Exploring variation in water use efficiency in barley (*Hordeum vulgare L.)* among wild and circadian mutant varieties, and its impact on responses to drought

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This thesis had a difficult gestation, as captured by Ted Hughes' The Thought Fox:

I imagine this midnight moment's forest:
Something else is alive
Beside the clock's loneliness
And this blank page where my fingers move.

Through the window I see no star:
Something more near
though deeper within darkness
Is entering the loneliness:

Cold, delicately as the dark snow
A fox's nose touches twig, leaf;
Two eyes serve a movement, that now
And again now, and now, and now

Sets neat prints into the snow
Between trees, and warily a lame
Shadow lags by stump and in hollow
Of a body that is bold to come

Across clearings, an eye,
A widening deepening greenness,
Brilliantly, concentratedly,
Coming about its own business

Till, with a sudden sharp hot stink of fox,
It enters the dark hole of the head.
The window is starless still; the clock ticks,
The page is printed

For Lucy, Ben & Theo Stevens.

Summary

Climate change will drive substantial changes in water resources and uses across the world, while population growth, changing dietary preferences and biofuel use will require greater agricultural output. Yet farmland is not likely to increase, therefore yields must rise. Episodes of drought arising from an increasingly-unstable climate will also become more frequent, severe and persistent. As a result, water use in agricultural production must be managed both where water availability appears satisfactory and where water stress exists. Finding novel phenotypes which blend high yield and parsimonious water use could help water-efficient production, and two approaches were considered for the major cereal crop, barley.

Circadian clock mutants introgressed into the elite variety, Bowman, were interrogated for their stomatal anatomical and physiological performance in relation to water use. In the short-term, Bowman A & g_s peaked later, and WUE_i earlier in the day than mutants under steady-state light, although dynamic responses to fluctuating light were not affected. Steady-state performance did not arise from anatomical causes, and were more likely to have a functional cause, where Bowman was sink-limited while clock mutants were source-limited. Drought of increasing severity was introduced to the clock mutants, and a mechanistic explanation of patterns of water use was sought in the production of the chloroplast-nuclear signalling molecule, phospho-adenosine phosphate (PAP). PAP concentration tends to be elevated in clock mutants compared to Bowman, and is elicited by drought stress. Clock mutants have better WUE_i under drought than Bowman, and are more responsive to fluctuations in light, leading to improved relative yield.

The water-use efficiency of a selection of wild barleys and landraces were also characterised relative to elite varieties. Stomatal anatomy was linked to g_s, A and WUE_i. Wild barleys and landraces sometimes produced better yield components (such as tillering) than elite varieties despite lacking targeted breeding. Meanwhile, phenotypic responses of the wild barleys were compared with the drought-tolerant variety, Bowman, under water stress conditions at germination and at tillering. Drought is an extreme form of WUE_i risk, and water stress initiated a range behaviours in wild barleys that ranged between conservative and non-conservative but were seldom clear-cut in their overall phenotype. Drought affects stomatal density and size when initiated early in development. Physiological responses were more-important at tillering. Tiller number, leaf area and growth stage effectively differentiated between varieties, and all are valuable yield components that could be targeted by breeders.

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Abbreviations

A	Assimilation rate of CO_2 (µmol m^{-2} s^{-1})
A_{max}	Maximum assimilation rate of CO_2
ABA	Abscisic Acid
C_i	Leaf internal CO_2 concentration
[CO_2]	CO_2 concentration
DAS	Days After Sowing
ET	Evapotranspiration
FC	Field Capacity
Fq'/Fm'	PSII Operating Efficiency
Fv/Fm	Maximum PSII Quantum Efficiency
Fv'/Fm'	Maximum PSII Quantum Efficiency in the light
GC	Guard Cell(s)
g_{max}	Maximum rate of stomatal conductance
g_{min}	Minimum rate of stomatal conductance
g_s	Stomatal conductance to water vapour (mmol or mol m^{-2} s^{-1})
HI	Harvest Index
IRGA	Infra-Red Gas Analyser
J	Rate of electron transport for the regeneration of RuBP
LED	Light Emitting Diode
NILs	Near-Isogenic Lines
NPQ	Non-Photochemical Quenching
PAP	Phospho-Adenosine Phosphate
PAR	Photosynthetically Active Radiation
Φ	Phi, the initial slope of a light response curve
RWC	Relative Water Content
SD	Stomatal Density
SLA	Specific Leaf Area
Sl_{max}	Maximum slope of g_s or A under a change of light intensity
SS	Stomatal Size
SI	Stomatal Index
ROS	Reactive Oxygen Species
τ	Tau, a constant for the time taken to reach 63% of a saturation value
TGW	Thousand-Grain Weight
Θ	Theta, the curvature of a slope in a light response curve
Vc_{max}	Maximum rate of Rubisco activation
VPD	Vapour Pressure Deficit
WUE_i	Intrinsic Water Use Efficiency (A g_s^{-1})
XRN	Exoribonuclease

Chapter 1: Introduction to the physiology and anatomy of wild and clock mutant barleys, and their relationship to drought.

1.1. General Introduction

Climate change is the lens through which all discussions of future environmental behaviour must be seen (IPCC, 2014) but there are other factors that are also important to human flourishing. There are around 7.2 billion people on earth at present, with that population expected to rise to 9.6 billion by 2050 (McGuire, 2013). Population growth is just one element of changing demand for food. Replacement of fossil fuels with biofuels and changing dietary preferences also have a part to play (Amin et al., 2006), along with rising urbanisation (Jones and Kandel, 1992). The consequence is that more land is probably not going to be made available for farming, so crop productivity will have to rise, with an urgent requirement for a doubling of yields by 2050 (Ray et al., 2013). A small number of monocots are responsible for the vast majority of all calories consumed globally – rice, wheat and maize are staples for 80% of humanity (Maclean et al., 2013).

Population pressures aside, climate change in itself presents a number of additional threats that may interact with human and plant behaviour. Rising CO_2 concentrations ([CO_2]) along with other greenhouse gases are expected to lead to higher global mean surface temperatures (IPCC, 2014), and recent months have witnessed a slew of reports highlighting the critical decision points now faced by politicians and the risks of inaction (Moore et al., 2019, Watts et al., 2018). Not only are mean temperatures expected to rise, but climate extremes are becoming more-common, with heat waves, droughts and extreme precipitation events expected to occur more frequently, for longer and with greater severity (IPCC, 2014). Although there is a fertilisation effect of higher atmospheric [CO_2] (Ainsworth and Long, 2005, Leakey, 2009) helping plants deliver higher yields, there are secondary countervailing effects on ambient and leaf temperature and water use / availability which tend to reduce the positive impact of fertilisation (Hatfield et al., 2011, Cai et al., 2016).

One critical manner in which plants are linked with water us through evapotranspiration – the process by which liquid water enters the gas phase either from the soil (evaporation) or from plants (transpiration). The impact on climate change on evapotranspiration is of grave importance. Greater evapotranspiration owing to higher temperatures will lead to lower soil moisture content: there is a link between stomatal conductance in the leaves and hydraulic conductance in the stem mediated by water potential as well as direct evaporation from the soil (Aasamaa et al., 2001). *Ceteris paribus*, higher temperatures will have a negative effect on yield, even with greater transpiration through the direct thermal damage as well as the reduction in water availability in the soil (Hatfield and Prueger, 2015). One of the key determinants of the rate of transpiration is the number, size and position of stomata, small pores on plant external surfaces (Hetherington and Woodward, 2003).

1.1.1 Evolution, development and morphology of stomata

Stomata are microscopic pores on the aerial surfaces of plants, primarily leaves, that are the nexus between the internal (leaf) spaces and the bulk atmosphere (Hetherington and Woodward, 2003). Stomata act as valves, balancing the plant's requirement for CO_2 for autotrophy with the need to maintain high internal water potential and manage water loss via transpiration (Morison et al., 2008). Stomata have been estimated to have evolved around 410 million years ago, along with a waterproof cuticle, and may be important in the emergence of vascular plants (Hetherington and Woodward, 2003, Edwards et al., 1998). The general morphology of stomata has remained essentially unchanged since then (Beerling and Woodward, 1997). Initially, stomata were surrounded by a pair of guard cells, but more recent evolution has included the development of subsidiary cells (Edwards et al., 1998), which allow rapid shuttling of solutes with guard cells for the adjustment of osmotic pressure and thus stomatal pore size (Franks and Farquhar, 2007). Still more recently (about 50-70 million years ago) the grasses evolved a distinctive dumbbell-shaped stoma which facilitated still-more rapid responses to changing environmental conditions (Schafer et al., 2018). That the stomatal complex of guard cells and subsidiary cells is evolutionarily conserved suggests its usefulness in fine, rapid control of stomatal

aperture (Bertolino et al., 2019), with dumbbell-shaped guard cells having rapid stomatal responses owing to their high surface area-to-volume ratio (Hetherington and Woodward, 2003) permitting faster osmotic shuttling (Franks and Farquhar, 2007).

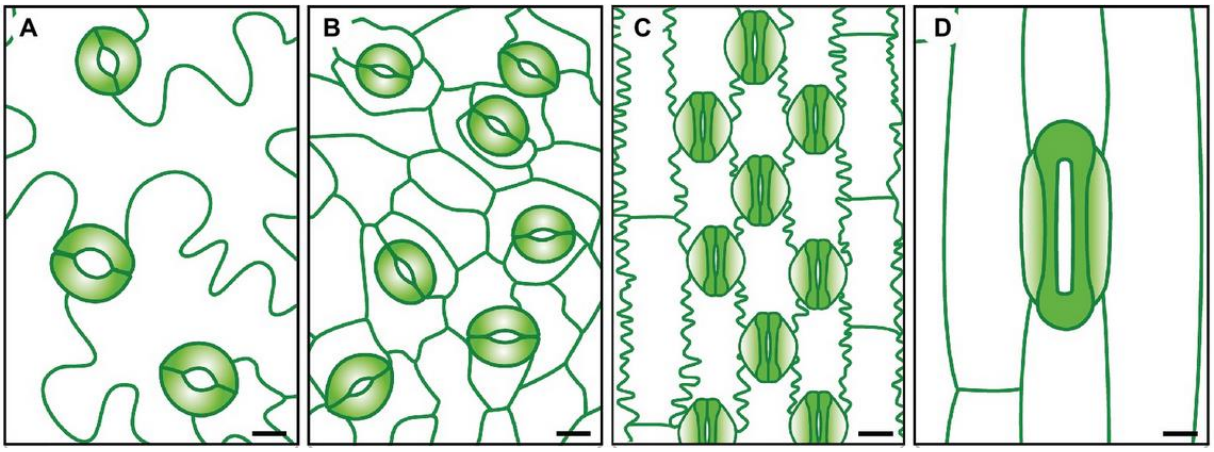

Figure 1.1. The variety of sizes, densities and morphologies of stomata. Kidney-shaped guard-cells in A) *Arabidopsis thaliana* and B) *Phaseolus vulgaris*. Dumbbell-shaped guard with subsidiary cells in C) *Oryza sativa* and D) *Triticum aestivum*. Scale of 10 µm. Reproduced from Bertolino et al. (2019).

From a developmental perspective, the patterning of stomata on the epidermis is under tight genetic control (Dow and Bergmann, 2014). Grass leaves grow in a polarised fashion from leaf base at the meristem to tip, with the epidermis consisting of longitudinal files of cells, in some of which are regularly-spaced stomata (Fig. 1.1); the developmental stage of the epidermal cells as a whole is of young cells at the base and mature cells at the tip (Croxdale, 2000). Six stages have been identified in the development of stomata in grasses (Hepworth et al., 2018). Undifferentiated cells proliferate in files at the base of the developing leaf, and alternate cells enter the stomatal development pathway (Hepworth et al., 2018). Protodermal cells that adjoin guard mother cells divide asymmetrically to produce subsidiary cells which then mature, while the guard mother cells divide symmetrically to form guard cells proper by elongation and maturation (Bergmann and Sack, 2007). Stomatal development is commonly arrested in monocots, leading to regular spacing of stomata in their files (Bergmann and Sack, 2007), a phenomenon that is thought to be important for efficient gas exchange (Sachs, 1991). Stomatal development is determined in given $leaf_n$ by environmental sensing in $leaf_{n-1}$ (the next oldest leaf) and other previous leaves (Lake et al., 2001), with earlier-emerging leaves more-susceptible to

water stress (Jordan et al., 1975). The major abiotic stress hormone, abscisic acid, accumulated most in young leaves, while stomatal conductance decreased most in older cotton leaves under water stress (Jordan et al., 1975) and the plastochron (the rate at which new leaves emerge) is invariant under constant temperature, with leaves being initiated at regular intervals (Dale and Milthorpe, 1981).

The number and size of stomata is thus under genetic control, and the range for stomatal conductance lies between 0 and some maximum value gs_{max}, a theoretical number determined by physical constants (e.g. the diffusivity of water in air), stomatal density and aperture, and guard cell size (Doheny-Adams et al., 2012, Lawson and Blatt, 2014). Anatomical gs_{max} can guide insights about leaf g_s at high light intensities. Light intensity, ambient [CO_2] and water availability are the major environmental determinants of stomatal density and size (Hetherington and Woodward, 2003, Doheny-Adams et al., 2012). Consequently, environmental conditions during development will have an impact on subsequent stomatal patterning (Doheny-Adams et al., 2012). Smaller stomata are expected to respond more-rapidly to short-term changes in environmental conditions (for instance, sun-flecks) (Franks and Farquhar, 2007, Lawson and Blatt, 2014, Drake et al., 2013), which should reduce the mismatch between the rate of change of photosynthesis and that of stomatal conductance to water (Lawson and Vialet-Chabrand, 2019), where changes in g_s may occur as much as an order of magnitude slower than for *A* (Lawson et al., 2010). Furthermore, stomatal density has been associated with changes in maximal rates of gas exchange (Franks et al., 2015, Mohammed et al., 2019). Furthermore, studies have repeatedly shown a trade-off between size and density (Franks and Beerling, 2009a, Lawson and Blatt, 2014) although the trade-off differs across taxa and furthermore, there may be other influences on speed of response than stomatal size (Franks and Farquhar, 2007, McAusland et al., 2016). It is usual to distinguish the impact of larger stomata from higher stomatal density, with size negatively correlated to speed of response to changing environmental conditions through faster shuttling of osmotica between guard and subsidiary cells (Franks and Farquhar, 2007) as well as shorter diffusional pathways (Franks and Beerling, 2009b). Stomatal density is expected to correlate positively with greater gs, lower leaf

temperature, particularly around mid-day and thus maintenance of assimilation and yield (Fischer et al., 1998).

1.1.2 Control of stomatal aperture

The primary abiotic stresses faced by plants are changes in light, temperature, vapour pressure deficit (VPD, air humidity relative to saturation), CO_2 concentration and water stress (Fig. 1.2), and stomata are responsive to changes in all these (Blatt, 2000). There is disparity in stomatal sensitivity and responsiveness among different species (Lawson et al., 2012, Lawson et al., 2003, Lawson and Vialet-Chabrand, 2019). Typically, stomata in C3 (and C4) species open in response to increasing or high light intensity, low internal [CO_2], high temperatures and low VPD. Conversely, stomatal closure is driven by low or decreasing light, high internal [CO_2] and high VPD as well as hormones such as ABA (Outlaw, 2003, Berry et al., 2010, Elliott-Kingston et al., 2016, Franks and Farquhar, 2001, Inoue and Kinoshita, 2017, Mott and Peak, 2013, Poole et al., 2000, Shimizu et al., 2015, Vialet-Chabrand et al., 2017b, Vialet-Chabrand et al., 2017c, Wang et al., 2008, Woodward, 1987). Signal transduction from abiotic stresses is often intermediated by kinase / phosphatase pathways, by secondary metabolites and by regulation of ion channels (Araujo, Fernie & Nunes-Nesi, 2011).

