ABA and ET separately induce stomatal closure, the presence of both hormones induces antioxidant activity, which results in stomatal opening via downregulation of H_2O_2 production (Fig. 3).

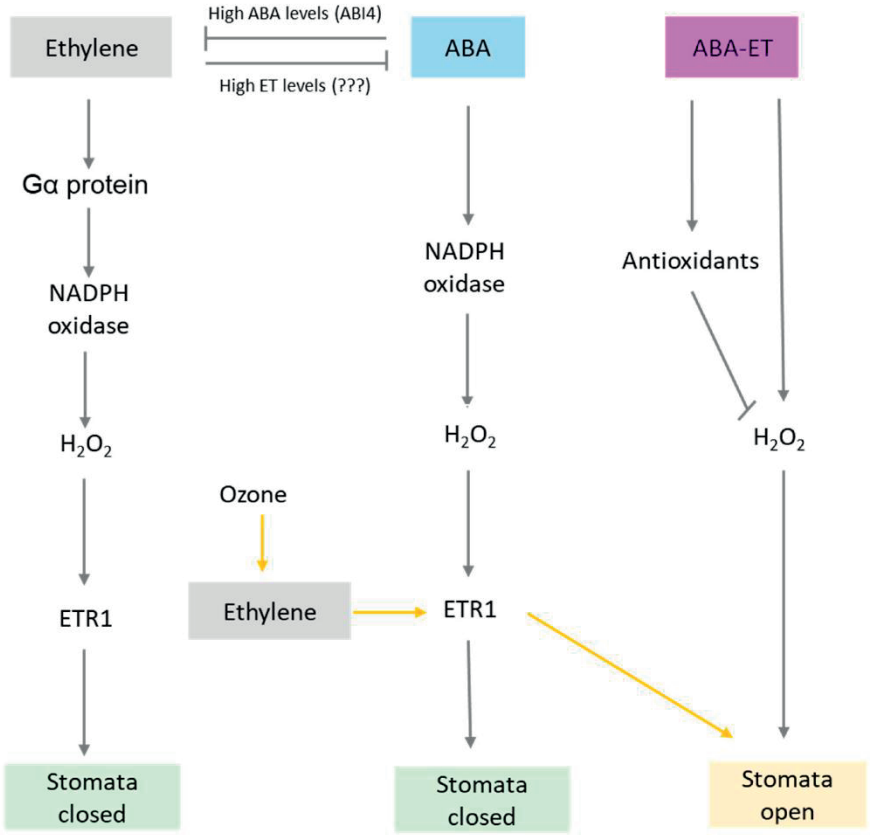


Fig. 3. Separate and combined effects of ET and ABA on stomatal movement regulation. Separately, ABA and ET both induce stomatal closure via H_2O_2 and/or ETR1 upregulation, while presence of both hormones inhibits H_2O_2 via antioxidant production, resulting in stomatal opening. Figure adapted from Müller (2021).

5.5 Role of ABA in high RH responses at a genetic level

Generally, the genetic control of transpiration is attributed to ABA and ABA-activated genes and signalling cascades (Nilson & Assmann 2007). Yet while many ABA-related genes are involved in regulating stomatal

movement and water flux, ABA-dependence in transpiration is not universal (Hsu et al. 2018; McAdam & Brodribb 2015). The different pathways involved in controlling stomatal movement have been investigated at a genetic level rather extensively, yet studies of responses to changes in RH (or VPD) have concentrated mainly on the effects of decreased RH, or increased evaporative demand (McAdam et al. 2016; Merilo et al. 2018). While comprehensive work has attempted to elucidate the mechanisms behind plant high RH responses, few have been performed at the -omics level. Panchal et al. (2016) analysed transcriptomic responses of *Arabidopsis thaliana* to high RH with a focus on biotic stress resistance, and found that high RH activates the JA signalling pathway in the short term (1 h exposure to high RH), yet suppresses it after 8 h exposure, and suppresses SA signalling after 1 h exposure. While these results provide useful insights, they do not indicate how growth in high RH would affect either of these pathways. While gene expression of selected ABA 8'-hydroxylase genes (CYP707As) using quantitative real-time PCR (qRT-PCR) determined the role of ABA catabolism in high RH effects (Arve et al. 2015; Okamoto et al. 2009), neither study investigated responses of the whole transcriptome. Thus, the final aim of this study was to investigate the role of ABA in high RH effects at a transcriptomic level, by analysing differential gene expression of the entire transcriptome of WT and *flc* plants grown in moderate or high RH (paper III). In addition to the long-term effects on gene expression in different growth conditions, short term responses and growth condition artefacts were analysed by reciprocal transfer of the plants between RH treatments for 24 h before further gene expression analyses. It was hypothesised that a different strategy for the control of water loss in ABA-deficient *flc* compared to WT plants under

changing evaporative demand would be detectable at a genetic level, thus allowing us to determine a genetic basis for control of water relations under low [ABA].

Objectives

The main objective of this study was to improve the understanding of the effects of high relative air humidity (RH) on plant growth and water relations, with specific focus on the effects on stomatal function and regulation and the involvement of ABA.

The specific objectives and hypotheses were:

- To investigate the effect of exposure to UV radiation on growth and stomatal responses of pea plants to growth in high RH. It was hypothesised that, since UV radiation induces stomatal closure, exposure to UV radiation during growth in high RH might mitigate the adverse effects of high RH previously found in stomatal functioning.

- To compare the growth and hydraulic responses of two important crop species – tomato and barley - to growth in high RH, and to investigate the role of ABA in these responses using ABA-deficient mutants with lesions in the same locus. It was hypothesised that growth in high RH would promote growth and water status of the ABA-deficient mutants due to diminished evaporative demand and excessive water loss.

- To investigate more thoroughly the role of ABA in hydraulic and genetic responses, using the transcriptome and analysing differentially expressed genes (DEGs) in response to long- and short- term exposure to high RH (analysed here as low VPD). It was hypothesised that *flc* plants would compensate for a lack of ABA by regulating different genes and regulatory pathways.

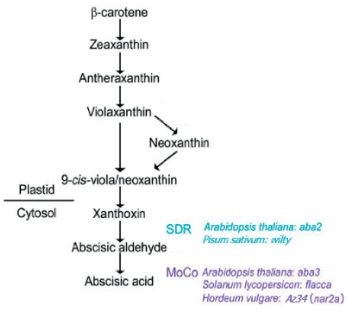
Materials and methods

7.1 Plant material

Three plant species were used in this study: *Pisum sativum* L., cv 'Torsdag' (pea, paper I), *Solanum lycopersicum*, cv 'Ailsa Craig' (tomato, paper II and III), and *Hordeum vulgare* cv. 'Steptoe' (barley, paper II). ABA-deficient mutants of tomato (*flc*) and barley (Az34, also called *nar2*) were used in paper II (both genotypes) and paper III (only tomato). Both ABA-deficient mutant genotypes are deficient in the synthesis of a molybdenum cofactor (MoCo) necessary for the activation of abscisic aldehyde oxidase, which catalyses the final step of ABA biosynthesis (Sagi et al. 2002; Walker-Simmons et al. 1989). Originally, *Pisum sativum* (pea, lines A10 and A1-22 *wilty*, as described in (Dodd 2003b)) were included in analyses for paper II, but were not included in the final publication due to differences in the mutated locus between mutants, affecting a different step in ABA biosynthesis. ABA-deficient *wilty* peas have a mutation which causes deficiency in the short-chain alcohol dehydrogenase/reductase (SDR) that facilitates conversion of xanthoxin to abscisic aldehyde (McAdam et al. 2015). Table 1 indicates the ABA-deficient mutants used throughout this study and illustrates where the mutations affects the ABA biosynthetic pathway, as well as their *Arabidopsis thaliana* counterparts. All three chosen species are important crops with available ABA-deficient mutants. Additionally, while tomato and pea are both eudicot species, peas are also legumes. Barley, on the other hand, is a monocot species. This allowed ABA-deficient eudicot and monocot species to be compared, and in the case of tomato and barley, genotypes that are mutated in the same locus.

Table 1. Species, genotype and mutation description of the ABA-deficient mutants used in this experiment. Schematic indicates where the mutant plants have mutations in the ABA biosynthesis pathway, as well as the corresponding *Arabidopsis thaliana* mutants. Figure adapted from McAdam et al. (2015).

Species	Genotype	Mutation Description
Tomato (*Solanum lycopersicum)*	Wild type cv. 'Ailsa Craig'	
	flacca	MoCo mutation
Pea (*Pisum sativum*)	Wild type line JI 1194	
	wilty line JI 1069	SDR mutation
Barley (*Hordeum vulgare*)	Wild type cv. 'Steptoe'	
	Az34	MoCo mutation



7.2 Growth conditions

In all three papers plants were grown at 60% or 90% RH, which corresponded to 0.94 and 0.23 kPa VPD for paper 1, and 1.06 and 0.26 kPa VPD for papers II and III due to differences in growth temperature. These, or similar humidity levels have previously been used in analyses of high humidity effects (Aliniaeifard et al. 2014; Arve et al. 2013; Arve et al. 2014; Arve et al. 2015; Arve & Torre 2015; Arve et al. 2017; Fanourakis et al. 2011; Fanourakis et al. 2013; Innes et al. 2018a; Innes et al. 2018b; Mortensen & Gislerød 1990; Mortensen & Fjeld 1998; Rezaei Nejad & van Meeteren 2008; Torre & Fjeld 2001; Torre et al. 2003), and were chosen as both lie within the range often found in greenhouse production (Shamshiri et al. 2018). The moderate level (60%) is not low enough to result in turgor

loss, while the high level (90%) is quite commonly found in production, inducing undesirable consequences, such as reduced functionality of stomata and increased susceptibility to biotic stress.

In paper I the plants were exposed to UV radiation. The UV radiation was provided by unscreened fluorescent tubes that emit UV-A, UV-B and some UV-C radiation (Fig. 4). In many greenhouses, the cladding material used either does not transmit, or transmits very little UV-B radiation, despite the important role UV radiation plays in signalling, photomorphogenesis and water relations (Robson et al. 2015). Furthermore, artificial UV radiation from similar tubes can be used to control pathogens, helping combat both powdery mildew and *Botrytis cinerea* (Demkura & Ballaré 2012; Suthaparan et al. 2012). It was this characteristic that determined the UV radiation regime used in the experiment, where UV radiation was given during darkness, as fungal photolyase requires UV-A and blue light to combat DNA damage (Suthaparan et al. 2014).

In paper III the plants were reciprocally transferred between RH levels for 24 h before measurements were taken. This was done to mimic production regimes, where humidity may change for a short period of time, for example after rain in field conditions or during daily changes in weather in a greenhouse setting. While changing VPD can rapidly (within 15 minutes) induce gene expression (McAdam et al. 2016), the longer duration of this experiment allowed plants to stabilise after adjusting to the different conditions. Thus, it could be determined whether the growth conditions played a role in how plants function after a change in the aerial environment.

7.3 Analysis of single and combined effects of high RH and UV radiation (paper I)

In 2014 and 2015, pea plants were germinated in a greenhouse compartment to the two-leaf stage, before being moved to controlled environment growth chambers for factorial analysis of the separate and combined effects of humidity and UV radiation. Two chambers were maintained at each of 60% and 90% RH, and in one chamber of each the plants were exposed to 0.15 W m^{-2} unscreened UV radiation (see Fig. 4 for spectrum) for 40 minutes in the middle of the dark period, following a pathogen-control protocol described by Suthaparan et al. (2012). Detached leaf desiccation tests were performed to investigate the closing ability of stomata during extreme water loss, and stomatal conductance was measured at intervals over 8 h to determine the stomatal closing ability of intact plants to lower RH (40%) and darkness. Some leaf damage was noticed in UV-exposed leaves grown in high RH, following which visible injuries were recorded in all treatments and cyclobutane pyrimidine dimer (CPD-DNA) quantification was performed using an enzyme linked immunosorbent assay (ELISA). Chlorophyll fluorescence measurements were taken on fully expanded, undamaged leaves after dark adaptation. Leaflet morphology was examined using cross-sections embedded in LR White resin and examined using light microscopy. To determine whether the treatments affected plant protective mechanisms, flavonoid quantification was performed on leaflet samples using high-pressure liquid chromatography (HPLC). Furthermore, analyses of the chemical composition of leaf cuticles were performed on both leaves with intact cuticles and leaves from which the epicuticular wax had been removed using warm chloroform. The analyses were performed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-

FTIR). Finally, leaflets were analysed for total antioxidant power using the Ferric Reducing Antioxidant Power (FRAP) assay, which determines the total reducing capacity of a sample to reduce a ferric complex into its ferrous form.

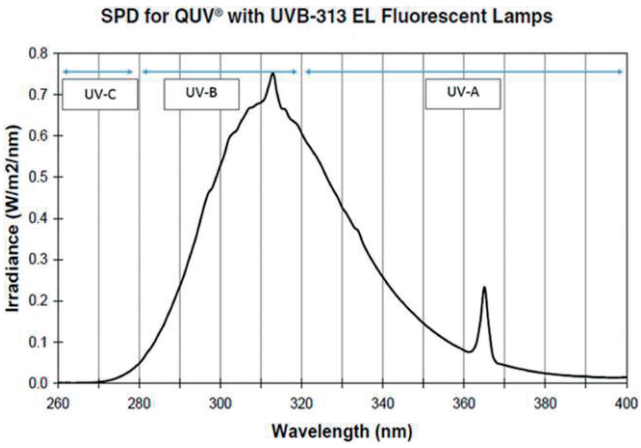


Fig. 4. Spectral power distribution (SPD) for Q-panel UV 313 lamps (Q-Lab Corporation, Ohio, USA) measured in W m^{-2} nm^{-1}. Adapted from Q-Lab corporation (www.q-lab.com). UV-A, UV-B and UV-C regions are indicated.

To determine main and interaction effects of RH and UV radiation, the data were analysed using generalised linear models (GLM) and two-way ANOVA, followed by Tukey's HSD post-hoc tests for differences between means. All data were tested for normality using normal-quantile plots and Shapiro-Wilk normality tests, as well as for homoscedasticity using Levene's test for equality of variances. Differences with $P < 0.05$ were considered significantly different. For the infrared spectral analyses, multiplicative signal correction (MSC) was used to process data from the 4000-600 cm^{-1} region, before being analysed using principle component

analysis (PCA). Mann-Whitney U-tests were used to calculate the statistical differences between principle component scores between samples. Partial least-squares-discriminant analyses (PLS-DA) were used to determine the effects of RH or UV radiation on the samples. Calibration models were evaluated using the PLS coefficient of determination, and biochemical similarities between samples were estimated by variability tests based on Pearson correlation coefficients for the spectral region of 1900-700 cm^{-1}.

7.4 Growth and hydraulic responses of two important crops to ABA-deficiency and high RH (paper II)

Two species, tomato and barley, and their ABA-deficient counterparts, *flc* and Az34 respectively, were selected to analyse growth and hydraulic responses to high RH. After germination in a greenhouse to the two-leaf stage, the plants were moved to controlled environment growth chambers for treatment. The experiments were set up in a factorial manner, with both WT and ABA-deficient genotypes of both species grown in 60% or 90% RH, corresponding to VPD levels of 1.06 or 0.26 kPa respectively, in order to test the role of ABA in the plants' response to high RH. An ABA radioimmunoassay assessed foliar ABA content and confirm the genotypes. The assay uses the monoclonal antibody AFRC MAC 252 to measure ABA concentration on a dry weight (DW) basis, following overnight extraction of freeze-dried samples in deionised water (Quarrie et al. 1988). Plant water relations were assessed in several ways. Leaf relative water content (RWC) was measured using fresh weight (FW), turgid weight (TW) and DW, whole plant transpiration (WPT) rates were determined gravimetrically for day and night values over a 72 h period wherein the soil was covered to prevent evaporative loss, stomatal morphology as SD and stomatal areas were

assessed from scanning electron micrographs taken from leaf samples, and foliar gas exchange measurements were made using an infrared gas analyser (LI-6400 Portable Photosynthesis System) to determine rates of photosynthesis (A), stomatal conductance (g_s) and the concentration of internal CO_2 (Ci). Growth responses were then determined in the form of morphological measurements: number of leaves, leaf area, stem and leaf DWs, which were used to calculate the shoot relative growth rate (RGR_{SHOOT}) using the following formula:

2) $RGR_{SHOOT} = (-)/(t_2 - t_1)$,

where: WT2 = total shoot DW at time point 2,
WT1 = total shoot DW at time point 1,
t_2 = time point 2 (14 days of growth),
t_1 = time point 1 (beginning of growth treatments).

Using the same method, growth rates were calculated for relative leaf expansion rate (RLER) using *ln* transformed leaf area.

Factorial data were analysed in the same manner as described for paper I, using two-way ANOVA and Tukey HSD post hoc analyses, and tested for normality and homoscedasticity. Gas exchange data were analysed for correlation using Pearson's test for correlation between paired samples.

7.5 Analysis of long and short-term effects of humidity on water relations and transcriptomics of a WT and an ABA-deficient genotype (paper III)

Water relations and gene expression responses were analysed in wild-type and ABA-deficient *flc* plants during both long- and short-term exposure to different humidities. The plants were grown as described for paper II, and

after 14 days growing at different VPD levels, half of the plants grown in low VPD were transferred to high VPD and *vice versa*. The transfer took place one hour after the beginning of the light period and all the plants were harvested 24 h after the transfer. Foliar ABA radioimmunoassays were carried out in the same way described for paper II, as were analyses of leaf water content. Stomatal morphology as SD, stomatal pore length and aperture width were measured from light micrographs taken from stomatal imprinting using Suzuki's Universal Micro-Printing (SUMP) method. WPT was determined gravimetrically for two days prior to transfer (control treatments, long term response) and during transfer (short term response). Whole plant gas exchange was measured for 1 h using a Li-Cor 6400 Portable Photosynthesis System connected to an external chamber as described in Jauregui et al. (2018). The plants were exposed to their growth VPD for five minutes to acclimate to the chamber, then the VPD was either increased or decreased (depending on growth VPD) for the remainder of the hour. More foliar ABA samples were taken at the beginning and end of this analysis to investigate these short-term responses to VPD.

Statistical analysis of the data was performed in the same manner as described for papers I and II for factorial analyses. Time course measurements of WPT were analysed as start- and end-status analyses, i.e. two-way ANOVA used to analyse data taken from the first five minutes vs the last five minutes of measurement.

RNA was extracted from leaf samples taken from both control and transfer plants 24 h after reciprocal transfer was performed. After DNA removal and RNA purification, the samples were sequenced using the BGISEQ-500

platform at BGI Tech Solutions (Hong Kong) CO., Ltd. (Tai PO, N. T., Hong Kong). Differential expression analysis was then performed to determine differential gene expression between genotypes at each VPD level, between VPD levels in each genotype, and the effects of transfer in both directions on both genotypes. A false discovery rate (FDR) of < 0.05 was used to classify DEGs. GO enrichment analysis and enrichment of KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways was performed using the software ShinyGO (v0.61 http://bioinformatics.sdstate.edu/go/).

Main results and discussion

8.1 Effects of high humidity on growth and morphology and the role of ABA thereupon

Long term exposure to high RH affects plant morphology in several ways, as previously determined in many species (Bakker 1991; del Amor & Marcelis 2005; Hand et al. 1996; Hoffman et al. 1971; Holder & Cockshull 1990; Hovenden et al. 2012; Innes et al. 2018a; Innes et al. 2018b; Leuschner 2002; Lihavainen et al. 2016; Mortensen & Gislerød 1990; Mortensen & Gislerød 1999; Mortensen 2000; Mortensen et al. 2001; Roriz et al. 2014; Suzuki et al. 2015; Torre et al. 2003). Analysis of previously reported results, as well as findings from this study (Papers I and II) have indicated that humidity effects on plant morphology are somewhat species-dependent, with the mechanisms affecting humidity response differing between plant families (Paper II).

High RH commonly increases plant height and number of leaves, as was found in pea plants in paper I and tomato plants in paper II, showing agreement with several previous findings (Grange & Hand 1987; Hoffman et al. 1971; Mortensen & Gislerød 1990; Mortensen & Fjeld 1998; Mortensen 2000). On the other hand, barley plants showed no effect of high RH on plant height or number of leaves (paper II), though this is not uncommon, as some variation between species is reported in the literature (Mortensen & Gislerød 1990; Mortensen 2000). Leaf morphology is another commonly affected parameter, though results indicate both increased and decreased leaf area (Mortensen 2000; Oksanen et al. 2019), both decreased and increased leaf thickness (Hovenden et al. 2012; Leuschner 2002; Torre et al. 2003) and overall changes in leaf anatomy

(Leuschner 2002; Torre et al. 2003). In paper II, high RH did not affect total leaf area of either tomato or barley. While this may result from different mechanisms, it is also possible that different results would be seen over a longer period of time. The experiments performed here used young plants (~28 days after sowing), and results may differ with older plants.

Increased plant height and leaf morphology under high RH have been attributed to increases in leaf water status (Leuschner 2002; Lihavainen et al. 2016; Mortensen 2000), yet growth in high RH can also decrease leaf area, most notably in tomato, due to decreased foliar [Ca^{2+}] as a result of decreased nutrient uptake (Leuschner 2002; Mortensen 2000; Oksanen et al. 2019). In our study, ABA-deficient mutants were used to determine the role of ABA in growth responses to high RH (paper II), as ABA-deficient mutants are commonly smaller plants with smaller leaves than their WT counterparts (Sharp et al. 2000). The results indicated that the effects of high RH on growth were ABA-independent in tomato, being rather dependent on water status (paper II). Yet high RH effects on growth of barley were ABA-dependent (paper II). This was shown by examining the components that make up RGR_{SHOOT}, as indicated in equation 1 and calculated in equation 2. By this, it was shown that growth in high RH could attenuate the negative effects of ABA-deficiency on RGR_{SHOOT} in tomato by enhancing RWC. Yet high RH decreased RWC of ABA-deficient barley, indicating an alternative mechanism of growth regulation than leaf water status. Similar results have previously indicated that leaf growth inhibition in Az34 barley was not attributed to compromised water relations when the plants were grown in compacted soil and exposed to high RH, indicating

that ABA-deficiency acts via a non-hydraulic mechanism to inhibit barley leaf growth (Mulholland et al. 1996a; Mulholland et al. 1996b).

Conversely, despite high RH decreasing barley RWC, neither RGR_{SHOOT} nor leaf morphological characteristics were affected, indicating that water status of non-expanding tissues does not play a dominant role in barley shoot growth. It is possible that the differences seen between tomato and barley in growth regulation by water status are brought about by the basic differences in morphology between eudicots and monocots, as has previously been indicated by differences in hydraulic properties in response to [CO_2] between tomato and barley (Wei et al. 2020). Eudicot transpiration and leaf expansion zones are congruent, while being spatially separated in monocots (Radin 1983). Further experiments testing the effects of RWC on leaf morphological characteristics and shoot growth in several monocot and eudicot species would be beneficial in confirming this speculation.

High RH not only affects leaf growth and morphology, but also leaf anatomy and cuticle composition (Hovenden et al. 2012; Leuschner 2002; Shepherd & Wynne Griffiths 2006; Torre et al. 2003). However, both the effects and underlying mechanisms seem to be species dependent. Pea plants showed no difference in leaf structural anatomy between moderate and high RH, despite clear differences in morphology (paper I).

Interestingly, pea plants that were originally included in results for paper II (wild-type line A10 and *wilty* line A1-22, as described in Dodd (2003b)) indicated no effect of high RH on growth or leaf morphology (see Appendix 1, Table A1), but thicker leaves (lower SLA) in ABA-deficient *wilty* grown

in high relative to moderate RH. This indicates some involvement of ABA in maintaining leaf structural properties in peas in high RH conditions. However, the difference in morphological response (plant height and number of leaves) between the different pea WT genotypes in our two experiments indicates either an underlying genetic mechanism resulting in different responses to high RH, or a difference in growth conditions that modifies pea plant responses to high RH in a manner previously overlooked. Both temperature and light source differed between experiments, where plants from paper I were exposed to 20°C and high pressure sodium (HPS) lamps which contain only ~5% blue light, while plants from paper II were exposed to 22°C and HQI-BT metal halide lamps which contain ~20% blue light. Previous work has shown that exposure to blue light during growth in high RH improved plant growth, leaf quality and stomatal functioning in several species (Innes et al. 2018a; Terfa et al. 2020), and the light quality may have altered pea responses to high RH. Furthermore, temperature has been shown to be an important factor in the development and yield of pea plants, and functions independently of humidity (Nonnecke et al. 1971). However, further investigation into the role of light and temperature in humidity response falls out of the scope of this study.

Interestingly, tomato plants showed no significant effect of high RH or ABA-deficiency on leaf thickness (as SLA), though *flc* had smaller leaves than WT in both RH levels (paper II). On the other hand, both WT and Az34 barley showed thinner leaves in high relative to moderate RH, but no effects on leaf area (paper II). Furthermore, Az34 barley showed significantly thicker leaves (lower SLA) than WT, but only in high RH (paper II). This indicates that the role of ABA in leaf morphological and anatomical features

differs between species, though differences in the ABA-dependence in response to RH between *flc* tomato plants and Az34 barley were possibly a result of the additional deficiency of NR in Az34 (paper II). Overall, changes in leaf anatomical features may play a role in stomatal development, size and density (Hovenden et al. 2012), thus affecting plant water relations, and this will be discussed in the following section.

In summary, the effects of growth in high RH on plant growth, morphology and anatomy are driven by different mechanisms in different species and genotypes, and is most likely dependent on several extrinsic (e.g. light and temperature) and intrinsic (e.g. ABA) factors.

8.2 Effects of high humidity on stomatal morphology and water relations

Total stomatal transpiration is determined by SD, size and the response of stomatal aperture to closing stimuli, such as changes in light availability or the aerial environment (Fanourakis et al. 2020a). Due to the low evaporative demand in high RH, transpiration at both the whole plant and single leaf level was lower in these conditions than in moderate RH (paper II and III). However, transferring plants to an environment of lower RH immediately and significantly increased transpiration, to a level considerably higher than that of plants grown in a lower RH environment in both pea and tomato (paper I and III). Pea plants transferred from high RH to a low light, low RH environment transpired significantly more than plants transferred from moderate RH, which lasted for three hours following transfer (paper I). Furthermore, the amount of water lost from detached pea leaves was significantly higher from leaves grown in high RH than moderate RH when

subjected to a desiccation test (paper I). Tomato plants grown in high RH and transferred to moderate RH for 24 h (indicated as low VPD and high VPD in the manuscript, respectively) showed significantly higher transpiration rates after 24 h than plants grown in moderate RH (paper III). Furthermore, transpiration rates of plants transferred from high to moderate RH reached the same rate as plants grown in moderate RH within 1 h of transfer, as demonstrated by continuous measurements of gas exchange (paper III).

However, in peas, transpiration rates of plants from high RH decreased to the same level as those from moderate RH after three hours in a low light, low RH environment (paper I). Moreover, stomatal aperture measurements of tomato plants, indicated as a ratio of stomatal length/stomatal width (L/W) so as to account for size differences, showed that stomatal apertures of both WT and *flc* transferred from high to moderate RH for 24 h were more closed than those of plants grown in moderate RH, thereby not accounting for the increased transpiration (paper III). Thus, neither the pea nor tomato plants grown in this study showed reduced stomatal function as a result of growth in high RH (discussed below). The increased transpiration, therefore, was most likely a result of differences in stomatal anatomical features, though may be due to increased driving force in lower RH despite more closed stomata.

As indicated in the previous section, leaf anatomical features may affect stomatal development and SD. In general, increased leaf expansion results in decreased SD by reducing the number of epidermal cells per unit area – assuming an increase in epidermal cell size, as opposed to increased cell

division, is the mechanism by which leaf area is increased (Hovenden et al. 2012). However, *flc* plants grown in high and moderate RH showed no difference in leaf area or SD, while high RH significantly increased leaf area of WT plants but decreased SD (paper II). This suggests epidermal cell expansion in high RH in WT tomato plants, but not enough to result in increased leaf area or cell number. Greater differences in RH than between 60% and 90% might significantly alter leaf area, but further investigation would need to be performed to confirm this. Nevertheless, the difference in response between WT and *flc* indicates the involvement of ABA in stomatal developmental responses to high RH in tomato plants, where the presence of normal [ABA] may inhibit stomatal formation in conditions of low evaporative demand. Analyses taking into account both stomatal density and stomatal frequency (number of stomata relative to epidermal pavement cells) would help confirm this.

Results from paper II indicated that both WT and *flc* had larger stomata (measured as stomatal pore area in μm^2) when grown in high RH. On the other hand, both WT and *flc* plants had lower SD in high RH relative to low RH (paper II). This indicates that in both WT and *flc* the effect of larger stomata may have been somewhat attenuated by lower SD in plants grown in high RH, leaving the significantly higher transpiration rates of plants grown in high RH upon removal to lower RH conditions somewhat inexplicable. There are two more factors that may have contributed to differences in total transpiration rates: 1) boundary layer resistance effects as a result of trichome density (TD), and 2) changes in SD on the adaxial leaf surface.

In paper II, TD was measured, though the results were not included in the final version (see appendix 1, Table A2). While *flc* had remarkably low numbers of trichomes compared to WT, TD did not significantly differ between RH levels. WT plants, on the other hand, had significantly lower TD in high RH than moderate RH (appendix 1, Table A2). By increasing leaf-air boundary layer resistance, TD plays a role in plant adaptation to water stress by limiting transpirational water loss (Galdon-Armero et al. 2018). While the results were not explored further in paper II, a decrease in boundary layer resistance in plants grown in high RH may account for some of the increased transpiration seen upon transfer to conditions of higher evaporative demand, though it is likely that the difference in the driving force between RH levels was the main cause of the differences in transpiration.

Both tomato and pea plants are furthermore described as amphistomatous, meaning that they have stomata on both epidermal surfaces, with environmental conditions during growth affecting not only the total SD, but the SD on both sides of the leaf in different ways (Driscoll et al. 2005; Gay & Hurd 1975; Wang et al. 1998). For instance, Gay and Hurd (1975) showed that low light conditions (20 W m^{-2}) changed tomato plants from being amphistomatous to being hypostomatous, indicating at least ten times more stomata on abaxial than adaxial leaf surfaces. Similarly, maize and *Vicia faba* leaves exposed to different levels of [CO_2] (Driscoll et al. 2005) or ABA and Ca^{2+} (Wang et al. 1998), respectively, showed differential responses in abaxial and adaxial stomata. This shows that differential responses of ab- and ad-axial stomata to differing conditions may have contributed to differences in total transpiration seen in tomato plants in this

study. Whether ab- and ad-axial stomata respond differently to humidity regimes requires further investigation.

Many studies have shown that exposure to long term high RH, especially during leaf development, results in reduced responsiveness of stomata to a range of closing stimuli (see Fanourakis et al. 2020a for review). Interestingly, while both the pea and tomato plants studied here showed increased transpiration after growth in a high RH environment, this was more likely driven by differences in the driving force of transpiration than reduced functionality of stomata (discussed above). Determining the effect of a single growth parameter is challenging, given the intricate way in which growth parameters interact to determine plant responses. For instance, the only difference in growth conditions between the experiments in this study (paper III) and the study performed by Arve and Torre (2015) was the light source used in the growth chambers at NMBU, where in this study plants received approximately 15% more blue light (400-500 nm) than in their study. Transpiration rates were similar between the two studies (see Appendix 1, Table A3), and while stomatal aperture during darkness was not measured in paper II as it was in Arve and Torre (2015), desiccation tests indicated 5% difference in leaf relative weight (%) after 3 h in this study (data not shown in paper II), compared to 15% difference in their study, as well as lower foliar [ABA] in this study compared to their study (Table A3). Although light-mediated effects fall outside the scope of this study, exposure to increased blue light has been shown to alter leaf anatomy (Terfa et al. 2013) and decrease [ABA] (Boccalandro et al. 2011), as well as affect stomatal parameters, especially in conditions of high humidity (Innes et al. 2018a; Terfa 2013; Terfa et al. 2020). Thus, the difference in

the effects of high RH on desiccation rate and foliar [ABA] may be a result of a higher level of blue light given in this study, and this small change in a single growth parameter affected the plant responses.

8.3 The role of ABA in stomatal responses to high RH

The results presented in this study indicate that growth in high RH affected SD differently in tomato and barley (paper II), where ABA played a role in SD response in tomato, but barley showed no effect of either high RH or ABA-deficiency on SD. In tomatoes, ABA-deficiency resulted in increased stomatal size and density (paper II), as well as decreased TD (appendix 1, Table A2) in *flc* compared to WT in both RH levels, which accounts for the significant difference in transpiration rates between the two genotypes (papers II and III). However, the decreased [ABA] in *flc* compared to WT (papers II and III) did not result in significantly reduced function of stomata, as their transpiration decreased (albeit to a lesser extent) in response to darkness (paper II), as did their stomatal aperture following transfer between RH levels in both directions (paper III).

Many studies have reported that growth in or long-term exposure to high RH decreased [ABA] (Arve et al. 2013; Okamoto et al. 2009; Rezaei Nejad & van Meeteren 2006; Rezaei Nejad & van Meeteren 2008). Consequently, reduced stomatal functioning of plants grown in high RH has been attributed to decreased [ABA], via increased hydroxylation to phaseic acid (Okamoto et al. 2009) or decreased deconjugation from ABA glucose ester (ABA-GE) (Arve et al. 2013). While some studies have attributed the reduced functionality of stomata to changes in stomatal features such as size and density (Giday et al. 2013b; Torre et al. 2003), others have refuted this

(Aliniaeifard et al. 2014), and the link between stomatal functioning and evaporative demand remains equivocal (Fanourakis et al. 2016; Fanourakis et al. 2020a). Based on the findings presented here, increased transpiration in tomato plants grown in high RH when transferred to a lower RH environment was not the result of significantly reduced stomatal function, showing agreement with previous findings in pea, where WT and *wilty* peas showed no difference in stomatal closure characteristics in response to changes in VPD (Merilo et al. 2018). Furthermore, *flc* plants with severely reduced [ABA] showed no difference to WT in either transpirational or stomatal responses to either long- or short-term exposure to high RH (papers II and III).

Both ABA-dependent and ABA-independent pathways have been proposed to regulate stomatal closure (Aliniaeifard et al. 2014). One such proposed ABA-independent pathway is CO_2-induced stomatal closure initiated via a signalling cascade induced downstream of OST1/SnRK2.6/SRK2E to activate GC S-type anion channels (SLAC1, SLAH2 and SLAH-like), though which requires both basal levels of ABA and OST1/SnRK2.6/SRK2E protein kinase activity (Hsu et al. 2018). ABA-deficient *flc*, with their higher SD and larger, more open stomata have higher internal CO_2 concentrations (paper II), indicating this as a possible ABA-independent mechanism initiating stomatal closure despite significantly low [ABA], though A/Ci analysis would need to be performed to determine cause and effect in this situation. Another ABA-independent pathway is the passive hydraulic control of stomatal movement found in basal lineages of vascular plants, which served as a means of water regulation in the earliest land plants (McAdam & Brodribb 2015). It is

possible that due to their low [ABA], *flc* plants revert to hydraulic regulation of stomatal movement to control water loss. It is not possible to determine the mechanism used by ABA-deficient plants to control their stomata from our results alone, though transcriptomic analyses (paper III) indicated upregulation of SLAC1/SLAH2 and SLAH2-like in *flc* in high relative to moderate RH, and upon transfer from moderate to high RH (paper III), indicating that direct activation of these channels by CO_2 is likely not the case.

