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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,

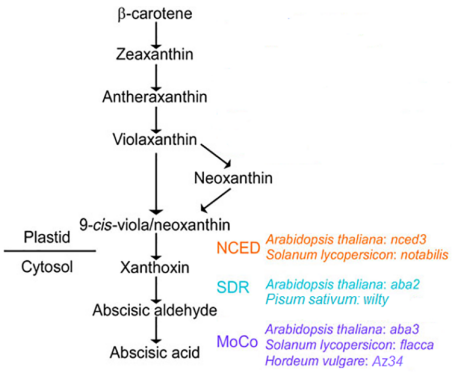
LMR = leaf mass ratio: the ratio of leaf mass to total plant mass and

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

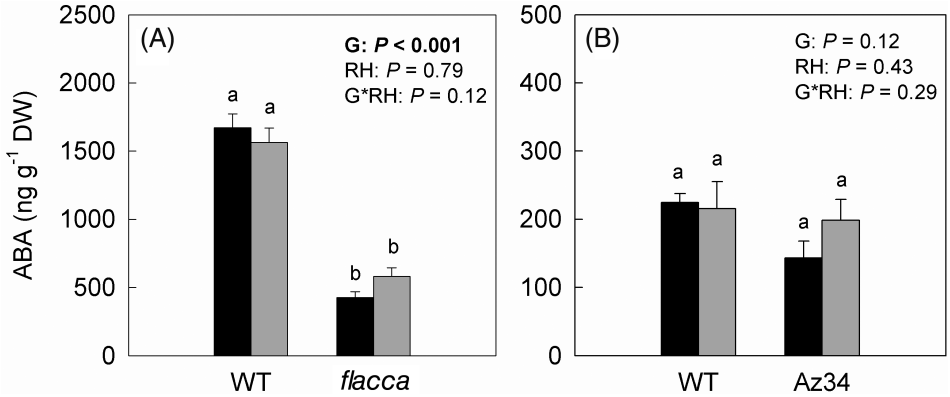


FIGURE 1 Foliar abscisic acid (ABA) concentrations in wild-type and ABA-deficient tomato (A) and barley (B) genotypes grown in 60% (black bars) or 90% (grey bars) RH in environmentally controlled growth chambers. Means ± SE shown, ABA: $n = 18$ for tomato WT and *flacca*, $n = 3$ for barley WT and Az34. 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

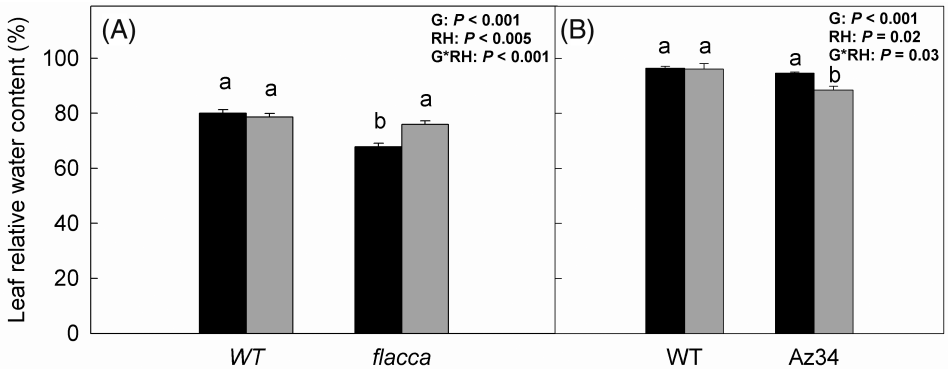


FIGURE 2 Leaf relative water contents of wild-type (WT) and abscisic acid-deficient tomato (A) and barley (B) genotypes grown in 60% (black bars) or 90% (grey bars) RH in environmentally controlled growth chambers. Means ± SE shown, $n = 12$. Different letters indicate significant differences between 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).