It can be tricky to separate stomatal temperature and VPD responses as saturation itself is temperature dependent (Mott & Peak, 2013). In terms of VPD itself, there are two broad mechanisms that may overlap (Mott & Peak, 2013, McAdam & Brodribb, 2015, Merilo et al., 2018). Lower plants generally use passive regulation at the leaf either by lower bulk leaf water status reducing guard cell turgor, or direct action on the guard cell itself (Mott & Peak, 2013, Oren et al., 1999). In angiosperms, there is a clear relationship between foliar ABA levels and stomatal aperture. This relationship may be of guard cell origin (an ABA biosynthesis pathway exists), and is also not from the xylem sap, but equally could have another leaf tissue origin (Bauer et al., 2013, Merilo et al., 2018). Stomatal conductance increases under higher temperatures at constant VPD, but this may be due to lowered viscosity of water and increased mesophyll conductance leading to increased guard cell turgor and stomatal aperture (Urban et al.,

2017a). Photosynthetic rate is also decoupled from stomatal conductance at higher temperatures Urban et al., 2017b).

There has been extensive study of the response of stomata to elevated [CO_2]. Higher levels of atmospheric CO_2 lead to a reduction in stomatal aperture by the activation of anion channels and K^+ efflux channels in the guard cell along with Ca^{2+} (Araujo, Fernie & Nunes-Nesi, 2011). Malate mediates between increased [CO_2] and the anion channel activator by an apoplastic route and this could be directly from the guard cell or be mesophyll signalling (Araujo, Fernie & Nunes-Nesi, 2011, Azoulay-Shemer et al., 2015). Reduction of gs under elevated [CO_2] is mainly due to increased activity of K^+ channels, the stimulation of Cl^- release from guard cells and increases in Ca^{2+} concentration causing stomatal closure (Brearley et al., 1997). Genes directly affecting signalling pathways under elevated [CO_2], include the *SLAC*1 (*Slow Anion Channel Associated 1*) gene which has been extensively associated with stomatal closure (Laanemets et al., 2013, Vahisalu et al., 2008). Several recent studies have also suggested a central role by carbonic anhydrase as a key regulatory factor in stomatal dynamics, with the bicarbonate ion activating SLAC1 anion channels (Xue et al., 2011, Hu et al., 2010). The hormone ABA is also involved, mainly through triggering the activation of the *OST1* (*Open STomata 1*) gene, a positive downstream regulator of ABA signalling that ultimately modulates ion channel activity in the guard cell (Chater et al., 2015, Merilo et al., 2015) although there is evidence of further ABA-independent pathways (Yamamoto et al., 2016). Other hormones known to be involved in partial stomatal closure under elevated [CO_2] include jasmonic acid (Geng et al., 2016). Translocation of sugars via phloem and the apoplast to stomata may be responsible for a feedback phenomenon via increased apoplastic osmolarity (Kang et al., 2007) or via guard cell sensing of internal sucrose concentrations, and the expression of ABA-related genes (Kelly et al., 2013, Bauer et al., 2013, Waadt et al., 2015). Sucrose may not be the only carbohydrate signal, and a number of other signals originating in the mesophyll may be involved in the process, including malate, pH changes, redox state signalling or a vapour-phase ions (Lawson et al., 2014, Matthews & Lawson, 2019).

Stomatal control of aperture in response to light comes via 2 or 3 separate routes (Fig. 1.2). Blue light is detected by phototropins while red light may be detected either as a result of photosynthesis in the

mesophyll or from the guard cell itself with sucrose accumulation in the guard cell being the result, or via starch degradation in the guard cell chloroplast, also resulting in sucrose or malate accumulation (Jezek & Blatt, 2017, Matthews & Lawson, 2016). High temperature cues at the guard cell level have recently been elucidated with isolated guard cells responding to high temperature with phototropins, H^+-ATPases and regulatory 14-3-3 proteins all involved, but not Ca^{2+} import (Kostaki et al., 2020).

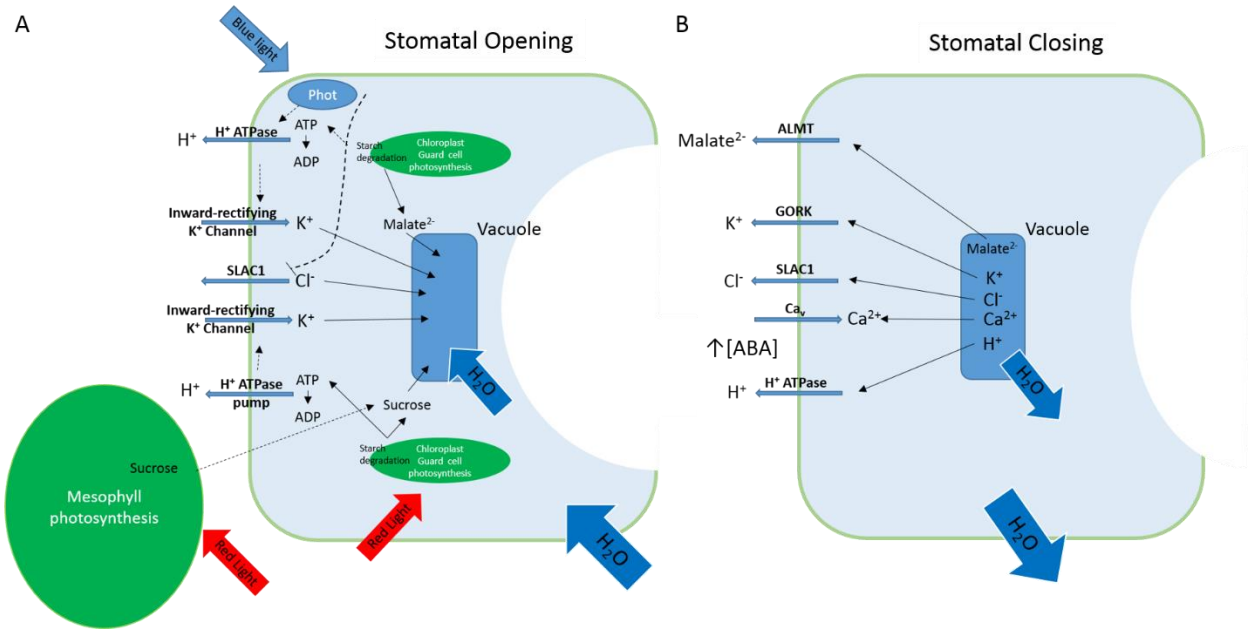

Figure 1.2. Guard cell control of stomatal aperture A. Guard cell control of stomatal aperture A. Around dawn, short wavelengths of light are detected by PHOT proteins which initiates a signal transduction cascade involving the phosphorylation of a range of intermediates including Blue Light Sensitive (BLUS) and finishing with an H^+-ATPase active transporter pumping protons out of the cytosol into the apoplast. ATP used by the pump may come from the mitochondria or from guard cell chloroplasts. In turn the hyperpolarised membrane potential causes an inward-rectify K^+ transporter, (KAT) to allow potassium ions to enter the cell. PHOT also prevents Cl^- ions from leaving the cell by downregulating the activity of Slow Anion Channels (SLAC) and other anion transporters. $Malate^{2-}$ ions may be generated by starch degradation, through the activity of PEP carboxylase and reduction of OAA. Red light induced guard cell photosynthesis may decrease C_i to some extent, but photosynthesis in the mesophyll is likely to be more important. In either case, sucrose concentration in the guard cell is increased, which prompts the phosphorylation of the H^+-ATPase pump. A low C_i signal is induced by red light and is communicated to Cl^- channels and SLAC1 by the protein High Temperature 1 (HT1) and CBC1/2 (Convergence of Blue light and CO_2) thereby linking both blue light and red light signalling pathways. A range of additional transporters such as K+/H+ exchangers, chloride channel and malate importers are present on the vacuolar membrane to allow solutes to move into the vacuole, and water follows owing to the lower water potential, which generates increased turgor pressure and thereby stomatal opening. B. The plant growth regulator Abscisic Acid (ABA) induces stomatal closure, initiating the process within minutes of the initiation of stress. ABA is sensed within the guard cell by a cytosolic receptor (*PYR1*) which initiates either a phosphorylation cascade or transient increases in cytosolic calcium, which is either released from internal stores (vacuole, endoplasmic reticulum) or taken up from the apoplast. These responses initially target the activity of an anion-out channel (*SLAC*), releasing Cl^- from the cell, and leading to a depolarisation of the membrane potential. These processes lead to inhibition of the plasma membrane H^+-ATPase and K^+ uptake via KAT (*Arabidopsis* K^+ channel), and stimulates activity of GORK (voltage-Gated Outward Rectifying K^+ channel), removing from cell. Additionally, Aluminium-activated Malate Transporters (ALMT) move malate ions out of the cell. In parallel, an equivalent set of channels operate in the vacuolar membrane to reduce solute concentration. The change in solute concentration and reduction in solute potential (becoming less negative) allows water to leave the cell by osmosis and reduces cell turgor pressure, leading to the reduction in stomatal aperture. Adapted from Santelia & Lawson (2016), Matthews, Vialet-Chabrand & Lawson (2019), Jezek & Blatt (2017) and Inoue & Kinoshita (2017).

1.1.3 Stomata and water-use efficiency

Since stomata must simultaneously control potentially conflicting demands – for CO_2 to diffuse into the plant for use in assimilation, and for water leave the plant to maintain the transpiration stream (Farquhar

and Sharkey, 1982), there is considerable interest in targeting intrinsic water use efficiency (WUE_i = CO_2 in for assimilation / H_2O out as stomatal conductance) as a means of optimising plant performance in the context of constrained agricultural water availability for improved yield (Condon et al., 2002). However, this approach has not been without its critics, who believe that the focus on WUE (sometimes also defined as biomass / total water used) is misleading and where effective use of water, irrespective of the quantity involved, is the relevant dimension (Blum, 2009). However, it has been shown that reducing stomatal density and thereby g_s can give enhanced biomass for lower water use in rice (Caine et al., 2019) and barley (Hughes et al., 2017), and strategies for improving WUEi for enhanced yield (such as increasing assimilation rates through genetic manipulation of photosynthetic pathways) are well known (Condon et al., 2004).

Steady-state measurements in the field have determined that g_s is directly proportional to A, and that furthermore, g_s is proportional to final yield mainly due to maintenance of canopy temperature depression around the middle of the day, which should improve photosynthetic reaction kinetics and possibly stress recovery through non-photochemical quenching (Guan et al., 2015, Kromdijk et al., 2016). 'Steady-state' measurements are defined here as those taken under constant conditions (typically mid-day in bright sunlight in the field; in the growth chamber or glasshouse, steady state conditions are easier to achieve). Such measurements have remained the mainstay of physiological research into WUE_i and its components (Ainsworth and Rogers, 2007), leading directly to insights of relevance to breeders, for instance the link between high g_s and yield (Fischer et al., 1998). Nevertheless, plant g_s and A responses even to steady-state light are not constant (Ng and Jarvis, 1980, Vialet-Chabrand et al., 2013) and more recent work has revealed a diurnal pattern of response in which A approximated a square wave and g_s a Gaussian curve (Matthews et al., 2018) modulated by negative feedback from sugar accumulation (Vialet-Chabrand et al., 2016, Graf et al., 2010, Haydon et al., 2013), leading to reduced stomatal aperture and slowed responses later in the afternoon (Vialet-Chabrand et al., 2016).

However, in line with Buckley's (2017) observation that it is instantaneous, marginal WUE ($\delta A\ \delta g_s^{-1}$) that is the key determinant of productivity, we can consider kinetic responses of stomata as equally valid

sources of productivity gain (Lawson et al., 2010). The assimilation rate adjusts within minutes in response to changing light (Fig. 1.2) or other environmental conditions (Ng and Jarvis, 1980, Whitehead and Teskey, 1995), while stomatal conductance to water vapour changes but slowly, and may be as much as an order of magnitude slower to respond (Lawson 2010). In the context of changing light conditions, therefore, the speed at which stomatal aperture can be adjusted becomes a critical determinant of potential productivity. A number of factors influence the rapidity of stomatal responses to environmental change, of which stomatal size is commonly-cited as the most important; small stomata may be faster to respond owing to greater surface area to volume ratio, although the presence and number of subsidiary cells may also be important and whether stomata are dumbbell or kidney-shaped, while stomatal clustering may also be a consideration (Lehmann and Or, 2015). Stomatal kinetic responses arise where environmental conditions change. Stomata change aperture to accommodate the new demands. By measuring the rate of change of g_s and A, greater understanding is reached and novel targets for breeding are generated (Raven, 2014, Lawson and Vialet-Chabrand, 2019, Lawson and Blatt, 2014). Fluctuating light intensity, VPD (vapour pressure deficit, the difference between the levels of moisture in the air and saturation) and temperature are the variables that a plant is most likely to experience during the day (Fig. 1.2); stomata open and close much more slowly in response to the changing environment (by around an order of magnitude) relative to the speed of photosynthetic responses (Qu et al., 2016, Lawson and Blatt, 2014, McAusland et al., 2016, Lawson et al., 2010). These response rates can be modelled and parameterised, for instance by a simple exponential growth model with start value, finish value and time constant (τ), being readily captured as parameters (Vialet-Chabrand et al., 2017b, Vialet-Chabrand et al., 2013). More recently this work has been extended beyond simple step changes in an environmental variable to continually fluctuating values (for instance in light levels) to mimic natural conditions, although this work remains in its infancy (Matthews et al., 2018, Vialet-Chabrand et al., 2017a).

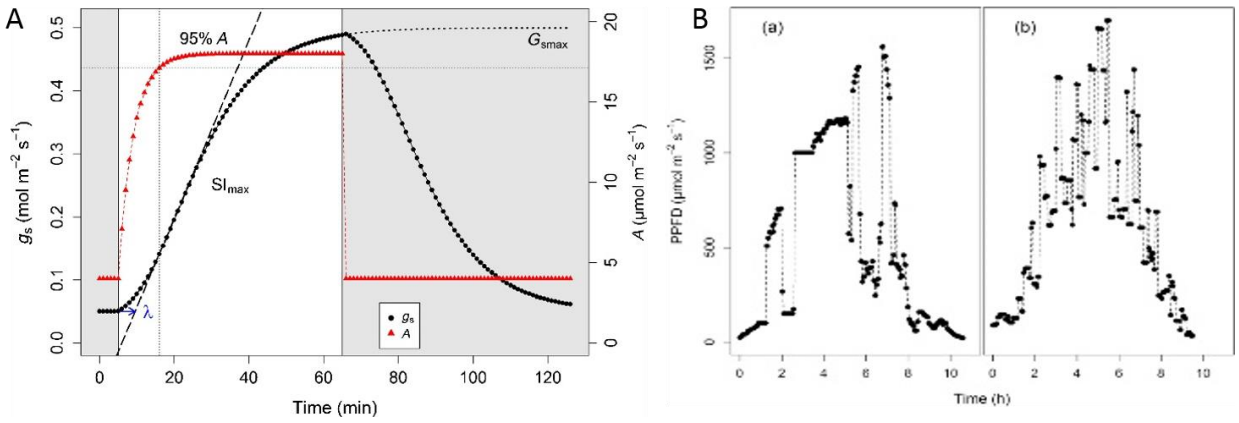

Figure 1.3. Modelling light regimes. A) Model of a step change in light from 100 to 1000 µmol m^{-2} s^{-1} PAR illustrating the lag between changes in assimilation induced by changes in Par, and changes in stomatal conductance. Reproduced from McAusland *et al*, 2016. B) Recording of fluctuating light intensity on the roof of the University of Essex as an example of the regimes used in fluctuating light experiments. Reproduced from Vialet-Chabrand *et al*, 2016.