In summary, while transferring both pea and tomato plants out of high RH environments (papers I and III) substantially increased transpiration rates, this could not be attributed to significantly reduced stomatal function in either species. Instead, changes the increased transpiration, while most likely driven by differences in driving force between the two RH levels, might also be attributed to differences in stomatal development and anatomy, such as size and density, and in tomato plants, these were significantly affected by ABA.

8.4 Transcriptomic responses to high humidity

While several studies examined the role of ABA in humidity responses, to the best of my knowledge no analyses have explored the underlying genetic changes involved in growth in high RH and how ABA affects these. The findings presented here (paper III) were surprising in several ways. The diminished [ABA] of *flc* plants led to the up- and down-regulation of thousands of genes when plants grown in different RH levels were compared, where the DEGs between RH levels in WT numbered in the hundreds. Moreover, when comparing DEGs between *flc* and WT in

different RH levels, the difference was in the thousands in moderate RH, yet zero in high RH, i.e. not a single gene was significantly up- or down-regulated in *flc* compared to WT plants when they were grown in high RH. The most interesting part of this, however, was that the difference in gene expression was found to be indirect, due to the effect of diminished [ABA] in the foliar water content in *flc*, rather than direct effects of ABA.

8.5 Effects of high humidity and ABA on abiotic and biotic defence responses

The results of this study indicated that growth in high RH induced both susceptibility to abiotic stress from UV radiation exposure in peas (paper I), as well as to pathogen infection and biotic stress in both WT and *flc* tomato plants (paper III). In paper I, growth in high RH induced susceptibility to UV-induced damage in peas by decreasing foliar antioxidant power and changing the cuticle composition. Pea plants showed both lower foliar flavonoid content and total antioxidant power when grown in high RH, which contributed to increased visible damage and the presence of CPD-DNA upon exposure to UV radiation (paper I).

The plant cuticle plays a role in cuticular transpiration resistance, plant-pathogen interactions and abiotic stress resistance (Macková et al. 2013; Schreiber et al. 2001; Schreiber 2005; Zeisler-Diehl et al. 2018). While no structural differences were found between leaves grown in different RH levels, pea plants grown in high and moderate RH showed differences in the chemical composition of the leaf cuticle (paper I). An increase in the ratio of cuticular waxes compared to proteins and uronic compounds was found in high RH relative to moderate RH. Unfortunately, the method used

measured only the ratio and was unable to indicate whether the difference between treatments was due to an increase in cuticular waxes or a decrease in proteins and uronic compounds. A reduction in proteins and uronic compounds may reduce potential antioxidant power in the cuticle and upper epidermis, as uronic compounds are precursors in the biosynthesis of ascorbates (Davey et al. 1999; Isherwood et al. 1954; Mapson & Isherwood 1956). Ascorbates act as reducing agents, mitigating the effects of oxidative stress and a reduction may render leaves more susceptible to both abiotic stress, such as UV radiation and pathogen attack. Indeed, the pea plants grown in high RH and exposed to UV radiation were significantly more susceptible to oxidative stress and CPD-DNA damage, though whether this was due to a loss of protection in the cuticle or a reduction in flavonoids and antioxidant power in whole leaves grown in high RH relative to moderate (paper I) remains equivocal.

In paper III, analyses of KEGG pathway enrichment by differential gene expression indicated upregulation of the plant-pathogen interaction pathway in high RH in WT plants, but downregulation in *flc* both grown in and transferred to high RH. In general, the gene expression pathways in *flc* relative to WT indicate a higher resistance to biotic stress, through upregulation of ET biosynthesis and response genes and JA biosynthesis genes, and upregulation of both autophagy and phagosome pathways (paper III). Stomata provide a natural opening for pathogen infection in plants (Panchal et al. 2016), and the larger, more open stomata in *flc* leave these ABA-deficient mutants more susceptible to infection than WT as a baseline. The gene expression patterns mentioned above suggest the possibility that defence mechanisms against pathogen infection are induced to a greater

degree in *flc*, due to their higher baseline susceptibility. However, when grown in high RH, the plant-pathogen interaction pathway was upregulated in WT plants, but not *flc*, and in *flc* further downregulation of ET biosynthesis and response genes and the glutathione pathway indicate attenuation of the abovementioned increased biotic stress resistance when the plants were exposed to both long- and short-term high RH (paper III). Additionally, WT plants showed increased ET biosynthesis and signalling in high RH, showing agreement with previous findings (Arve & Torre 2015). The difference in ET regulation between WT and *flc* suggests that high RH may perturb the already complex ABA-ET relationship, and that ABA may be a requirement for ET production in high RH conditions.

Upregulation of genes involved in phosphatidylinositol signalling system (PSS) in *flc* relative to WT plants in moderate RH, as well as *flc* plants when transferred from high to moderate RH, (paper III) may indicate a response to turgor loss being experienced by *flc* plants in moderate RH (Zhu 2002). The higher rate of water loss, lower RWC and wilting tendency of *flc* plants (papers II and III), suggest they experience turgor loss in moderate RH and be reacting accordingly with corresponding changes in gene expression. The PSS and products thereof (inositol 1,4,5-trisphosphate and phospholipase C, specifically) are important for ABA-mediated stomatal regulation but also act in an ABA-independent manner in response to abiotic stress (Zhu 2002). Furthermore, compared to WT grown in moderate RH, *flc* showed downregulation of genes involved in amino acid metabolism, vitamin and cofactor metabolism and carbohydrate metabolism, many of which were also downregulated upon transfer from high to moderate RH, but upregulated when transferred in the other direction (paper III). This

agrees with Bradford (1983), who found that *flc* plants did not osmotically adjust despite experiencing daily water stress. Additionally, while starch and sucrose metabolism genes were downregulated compared to WT in moderate RH and when transferred from high to moderate RH, these were upregulated in both growth and transfer to high RH (paper III). Stressful conditions stimulate the breakdown of starch content into soluble sugars (Khan et al. 2020) and the downregulation of starch and sucrose metabolism pathway may indicate a decrease in the rate of this breakdown, resulting in fewer soluble sugars in *flc* compared to WT and in moderate compared to high RH. Free amino acids and soluble sugars comprise some of the molecules collectively known as compatible solutes, or osmoprotectants, which function in several ways to maintain cell turgor under water deficit and act as messengers in stress defence (Cushman 2015; Khan et al. 2020). Finally, glutathione metabolism was downregulated in *flc* relative to WT in moderate RH, as well as in *flc* transferred from moderate to high RH, indicating possible decreased osmotic regulation (Hasanuzzaman et al. 2017). Glutathione is a non-enzymatic antioxidant, which plays a role in ABA-induced stomatal closure and functions in osmotic stress regulation and conferring drought tolerance by ameliorating ROS-induced damages (Hasanuzzaman et al. 2017). Thus, in agreement with Bradford (1983), it seems that the *flc* plants in this study did not undergo osmotic adjustment, despite experiencing high rates of transpiration and low RWC in moderate RH. However, further investigation into the osmotic status through measurement of osmotic potential at full turgor would assist in confirming this. Furthermore, a full metabolomic study looking into the availability of specific compatible solutes and the concentration of starch in WT and *flc*

would provide insight into the mechanisms of osmoregulation between these two genotypes.

8.6 Comments on experimental setup

Several differences in experimental setup and data collection may have enhanced the conclusions drawn from this study. For example, different measurements were carried out in the different experiments, decreasing the utility of comparisons. For instance, regarding water relations, pea plants were subjected to a desiccation test and transpiration was measured in a room with low RH (40%) and darkness (paper I). Tomato and barley transpiration was measured in paper II, but it was measured over the period of several days, with no change in conditions, while tomato transpiration was measured again in paper III under changing conditions, but via reciprocal transfer in the light, as opposed to low RH and darkness as in paper I. While all three measurement strategies gave valuable information in their own right, it decreased somewhat comparability between experiments.

Furthermore, using different growth chambers with different light sources may result in different responses. As mentioned, light quality may affect plant responses to different RH levels. Thus, slight differences in light spectra between the HPS (paper I), HQI-BT metal halide (papers II and III) and daylight and red-biased fluorescent tubes (5x 58W colour 2023, 14x 58W colour 2084, 4x18W colour 840, paper III) may have induced slightly different responses in stomatal functioning, though the morphological and anatomical responses examined here did not differ between light conditions.

Future perspectives

Deeper investigation into the differences between tomato and barley growth mechanisms, such as the role of RWC in monocots vs eudicots, as well as the role of NR-deficiency in ABA-deficient Az34 compared to *flc* would help shed light on how both water status and hormones differentially affect growth in these two important crop species. To do this, several eudicot and monocot species should be tested simultaneously with different levels of RWC to determine the role of water content on growth between the two plant clades. Testing the role of NR-deficiency in ABA-deficient Az34 compared to *flc*, or other molybdenum cofactor mutants such as *Arabidopsis aba3*, might be performed by including NR-deficient tomato and *Arabidopsis* mutants and analysing their responses of growth and water relations to RH and/or water status.

An investigation into all of the components of transpiration, i.e. cuticular transpiration, trichome density and stomatal size, density, frequency and function, and how they are affected by high RH will help elucidate the role of more nuanced parameters, such as boundary layer resistance, any differential response of ab- vs. ad-axial stomata, and cuticular composition to changing RH. Furthermore, investigating the combined roles of light and RH in determining stomatal anatomy and function may help clarify the differences in results found between studies and improve the physiological understanding of air humidity responses in general. This may be done by factorial analysis of RH and light quality using different levels of blue light.

To elucidate a clearer picture of the genes involved in RH responses under low [ABA], qPCR analyses of specific gene transcripts should be performed

to indicate absolute, as opposed to relative, transcript abundances in WT and *flc* plants. Examples of such gene transcripts may include ABA response genes such as PYR/PYL/RCAR receptors, PP2Cs and SnRK2s, guard cell ion channel genes such as SLAC1/SLAH, CPKs and GORKs, ABA catabolism genes such as CYP707A and β-glucosidase, ethylene biosynthesis and response genes such as SAM, ACO, ACS and ETR, among others. Metabolomic analyses to determine up- and down-regulation of specific metabolites, including free amino acids, soluble sugars and antioxidants, both enzymatic and non-enzymatic, should be conducted to determine their role and relationship with ABA. Further hormone analysis would also be beneficial to determine the role of ET in high RH, as well as elucidate the relationships between the so-called "stress hormones" – ABA, ET, JA and SA both in the presence of lower ABA concentrations and the effects of high RH.

The role of ABA in biotic stress resistance in response to growth in high RH may be investigated using ABA-deficient mutants, with focus on pathogens that enter through the stomatal pore. As high RH leaves plants more susceptible to pathogen attack as a result of open stomata, focus on immunity under high RH has become prominent in protected plant cultivation. While ABA has been found to attenuate plant defence against some pathogens, many studies focus on hormone responses to specific stresses. Analysing responses to pathogens under different humidity regimes may indicate whether humidity affects pathogen infection severity via effects on the pathogen itself or by changing the susceptibility of the host plant. Knowledge from such studies would be beneficial in combatting pathogen infection in greenhouse crop production.

As mentioned, a full metabolomic study of WT and *flc* tomato plants may provide a more complete picture regarding the mechanisms used in response to different RH levels, and as shown here, different levels of water status. Gene expression patterns indicate changes happening as a result of different conditions, but a metabolic study would indicate the baseline metabolic state on which changes in gene expression are happening. Thus, while no difference was seen here in gene expression between WT and *flc* plants grown in high RH, the genotypes may have different metabolic baselines, which would provide insight into the differences seen between the phenotypes and their responses to changes in RH/water status.

Conclusions

The first aim of this study was to investigate the effect of exposure to UV radiation on growth and stomatal responses of pea plants to growth in high RH. Since UV radiation induces stomatal closure, it was hypothesised that exposure to UV radiation during growth in high RH might mitigate the adverse effects of high RH on stomatal functioning, but this was refuted. Instead, exposing pea plants to UV radiation decreased instantaneous transpiration rates, with no effect on long-term stomatal functioning and response to closing stimuli. Additionally, growth in high RH decreased foliar flavonoid levels and UV-protective compounds in the cuticle, leaving plants susceptible to UV-induced foliar and DNA damage from an otherwise non-harmful UV dose (paper I).

The second aim of this study was to compare the growth and hydraulic responses of tomato and barley to growth in high RH, and to investigate the role of ABA in these responses using ABA-deficient mutants. The hypothesis that high RH would promote growth and water status of the ABA-deficient mutants was supported in both species, yet the mechanisms by which this occurred differed between the species. High RH alleviated the impacts of ABA-deficiency in tomato plants by enhancing plant water status, while in barley an unknown non-hydraulic mechanism was involved. These apparent species differences may arise because transpiration and leaf expansion areas are spatially separate in monocots, or because of an additional NR-deficiency in the ABA-deficient Az34 barley, that is absent in ABA-deficient *flc* tomato plants.

The final aim of the study was to investigate the role of ABA in hydraulic and genetic responses to high RH, by analysing leaf transcriptomic responses to long- and short- term exposure to high RH. The hypothesis that *flc* plants would compensate for a lack of ABA by regulating different genes and regulatory pathways was confirmed. Moreover, *flc* differentially expressed thousands of genes in response to RH, compared to the few hundred differentially expressed in WT. This genotypic contrast seems to be an indirect response of low [ABA] affecting leaf water status, rather than ABA status directly altering gene expression. This was because in high RH, neither water status nor gene expression differed between the genotypes. Finally, high RH perturbs the ABA-ET relationship in tomato plants, with ABA possibly required for ET production in high RH.

In conclusion, the effects of high RH on plant growth and water relations are somewhat species-dependent and the mechanisms of high RH perception, signalling and response remain equivocal. ABA plays a role in high RH responses in tomato plants, most likely by mediating plant water relations. Nevertheless, growth in high RH increases plant susceptibility to both abiotic and biotic stress via several mechanisms, and this has potential consequences for both controlled environment production and ecosystems in the face of a changing climate.

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Appendix 1

Table A1. Pea leaf morphological parameters measured in wild-type (WT) and ABA-deficient genotypes grown in 60% or 90% RH in environmentally controlled growth chambers. Means ± SE shown, as well as main effects (genotype, RH) and interaction effects from two-way ANOVAs. Different letters indicate significant differences between genotypes and RH levels for a given time of day, as determined by post-hoc Tukey HSD analyses ($P < 0.05$).

Genotype	**RH (%)**	**Number of leaves**	**leaf area (cm^2)**	**SLA (cm^2g^{-1})**
WT				
	60	21.25 ± 1.31[a]	586.0 ± 36.8[a]	326.2 ± 7.1[a]
	90	19.00 ± 0.41[a]	515.2 ± 38.6[a]	314.4 ± 13.8[a]
wilty				
	60	21.00 ± 1.83[a]	525.1 ± 78.4[a]	326.4 ± 15.9[a]
	90	24.00 ± 1.00[a]	547.6 ± 35.3[a]	252.2 ± 14.8[b]
P values				
Genotype		0.081	0.783	**0.039**
RH		0.769	0.642	**0.007**
Genotype*RH		0.057	0.376	**0.038**

Table A2. Stomatal length and trichome density of wild-type (WT) and ABA-deficient (*flc*) tomato genotypes grown in 60% or 90% RH in environmentally controlled growth chambers. Means ± SE shown, as well as main effects (genotype, RH) and interaction effects from two-way ANOVAs. Different letters indicate significant differences between genotypes and RH levels for a given time of day, as determined by post-hoc Tukey HSD analyses ($P < 0.05$).

Genotype	**RH (%)**	**Length (µm)**	**Trichome density (per 0.14 mm^2)**
WT			
	60	16.59 ± 0.17^{d}	13.70 ± 0.57^{a}
	90	18.11 ± 0.20^{c}	10.45 ± 0.43^{b}
flc			
	60	24.87 ± 0.30^{a}	2.00 ± 0.34^{c}
	90	23.45 ± 0.25^{b}	1.35 ± 0.21^{c}
P values			
Genotype		**< 0.001**	**< 0.001**
RH		0.767	**< 0.001**
Genotype*RH		**< 0.001**	**< 0.001**

Table A3. Comparison of results between this study (paper II) and a previous study performed on the same genotype, under the same experimental conditions excepting light quality.

	This study (paper II)		**Arve and Torre (2015)**	
	60% RH	**90% RH**	**60% RH**	**90% RH**
Transpiration: day (mmol m^{-2} s^{-1})	1,2	0,7	1,2	0,7
Transpiration: night (mmol m^{-2} s^{-1})	0,6	0,3	0,6	0,4
Stomatal aperture: day (µm)	-	-	5	7
Stomatal aperture: night (µm)	-	-	4	6
Relative leaf weight (% after 3 h)	77	73	83	70
[ABA] (ng g^{-1} DW)	1228	1326	2250	2300

Paper I

Photochemical & Photobiological Sciences

PAPER

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Elevated air humidity increases UV mediated leaf and DNA damage in pea (*Pisum sativum*) due to reduced flavonoid content and antioxidant power

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Growth in high relative air humidity (RH, >85%) affects plant morphology and causes diminished response to stomatal closing signals. Many greenhouses are prone to high RH conditions, which may negatively affect production and post-harvest quality. UV radiation induces stomatal closure in several species, and facilitates disease control. We hypothesised that UV exposure may trigger stomatal closure in pea plants (*Pisum sativum*) grown in high RH, thereby restoring stomatal function. The effects of UV exposure were tested on plants grown in moderate (60%) or high (90%) RH. UV exposure occurred at night, according to a disease control protocol. Lower stomatal conductance rates were found in UV-exposed plants, though UV exposure did not improve the rate of response to closing stimuli or desiccation tolerance. UV-exposed plants showed leaf curling, chlorosis, necrosis, and DNA damage measured by the presence of cyclobutane pyrimidine dimers (CPD), all of which were significantly greater in high RH plants. These plants also had lower total flavonoid content than moderate RH plants, and UV-exposed plants had less than controls. Plants exposed to UV had a higher content of cuticular layer uronic compounds than control plants. However, high RH plants had a higher relative amount of cuticular waxes, but decreased proteins and uronic compounds. Plants grown in high RH had reduced foliar antioxidant power compared to moderate RH. These results indicate that high RH plants were more susceptible to UV-induced damage than moderate RH plants due to reduced flavonoid content and oxidative stress defence.

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1 Introduction

Ultraviolet (UV) radiation has the highest energy per photon of the portion of the solar spectrum reaching the surface of the Earth. The electromagnetic spectrum of UV radiation reaching the Earth's atmosphere can be divided into vacuum UV (<200 nm), UV-C (200 to 280 nm), UV-B (280 to 315 nm), and UV-A (315 to 400 nm), though the stratospheric ozone layer absorbs all of the vacuum- and UV-C, as well as much of the UV-B radiation.[1,2] UV radiation is biologically active at low doses and may induce signalling cascades that trigger a range of photomorphogenic responses in plants. However, at high or chronic doses, UV radiation is a stressor and may cause damage to DNA, protein- and membrane lipids and the photosynthetic apparatus.[3] In the natural environment, plants rarely show signs of UV-induced damage, and while many previous studies have focused on plant responses to excessively high UV radiation doses or exposure durations (*e.g.* Jansen *et al.*, 1998,[4] and references cited therein), a shift has been seen in the last decade to more realistic experimental designs and a focus on UV-induced changes in morphology, physiology, metabolics, and gene expression.[2,3] UV radiation induces photomorphogenic responses in plants *via* the UVR8 photoreceptor pathway[1,5] and the most well documented photomorphogenic response to UV radiation is the biosynthesis of UV-screening compounds, such as flavonoids and anthocyanins,[6] through transcription of genes encoding the chalcone synthase (CHS) enzyme, a key enzyme in the phenylpropanoid pathway.[7]

UV radiation has also been found to affect plant water relations through effects on stomatal movement, though the magnitude and direction of such effects are dependent on several factors, and previously reported results are often contradictory.[4,8–11] While Eisinger *et al.*[12] reported that the peak of the stomatal opening action spectrum in *Vicia faba* leaves lies in the UV range with a major peak at 280 nm, Tossi

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et al.[13] proposed a signalling model for stomatal closure in response to UV-B radiation, involving both abscisic acid (ABA)-dependent and -independent pathways. In both pathways, exposure to UV-B resulted in stomatal closure, though this effect may be species-dependent.[9,14] The increased ABA concentration frequently associated with UV-B exposure is often a stress-related response, wherein both drought and UV-B tolerance are enhanced.[14] Indeed, several authors have reported increased drought tolerance upon exposure to UV-B radiation.[8,15,16] Furthermore, UV-B radiation has been shown to induce thickening of the plant cuticle and cuticular wax,[17,18] though the correlation between increased cuticular thickness and cuticular water loss is questionable.[19]

In greenhouse production the environment may be closely regulated for optimal growth. However, in the northern latitudes during winter, high relative air humidity (RH) is almost unavoidable due to a trade-off between ventilation and energy saving. It has previously been shown that continuous growth of plants in high RH (>85%) has a strong impact on plant transpiration, photosynthesis, growth and desiccation tolerance.[20–27] High RH normally induces stomatal opening, and long term high RH results in larger stomata that are unable to close when exposed to environmental closing signals, such as darkness, drought and ABA.[21,23,28,29] The reasons for the loss of functionality of stomata developed in high RH have been hypothesised to involve changes in the guard cell wall flexibility or altered ABA level and signalling, though other signals are also likely to be involved.[30–33] Environmental changes that trigger stomatal movements, like changes in RH and/or temperature, have been shown to improve stomatal function in high RH.[27,33] Furthermore, high RH has been reported to increase cuticular transpiration and soften epicuticular waxes.[34] However, how RH affects the wax structure and/or thickness or the chemical composition of the cuticle is inconclusive and species-dependent.[35,36] Cuticular water loss *via* diffusion is generally considered negligible.[37] However, under conditions of stomatal closure, cuticular transpiration accounts for the majority of water loss and becomes increasingly important.[35]

Many greenhouses have cladding material that either does not transmit UV-B radiation, while at least partially transmitting UV-A radiation, or does not transmit UV radiation at all. Given that UV has been shown to have a role in plant signalling, photomorphogenesis and plant water relations, the use of artificial UV radiation may prove beneficial in the control of plant growth. UV radiation has, furthermore, been found to have positive effects in the control of plant pathogens, such as powdery mildew[38,39] and *Botrytis cinerea*,[40,41] and could therefore be an important tool in plant production systems. We decided to expose plants to UV during the dark period, as UV-exposure in darkness is more efficient at the control of powdery mildew since fungal photolyase needs UV-A or blue light for repair of DNA damage.[42] In addition we used unscreened UV-B tubes with a spectral range of UV slightly below 280 nm, as UV wavelengths below 300 nm are necessary for control of powdery mildew.[43] This UV radiation was used to test the effects of a UV exposure protocol which can also be used to control powdery mildew.

It is important to understand the positive and negative effects UV radiation has on the specific plant species both during production and post-harvest, as responses to UV radiation vary between species.[4] We therefore investigated the effect of UV radiation on plant growth, transpiration and flavonoid content as well as UV induced damage in a background of moderate and high air humidity in *Pisum sativum*. Exposure to UV radiation affects stomatal movements, plant cuticle structure and chemical composition and could therefore play an important role as a trigger to improve stomatal closure in response to closing signals and desiccation tolerance in plants developed in high RH. Hence we also investigated the effect on stomatal responses and the chemical composition of the cuticle. We hypothesised that exposure to UV radiation may contribute towards combatting the negative effects of plants grown at high RH and improving stomatal function and desiccation tolerance.

2 Materials and methods

2.1 Plant material and growth conditions

Pea plants of the wild type pea, *Pisum sativum* L., cv Torsdag were used in this study. The plants were germinated in 12 cm pots containing peat (L.O.G. Gartnerjord, Rakkested, Norway). The plants were grown in a greenhouse with polyacrylic walls and a glass roof at 20 °C, with 80% relative air humidity (RH), and 20 h daily supplementary light of 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR: 400–700 nm) supplied by high pressure sodium lamps (HPS, Osram NAVT-400W, Munich, Germany) at the Norwegian University of Life Sciences, Ås, Norway (N59°40.120′, E10°46.232′). The plants were grown during August and September in 2014 and 2015, during which time the plants received between 10 and 16 h of daylight.[44] The plants were kept in the greenhouse until they were approximately 10 cm tall.

The plants were then transferred to four environmentally controlled growth chambers for experimental treatments. A factorial 2 × 2 design (two RH levels: 60% and 90%, and two UV radiation levels: UV-exposure and no-UV control) was used with 5–8 plants per treatment grown in five repeated experiments. The chambers were maintained at 20 °C and 60% or 90% RH throughout the experiment using a PRIVA system (Priva, Ontario, Canada). The plants received 150 ± 10 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR: 400–700 nm) from HPS lamps, as measured at the top of the canopy using a LI-Cor Quantum sensor attached to a LI-250 Light Meter (Li-Cor Inc., Lincoln, NE, USA) for a 20 h photoperiod each day. This gave a daily light integral (DLI) of 10.8 ± 0.7 mol m^{-2} d^{-1}, just slightly higher than the recommended DLI for the best integrated quality of pea plants.[45] UV radiation (Fig. 1) was provided by unscreened fluorescent tubes (Q-panel UV 313, Q-Lab Corporation, Ohio, USA) at 0.15 W m^{-2} UV-B for 40 minutes every night in the middle of the dark period, according to a method adapted from Suthaparan *et al.*[38] for control of powdery mildew by UV-B. UV radiation was

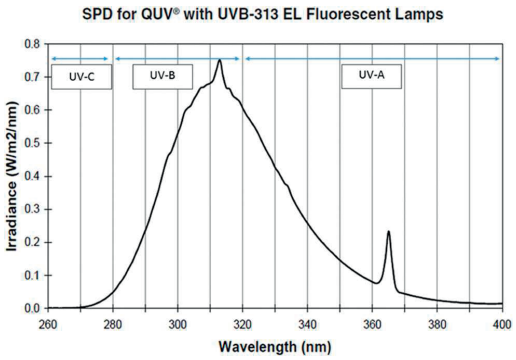

Fig. 1 Spectral power distribution (SPD) for Q-panel UV 313 lamps (Q-Lab Corporation, Ohio, USA) measured in W m^{-2} nm^{-1}. Adapted from Q-Lab Corporation (www.q-lab.com). UV-A, UV-B and UV-C regions are indicated.

measured at the top of the canopy using a Skye SKU 430/SS2 UVB Sensor connected to a Skye SpectroSense2 Meter (Skye Instruments Ltd, Llandrindod Wells, Powys, UK), which was calibrated using an Optronic OL756 Spectroradiometer (Optronic Laboratories, Inc., Florida, USA). Green's weighting spectrum for DNA damage,[46] normalized to 1 at 300 nm, was used to estimate biologically effective UV-B (UV-B_{BE}) at 0.22 W m^{-2}. Measurements are specified for UV-B here, as the measurements were taken using a UV-B sensor.

The plants were watered daily and fertilized twice a week using a 50/50 mixture of YaraLiva CALCINIT and YaraTera KRISTALON INDIGO (both Yara Norge AS, Oslo, Norway), with EC level 1.5 mS cm^{-1}. The plants were subjected to experimental conditions for 15 days before plant growth parameters were measured and further sampling began. The plant height was measured from the rim of the pot to the shoot apical meristem, and the number of leaves (as petiole, leaflets and a tendril) per plant was counted for each plant when the plants were harvested at the end of four of the experiments.

2.2 Water relations

2.2.1 Detached leaf desiccation. After 15 days of growth in the chambers, one fully expanded, undamaged leaflet was sampled from the third or fourth leaf from the base of five plants from each treatment. The analysis was repeated in all five of the experimental rounds. The leaflets were detached 1 h before the end of the light period, placed adaxial side-down on a clean workbench, and weighed after 0 and 180 minutes. The test was performed in a room with 40% RH, 20 °C, and 15 μmol m^{-2} s^{-1} irradiance at the surface of the leaves. The relative water content at time 0 was set at 100% and the relative water loss after three hours was calculated (weight after 180 min/original weight × 100).

2.2.2 Stomatal conductance measurements: time series in the dark. Stomatal conductance measurements were repeated in time series on plants that were transferred to a different, dark environment (40% RH, 20 °C, in the dark) during the light period. Three plants from each treatment were transferred to a dark environment 1 h before the start of the dark period. Conductance rates were recorded on leaflets from the third and fourth leaves from the base of the plants immediately, 1 h, 3 h and 8 h post transfer. The analysis was performed in two replicate experiments.

2.3 Plant injury quantification

2.3.1 Visible symptoms of leaflet injury. Visible plant injuries in the form of leaflet curling, leaflet chlorosis and leaflet necrosis were quantified by counting the number of >10 mm leaflets showing visible injuries on each plant. Leaflets were considered chlorotic/necrotic when >30% of the surface of the leaflet indicated chlorosis/necrosis.

2.3.2 Cyclobutane pyrimidine dimer (CPD-DNA) quantification. DNA damages in the form of cyclobutane pyrimidine dimers (CPDs) were quantified by the enzyme-linked immunosorbent assay (ELISA) using OxiSelect UV-Induced DNA damage kits for CPD quantification (Cell Biolabs, Inc., USA). Fully expanded, undamaged leaflets from the fourth leaf from the base of three plants per treatment were sampled 1 h before the start of the dark period and were immediately placed in liquid N_2, followed by storage at −80 °C. Frozen tissue (100 ± 0.5 mg) was disrupted from each leaflet sample in a Tissue Lyzer (Mixer Mill Type MM301, Retsch GmbH, Haan, Germany). DNA was extracted using a DNeasy Plant Minikit (Qiagen GmbH, Hilden, Germany) in a darkened room, with a yellow filter over the light. Standards were prepared according to the ELISA protocol. DNA samples were diluted to 0.75 μg ml^{-1} using a cold phosphate-buffered saline (PBS) solution. Samples were then converted to single-stranded DNA by heating to 95 °C for 10 min, followed by 10 min on ice. The ELISA assay protocol was followed for the reaction between DNA and anti-CPD antibody solution. The absorbance of the reaction mixture was measured on a microplate reader (Biochrom Asys UVM 340 with KIM, UK) with 450 nm as the primary wavelength. The analysis was performed in two replicate experiments.

2.3.3 Chlorophyll fluorescence. The maximal photosystem II (PSII) efficiency (variable fluorescence [F_v]/maximum fluorescence) was measured on fully expanded, visibly undamaged leaflets from the fifth leaf from the base of the plant. This was performed using a portable chlorophyll fluorometer (Plant Efficiency Analyzer, Hansatech Instruments, Norfolk, UK) using excitation light of approximately 3500 μmol m^{-2} s^{-1} (PAR: 400–700 nm) after dark adaptation. This analysis was performed in one of the experimental rounds. Undamaged leaflets were used specifically to determine any PSII core damage arising as a direct result of UV exposure.

2.3.4 Leaflet morphology from cross-sections. Leaflet cross-sections were examined to determine RH or UV-induced changes to leaflet morphology. Leaflets from the fully expanded sixth leaf from the base of four plants were detached and cut into approximately 3 × 3 mm pieces, which were immediately submerged in fixation medium (1.2% glutaralde-

hyde, 2% paraformaldehyde, and 0.1% (v/v) Tween 20 in 0.01 M sodium phosphate buffer, pH 7.2) and stored at 4 °C. The samples were dehydrated through a graded ethanol series before being infiltrated with resin LR White (Electron Microscopy Sciences, Hatfield, PA, USA) in a further graded series, with a progressively increasing ratio of LR White resin to ethanol. The sections were then placed in an embedding mould with 100% LR White, which was polymerized overnight at 50 °C. Samples embedded in LR White blocks were sectioned using a Micro Star diamond knife (Micro Star Technologies, Huntsville, TX, USA) on a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Cross sections (2 μm thick) were mounted onto slides and stained using Stevenel's Blue. Coverslips were sealed onto the slides using Depex mounting medium before the slides were viewed using a Leica DM 5000 B light microscope connected to a Leica DFC 425 digital microscope camera with a Leica 10445929 0.5× video objective. Leica Application Suite v4.3.0 software (all Leica Microsystems GmbH, Wetzlar, Germany) was used for image capture and analysis. The analysis was performed in one of the experimental rounds.

2.4 Protective compound analyses

2.4.1 Flavonoid quantification by HPLC. Fully expanded leaflets from the fifth leaf from the base of 4–5 plants per treatment were detached and placed immediately in liquid N_2 for storage before freeze-drying. Samples were freeze-dried using a Telstar LyoQuest (Telstar, Terrassa, Spain). 20 mg of dried, crushed plant material was extracted five times with 600 μg of methanol (MeOH) before the MeOH was evaporated under vacuum and the dried residue was frozen. The residue was redissolved in MeOH and water (200 + 200 μl) before being centrifuged, poured into syringe filters and sealed in HPLC vials. Phenolic acids and flavonoids were analysed by HPLC (Agilent, Series 1100, Germany), which consisted of a binary pump (G1312A), a thermostated autosampler (G1329A), a thermostated column oven (G1316A) and a diode array detector (G1315B). The metabolites were separated using an ODS Hypersil C18 (4.6 × 50 mm) HPLC column (Thermo Scientific, Waltham, Massachusetts, USA). The samples were and eluted (flow rate 2 ml min^{-1}) using a MeOH : water gradient.[47] The auto-injection volume was 20 μl and all runs were performed at 30 °C. Identification of metabolites was completed by comparison of retention times and UV spectra with commercial standards. The analysis was repeated in three of the experimental rounds.

2.4.2 Chemical composition of the cuticle. Cuticular chemical composition was analysed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). A pair of fully expanded leaflets was removed from the third leaf from the base of five plants per treatment and air dried in a warming cupboard at 60 °C. Epicuticular wax was removed from one leaflet from each pair of leaflet samples. This was performed by washing each leaflet twice in warm (40 °C) chloroform for 60 s per wash. Each leaflet sample was measured at three different positions on both adaxial and abaxial sides (6 measurement points per leaf). On both the ab- and adaxial sides, two measurement points on each leaflet were basal and close to either side of the midrib. The third measurement was distal and close to the midrib. Samples were measured using a Vertex 70 FTIR spectrometer (Bruker Optik, Germany) with the single-reflection attenuated total reflectance (SR-ATR) accessory. The ATR IR spectra were recorded with 32 scans using a horizontal SR-ATR diamond prism with a 45° angle of incidence on a High Temperature Golden Gate ATR Mk II (Specac, United Kingdom). Spectra were recorded in the region between 7000 and 600 cm^{-1} with a spectral resolution of 4 cm^{-1}. Each spectrum was recorded as the ratio of the sample spectrum to the spectrum of the empty ATR plate. The penetration depth of infrared light in ATR-FTIR measurements is 0.5–5 μm, depending on the wavelength.[48] Thus, the FTIR spectra of leaves predominantly contain information on the leaf cuticle, while the underlying epidermal cells contribute to a lower degree. The analysis was performed during one of the experimental rounds.