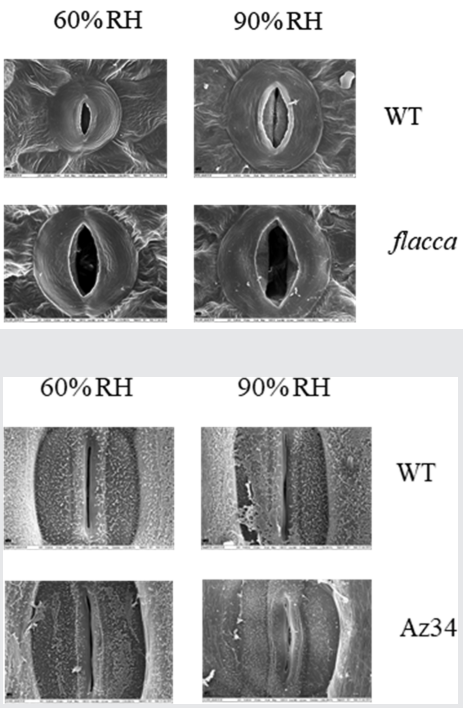
Both WT and *flacca* tomatoes had fewer, larger stomata in 90% compared to 60% RH (Table 2). In barley, RH did not affect the stomatal number of either genotype, but high RH decreased stomatal size. In tomatoes, WT and *flacca* stomata were 47 and 6% larger in 90% compared to 60% RH, respectively, while 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,