Crops with C3 metabolism, from legumes to cereals, showed a wide diversity of kinetic responses when modelled (McAusland et al., 2016). Furthermore, modelling suggested further interactions between species and the speed of opening of stomata compared to speed of closing (McAusland et al., 2016), which in turn led to the proposal that there are a wide range of options available to manage dynamic water requirements for cooling alongside CO_2 demands for assimilation. Stomata in legumes, for instance, appear to prioritise limiting water loss when closing, while failing to prevent stomatal limitation of *A* when light levels increase (McAusland et al., 2016, Lawson and Blatt, 2014). Grasses have rapid responses, both in opening and closing in well-watered conditions compared to many dicots (Kumar et al., 2017, Munns et al., 2010, Qu et al., 2016).

1.1.4 Artificial selection in crops – major developments and bottlenecks

The Fertile Crescent of the Near East witnessed an astonishing revolution in technology between 10,000 and 8,000 BC. The emergence of settled farming populations from nomadic pastoralists has been associated with the domestication of a number of crops (Allaby et al., 2017), a process of cultural change for humans and change in allele frequency for plants. Data are emerging that suggest the rate of evolution of domestication traits (for instance, non-shattering spikes on the ear) was not constant for crops such as wheat (*Triticum monococcum*) or barley (*Hordeum vulgare*) (Allaby et al., 2017).

Furthermore, desirable traits did not emerge simultaneously. Changes in grain size and shape were two of the earlier features of domestication, appearing to evolve within centuries. Meanwhile the classic domestication trait - non-shattering spikes - only evolved some 1-2000 years later (Fuller, 2007).

Added in to this mix is the debate over the number of times that a cereal like barley has been domesticated. In the earlier years of the 20th Century, a multi-origin domestication was given wide credence (Åberg, 1938, Harlan and Zohary, 1966), but by the late 20th Century, this view had changed to one that saw the Fertile Crescent as the origin of all subsequent barley varieties, which gradually spread East and West towards Europe and Central Asia, and finally to South Asia, Africa and China (Badr et al., 2000). This interpretation remained somewhat contentious, and more recent genetic work has identified at least one other centre of barley domestication East of the Zagros mountains in Central Asia where the range of wild barley (*H. vulgare ssp. spontaneum*) extends as far as Kyrgyzstan, Afghanistan and western Pakistan (Morrell and Clegg, 2007). European, Near Eastern and Ethiopian barleys certainly appear distinct from those found in Central, Eastern and Southern Asia based on haplotype frequencies (Morrell and Clegg, 2007), with additional claims for independent domestication extending as far East as the Tibetan plateau (Dai et al., 2012), in line with Aberg's original discovery of a 6-rowed wild barley there (Åberg, 1938).

Following the earliest domestication and adoption of fortuitous mutations such as non-shattering spikes (Fuller, 2007), crops such as barley enjoyed increasing specificity to locales, environments and farming methods by selection (Newton et al., 2010). Before the advent of planned breeding programmes, which were initiated toward the end of the nineteenth century, relative to modern elite varieties these diverse cultivars were characterised by genetic heterogeneity both within and across genotypes (Newton et al., 2010). These varieties are commonly known as landraces (Zeven, 1998). They are distinguished today not by high yield but yield stability, particularly through adaptation to the local environment and disease resistance (Piffanelli et al., 2004). An example of the rediscovery of desirable adaptations is the

discovery of the *mlo* powdery mildew resistance gene in Ethiopian barley landraces (Piffanelli et al., 2004), now being introgressed into elite European cultivars.

With planned breeding programmes came a focus on high yield *per se* over other indicators of breeding success such as disease resistance (Zeven, 1998). Yet despite a broad expectation that landraces would rapidly disappear from the global seedbank, in fact cultivation of landraces persisted in more-marginal areas. For the reasons cited above, including disease resistance, adaptation to local environments and general yield stability, the last being an important consideration for subsistence farmers (Zeven, 1998, Newton et al., 2010). Nevertheless, in regions where the environment was favourable for cultivation of crops such as barley, landraces did largely disappear in the early decades of the twentieth century (Zeven, 1998). Today these landraces provide an important potential resource for modern breeders, but care must be exercised as it is known that diversity has been lost in a number of collections, and that duplications are very likely (Newton et al., 2010). Nevertheless, many of the landraces that now exist in global collections offer the potential to overcome abiotic stress arising due to climate change in regions as diverse as the north of Scotland and the Mediterranean basin (Monteagudo et al., 2019, Mahon et al., 2016, Dawson et al., 2015).

From the middle of the last century, crop improvement programmes made the next series of advances (Backes et al., 2003) which were instrumental in improving global access to calories, particularly in developing nations such as Mexico, The Philippines and India (Evenson and Gollin, 2003) even though there was considerable heterogeneity in benefits to consumers compared to producers and in regional terms (Evenson and Gollin, 2003). The Green Revolution targeted harvest index (the proportion of biomass used by humans) through the use of semi-dwarfing genes, and targeted nitrogen use efficiency to improve yields along with increased inputs of fertilizers, pesticides and water and other farm management practices (Evenson and Gollin, 2003, Bingham et al., 2012, Newton et al., 2011). More recently, the limits of the advances of the Green Revolution have become clear with yield plateaus identified in many critical production regions (Dawson et al., 2015, Grassini et al., 2013, van Ittersum et

al., 2013). The introduction of the semi-dwarfing gene has had downstream consequences, with the growth-inhibiting DELLA protein also reducing the capacity of plants to absorb nitrogen (Li et al., 2018). Redressing that imbalance would be a great boon to farmers, since use of inputs such as fertilizers must fall to meet climate change goals around fossil fuel use, maintenance of soil health and water quality (FAO, 2015, IPCC, 2019, van Ittersum et al., 2013, Li et al., 2018, Newton et al., 2010).

It is against this backdrop that breeders have turned to the diversity in older (pre-Green Revolution) germplasm as a potential source of novel opportunities to escape from the trap of arithmetic yield gains where geometric ones are needed (Grassini et al., 2013). in an attempt to bridge the yield gap identified by Ray et al. (2013), the search for novel germplasm in crops has therefore led to a resurgence of interest in the landraces and wild relatives of major crops, including barley (Backes et al., 2003, Brozynska et al., 2016, Lopes et al., 2015, Malysheva-Otto et al., 2007, Maroof et al., 1995, Newton et al., 2009, Wang et al., 2017). Along with wild barley accessions in global collections, landraces offer exciting possibilities to introduce resistance to biotic and abiotic stresses into modern varieties in light of breeding bottlenecks (Backes et al., 2003, Brozynska et al., 2016, Dawson et al., 2015), with some evidence of success already as genetic diversity in crops may have increased in recent years (van de Wouw et al., 2010). One of the difficulties with the existing collections of wild barleys and landraces, is the lack of genotyping and phenotyping that has been carried out (Newton et al., 2010), although the publication of the barley genome (Mayer et al., 2012) will assist gene discovery and identification, and by extension marker assisted breeding (Newton et al., 2010).

1.1.5 The impact of drought on anatomy, physiology and productivity in cereals

One of the predicted consequences of climate change is an increase in the severity, frequency and duration of water stress episodes (IPCC, 2014). Extreme water stress is often considered to be 'drought', but terms are loosely defined and vary from species to species. For instance, in the model C3 dicot *Arabidopsis thaliana*, mild to moderate drought is defined as 30% of field capacity (actual mass of water compared to mass of water in saturated, free-drained pots) (Harb et al., 2010), but this may not apply to

plants that have a pre-existing ability to withstand drought. What can be stated is that plants' responses to abiotic stress such as limited water availability can often be resolved into two contrasting behaviours: plants with *conservative* phenotypes prioritise survival over growth, while plants with *non-conservative* phenotypes do the opposite (Caine et al., 2019, Chapin, 1980, Valladares et al., 2000). Within the conservative phenotype, two further behaviours can be distinguished: *escape* from stress (or potential stress) and *avoidance* of stress (Farooq et al., 2009). Plants that use the escape behaviour favour early maturation to ensure that the annual life-cycle is complete ahead of late season droughts. This ensures survival at the cost of a shorter growing season and thus lower yield. By contrast, the avoidant plant will use mechanisms that reduce water loss (the first of which is to close stomata) as well as seeking to maintain turgor; these behaviours also have clear yield consequences (Farooq et al., 2009). Non-conservative plants seek to maintain higher water potentials by continuing to use the available soil moisture. Therefore, the non-conservative phenotype witnesses open stomata under drought, along with enhanced reactive oxygen species detoxification and increased used of osmoprotectants such as proline (Claeys and Inze, 2013, Harb et al., 2010). For breeders seeking to improve yield, non-conservative traits are preferred. Higher g_s, a non-conservative trait, is known to drive higher assimilation and by extension yield under well-watered conditions (Fischer et al., 1998, Wong et al., 1979). But work on water stress has recently shown in species such as *Arabidopsis*, rice and barley that plants with reduced stomatal density (SD) are capable of maintaining yield compared to high SD plants through a reduction in g_s (Hepworth et al., 2015, Caine et al., 2019, Hughes et al., 2017). It may well be that since stomatal density was also accompanied by a reduction in stomatal size this could explain the impact on yield, since smaller stomata are expected to react more-rapidly to changing conditions, offering an alternate route to yield maintenance (but not increase) via increased conservatism (Qu et al., 2016, Bertolino et al., 2019, Caine et al., 2019, Hughes et al., 2017, Lawson and Blatt, 2014). To reiterate, the behaviour of the non-conservative plant serves to improve yield under milder droughts relative to conservative plants and is therefore a desirable breeding trait, but under severe droughts,

non-conservatism may end in plant death and thus no yield at all (Munns et al., 2010, Claeys and Inze, 2013), although *in extremis*, grasses have the ability to switch strategies (Munns et al., 2010).

As has been discussed, stomatal responses diverge according to the conservative–non-conservative phenotype under water stress which may vary not only between species but even within genotypes for *T. aestivum* or *H. vulgare* (Munns et al., 2010). Skelton et al. (2015), using a different but analogous schema (isohydry- anisohydry), also identified conservative behaviours with rapid stomatal responses and the maintenance of higher leaf water potential compared to behaviours that appear more non-conservative that include open stomata and higher rates of carbon assimilation. Non-conservatism is more-closely associated with slower stomatal responses under drought compared to conservative rice cultivars, which had more-rapid responses (Qu et al., 2016).

It is clear that barley is non-conservative in general at the onset of stress (Munns et al., 2010) but whether that behaviour can change as drought progresses and becomes more-severe is not so certain, although some evidence exists that a variable phenotype along these lines exists in some grasses (Balachowski et al., 2016) including barley (Munns et al., 2010). There is certainly pressure on researchers to identify ideotypes that emphasise conservative behaviours under the threat of climate change (Bertolino et al., 2019), where more-extreme conditions require cultivars that prioritise survival as much as growth (Claeys and Inze, 2013).

1.1.6 The plant circadian clock

The earth is locked into a 24 hour cycle of days and nights of varying length, and this cycle contains important information that can be used by organisms to anticipate future environmental changes over periods from hours to days up to seasons and years. Substantially all eukaryotes as well as cyanobacteria possess a circadian clock – a series of interlocking negative and positive feedback loops (Fig. 1.3) - that allow an organism to monitor and interact with this 24 hour (diel) cycle (Goldbeter, 2008, Kondo and Ishiura, 1999). The existence of the clock in plants has been appreciated since Darwin's time with

multiple independent evolutions of the clock in different phyla suggestive of its importance (Kondo and Ishiura, 1999). In plants, the circadian clock consists of up to four major groups of transcription factors that are elicited at specific times of day (Fig. 1.3) and that operate as an entire mutually-dependent system (Goldbeter, 2008). Around dawn, the proteins LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) repress the transcription of the *Pseudo-Response Regulator* (*PRR*) genes *PRR5*, *PRR7* and *PRR9* whose products have expression peaks through the middle of the day; of the Evening Complex (EC) comprising *Lux Arrythmo* (*LUX*), *Early Flowering 3* (*ELF3*) and *Early Flowering 4* (*ELF4*); and of the *Timing Of Chlorophyll A/B Expression 1* (*TOC1*) (Hicks et al, 1996, Doyle et al., 2002, Nusinow et al., 2011, Hsu and Harmer, 2014). The midday group reciprocally represses *CCA1* and *LHY*, while the Evening Complex downregulates expression of the midday group as well, and TOC1 represses transcription of elements of all the other three main components of the clock; finally, the EC indirectly promotes LHY and CCA1 activity (Hsu and Harmer, 2014, Huang and Nusinow, 2016).

Table 1.1: Phenotypes of mutants of key circadian clock genes in *A. thaliana* and timing of peak expression of those genes. Adapted from Hsu & Harmer, 2014

Gene	Timing of peak expression	Function	Overexpression phenotype	Loss-of-function phenotype
CCA1	Morning	Transcription factor	Arrhythmic	Short period
LHY	Morning	Transcription factor	Arrhythmic	Short period
PRR5	Afternoon	Transcription factor	Long period	Short period
PRR7	After dawn	Transcription factor	Long period	Long period
PRR9	After dawn	Transcription factor	Short period	Long period
RVE8	Afternoon	Transcription factor	Long period	Long period
ELF3	Evening	Transcription regulator	Long period	Arrhythmic
ELF4	Evening	Transcription regulator		Arrhythmic
LUX	Evening	Transcription factor	Arrhythmic	Arrhythmic
TOC1	Evening	Transcription factor	Arrhythmic	Short period
ZTL	Evening	F-box protein, blue light receptor		Long period

The plant circadian clock can receive inputs from multiple sources, both external (such as light and temperature) and internal (the concentrations of hormones such as ABA, sugars or metal ions) (Hanano et al., 2006, Hayden et al., 2013, Kusakina et al., 2014, Ruiz et al., 2018, Somers, Devlin & Kay, 1998, Webb et al., 2019). This work will focus in particular on the impact of light on the clock as that is the biggest environmental stimulus to the clock (Millar, 2003). Light reception is mediated by a number of receptors tuned to specific wavelengths of light – Phytochromes (PHY), Cryptochromes (CRY), Zeitlupe (ZTL) and Ultraviolet Receptor 8 (UVR8) (Ahmad & Cashmore, 1993, Bognar et al., 1999, Millar, 2003, Sharrock & Quail, 1989). Variously, the phytochromes detect red and far red light, blue by crytochromes, blue for Zeitlupe and phototropins while UV-B is detected by UVR8 (Ahmad & Cashmore, 1993, Bognar et al., 1999, Kim et al., 2007, Sharrock & Quail, 1989, Oakenfull & Davis, 2017). Furthermore, it is clear that the detection of light entrains the circadian clock in order to synchronise it with the external environment notably at dawn and dusk, with phytochromes and cryptochromes standing out for the extent of their effect on the clock (Millar, 2003, Oakenfull & Davis, 2017). While mechanisms are yet to

be elucidated in full, these photoreceptors pass information to the central oscillator causing an acceleration of the clock proportional to light intensity (Oakenfull and Davis, 2017).