2.4.3 Antioxidant power in leaves. The antioxidant power in whole leaflets was determined using an OxiSelect Ferric Reducing Antioxidant Power (FRAP) Assay Kit (Cell Biolabs, Inc., CA, USA). Studies analysing antioxidant capacity using several methods (*e.g.* FRAP and 2,2-diphenyl-1-picrylhydraziyl (DPPH)) have indicated significant correlation between the methods,[49–51] leading Clarke *et al.*[51] to conclude the use of a single method to be sufficient. As a result of this, only FRAP was used to analyse antioxidant capacity in this study. Fully expanded leaflets from the sixth leaf from the base of three plants per treatment were removed and immediately placed in liquid N_2, followed by storage at −80 °C. Tissue samples were weighed out (10 mg) and homogenised in 1 mL cold assay buffer. The absorbance values of the reaction mixtures were measured on a microplate reader (Biochrom Asys UVM 340 with KIM, UK) using 540 nm as the primary wavelength. One leaflet from three separate plants in each treatment was sampled, and three technical replicates from each leaflet were analysed (total $n = 36$ including biological and technical replicates). Samples were measured against iron(II) standards. The results were converted to relative amounts with moderate RH antioxidant power normalized to 100%. The analysis was performed during one of the experimental rounds.

2.5 Data analysis

Significant differences between the means were determined for all data using generalised linear models (GLM) and two-way ANOVA followed by Tukey's HSD *post-hoc* tests. Data were tested for normality using normal-quantile plots and Shapiro–Wilk normality tests, and for homoscedasticity using Levene's test for equality of variances. Differences with $p < 0.05$ were considered significantly different, unless otherwise stated in the text. Statistical tests were performed using Minitab 16.2 (Minitab 16.2.2, Windows version, State College, PA, USA) and RStudio version 1.0.44 (© 2009–2016 RStudio, Inc.).

For the analyses of infrared spectral data, the spectral region of 4000–600 cm^{-1} was selected, and processed using

multiplicative signal correction (MSC). The processed spectra were analysed initially using principal component analysis (PCA) to determine which treatment variables could explain the highest proportions of the data. Mann–Whitney *U* tests were then used to calculate the statistical significance of differences in the PCA principal component scores between samples. Partial least-squares-discriminant analysis (PLS-DA) was conducted in order to evaluate the effect of RH or UV exposure on samples. The optimal number of components (*i.e.*, PLS factors) of the calibration models (A_{Opt}) was determined using full cross-validation. Since the majority of the models had 4 as an optimal number of components, 4 components were used in all PLS-DA models in order to compare models and avoid over-fitting. The PLS coefficient of determination (R^2) between the taxa was used to evaluate the calibration models. Biochemical similarities between individual leaf samples were estimated by variability tests based on Pearson correlation coefficients (PCCs) for the spectral region of 1900–700 cm^{-1}. All spectroscopy processing methods and data analyses were performed using The Unscrambler X 10.3 (CAMO Software, Oslo, Norway) and using functions and in-house developed routines written in MATLAB 2014a, 8.3.0.532 (The MathWorks, Natick, MA, USA).

3 Results

3.1 Effect of RH and UV on stomatal function and conductance in pea plants

After 15 days of growth under experimental conditions in the chambers, water loss from detached leaves after three hours under a common RH environment (40% RH) was significantly affected by both RH level and UV exposure (Fig. 2A). No significant interaction was found between RH and UV exposure. Leaves grown in high RH lost 40–50% more water than leaves grown in moderate RH, both with and without UV radiation.

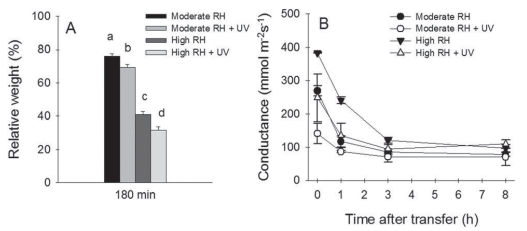

Fig. 2 Water loss and stomatal conductance in pea plants grown under moderate (60%) and high (90%) relative humidity (RH) with (+UV) or without UV radiation. (A) Water loss from detached leaves 3 h after detachment and transfer to a common environment (40% RH, 15 μmol m^{-2} s^{-1}). The means ± SE are shown, n = 5 for five replicate experiments, total n = 25; (B) stomatal conductance measurements in a time course after removal to a dark environment (40% RH). The means ± SE are shown, n = 3 for two replicate experiments, total n = 6. Different letters indicate statistically significant differences ($p \leq 0.05$) as analysed by analysis of variance followed by Tukey's HSD test.

Moreover, at both RH levels, leaves exposed to UV radiation lost significantly more water than leaves not exposed to UV (Fig. 2A).

Initial time course measurements of stomatal conductance after transfer of plants to the 40% RH environment and darkness indicated that plants grown in high RH had significantly higher instantaneous stomatal conductance rates than plants grown in moderate RH (Fig. 2B). Moreover, plants that had been exposed to UV had significantly lower conductance rates than those not exposed to UV. No significant interaction was found between RH and UV exposure. After 1 h, plants grown in high RH without UV still had significantly higher instantaneous conductance than any other treatment (Fig. 2B). After three hours there were no longer any significant differences seen in conductance rates between the treatments, though conductance rates remained between 70 and 110 mmol m^{-2} s^{-1} even after eight hours in darkness. No significant interaction was found between RH and UV exposure.

3.2 Visible injury and CPD-DNA damage

Exposure to UV radiation caused some leaf curling in moderate RH (Fig. 3A), but had a severely damaging effect with extensive leaf curling on the plants when grown in high RH (Fig. 3). RH and UV exposure had a significant interaction in visible injury and quantified CPD-DNA ($p < 0.05$). Plants exposed to UV radiation showed no chlorosis or necrosis, and very little UV-induced CPD-DNA damage when grown in moderate RH, yet plants grown in high RH had severe visible damage when exposed to UV radiation (Fig. 3). Damage to high RH + UV plants included severe leaf curling (Fig. 3A), leaf chlorosis (Fig. 3B), some leaf necrosis (Fig. 3C), and a significant amount of CPD-DNA damage (Fig. 3D). Structurally, neither RH nor UV radiation affected cellular leaf morphology (Fig. 3E).

3.3 Effect of RH and UV on plant growth and photosynthesis

Growth of pea was significantly affected by RH, but not by UV radiation (Fig. 4). No significant interaction was found between RH and UV exposure. Plants grown in high RH were, on average, 10% taller than plants grown in moderate RH ($p < 0.01$), regardless of UV exposure. A similar result was seen in the number of leaves per plant, with plants grown in high RH having a greater number of leaves than plants grown in moderate RH ($p < 0.03$).

Maximal photosystem II efficiency (F_v/F_m) was measured on leaves with no visible damage and the results indicated no damage to the photosynthetic apparatus in any of the treatments. All treatment measurements were between 0.83 and 0.85, within the optimal range,[52] and no differences between the treatments were found (data not shown).

3.4 Plant protective compounds

3.4.1 Flavonoid content in whole leaves. As expected from previous studies of pea leaves, quercetin-glycosides were the most prominent flavonoid compounds present (Table 1). UV exposure under high RH significantly reduced the phenolic

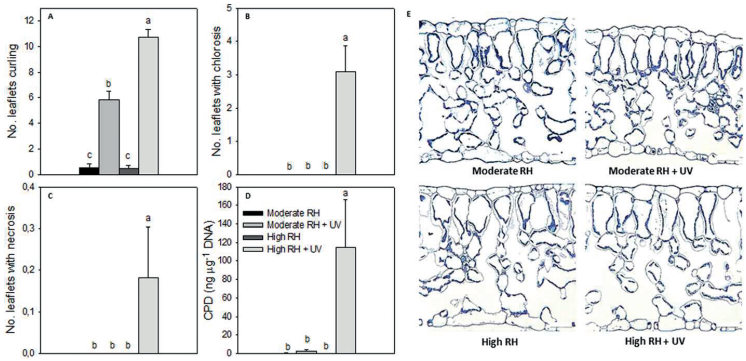

Fig. 3 Injuries (A–D) and cross sectional leaf anatomy (E) in pea plants grown under moderate (60%) and high (90%) relative humidity (RH) with (+UV) or without UV radiation. (A) The number of leaflets showing leaf curling; (B) the number of leaflets showing leaf chlorosis; (C) the number of leaflets showing necrosis; and (D) CPD-DNA damage in leaflets. The means ± SE are shown, n = 3 for two replicate experiments, total n = 6. Different letters indicate significantly different values ($p \leq 0.05$) as analysed using ANOVA followed by Tukey's HSD test. (E) Cross sections of leaflets embedded in LR-White stained with Stevenel's Blue taken using a 40× objective.

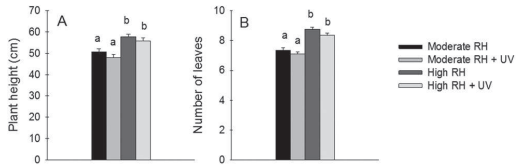

Fig. 4 (A) Plant height; and (B) the number of leaves on plants growing in moderate or high RH, with or without UV radiation. The means ± SE are shown, n = 5–8 for four replicate experiments, total n = 24–27. Different letters indicate significantly different values as analysed using GLM followed by Tukey's HSD test.

acid concentration ($p < 0.01$, Table 1). However, one group of phenolic acids, the chlorogenic acids, showed an opposite trend upon UV exposure in high RH, as well as an increase in chlorogenic acids in high RH plants compared to moderate RH plants (Table 1). Due to the opposite trends in phenolic and chlorogenic acids, total phenolic acid concentration showed no significant effect of either RH or UV exposure (Table 1). RH had a significant effect ($p < 0.01$) on the total quercetin-glycoside concentration, strengthened by exposure to UV, resulting in −UV plants grown in moderate RH having significantly higher concentrations of quercetin-glycosides than UV-exposed plants grown in high RH (Table 1). Kaempferol-glycosides were found in very low concentrations, and neither RH nor UV affected their concentration (Table 1). Total flavonoid concentrations reflect the pattern seen in quercetin-glycoside concentrations, as the concentration of the latter was so much greater than kaempferol-glycosides (Table 1). No significant interaction was found between RH and UV exposure for any of the flavonoids.

3.4.2 Chemical composition of the leaf cuticle. The infrared spectra of control leaves (detached leaves, dried and unwashed) showed characteristic signals related to alkyl groups, which are the predominant functional groups in the long-chain chemical constituents of cuticular waxes (Fig. 3a). The spectra showed alkyl-related vibrational bands at 2914 and 2846 cm^{-1} (C–H stretch in alkyl groups), 1472 cm^{-1} (CH_2 bending), 1462 and 1365 cm^{-1} (CH_3 deformations).[53,54]

Table 1 Concentration of phenolic compounds in pea leaves grown under moderate (60%) or high (90%) relative humidity RH, with (+UV) or without (−UV) UV radiation. The means ± SE are shown, n = 4–5 for three replicate experiments, total n = 12–15. Different letters indicate significantly different values

	60% RH		90% RH	
	−UV	+UV	−UV	+UV
Phenolic acids (mg g^{-1})	0.74 ± 0.04^{ab}	0.58 ± 0.06^{ab}	0.76 ± 0.06^{a}	0.54 ± 0.03^{b}
Chlorogenic acid (mg g^{-1})	0.12 ± 0.02^{b}	0.11 ± 0.01^{b}	0.14 ± 0.01^{ab}	0.18 ± 0.01^{a}
Total phenolic acids (mg g^{-1})	0.85 ± 0.05^{a}	0.69 ± 0.06^{a}	0.91 ± 0.07^{a}	0.73 ± 0.04^{a}
Quercetin-glycosides (mg g^{-1})	16.17 ± 0.67^{a}	14.90 ± 0.54^{ab}	13.42 ± 0.93^{ab}	12.57 ± 0.74^{b}
Kaempferol-glycosides (mg g^{-1})	0.03 ± 0.004^{a}	0.03 ± 0.002^{a}	0.03 ± 0.003^{a}	0.02 ± 0.006^{a}
Total flavonoids (mg g^{-1})	16.20 ± 0.67^{a}	14.92 ± 0.54^{ab}	13.45 ± 0.93^{ab}	12.59 ± 0.74^{b}

Significance based on two-way ANOVA followed by *post-hoc* Tukey's HSD analyses. Significance level: $p \leq 0.05$.

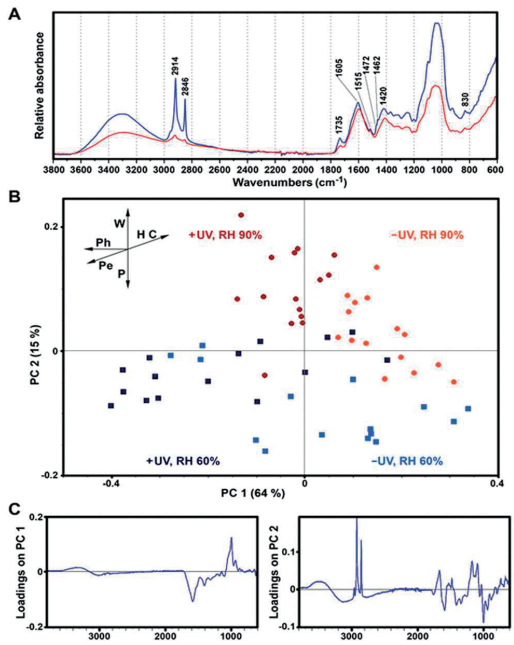

Fig. 5 (A) Averaged and preprocessed FTIR spectra of control (blue) and chloroform-washed (red) pea leaves grown under 60% relative humidity (RH) and exposed to UV radiation; (B) principal component analysis (PCA) score plot of FTIR spectral data set comprising measurements on the adaxial side (representative of results for both leaf sides) of the control leaves, with depiction of growth conditions (with (+UV) or without (−UV) UV radiation under moderate (60%) or high (90%) RH). The vectors approximate the increase in relative amounts of wax (W), phenolics (Ph), pectin (Pe), proteins (P), hemicellulose (H) and cellulose (C). The percent variances for the first five principal components (PCs) were 64.40, 15.06, 6.92, 4.34, and 2.28. (C) Loading plots on the first two PCs of the PCA.

The spectra of chloroform-washed leaves were devoid of these signals, signifying that the cuticular waxes were removed by the washing treatment (Fig. 5a). The spectra of the washed leaves were dominated by the strong signals related to carbonyl groups characteristic of uronic acids and esters, such as glucuronic and galacturonic acids and esters of pectin: a carboxylic ester band at 1735 cm^{-1} (C═O stretch in esters), and two carboxylate bands at 1605 cm^{-1} (COO^- antisymmetric stretch) and 1420 cm^{-1} (COO^- symmetric stretch).[54] There was lower absorbance in the 1735 cm^{-1} band than in the band at 1605 cm^{-1}, indicating that the majority of the uronic compounds are in acid or salt form, such as galacturonic acid, and not in ester form, such as methyl esters, which are common groups in pectic polysaccharides. The remaining principal feature in the FTIR spectra of washed leaves was strong absorbance in the 1200–900 cm^{-1} region related to stretching and bending of C–O–C and C–OH bonds characteristic of cuticular saccharides, including monosaccharides, such as arabinose, xylose, mannose, glucose, galactose and uronic acids, as well as polysaccharides, predominantly pectin, hemicellulose and cellulose.[54] Finally, the spectra showed distinctive bands at 1515 and 830 cm^{-1} associated with the vibrations of aromatic rings of phenolic compounds.[54,55]

Principal component analyses of FTIR spectral data indicated strong effects of both UV and RH on leaf cuticle chemical composition (Fig. 5). UV treatment correlated with PC1, while RH correlated with PC2 (Fig. 5b). PC1 loadings indicated that plants exposed to UV radiation had a higher relative content of uronic acids and phenolics, and lower content of cellulose and non-uronic based hemicellulose, than plants not exposed to UV (Fig. 5C). PC2 loadings indicated that high RH plants had a higher relative amount of cuticular waxes and lower amounts of proteins (probably cell wall glycoproteins) and uronic compounds than moderate RH plants (Fig. 5C). Mann–Whitney *U* tests, based on the PCA principal component scores between samples, confirmed that the UV effect on total chemical composition of leaves was significant at both moderate and high RH.

The PLS-DA results indicated that the effect of UV on cuticle chemical composition was stronger in both control and washed leaves from high RH-grown plants compared to moderate RH-grown plants (Table 2, +UV *vs.* −UV). This effect was stronger on the adaxial than on the abaxial side of the leaves. The effect of RH on the cuticle chemical composition was stronger in plants exposed to UV compared to plants not exposed to UV (Table 2, 60% *vs.* 90%). This was seen in both control and washed leaves on both adaxial and abaxial leaf sides (Table 2). Variability analyses based on Pearson correlation coefficients (PCCs) indicated that growth in high RH resulted in a more uniform cuticular chemical composition between individual leaves compared to moderate RH, irrespective of UV treatment (Table 3).

3.4.3 Antioxidant power of whole leaves. The total antioxidant capacity of whole leaves was tested using a FRAP assay (Fig. 5). Plants grown in high RH had significantly lower total antioxidant capacity than plants grown in high RH (p = 0.0133). No effect of UV radiation was found, and no signifi-

Table 2 Comparison of ATR-FTIR data for the chemical composition of leaf surfaces (dried leaves) of pea plants grown under moderate (60%) and high (90%) relative humidity (RH) and exposed (+UV) or not exposed (−UV) to UV radiation. Three measurements were taken on each of ad- and ab-axial sides of each leaf, taken on five leaflets from different individuals in each treatment. R^2 values, shown for adaxial and abaxial sides of control (detached, dried) and chloroform-washed leaves, indicate the degree of difference between the different treatments

		+UV *vs.* −UV (R^2)		60% *vs.* 90% RH (R^2)	
Leaf side	Wash treatment	60% RH	90% RH	+UV	−UV
Adaxial	Control	0.82	0.90	0.86	0.74
	Washed	0.74	0.85	0.95	0.89
Abaxial	Control	0.68	0.77	0.87	0.83
	Washed	0.67	0.83	0.91	0.76

Table 3 Comparison of variability values for dried leaflets of pea plants grown in moderate (60%) or high (90%) relative humidity (RH) and either exposed (+UV) or not exposed (−UV) to UV radiation. Three measurements were taken on each of ad- and ab-axial sides of each leaf, taken on five leaflets from different individuals in each treatment. Variability values, calculated from Pearson correlation coefficients, indicate the degree of variability between individual leaves based on leaf side (ad- or abaxial), wash treatment for each leaf side (control or chloroform-washed, RH with UV radiation notwithstanding), RH, and between +UV and −UV for each RH level. Partial least-squares-discriminant analysis (PLS-DA) was performed based on ATR-FTIR data

			Variability				
Leaf side	Wash treatment	RH	Leaf side	Wash treatment	RH	+UV	−UV
Adaxial	Control	Moderate	105	84	104	78	76
		High			38	18	16
	Washed	Moderate		120	152	89	76
		High			39	18	31
Abaxial	Control	Moderate	85	80	108	68	105
		High			36	12	21
	Washed	Moderate		84	107	70	77
		High			36	12	29

Pearson correlation coefficient (PCC): 1900–700 cm^{-1}. Variability = $(1 - PCC^*) \times 10^{-4}$.

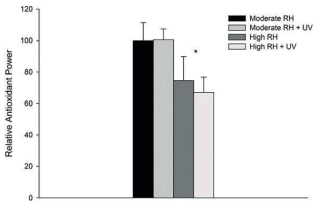

Fig. 6 Relative antioxidant capacity of whole leaves from pea grown in moderate (60%) or high (90%) RH with (+UV) or without UV radiation and tested using a FRAP assay. The values for the other treatments were normalized to the value for the moderate RH antioxidant capacity ± relative SE. Three technical replicates were measured from three leaflets from separate individual plants per treatment. This was performed in a single experiment * indicates significant difference due to RH (RH: $p < 0.05$).

cant interaction was found between RH and UV exposure (Fig. 6).

4 Discussion

4.1 UV radiation induced damage in plants grown at high RH, but did not affect growth or photosynthetic capacity

Night-time exposure to UV during growth caused plant injuries. There was no visible leaf damage in either RH treatment that had not received UV radiation, yet there was leaf curling in leaves exposed to UV radiation (Fig. 3A), with significantly more in high RH than in moderate RH. More severe damage, in the form of chlorosis, some necrosis, and significant CPD-DNA damage were found in UV-exposed leaves from high RH, but not in any of the other treatments (Fig. 3B–D). This clearly shows that growth over time in high RH makes plants more susceptible to UV-induced stress than growth in moderate RH. CPD-DNA damage is repaired by blue light-dependent photolyase,[56] and Li *et al.*[57] found repair of 83% of CPD after 2 h irradiation with white light. The presence of a significant amount of CPD may be due to the small amount of blue light present in HPS lamps (approximately 5%). Additionally, exposure to UV radiation during the night, as opposed to simultaneous exposure to daylight and UV, may have further decreased the plants' ability to repair DNA damage.[56]

In spite of the visible and CPD-DNA damage caused by exposure to UV radiation, neither RH nor UV radiation had an effect on the maximum efficiency of photosystem II in pea plants, indicating that UV radiation did not induce stress on photosystem II in either RH treatment. Furthermore, no significant differences in cellular leaf structure were seen as a result of either RH or UV radiation (Fig. 3E).

Taller pea plants with more leaves in high RH as compared to moderate RH are similar to previous findings in *Rosa hybrida*,[58] *Gossypium hirsutum*,[59] and several foliage species.[60] However, while previous findings have shown a reductive effect of UV radiation on plant height,[39,61,62] exposure to UV radiation during the night had no significant effect on plant height in this experiment (Fig. 4A). Roro *et al.*[63] showed that the UV-B induced reduction in stem elongation in pea was mediated through a reduction in bioactive gibberellin (GA), which acts on cell division and cell elongation in the subapical meristem. In the present experiment, the lack of UV effects on growth may be due to differences in experimental growth conditions, such as light and temperature,[3] or the time and dose of UV radiation exposure.

4.2 Flavonoid content and antioxidant power are reduced in high RH leaves

The results indicated a trend towards decreased phenolics and flavonoids in response to high RH and UV exposure, most prominently in the 'strong antioxidant'[64] quercetin-glycoside (Table 1). This may be due to the light conditions during growth. According to Siipola *et al.*,[65] attenuation of solar blue light resulted in a greater reduction in leaf flavonoid content than attenuation of UV radiation in pea plants. Similar to the

lack of CPD-DNA damage repair by photolyases described above, the light provided by HPS lamps may have had insufficient blue light for flavonoid accumulation. This, coupled with UV radiation received during the dark period, as opposed to combination with other light, indicates some support for flavonoid accumulation being more dependent on light, rather than UV radiation. Enzymes involved in the synthesis of several flavonoids are found to be highly responsive in plants exposed to a wide range of environmental stresses,[66] including drought stress.[8] In our experiment, RH was shown to affect flavonoid accumulation: plants grown in high RH had significantly lower flavonoid content than plants grown in moderate RH (Table 1). This indicates a possibility that high RH is construed as well-watered conditions by the plant, thereby diminishing the need for flavonoid accumulation. Such a scenario is compounded by the results of the FRAP assay (Fig. 6), which showed a decrease in the total antioxidant power in leaves grown in high RH. Antioxidant power, measured in the FRAP assay as the power of a sample to reduce a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex into a ferrous (Fe^{2+}) form,[67] indicates the ability of the sample to scavenge excess ROS, which have the potential to cause oxidative damage. Taken together, these results show that plants grown in high RH were more susceptible to oxidative damage by UV radiation due to decreased leaf flavonoids, and total antioxidant power, and may explain the increased visible damage and presence of CPD-DNA in high RH + UV plants (Fig. 3).

4.3 UV exposure increases content of phenolic and uronic compounds in leaf cuticles, while high RH increases epicuticular wax

The FTIR-based chemical characterization of leaf cuticles was in accordance with the published data.[68–71] These analyses indicated an increase in content of phenolic and uronic compounds, as well as a decrease in content of cellulose and non-uronic based hemicellulose (such as arabinans and xyloglucans), in leaves exposed to UV radiation at both RH levels (Fig. 5). It is important to note that, although the total concentration of phenolic compounds in whole leaves of UV exposed plants decreased (Table 1), the phenolic content in the leaf cuticular layer actually increased (Fig. 5). Therefore, in leaves grown with high UV exposure the epidermis probably has better UV protection by phenolics than control group leaves.

However, the main difference in cuticular chemistry between the control and UV-exposed leaves lies not in the phenolic content, but rather in the content of uronic compounds. In leaves grown with UV exposure, the cuticular layer had higher content of uronic compounds than in control group leaves. Uronic acids and esters are the principal components of plant cuticles.[71] They are embedded in the cuticle layer either as monosaccharides, such as glucuronic and galacturonic acids, or as monomer units incorporated into backbone chains in pectic polysaccharides, such as pectin, and side chains in hemicellulosic polysaccharides, such as arabinogalactans and xylans.[71,72] It has been proposed that UV exposure of plant tissue leads to pectin degradation into methane and galacturonic acid in the plant cell wall.[70,73] Though this mechanism potentially leads to the production of superoxide, it may also have a beneficial effect by: (1) the release of methane and superoxide as stress-signalling molecules, and (2) accumulation of uronic acid as a precursor in the biosynthesis of ascorbates.[74,75] Ascorbates can have an essential role in stress mitigation as they act as reducing agents, protecting plants against oxidative stress. The amount of reactive oxygen species (ROS) may increase dramatically under increased UV irradiation and lead to a high level of oxidative stress. Therefore, a high content of uronic acids, either as free chemicals or as monomers in pectic polysaccharides, may have great protective potential as a build-up of antioxidant precursor chemicals.[76] Moreover, constrained generation of ROS in cuticles and the outer cell wall, where ROS concentration can be regulated by ascorbate biosynthesis, is favoured when compared with considerably more harmful intracellular build-up of ROS.

Growth in high RH was seen to increase content of cuticular waxes, and decrease the content of proteins and uronic compounds. It is difficult to assess whether this observation was due to higher production of waxes or lower production of proteins and uronic compounds in plants grown under high RH. Previous studies have indicated that high RH may and may not affect wax coverage and morphology.[36] Should this be the case, the content of proteins and uronic acids is decreased in plants grown in high RH. This may cause a reduction in potential antioxidant power in the cuticle and upper epidermis, which reflects the situation found in whole leaves grown in high RH (Fig. 6).

4.4 UV radiation did not improve stomatal function in plants produced at high RH but reduced conductance in intact plants and increased water loss in detached leaves

We hypothesised that exposure to UV radiation may trigger stomatal closure in pea plants grown in high RH, and thereby re-establish stomatal function. Indeed, our results showed that pea exposed to UV had lower instantaneous conductance rates immediately after removal from light conditions than plants developed without UV (Fig. 2B). While Jansen and Van den Noort[11] reported that UV exposure may induce stomatal opening or stomatal closure, dependent on the metabolic state of the guard cells, several other studies have previously reported stomatal closure as a response to UV radiation.[13,77,78] This takes place either through an increase in ABA concentration or *via* regulation by the UVR8 photoreceptor in a signalling cascade involving COP1 and HY5 in *Arabidopsis* in a NO-dependent mechanism.[13] In this study, the degree of stomatal closure due to UV exposure was similar in both moderate and high RH (Fig. 2B). As previously shown,[21,32] plants developed in continuous high RH had higher instantaneous conductance rates than plants developed at lower RH (Fig. 2B). Previous studies on *R. hybrida*, *Arabidopsis thaliana*, *Vicia faba* and *T. virginiana* have shown that stomata developed in continuous high RH are unable to close when exposed to environmental closing signals, such as darkness or exogenous ABA

treatment.[21–24,31] However, the results presented here show closure of stomata in a dark, low RH environment, given sufficient acclimation time, in all treatments (Fig. 2B).

Stomatal conductance was higher in plants grown in high RH compared to moderate RH, showing agreement with previous findings in other species.[21,23] Though stomatal closure was eventually induced in plants grown in high RH (significant reduction after three hours), exposure to UV did not improve the response time. We hypothesised that due to the stomatal closure response induced by UV exposure,[13] UV radiation would improve stomatal responsiveness after growth in high RH. Despite UV-exposed plants having a lower instantaneous conductance than plants not exposed to UV, UV exposure resulted in a decreased rate of responsiveness to closing stimuli, indicating that exposure to UV does not improve stomatal responsiveness.

Finally, we found that pea leaves developed in high RH lost more water during a three hour desiccation test than leaves from moderate RH (Fig. 2A). This shows that the stomata do not close properly in leaves from high RH in response to desiccation alone. Furthermore, despite lower instantaneous conductance rates, UV-exposed detached leaves lost more water than controls at both RH levels, indicating no improvement in stomatal closure as a result of UV exposure.

4.5 Conclusions

The present study shows that in pea plants grown in continuous high RH, stomata are more open and less responsive to closing stimuli. The hypothesis that UV exposure would trigger stomatal movement and thereby increase responsiveness has been refuted. While plants grown in both moderate and high RH and exposed to UV had lower instantaneous stomatal conductance rates, the rate of responsiveness to closing stimuli was not improved. Furthermore, plants grown in continuous high RH were more susceptible to UV-induced damage than plants grown in moderate RH. This was due to a reduction in leaf flavonoid content and a reduction in leaf antioxidant power, though the mechanisms behind this remain undetermined. UV radiation is a potentially powerful tool in protected plant production but background humidity conditions need to be taken into consideration.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Paper II

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ECOPHYSIOLOGY, STRESS AND ADAPTATION

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Different abscisic acid-deficient mutants show unique morphological and hydraulic responses to high air humidity

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Abstract

High relative humidity (RH) perturbs plant growth, stomatal functioning and abscisic acid (ABA) homeostasis, but the role of ABA in this physiological regulation is equivocal. To determine the role(s) of ABA in plant responses to high RH, wild-type (WT) tomato and barley plants and their respective ABA-deficient mutants *flacca* and Az34 (which are mutated in the same locus of the ABA biosynthesis pathway) were grown in contrasting RHs (60% and 90%) to measure biomass partitioning, stomatal traits and water relations. Surprisingly, growth RH did not affect foliar ABA levels in either species. While Az34 showed similar stomatal size and density as WT plants, *flacca* had larger and more abundant stomata. High RH increased stomatal size in tomato, but decreased it in barley, and decreased stomatal density in tomato, but not in barley. Altered stomatal responses in ABA-deficient plants to high RH had little effect on tomato photosynthesis, but Az34 barley showed lower photosynthesis. ABA deficiency decreased relative shoot growth rate (RGR_{SHOOT}) in both species, yet this was counteracted by high RH increasing leaf water status in tomato, but not in barley. High RH increased RGR_{SHOOT} in *flacca*, but not in WT tomatoes, while having no effect on RGR_{SHOOT} in barley, but affecting barley net assimilation rate, leaf area ratio (LAR) and specific leaf area in an ABA-dependent manner. ABA-RH interaction affected leaf development in tomato only. Thus, different crop species show variable responses to both high RH and ABA deficiency, making it difficult to generalise on the role of ABA in growth regulation at contrasting RHs.

1 | INTRODUCTION

Plant responses to low air relative humidity (RH, corresponding to high vapour pressure deficit, VPD, provided no change in temperature) are important to prevent excessive water loss, yet responses to high RH (> 85%) (Torre et al., 2003) are arguably as important. In protected plant production systems at high latitudes, a trade-off between ventilation and energy-saving often leads to a high RH environment during growth, affecting not only plant morphology and water relations, but also post-harvest keeping quality (Fanourakis et al., 2016; Innes et al., 2018; Innes et al., 2019; Mortensen, 2000; Torre et al., 2003). High RH increased biomass, leaf area and the number of leaves of several species (Innes et al., 2019; Oksanen et al., 2019) by increasing the leaf water status (Leuschner, 2002; Lihavainen et al., 2016; Mortensen, 2000). However, decreased leaf area has also been found in several species, including tomato, grown in high (> 90%) RH (Mortensen, 2000; Oksanen et al., 2019). In tomato, this was attributed to low leaf calcium concentrations, in agreement with Leuschner (2002) and Oksanen et al. (2019), who reported nutrient dilution in temperate woodland herbs and northern

forest trees grown in high (> 90%) RH, respectively. Growth in high RH also affects morphological characteristics, such as increasing both the number and size of stomata, as well as a decreasing stomatal functionality in response to closing signals (Arve et al., 2014; Fanourakis et al., 2011; Fanourakis et al., 2016; Nejad & Van Meeteren, 2005; Torre et al., 2003). However, lower stomatal frequency has also been reported as a result of increased leaf expansion due to high RH (Leuschner, 2002). Further investigations into morphological and hydraulic responses to growth in high RH are needed as responses are inconsistent, and the regulatory mechanisms not always elucidated.

As abscisic acid (ABA) is strongly implicated in plant responses to both low and high RH (Aliniaeifard & van Meeteren, 2013; Arve et al., 2013; Arve et al., 2014; Arve et al., 2015; Bauer et al., 2013; McAdam et al., 2015; McAdam & Brodribb, 2016; Merilo et al., 2018; Nejad & Van Meeteren, 2005; Okamoto et al., 2009), and different genotypes vary in their responses to increased air humidity (or decreased VPD) (Mortensen & Gislerød, 1990; Oksanen et al., 2019), it is important to understand the ABA-RH interactions and their effects on different species. The availability of many ABA-deficient mutants (summarised by McAdam et al., 2015, including their lesions in the ABA biosynthesis pathway) has allowed many investigations regarding their growth and physiology. ABA-deficient mutants have characteristically smaller leaves than their wild-type (WT) counterparts (Sharp et al., 2000), and considerably higher transpiration rates, often with impaired stomatal closure in response to darkness or desiccation (Sagi et al., 2002; Tal, 1966; Walker-Simmons et al., 1989). Tomato *flacca* and barley Az34 mutants both carry mutations in the molybdenum cofactor (see Table 1). The molybdenum cofactor is found at the catalytic sites in several molybdoenzymes present in higher plants: nitrate reductase (NR), xanthine dehydrogenase (XDH) and aldehyde oxidase (AO) (Zdunek-Zastocka & Lips, 2003). However, while Az34 lacks activity of all three enzymes, *flacca* only lacks XDH and AO activity (Sagi et al., 1999). Using two important crops comprising both eudicot and monocot species, these contrasting mutations allow the effects of ABA deficiency to be investigated and compared.

While formal growth analyses, as described by Poorter (2002), have been widely used to determine growth regulation in response to different environmental factors, the relative importance of the components affecting relative growth rate (RGR) varies.

RGR is defined as:

$$\mathrm{RGR} = \mathrm{NAR} \times (\mathrm{SLA} \times \mathrm{LMR}) \quad (1)$$

where NAR = net assimilation rate: the rate of mass increase per unit leaf area,

SLA = specific leaf area: the ratio of leaf area to leaf mass,

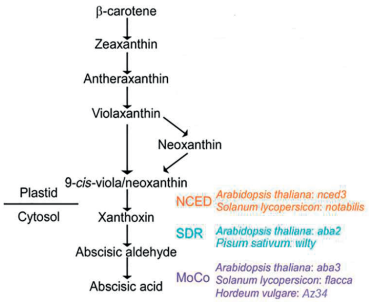
LMR = leaf mass ratio: the ratio of leaf mass to total plant mass and

LAR = SLA × LMR.Few formal growth analyses have partitioned the relative importance of the components of RGR in ABA-deficient mutants. Decreased RGR of the ABA-deficient tomato mutant *sitiens* (compared to WT plants) resulted from lower SLA, while NAR and LMR were unaffected (Mäkelä et al., 2003; Nagel et al., 1994). In contrast, decreased RGR of *flacca* tomatoes was attributed to decreased NAR, as LAR was significantly higher than in WT plants, and SLA was unaffected (Coleman & Schneider, 1996). In barley, Mulholland, Black, et al. (1996) reported a higher SLA in ABA-deficient plants than cv. Steptoe WT, though RGR was not measured. There are few, often incomplete, comparative analyses of the effects of ABA deficiency on growth of different species, often with contrasting results.