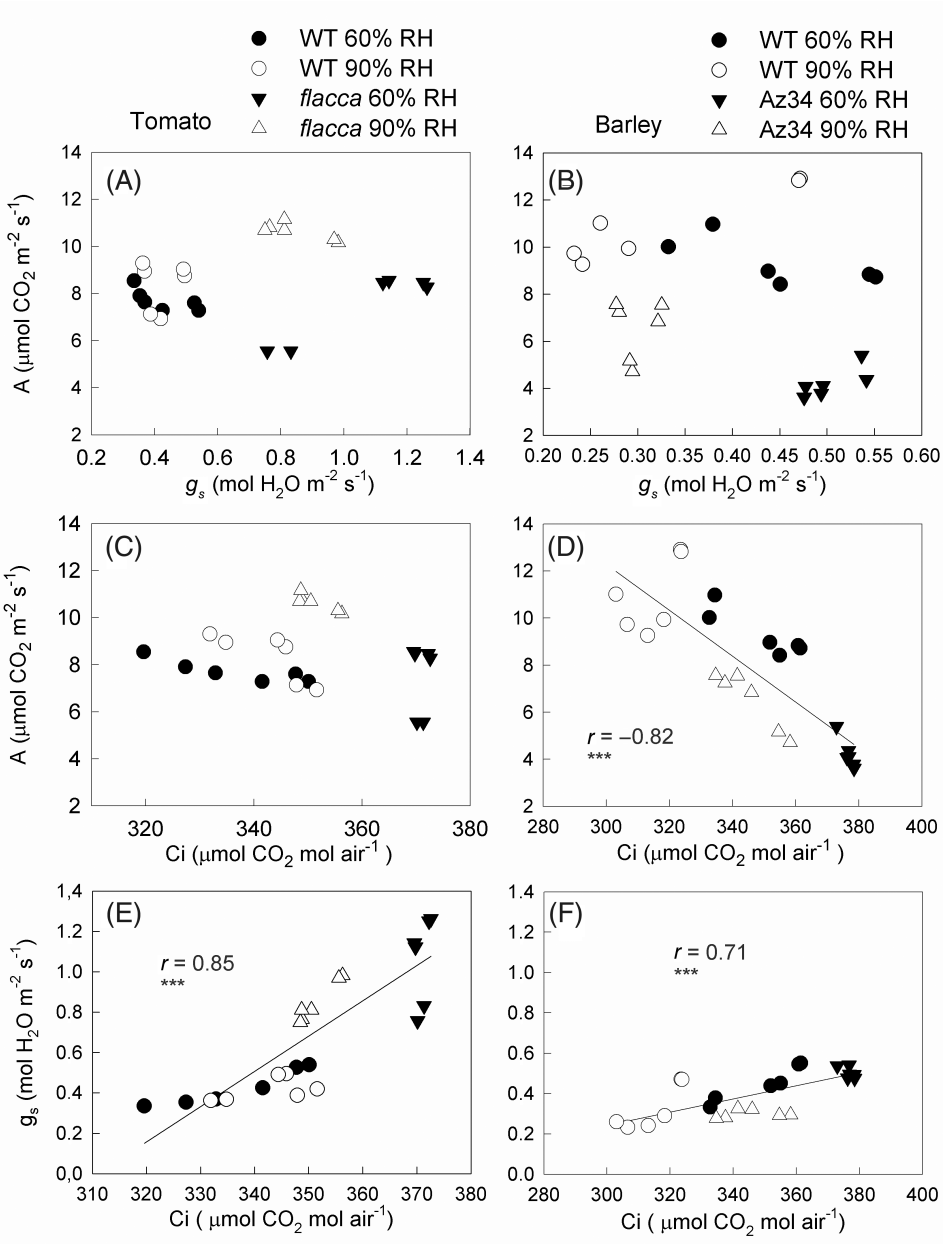
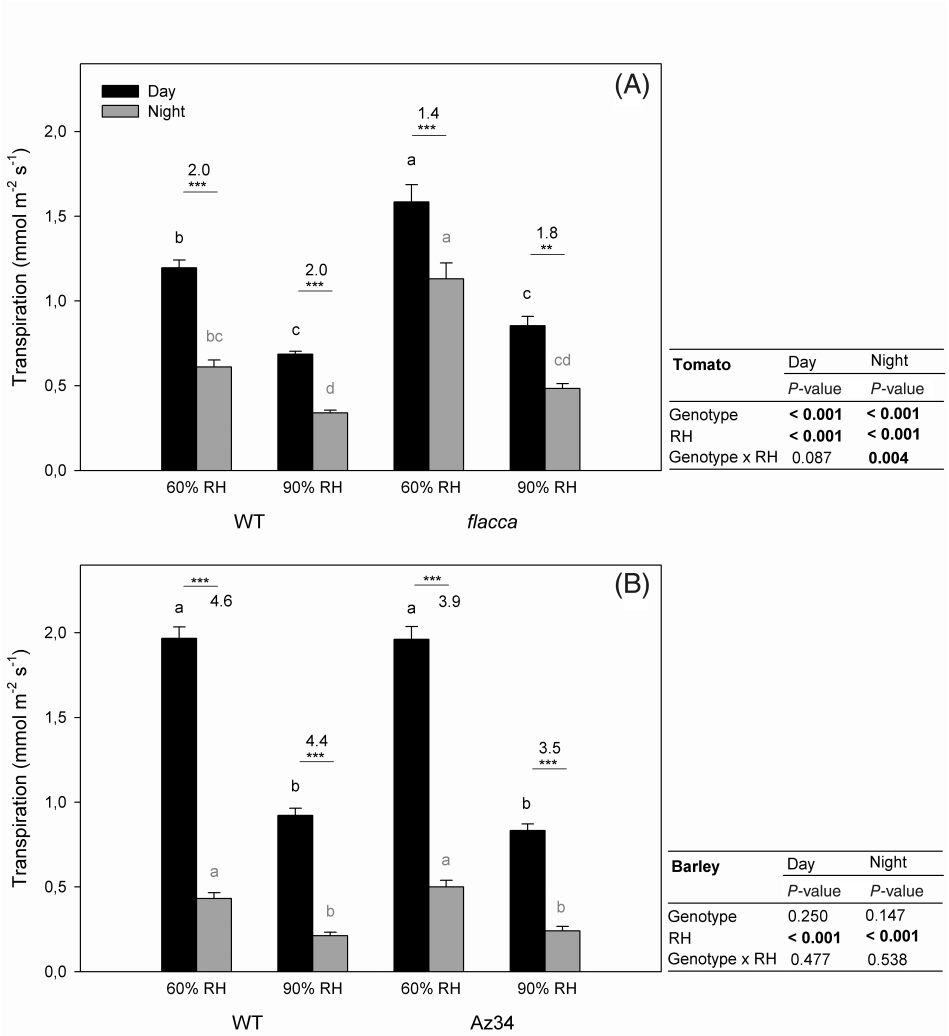


FIGURE 3 Leaf gas exchange of wild-type (WT) and abscisic acid-deficient tomato (A, C, E) and barley (B, D, F) genotypes grown in 60% or 90% RH in environmentally controlled growth chambers. Photosynthetic assimilation rate (A) plotted against stomatal conductance (A, B) (g_s) and internal CO_2 concentration (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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