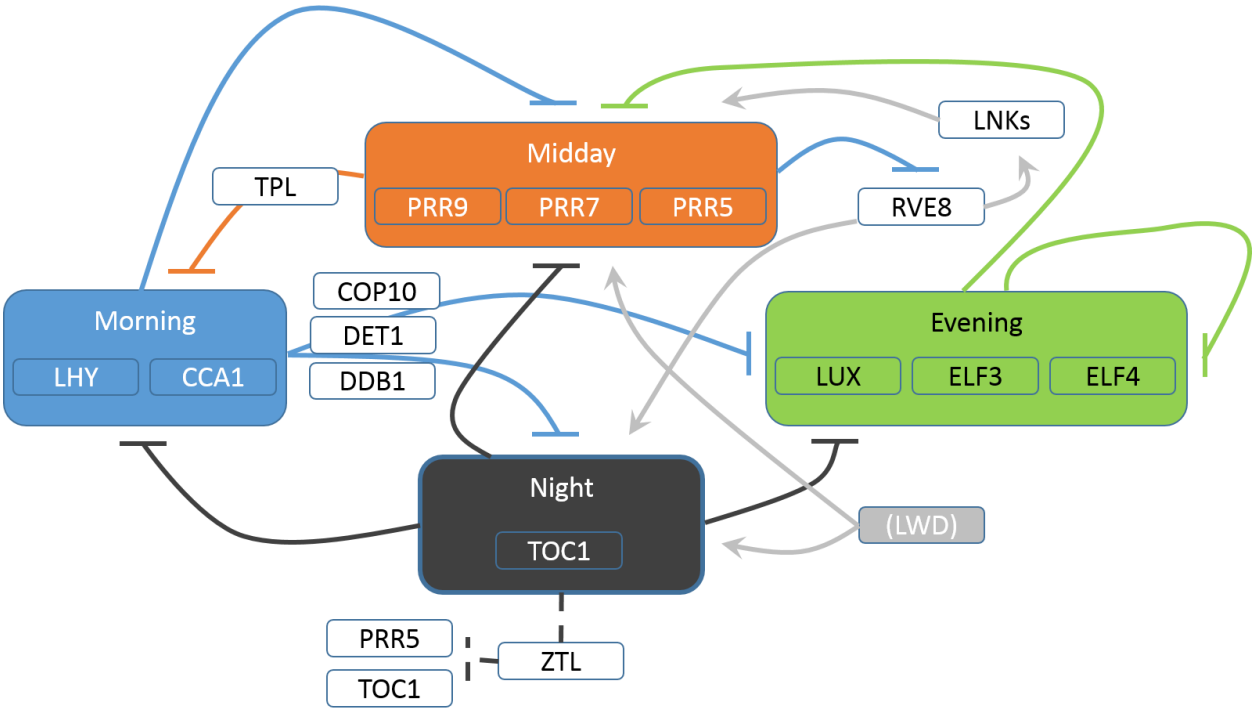

Figure 1.4. The core oscillator of the plant circadian clock. At the start of the day, CCA1 and LHY (blue) bind to and repress promoters of mid-day (orange) and evening (green) genes including *LUX*, *ELF3* and *ELF4*, a process mediated by a complex formed of COP10, DET1 and DDB1. Moving further into the day, the pseudo-response regulators PRR9, PRR7 and PRR5 in association with TPL repress *LHY* and *CCA1* expression. The evening complex, formed of ELF3, ELF4 and LUX repress *PRR9* and *LUX* expression. However, LWD, which binds to ELF3 upregulates *PRR9*, *PRR5* and *TOC1* expression. TOC1 itself is maximally expressed at night, and represses the main elements of the morning, midday and evening genes *CCA1, LHY, PRR9, PRR7, TOC1, ELF4* and *LUX*. The transcription factor RVE8 upregulates *PRR5* and *TOC1* expression as well as other evening complex elements. PRR5 represses *RVE8* transcription. LNK genes work with RVE8 to induce *TOC1* and *PRR5* expression. Finally, degradation of PRR5 and TOC1 is associated with ZTL.

The red light receptors (PHYA-E), are broadly distinguished by two isoforms based on the ratio of red to far-red light, with the inactive P_r form converted to the active P_{fr} form in the presence of red light (Oakenfull & Davis, 2017, Sharrock & Quail, 1989). Interestingly, PHYA signals are integrated into the clock by ELF4 while PHYB interacts directly with the evening complex proteins, ELF3 and LUX and along with PHYA, PHYD and PHYE mediates high intensity red light signalling to the clock (Kolmos et al., 2011, Li et al., 2011, Oakenfull & Davis, 2017, Yeom et al., 2014). Meanwhile, the cryptochromes (CRY1, CRY2) have homology with similar animal light receptors in sensing blue (and UV-A) light, and at low red or

blue light intensity, appear to interact with PHYA (Ahmad & Cashmore, 1993, Lin et al., 2008, Lin & Todo, 2005, Millar, 2003). There are two phototropins (PHOT1 & PHOT2) which are also blue light sensors that affect plant behaviour although they affect circadian rhythms independently of the core oscillator (Litthauer, Battle & Jones, 2016, Li et al., 2015).

Having detected and integrated external signals, the circadian clock is in a position to modulate multiple outputs that affect a wide range of plant activities (Hsu & Harmer, 2014). This may be to respond to biotic stresses such as pathogens or abiotic stresses such as drought or cold via C-Repeat Binding Factor proteins (CBF1-3) as well as managing developmentally important events such as flowering time (Hsu & Harmer, 2014, Shim et al., 2017, Wang et al., 2011). But the timescales can be short; for instance the daily cycle of starch storage and use is under clock control (Graf et al., 2010, Horrer et al, 2016). At even shorter timescales, stomatal opening and photosynthesis are also subject to circadian influence (Dodd et al., 2005, Hassidim et al., 2017, Hennessy & Field, 1991, Horrer et al., 2016). Stomatal control of aperture is influenced by a specific pathway that is responsive to blue light and involves the degradation of starch granules in the guard cell chloroplast which would suggest a link to the circadian clock, and which, as outlined below, has a different phase to the surrounding mesophyll (Horrer et al., 2016, Endo et al, 2016). It has emerged that individual tissues have clocks that operate at differing period lengths, in particular, that mesophyll and stomatal guard cells in the leaf differ in their peak amplitude by up to 11 hours in free-run (i.e. constant light) conditions, possibly as a result of symplastic isolation owing to the lack of plasmodesmata (Endo et al., 2016, Yakir et al., 2011). Furthermore, the Evening Complex genes are generally expressed at higher levels in the vasculature than in the mesophyll of leaves with the vasculature clock dominating the mesophyll clock while they operate on different phases (Endo et al., 2016). Other work has shown that ZTL, which is both a light receptor and a component of the clock has an important role to play in controlling the photoperiod of g_s and A (Dodd, Parkinson & Webb, 2004). The *ztl* mutation in *Arabidopis* led to a longer photoperiod in LL conditions for both g_s and A responses, but stomatal conductance's period was extended by 5 hours compared to a 2.5 hour extension for

assimilation (Dodd, Parkinson & Webb, 2004). More recently, Simon et al. (2020) have shown the importance of the clock to in managing WUE across a range of *Arabidopsis* mutants. In particular, the EC mutants *elf3* and *lux* had variously a significantly lower WUE and unchanged WUE compared to WT, with significant declines in accumulated biomass and water use for both mutants (Simon et al., 2020). A key driver of differences in transpiration was changes in rosette area (Simon et al, 2020). Elsewhere, successive studies have shown the importance of circadian control of plant productivity under LL conditions for a range of crop species with peak g_s, *A* and WUE varying widely (Resco de Dios, 2017).

Control of the *Flowering Time (FT)* gene is one of the major regulatory activities of the clock (Valverde et al., 2004).The Evening Complex (EC) is closely associated with a number of plant activities, and loss-of-function mutations in any of its core elements *lux*, *elf3* or *elf4* give rise to an arrhythmic phenotype in LL conditions (Huang & Nusinow, 2019). ELF3 associates with Constitutive Photomorphogenic 1 (COP1) and Gigantea (GI) promoting GI degradation which provides a link to (FT) via Constans (CO) (Hsu & Harmer, 2014, Onouchi et al., 2000). Evening Complex (EC) proteins directly repress expression of *Phytochrome Interacting Factor 4 & 5* (*PIF4* & *PIF5*) in relation to hypocotyl extension (Hsu & Harmer 2014, Nusinow, 2014). Elsewhere, *Arabidopsis elf4* mutants flower early in long days and are etiolated, while they are generally arrhythmic under constant light with individual seedlings continuing to show weak rhythmicity (Doyle et al., 2002). *GI* is epistatic to *ELF4* in the control of flowering time (Kim et al., 2012). Generally, the Evening Complex is required to suppress to pathway to flowering until it is seasonally appropriate (Huang & Nusinow, 2016).

1.1.7 The circadian clock and stomata

From a short-term perspective, stomata must integrate multiple internal and external signals (Hetherington and Woodward, 2003), in which guard cells appear to be relatively isolated from the rest of the leaf, where the onset of dawn and dusk are anticipated by stomata (Dodd et al., 2004), and where the circadian clock directly affects and improves the plant's water use efficiency (Dodd et al., 2005). This process, in which responses are said to be *gated*, (i.e. modified, in this case by the circadian clock) is

closely linked to stomatal aperture (Hotta et al., 2007). Stomata open ahead of dawn in the expectation of increasing photosynthesis, and close long before dusk, possibly owing to the accumulation of sugars by day or the consumption of starch by night (Graf et al., 2010, Dodd et al., 2015). Furthermore, the evidence is clear that a coincidence of internal clock time and environmental clock time is critical in maximising yield (Dodd et al., 2005). On this basis, plants lacking or heavily impaired in circadian clock function might be presumed to suffer a lack of fitness, for instance *lux* mutants have recently been shown to be unable to open stomata in a clock-dependent manner as part of a defence response (Zhang et al., 2019). However, in non-natural environments such as agriculture, where otherwise non-favourable genes can proliferate (consider non-shattering spikes in cereal crops, for instance, where human control of the environment allows these deleterious genes to survive), the clock is known to have an impact on cereals such as wheat and barley (Turner et al., 2005, Beales et al., 2007, Allaby et al., 2017). One of the more interesting introgressions into Bowman as described above was at the *mat-a* locus, which was identified with an early-maturity phenotype (Zakhrabekova et al., 2012, Lundqvist, 2014). A number of alleles were identified at *eam-10* and *eam-8* (Druka et al., 2011), and in the 1960s, the variety Mari was developed from one of the *eam-8* mutants, a variety whose early maturing characteristics were advantageous in the long but cool summers of Sweden but perhaps surprisingly also the Colombian highlands, where avoiding late summer drought was more important (Lundqvist, 2014); in either event, flowering time is an important determinant of final yield in conjunction with environmental variables (Wiegmann et al., 2019).

More recently, these mutations have been associated with specific genes – *HvELF3* in the case of *eam-8* and *HvLUX* in the case of eam-10, with the underlying genes orthologs of Arabidopsis (Faure et al., 2012, Campoli et al., 2013). The *elf3* and *lux* mutants in barley had notably disrupted expression of floral regulator genes such as FT and concurrent impairments in gene expression of the core circadian oscillator under constant light (Faure et al., 2012, Campoli et al., 2013). The *lux* mutation gave rise to non-rhythmic reduced expression of HvCCA1 but upregulation of HvPRR37 under LL while the *elf3*

mutation shifted the phase and reduced *GI* expression and severely attenuated *HvCCA1* expression also in LL (Faure et al., 2012, Campoli et al., 2013). Nevertheless, residual rhythmicity was observed in gene expression levels of *CCA1* and *TOC1* in the *eam-8* barley lines, and in the *eam-10* mutants, the pseudo-response regulators PRR37 and PRR73 retained some functionality under LL conditions (Faure et al., 2012, Campoli et al., 2013). Both these results suggest an impairment but not elimination of the oscillator of the clock.

1.1.8 Stomatal responses to environmental stress

Stomatal conductance (and by extension, average stomatal aperture) has long been correlated with assimilation and by extension, yield (Condon et al., 2002, Fischer et al., 1998, Faralli et al., 2019) via two mechanisms – open stomata allow greater CO_2 diffusion into the plant while the exit of water permits greater evaporative cooling to take place (Farquhar and Sharkey, 1982), improving the reaction kinetics of the photosynthetic apparatus which are hampered at high temperatures (Farquhar et al., 1980). That stomatal aperture is progressively reduced under drought is a well-established phenomenon (Farquhar and Sharkey, 1982). Assimilation (*A*) may not be notably limited by stomatal aperture in unstressed conditions, but that aperture does become the major limitation on *A* under moderate drought (Farooq et al., 2009), where the size of the pore restricts CO_2 diffusion into the leaf, leading to lower internal [CO_2] and thereby, lower assimilation (Farquhar and Sharkey, 1982), while higher leaf temperature at lower stomatal conductance (via smaller aperture) is another cause of limitation (Farquhar and Sharkey, 1982). Observations in the field have shown declining transpiration and *A* under mild drought in barley and other cereals (Lipiec et al., 2013), but the link between water use and photosynthetic output is not always clear even when yield declines, suggesting a conservative phenotype is at work, with lower biomass itself an adaptive response (Rollins et al., 2013).

There are other drivers of plant behaviour above and beyond the conservative-non-conservative dichotomy. Sensitivity to drought may have changed over time, as modern cultivars appear to have

stronger responses to drought than older spring wheat varieties (Guan et al., 2015) for instance, showing relatively lower g_s under water stress. Meanwhile, mature leaves generally drive g_s through local sensing of water status, while in young leaves, root-to-shoot signalling of soil moisture status prevails (Chen et al., 2013), again modulating emergent phenotypes even under an overall preferences for one behaviour (such as conservatism) over another.

1.1.9 Mechanisms of response to water stress

Abscisic acid (ABA) is the best-known hormone related to water stress (Bradford & Hsiao, 1982). ABA is released by the roots and elicits a range of responses in plants, although a number of other hormones are known to vary in abundance as water potential falls, with auxins and gibberellins generally decreasing in concentration while ethylene and jasmonic acid rise(Bradford & Hsiao, 1982, Geng et al., 2016). One of the earliest plant responses to stress is stomatal closure, in conjunction with a range of other effects such as reduced shoot growth and leaf size, and maintenance of root elongation, although direct sensing of osmotic potential is also a possible route by which water stress is detected at the leaf (Franks & Farquhar, 2001, Bradford & Hsiao, 1982). Stomatal closure is primarily driven by anion channel activity (Yamamoto et al., 2016). Among the channels affected is the SLAC1 protein with carbonic anhydrase playing a central regulatory role with the bicarbonate ion activating the SLAC1 channel (Vahasilu et al., 2008, Yamamoto et al., 2016). ABA triggers the activation of Open Stomata 1 (OST1) which regulates ABA activity downstream at the guard cell ion channels, although another pathway exists (Chater et al., 2015, Merilo et al., 2015, Yamamoto et al, 2016).

One emergent area of research into responses to drought has emerged in Phospho-adenosine-phosphate [PAP] signalling. It has been clear for many years that there must be signalling pathways between the nucleus and plastids (such as the chloroplast: so-called anterograde signalling) by messengers such as transcription factors (Dodd et al., 2005, Dodd et al., 2015). Equally, the loop must be completed for signalling from the plastid to the nucleus (retrograde signalling), and small metabolites are often believed to be responsible, including sugars, DHAP and MEcPP (Dodd et al., 2015, Phua et al.,

2018). One such small signalling molecule turns out to be PAP, invoked in the PAP-SAL1 pathway (Litthauer et al., 2018, Estavillo et al., 2011) under environmental stress such as drought or elevated light intensity (Estavillo et al., 2011) and may turn out to be a major integrator of retrograde signalling with impacts on growth and development (Phua et al., 2018).

PAP is a by-product of the assimilation of sulphates from the environment for the production of cysteine or glutathione (Bohrer et al., 2015) that is present in low concentrations throughout the cell (Estavillo et al., 2011). SAL1 (SALt1) phosphatase is responsible for preventing the accumulation of PAP, degrading it to AMP and P_i at the chloroplast and the mitochondria (Phua et al., 2018). However, SAL is inactivated by oxidative stress, such as occurs during drought. The inactivation of SAL1 leads to an increase in PAP concentration` which diffuses throughout the cell. PAP binds with exoribonucleases (XRNs) and causes repression of XRN activity at the nucleus, causing up-regulation of stress-response genes (Litthauer et al., 2018).