Understanding the physiological mechanisms regulating the growth of ABA-deficient mutants is complicated by their poor stomatal regulation, causing low leaf turgor and relative water content (RWC, Bradford, 1983; Sharp et al., 2000; Tal, 1966; Walker-Simmons et al., 1989). To compensate for the high rates of water loss in the mutants, the ABA-deficient and the WT plants can be grown at different RHs to ensure the effects of ABA deficiency are compared between leaves of the same RWC and/or leaf water potential (Mäkelä et al., 2003; Okamoto et al., 2009; Sharp et al., 2000; Yaaran et al., 2019).

TABLE 1 Species, genotype and mutation description of the abscisic acid (ABA)-deficient mutants used in this experiment

Species	Genotype	Mutation description
Tomato (*Solanum lycopersicum*)	cv. 'Ailsa Craig'	Wild type
	flacca	MoCo mutation
Barley (*Hordeum vulgare*)	cv. 'Steptoe'	Wild type
	Az34	MoCo mutation



Note: Schematic indicates mutations in the ABA biosynthesis pathway, as well as the corresponding *Arabidopsis thaliana* mutants. Figure adapted from McAdam et al. (2015).

Since growth in high RH affects plant morphology and water relations (Fanourakis et al., 2016; Innes et al., 2018; Innes et al., 2019; Torre et al., 2003), and high RH decreases ABA concentration (Aliniaeifard et al., 2014; Arve et al., 2013; Fanourakis et al., 2011; Okamoto et al., 2009), separating the effects of these two main factors is important but has not been previously investigated. For example, Mulholland, Black, et al. (1996) grew plants at a single, high RH (100%) to minimise the effects of leaf water deficit on growth, while Sharp et al. (2000) grew WT and ABA-deficient mutants at two different RH levels to minimise differences in leaf water status between WT and ABA-deficient mutants. Neither of these experiments were factorial for RH and ABA status, thus our factorial experiments allowed us to separate the RH and ABA effects in order to investigate whether RH modulates growth and hydraulic responses to ABA deficiency. We hypothesised that high RH would promote growth and water status of ABA-deficient mutants. To determine if these responses are conserved across species, we grew ABA-deficient mutants and their corresponding WTs of two important crop species (both eudicot and monocot origin) at two different relative humidities.

2 | MATERIALS AND METHODS

2.1 | Plant material

In this investigation, one eudicot species, *Solanum lycopersicum* cv. 'Ailsa Craig' (tomato), and one monocot species, *Hordeum vulgare* cv. 'Steptoe' (barley), were used. ABA deficient mutants (described in Table 1) of tomato (*flacca*) and barley (Az34) were used to investigate the relationship between ABA and growth in continuous high RH. The tomato *flacca* mutant is deficient in the synthesis of a molybdenum cofactor necessary for activating abscisic aldehyde oxidase (AAO). The barley mutant, Az34 (*nar*2a), was initially characterised as a NR-deficient mutant, but the *nar*2 locus codes for the same molybdenum cofactor of the molybdoenzyme AO (Walker-Simmons et al., 1989), indicating that both *flacca* and Az34 are deficient in the enzyme which catalyses the conversion of abscisic aldehyde to ABA in the final step of ABA biosynthesis (Bauer et al., 2013; McAdam et al., 2015; Sagi et al., 2002). These mutations decrease leaf ABA concentrations by up to 60% in *flacca* (Netting et al., 2012) and 25–53% in Az34 (Mulholland, Black, et al., 1996).

2.2 | Growth conditions

The experiments were performed at the Norwegian University of Life Sciences (NMBU), Ås (59.7°N), Norway in the winter of 2017/2018 and the summer of 2019. The seeds were germinated in Sphagnum peat growth medium, 6% ash, pH 5.0–6.0 (Degernes Torvstrøfabrikk AS) in 17 cm diameter, 2-L (tomato) or 12 cm diameter, 1.5-L pots (barley). The plants were grown in a single greenhouse compartment at a constant 20 ± 1°C and 70% ± 5% RH controlled by a PRIVA system (Priva, De Lier). During the experiments, natural daylight ranged from 6 to 10 h (timeanddate.com, 2018), so 100 μmol m^{-2} s^{-1} of supplementary light was supplied by high pressure sodium lamps (HPS, Osram NAVT-400 W) to extend the photoperiod to 20 h. The plants were watered daily to drip point and were kept in the greenhouse for 14 days.

Following germination, the plants were moved to controlled environment growth chambers at the two-leaf stage for growth treatments. Four growth chambers were used. All chambers were maintained at 22 ± 1°C using a PRIVA system. Two of the chambers were maintained at moderate (60%) RH, while the other two had high (90%) RH, corresponding to VPDs of 1.06 and 0.26 kPa, respectively. The plants were exposed to a 20-h photoperiod, with light supplied at 220 ± 20 μmol m^{-2} s^{-1} at plant height by Powerstar HQI-BT metal halide lamps (Ledvance GmbH) as measured using a Li-Cor quantum sensor connected to a Li-Cor LI-250 light meter (Li-Cor Inc.). The plants were watered daily using a 50/50 mixture of YaraLiva® Calcinit™ calcium nitrate solution (14.4% NO_3, 1.1% NH_4, 19.0% Ca, Yara Norge AS, Oslo, Norway) and Kristalon™ Indigo (7.5% NO_3, 1% NH_4, 4.9% P, 24.7% K, 4.2% Mg, 5.7% S, 0.027% B, 0.004% Cu, 0.06% Mn, 0.2% Fe, 0.004% Mo, 0.027% Zn, Yara Norge AS), EC level 2.0 mS cm^{-1}.

2.3 | Foliar ABA radioimmunoassay

Foliar ABA concentration was measured using a radioimmunoassay as described by Quarrie et al. (1988). Fully expanded leaflets from 3 to 5 plants per genotype per treatment were removed 1–2 h after the start of the light period and immediately placed in tubes and frozen in liquid N_2. Samples were freeze-dried using a Telstar LyoQuest (Telstar). Freeze-dried tissue was ground to powder and extracted in distilled de-ionised water on a shaker at 4°C overnight. The extracted aqueous solutions were measured for ABA concentration using the monoclonal antibody AFRC MAC 252.

2.4 | Water relations

2.4.1 | Leaf RWC

Detached leaves (two leaves per plant, four plants per treatment, $n = 8$) were cut under water and immediately fresh weighed (FW) before the petiole was submerged in water for at least 1 h. The turgid weight (TW) of each leaf was measured before the leaves were placed in a drying cabinet at 60°C for at least 24 h. Dry weight (DW) was measured for each leaf, and the following equation was used to calculate the RWC for each leaf:

$$\mathrm{RWC} = ([\mathrm{FW} - \mathrm{DW}]/[\mathrm{TW} - \mathrm{DW}]) \times 100 \quad (2)$$

2.4.2 | Day and night whole plant transpiration

Plant water usage was determined gravimetrically during three days and three nights on four or five plants per genotype in each RH

treatment. Each pot was sealed in plastic to prevent water loss from the soil, and the plants were weighed at the end of each day and each night. Plants were watered at the end of each night (to replace evapotranspirational losses) and weighed both before and after watering. Weight differences and leaf area, measured using a LI-3011 Leaf Area Meter (Li-Cor, Inc.), were used to determine total water use (g cm^{-2} h^{-1}) for each day and each night. These data allowed the rate of water loss (as mmol H_2O m^{-2} s^{-1}) to be calculated for each plant for each day and night using mol = g molar $mass^{-1}$, where the molar mass of water = 18.01528.

2.4.3 | Stomatal morphology

Three leaf samples were taken from all four genotypes and immediately placed in a fixation solution (1.25% glutaraldehyde, 2.5% paraformaldehyde in PIPES buffer). The leaves used for gas exchange measurements were removed from each plant, and 2 × 2 mm pieces were cut from close to the mid-rib using a scalpel blade. The pieces were placed immediately in fixation medium and stored at 4°C until microscopy preparation. For microscopy, the samples were washed twice for 15 min with PIPES buffer before being dehydrated using a graded ethanol series. Once dehydrated, the plants were critical point dried using a BAL-TEC CPD 030 (BAL-TEC AG). The dried samples were mounted onto stubs and sputter coated with a gold–palladium mix using a Polaron SC 7640 Sputter Coater (Quorum Technologies Ltd.). The coated samples were analysed using a Zeiss EVO 50 scanning electron microscope. Electron micrographs were taken at 400× and 700× magnification for measurements of stomatal anatomy. Stomata and trichomes were counted on 10 fields of view per treatment per genotype, and stomatal areas were measured on 100 or 57 stomata for each tomato and barley genotype respectively. Stomatal areas were measured using ImageJ software (ImageJ 1.49g, National Institutes of Health). Further electron micrographs were taken at 7000× magnification to compare single stomata between genotypes and treatments.

2.4.4 | Leaf gas exchange

Leaf photosynthesis (*A*), conductance (g_s) and internal CO_2 concentration (Ci) were measured on all genotypes using a LI-6400 Portable Photosynthesis System. The system was connected to a 6400-40 Leaf Chamber Fluorometer (LCF; Li-Cor, Inc.), in which LEDs provided 87% red, 10% blue and 3% far-red light at 200 μmol m^{-2} s^{-1}. RH in the cuvette was maintained as close to growth RH as possible (±15% during measurement), CO_2 was maintained at 400 ppm, and block temperature was set at 22°C. Young, fully expanded leaves from four plants per treatment were measured in all genotypes. Leaves were acclimatised in the chamber for at least 3 min until variables had stabilised. Leaf temperature was 20 ± 2°C and only below 20°C in *flacca* in 60% RH. Measurements were taken 1 h after the start of the light period.

2.5 | Growth measurements

2.5.1 | Morphology

Four to five replicates per treatment were randomly selected, starting 2 weeks after sowing and harvested weekly for 2 weeks. For each plant, the number of leaves (> 1 cm length) was counted, and leaf area was determined using a LI-3100 Area Meter (Li-Cor, Inc.). The stem and leaf materials were dried separately at 60°C for a minimum of 48 h before DWs were determined. Specific leaf area (SLA = leaf area/leaf DW), leaf mass ratio (LMR = leaf DW/shoot DW) and leaf area ratio (LAR = LMR × SLA) were calculated for each plant, to conduct a formal growth analysis. Roots were not recovered from the substrate.

2.5.2 | Relative growth rate

Relative shoot growth rates (RGR_{SHOOT}) were calculated using the mean of natural logarithm (ln) transformed total shoot DW data, according to Hoffmann and Poorter (2002) RGR_{SHOOT} was calculated using:

$$RGR_{SHOOT} = \left(\ln\bar{W}T2 - \ln\bar{W}T1\right)/(t_2 - t_1), \quad (3)$$

where: WT2 = total shoot DW at time point 2,
WT1 = total shoot DW at time point 1,
t_2 = time point 2 (14 days of growth),
t_1 = time point 1 (beginning of growth treatments).

Using the same method, growth rates were calculated for the relative leaf expansion rate (RLER) using ln transformed leaf area.

2.6 | Statistical analysis

All statistical analyses were performed in R (version 4.0.3, The R Foundation for Statistical Computing). Growth data and water relations data were collected from two independent experiments (Table S1). Data from replicate experiments were checked for differences between replicates and then pooled. Data were analysed factorially using two-way ANOVAS (main effects: genotype and RH), with statistical significance assigned to $P \leq 0.05$. The data were tested for normality using Shapiro–Wilk normality tests, and for homoscedasticity using Levene's test for homogeneity of variance. Gas exchange data were analysed for correlation using Pearson's test for correlation between paired samples.

3 | RESULTS

3.1 | Foliar ABA concentration

Foliar ABA concentration of *flacca* plants was 69% less than in WT plants averaged across the two RH levels (Figure 1A, $P < 0.001$ in

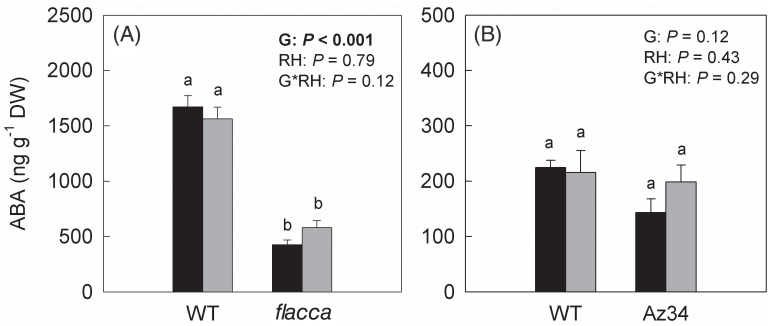
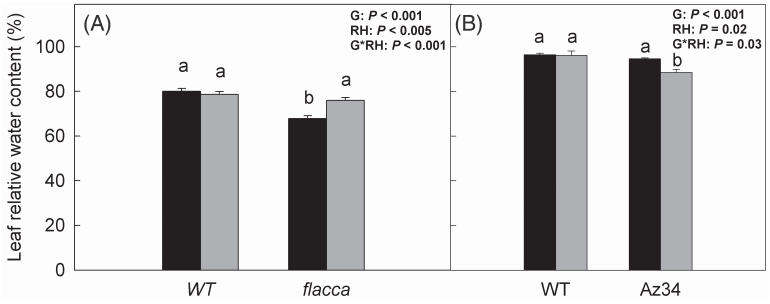


FIGURE 1 Foliar abscisic acid (ABA) concentrations in wild-type and ABA-deficient tomato (A) and barley (B) genotypes grown in 60% (black bars) or 90% (grey bars) RH in environmentally controlled growth chambers. Means ± SE shown, ABA: $n = 18$ for tomato WT and *flacca*, $n = 3$ for barley WT and Az34. Different scales used for different genotypes due to the large interspecific difference in ABA content. Different letters indicate significant differences between treatments ($P < 0.05$) as determined by two-way ANOVA (insert, G: genotype, RH: RH, G*RH: interaction) and post-hoc Tukey HSD analyses

FIGURE 2 Leaf relative water contents of wild-type (WT) and abscisic acid-deficient tomato (A) and barley (B) genotypes grown in 60% (black bars) or 90% (grey bars) RH in environmentally controlled growth chambers. Means ± SE shown, $n = 12$. Different letters indicate significant differences between treatments ($P < 0.05$) determined by two-way ANOVA (insert, G: genotype, RH: RH, G*RH: interaction) and post-hoc Tukey HSD analyses



both RH levels). WT and Az34 barley plants had statistically similar leaf ABA levels (Figure 1B). Furthermore, RH did not affect ABA levels in any of the genotypes analysed (Figure 1A,B).

3.2 | Relative water content

In tomatoes, *flacca* leaves had lower RWC than WT leaves at 60% RH, but not at 90% RH (Figure 2A). In barley, Az34 leaves had lower RWC in 90% RH, but not 60% RH (Figure 2B). Growth RH did not affect RWC of either WT genotype, but the ABA-deficient genotypes showed opposite effects since 90% RH increased RWC of *flacca* leaves but decreased RWC of Az34 leaves compared to 60% RH. Thus, growth at high RH did not always normalise leaf water relations of the ABA-deficient mutants.

3.3 | Stomatal morphology and gas exchange

In tomatoes, *flacca* leaves had more stomata than WT plants in both RH levels, and their stomata were 87% and 35% larger than WT stomata in 60% and 90% RH, respectively (Table 2). In barley, WT and Az34 leaves had similar stomatal counts, with Az34 stomata being 17% larger than WT in 60% RH, but 18% smaller than WT in 90% RH (Table 2).

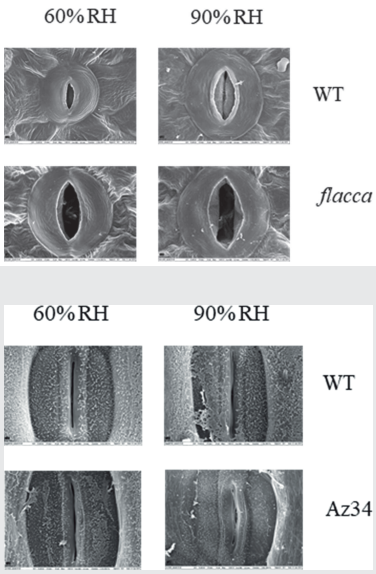
Both WT and *flacca* tomatoes had fewer, larger stomata in 90% compared to 60% RH (Table 2). In barley, RH did not affect the stomatal number of either genotype, but high RH decreased stomatal size. In tomatoes, WT and *flacca* stomata were 47 and 6% larger in 90% compared to 60% RH, respectively, while in barley, WT and Az34 stomata were 4 and 33% larger. This indicates that ABA and RH affect tomato (but not barley) stomatal number, and the interaction between ABA and RH on stomatal pore area affects tomato and barley differently.

3.4 | Gas exchange

In tomato, WT and *flacca* had similar assimilation rates (A) in 60% RH, but in 90% RH *flacca* had 38% higher A than WT (Table S2). A was not correlated with stomatal conductance (g_s, Figure 3A), which was 127% higher in *flacca* than WT plants averaged across RH levels (Table S2), but instantaneous water-use efficiency (iWUE, calculated as A/g_s) was 50% lower in *flacca* than WT plants (Table S2). A was not

TABLE 2 Stomatal morphology data from wild-type (WT) and abscisic acid-deficient tomato and barley genotypes grown in 60% or 90% RH in environmentally controlled growth chambers

Genotype	RH (%)	Stomatal density (per 0.14 mm^2)	Stomatal area (μm^2)	Single stomate 7000× mag.
Tomato				
WT	60	52.05 ± 0.90^b	268.0 ± 4.0^d	
	90	47.25 ± 1.01^c	393.3 ± 6.3^d	
flacca	60	65.40 ± 1.49^a	502.4 ± 10.7^b	
	90	61.60 ± 1.26^a	534.7 ± 12.3^a	
P values				
Genotype		**< 0.001**	**< 0.001**	
RH		**< 0.001**	**< 0.001**	
Genotype*RH		0.092	**< 0.001**	
Barley				
WT	60	9.1 ± 0.8^a	734.9 ± 25.8^b	
	90	9.2 ± 0.8^a	703.1 ± 16.7^b	
Az34	60	8.8 ± 0.8^a	861.8 ± 37.8^a	
	90	10.8 ± 1.1^a	575.4 ± 19.4^c	
P values				
Genotype		0.459	0.050	
RH		0.235	**< 0.001**	
Genotype*RH		0.281	**< 0.001**	



Note: Stomatal pore area (μm^2) and stomatal counts, and scanning electron micrographs of single stomata taken at 7000× magnification. Scale bars = 10 μm. Measurements were taken from scanning electron micrographs. Means ± SE shown, as well as main effects (genotype, RH) and interaction effects from two-way ANOVA, $n = 57–100$ for stomatal area, $n = 20$ for stomata counts. Different letters indicate significant differences between treatments ($P < 0.05$) as determined by post-hoc Tukey HSD analyses.

correlated with internal CO_2 concentration (Ci, Figure 3C), which was 10% and 3% higher in *flacca* than WT plants in 60 and 90% RH, respectively (Table S2). Tomato g_s and Ci showed a strong positive correlation (Figure 3E). In barley, Az34 plants had 48% lower *A* than WT plants averaged across RH levels (Table S2). *A* was not correlated with g_s (Figure 3B), which was statistically similar in WT and Az34 plants in both RH levels (Table S2), but iWUE was 50% lower in Az34 than WT plants (Table S2). *A* was strongly and negatively correlated with Ci (Figure 3D), which was 9% higher in Az34 than WT plants. Barley g_s showed a strong positive correlation with Ci (Figure 3F). Thus, ABA deficiency affects gas exchange responses differently in tomato and barley plants, most notably in *A* and g_s.

Tomato *flacca* plants had 42% higher *A*, 20% lower g_s, 5% lower Ci, and 80% higher iWUE in 90% RH compared to 60% RH, respectively, while WT showed no effects of RH on gas exchange parameters (Table S2). Barley Az34 plants had 54% higher *A* in 90% RH, while WT showed no impact of RH on *A* (Table S2). Barley WT and Az34 had 27 and 40% lower g_s, 10 and 8% lower Ci and 62 and 162% higher iWUE in 90% RH compared to 60% RH, respectively. These results show that tomato and barley WT and ABA-deficient mutants respond similarly to high RH.

3.5 | Whole plant transpiration during day and night

In tomatoes, *flacca* plants had higher transpiration rates compared to WT plants in 60% RH, but not 90% RH during both day and night (Figure 4A). However, in barley, WT and Az34 plants had similar transpiration rates during both day and night (Figure 4B).

Both WT tomatoes and *flacca* tomatoes had lower transpiration rates in 90% RH compared to 60% RH, during both day and night. However, WT plants had higher response indices (calculated as day/night ratio of transpiration rates) than *flacca* plants in both RH levels, indicating that WT plants responded more strongly to darkness as a stomatal closing signal (Figure 4A). Barley showed similar results; both genotypes decreased transpiration in darkness in both RH levels, though response indices were greater in WT than Az34 plants (Figure 4B).

3.6 | Growth rates and morphology

Genotypic and RH effects on RGR_{SHOOT} components (Equation 1) differed between species (Table 3, Figure S1). Averaged across RH levels,

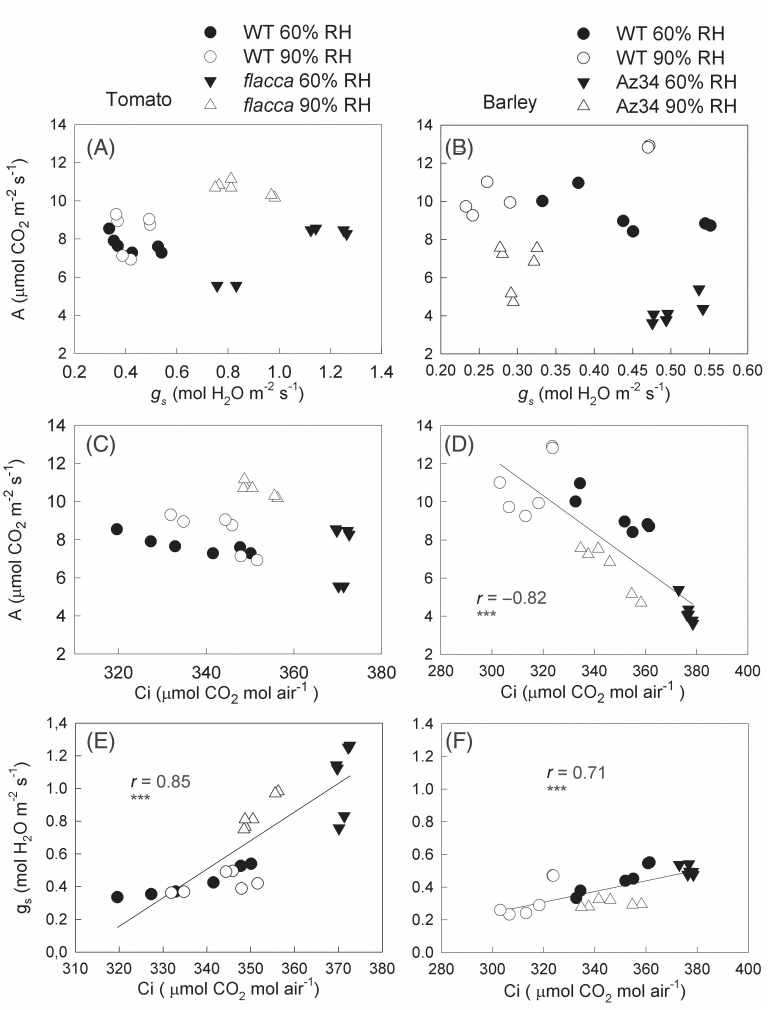
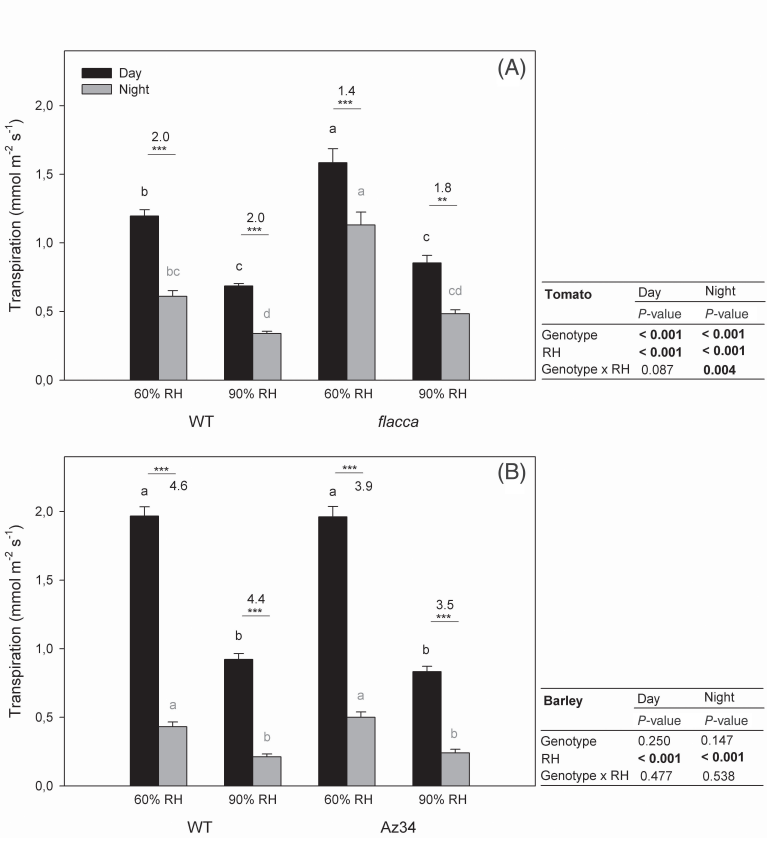


FIGURE 3 Leaf gas exchange of wild-type (WT) and abscisic acid-deficient tomato (A, C, E) and barley (B, D, F) genotypes grown in 60% or 90% RH in environmentally controlled growth chambers. Photosynthetic assimilation rate (A) plotted against stomatal conductance (A, B) (g_s) and internal CO_2 concentration (Ci); (C, D) along with g_s plotted against Ci (E, F). Pearson's correlation coefficient (r) and statistical significance of correlation indicated when significant. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

RGR_{SHOOT} of *flacca* was 15% less than WT tomatoes. The differences in tomato growth components were not significant in *flacca* compared to WT, but the components that contributed most to the change in RGR_{SHOOT} were LAR and SLA in 60% RH, and SLA in 90% RH (Table 3). Averaged across RH levels, RGR_{SHOOT} of Az34 was 20% less than WT barley, with both LAR and NAR significantly less than WT plants in 60% RH, by 40% and 21% respectively. Az34 had decreased SLA, though this was only significant in 90% RH (−24%). LMR showed a slight increase in Az34, though it was not significant in either RH level (Table 3).

High RH significantly increased *flacca* RGR_{SHOOT} by 8% but did not affect WT. It furthermore decreased LMR by 3.5%, averaged across tomato genotypes, though no other components significantly changed with RH (Table 3). High RH decreased NAR (by 14%) and increased SLA (by 40%) in WT barley, but no other growth components were significantly affected by RH in WT barley. Az34 RGR_{SHOOT} was not affected by high RH despite significantly increased SLA (24%), LAR (73%) and NAR (7%) (Table 3). Thus, the growth response to high RH is somewhat ABA-independent in tomatoes, but ABA-dependent in barley.

At both RH levels, *flacca* plants had fewer leaves than WT plants, thereby decreasing RLER and total leaf area of these plants (Table 4). A similar response occurred in barley (Table 4). High RH did not affect either tomato or barley leaf number or area, with no significant main effect or interaction between RH and ABA status. However, in tomatoes, the effect of high RH on RLER depended on ABA status, with 90% RH increasing RLER in *flacca*, but not WT tomatoes (Table 4). Thus, ABA status alters tomato leaf development by interacting with RH, but not in barley.



Tomato	Day	Night
	P-value	*P*-value
Genotype	**< 0.001**	**< 0.001**
RH	**< 0.001**	**< 0.001**
Genotype x RH	0.087	**0.004**

Barley	Day	Night
	P-value	*P*-value
Genotype	0.250	0.147
RH	**< 0.001**	**< 0.001**
Genotype x RH	0.477	0.538

FIGURE 4 Whole plant transpiration rates (mmol m^{-2} s^{-1}) of wild-type (WT) and abscisic acid-deficient tomato (A) and barley (B) genotypes grown in 60% or 90% RH in environmentally controlled growth chambers. The plants were measured over day (20 h) and night (4 h) periods. Means ± SE, $n = 4–5$. Different letters indicate significant differences between genotypes and RH levels for a given time of day, as determined by two-way ANOVA (insert) and post-hoc Tukey HSD analyses ($P < 0.05$). Black letters indicate day, grey letters indicate night. Horizontal brackets indicate significant differences between day and night transpiration for each genotype in each treatment. Statistical significance: *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Numbers above brackets indicate response index (day/night transpiration)

TABLE 3 Change (%) in relative shoot growth rate (RGR_{SHOOT}) and its components in wild-type (WT) and abscisic acid-deficient genotypes of tomato and barley genotypes grown in 60% or 90% RH in environmentally controlled growth chambers

	Tomato		Barley		Tomato		Barley	
	flacca vs. WT		Az34 vs. WT		90% vs. 60% RH		90% vs. 60% RH	
	60% RH	90% RH	60% RH	90% RH	WT	Flacca	WT	Az34
RGR_{SHOOT} (g g^{-1} day^{-1})	−17	−12	−22	−17	1	8	−4	2
NAR (mg cm^{-2} day^{-1})	−6	−8	−21	−2	1	−2	−14	7
LAR (cm^2 g^{-1})	−14	−4	−40	−2	−8	3	6	73
SLA (cm^{-2} g^{-1})	−16	−17	−14	−24	−2	−3	40	24
LMR (g g^{-1})	−2	−2	−3	−4	−4	−3	7	6

Note: Values are relative to WT plants and 60% RH, respectively. ■ indicates significant increase, ■ indicates significant decrease and ■ indicates no significant change as determined by two-way ANOVA and post-hoc Tukey analyses, where significance was assigned to $P < 0.05$.
Abbreviations: LAR, specific leaf area × leaf mass ratio; LMR, leaf mass ratio; NAR, net assimilation rate; SLA, specific leaf area.

4 | DISCUSSION

We hypothesised that high RH would promote growth and water status of ABA-deficient mutants of tomato and barley. This was confirmed for tomato, but not for barley (Figure 2, Table 3). Commercially growing tomatoes at high humidities (up to 90%) increased their biomass and yield (Lu et al., 2015; Shamshiri et al., 2018), but barley did not show the same response. While tomato growth responses to high RH were ABA-independent, they were ABA-dependent in barley (Table 3). Furthermore, tomato gas exchange responses to high RH were ABA-dependent,

TABLE 4 Morphological parameters measured in wild-type (WT) and abscisic acid-deficient genotypes of tomato and barley genotypes grown in 60 or 90% relative humidity (RH) in environmentally controlled growth chambers

Genotype	RH (%)	Number of leaves	Total leaf area (cm^2)	RLER ($g\ g^{-1}\ day^{-1}$)
Tomato				
WT	60	17.7 ± 1.1^a	875 ± 57^a	0.297 ± 0.003^a
	90	18.0 ± 0.7^a	906 ± 84^a	0.296 ± 0.002^a
flacca	60	12.0 ± 0.0^b	324 ± 19^b	0.237 ± 0.004^c
	90	14.5 ± 2.1^{ab}	388 ± 71^b	0.261 ± 0.005^b
P-values				
Genotype		**0.004**	**< 0.001**	**< 0.001**
RH		0.176	0.462	**0.003**
Genotype*R H		0.549	0.796	**0.002**
Barley				
WT	60	16.2 ± 2.5^a	185 ± 32^a	0.173 ± 0.004^a
	90	15.9 ± 2.2^a	194 ± 36^a	0.177 ± 0.004^a
Az34	60	13.1 ± 1.9^a	103 ± 12^a	0.130 ± 0.003^b
	90	11.0 ± 1.4^a	107 ± 10^a	0.129 ± 0.004^b
P-values				
Genotype		**< 0.001**	**< 0.001**	**< 0.001**
RH		0.246	0.626	0.783
Genotype*RH		0.397	0.819	0.414

Note: Means ± SE shown, as well as main effects (genotype, RH) and interaction effects from two-way ANOVAs. Different letters indicate significant differences between genotypes and RH levels for a given time of day, as determined by post-hoc Tukey HSD analyses ($P < 0.05$).
Abbreviation: RLER, relative leaf expansion rate.

while those of barley were not. Overall, despite similar lesions in the ABA biosynthetic pathway, ABA-deficient mutants of tomato and barley responded differently to their aerial environment, caused by differences in the relative magnitude of ABA deficiency and/or the specific enzymes impaired by mutations in the molybdenum cofactor.

As expected, *flacca* plants had 60%–70% less ABA than WT tomato (Tal & Nevo, 1973), while Az34 and WT barley plants had similar foliar ABA concentrations (Figure 1) (Walker-Simmons et al., 1989). In leaky mutants such as Az34 barley, end product quantification (here ABA) in plant tissues may not adequately indicate plant function (Walker-Simmons et al., 1989). Instead, xylem sap ABA concentration of Az34 was only half that of WT plants (Martin-Vertedor & Dodd, 2011), consistent with the decreased growth rate of Az34 compared to WT plants (Table 3).

4.1 | ABA deficiency affects tomato water relations independently of RH, but is RH-dependent in barley

Higher g_s of *flacca* was consistent with its larger, more abundant stomata independent of RH (Table 2). This agrees with similar results from guard cell-specific ABA-insensitive *Arabidopsis* mutants (Yaaran et al., 2019), suggesting that ABA status influences stomatal traits under differing RH levels. Nevertheless, high RH diminished genotypic differences in both stomatal size and g_s in tomatoes (Table 2, Table S2). In barley, Az34 had smaller, more abundant stomata than WT in 60% RH, but it had fewer, larger stomata than WT in 90% RH (Table 2). Despite these morphological differences, g_s of both genotypes was similar at either RH level, as previously found when these genotypes were grown under control and salt-stressed conditions (Zuo et al., 2019). Previous findings in *Arabidopsis* indicate that *aba3* mutants, which have a similar lesion to *flacca* and Az34 (see Table 1), had higher stomatal density than Col-0 WT plants (Jalakas et al., 2018). Thus, ABA deficiency influences stomatal traits in tomatoes, though whether these are direct (e.g. regulation of stomatal conductance) or indirect (e.g. an artefact of low leaf turgor constraining cellular expansion) consequences of ABA deficiency remains equivocal. Further analyses into the mechanisms involved in RH responses in WT and ABA-related (biosynthesis and receptor) mutants would help clarify this.