It has been clear for some time that the circadian clock aided plants in responding appropriately to stress (Dodd et al., 2005, Hsu and Harmer, 2014), and thus ought to mediate between drought stress and plant activities. Yet although some signals were known which improve drought tolerance (Hundertmark and Hincha, 2008) – the best understood is probably abscisic acid (ABA), which downregulates ion channel activity in guard cells (Merilo et al., 2015) - and although non-ABA channels are also known (Yamamoto et al., 2016), the problem has been to understand how the chloroplast can signal stress to the nucleus (retrograde signalling) given the integration of plastid and nuclear genes (Estavillo et al., 2011). Pornsiriwong et al. (2017) extended our understanding of ABA signalling and showed that the SAL1-PAP pathway linked to abscisic-acid induced stomatal closure.

It is known that osmotic stress (such as that induced by drought) induces accumulation of PAP and extends the circadian period (Litthauer et al., 2018). Litthauer et al. (2018) observed that SAL1 expression was probably a result of the diel cycle rather than constitutive nuclear clock expression. They showed that the *sal1* mutant, similar to *xrn* mutants had delayed expression of the clock gene *CCA1*

showing the influence of [PAP] on clock activities. And it appears that increasing [PAP] itself is enough to make the clock run slower (Litthauer et al., 2018), where the hormone was sprayed directly on the leaf. However it is not known whether the clock induces changes in [PAP], hence interacting with responses to abiotic stress such as drought. Litthauer et al. (2018) did show that PAP concentration was increased in plants subjected to osmotic stress, and that the circadian period was extended. What was not attempted was the use of clock mutants with no / limited clock function in a drought experiment to understand the impact of the clock directly on [PAP] and hence the status of retrograde signalling in those mutants. This is important to consider as stomatal responses to drought are influenced by the clock, which promotes the production of stress-related genes ahead of water deficits in the afternoon in order to extend stomatal opening (Harmer et al., 2000), and it is reasonable to suppose that in a source-limited crop such as barley (Serrago et al., 2013), extending stomatal opening to increase total assimilation would be a desirable strategy.

Climate change, population growth, changing dietary preferences and availability of water remain set to be the key drivers of breeding efforts in the coming decades. Improving water use efficiency by gaining a better understanding of the coordination between g_s and A under changing conditions as mediated by understanding stomatal anatomy and behaviour remains critical to improving crop varieties. Barley is an excellent model cereal crop as well as being an important food in its own right, and many important collections exist of mutant NILs, which includes lines impaired in their circadian clock function as well as landraces and wild barleys, giving the researcher multiple avenues to consider in the search for novel phenotypes.

Meanwhile, climate change will drive more frequent, severe and persistent drought episodes, and thus is a significant threat to farmers, particularly in more-arid regions. Plants are often categorised by their level of conservatism under drought, and non-conservative types are generally preferred under current conditions. Nevertheless, future drought risks may give rise to selection pressure for alternative phenotypes, including more-conservative ones while yield must be maintained or improved. Elsewhere,

a pathway has been identified which links the signalling molecule, PAP to the up-regulation of stress response genes under drought; it remains to be seen whether it is possible to identify the impact of the clock on PAP expression and by extension, the impact on stomatal behaviour.

The intention of the series of experiments described below was to explore some of the ways in which light and water availability affected g_s, A, WUE_i, yield and transpiration. Chapter 3 describes the process of exploring the impact of mutations in *Hvlux* and *Hvelf3* cultivars to LL constant light and LD conditions to mimic the work of Faure et al. (2012) and Campoli et al. (2013) on gene expression. Next, an attempt was made to explore the phenotype of the barley mutants over developmental time, again in line with the work of Faure et al. (2012) and Campoli et al. (2013). To extend our understanding of the extent of the photosynthetic / stomatal phenotype, further manipulations involved progressively more-complex diurnal light treatments that involved exploring the effect of acclimation to morning light conditions in the afternoon while the impact of driving photosynthesis / stomatal opening and closing through changes in light intensity were also considered. Chapter 4 considered the natural variation in g_s, A and WUE_i and overall yield of a range of wild, landrace and elite barleys in a polytunnel and well as growth chamber with the general aim of establishing water-use phenotypes. A variety of light regimes were used to explore the phenotypes, including step changes from low to high light, light response curves and C_i response curves. The next two chapters sought to extend the work on water use efficiency to drought responses in the barley mutants (Chapter 5) and wild barleys / landraces (Chapter 6). Chapter 5 considers the effect of drought stress of varying degrees on developmental as well as physiological phenotypes in the EC mutants along with examining the possibility of a retrograde signalling (PAP) pathway existing in barley. Chapter 6 also explores the impact of transient and developmental drought on development, physiology and yield, this time in the wild barleys. Here, diurnal light regimes and light response curves were used to examine the impact of light on g_s, A and WUE_i notably in terms of short-term stomatal responses under drought.

1.1.10 Aims and objectives

With climate change, population growth, dietary preferences and water availability all impinging on food production requirements, there is a clear need to develop new varieties of crops which are more productive – gaining more 'crop per drop'. One key risk of climate change is that drought episodes become more frequent, severe and persistent, and near-isogenic clock mutant barleys, wild barleys and landraces all offer the possibility to find a diversity of responses in behaviour to changing environmental conditions in balancing assimilation and stomatal conductance. Stomata are critical in regulating CO_2 uptake and water loss, and make their presence felt in the short term in physiological responses, but in the longer term, are part of the integrated processes that lead to overall yield.

The studies described below aimed to develop an understanding of natural variation in wild and landrace barleys compared to elite varieties in their physiological responses. A second aim was to develop our understanding of the role of the clock in governing physiological processes. Both these aims were pursued in the context of a need to improve yield through the discovery of novel traits. The aims were met by the following objectives:

1. To use gas exchange measurements to explore steady state and kinetic physiological responses to light intensity in clock mutants and wild / landrace barleys (Chapters 3 & 4).
2. To employ water stress to understand the interaction between drought and light on developmental and instantaneous physiological responses in clock mutants and wild / landrace barleys (Chapters 5 & 6).
3. To assess the variation in yield outcomes resulting from genotypic differences either in clock mutant or wild / landrace barleys (Chapters 3-6).
4. To understand the effect of the clock on retrograde signalling in plants under drought stress through PAP assays (Chapter 5).

Chapter 2: Materials and Methods

2.1. Plant materials

Barley, a model organism for crop research owing to its relatively small diploid genome (Dawson et al., 2015), was selected for the series of studies described below. It is a desirable model in the context of this research owing to its extensive North-South range from Sweden to Ethiopia, and its ubiquity as a crop in Asia, Europe and the Americas (Badr et al., 2000).

2.1.1 Circadian clock mutants

Near Isogenic Lines (NILs) were created in a Bowman background in the 1980s for a range of mutants assembled in Sweden and elsewhere, largely as a consequence of electromagnetic mutagenic research (Lundqvist, 2014). Bowman itself is a 2-row spring barley developed in the US as a drought-tolerant plant and registered in 1985 (Franckowiak et al., 1985). Two NIL mutants were investigated here. BW284 is an early maturing mutant at the *eam10* locus, later identified as a mutation in the *HvLUX* gene (Campoli et al., 2013). BW289 is an even earlier-flowering mutant associated with the *eam8* locus, which has more recently been cloned and found to be *HvELF3* (Faure et al., 2012).

2.1.2 Elite, landrace and wild barleys

A second thread in these studies was to identify natural variation in a range of elite, landrace and wild barleys. Initially this work was conducted with the help of the commercial breeding firm KWS UK Ltd, including varietal selection. Where possible, a wide combination of two and six-row types, spring or winter habits and malt vs feed varieties was selected. Material was sourced from KWS UK Ltd, The University of Adelaide, Australia and John Innes Centre Germplasm Resources Unit.

Table 2.1: List of elite, wild and landrace barleys, and their key selection criteria

Variety	Type	Rows	Habit	Use	Source
B3733	Wild	2	Winter	-	JIC GRU
B3736	Wild	2	Winter	-	JIC GRU
B3745	Wild	2	Winter	-	JIC GRU
Alpha	Landrace	2	Spring	Malt	KWS UK Ltd
Bere	Landrace	6	Spring	Malt	JIC GRU
Dea	Landrace	6	Winter	Feed	JIC GRU
Eire-6-Row	Landrace	6	Spring	Malt	KWS UK Ltd
Golden Archer	Landrace	2	Spring	Malt	JIC GRU
Hatif de Grignon	Landrace	6	Winter	Feed	JIC GRU
KWS Irina	Elite	2	Spring	Feed / Malt	KWS UK Ltd
KWS Kosmos	Elite	6	Winter	Feed	KWS UK Ltd
KWS Orwell	Elite	2	Winter	Feed	KWS UK Ltd
KWS Sassy	Elite	2	Spring	Malt	KWS UK Ltd
KWS Wintmalt	Elite	2	Winter	Malt	KWS UK Ltd
Maritime	Elite	6	Spring	Feed	University of Adelaide

2.2. Plant growth conditions

All plants were planted into Levington F2+S seed and modular compost except in the case of the polytunnel trial, where plants were initially grown in F2+S, but were pricked out into bare soil.

2.2.1 Polytunnel field trials

15 varieties were grown in 6 randomised blocks with double guard rows surrounding them at the KWS field site at Thriplow in Cambridgeshire between March and July 2016. Three groups of varieties were selected: 4 wild barleys originating in Central Asia; 5 Northern European landraces and 6 elite cultivars. The landrace and elite cultivars were selected to offer a range of ear types (2 or 6-row), habits (winter / spring) and end-uses (malt / feed). The plants were germinated in modular pots in Levington F2+S at the site including an initial 5 day stratification in the dark at 4^{o}C. They were then transplanted to a polytunnel for the duration of the experiment, being randomised into 6 blocks with one biological replicate per block. They were not given any additional fertiliser, but they were regularly watered.

2.2.2 Glasshouse trials

Multiple trials were conducted at the glasshouse facility of the University of Essex, Wivenhoe Park, Essex. The unit had light augmentation to a minimum of 200 μmol m^{-2} s^{-1} light between 7am and 7pm daily using sodium vapour lamps with a colour temperature of 2700K (General Electric Inc, Boston, Mssacheussetts, USA). All experiments were randomised in 6 or 8 block designs, with one biological replicate per block.

2.2.3 Growth chamber trials

Additional trials relating to Chapter 4 (see below) were carried out in a growth chamber at the University of Essex. 6 replicates of 4 landraces (Eire 6-Row, Golden Archer, Hatif Dea, and Hatif de Grignon) and of the elite cv KWS Irina were grown in a Fitotron growth chamber (Sanyo Electric Co. Ltd., Kadoma, Osaka, Japan) with light delivered by fluorescent lamps with a colour temperature of 5000K. They were grown in two batches, one of Eire and Archer with Irina, and one of Dea and Hatif de Grignon with Irina, under 840 μmol m^{-2} s^{-1} square wave light for 12h light cycles at 24°C, 50% relative humidity 12h dark cycles at 16°C and 50% humidity.

2.2.4 Phenotyping platform trials

A number of trials relating to the impact of drought on physiology were carried out in the plant phenotyping platform (PSI, Drasov, Czech Republic) at the University of Essex. In some experiments, plants were automatically watered to a target weight using an integral balance. In others, plants were hand-weighed and rewatered. In addition, in some experiments pots were allowed to dry at their own rate; in others, pots were rewatered relative to the rate of drying of the slowest drying pot. The experimental conditions in the platform were 20°C temperature in the light and dark under 12h light and 12h dark, and at 50% humidity. Light intensity was limited to 400 μmol m^{-2} s^{-1} Photosynthetically Active Radiation (PAR) using Valoya NS1 lights (Valoya Oy, Melkonkatu, Helsinki, Finland) at a colour temperature of 4800K.

Experiments involving thermal imaging used the integrated FLIR A655sc (FLIR System AB, Täby, Sweden) thermal camera as part of the phenotyping platform. Low light conditions were 400 µmol m^{-2} s^{-1} PAR used elsewhere on the phenotyping platform. High light conditions were 700 µmol m^{-2} s^{-1} PAR (the limit of the Heliospectra LX601C (Heliospectra AB, Göteborg, Sweden) light source available).

2.3. Light Regimes

The mainstay of these studies were controlled measurements of plants in IRGAs. A number of different approaches were used according to the requirements of the experiment.

2.3.1 Constant light

To gain an insight into the physiology of the circadian clock mutants, a constant-light setup was used. The middle portion of the third leaf of the main tiller was placed in the chamber of an ADC LCpro IRGA (ADC Bioscientific Ltd, Hoddesdon, Herts, UK) and illuminated under continuous 100 µmol m^{-2} s^{-1} PAR. The rest of the plant was simultaneously illuminated at the same light intensity using a Heliospectra LX601C LED lamp; plants were not otherwise exposed to external light sources. External air was supplied to the IRGA via a carboy to maintain constant [CO_2]. While no attempt was made to manage humidity, leaf temperature was controlled to 22^{o}C. Each run lasted 5 days, with data for the first day being discarded while the plant acclimatised to the new conditions.

2.3.2 Diel light

An extension of the constant light design was to include periods of square wave light on 12h 12h light-dark cycles. Experimental set-up was as per section 2.3.1, except that after acclimatisation overnight in the ADC LCpro IRGAs, plants were exposed to alternating 12h:12h cycles of light (100 µmol m^{-2} s^{-1}) and dark (0 µmol m^{-2} s^{-1}) over two days. The LED light source was also timed to run concurrently with the light-dark cycles in the IRGA.

2.3.3 Fluctuating light

Physiological responses to changing light levels were the mainstay of this work. Four fluctuating light regimes were used:

1) In the first, the plant was acclimated in a LiCOR 6400 IRGA (LiCOR Inc., Lincoln, Nebraska) to low light intensity (100 µmol m^{-2} s^{-1} PAR) for 0.5 hours before light was increased to 1000 µmol m^{-2} s^{-1} PAR for eleven hours. PAR was then decreased to 100 µmol m^{-2} s^{-1} PAR for a final 0.5 hours.
2) In addition, plants were acclimated at 100 µmol m^{-2} s^{-1} PAR in a LiCOR IRGA for 0.5 hours before the intensity was increased to 1000 µmol m^{-2} s^{-1} PAR for 4.5 hours. The intensity was then decreased to 100 µmol m^{-2} s^{-1} PAR for 0.5hrs before being increased again to 1000 µmol m^{-2} s^{-1} PAR for 4.5 hours before finally being reduced again to 100 µmol m^{-2} s^{-1} PAR for the last 0.5 hours.
3) Plants were acclimatised at 100 µmol m^{-2} s^{-1} PAR in a LiCOR IRGA before the start of the measurement period. Light intensity remained at 100 µmol m^{-2} s^{-1} PAR for six hours. Then light intensity was increased to 1000 µmol m^{-2} s^{-1} PAR for 1 hour and back down to 100 µmol m^{-2} s^{-1} PAR for 1 hour three times, before a final decrease to 100 µmol m^{-2} s^{-1} PAR for 0.5 hours.
4) Plants were acclimatised for 0.5 hours in a LiCOR IRGA at 100 µmol m^{-2} s^{-1} PAR for 0.5 hours. 6 periods of alternating high (1000 µmol m^{-2} s^{-1} PAR) and low (100 µmol m^{-2} s^{-1} PAR) were then applied, before a final 0.5 hours at 100 µmol m^{-2} s^{-1} PAR.

2.4. Modelling leaf gas exchange, chlorophyll fluorescence and thermal imaging

2.4.1 Gas exchange - IRGA

Four main methods were used to model leaf gas exchange:

2.4.1.1 Assimilation responses to changing light – A-Q curves

Plants were acclimated at high light (1500 µmol m^{-2} s^{-1} PAR) and then exposed to steadily declining light levels of 1500, 1250, 1100, 900, 700, 600, 500, 400, 200, 100, 50 and 0 µmol m^{-2} s^{-1} PAR, being given up to two minutes for assimilation to decrease after each step change down in light. VPD was maintained at 1 KPa, temperature at 22°C, and [CO_2] at 400 µmol mol^{-1}. Data were modelled using the methods outlined in Buckley and Diaz (2015) into Φ, the initial slope between light intensity and *J*, the calculated rate of electron transport; Θ, the convexity of the curve of light against *J,* and *J* itself.

2.4.1.2 Assimilation responses to changing internal [CO_2] – A-C_i curves

Plants were acclimated at saturating light (1250 µmol m^{-2} s^{-1} PAR, 400 µmol mol^{-1} CO_2) and then exposed to regularly changing reference [CO_2] values of 400, 250, 150, 100, 50, 400, 500, 600, 800, 1000, 1250, 1500. Plants were given up to 3 minutes to settle following a change in [CO_2]. Temperature was set to 22°C and VPD to 1 KPa. The data derived from the measurements (C_i, the sub-stomatal [CO_2], and *A*) were modelled according to the equations and methods suggested by Sharkey (2007, 2016) using the Farquhar et al. (1980) model of photosynthesis. Five parameters were extracted. Vc_{max}, which models the first phase of the increase in *A* following an increase in C_i; *J*, the electron transport rate which describes the curve after its inflection point; and *TPU*, or triose phosphate utilisation, which describes the final part of the curve as it approaches steady-state. The final two parameters were R_d, background respiration during the day and g_m, mesophyll conductance, the latter presumed to be limiting. Of these, the first two parameters were considered of particular importance, as Vc_{max} describes the level of activation of Rubisco and *J* the RuBP regeneration-limitation of the Calvin Cycle.

2.4.1.3 A, g_s and responses to step changes in light intensity

Step changes were run either as a standalone measurement, at 22°C, 1 KPa VPD and 400 µmol mol^{-1} where, following a period of acclimation, light intensity in a LiCOR 6400 IRGA was increased from 100 µmol m^{-2} s^{-1} PAR to 1000 µmol m^{-2} s^{-1} PAR for up to an hour *or* as part of an ongoing fluctuating light experiment (in particular, see 2.3.3.3 and 2.3.3.4 above). The changes were modelled in a simplified version of the equations used by Vialet-Chabrand et al. (2017b):

$$f(g) = g_{min} + (g_{max} - g_{min})e^{(-t/\tau)}$$

Where g_{min} is the minimum predicted steady-state value of *A* or g_s, g_{max} the maximum predicted value of *A* or g_s and τ is the time constant and can be thought of as the time taken to increase the dependent variable from g_{min} to 63% of g_{max}. Naïve Bayesian estimators of initial values for the g_{min} and g_{max} were derived from the first and last values of the step change. The parameter τ was initially estimated at 5 minutes. From these data, two further parameters can readily be derived; the first gives the maximum slope of the equation:

$$f(x) = \tau\left(\frac{g_{max} - g_{min}}{e}\right)$$

And the second the stomatal limitation on *A*, calculated by finding the time taken to reach 95% of g_{max} for assimilation. The time taken to reach 95% *A* must be:

$$1 - e^{-(t/\tau)} = 0.95$$

Giving the value of t ≈ 3τ as the solution to the equation and the value of *A* must be:

$$f(g) = 0.95 * (g_{max} - g_{min}) + g_{min}$$

These equations can be used with step changes of increasing or decreasing light, which may have different profiles (McAusland et al., 2016), particularly for g_s, and consequently different meanings (Lawson and Blatt, 2014).

2.4.1.4 A, g_s and WUE_i responses to steady-state light intensity.

There are a number of possible approaches to descriptive modelling of steady-state behaviour of gas-exchange through time under constant conditions. One method is Gaussian modelling (Matthews et al.,

2018). An alternative approach, used in climate modelling is to partition the curves into their mean, standard deviation and centroid, (described as their mean, amplitude and phase), and use these parameters to compare experimental conditions (Yin and Porporato, 2017). The advantage of this approach is that, unlike Gaussian modelling, it does not presuppose any form of distribution, Gaussian or otherwise.

2.4.2 Gas exchange – porometry

A Delta-T AP4 porometer (Delta-T Devices, Burwell, Cambs, UK) porometer was used to measure stomatal conductance during the first study comparing the drought responses of clock mutants (see Chapter 5). The device was recalibrated before each batch of measurements.

2.1.1 Chlorophyll fluorescence

Three techniques were used for chlorophyll fluorescence that gave an understanding of each plant's photosynthetic capacities and efficiencies. Dark-adapted measurements of *Fv/Fm* typically lie close to 0.83 (Baker, 2008) and values much below that suggest a plant is stressed, in all cases, the plants were dark-adapted for at least 25 minutes and up to one hour. Under actinic light, (500 µmol m^{-2} s^{-1} or 1000 µmol m^{-2} s^{-1} depending on the study), the main parameter of interest is *Fq'/Fm'*, the operating efficiency of Photosystem II, but *Fv'/Fm'*, the maximal quantum efficiency of Photosystem II is also of interest, as the efficiency factor, *Fq'/Fv'* relates the two. Finally, the rate of heat loss (Non-Photochemical Quenching, NPQ), calculated as *Fm'/Fm* draws energy-dependent quenching, state transitions and photoinhibition processes that dissipate excess energy (Genty et al., 1989, Baker, 2008).

Point measurements of fluorescence were made using the Hansatech FMS system (Hansatech Instruments Ltd, Pentney, Norfolk). Whole-leaf measurements were made on a Technologica FluorImager (Technologica, Colchester, Essex).

2.5. Leaf and stomatal characteristics

A number of estimates were made consistently throughout these studies of stomatal anatomical characteristics, as well as a number of estimates of leaf area, specific leaf area and leaf thickness. Stomatal anatomical features were captured using dental silicone impressions, which were painted with a thin coat of nail varnish when dried. The nail varnish was removed from the impression using Sellotape and mounted directly on to a slide. Slides were then viewed at 100x magnification for stomatal and epithelial counts, and at 400x magnification for measurements of stomatal pore length and guard cell length on an Olympus BX60 microscope (Olympus Corp., Shinjuku, Tokyo, Japan), the method having first been described by Weyers and Johansen (Weyers and Johansen, 1985).

It was possible to calculate the maximum expected value of g_s from the anatomical data – the anatomical gs_{max}. This links stomatal size and density with physical diffusion constants of water vapour:

$$Anatomical\ gs_{max} = (d \cdot D \cdot A_{max}) / \left(v \cdot \left(l + (\pi/2) \cdot \sqrt{(A_{max}/\pi)}\right)\right)$$

Where d is the diffusivity of air in water, D the stomatal density, A_{max} the maximal rate of assimilation(calculated from an A-Q response curve), v the molar volume of air and l the stomatal pore length (Dow et al., 2014). Stomatal index (SI) was calculated as:

$$SI = 100 \cdot Stomatal\ density \cdot (Stomatal\ density + Epithelial\ density)^{-1}$$

Leaf area was calculated in one of two ways. In intact plants, *in vivo* estimates were made using Kemp's method (Kemp, 1960):

$$Leaf\ Area = 0.905 \cdot Length \cdot Breadth\ (at\ the\ midpoint)$$

Alternatively, for harvested samples, a LiCOR LI3100C Area Meter (LiCOR Inc., Lincoln, Nebraska, US) was used. If the fresh mass of a leaf was known, then its specific leaf area could be calculated:

$$Specific\ Leaf\ Area\ (SLA) = Area \cdot Fresh\ Mass^{-1}$$

And by extension, assuming the density of the leaf ≈ 1 g cm^{-3}, then leaf thickness is:

$$Leaf\ Thickness\ (LT) = SLA \cdot (Dry\ Mass \cdot Fresh\ Mass^{-1})^{-1}$$

Using the methods outlined in Vile et al. (2005).

2.6. Yield and yield components

These studies focused solely on above-ground harvests for assessments of biomass. For immature plants, fresh biomass, dry biomass and number of tillers were measured. Tillering is an important element of yield in barley where floret number is fixed (unlike in wheat) (AHDB, 2018). Later shoots are often aborted, so the number of fertile ears tiller^{-1} is also of interest. When plants were allowed to develop to maturity, fresh biomass measurements were irrelevant, but a number of additional measures could be used. Harvest Index (grain mass / biomass) is a key indicator of yield potential (Evenson and Gollin, 2003), while thousand grain weight (TGW) is a widespread measure of plumpness in grain – a desirable characteristic, particularly in malting barleys, and is closely related to grain number per plant and grain mass per plant (AHDB, 2019).

2.7. Measuring the concentration of phospho-adenosine phosphate

PAP was extracted from leaves as described in Estavillo et al. (2011). Metabolites were extracted from 150-300 mg tissue ground in a pestle and mortar chilled in liquid N_2 using 1 mL 0.1 M HCl with incubation on ice for 15 min and centrifuged twice at 16,000*g* at 4°C for 5 min. The supernatant (150 µL) was added to 770 µL CP buffer (620 mM citric acid and 760 mM Na_2HPO_4, pH 4) and derivatised using 80 µL 50% (w/v) chloroacetaldehyde solution with incubation at 80°C for 10 min, and centrifuged for 45 min at 16,000*g* at 4°C as described in Litthauer et al.(2018). Twenty microliters of the supernatant was injected into an Agilent 1100 HPLC system connected to a FLD G1321A (Agilent) fluorescent detector. PAP was analysed by reverse-phase HPLC using a Luna 5 µm C18(2) 100 Å column (Phenomenex). The column was equilibrated for 0.2 min with 95% (v/v) of buffer A (5.7mM $[CH_3(CH_2)_3]_4NHSO_4$ and 30.5 mM KH_2PO_4, pH 5.8) and 5% (v/v) buffer B (67% [v/v] acetonitrile and 33% [v/v] buffer A), followed by a linear gradient for 53 min up to 50% (v/v) of buffer B. The column was re-equilibrated for 7 min with 5% (v/v)

buffer B. PAP concentration was calculated relative to a commercially available standard (Santa Cruz Biotechnology; sc-210760) as described in Litthauer et al. (2018).

2.8. Statistical analysis

All statistical analyses were conducted in the R statistical computing environment using R as developed in Rstudio (R Core Team, 2018). Data were assessed for normality using the Shapiro-Wilk Test. All statistical analyses were modelled using generalised linear models. The dependent variable in each case was compared to between one and three possible factors in the fixed effects (typically variety, where appropriate, treatment and occasionally time-of-day if repeated measures were used), and minimal models derived. In addition, block was always considered a random effect. In certain circumstances, time-of-day, day and ambient temperature were also considered as random effects if the circumstances dictated, and these are described in the relevant chapters.

Chapter 3: Circadian clock mutants of the barley Bowman may have novel phenotypes relating to water use efficiency

3.1. Introduction

From the 1920s, a number of mutants, often but not always, induced by irradiation with x-rays, were discovered, and desirable mutations bred into commercial cultivars – for instance the successful early-flowering variant Mari, which was widely planted in Sweden from the 1960s. A mutant population was established from the early 1980s which created Near Isogenic Lines (NILs) of key mutants crossed into a Bowman background (Druka et al., 2011), the latter being a commercial spring variety developed in the late 1970s (Franckowiak et al., 1985). Two mutants in this population that are of particular interest are BW289 and BW284. The BW284 variant containing the mutation found in Mari, and which drives heading 3-12 days earlier than Bowman, depending on day length (Francowiak, 2014). They are impaired respectively at the evening clock genes *Hvelf3* (Faure et al., 2012) and *Hvlux1* (Campoli et al., 2013), both of which are homologues of circadian clock genes (Calixto et al., 2015) that found as part of the EC in *Arabidopsis thaliana* (Nusinow et al., 2011). Mutations of this sort are known to invoke an early flowering, photoperiod-insensitive phenotype (Cockram et al., 2007) – breeding for this locus extended the range of barley into more-northerly climates such as Sweden and at the same time allowed farmers in hotter regions to harvest earlier, reducing the risk from summer droughts (Cockram et al., 2007).

In young barley seedlings, clock genes were found to respond to stress gene output (such as the ABA-response gene, *ABA Insensitive 5* (*HvABI5*), but themselves had a limited impact on physiological traits g_s and *A* (Habte et al., 2014). It is not known if these physiological responses in seedlings persist further into development, but it appears unlikely since clock mutants have a well-established early-flowering phenotype. The Evening Complex is known to be the locus for the integration of endogenous and environmental signals, including key genes regulating photosynthesis (Ezer et al., 2017). Meanwhile, circadian-type rhythms have been observed in plants over diurnal timeframes, with significant heterogeneity observed between herbaceous crop species in sinusoidal light regimes (Matthews et al., 2017). Other recent work has shown the importance of stomatal anatomy in stomatal kinetic responses

to changing light levels (Vialet-Chabrand et al., 2017b). We expect mutations in clock genes to have an impact on the manner in which dawn and dusk are predicted; clock gene expression is clearly cyclical in many pathways (Harmer et al., 2000) and is closely related to physiological outputs (Dodd et al., 2005) such as stomatal aperture (Hassidim et al., 2017) with a consequent impact both on kinetic and steady-state performance of g_s , A and WUE_i.

The aim of this study was to investigate the impact of the clock on stomatal conductance and assimilation rate, and by extension WUE_i in Bowman and the two clock mutants described above. Two possible routes to improving WUE_i include improving stomatal responses to changes in light levels or peak performance over longer (diurnal) timescales. Stomatal conductance and assimilation were characterised early in development (pre Growth Stage 31) over the course of a day. Kinetic and steady-state performance were assessed separately. In the first part, the intention was to study the impact of cultivar (Bowman, BW284 and BW289) on steady-state physiological responses to confirm the relationship with gene expression levels (Faure et al., 2012, Campoli et al., 2013). In the second, to complement this with kinetic response data to changing light levels in terms of cultivars. Finally, an attempt was made to characterise developmental phenotypes such as early flowering in the context of tiller, leaf formation and biomass in the light of the early maturing phenotype of these mutations (Faure et al., 2012, Zakhrabekova et al., 2013, Campoli et al., 2013). These data were used to relate physiology to phenotype, notably in terms of stomatal anatomy and also leaf water content. With the impairment to their circadian system, the barley clock mutants should have a reduced ability to anticipate time of day, and therefore their rates of assimilation and stomatal conductance could differ from Bowman over diurnal timescales. It was also hypothesised that the impaired ability to predict dawn would influence the speed of response of stomata to changing conditions, and this should interact with time of day. The impaired adaptation to diurnal cycles should drive the clock mutants to exhibit lower biomass and phenotypes linked to poor synchronisation with photoperiod such as etiolation and smaller leaves.

3.2. Methods

Thirty-six plants of the species *H. vulgare L* (cv Bowman, cv BW284 and cv BW289) were grown in 2 separate batches between 21/11/2017 and 23/01/2018 and 20/02/2018 and 26/03/2018 each randomised into 6 blocks with one biological replicate per block, in the glasshouse of the University of Essex, Wivenhoe Park, Colchester. Supplementary lighting was supplied by sodium vapour lamps if PAR fell below 200 µmol m^{-2} s^{-1} light between 0700 and 19:00 daily. Minimum daytime temperatures were 20°C and 15°C at night.