Changes in stomatal morphology in response to high RH affected leaf gas exchange responses in WT and *flacca* tomatoes (Table S2). While fewer, larger stomata decreased g_s and Ci of *flacca* plants at high RH, RH did not change g_s and Ci in WT tomatoes, again indicating stomatal responses of tomatoes to high RH are ABA-dependent. The stability of leaf ABA concentration in different RHs in WT tomato (Figure 1A) likely explains why RH did not change g_s and Ci. High RH decreased stomatal pore areas of both barley genotypes, to a greater extent in Az34 than WT (Table 2), indicating an ABA-dependent RH response. However, RH did not affect the stomatal number of either

barley genotype. The stomatal number varied between *Arabidopsis* WT and guard cell-specific ABA insensitive mutants when grown in 90% RH, where the mutants had fewer stomata while WT plant showed no difference in stomatal number (Yaaran et al., 2019). High (92%) RH decreased leaf ABA concentration in roses compared to moderate (61%) RH, thereby increasing stomatal aperture (Carvalho et al., 2015) with no effect on stomatal density. Taken together, ABA deficiency affects stomatal number responses similarly in tomato and *Arabidopsis* at high RH, but barley showed opposing effects of high RH on stomatal number in both genotypes.

Photosynthesis was not related to g_s across our range of conditions (Figure 3A,B), but stomatal closure at lower g_s would likely induce stomatal limitations to photosynthesis (Flexas et al., 2004). However, while neither species showed stomatal limitations to photosynthesis, non-stomatal factors such as lower foliar total soluble protein content and total Rubisco activity (Jauregui et al., 2018) likely limit photosynthetic assimilation in ABA-deficient barley plants (Jiang et al., 2006). However, the strong negative correlation between *A* and Ci in barley (Figure 3D) may result from NR deficiency, as opposed to ABA deficiency, in this genotype. An NR-deficient *Nicotiana plumbaginifolia* accumulated starch, which led to a disruption of the thylakoid structure, disorientation of grana and pigment deficiency, all of which decreased RuBP carboxylase activity and photosynthetic carbon assimilation rates (Saux et al., 1987). As NR-deficiency is an artefact of the molybdenum cofactor (MoCo) mutation in barley (Walker-Simmons et al., 1989), but not tomato (Sagi et al., 1999), this may explain interspecific differences in the *A*:Ci relationships (Figure 3D). In contrast, tomato photosynthesis responds little to changes in stomatal size and movement in response to ABA and humidity (Flexas et al., 2004), and neither ABA deficiency nor high RH limits photosynthesis (Long & Bernacchi, 2003).

The stomata of ABA-deficient mutants of both species closed in response to darkness (Figure 4), though the degree of closure (response index) was higher in WT than ABA-deficient mutants of both species (Figure 4). Consistent differences in response index also occurred when comparing WT and *flacca* tomatoes (Bradford et al., 1983; Neill & Horgan, 1985), yet *Arabidopsis* plants with guard cell-specific ABA-insensitivity showed a WT-like response to darkness (Yaaran et al., 2019). As darkness has been a constant, unchanging factor affecting gas exchange since plants colonised land, Costa et al. (2015) postulated that the dark response of stomata is a 'primitive regulatory backbone' upon which other mechanisms have evolved in order to respond to an increasing number of stimuli over time. While ABA signalling is required for stomatal responses to environmental stimuli such as elevated CO_2, O_3 and decreased RH (Chater et al., 2015; Merilo et al., 2013), it has been proposed that stomatal response to darkness may occur, at least partially, via an ABA-independent pathway (Costa et al., 2015; Merilo et al., 2013). Our results support this, though the greater response of WT plants than ABA-deficient mutants (Figure 4 response indices) indicates some involvement of ABA.

4.2 | Effects of ABA deficiency and RH on growth rate components is not conserved across species

Both *flacca* and Az34 had lower RGR_{SHOOT} than their respective WT plants, as reported previously for tomato (Coleman & Schneider, 1996; Nagel et al., 1994) and barley (Mulholland, Black, et al., 1996). However, the underlying components of RGR_{SHOOT} differed in their response between ABA-deficient genotypes, with NAR similar in *flacca* and WT tomatoes, but strongly reduced in Az34 (Table 3). NAR indicates the efficiency of leaves in generating biomass, and is related to photosynthesis as the basis of dry matter production in plants (Sudhakar et al., 2016). Here, photosynthesis strongly decreased in Az34 compared to WT barley, but was similar in both tomato genotypes (Table S2). NAR usually best predicts RGR (Li et al., 2016; Shipley, 2006), as in barley (Table 3), though SLA better predicted RGR in herbaceous plants experiencing low irradiance (Shipley, 2006). Low light levels may account for SLA being a stronger determinant of RGR_{SHOOT} than NAR in the tomatoes studied here.

Growing crops in high RH decreased transpiration and increased leaf water status, while also impairing stomatal functioning upon removal to a lower RH environment (Aliniaeifard & van Meeteren, 2013; Arve et al., 2013; Fanourakis et al., 2011; Fanourakis et al., 2016). ABA deficiency inhibits stomatal closure and alters stomatal anatomy, thereby increasing transpiration and decreasing leaf water status which in turn may inhibit cell expansion and decrease leaf growth (Bradford, 1983; Coleman & Schneider, 1996; Mäkelä et al., 2003; Nagel et al., 1994; Radin, 1983; Tal & Nevo, 1973). Here, high RH attenuated the negative effect of ABA deficiency on tomato RGR_{SHOOT} by improving leaf RWC (Figure 2, Table 3). In contrast, Az34 plants had lower RWC than WT barley in 90% RH, but not 60% RH (Figure 2), indicating alternative mechanisms of growth regulation than leaf water status. Furthermore, high RH attenuated the negative effect of ABA deficiency on tomato, but not barley RLER (Table 4). Previously, leaf growth inhibition of Az34 mutant was not attributed to compromised water relations when grown in compacted soil at high RH (Mulholland, Black, et al., 1996; Mulholland, Taylor, et al., 1996). Indeed, ABA deficiency is considered to inhibit shoot growth by non-hydraulic mechanisms (Bradford, 1983; Sharp et al., 2000) such as enhanced emission of the growth inhibitor ethylene (Sharp et al., 2000; Dodd et al. 2009), even if RH did not affect ethylene emission of *flacca* tomato (Arve & Torre, 2015). Furthermore, leaf water deficits induced by high transpiration rates may affect eudicots more severely than monocots, as monocot transpiration and leaf expansion zones are spatially separate (Radin, 1983).

Growth in high RH increased NAR, SLA, and thereby LAR of Az34 (Table 3), with almost complete phenotypic reversion of these growth components in Az34 (Figure S2). While high RH significantly increased SLA of WT barley, both LAR and RGR_{SHOOT} were unaffected. Thus, barley growth responses to high RH were ABA-dependent, with high RH allowing partial recovery from the negative effects of ABA deficiency via a non-hydraulic mechanism. Overall, the effects of ABA deficiency on tomato, but not barley growth seem partially dependent on leaf water status, while high RH effects on growth are ABA-independent in tomato, but ABA-dependent in barley.

While *flacca* and Az34 are both molybdenum cofactor mutants and have similar lesions in the ABA biosynthetic pathway, *flacca* plants retain NR activity (Sagi et al., 1999), yet this is impaired in Az34 barley (Walker-Simmons et al., 1989). Differences in ABA-dependent responses to RH may be related to NR activity, with NR activity playing a crucial role in stomatal movement in response to UV-B radiation downstream of ABA responsive genes (Tossi et al., 2014). This same pathway indicates the importance of NR in producing NO, which is critical to regulating stomatal movement (Cheeseman & Tankou, 2005; García-Mata & Lamattina, 2003). Furthermore, the *Arabidopsis aba3* MoCo mutants, which retain NR activity (Sagi et al., 1999), have a similar stomatal phenotype to *flacca*, with higher stomatal density than WT counterparts (Chater et al., 2015; Jalakas et al., 2018), which was not found in Az34 barley. *Arabidopsis* NR mutants (*nia1nia2*) are impaired in stomatal closure due to alterations in genes of ABA signalling components (Zhao et al., 2016), though they do not have a wilty phenotype and close their stomata in response to stimuli such as darkness and H_2O_2 (Desikan et al., 2002). Further investigation into the effects of NR impairment and its involvement in ABA responses to RH, for example by comparing responses of NR and NCED mutants, may help understand the differences between *flacca* and Az34 mutants.

5 | CONCLUSIONS

Although *flacca* tomato and Az34 barley both have molybdenum cofactor mutations and similar phenotypic responses to ABA deficiency and high RH, these species varied in the mechanisms underlying the responses. High RH alleviated the effects of ABA deficiency on tomato growth, likely by increasing leaf water status. However, growth responses to high RH varied with ABA status, indicating that high RH responses are ABA-independent in this species. High RH also alleviated the effects of ABA deficiency on barley growth, but independently of leaf water status. Furthermore, lower photosynthesis in ABA-deficient barley, likely related to lower Rubisco activity, did not occur in tomato. Comparing different species highlights that similar phenotypic responses to ABA deficiency do not necessarily indicate similar mechanisms, which may be important to crop improvement efforts within a changing climate.

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AUTHOR CONTRIBUTIONS

The authors have all contributed substantially to the underlying research and drafting of this manuscript, and declare no conflict of interest, financial or otherwise. Conceptualisation and planning were performed by Sheona N. Innes, Sissel Torre, Knut A. Solhaug and Ian C. Dodd. Data collection and analysis were performed by Sheona N. Innes and Sissel Torre, with input and advice from Ian C. Dodd and Knut A. Solhaug. Manuscript drafting was performed by Sheona N. Innes, Ian C. Dodd and Sissel Torre.

DATA AVAILABILITY STATEMENT

Data are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Supplementary Information

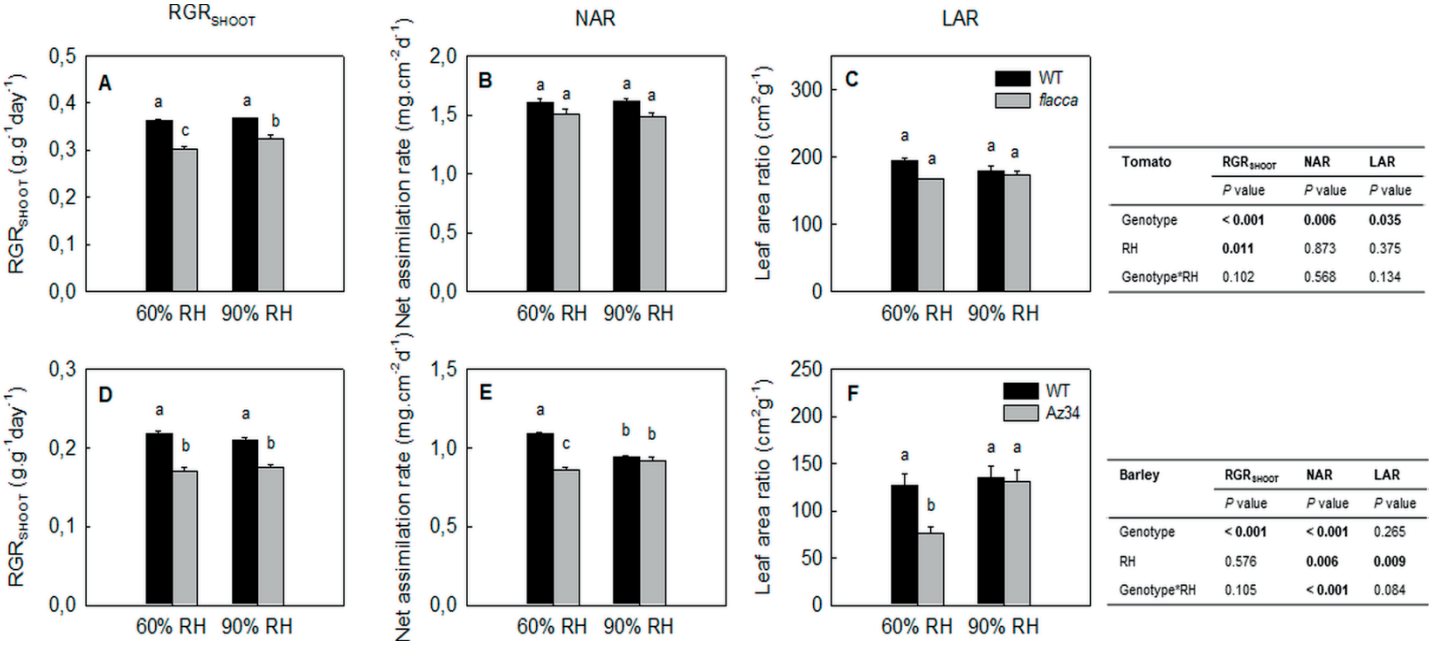
Appendix S1

Table S1. Overview of experimental data collection for tomato (t) and barley (b) genotypes during the two independent experiments. Double letters indicate data collected from two replicate measurements during the experiment. Data from such replicates were pooled prior to analysis.

	Experiment	Growth	Whole plant transpiration	Relative water content	Foliar gas exchange	Stomatal anatomy	ABA
1	Winter 2017/2018	tt bb					tt b
2	Summer 2019		t b	t b	t b	t b	

Table S2: Gas exchange parameters measured on tomato and barley wild-type and ABA-deficient plants. A: Photosynthesis; g_s: stomatal conductance; Ci: internal CO_2 concentration. Means ± SE shown, as well as main effects (genotype, RH) and interaction effects from two-way ANOVAs. Different letters indicate significant differences between means, as according to post-hoc Tukey HSD analyses.

Genotype	RH (%)	A (µmol CO_2 m^{-2} s^{-1})	g_s (mol H_2O m^{-2} s^{-1})	Ci (µmol CO_2 mol air^{-1})	iWUE (µmol CO_2 / µmol H_2O)
Tomato					
WT	60	7.71 ± 0.19^{b}	0.42 ± 0.04^{c}	336.5 ± 4.9^{c}	18.93 ± 1.92^{a}
	90	8.34 ± 0.42^{b}	0.42 ± 0.02^{c}	342.7 ± 3.2^{bc}	20.15 ± 1.56^{a}
flacca	60	7.47 ± 0.61^{b}	1.06 ± 0.09^{a}	370.9 ± 0.5^{a}	7.05 ± 0.18^{c}
	90	10.63 ± 0.15^{a}	0.85 ± 0.04^{b}	351.4 ± 1.5^{b}	12.71 ± 0.72^{b}
P values					
Genotype		**0.016**	**< 0.001**	**< 0.001**	**< 0.001**
RH		**< 0.001**	**0.055**	**0.039**	**0.015**
Genotype*RH		**0.004**	0.064	**< 0.001**	0.102
Barley					
WT	60	9.32 ± 0.40^{a}	0.45 ± 0.04^{a}	349.3 ± 5.2^{b}	21.70 ± 2.57^{b}
	90	10.94 ± 0.65^{a}	0.33 ± 0.05^{b}	314.7 ± 3.5^{c}	35.20 ± 2.76^{a}
Az34	60	4.22 ± 0.26^{c}	0.50 ± 0.01^{a}	376.6 ± 0.8^{a}	8.36 ± 0.37^{c}
	90	6.50 ± 0.51^{b}	0.30 ± 0.01^{b}	345.4 ± 3.8^{b}	21.87 ± 1.81^{b}
P values					
Genotype		**< 0.001**	0.686	**< 0.001**	**< 0.001**
RH		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Genotype*RH		0.500	0.178	0.647	0.999



Tomato	RGR$_{SHOOT}$	NAR	LAR
	P value	*P* value	*P* value
Genotype	**< 0.001**	**0.006**	**0.035**
RH	**0.011**	0.873	0.375
Genotype*RH	0.102	0.568	0.134

Barley	RGR$_{SHOOT}$	NAR	LAR
	P value	*P* value	*P* value
Genotype	**< 0.001**	**< 0.001**	0.265
RH	0.576	**0.006**	**0.009**
Genotype*RH	0.105	**< 0.001**	0.084

Figure S1. Shoot relative growth rate (RGR$_{SHOOT}$), net assimilation rate (NAR) and leaf area ratio LAR) of tomato (A-C) and barley (D-F) WT and ABA-deficient mutants. After germinating in a greenhouse, plants were grown at 60% or 90% RH from the 2-leaf stage, with measurements at the start, 7 and 14 days after the start of the growth treatments. Means ± SE shown. Different letters indicate significant differences between means, as according to two-way ANOVA and post-hoc Tukey HSD analyses.

Paper III

Foliar ABA content mediates transcriptomic responses to evaporative demand solely by altering leaf water status of tomato (*Solanum lycopersicum*)

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Abstract

The role of ABA in plant responses to increasing vapour pressure deficit (VPD) have been thoroughly explored, yet plant responses to low evaporative demand are arguably as important. Thus we examined foliar ABA concentration, water relations and differential gene expression in wild-type (WT) and ABA-deficient *flacca* (*flc*) tomatoes to long-term (growth) and short-term (24 h reciprocal transfer) exposure to 1.06 (high) and 0.26 (low) kPa VPD. Although leaf relative water content (RWC) remained constant, [ABA], stomatal aperture and transpiration of WT were affected by changes in VPD. As expected, VPD did not affect *flc* [ABA], yet affected stomatal aperture, transpiration and RWC. This co-occurred with regulation of thousands of genes, an order of magnitude higher than WT, in response to both steady-state and changing VPD, indicating that differential gene expression in *flc* in response to VPD is dependent on water status, rather than [ABA]. Although high VPD caused high rates of transpiration and turgor loss of *flc*, it caused downregulation of genes involved in osmotic adjustment compared to the WT. Interestingly, high VPD upregulated pathogen defence genes of *flc* in comparison to WT, most notably ethylene (ET) synthesis, possibly indicating increased defence against pathogen infection. Contrastingly, these genes were downregulated in *flc* exposed to low VPD in an opposite pattern to WT plants, indicating that high RH may perturb ABA-ET relations.

Introduction

One of the strongest drivers of plant transpiration rate is atmospheric evaporative demand, which is determined by relative air humidity (RH) and temperature. In natural growing conditions, these variables fluctuate throughout the day (Assmann et al. 2000), with decreased transpiration rates associated with elevated humidity, The vapour pressure difference (VPD) between the surrounding air and the leaf provides the driving force for water movement (Bakker 1991), with actual transpiration rates modulated by stomatal conductance, as a function of stomatal density, size and pore aperture (Fanourakis et al. 2020).

Growth in high RH negatively affects the morphology and functioning of stomata, resulting in larger, more abundant stomata that either fail to respond or respond sluggishly or to a lesser degree to closing stimuli such as darkness and desiccation (Arve et al. 2013; Fanourakis et al. 2011; Mortensen 2000; Rezaei Nejad & Van Meeteren 2005; Torre & Fjeld 2001; Torre et al. 2003). The mechanisms of this reduced responsiveness remain equivocal, though combative measures have been assessed and reviewed (Fanourakis et al. 2016). Micropropagated rose plants showed no difference in the stomatal ultrastructure between *in vitro* and greenhouse grown plants, but had decreased physiological function and water movement (Sallanon et al. 1993). Fanourakis et al. (2020) attribute stomatal (mal)functioning in plants grown in low VPD to decreased endogenous abscisic acid concentrations ([ABA]), related to abnormally high water potentials found under conditions of low evaporative demand. Indeed, while high VPD induced increased expression of the gene encoding the carotenoid cleavage

enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED), which is also the rate-limiting step in the ABA biosynthetic pathway (McAdam et al. 2016); several species have shown decreased [ABA] during growth in low VPD (Arve et al. 2013; Giday et al. 2013; Giday et al. 2014; Rezaei Nejad & van Meeteren 2006). Endogenous [ABA] is regulated by a balance between biosynthesis and catabolism/conjugation (Nambara & Marion-Poll 2005). In *Arabidopsis*, short-term growth in high RH increased expression of CYP707A genes, which catabolise ABA to phaseic acid (Okamoto et al. 2009), and decreased activity of β-glucosidase in roses, which releases ABA from its conjugated form, ABA glucosyl ester (ABA-GE) (Arve et al. 2013). Both ABA catabolism and conjugation result in less available ABA for inducing stomatal closure, and lower content of ABA may contribute to the less responsive stomata found during growth in low VPD. Furthermore, ABA perception by PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) is crucial for basal ABA signalling, and sucrose non-fermenting 1-related subfamily2 (SnRK2) protein kinases, specifically SnRK2.2/D, SnRK2.3/I and SnRK2.6/OST1/SRK2E, form a bottleneck of ABA signalling that is required for the regulation of steady state transpiration (Gonzalez-Guzman et al. 2012). This highlights that both the availability of ABA and the plant sensitivity to it are important for ABA-induced responses to environmental conditions. Indeed, OST1 has been shown to be more important in VPD-induced stomatal responses than [ABA] (Merilo et al. 2018), highlighting the importance of intact signalling pathways.

One of the main differences between WT and ABA-deficient mutants is that the latter have constitutively higher transpiration rates (Bradford 1983; Tal

1966), and in general the genetic control of transpiration is highly attributed to ABA and ABA-activated genes and signalling cascades (Nilson & Assmann 2007). However, basal lineages of plants such as lycophytes, ferns and conifers utilise ABA-independent mechanisms of controlling water loss (McAdam & Brodribb 2015), indicating that ABA is not the sole controlling factor in transpirational water loss. In bryophytes, lycophytes and ferns, stomatal regulation is primarily based on leaf hydraulics (Sun et al. 2020), with species-dependent effects of ABA. For instance, the stomata of several fern and lycophyte species were ABA insensitive (Brodribb & McAdam 2011; McAdam & Brodribb 2012; Soni et al. 2012), while two fern species showed weak ABA responses, albeit dependent on humidity conditions during growth (Hõrak et al. 2017). Recently, Merilo et al. (2018) found that the stomatal VPD response of ABA-deficient *Arabidopsis* mutants is hydraulically regulated and dependent on the stomatal conductance prior to VPD treatments. They showed that the ABA-deficient mutants closed their stomata in response to increased VPD, in some cases more intensely than WT plants. This indicates that plants with diminished ability to synthesise ABA may revert to primitive signalling systems to regulate water loss.

High RH-induced stomatal opening increases susceptibility to pathogen infection via the stomatal pores (Panchal et al. 2016). The so-called stress response hormones – ABA, ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) – play diverse roles in defence against both biotic and abiotic stresses (Blanco-Ulate et al. 2013). Environmental conditions determine interactions between these phytohormones, thereby affecting all aspects of plant growth, development and environmental responses. ET and JA function together in pathogen defence responses, but SA is antagonistic to

JA. ABA is involved in abiotic stress response, yet suppresses pathogen defences through antagonising ET/JA and SA immune responses to pathogen attack (Blanco-Ulate et al. 2013). ABA-deficiency stimulates ET evolution rates (Arve & Torre 2015; Dodd et al. 2009; LeNoble et al. 2004; Sharp & LeNoble 2002), thereby inducing the ET-JA system for pathogen defence (Blanco-Ulate et al. 2013). Moreover, tomato grown in high RH (low VPD) has higher rates of ET evolution, associated with high RH-induced stomatal opening (Arve & Torre 2015), though high RH dampens ABA-deficiency-induced ET induction in *flc* (Arve & Torre 2015; Tal et al. 1979). Thus, ABA-deficient *flc* plants may be less susceptible to biotic stress than WT plants as a result of increased ET evolution, yet this may be dampened with growth in high RH. The relationship between ABA and ET is highly complex, and antagonism between the two hormones seemingly depends on their endogenous concentrations (Müller 2021). A low concentration of one will activate biosynthesis of the other, despite mutual antagonism when concentrations of both are high (Müller 2021). Thus, the ABA-ET relationship and the effects of VPD thereupon may influence plant responses in unexpected ways. Mitogen activated protein kinases (MAPKs) are signalling molecules involved in almost all aspects of plant signal transduction in response to both abiotic and biotic stress (Danquah et al. 2014), and their role in ABA signalling has been well studied (Danquah et al. 2014; Takahashi et al. 2020).

Specific, independent MAPK pathways are involved in pathogen response and ABA signalling, and both pathways induce stomatal closure via activation of SLOW ANION CHANNEL-ASSOCIATED1/HOMOLOGUES (SLAC1/SLAH) anion efflux channels

(Danquah et al. 2014; Montillet et al. 2013). Given their differences in both ABA biosynthesis and biotic stress response, the involvement of specific MAPK pathways may indicate different mechanisms used in VPD response between WT and ABA-deficient *flc*.

Tomato responses to ABA-deficiency have been thoroughly investigated from genetic to whole-plant levels at variable RHs (Dodd et al. 2009; Sharp et al. 2000; Tal 1966). Tomato is both a commercially important crop species worldwide and a model species for genetic and -omics based studies due to its sequenced genome and availability of mutants. Previous studies indicate that long-term growth of tomato plants at different VPDs does not result in different foliar [ABA] (Arve & Torre 2015; Innes et al. 2021), yet the plants still show phenotypes typical of low VPD, including fewer, larger, more open stomata (Innes et al. 2021), decreased stomatal response to darkness and higher rates of detached leaf water loss (Arve & Torre 2015). Despite no change in [ABA] in WT or *flc* in low VPD, the hydraulic responses to low VPD were ABA-dependent, given the differences in response between WT and *flc* (Innes et al. 2021), possibly due to the interplay between ABA and ET (Arve & Torre 2015). The present study investigated the genetic control of water relations and stomatal responses in WT and ABA-deficient *flc* plants to determine which genes or biological pathways are up- and down-regulated in response to both increased and decreased VPD. We determined whether steady-state (long-term) responses to growth in different VPD levels differed from short-term responses to a change in VPD over 24 h. Thus, we tested the separate and combined effects of long-term growth in high or low VPD using a reciprocal transfer of plants between VPD levels for 24 h. We hypothesised that *flc* plants would employ

different genetic strategies for controlling water loss in response to changing evaporative demand. To the best of our knowledge, transcriptomic effects of long- and short- term VPD exposure and the role of ABA in this response has not been performed.

Materials and Methods

Plant Material and growth conditions

Tomato (*Solanum lycopersicum* Mill. cv. 'Ailsa Craig') wild-type (WT) and ABA-deficient mutant *flacca* (*flc*) genotypes were used in experiments performed at the Norwegian University of Life Sciences (NMBU), Ås, Norway in winter 2019, and replicated at the Lancaster Environment Centre at Lancaster University (LU), UK during spring 2019.

Experiments at NMBU

Plant growth and RH treatments at NMBU were performed as described in a previous paper (Innes et al. 2021). Briefly, seeds were germinated in Sphagnum peat growth medium, 6% ash, pH 5.0–6.0 (Degernes Torvstrøfabrikk AS, Degernes, Norway) in 17 cm, 2 L pots. The plants were grown in a single greenhouse compartment at a constant 20±1°C and 70±5% RH controlled by a PRIVA system (Priva, De Lier, The Netherlands). As Ås lies at 59.7°N, the natural daylight during the experiments ranged from 6-10 h (timeanddate.com 2018), so 100 μmol m^{-2} s^{-1} of supplementary light was supplied by high pressure sodium lamps (HPS, Osram NAVT- 400W, Munich, Germany) to extend the photoperiod to 20 h. The plants were watered daily to drip point and were kept in the greenhouse for 14 days.

The plants were then moved to controlled environment growth chambers for growth treatments. Four growth chambers were used. All of the chambers were maintained at 22 ± 1°C using a PRIVA system. Two of the chambers were maintained at moderate (60%) RH, while the other two had

high (90%) RH, corresponding to VPDs of 1.06 (high VPD) and 0.26 kPa (low VPD) respectively. The plants were exposed to a 20 h photoperiod, wherein light was supplied at 220 ± 20 μmol m^{-2} s^{-1} by Powerstar HQI-BT metal halide lamps (Ledvance GmbH, Munich, Germany) as measured using a Li-Cor quantum sensor connected to a Li-Cor LI-250 light meter (Li-Cor Inc., Lincoln, NE, USA). The plants were watered daily using a 50/50 mixture of YaraLiva® Calcinit™ calcium nitrate solution (14.4% NO3, 1.1% NH4, 19.0% Ca, Yara Norge AS, Oslo, Norway) and Kristalon™ Indigo (7.5% NO3, 1% NH4, 4.9% P, 24.7% K, 4.2% Mg, 5.7% S, 0.027% B, 0.004% Cu, 0.06% Mn, 0.2% Fe, 0.004% Mo, 0.027% Zn, Yara Norge AS, Oslo, Norway), EC level 2.0 mS cm^{-1}.

Experiments at LU

Seeds were germinated in seed trays (single seed in each well, ca. 3 cm deep) in peat-based growth medium (Levingtons M3, Levington Horticulture Ltd., Ipswich, UK) and placed in a walk-in controlled environment room with a constant temperature of 22±1°C and 20 h photoperiod. Relative humidity was 50±5 %, corresponding to a vapour pressure deficit of 1.3 kPa. The light intensity at bench height was 180±10 μmol m^{-2} s^{-1}, provided by metal halide lamps (HQI-T 400N, Osram, St Helens, UK). The plants were kept in these conditions for two weeks before being re-potted into 15 cm, 2 L pots and transferred to controlled environment growth chambers (Snijders Scientific Microclima 1750, Snijders Labs, Tilburg, The Netherlands). The chamber settings were maintained as described for NMBU, with one 60% RH and one 90% RH chamber. The chambers were kept at 22±1°C and light was provided by

daylight and red-biased fluorescent tubes (5x 58W colour 2023, 14x 58W colour 2084, 4x18W colour 840) at 200 µmol m^{-2} s^{-1}. The plants were watered daily for two weeks before measurement commenced.

Reciprocal transfer

Reciprocal transfers were performed at both NMBU and LU, and sampling and measurements were carried out in the same way at both institutes unless specified. For reciprocal transfer, plants grown in high VPD remained there until measurement (HH), while half were transferred to low VPD (HL). Similarly, half of the plants grown in low VPD remained there (LL) and half were transferred to high VPD (LH). The plants were grown in their growth environment for 14 days before transfer, which took place 1 h after the start of the light period. Measurements and sampling were then carried out on all plants (both transferred and not transferred, 24 h later.

Foliar ABA radioimmunoassay

Young, fully expanded leaflets (6^{th} or 7^{th} leaf from the base) from 3-5 plants per genotype per treatment were removed and immediately placed in tubes and frozen in liquid N_2. One hour after the start of the light period, samples were taken 15 min, 1 h, 4 h and 24 h after reciprocal transfer from control plants of each genotype from each VPD chamber, and from reciprocally transferred plants. Samples were freeze-dried using a Telstar LyoQuest (Telstar, Terrassa, Spain). Freeze-dried tissue was ground to powder and extracted in distilled de-ionized water on a shaker at 4°C overnight. The extracted aqueous solutions were measured for ABA concentration using the monoclonal antibody AFRC MAC 252 in a radioimmunoassay as described by Quarrie et al. (1988).

Day/Night transpiration of whole plants

Whole plant transpiration was determined gravimetrically over the course of three days and three nights, during which reciprocal transfer took place after two days. Pots were sealed with plastic to prevent evaporation from the soil and plants were weighed at the start and end of the light period, as well as before and after daily watering which took place at the end of the dark period. Watering compensated for evapotranspirational losses. Upon harvest, which occurred 1 h after the start of the light period, 24 h after reciprocal transfer, leaf area was measured using an LI-3100 Leaf Area Meter (Li-Cor, Inc., Lincoln, NE, USA), and used to determine total water use (g cm^{-2} h^{-1}) for each day and night. This was converted to rate of water loss (mmol H_2O m^{-2} s^{-1}) using mol = g/molar mass, where the molar mass of water is 18.01528.

Leaf relative water content

Young, fully expanded leaflets (from the 6^{th} or 7^{th} leaf from the base of the plant, opposite the leaflets detached for ABA immunoassay) were detached from five plants per genotype per treatment, 24 h after reciprocal transfer, which took place 1 h after the start of the light period, for determination of relative water content (RWC). Leaf petioles were cut under water after leaf detachment, before being immediately weighed (FW) and placed in water in a sealed container in the dark for at least 1 h to determine turgid weight (TW), before being placed in a drying cabinet at 60°C for at least 24 h before dry weight (DW) was determined. RWC was determined as (FW-DW)/(TW-DW)*100.

Gas Exchange

Whole plant gas exchange measurements at LU were made using a Li-Cor 6400 Portable Photosynthesis System connected to an external chamber as described in Jauregui et al. (2018). Whole plant measurements were made over two consecutive days, where 4 plants per genotype per treatment were measured per day. Plants were removed from their growth chamber beginning 1 h after the start of the light period and immediately placed into the gas exchange chamber, which had been prepared at the same temperature, light intensity and humidity as the growth chamber (high or low VPD). The plant was acclimatised to the chamber for 5 mins to stabilise gas exchange before the VPD was changed from high to low or low to high, depending on the growth humidity of the plant. Photosynthesis (A) and transpiration (Tr) measurements were made every 30 s for 1 h at the new VPD. Results were checked for differences resulting from measurement time of day and none were found.

SUMP stomatal imprints

Suzuki's Universal Micro-Printing Method (SUMP Laboratory, Tokyo, Japan) was used to take leaf impressions (approx. 0.5 cm^2) for measurement of stomatal morphology. Young, fully expanded leaflets (from the 6^{th} or 7^{th} leaf from the base of the plant, the same leaf used for ABA and RWC measurements) were used. Four leaflets per genotype per treatment were used. Cellulose plates were covered in a thin layer of SUMP liquid, pressed onto the abaxial sides of the leaves and left to dry for approximately five minutes. Once dry, the cellulose plates were carefully detached from the leaves and attached to slides using double sided tape. The imprints were examined, and five micrographs per imprint (total 20 micrographs per

genotype per treatment) were taken using a Leica DM 5000 B light microscope connected to a CTR 5000 electronics box. This was attached to a Leica DFC 425 digital microscope camera with a Leica 10445929 0.5x video objective (all Leica Microsystems GmbH., Wetzlar, Germany). Stomatal counts and pore area measurements were carried out using ImageJ software (ImageJ 1.49g, National Institutes of Health, USA). All imprints were taken 1 h after the start of the light period.

Statistical analysis

Data were analysed for main effects of, and interaction effects between, ABA and RH (with transfer) using two-way ANOVAs, and significant differences between means were tested using post-hoc Tukey HSD analyses. All data were checked for normality using normal-quantile plots and Shapiro-Wilk Normality Tests, and for homoscedasticity using residuals vs. fitted plots. All data were analysed using R (version 4.0.3, The R Foundation for Statistical Computing).

RNA extraction and sequencing

One day (24 h) after the reciprocal transfers and 1 h after the start of the light period, samples were taken from control plants of both genotypes from high and low VPD, as well as transfer plants (high to low VPD, and low to high VPD). Three young, fully developed, but not yet fully expanded leaflets (6^{th} or 7^{th} leaf from the base) were taken from each genotype from each treatment, immediately placed in 50 ml tubes and transferred to liquid nitrogen, then stored at -80°C until analysis. Leaf samples were crushed using autoclaved (121°C, sterile time 20 min) mortar and pestle with liquid nitrogen Total RNA was extracted from the crushed leaf matter using the

RNeasy Plant Mini Kit (Qiagen GmbH, Germany). DNA was removed from the extracted RNA using an Ambion TURBO DNA-*free* Kit and RNA was purified using an Ambion PureLink RNA Mini Kit (both Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA quantities were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and RNA quality was evaluated using an Agilent 2100 Bioanalyzer with an RNA 144000 NanoKit (Agilent Technologies, Palo Alto, CA, USA).