3.2.1 Assessment of functioning of the circadian clock

The functioning of the circadian clock was assessed according to the methods outlined in Chapter 2.

3.2.2 Functioning of clock mutants in low-light diel cycles

Eight seeds each of Bowman, BW284 and BW289 were planted on 1/11/17 in 0.5L pots and stratified for 4 days at 4°C before being placed in the glasshouse at the University of Essex. Each plant was assigned to one of eight blocks, with one replicate per block.

3.2.3 Steady state measurements of g_s and *A* under constant high light

Thirty-four individual plants of three different cultivars of the species *H. vulgare* L were grown; respectively twelve, eleven and eleven individuals of cv Bowman, cv BW284 and cv BW289, in a glasshouse at the University of Essex, between 11/2/2017 and 28/6/2017 and between 7/4/2017 and 3/9/2017.

Between 32 and 47 days after sowing (DAS), eight plants from each cultivar were selected, and the middle portion of the 3rd leaf of the main stem placed in the cuvette of an ADC LcPRO IRGA. Plants were allowed to settle for up to 0.5 hours at 0 µmol m^{-2} s^{-1} PAR, then exposed to 10 hours of 1000 µmol m^{-2} s^{-1} PAR, before finishing with a final 0.5 hours at 0 µmol m^{-2} s^{-1} PAR.

3.2.4 Evaluation of kinetics of g_s and *A* under step changes in light

Separately, eighteen individuals of three different cultivars of the species *H. vulgare* L were grown in a blocked design; six each of cv Bowman, cv BW284 and cv BW289, in the University of Essex greenhouse, between 2/10/15 and 12/2/16 and between 18/12/2015 and21/4/2016. Between 21 and 32 DAS, each plant was run through a diurnal step change protocol using a Li-Cor 6400 IRGA. Two variants on the method were used. Plants were exposed to an alternating regime of 1 hour at 100 µmol m^{-2} s^{-1} PAR before the light intensity was increased to 1000 µmol m^{-2} s^{-1} PAR for 1 hour. This process was repeated for 6 times, with 1 hour at the end of 100 µmol m^{-2} s^{-1} PAR. In the other variant, plants were exposed to constant light at 100 µmol m^{-2} s^{-1} for 6 hours before 3 cycles of low-high light were applied with 1 hour of low light at the end as before.

3.2.5 Data modelling of kinetic and steady-state physiological data

3.2.5.1 Kinetic modelling

The step changes sections 3.2.2 and 3.2.3 above were parameterised using a simplified version of the growth model derived by Vialet-Chabrand (2017b) as follows:

$$f(g) = g_{min} + (g_{max} - g_{min})e^{(-t/\tau)}$$

Where g_{min} was the predicted value observed at low light, g_{max} the predicted maximal value and τ (tau) the time constant associated with an increase of 63% from the minimum to the maximum value with naïve Bayesian estimators applied to each value.

3.2.5.2 Steady-state modelling

Values during the steady-state periods for section 3.2.2 were parameterised using a simple model devised by Yin and Porporato (2017) to avoid sinusoidal modelling of information-deficient datasets, or the use of the Fast Fourier Transform for similar reasons. Each steady state dataset for *A* or g_s was broken down into its mean, its phase (as measured by the centroid) and its amplitude (as measured by standard deviation).

3.2.6 Harvest

Plants involved in the 'square wave diurnal experiment' as outlined in section 3.2.2 were harvested at 11, 21, 32, 47 and 68 DAS, with a final harvest being made at 121 DAS. Total dry biomass, total grain mass, total grain number, number of ears and number of tillers were established at the final harvest. In addition, at each harvest, point measurements of stomatal conductance using an AP1 porometer and of chlorophyll fluorescence using a FMS2 fluorimeter were made.

3.3. Results

The studies described below aimed to assess the physiological consequences of mutations to the circadian clock genes. Having done so, further work sought to identify more-detailed physiological traits to understand short term differences between the clock mutants and Bowman, and to consider anatomical features that might underpin those differences. Elsewhere, developmental impacts of the clock mutations were assessed to understand the timing, nature and extent of physical changes from seedling to harvest.

3.3.1 Assessment of the functioning of the circadian clock

It is usual to explore clock function by exposing a plant previously grown in day:night conditions to constant light to understand the extent to which circadian rhythms are able to persist in the absence of a regular reset at dawn or dusk. To test the assertion that the mutants BW284 and BW289 had impairment to their clock function, they along with Bowman were placed in constant light conditions for 96 hours (Fig. 3.1), and stomatal conductance measured every minute with an IRGA. The first day's measurement was not used, and only the data for the ensuing four days considered. The periodicity parameterised (Fig. 3.2) to understand the relationship between cyclical amplitude and period length.

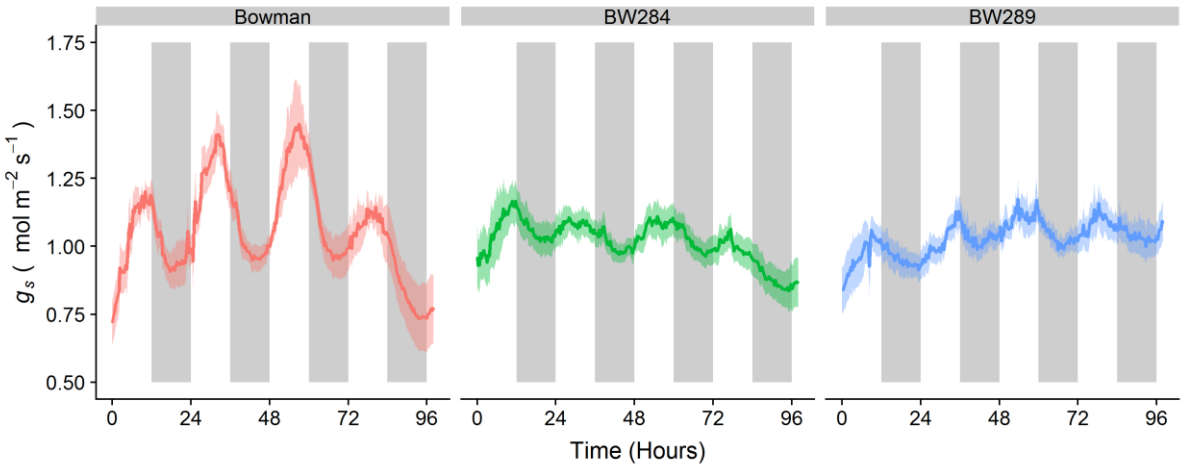

Figure 3.1: Time series of stomatal conductance (g_s) for Bowman and clock mutants BW284 & BW289. Plants were exposed to constant PAR of 100 µmol m^{-2} s^{-1} for 5 days with the first day's measurement's discarded, and subsequent four days' data displayed above, starting from apparent dawn (h=0). Vertical grey bars denote apparent night based on plants' growth environment. Data shown are normalised to mean = 1 mol m^{-2} s^{-1} across the entire period, but are not otherwise scaled. Data are shown are means at each time point +/- SE, N=7-8.

The data were rescaled to have a mean of 1 mol m^{-2} s^{-1} (Fig. 3.1), and show attenuated and inconsistent but not eliminated circadian rhythms of stomatal conductance in the clock mutants, while for the control, a strong rhythm persists with g_s peaking consistently in the subjective afternoon. The differences are underlined (Fig. 3.2) by Bowman appearing to form a separate group compared to the clock mutants, with a peak of g_s at 23.9 hours and an RAE of below 0.2 mmol m^{-2} s^{-1}. The mutants appear to retain some periodicity, albeit with high variability in the period between replicates which may reflect residual changes in temperature and humidity in the experimental area. There were no differences between mutants and Bowman (1-way ANOVA, $p>0.05$). Bowman had a significantly smaller RAE than either mutant (1-way ANOVA, $F_{(2, 24)}=5.14$, $p=0.01$).

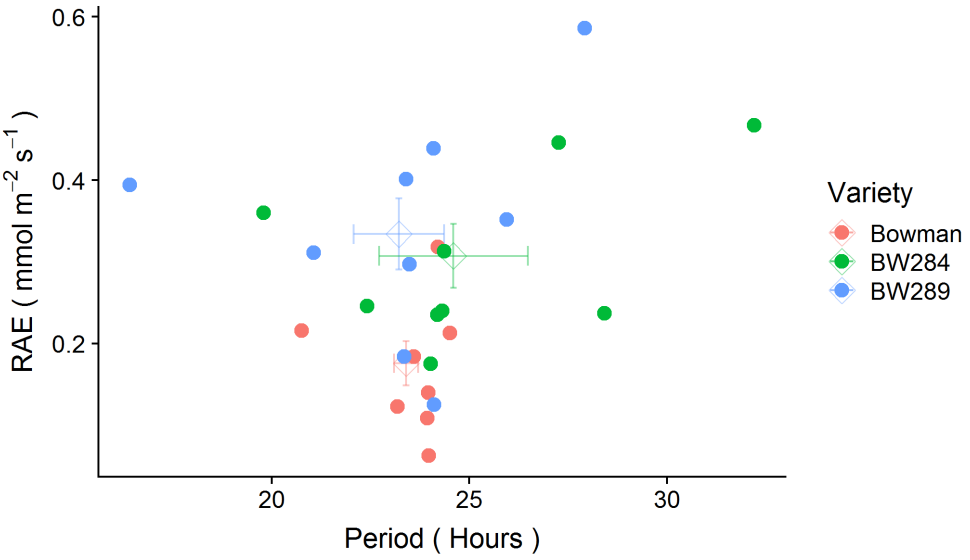

Figure 3.2: Scatterplot of calculated photoperiod versus relative amplitude error (RAE) for Bowman and the clock mutants BW284 and BW289. Both parameters were calculated using the BRASS software. Each point represents one biological replicate. Diamonds represent mean values +/- SE, N=7-8.

The next step was to test the physiological performance of the mutants versus control over square-wave diel cycles. To this end, plants were run over two days of 12h:12h light dark cycles, still at low light levels (PAR = 100 µmol m^{-2} s^{-1}, Fig. 3.3). With the opportunity for a daily reset at dawn and dusk, plant behaviour is entrained regardless of the presence or absence of clock mutations. The clock mutant BW289 had consistently the highest assimilation (Fig. 3.3A), and BW284 the lowest. Similarly, BW289 had consistently the highest g_s, while that for Bowman and BW284 was similar overall. There appeared to be a distinct delay in the rise in g_s after apparent dawn for Bowman compared to BW284 and BW289, with Bowman also appearing to have a distinct peak of g_s later in the day. The result was that Bowman had consistently higher WUE_i compared to the clock mutants, with all cultivars experiencing a decline in WUEi during the course of the day. For a more-complete picture of behaviour, these data were therefore parameterised into mean, phase and amplitude components (Fig. 3.4).

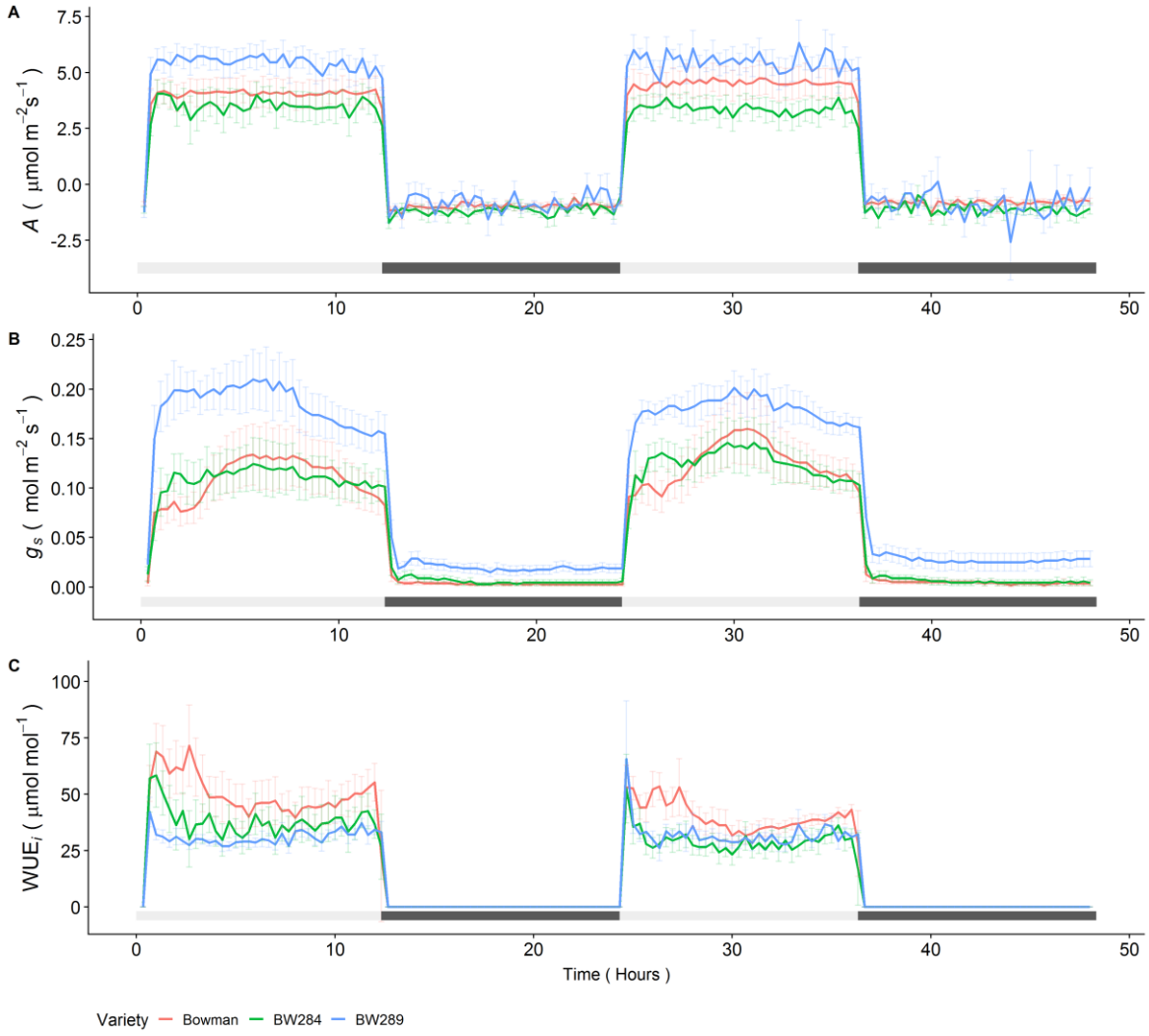

Figure 3.3: Time series of assimilation (A), stomatal conductance (B) and WUE_i (C) for Bowman and the clock mutants BW284 and BW289. Plants were exposed to alternating periods of light (PAR = 100 µmol m^{-2} s^{-1}) and dark (PAR = 0 µmol m^{-2} s^{-1}) for 12 hours each over two days. Data shown are means for each cultivar +/- SE, N=7-9.

Comparisons were made for each of mean, phase and amplitude for each of *A*, g_s and WUE_i curves (Fig. 3.4). The mean was the simple average of all points, the amplitude for each curve was approximated by the standard deviation and the phase was the difference between the centroid and the mid-point of each diurnal period (6 hours).