The transcriptomes of the leaf material were sequenced using a BGISEQ-500 platform at BGI Tech Solutions (Hong Kong) CO., Ltd. (Tai PO, N. T., Hong Kong). Oligo (dT)-attached magnetic beads were used to purify mRNA, which was fragmented using fragment buffer. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand synthesis. A-Tailing mix and RNA Index Adapters were then added by incubating to end repair. The cDNA fragments were amplified by PCR and purified using Ampure XP Beads dissolved in EB solution. Double stranded PCR products were denatured by the splint oligo sequence to get single strand circle DNA (ssCir DNA), which was formatted as the final library. This was amplified with phi29 to make DNA nanoballs, which were loaded onto the patterned nanoarray and paired-end 100 base reads were generated on the BGISEQ-500 platform.

Differential gene expression analysis

Reads were mapped to the *S. lycopersicum* genome (version 3.0; Sato et al. (2012)) using the BWA mapper (version 0.7.17; Li and Durbin (2009)). Transcripts were quantified using RSEM (version 1.3.2; Li and Dewey

(2011)) and differentially expressed genes (DEGs) were identified using the R package SARTOOLS (Varet et al. 2016) as a wrapper for DESeq2 (version 1.26.0; Love et al. (2014)). Transfer samples HL and LH were compared to control (growth RH) samples for both genotypes, i.e. HL to HH and LL to LH, to determine how changes in humidity altered gene expression. A false discovery rate (FDR) and adjusted p-value of < 0.05 was used to classify DEGs.

Gene ontology (GO) enrichment analysis and classification (KEGG) of DEGs

GO enrichment analysis and enrichment of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways was performed using enrichment software ShinyGO (v0.61 http://bioinformatics.sdstate.edu/go/). Enrichment lists, trees and network diagrams detailing the biological processes, cellular components and molecular functions of DEGs, as well as the enriched KEGG pathways, were obtained for all up- and down-regulated genes from each transfer treatment in both genotypes.

Results

VPD effects on ABA concentration

Foliar ABA concentrations of *flc* plants averaged 75% less than WT plants when grown at high and low VPD, respectively at NMBU (Fig. 1, $P < 0.001$), and similar results (79% less) were found at Lancaster. When measured 24 h after transfer, ABA concentrations at low VPD were independent of initial growth RH in both genotypes. Compared to plants maintained at low VPD, LH treatment increased WT ABA by 34%, but had no significant effect on *flc* ABA (Fig. 1, RH effect: $P < 0.001$, Genotype*RH effect: $P = 0.035$). Thus steady-state ABA levels did not differ between plants grown at different VPD levels, but transferring plants from low to high VPD for 24 h increased their ABA levels.

Leaf relative water content, transpiration and stomatal response to changing VPD

Growing plants at high VPD resulted in *flc* plants with18% lower RWC than WT plants, but this genotypic difference was abolished when plants were grown at low VPD, where *flc* RWC increased (Fig. 2, Genotype*RH effect: $P < 0.001$). Transferring plants from high to low VPD did not affect RWC of WT plants, but *flc* RWC increased by 12%. Similarly, the LH treatment did not affect RWC of WT plants, but decreased *flc* RWC by 9% (Fig. 2). Thus, *flc* RWC was more responsive to changes in VPD.

Transpiration rate of *flc* plants was 1.4-fold higher than WT plants at high VPD, but there were no genotypic differences at low VPD (Fig. 3A, Genotype*RH effect: $P < 0.001$), which decreased WT and *flc* transpiration

by 58 and 76% respectively. Transferring plants from high to low VPD decreased *flc* transpiration 3.2-fold, but did not affect WT. However, the LH treatment increased WT and *flc* transpiration 2.6-fold and 3.8-fold, respectively (Fig. 3A). The responses were the same for night-time transpiration (data not shown). Thus, *flc* transpiration was more strongly affected by changes in VPD.

Stomatal length/width (L/W) ratio of *flc* leaves was 18 and 6% less than WT stomata in high and low VPD, respectively (Fig. 3B, Genotype effect: $P < 0.001$), indicating greater stomatal opening in *flc*. Growth in low VPD decreased L/W ratio in WT leaves by 6%, indicating stomatal opening, but increased L/W ratio in *flc* leaves by 8%, indicating stomatal closure (Fig. 3B, Genotype*RH effect: $P < 0.001$). Transferring plants from high to low VPD did not affect L/W ratio of WT leaves, but increased L/W ratio of *flc* leaves by 8%, indicating stomatal closure (Fig. 3B). Transferring plants from low to high VPD increased L/W ratio by 22 and 11% in WT and *flc* respectively, indicating stomatal closure (Fig. 3B, RH effect: $P < 0.001$). Thus, stomatal aperture responses to VPD varied with foliar ABA status.

Whole plant transpiration rate was measured for 1 h (Fig. 3B-E), comprising exposure to growth VPD for 5 min, before the VPD was changed from either low to high (LH, Fig. 3B) or high to low (HL, Fig. 3C). Transpiration of *flc* was consistently higher than WT transpiration when the transpiration rates were stable at the start and end of the measurements (graph inserts, Genotype effect: $P < 0.001$, RH effect: $P < 0.001$, Genotype*RH effect: $P < 0.001$ for both start and finish measurements). Transpiration was more variable when plants were exposed to increased VPD than decreased VPD.

Increasing VPD enhanced transpiration of both WT and *flc* plants, though *flc* transpiration plateaued between 10 and 20 mins after VPD change, before continuing to increase, while this was not the case for WT (Fig. 3B). Transpiration response to increasing VPD was delayed in both genotypes (Fig. 3B), and while *flc* response started sooner than WT, the time to maximum transpiration (slope of the graph between minimum and maximum transpiration) was longer than WT, indicating a slower total response. Both WT and *flc* responded immediately to decreased VPD (Fig. 3C).

WT had an average of 75% higher foliar ABA concentration than *flc* plants across all the treatments (Fig. 3D-E, Genotype effect: $P < 0.001$). LH transfer increased WT ABA concentration by 24%, but did not affect *flc* ABA (Fig. 3D, Genotype*RH effect: $P = 0.03$). HL transfer did not affect WT or *flc* ABA concentration (Fig. 3E).

Differential gene expression in WT and ABA-deficient mutants in response to VPD

At high VPD, ABA-deficiency resulted in differential expression of 8027 genes in *flc* compared to WT (Fig. 4A). However, in low VPD there were no differences in gene expression between the two genotypes (Fig. 4A). In *flc*, growth in low VPD induced differential expression of 6743 genes compared to high VPD (Fig. 4B), while WT only showed differential expression of 695 genes when grown in low compared to high VPD (Fig. 4B). Of the 6743 and 695 DEGs in *flc* and WT, respectively, 497 genes were differentially expressed in both genotypes (Fig. 4D).

A 31-38-fold difference in the number of DEGs from each transfer treatment was found between WT and *flc* plants (Fig. 4C). In WT plants, 155 and 226 genes were differentially expressed in LH and HL transfer treatments, respectively, and of these, 27 were differentially expressed in both treatments (Fig. 4E). In *flc* plants, 4763 and 8579 genes were differentially expressed in LH and HL transfer treatments, respectively, and 4177 were differentially expressed in both treatments (Fig. 4E). In the HL treatment, WT and *flc* had 131 DEGs that were common to both genotypes, while in the LH treatment only 68 were differentially expressed in both genotypes (Fig. 4E). Only 11 genes were differentially expressed in both genotypes in both transfer treatments (Fig. 4E).

KEGG pathways affected by differential gene expression

KEGG pathway analysis revealed a total of 49 biological pathways were affected by DEGs in *flc* compared to WT (Table 1). Interestingly, these differences were only found in high VPD, as no significant DEGs were found between WT and *flc* plants in low VPD.

In addition, 48 biological pathways were affected by growth in high RH in WT and *flc* plants (Table 1). Of these, 7 pathways were affected in WT, while 46 were affected in *flc*. The pathways affected in WT plants were related to carbohydrate metabolism, biosynthesis of secondary metabolites and signal transduction, while those affected in *flc* spanned all aspects of metabolism except biosynthesis of secondary metabolites, as well as genetic information processing, signal transduction, cellular processes related to transport and catabolism, and environmental adaptation related to plant-pathogen interaction (Table 1).

Furthermore, 55 biological pathways were affected by reciprocal transfer (Table 2). Of this, 9 pathways were affected in WT, while 54 were affected in *flc*. The pathways affected in WT responses to VPD change were related to metabolism (amino acid metabolism and biosynthesis of secondary metabolites) and environmental information processing in the form of signal transduction. The pathways affected by VPD change in *flc* were related to metabolism, genetic and environmental information processing, cellular processing in the form of transport and catabolism, and organismal systems related to environmental adaptation (Table 2).

Interestingly, all of the shared pathways affected by ABA-deficiency and growth in low VPD in *flc* showed opposite regulation, i.e. those that were upregulated in *flc* compared to WT were downregulated in *flc* grown in low compared to high VPD (Table 1). Furthermore, almost all of the pathways that were downregulated in *flc* compared to WT in high VPD (Table 1) were found to be upregulated in response to decreased VPD (Table 2) and *vice versa*, indicating the effects of ABA-deficiency were attenuated in low VPD. Several pathways were involved in steady state, but not short-term ABA and VPD responses (Table 1, bold italic), while others were solely involved in short-term responses to changing VPD, but not steady state responses to ABA or VPD (Table 2, bold italic).

Hormonal gene expression analyses

Effects of ABA-deficiency on hormone-related gene expression due to growth in high VPD

Gene expression tables indicating hormone biosynthesis (Tables 3 and 4) and response (Tables 5 and 6) genes in the different treatments agree with the KEGG pathway analyses, indicating more *flc* than WT hormone biosynthesis and response genes were differentially expressed in both steady-state and reciprocal transfer treatments. In *flc* relative to WT grown in high VPD, ABA biosynthesis genes were mostly upregulated (Table 3). Additionally, 8'-hydroxylase genes (cytochrome P450 CYP707A1, LOC101267805 and LOC101266767) were both up- and down-regulated, with stronger downregulation, indicating decreased ABA catabolism from ABA to phaseic acid (PA) (Table 3); ABA conjugation to ABA-glucose ester (ABA-GE) by ABA uridine diphosphate glucosyltransferase (SlUGT75Cl) and deconjugation by beta-glucosidase 18 (LOC104645804 and LOC104645805) were mostly upregulated (Table 3), indicating both increased conjugation and de-conjugation to the active form of ABA; and ABA signalling and response genes were generally upregulated (Table 5). The same treatment caused upregulation of ET biosynthesis and response genes (Tables 3 and 5). Genes involved in early JA biosynthesis were downregulated in *flc* relative to WT grown in high VPD, while later biosynthesis genes were upregulated (Table 3) and JA response genes were mostly upregulated (Table 5). SA biosynthesis and response genes were both up- and down-regulated in *flc* relative to WT plants grown in high VPD (Table 3 and 5).

Differences in hormone-related gene expression due to growth in low VPD

One beta-glucosidase 18 gene (LOC104645804) was upregulated in WT grown in low vs high VPD, while no other ABA biosynthesis genes were affected (Table 3). In *flc* plants grown in low VPD, one ABA DEFICIENT 4 (ABA4) gene (LOC101268051) was upregulated, but other ABA biosynthesis and catabolism genes were downregulated (Table 3). Both WT and *flc* grown in low VPD showed general upregulation of ABA response genes relative to plants grown in high VPD (Table 5). WT plants showed upregulation of ET biosynthesis genes, while *flc* showed both up- and down-regulation in low compared to high VPD (Table 3). Few ET response genes were differentially expressed in WT in low compared to high VPD, yet those that were affected were mostly upregulated, while in *flc* plants ET response genes were mostly downregulated (Table 5). Only one lipoxygenase (LOX1.1), involved in early JA biosynthesis, was downregulated in WT plants grown in low relative to high VPD (Table 3), and a single WRKY transcription factor 1 (LOC100301944) was upregulated (Table 5). Growing *flc* plants at low VPD caused downregulation of early JA biosynthesis genes and upregulation of JA biosynthesis genes further downstream compared to high VPD (Table 3), yet strong upregulation of JA response genes in low relative to high VPD (Table 5). Growing WT plants at low VPD caused upregulation of two SA response genes, despite no response in biosynthesis (Tables 5 and 3, respectively), while in *flc* SA biosynthesis genes were both up- and down-regulated (Table 3) and SA response genes were mostly downregulated (Table 5).

Changes in hormonal gene expression in response to 24 h of decreased VPD

Decreasing VPD did not significantly affect ABA biosynthesis genes of WT plants (Table 4), but caused up- and down-regulation of various genes in *flc* (Table 4). Additionally, in *flc*, an ABA 8'-hydroxylase (LOC101249565) was strongly upregulated and a beta-glucosidase 18 (LOC101257526) was downregulated, indicating increased ABA catabolism to PA and decreased de-conjugation from ABA-GE in response to decreased VPD. This also resulted in both up- and down-regulation of ABA response genes in both WT and *flc* plants (Table 6). While decreased VPD did not significantly affect ET biosynthesis genes of WT plants, two ET response genes were upregulated (Table 6). In *flc* plants, decreased VPD mostly caused downregulation of ET biosynthesis genes (Table 4), with up- and down-regulation of various ET response genes. This treatment also induced upregulation of most JA biosynthesis genes in *flc,* yet only upregulation of a single lipoxygenase (TomloxC) gene in WT (Table 4). Decreased VPD caused both up- and down-regulation of JA response genes of *flc* (Table 6), yet this had no effect on JA response genes in WT. Only *flc* showed differential expression of SA biosynthesis genes in response to both transfer treatments (increased and decreased VPD), and both treatments induced up- and down-regulation of these genes (Table 4). Furthermore, both transfer treatments induced up- and down-regulation of SA response genes in *flc* plants, yet induced downregulation of a single PR1 protein (LOC100191111, involved in defence reactions against pathogens (The UniProt Consortium 2021)) in WT (Table 6).

Changes in hormonal gene expression in response to 24 h of increased VPD

Increased VPD only caused downregulation of a single beta-glucosidase 18 (LOC104645804) gene in WT, indicating decreased de-conjugation from ABA-GE to active ABA (Table 4). In *flc,* tomato nine-cis-epoxycarotenoid dioxygenase (LeNCED1, the rate-limiting step in ABA biosynthesis) and two molybdenum cofactor sulfurases (FLACCA and LOC101249114) were upregulated, indicating increased ABA biosynthesis in response to increased VPD (Table 4). The same treatment caused downregulation of early ABA receptor genes and upregulation of genes involved further downstream in ABA response in both WT and *flc* plants (Table 6). Increased VPD caused upregulation of ET biosynthesis genes in *flc* (Table 4), yet only one ET biosynthesis gene was upregulated in WT, a 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE HOMOLOG (LOC101244528, Table 4). Increased VPD resulted in strong upregulation of ET response genes in *flc* (Table 6), but downregulation in only one ET response factor 4 (ERF4) and upregulation of an ethylene-responsive transcription factor ERF061 (LOC101258880) in WT (Table 6). Similarly, this treatment caused upregulation of most JA biosynthesis genes and strong upregulation of JA response genes in *flc* (Table 6), yet had no effect on JA biosynthesis or response genes in WT (Table 4).

Discussion

Although foliar ABA status affects steady-state transpiration, other factors are involved in responses to changing VPD. Similarly, neither foliar ABA concentration ([ABA]) nor rate of delivery played an obligate role in the humidity response of *Arabidopsis thaliana* (Assmann et al. 2000), while the protein kinase OST1 was more important than ABA in VPD-induced stomatal responses to both increased and decreased VPD (Merilo et al. 2018; Xie et al. 2006). In this study, transpiration of *flc* plants was more sensitive than WT to changes in VPD (Fig. 3), showing agreement with previous findings (McAdam et al. 2016; Merilo et al. 2018). Differential gene expression between the two cultivars was more dependent on water status than ABA, as no DEGs were found between the two genotypes in low VPD, where *flc* water status was increased to WT levels. The results of GO enrichment KEGG analyses presented here indicate that genes involved in almost all biological processes were up- and down-regulated in *flc* plants (Fig. 6), an order of magnitude higher than WT plants under both steady-state (Table 1) and changing (Table 2) VPD conditions. Furthermore, ABA-deficiency and changing VPD affected gene expression involved in similar KEGG pathways (Tables 1 and 2), supporting the result that gene expression was dependent on water status, rather than ABA. Thus, growth in low VPD alleviates the effects of ABA-deficiency at the level of gene expression, yet seems to regulate gene expression indirectly as a result of changes in water status.

ABA content and effect on water relations

As expected, *flc* had lower foliar [ABA] than WT plants irrespective of RH (Tal & Nevo 1973). Growth VPD did not affect either WT or *flc* steady-state [ABA], as is typical of tomato (Arve & Torre 2015; Innes et al. 2021). That *flc* had lower RWC than WT plants in high VPD, but not low VPD (Fig. 2) indicates that growth in low VPD ameliorates the effects of ABA-deficiency on leaf water status, as previously reported (Sharp et al. 2000). Similarly, *flc* and WT transpiration rates were equivalent in low VPD (Fig. 3A) and there were no significant DEGs between WT and *flc* grown in low VPD (Fig. 5, Table 1). Thus, growth at low VPD not only enhances leaf water status of *flc*, but indicates that differential gene expression between the two cultivars depends on leaf water status rather than ABA.

Transferring WT plants from low to high VPD did not perturb foliar RWC (Fig. 2A), but increased foliar [ABA] (Fig. 1A) accompanied by pronounced stomatal closure (22% more closed after transfer, Fig. 3B). Since gene expression analyses indicated no significant upregulation of ABA biosynthesis, catabolism or response genes (Tables 4 and 6), foliar ABA accumulation was probably mediated by greater delivery of xylem-borne ABA from root to shoot (Gowing et al. 1993). Although *flc* showed upregulation of both ABA biosynthesis and response genes (Tables 4 and 6), there was no foliar ABA accumulation in response to decreased foliar RWC (Fig. 2B), yet significant stomatal closure (Fig. 3B) consistent with previous reports (Merilo et al. 2018). While stomatal closure in *flc* was seemingly a hydropassive response, i.e. no change in [ABA], this was augmented by ABA-dependent stomatal closure in WT plants.

While transpiration of *flc* was twice that of WT in high VPD conditions, both genotypes showed equivalent transpiration after 5 mins of exposure to low VPD (Fig. 3D), analogous to a stomatal opening response of *flc* than WT plants when VPD decreased (Merilo et al. 2018). Compared to their ABA-deficient mutants, WT plants showed pronounced hysteresis in the re-opening of stomata and increase of stomatal conductance of WT tomato, pea (*Pisum sativum*) and *Arabidopsis thaliana* plants when transferred from high to low VPD (McAdam et al. 2016; Merilo et al. 2018). This hysteresis (i.e. the "slowness" of the stomatal response) was ABA-dependent, with higher [ABA] delaying stomatal opening (Merilo et al. 2018). Such a mechanism may account for *flc* adjusting its transpiration more rapidly than WT when transferred from high to low VPD (Fig. 3D).

Decreased VPD did not alter [ABA], RWC, transpiration or stomatal aperture after 24 h in WT, yet caused stomatal closure, decreased transpiration and increased RWC in *flc* despite no change in [ABA]. Thus, stomatal response to changing VPD is ABA-independent in low [ABA] conditions. In this study, a serine/threonine-protein kinase SRK2E (LOC101268620), which is the tomato homolog for OST1 (a member of the SnRK2 family), was only affected by VPD in *flc*, being upregulated in low relative to high VPD, as well as after transfer from high to low VPD (Tables 5 and 6). This was unexpected result, as OST1 rather than ABA is crucial for stomatal responses to both increased and decreased VPD (Merilo et al. 2018). The similar lack of differential expression of SRK2E in both steady-state and short-term high VPD exposure suggests different mechanisms drive stomatal closure and decreased transpiration, such as the passive hydraulic regulation described in several studies (McAdam & Brodribb

2015; McAdam & Brodribb 2016; Merilo et al. 2018), or a CO_2-induced signalling cascade initiated downstream of OST1 (Hsu et al. 2018). The passive hydraulic mechanism is primarily found in basal lineages of vascular plants, including gymnosperms, and is independent of foliar ABA as it was the mechanism for regulation of water loss in the earliest land plants (McAdam & Brodribb 2015). Elevated [CO_2] activates guard cell S-type anion channels in both ABA biosynthesis and receptor mutants (Hsu et al. 2018), with higher internal [CO_2] (Ci) in *flc* relative to WT plants (Bradford et al. 1983; Innes et al. 2021), possibly inducing stomatal closure of ABA-deficient plants. Although exogenous or root-supplied ABA can phenotypically revert ABA-dependent stomatal control of *flc* plants (Dodd et al. 2009; Merilo et al. 2018; Sharp et al. 2000; Tal et al. 1979), the lower [ABA] in *flc* results in hydraulic regulation of water relations, as found in primitive plant lineages (Sun et al. 2020). Recovery of [ABA] to WT levels allows ABA-dependence in controlling water loss, indicating *flc* maintains downstream signal transduction pathways.

Plant immune responses via hormones and MAPKs

Several MAPK pathways that were upregulated in *flc* compared to WT in high VPD were involved in defence against pathogen infection and attack, and wounding responses (Fig. S2). ET synthesis and ET and JA signalling were important in these responses, and genes involved in biosynthesis of both were upregulated in *flc* relative to WT in high VPD (Table 3). This indicates upregulation of biotic defence genes in ABA-deficient *flc* under high VPD and agrees with upregulation of the α-linolenic acid KEGG pathway (Table 1), a precursor of JA (Mata-Pérez et al. 2015), and with studies showing the antagonistic relationship between ABA and ET-JA

systems (Anderson et al. 2004). Furthermore, this supports findings that ABA attenuates plant tolerance to pathogens (Audenaert et al. 2002; Zhang & Sonnewald 2017), and that *flc* are better equipped for biotic stress defence than WT plants (Kettner & Dörffling 1995). The KEGG pathway up- and down-regulation patterns (Tables 1, 2) show upregulation of the plant-pathogen interaction pathway in *flc* relative to WT in high VPD. Gene expression patterns of this pathway oppose those of the carotenoid synthesis pathway in the different treatments, suggesting that changes in ABA biosynthesis occur concurrently to changes in plant-pathogen interactions (Tables 1, 2). Some agreement was shown in ABA, ET and JA biosynthesis patterns, where *flc* showed both up- and down-regulation of ABA biosynthesis concurrent with strong upregulation of ET and JA relative to WT in high VPD (Table 3). However, both ABA and ET response genes were strongly upregulated in *flc*, while JA response genes showed both up- and down-regulation relative to WT in high VPD, suggesting a more complex ABA-ET-JA relationship. Interestingly, the FLACCA gene was upregulated in *flc* upon transfer from low to high VPD (Table 4), despite *flc* having a mutation in this gene. This supports previous findings that the *flc* mutant is somewhat leaky (Linforth et al. 1990; Neill & Horgan 1985). It should be noted that although there was significant upregulation of this and another ABA biosynthesis gene upon transfer from low to high VPD, this was relative and did not significantly increase the total foliar [ABA]. Upregulation of genes involved in autophagy and phagosome in *flc* relative to WT in high VPD may also indicate increased plant defence against pathogens (Avin-Wittenberg et al. 2018; Bozhkov 2018). Furthermore, the downregulation of ascorbate and aldarate metabolism and valine, leucine and isoleucine biosynthesis in the same conditions may also affect plant

pathogen resistance, as both pathways form integral parts of the plant immune system (Zeier 2013). Given their larger, more open stomata (Fig. 3B), it is likely that *flc* plants are more susceptible to pathogen attack (Panchal et al. 2016) and counteract this by increasing defence responses towards pathogen attack.

The up-regulation of MAPK-related genes in *flc* relative to WT in high VPD (Fig. S2) and in *flc* transferred from low to high VPD (Fig. S5B) was reversed in *flc* both grown in and transferred to low VPD (Fig. S3B and S4B), yet steady-state and short-term MAPK responses to VPD did not substantially differ in *flc*. This supports the suggestion that low VPD alleviates the effects of ABA-deficiency in tomato, and that the differences in gene expression between WT and *flc* are mainly driven by water status *per se*, and not ABA. Moreover, this may attenuate the increased biotic stress resistance in *flc* compared to WT, leaving ABA-deficient plants more susceptible to biotic stress in low VPD environments. This is supported by downregulation of both plant-pathogen interaction responses and glutathione metabolism KEGG pathways in *flc* transferred from high to low VPD (Table 2). Glutathione has been implicated in plant pathogen immunity through controlling ROS, inducing defence genes by changing its redox state and induction of genes involved in ET synthesis (Zechmann 2020). The reduced form of glutathione (GSH) mediates ET synthesis by regulating ACS and ACO enzymes (Datta et al. 2015), with activation of ACS6 associated with stress-induced production of ET (Li et al. 2012) as indicated in Fig S1. ABA and ET appear to act both synergistically and antagonistically to one another, depending on tissue type, plant developmental stage, species and environmental conditions (Müller 2021).

Yet the mechanisms of interaction at the transcriptional level remain equivocal, and highly dependent on endogenous concentrations, whereby low concentration of one hormone induces biosynthesis of the other (Müller 2021). Thus, there was strong upregulation and signalling of ET in ABA-deficient *flc* compared to WT plants in high VPD (Tables 3 and 5). ET biosynthesis and signalling genes were furthermore upregulated in *flc* transferred from low to high VPD (Tables 3-6, Fig. S2), but downregulated in *flc* both grown in and transferred to low VPD relative to high VPD (Tables 3-6, Fig. S3B and S4B). While lower ABA levels appear to contribute to stress-induced increased ET production, this effect is alleviated by low VPD. This supports previous findings of greater ET evolution in *flc* compared to WT plants, but high humidity decreasing *flc* ET evolution (Arve & Torre 2015). On the other hand, ET evolution and signalling of WT plants increased in low VPD (Tables 3-6), as found in Arve and Torre (2015). We argue above that differential gene expression between WT and *flc* plants are regulated by plant water status *per se* and not ABA status. Yet the opposite patterns of ET regulation in WT and *flc* in high RH indicates that high RH may perturb the already complex ABA-ET relationship, with ABA possibly required for ET production, and thereby hormone-related biotic stress resistance, in high RH environments.

Plants typically trade-off growth and defence, achieving one conditionally at the expense of the other (Bozhkov 2018). The induction of ET biosynthesis and signalling, coupled with the upregulation of cellular responses in *flc* relative to WT in high VPD, indicates increased catabolism and energy release, suggesting that *flc* plants upregulate defence mechanisms at the expense of growth in response to high VPD. Several

pathways that were upregulated in *flc* both grown in and transferred to low VPD were associated with anabolism and increased growth (e.g. carbon fixation, photosynthesis, aminoacyl-tRNA biosynthesis Tables 1 and 2). The same pathways were downregulated both in *flc* compared to WT in high VPD, and in *flc* transferred from low to high VPD. Previous results from metabolic analyses of *Hydrangea macrophylla* indicated a more carbon-sufficient, anabolic state in leaves from plants grown in high (90%) compared to moderate (60%) humidity (S. Torre unpublished data), suggesting more favourable physiological status for growth and attenuation of stress responses. Interestingly, WT plants showed no indications in such a shift in metabolic state, possibly because 60% RH is not experienced as particularly stressful by WT plants (see Fig. 2) as they better regulate their water relations than *flc* (Bradford 1983).

Conclusions

Genotypic differences in transpiration and water status are important drivers of gene expression in tomatoes. Long term low VPD decreased transpiration and increased RWC of *flc* to the same levels as WT plants, resulting in no significant differential gene expression between the two genotypes. However, in high VPD, where *flc* transpiration rate was almost double and RWC significantly lower than WT, 8027 genes were differentially expressed between *flc* and WT. Analysis of transcriptomic responses to short- and long-term exposure to different VPD levels indicated that ABA-deficiency of *flc* plants induces differential expression of thousands of genes compared to WT plants, yet this results not from ABA-deficiency *per se*, but rather as a result of differences in water status. Thus, growing *flc* in low VPD alleviates the effects of ABA-deficiency by increasing plant water status and foliar turgor. Low VPD induced genes in *flc* that indicate a more carbon-sufficient, anabolic state, while the opposite was found in high VPD, indicating a shift from a growth to a defence state between low and high VPD. Moreover, the genes differentially expressed in *flc* compared to WT plants in high VPD seem to indicate that biotic stress defence responses, especially increased ET and JA biosynthesis and response, are induced. Low VPD caused downregulation of the same genes and pathways, supporting the transition to a growth state. Overall, ABA deficiency induces differential expression of thousands of genes, yet this was conditional on VPD modulating plant water status.

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Figure and table legends

Fig. 1. Foliar ABA concentration for A) wild-type (WT) and B) *flacca* (*flc*) tomatoes grown in continuously high (HH) or continuously low (LL) VPD and transferred from high to low (HL) or from low to high (LH) VPD for 24 h. Means ± SE, n = 4-8 per genotype per treatment. Note the difference in y-axis range between panels. Different letters indicate significant differences between treatments ($p < 0.05$) as determined by ANOVA and post-hoc Tukey HSD analyses.

Fig. 2. Leaf relative water content (RWC) for wild-type (WT) and *flacca* (*flc*) tomatoes grown in continuously high (HH) or continuously low (LL) VPD and transferred from to low (HL) or from low to high (LH) VPD for 24 h. Means ± SE, n = 5 per genotype per treatment. Different letters indicate significant differences between treatments ($p < 0.05$) as determined by ANOVA and post-hoc Tukey HSD analyses.

Figure 3. Water relations of wild-type (WT) and *flacca* (*flc*) tomatoes exposed to long-term short-term or very short-term high or low VPD. Transpiration rates (A) and stomatal closure index (aperture L/W ratio; B) during growth in continuously high (HH) or continuously low (LL) VPD and transferred from high to low (HL) or from low to high (LH) VPD for 24 h. Means ± SE, n = 6 per genotype per treatment (A), or n = 10-23 stomata per genotype per treatment (B). C-D: Time course of whole plant transpiration during very short term (1 h) exposure of plants to high (C) or low (D) VPD. E-F: ABA concentration from the start and end of the time course of plants reciprocally transferred from low to high VPD (E) or from

high to low VPD (F). VPD at the start of time of measurement was growth VPD, VPD follows same scale as transpiration (vertical axis). Inserts indicate means ± SE from the first and last five minutes of measurement. Different letters indicate significant differences between treatments ($p < 0.05$) as determined by ANOVA and post-hoc Tukey HSD analyses.

Fig. 4. Differentially expressed genes (DEGs) in wild type (WT) and *flacca* (*flc*) tomatoes continuously grown in high (HH) or low (LL) VPD (A, B, D), or transferred from low to high (LH) or from high to low (HL) VPD (C, E) for 24 h (FDR < 0.05). A) Steady-state effects of ABA-deficiency at two VPD levels showing DEGs in *flc* relative to WT, B) steady-state effects of growth in low compared to high VPD in each genotype. C) Short term effects (24 h) of transfer between VPDs from low to high (LH) or high to low (HL) in each genotype. Up- and down-regulated genes indicated as positive or negative on the y-axis, respectively. Colour shades indicate the level of log2 fold change (see legend). C) Number of DEGs (both up- and down-regulated) in WT and *flc* plants grown in low relative to high VPD, including the number of overlapping genes affected in both genotypes, D) Number of DEGs from transfer treatments in WT and *flc* plants and the number of overlapping genes affected between genotypes, treatments and both.

Table 1. KEGG pathways affected by DEGs in WT and *flc* plants, arranged according to BRITE Hierarchy. Arrows indicate (↑) up- and (↓) down-regulation of genes in *flc* compared to WT when grown continuously in high or low VPD, and regulation of genes in WT and *flc* grown in low VPD

compared to high VPD. Pathways highlighted in bold italics indicate pathways affected by steady-state conditions, but not by changing VPD.

Table 2. KEGG pathways affected by differential gene expression in WT and *flc* plants in response to 24h of increased or decreased VPD and arranged according to BRITE Hierarchy. Arrows indicate (↑) up- and (↓) down-regulation of genes involved in each biological pathway. Pathways highlighted in bold italics indicate pathways affected by short term VPD changes, but not steady state conditions.

Table 3. Stress hormone biosynthesis genes with significantly altered gene expression, shown as log2 fold change, between wild-type (WT) and *flacca* (*flc*) tomatoes grown for two weeks in high and low VPD, or between plants grown in high or low VPD within each genotype. Blue indicates downregulation, red indicates upregulation. Colour intensity indicates log2 fold change magnitude: lightest colour indicates $|0| <$ log2 fold change $< |1|$, medium colour indicates $|1| <$ log2 fold change $< |2|$, and most intense colour indicates log2 fold change $> |2|$. Blank squares indicate no significant log2 fold change. Significant log2 fold change classified as adjusted $P < 0.05$.

Table 4. Stress hormone biosynthesis genes with significantly altered gene expression, shown as log2 fold change, plants of each genotype grown for two weeks in high or low VPD followed by reciprocal transfer between VPD treatments for 24 h. Blue indicates downregulation, red indicates upregulation. Colour intensity indicates log2 fold change magnitude: lightest colour indicates $|0| <$ log2 fold change $< |1|$, medium colour indicates $|1| <$ log2 fold change $< |2|$, and most intense colour indicates log2

fold change > |2|. Blank squares indicate no significant log2 fold change. Significant log2 fold change classified as adjusted $P < 0.05$.

Table 5. Stress hormone receptor and response genes with significantly altered gene expression, shown as log2 fold change, between wild-type (WT) and *flacca* (*flc*) tomatoes grown for two weeks in high and low VPD, or between plants grown in high or low VPD within each genotype. Blue indicates downregulation, red indicates upregulation. Colour intensity indicates log2 fold change magnitude: lightest colour indicates |0| < log2 fold change < |1|, medium colour indicates |1| < log2 fold change < |2|, and most intense colour indicates log2 fold change > |2|. Blank squares indicate no significant log2 fold change. Significant log2 fold change classified as adjusted $P < 0.05$.

Table 6. Stress hormone receptor and response genes with significantly altered gene expression, shown as log2 fold change, plants of each genotype grown for two weeks in high or low VPD followed by reciprocal transfer between VPD treatments for 24 h. Blue indicates downregulation, red indicates upregulation. Colour intensity indicates log2 fold change magnitude: lightest colour indicates |0| < log2 fold change < |1|, medium colour indicates |1| < log2 fold change < |2|, and most intense colour indicates log2 fold change > |2|. Blank squares indicate no significant log2 fold change. Significant log2 fold change classified as adjusted $P < 0.05$.

Fig. 1

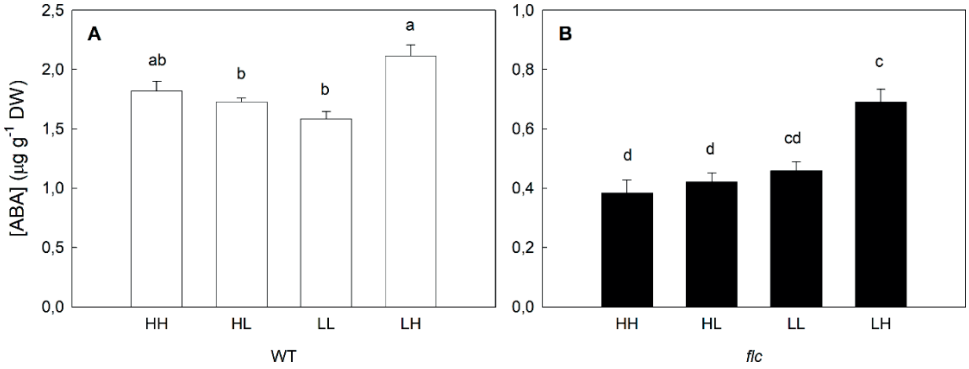
A
B
[ABA] (µg g-1 DW)
2,5
2,0
1,5
1,0
0,5
0,0
1,0
0,8
0,6
0,4
0,2
0,0
ab
b
b
a
d
d
cd
c
HH
HL
LL
LH
WT
flc

Fig. 2

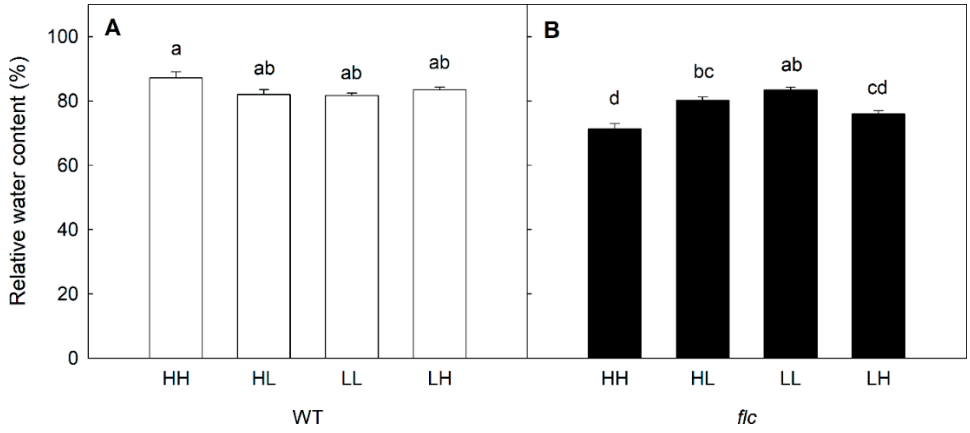
A
B
Relative water content (%)
100
80
60
40
20
0
a
ab
ab
ab
d
bc
ab
cd
HH
HL
LL
LH
HH
HL
LL
LH
WT
flc

Fig. 3

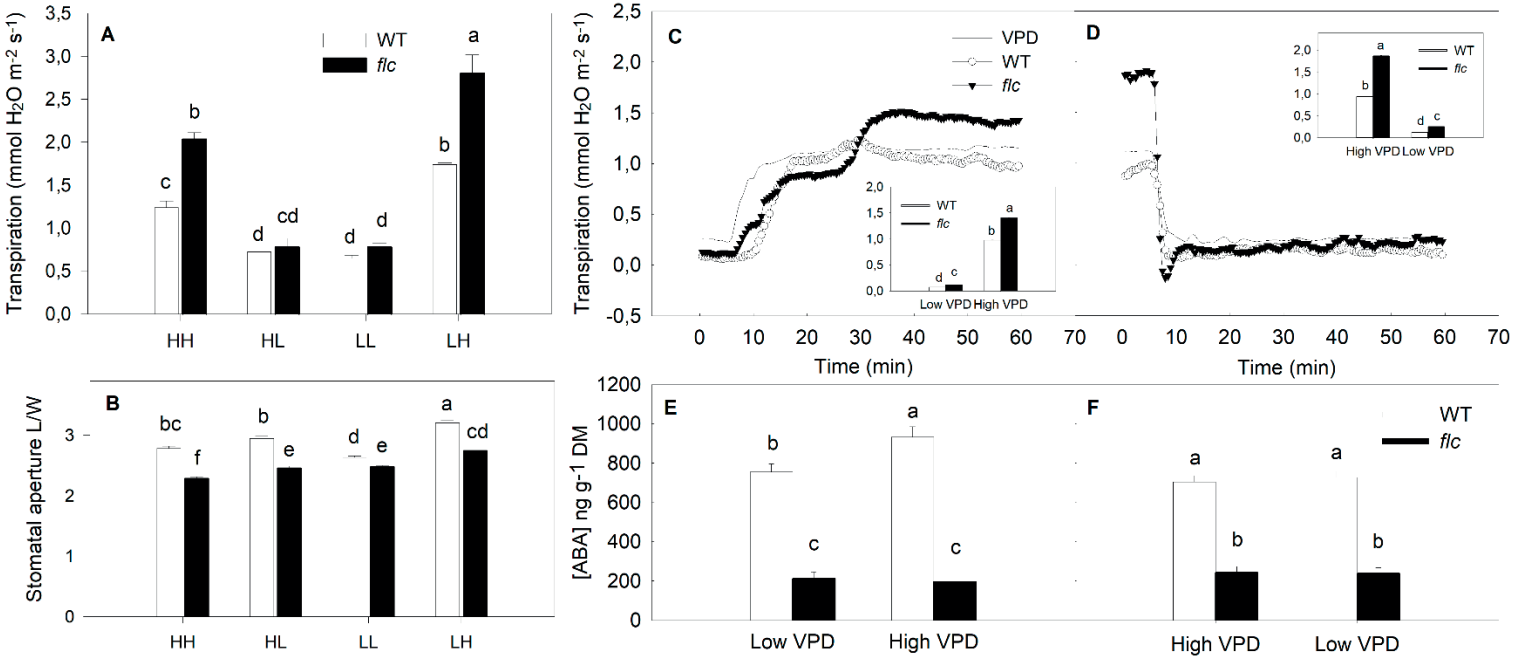
A
WT
flc
Transpiration (mmol H2O m-2 s-1)
HH
HL
LL
LH
B
Stomatal aperture L/W
C
VPD
WT
flc
Low VPD High VPD
Time (min)
D
High VPD Low VPD
Time (min)
E
[ABA] ng g-1 DM
Low VPD
High VPD
F
WT
flc
High VPD
Low VPD

Fig. 4

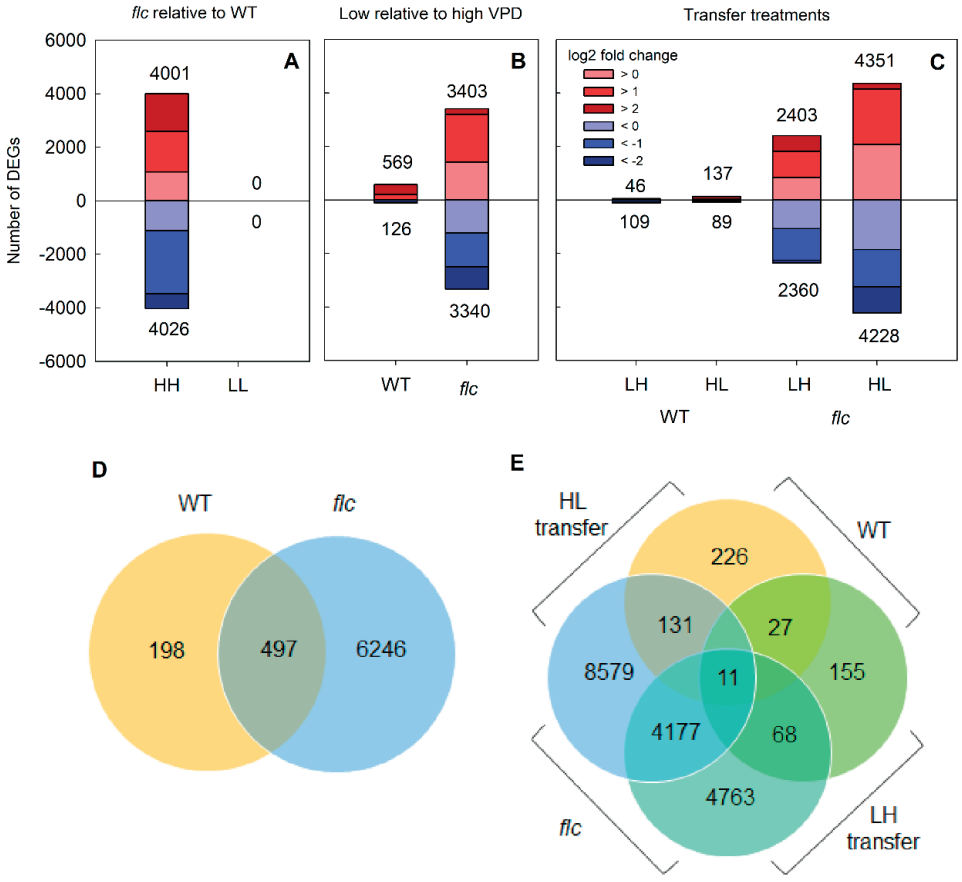
flc relative to WT
Low relative to high VPD
Transfer treatments
A
B
C
Number of DEGs
6000
4000
2000
0
-2000
-4000
-6000
4001
0
0
4026
HH
LL
569
126
3403
3340
WT
flc
log2 fold change
> 0
> 1
> 2
< 0
< -1
< -2
46
109
137
89
2403
2360
4351
4228
LH
HL
LH
HL
WT
flc
D
WT
flc
198
497
6246
E
HL transfer
WT
LH transfer
flc
226
131
27
8579
11
155
4177
68
4763

Table 1.

BRITE Hierarchy: *A*,B	BRITE Hierarchy: C KEGG pathway (total genes *S. lycopersicum*)	*flc* vs WT		Low vs high VPD	
		High VPD	Low VPD	WT	*flc*
Metabolism					
Carbohydrate metabolism	Glycolysis / Gluconeogenesis (80)	↓			↑
	Citrate cycle (TCA cycle) (38)	↑			↓
	Pentose and glucuronate interconversions (58)	↓			
	Ascorbate and aldarate metabolism (31)	↓			
	Starch and sucrose metabolism (62)	↓			↑
	Amino sugar and nucleotide sugar metabolism (82)	↑		↑	↓
	Pyruvate metabolism (52)	↓			↑
	Glyoxylate and dicarboxylate metabolism (44)	↓			↑
	Propanoate metabolism (23)	↑			↓
	Butanoate metabolism (18)			↑	
	Inositol phosphate metabolism (34)	↑			↓
Energy metabolism	Photosynthesis (34)	↓			↑
	Photosynthesis (13)	↓			↑
	Carbon fixation in photosynthetic organisms (39)	↓			↑
	Sulfur metabolism (17)				↑
Lipid metabolism	Fatty acid degradation (20)	↑			↓
	Steroid biosynthesis (21)	↓			↑
	Glycerolipid metabolism (51)				↑
	Glycerophospholipid metabolism (53)				↑
	Alpha-Linolenic acid metabolism (22)	↑			↓
Nucelotide metabolism	Purine metabolism (49)	↓			↑
Amino acid metabolism	Alanine, aspartate and glutamate metabolism (30)				↑
	Glycine, serine and threonine metabolism (42)	↓			↑
	Valine, leucine and isoleucine biosynthesis (12)	↓			
	Valine, leucine and isoleucine degradation (34)				↓
	Phenylalanine metabolism (37)	↓			
	Phenylalanine, tyrosine and tryptophan biosynthesis (31)	↓			↑
Metabolism of other amino acids	Beta-Alanine metabolism (34)				↓
	Glutathione metabolism (45)	↓			
Glycan biogenesis and metabolism	N-Glycan biosynthesis (18)	↑			↓
Metabolism of cofactors and vitamins	Riboflavin metabolism (13)	↓			↑
	Vitamin B6 metabolism (8)	↓			↑
	One carbon pool by folate (14)	↓			↑
	Porphyrin and chlorophyll metabolism (36)	↓			↑
Metabolism of terpenoids and polyketides	Terpenoid backbone biosynthesis (32)				↑
	Carotenoid biosynthesis (17)	↓			↑
Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis (98)	↑		↑	
	Flavonoid biosynthesis (34)	↓			
Genetic information and processing					
Translation	Aminoacyl-tRNA biosynthesis (27)	↓			↑
Folding, sorting and degradation	Protein export (20)				↓
	Protein processing in endoplasmic reticulum (107)	↑			↓
	SNARE interactions in vesicular transport (23)	↑			↓
	Proteasome (30)	↑			↓
Replication and repair	***DNA replication (22)***	↓			↑
	Homologous recombination (19)	↓			
Environmental information processing					
Signal transduction	MAPK signaling pathway (93)	↑		↑	↓
	Phosphatidylinositol signaling system (37)	↑			↓
	Plant hormone signal transduction (150)	↑			↑↓
Cellular processes					
Transport and catabolism	Endocytosis (106)	↑			↓
	Phagosome (57)	↑			
	Peroxisome (37)	↑			
	Autophagy (15)	↑			↓
Organismal systems					
Environmental adaptation	Plant-pathogen interaction (95)	↑		↑	↓

Table 2.

BRITE hierarchy: *A*, B	BRITE hierarchy: C KEGG pathway (total genes *S. lycopersicum*)	High to low VPD		Low to high VPD	
		flc	WT	*flc*	WT
Metabolism					
Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism (82)	↓	↑↓	↑	↓
	Citrate cycle (TCA cycle)	↓			
	Fructose and mannose metabolism (47)	↑↓			
	Glycolysis / Gluconeogenesis (80)	↑		↓	
	Glyoxylate and dicarboxylate metabolism (44)	↑		↓	
	Inositol phosphate metabolism (34)	↓		↑	
	Pentose and glucuronate interconversions			↓	
	Pentose phosphate pathway (29)	↑			
	Propanoate metabolism (23)	↓		↑	
	Pyruvate metabolism (52)	↑			
	Starch and sucrose metabolism (62)	↑		↓	
Energy metabolism	Carbon fixation in photosynthetic organisms (39)	↑		↓	
	Photosynthesis (34)	↑		↓	
	Photosynthesis (13)	↑		↓	
Lipid metabolism	Fatty acid degradation (20)			↑	
	Glycerolipid metabolism (51)	↑			
	Glycerophospholipid metabolism (53)	↑			
Nucleotide metabolism	Purine metabolism (49)	↑		↓	
Amino acid metabolism	Alanine, aspartate and glutamate metabolism (30)	↑	↑		
	Glycine, serine and threonine metabolism (42)	↑		↓	
	Phenylalanine metabolism (37)	↑	↑		
	Phenylalanine, tyrosine and tryptophan biosynthesis (31)	↑		↓	
	Valine, leucine and isoleucine degradation (34)			↑	
Metabolism of other amino acids	Beta-Alanine metabolism (34)			↑	
	Glutathione metabolism (45)	↓		↑	
Glycan biosynthesis	N-Glycan biosynthesis (18)	↓		↑	
Metabolism of cofactors and vitamins	One carbon pool by folate (14)	↑		↓	
	Porphyrin and chlorophyll metabolism (36)	↑		↓	
	Riboflavin metabolism (13)	↑		↓	
	Thiamine metabolism (14)			↑	
	Ubiquinone and other terpenoid-quinone biosynthesis (26)	↑			
	Vitamin B6 metabolism (8)	↑		↓	
Metabolism of terpenoids and polyketides	Carotenoid biosynthesis (17)	↑		↓	
	Terpenoid backbone biosynthesis (32)	↑			
Biosynthesis of other secondary metabolites	Phenylpropanoid biosynthesis		↓		↓
Genetic information processing					
Transcription	***RNA polymerase***	↓			
	Spliceosome	↓			
Translation	Aminoacyl-tRNA biosynthesis (27)	↑		↓	
	Ribosome biogenesis in eukaryotes	↓			
	RNA transport	↓			
Folding, sorting and degradation	Proteasome (30)	↓		↑	
	Protein export (20)	↓		↑	
	Protein processing in endoplasmic reticulum (107)	↓		↑	
	RNA degradation	↓			
	SNARE interactions in vesicular transport (23)	↓		↑	
Environmental information processing					
Signal transduction	MAPK signaling pathway (93)	↓	↑↓	↑	↓
	Phosphatidylinositol signaling system (37)	↓		↑	
	Plant hormone signal transduction (150)	↑↓	↑↓	↑	↓
Cellular processes					
Transport and catabolism	Endocytosis (106)	↓		↑	
Organismal systems					
Environmental adaptation	***Circadian rhythm (22)***	↑			
	Plant-pathogen interaction (95)	↓		↑	

Table 3.

Hormone	geneID	*flc* vs WT		Low vs high VPD		Annotation	Description
		High VPD	Low VPD	WT	*flc*		
Abscisic acid	Solyc02g085940.3.1				1,254	LOC101268051	protein ABA DEFICIENT 4, chloroplastic
	Solyc02g086050.3.1	-1,547				LOC101268051	protein ABA DEFICIENT 4, chloroplastic
	Solyc07g056570.1.1	0,94				LeNCED1	nine-cis-epoxycarotenoid dioxygenase
	Solyc08g080180.1.1	1,84				LOC101249114	molybdenum cofactor sulfurase
	Solyc07g066260.3.1				-0,808	FLACCA	molybdenum cofactor sulfurase
	Solyc07g066480.3.1	1,173				FLACCA	molybdenum cofactor sulfurase
	Solyc08g079910.2.1				-2,677	LOC101249114	molybdenum cofactor sulfurase
	Solyc04g078900.3.1	-3,991				CYP707A1	ABA 8'-hydroxylase
	Solyc04g080650.3.1	1,352				LOC101267805	abscisic acid 8'-hydroxylase 2
	Solyc01g108210.3.1	-2,846				LOC101266767	abscisic acid 8'-hydroxylase 4
	Solyc09g091670.3.1				-5,921	SlUGT75C1	ABA uridine diphosphate glucosyltransferase
	Solyc09g092500.1.1	1,545				SlUGT75C1	ABA uridine diphosphate glucosyltransferase
	Solyc02g080290.3.1	1,537		1,062		LOC104645804	beta-glucosidase 18
	Solyc02g080300.3.1	1,078				LOC104645805	beta-glucosidase 18
	Solyc02g080310.2.1	-1,545				LOC101257526	beta-glucosidase 18
Ethylene	Solyc09g031610.3.1				0,866	LOC101249120	ethylene-overproduction protein 1-like
	Solyc09g055310.3.1	0,931				LOC101249120	ethylene-overproduction protein 1-like
	Solyc12g005060.1.1				-1,719	LOC101255470	ethylene-inducing xylanase

	Solyc12g005620.1.1	3,618	3,203		LOC101255470	ethylene-inducing xylanase
	Solyc12g099000.2.1	-0,687			LOC101247506	S-adenosylmethionine synthase 2
	Solyc08g081310.3.1			0,971	ACS1A	1-aminocyclopropane-1-carboxylate synthase
	Solyc08g081555.1.1	1,587			ACS1A	1-aminocyclopropane-1-carboxylate synthase
	Solyc01g095080.3.1	9,562		-6,695	ACS2	1-aminocyclopropane-1-carboxylate synthase 2
	Solyc07g049260.3.1			-1,027	ACO1	1-aminocyclopropane-1-carboxylate oxidase 1
	Solyc07g049530.3.1	7,773	3,921		ACO1	1-aminocyclopropane-1-carboxylate oxidase 1
	Solyc12g005400.2.1			1,042	LOC101251255	1-aminocyclopropane-1-carboxylate oxidase
	Solyc12g005940.2.1	4,58	2,366		LOC101251255	1-aminocyclopropane-1-carboxylate oxidase
	Solyc02g036350.3.1	1,679	1,502		LOC100125909	1-aminocyclopropane-1-carboxylate oxidase
	Solyc07g049370.2.1			1,18	ACO4	ethylene-forming enzyme
	Solyc07g049550.3.1	3,829	1,319		ACO4	ethylene-forming enzyme
Jasmonic acid	Solyc08g066530.3.1			1,247	TPLD	phospholipase D alpha
	Solyc08g066800.3.1	-0,748			TPLD	phospholipase D alpha
	Solyc08g014000.3.1	-7,088	-1,862		LOX1.1	Lipoxygenase
	Solyc01g006540.3.1	-1,031			TomloxC	Lipoxygenase
	Solyc03g122140.3.1			1,119	loxD	linoleate 13S-lipoxygenase 3-1, chloroplastic
	Solyc03g122340.3.1	1,097			loxD	linoleate 13S-lipoxygenase 3-1, chloroplastic
	Solyc01g099160.3.1	2,702		-1,721	TomloxE	Lipoxygenase
	Solyc01g006560.3.1			-1,032	loxF	Lipoxygenase
	Solyc04g079640.3.1			-2,363	aos	allene oxide synthase
	Solyc04g079730.1.1	1,922			aos	allene oxide synthase
	Solyc11g069270.2.1			1,04	AOS2	allene oxide synthase 2

	Solyc11g069800.1.1	0,801		AOS2	allene oxide synthase 2
	Solyc01g103390.3.1	1,49		opr2	opr2 protein
	Solyc07g007700.2.1		0,914	opr3	12-oxophytodienoate reductase 3
	Solyc08g078390.3.1	0,695		Acx1A	peroxisomal acyl-CoA oxidase 1A
	Solyc12g098660.2.1		0,886	LOC101263592	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a
	Solyc12g099440.2.1	1,176		LOC101263592	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a
Salicylic acid	Solyc06g069780.3.1		-1,42	LOC778225	isochorismate synthase
	Solyc06g071030.3.1	-0,992		LOC778225	isochorismate synthase
	Solyc06g070960.2.1		-3,433	EDS1	protein EDS1
	Solyc02g088390.3.1		0,993	LOC101243843	chorismate mutase 1, chloroplastic
	Solyc02g088460.3.1	1,178		LOC101243843	chorismate mutase 1, chloroplastic
	Solyc09g007710.3.1		1,387	PAL3	phenylalanine ammonia-lyase 3
	Solyc09g007920.3.1	-0,781		PAL3	phenylalanine ammonia-lyase 3
	Solyc02g032850.3.1	3,055	-2,355	LOC101257028	lipase-like PAD4

Table 4.

Hormone	geneID	High to low VPD		Low to high VPD		Annotation	Description
		flc	WT	*flc*	WT		
Abscisic acid	Solyc11g011340.2.1	-2,122				ZEP	zeaxanthin epoxidase, chloroplastic
	Solyc02g086050.3.1			-1,367		LOC101268051	protein ABA DEFICIENT 4, chloroplastic
	Solyc12g021340.2.1	0,933				LOC101268051	protein ABA DEFICIENT 4, chloroplastic
	Solyc07g056570.1.1			1,238		LeNCED1	nine-cis-epoxycarotenoid dioxygenase
	Solyc06g083650.3.1	1,522				LOC101256252	abscisic-aldehyde oxidase-like
	Solyc07g066480.3.1			1,006		FLACCA	molybdenum cofactor sulfurase
	Solyc08g077760.3.1	-2,093				FLACCA	molybdenum cofactor sulfurase
	Solyc01g067360.3.1	0,959				LOC101249114	molybdenum cofactor sulfurase
	Solyc08g080180.1.1			0,995		LOC101249114	molybdenum cofactor sulfurase
	Solyc03g112130.1.1	2,185				LOC101249565	abscisic acid 8'-hydroxylase 1
	Solyc02g080290.3.1				-1,396	LOC104645804	beta-glucosidase 18
	Solyc02g080310.2.1			-1,222		LOC101257526	beta-glucosidase 18
	Solyc02g085570.3.1	-0,54				LOC101257526	beta-glucosidase 18
Ethylene	Solyc01g079220.3.1	0,591				LOC101255470	ethylene-inducing xylanase
	Solyc03g026060.3.1	-0,95				LOC101249120	ethylene-overproduction protein 1-like
	Solyc09g055310.3.1			0,641		LOC101249120	ethylene-overproduction protein 1-like
	Solyc04g076510.3.1	-0,694				ACS1A	1-aminocyclopropane-1-carboxylate synthase
	Solyc08g081555.1.1			1,712		ACS1A	1-aminocyclopropane-1-carboxylate synthase
	Solyc04g079350.1.1	-3,42				LE-ACS1B	1-aminocyclopropane-1-carboxylate synthase-like

	Solyc01g067290.2.1	1,267				ACS2	1-aminocyclopropane-1-carboxylate synthase 2
	Solyc01g095080.3.1			5,825		ACS2	1-aminocyclopropane-1-carboxylate synthase 2
	Solyc01g100570.3.1	-1,043				LOC101251255	1-aminocyclopropane-1-carboxylate oxidase
	Solyc12g005940.2.1			1,408		LOC101251255	1-aminocyclopropane-1-carboxylate oxidase
	Solyc07g049530.3.1			4,57		ACO1	1-aminocyclopropane-1-carboxylate oxidase 1
	Solyc10g009210.3.1	-0,516				ACO1	1-aminocyclopropane-1-carboxylate oxidase 1
	Solyc11g005920.2.1	-1,613				ACO3	1-aminocyclopropane-1-carboxylate oxidase homolog
	Solyc07g049550.3.1			1,891		ACO4	ethylene-forming enzyme
	Solyc08g074290.3.1	-0,995				ACO4	ethylene-forming enzyme
	Solyc09g089730.3.1				1,009	LOC101244528	1-aminocyclopropane-1-carboxylate oxidase homolog
Jasmonic acid	Solyc07g063850.3.1	0,819				TPLD	phospholipase D alpha
	Solyc01g006540.3.1		0,997			TomloxC	Lipoxygenase
	Solyc03g122340.3.1			1,411		loxD	linoleate 13S-lipoxygenase 3-1, chloroplastic
	Solyc09g082270.3.1	-5,326				loxD	linoleate 13S-lipoxygenase 3-1, chloroplastic
	Solyc01g099160.3.1			1,199		TomloxE	Lipoxygenase
	Solyc02g087880.3.1	1,074				TomloxE	Lipoxygenase
	Solyc01g081320.3.1	1,28				aos	allene oxide synthase
	Solyc04g079730.1.1			2,167		aos	allene oxide synthase
	Solyc01g087775.1.1	1,243				AOS2	allene oxide synthase 2
	Solyc11g069800.1.1			1,618		AOS2	allene oxide synthase 2
	Solyc02g085730.3.1			0,791		AOC	allene oxide cyclase
	Solyc01g103390.3.1			-1,49		opr2	opr2 protein

	Solyc07g056490.4.1	0,769		LOC101263592	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a
	Solyc12g099440.2.1		0,797	LOC101263592	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a
Salicylic acid	Solyc04g082740.3.1	1,414		LOC778225	isochorismate synthase
	Solyc06g071030.3.1		-1,118	LOC778225	isochorismate synthase
	Solyc03g111870.3.1	-0,94		EDS1	protein EDS1
	Solyc03g119810.1.1	-3,435		LOC101243843	chorismate mutase 1, chloroplastic
	Solyc08g007210.3.1	1,355		PAL3	phenylalanine ammonia-lyase 3
	Solyc09g007920.3.1		-1,105	PAL3	phenylalanine ammonia-lyase 3
	Solyc02g032850.3.1		1,606	LOC101257028	lipase-like PAD4
	Solyc04g012040.3.1	-1,07		LOC101257028	lipase-like PAD4

Table 5.

Hormone	geneID	*flc* vs WT		Low vs high VPD		Annotation	Description
		High VPD	Low VPD	WT	*flc*		
Abscisic acid	Solyc06g061180.1.1	0,995				LOC101267127	abscisic acid receptor PYR1
	Solyc08g076650.3.1				1,177	LOC101268417	abscisic acid receptor PYR1
	Solyc03g095720.3.1				-0,548	LOC101256856	abscisic acid receptor PYL4
	Solyc03g095780.2.1	2,59		2,797		LOC101256856	abscisic acid receptor PYL4
	Solyc06g050500.2.1	3,967		3,589		LOC101258963	abscisic acid receptor PYL4
	Solyc10g085310.1.1	1,998				LOC101250944	abscisic acid receptor PYL4
	Solyc10g076410.1.1			2,417		LOC101245998	abscisic acid receptor PYL4-like
	Solyc07g062700.3.1				-1,406	DIG3	protein phosphatase 2C
	Solyc07g062970.3.1	1,018				DIG3	protein phosphatase 2C
	Solyc06g051520.3.1				0,6	LOC101258071	protein phosphatase 2C
	Solyc06g051940.3.1			-3,145		LOC101258071	protein phosphatase 2C
	Solyc03g121760.3.1				1,256	PP2C-1	protein phosphatase 2C ABI2 homolog
	Solyc03g096380.3.1				0,591	PP2C-2	protein phosphatase 2C AHG3 homolog
	Solyc08g077150.3.1	-0,587				LOC101263835	protein phosphatase 2C 29-like
	Solyc01g079720.3.1	0,742				LOC101249298	protein phosphatase 2C 70
	Solyc01g108280.3.1				0,605	LOC101268620	serine/threonine-protein kinase SRK2E (OST1/SnRK2.6)
	Solyc02g090390.3.1	1,008				LOC101251432	serine/threonine-protein kinase SRK2I
	Solyc08g079545.1.1				1,358	LOC101256903	guard cell S-type anion channel SLAC1
	Solyc09g014215.1.1				0,782	LOC101266554	S-type anion channel SLAH2-like

	Solyc03g007770.3.1	1,579			LOC101261097	S-type anion channel SLAH2
	Solyc11g040390.2.1			0,589	SlABF4	ABA responsive transcription factor
	Solyc11g044560.2.1	1,064			SlABF4	ABA responsive transcription factor
	Solyc01g108087.1.1	1,32			LOC101263766	ABSCISIC ACID-INSENSITIVE 5-like protein 7
	Solyc02g063360.3.1	3,623	2,331	-1,233	LOC101251949	protein C2-DOMAIN ABA-RELATED 1
	Solyc02g091470.3.1			1,007	LOC101255351	protein C2-DOMAIN ABA-RELATED 3
	Solyc02g091520.3.1	1,393			LOC101255351	protein C2-DOMAIN ABA-RELATED 3
	Solyc04g007800.3.1	0,788			LOC101263994	protein C2-DOMAIN ABA-RELATED 7-like
	Solyc11g012120.2.1			1,831	LOC101267573	protein C2-DOMAIN ABA-RELATED 11
	Solyc11g012780.2.1	1,482			LOC101267573	protein C2-DOMAIN ABA-RELATED 11
	Solyc04g014900.3.1			-0,961	LOC101256762	protein C2-DOMAIN ABA-RELATED 11-like
	Solyc04g015070.3.1	-1,457			LOC101256762	protein C2-DOMAIN ABA-RELATED 11-like
	Solyc03g118570.3.1			0,722	LOC104644270	C2-domain ABA-related family protein
	Solyc02g088910.3.1	1,104	1,447		LOC101259390	ninja-family protein AFP3
	Solyc05g012210.3.1	-1,69			LOC101266721	ninja-family protein AFP3
	Solyc07g066220.3.1	0,797			SlWRKY2	WRKY transcription factor 2
	Solyc02g032950.3.1	3,208	1,81	-1,585	LOC101258801	WRKY transcription factor 6
Ethylene	Solyc12g010690.2.1			-1,624	ETR1	Ethylene receptor 1
	Solyc12g011330.3.1	1,283			ETR1	Ethylene receptor 1
	Solyc09g075120.3.1			-1,896	ETR3	ethylene receptor neverripe
	Solyc09g075440.3.1	0,827			ETR3	ethylene receptor neverripe
	Solyc06g053455.1.1			-0,591	ETR4	ethylene receptor homolog
	Solyc06g053500.3.1			-0,627	ETR4	ethylene receptor homolog

Solyc06g053710.3.1	2,3		ETR4	ethylene receptor homolog
Solyc06g053715.1.1	2,183		ETR4	ethylene receptor homolog
Solyc11g006180.2.1	1,028		ETR5	ethylene receptor homolog
Solyc05g055070.3.1	1,013		LOC101249081	protein EIN4-like
Solyc10g083290.4.1		-2,101	TCTR1	ethylene-inducible CTR1-like protein kinase
Solyc10g083610.2.1	1,279		TCTR1	ethylene-inducible CTR1-like protein kinase
Solyc01g097980.3.1	1,299	-1,127	TCTR2	TCTR2 protein
Solyc09g009090.3.1	1,066		CTR3	CTR1-like protein kinase
Solyc09g008700.2.1		-0,641	CTR3	CTR1-like protein kinase
Solyc07g008250.3.1	1,145		LOC101248690	EIN3-binding F-box protein 1
Solyc12g009080.2.1		0,812	EBF1	EIN3-binding F-box protein 1
Solyc12g009560.2.1	0,788		EBF1	EIN3-binding F-box protein 1
Solyc08g044260.3.1		0,799	EBF2	EIN3-binding F-box protein 2
Solyc08g060810.3.1	0,838		EBF2	EIN3-binding F-box protein 2
Solyc07g008107.1.1		-2,832	LOC101248690	EIN3-binding F-box protein 1
Solyc06g073730.2.1	0,805		EIL4	EIN3-like protein
Solyc01g014480.3.1	0,765		LOC101264242	ETHYLENE INSENSITIVE 3-like 3 protein
Solyc01g009170.3.1	0,953		EIL2	EIL2 protein
Solyc01g096810.3.1	1,823	-1,271	EIL3	EIL3 protein
Solyc08g078190.1.1	1,454		LOC101267548	ethylene-responsive transcription factor
Solyc05g050930.2.1		-0,699	ERF1	ethylene-responsive transcription factor 1
Solyc05g051200.1.1	4,44		ERF1	ethylene-responsive transcription factor 1
Solyc03g093610.1.1	1,019		LOC101267589	ethylene-responsive transcription factor 1
Solyc04g014530.1.1	1,78		LOC101267105	ethylene-responsive transcription factor 1B

Solyc11g011740.1.1	1,985	2,839		LOC101246590	ethylene-responsive transcription factor 1B
Solyc12g055855.1.1			-1,158	SlERFD2	ethylene-responsive transcription factor 2a
Solyc12g056590.2.1	5,254			SlERFD2	ethylene-responsive transcription factor 2a
Solyc10g008520.3.1			0,812	ERF3	ethylene response factor 3
Solyc10g009110.1.1	1,378			ERF3	ethylene response factor 3
Solyc05g052030.1.1	2,901	2,729		ERF4	ethylene response factor 4
Solyc03g093310.3.1			-1,124	ERF5	ethylene response factor 5
Solyc09g083440.3.1			1,945	EREB	ethylene responsive element binding protein
Solyc09g089930.2.1	6,375			EREB	ethylene responsive element binding protein
Solyc08g077980.3.1			-1,701	LOC101267548	ethylene-responsive transcription factor
Solyc05g052040.1.1	2,461	1,942		DDTFR10/A	ethylene-responsive transcription factor 5
Solyc04g071660.3.1			0,696	LOC101251084	ethylene-responsive transcription factor ABR1-like
Solyc08g081555.1.1			-1,6	LOC101264747	ethylene-responsive transcription factor CRF1
Solyc08g081960.2.1	-1,443			LOC101264747	ethylene-responsive transcription factor CRF1
Solyc07g053990.3.1			-0,711	LOC101264838	ethylene-responsive transcription factor ERF054
Solyc08g008020.1.1			1,388	LOC101258880	ethylene-responsive transcription factor ERF061
Solyc08g008305.1.1	1,773			LOC101258880	ethylene-responsive transcription factor ERF061
Solyc08g081730.3.1			-1,073	LOC101257992	ethylene-responsive transcription factor ERF061
Solyc08g082210.3.1	1,741			LOC101257992	ethylene-responsive transcription factor ERF061
Solyc05g009460.3.1	-0,867			LOC101256485	ethylene-responsive transcription factor ERF118
Solyc05g009450.2.1	-0,878			LOC101256485	ethylene-responsive transcription factor ERF118
Solyc05g050710.3.1			1,797	LOC101243711	ethylene-responsive transcription factor ERF038-like
Solyc06g008803.1.1	-1,96			LOC104647283	ethylene-responsive transcription factor ERF119-like

	Solyc06g053240.3.1	-2,935			SHINE3	ethylene-responsive transcription factor SHINE3
	Solyc01g090340.2.1	-2,368	-1,342	1,399	LOC101258696	APETALA2/ethylene response factor
	Solyc07g018070.3.1			0,687	LOC101262024	AP2-like ethylene-responsive transcription factor AIL5
	Solyc04g077360.3.1			-4,376	LeANT	AP2-like ethylene-responsive transcription factor ANT-like
	Solyc02g022920.1.1	0,879		-0,861	ERN1	ethylene-responsive nuclear protein
	Solyc01g104740.3.1	6,063	3,506	-3,355	ER24	ethylene-responsive transcriptional coactivator
	Solyc01g104750.3.1	2,738		-1,968	ER24	ethylene-responsive transcriptional coactivator
	Solyc05g009630.3.1			-1,641	SlRAV2	transcription factor RELATED TO ABI3/VP1 2
	Solyc05g009790.1.1	3,122	1,571		SlRAV2	transcription factor RELATED TO ABI3/VP1 2
	Solyc10g011910.3.1	2,279			LOC101258194	WRKY transcription factor 22-like
Jasmonic acid	Solyc12g008830.2.1			1,391	LOC100134911	jasmonate ZIM-domain protein 1
	Solyc12g009220.2.1	3,823			LOC100134911	jasmonate ZIM-domain protein 1
	Solyc05g007050.3.1			-1,014	LEJA1	jasmonic acid 12
	Solyc05g007180.3.1	-1,344			LEJA1	jasmonic acid 1
	Solyc12g010910.2.1			1,155	JA2	jasmonic acid 2
	Solyc12g013620.2.1	4,967			JA2	jasmonic acid 2
	Solyc10g011660.3.1	0,718			LOC101262053	jasmonic acid-amido synthetase JAR1
	Solyc04g080660.3.1	1,541			LOC101264819	jasmonate O-methyltransferase
	Solyc01g096370.3.1	-1,632		1,406	LOC101264068	transcription factor MYC2-like
	Solyc10g008740.3.1			1,153	LOC101267164	transcription factor MYC3-like
	Solyc12g014440.2.1			1,086	LOC101258337	RAP domain-containing protein, chloroplastic
	Solyc06g068060.3.1			-0,812	LOC100301944	WRKY transcription factor 1

	Solyc06g068460.3.1	2,486	1,719		LOC100301944	WRKY transcription factor 1
Salicylic acid	Solyc12g094440.2.1			1,826	LOC101264610	protein ENHANCED DISEASE RESISTANCE 2
	Solyc01g095590.3.1			-0,717	LOC104649423	protein ENHANCED DISEASE RESISTANCE 4
	Solyc02g065280.3.1	-0,748		0,705	LOC101260990	salicylic acid-binding protein 2
	Solyc02g065240.3.1	-0,849		1,034	LOC101261578	salicylic acid-binding protein 2
	Solyc04g011670.3.1	-1,186			LOC101255681	TGACG-sequence-specific DNA-binding protein TGA-1A
	Solyc10g080770.2.1	1,431			LOC543600	TGACG-sequence-specific DNA-binding protein TGA-2.1
	Solyc10g080780.2.1	-0,736			LOC101245511	TGACG-sequence-specific DNA-binding protein TGA-2.1
	Solyc12g056860.2.1	0,937			LOC101253982	transcription factor TGA1
	Solyc11g064950.2.1	1,116			LOC101262685	transcription factor TGA2.2
	Solyc01g008730.3.1	0,937		-0,648	LOC101260964	transcription factor TGA2.2-like
	Solyc05g009660.3.1	-1,255			LOC101247526	transcription factor TGA2.3-like
	Solyc01g106620.2.1	14,372	13,221		LOC100191111	PR1 protein
	Solyc01g106630.2.1	7,207			LOC100191111	PR1 protein
	Solyc00g174340.2.1			-2,663	PR1b1	pathogenesis-related leaf protein 6
	Solyc01g095630.3.1	3,846	1,799	-2,707	WRKY3	WRKY transcription factor

Table 6.

Hormone	geneID	High to low VPD		Low to high VPD		Annotation	Description
		flc	WT	*flc*	WT		
Abscisic acid	Solyc02g037530.3.1	0,758				LOC101268417	abscisic acid receptor PYR1
	Solyc08g076960.1.1			-1,039		LOC101268417	abscisic acid receptor PYR1
	Solyc03g082510.1.1	1,413				LOC101258886	abscisic acid receptor PYL3
	Solyc01g109100.2.1	-0,812				LOC101258963	abscisic acid receptor PYL4
	Solyc02g069250.3.1	-4,48				LOC101256856	abscisic acid receptor PYL4
	Solyc03g095780.2.1			-1,628	-3,68	LOC101256856	abscisic acid receptor PYL4
	Solyc06g050500.2.1		2,331		-2,662	LOC101258963	abscisic acid receptor PYL4
	Solyc10g085310.1.1				-1,666	LOC101250944	abscisic acid receptor PYL4
	Solyc05g052420.2.1			-1,967		LOC101261414	abscisic acid receptor PYL4-like
	Solyc09g015380.1.1			-1,638		LOC101263741	abscisic acid receptor PYL4-like
	Solyc10g076410.1.1		1,496	-0,871	-2,264	LOC101245998	abscisic acid receptor PYL4-like
	Solyc02g083835.1.1	-1,51				LOC101260372	protein kinase and PP2C-like domain-containing protein
	Solyc09g011010.3.1	1,493				DIG3	protein phosphatase 2C
	Solyc06g051940.3.1		-2,28	3,302	2,185	LOC101258071	protein phosphatase 2C
	Solyc02g080590.3.1	-0,722				LOC101248778	protein phosphatase 2C 37
	Solyc03g007230.3.1			1,436		LOC101249794	protein phosphatase 2C 51-like
	Solyc10g080740.2.1	-0,676				LOC101262900	protein phosphatase 2C 57
	Solyc03g121880.3.1			1,11		PP2C-1	protein phosphatase 2C ABI2 homolog
	Solyc07g053320.3.1	0,583				PP2C-1	protein phosphatase 2C ABI2 homolog

	Solyc02g091970.3.1	0,851				PP2C-2	protein phosphatase 2C AHG3 homolog
	Solyc03g096670.3.1		-1,071	2,349	1,628	PP2C-2	protein phosphatase 2C AHG3 homolog
	Solyc09g059170.2.1	1,758				LOC101268620	serine/threonine-protein kinase SRK2E
	Solyc03g118300.1.1	0,868				LOC101266554	S-type anion channel SLAH2-like
	Solyc05g013040.3.1	-0,519				SlABF4	ABA responsive transcription factor
	Solyc11g044560.2.1			0,696		SlABF4	ABA responsive transcription factor
	Solyc07g063580.3.1	-0,543				LOC101263766	ABSCISIC ACID-INSENSITIVE 5-like protein 7
	Solyc10g005200.3.1	0,878				LOC101251949	protein C2-DOMAIN ABA-RELATED 1
	Solyc02g064670.1.1	-0,452				LOC101267573	protein C2-DOMAIN ABA-RELATED 11
	Solyc11g012780.2.1			0,942		LOC101267573	protein C2-DOMAIN ABA-RELATED 11
	Solyc01g109560.3.1	-1,16				LOC101256762	protein C2-DOMAIN ABA-RELATED 11-like
	Solyc04g015070.3.1			-0,993		LOC101256762	protein C2-DOMAIN ABA-RELATED 11-like
	Solyc02g088910.3.1			-0,593		LOC101259390	ninja-family protein AFP3
	Solyc05g012210.3.1		-1,59		1,452	LOC101266721	ninja-family protein AFP3
	Solyc10g080680.2.1	0,835				LOC101266721	ninja-family protein AFP3
	Solyc02g032950.3.1			1,846		LOC101258801	WRKY transcription factor 6
	Solyc04g056580.3.1	1,412				LOC101258801	WRKY transcription factor 6
Ethylene	Solyc10g047140.2.1	-1,251				ETR1	Ethylene receptor 1
	Solyc05g047530.3.1	-0,948				ETR2	ethylene receptor 2
	Solyc05g007120.3.1	0,743				ETR3	ethylene receptor neverripe
	Solyc09g075440.3.1			0,697		ETR3	ethylene receptor neverripe
	Solyc06g053710.3.1			1,633		ETR4	ethylene receptor homolog
	Solyc06g074700.3.1	-0,558				ETR4	ethylene receptor homolog

Solyc09g091160.1.1	-1,048			ETR4	ethylene receptor homolog
Solyc03g005760.1.1	1,196			ETR5	ethylene receptor homolog
Solyc10g011815.1.1	-2,155			TCTR1	ethylene-inducible CTR1-like protein kinase
Solyc10g083610.2.1		0,829		TCTR1	ethylene-inducible CTR1-like protein kinase
Solyc01g097980.3.1		0,733		TCTR2	TCTR2 protein
Solyc04g080830.3.1	1,467			TCTR2	TCTR2 protein
Solyc02g082140.3.1	0,619			CTR3	CTR1-like protein kinase
Solyc09g009090.3.1		0,637		CTR3	CTR1-like protein kinase
Solyc03g119160.3.1	-1,089			LOC101248690	EIN3-binding F-box protein 1
Solyc02g082670.3.1	-1,67			EBF1	EIN3-binding F-box protein 1
Solyc10g054030.2.1	-1,134			EBF2	EIN3-binding F-box protein 2
Solyc01g010000.3.1	1,266			EIL3	EIL3 protein
Solyc01g096810.3.1		0,983		EIL3	EIL3 protein
Solyc05g051200.1.1		1,463		ERF1	ethylene-responsive transcription factor 1
Solyc08g061250.3.1	-2,963			ERF1	ethylene-responsive transcription factor 1
Solyc02g062385.1.1	-1,651			ERF3	ethylene response factor 3
Solyc10g009110.1.1		1,624		ERF3	ethylene response factor 3
Solyc05g052030.1.1			-1,69	ERF4	ethylene response factor 4
Solyc02g062130.3.1	2,021			LOC101249381	ethylene-responsive transcription factor 4
Solyc07g053740.1.1		0,776		LOC101249381	ethylene-responsive transcription factor 4
Solyc02g081530.1.1	1,653			ERF5	ethylene response factor 5
Solyc06g068620.3.1	-1,156			SlERFD2	ethylene-responsive transcription factor 2a
Solyc12g056590.2.1		3,319		SlERFD2	ethylene-responsive transcription factor 2a
Solyc07g040710.3.1	-6,77			LOC101267548	ethylene-responsive transcription factor

Solyc08g023270.3.1	-0,896				EREB	ethylene responsive element binding protein
Solyc09g089930.2.1			4,979		EREB	ethylene responsive element binding protein
Solyc02g036270.3.1	0,628				LOC101267105	ethylene-responsive transcription factor 1B
Solyc08g081960.2.1			-1,927		LOC101264747	ethylene-responsive transcription factor CRF1
Solyc01g100730.3.1	-1,11				LOC101264838	ethylene-responsive transcription factor ERF054
Solyc07g054220.1.1			3,097		LOC101264838	ethylene-responsive transcription factor ERF054
Solyc01g106390.3.1	-1,801				LOC101258880	ethylene-responsive transcription factor ERF061
Solyc02g086130.3.1	1,34				LOC101257992	ethylene-responsive transcription factor ERF061
Solyc08g008305.1.1			3,876	1,75	LOC101258880	ethylene-responsive transcription factor ERF061
Solyc08g082210.3.1			2,644		LOC101257992	ethylene-responsive transcription factor ERF061
Solyc02g069770.3.1	-1,746				LOC101268529	ethylene-responsive transcription factor RAP2-10
Solyc12g056980.1.1		0,817			LOC101250096	ethylene-responsive transcription factor RAP2-13
Solyc04g074490.3.1	-0,83				LOC101252772	ethylene-responsive transcription factor RAP2-7-like
Solyc02g080170.1.1	1,126				LOC101258696	APETALA2/ethylene response factor
Solyc01g044307.1.1	-1,177				LOC101262024	AP2-like ethylene-responsive transcription factor AIL5
Solyc07g032640.2.1	1,302				ER49	ethylene-responsive elongation factor EF-Ts precursor
Solyc01g104740.3.1		2,494	3,309		ER24	ethylene-responsive transcriptional coactivator
Solyc01g104750.3.1			2,055		ER24	ethylene-responsive transcriptional coactivator

	Solyc02g071200.3.1	0,974		ER24	ethylene-responsive transcriptional coactivator
	Solyc12g096560.2.1	-0,873		ER24	ethylene-responsive transcriptional coactivator
	Solyc01g100580.3.1	-0,868		SlRAV2	transcription factor RELATED TO ABI3/VP1 2
	Solyc05g009790.1.1		1,725	SlRAV2	transcription factor RELATED TO ABI3/VP1 2
	Solyc01g107320.3.1	0,644		LOC101258194	WRKY transcription factor 22-like
Jasmonic acid	Solyc06g076450.3.1	-2,243		LOC100134911	jasmonate ZIM-domain protein 1
	Solyc12g009220.2.1		3,292	LOC100134911	jasmonate ZIM-domain protein 1
	Solyc08g074630.2.1	-1,273		LEJA1	jasmonic acid 1%2C
	Solyc04g079110.1.1	0,836		JA2	jasmonic acid 2
	Solyc12g013620.2.1		4,592	JA2	jasmonic acid 2
	Solyc07g063410.3.1		1,896	JA2L	JA2-like
	Solyc03g098210.3.1	-2,7		LOC101262053	jasmonic acid-amido synthetase JAR1
	Solyc08g013720.3.1	1,401		LOC101268624	jasmonic acid-amido synthetase JAR1-like
	Solyc01g067480.3.1	0,622		MYC1	transcription factor MYC1
	Solyc03g077910.3.1	1,023		LOC101264068	transcription factor MYC2-like
	Solyc01g095900.3.1	1,451		LOC101267164	transcription factor MYC3-like
	Solyc10g009290.1.1		-1,424	LOC101267164	transcription factor MYC3-like
	Solyc05g052780.3.1	-0,798		LOC100301944	WRKY transcription factor 1
	Solyc06g068460.3.1		0,912	LOC100301944	WRKY transcription factor 1
Salicylic acid	Solyc05g010320.3.1	1,425		LOC101260990	salicylic acid-binding protein 2
	Solyc06g074220.3.1	-0,503		LOC101261578	salicylic acid-binding protein 2
	Solyc09g075010.3.1	-0,686		LOC101267790	serine/threonine-protein kinase EDR1-like

Solyc04g011670.3.1		-1,358		LOC101255681	TGACG-sequence-specific DNA-binding protein TGA-1A
Solyc10g080770.2.1		1,181		LOC543600	TGACG-sequence-specific DNA-binding protein TGA-2.1
Solyc10g080780.2.1		-0,608		LOC101245511	TGACG-sequence-specific DNA-binding protein TGA-2.1
Solyc11g068520.2.1	1,079			LOC101262685	transcription factor TGA2.2
Solyc07g066260.3.1	-0,84			LOC101262685	transcription factor TGA2.2
Solyc06g062550.4.1	-0,781			LOC101260964	transcription factor TGA2.2-like
Solyc01g106620.2.1			-6,227	LOC100191111	PR1 protein
Solyc01g095630.3.1		2,361		WRKY3	WRKY transcription factor
Solyc03g078080.3.1	1,432			WRKY3	WRKY transcription factor

Supplemental Information

Table S1. Stomatal aperture lengths and widths in wild type (WT) and *flacca* (*flc*) tomatoes grown in high (HH) or low (LL) VPD and transferred to low (HL) or high (LH) VPD for 24 h. Means ± SE, n = 10-23 stomata per genotype per treatment. Different letters indicate significant differences between treatments ($P < 0.05$) as determined by ANOVA and post-hoc Tukey HSD analyses.

	Stomatal aperture length (µm)	Stomatal aperture width (µm)
WT		
HH	16.59 ± 0.17^{d}	6.14 ± 0.09^{de}
LL	18.11 ± 0.20^{c}	7.11 ± 0.10^{c}
HL		6.60 ± 0.10^{cd}
LH		5.83 ± 0.09^{e}
flc		
HH	24.87 ± 0.30^{a}	11.27 ± 0.17^{a}
LL	23.45 ± 0.25^{b}	9.73 ± 0.12^{b}
HL		10.95 ± 0.15^{a}
LH		9.25 ± 0.15^{b}

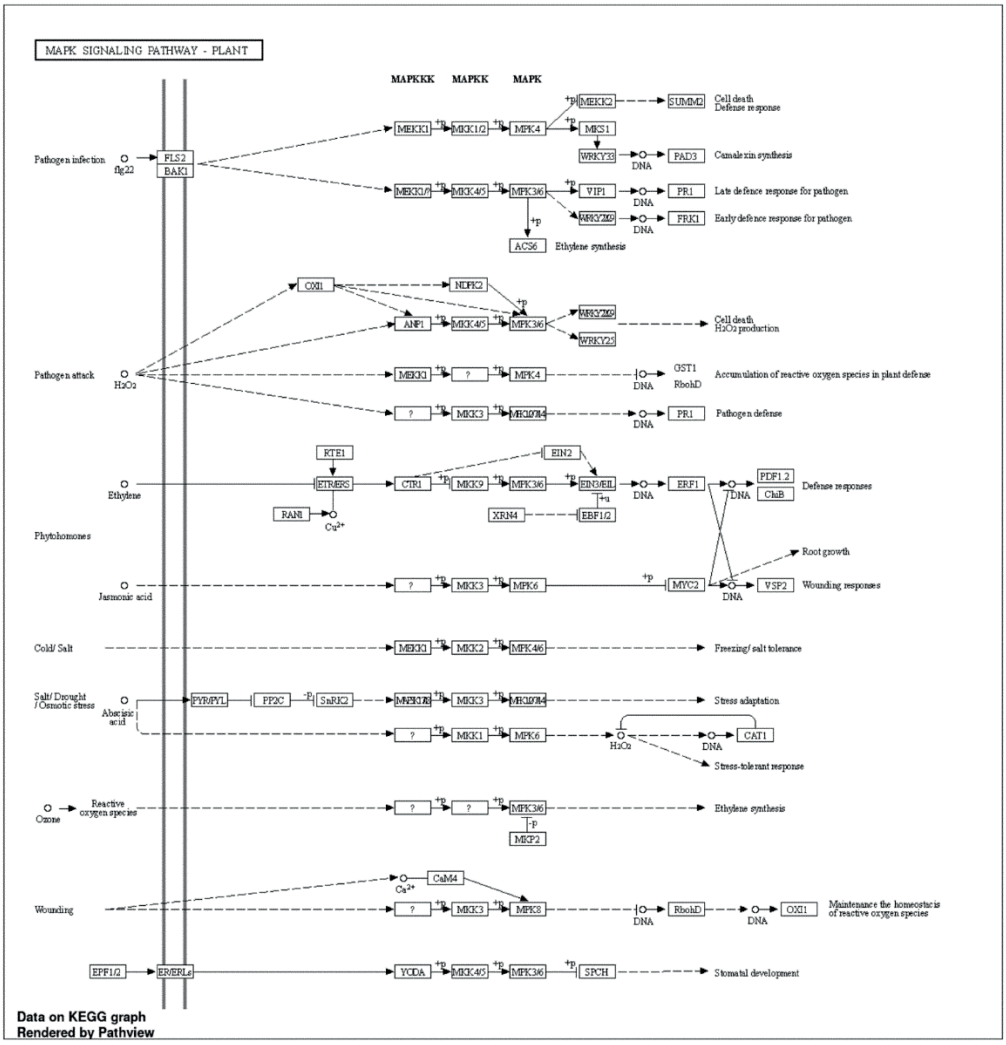

Fig. S1. Reference pathway from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) indicating MAPK Signaling Pathway regulation and downstream effects.

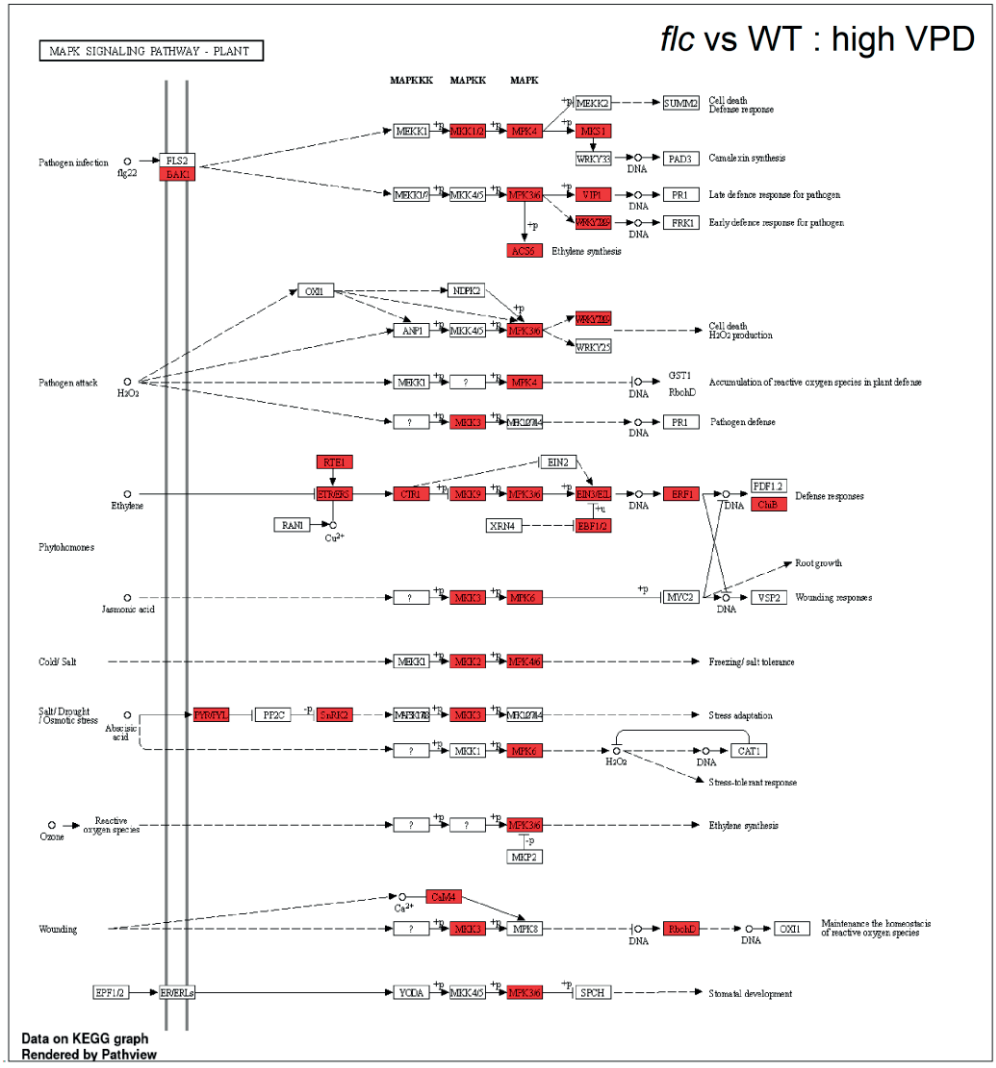

Fig. S2. Steady-state up- and down-regulation, indicated in red and blue respectively, of genes involved in the MAPK signalling pathway in *flc* relative to WT tomatoes grown in high VPD.

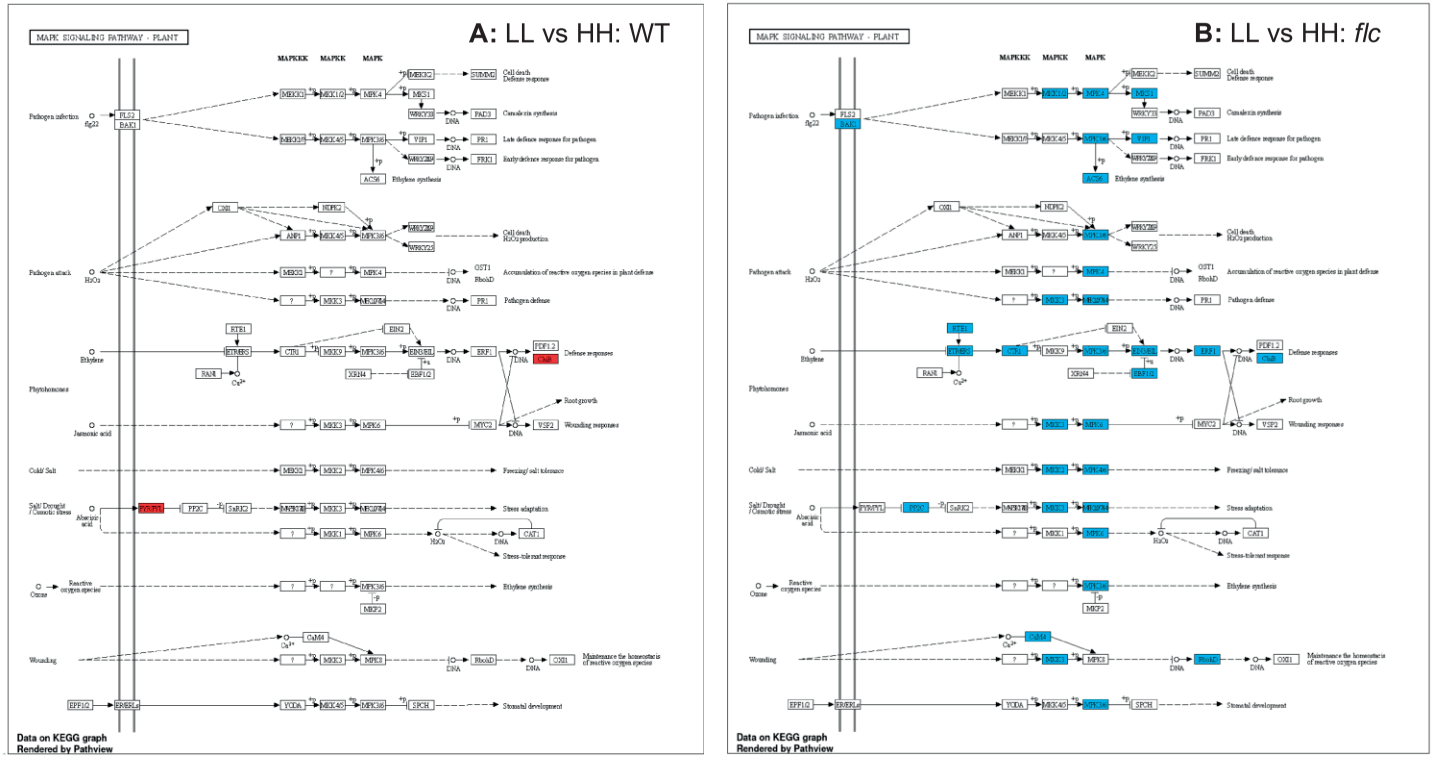

Fig. S3. Steady-state up- and down-regulation, indicated in red and blue respectively, of genes involved in the MAPK signalling pathway in A: WT plants grown in low VPD relative to high VPD, and B: *flc* plants grown in low VPD relative to high VPD.

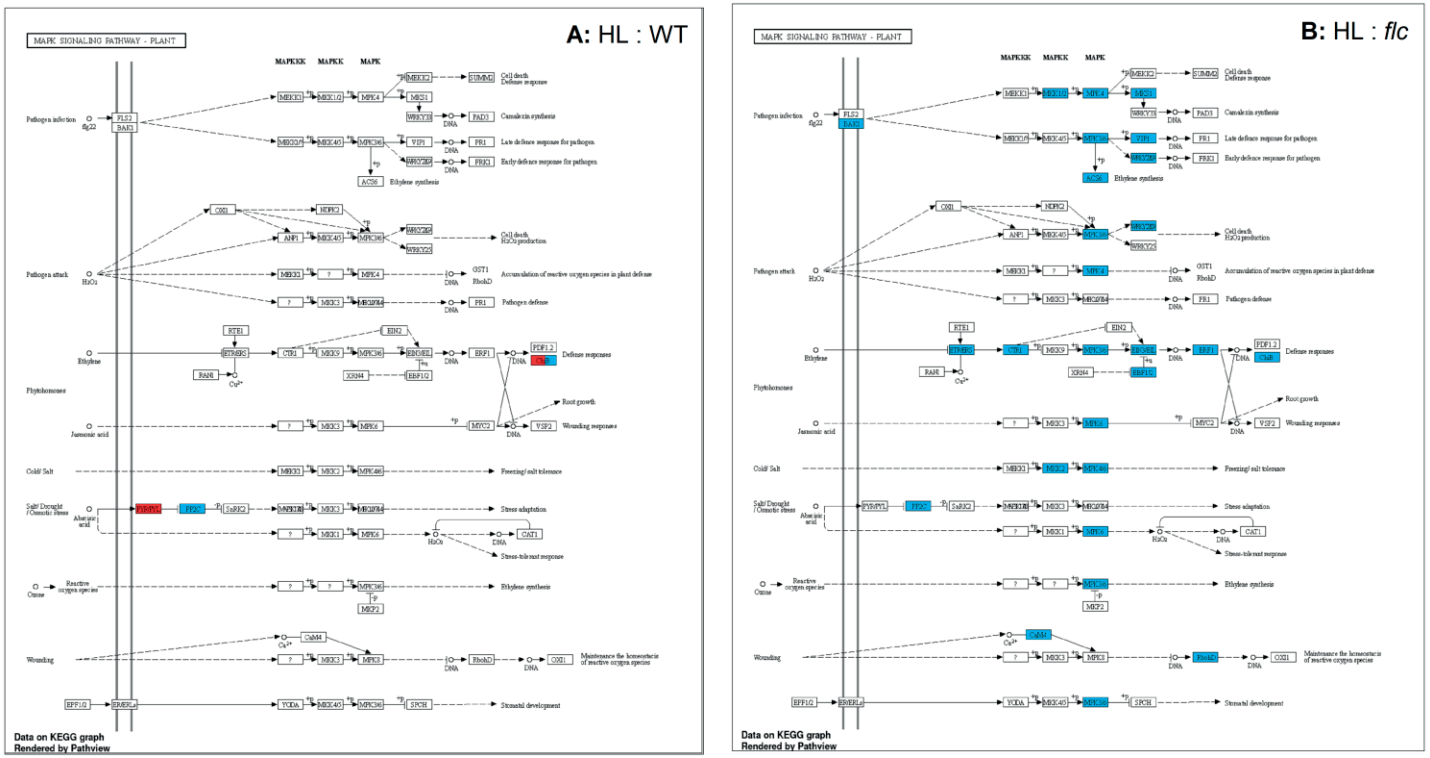

Fig. S4. Short-term up- and down-regulation, indicated in red and blue respectively, of genes involved in the MAPK signalling pathway in A: WT plants grown in high and transferred to low VPD, B: *flc* plants grown in high and transferred to low VPD.

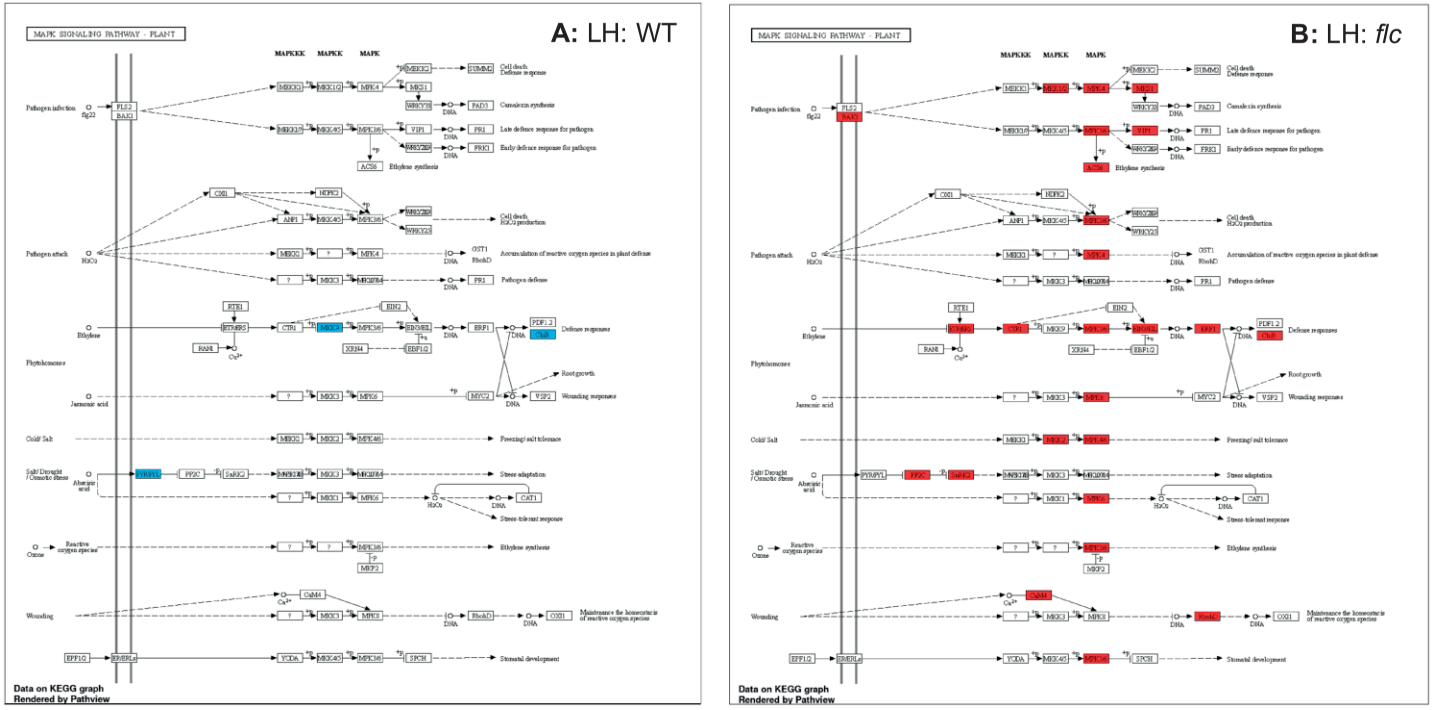

Fig. S5. Short-term up- and down-regulation, indicated in red and blue respectively, of genes involved in the MAPK signalling pathway in A: WT plants grown in low and transferred to high VPD, and B: *flc* plants grown in low and transferred to high VPD.

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