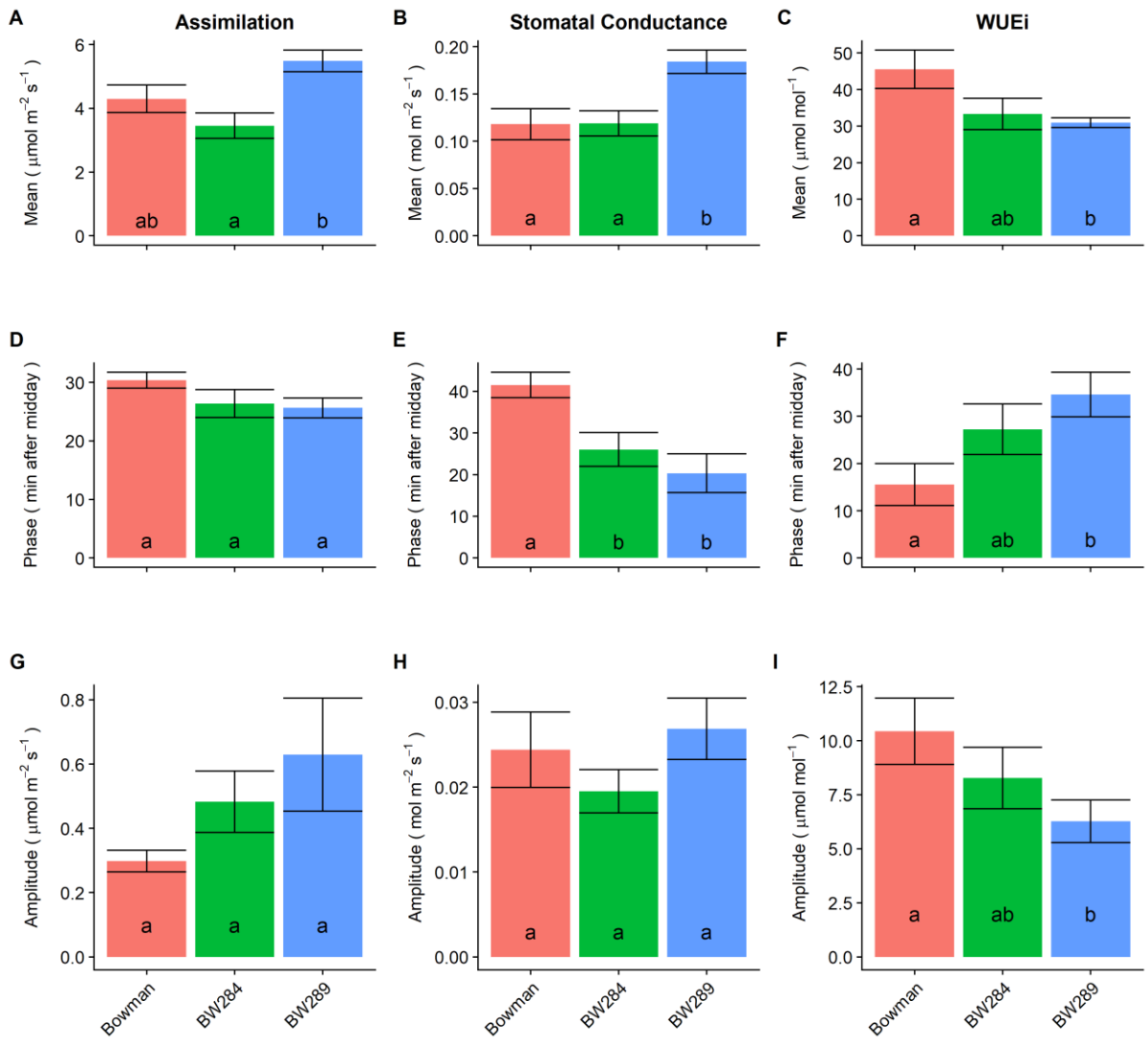

Figure 3.4. Parameterisation of daytime outputs of 2-day diel time series for Bowman, BW284 and BW289 for *A*, g_s and WUE_i. A) Mean *A*. B) Mean g_s. C) Mean WUE_i. D) Phase advance of *A* relative to midday. E) Phase advance of g_s relative to midday. F) Phase advance of WUE_i relative to midday. G) Amplitude of *A*. H) Amplitude of g_s. I) Amplitude of WUE_i. Means shown +/- SE. N=14-15. Mean = mean of relevant values. Phase = centroid of relevant values. Amplitude = standard deviation of relevant values. No significant differences on Tukey's test were found in pairwise comparisons with bars having identical letters at $p_{crit} = 0.05$.

The mutant BW289 (Fig. 3.4A) had significantly higher mean *A* than BW284 (5.48 µmol m^{-2} s^{-1} vs 3.45 µmol m^{-2} s^{-1}), and g_s than either BW284 or Bowman (Fig. 3.4B, 0.18 mol m^{-2} s^{-1} vs 0.12 mol m^{-2} s^{-1} for the others). As a result, Bowman had the highest mean WUE_I (45.5 µmol mol^{-1}, Fig. 3.4C), significantly greater than that of BW289 (31 µmol mol^{-1}, Tukey's test, $p<0.05$). While there was no difference in phase of *A* between varieties (Fig. 3.4D), the move of phase for g_s to 42 minutes later than midday for Bowman Fig. 3.4E) was 37% less in BW284 and 52% less in BW289 (Tukey's Test, $p<0.05$). The result was that BW289 has a phase for WUE_i (Fig. 3.4F) that was 35 minutes after midday, significantly more

advanced than Bowman's (16 minutes, Tukey's test, $p<0.05$). While BW289 had the greatest amplitude of response for *A* (0.63 µmol m^{-2} s^{-1}, Fig. 3.4G) and g_s (0.27 mol m^{-2} s^{-1}, Fig. 3.4H), it had the smallest amplitude for WUE_i of just 6.3 µmol mol^{-1} (Fig. 3.4I), significantly less than Bowman's (10.4 µmol mol^{-1}).

3.3.2 Assessing the impact of clock mutations on developmental phenotypes

Both BW284 and BW289 varieties passed the critical flowering stage of growth stage 31 (Fig. 3.5) many days before Bowman achieved the same level of maturity. BW284 already had a significantly later growth stage than Bowman at 21 Days After Sowing (DAS) (Fig. 3.5C, Tukey's test, $p<0.05$), and Bowman remained at a significantly earlier growth stage than both mutants at 32 and 47 DAS (Tukey's test, $p<0.05$). Similar observations were made for height, where BW289 was significantly shorter than Bowman at 68 DAS (Fig. 3.5A, Tukey's test, $p<0.05$) and tiller number, where Bowman had more tillers than BW289 at 47 and 68 DAS (Fig. 3.5C, Tukey's test, p<0/05), although the number of tillers produced had been the same up to that point.

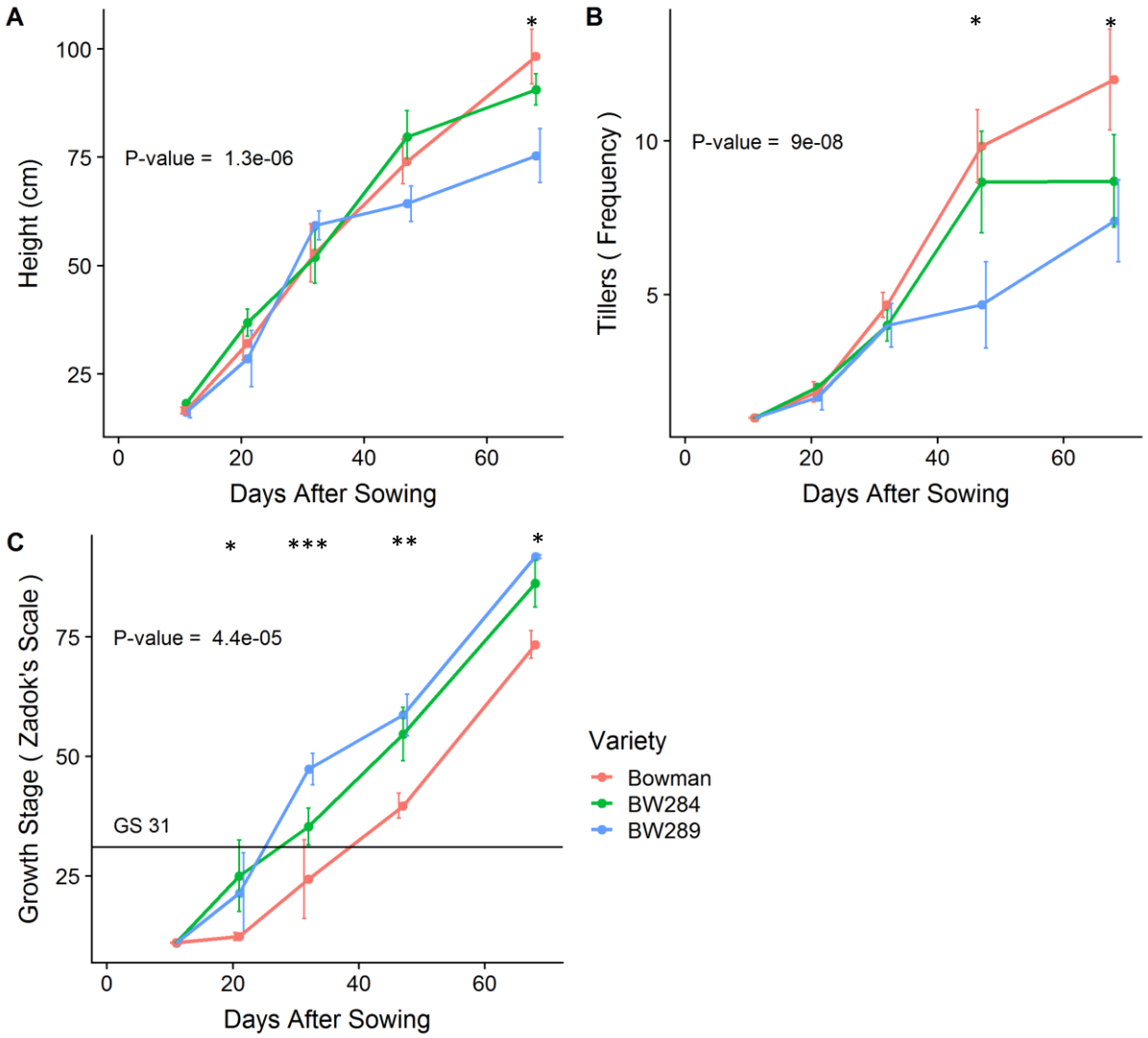

Figure 3.5: Stem height (A) and tiller number (B). Stars indicate where at least one mutant was significantly different from Bowman, * p<0.05. N=6, means shown +/- SE. (C) Growth stage of the main stem on the Zadok scale. X-axis shows the first five harvests of Bowman and the mutants BW284 and BW289. Growth stage 31 is indicated by the horizontal line, and marks the point at which vegetative growth becomes reproductive growth. Means shown +/- SE, N=6. Stars indicate where at least one mutant is significantly different from Bowman based on Tukey's HSD test, * p<0.05, ** p<0.001, *** p<0.0001.

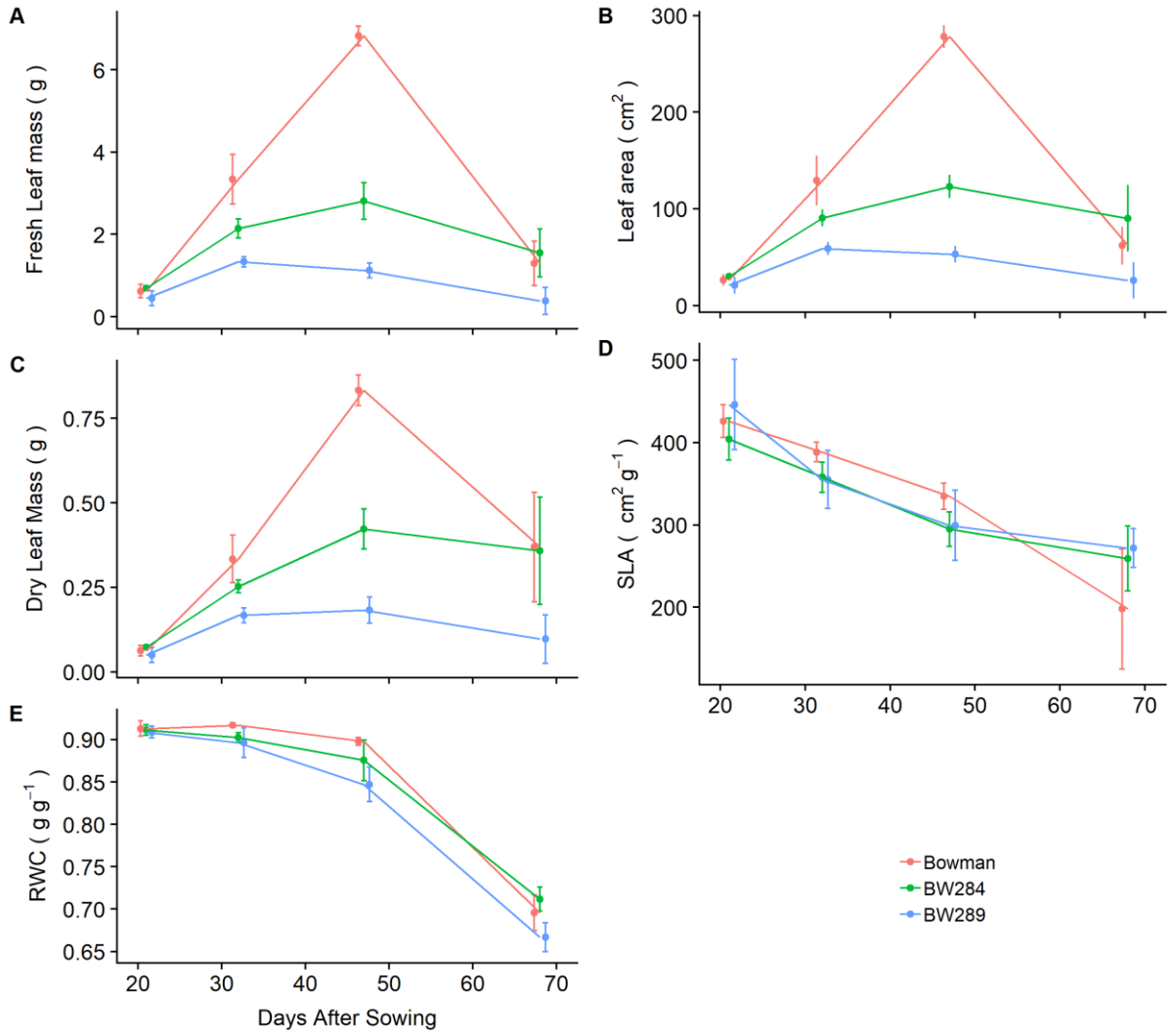

Figure 3.6: Summary plots of fresh leaf mass (A), total leaf area (B), dry leaf mass (C) Specific Leaf Area (D) and (E) Relative Water Content for the first four harvests of Bowman and the clock mutants BW284 and BW289. Means shown +/- se for each point, N=6.

Bowman, BW284 and BW289 were assessed on their leaf characteristics (Fig. 3.6), focusing on fresh and dry mass, and by extension, water content, and leaf area plus specific leaf area, which approximates to leaf thickness (Vile et al., 2005). There was a significant interaction (Fig. 3.6A) between harvest date and variety in predicting fresh leaf mass (2-way ANOVA, $F_{(2,55)}=57.6$, $p=2.2x10^{-16}$). At the first harvest, 21 DAS, there were no differences in fresh weight (Fig. 3.6A), however, Bowman accumulated significantly more fresh leaf mass than either mutant by the time of the second (32 DAS) and third (43 DAS) harvests (Tukey's test, $p<0.05$). By the time of the fourth harvest at 68 DAS, Bowman had significantly greater fresh leaf mass than BW289 (Tukey's test, $p<0.05$). There was a significant interaction between harvest date and

variety on leaf area (2-way ANOVA, $F_{(2,70)}$=57.6, p=2.2x10^{-16}, Fig. 3.6B); leaf area was identical at the first harvest, but Bowman had significantly more leaf area than BW289 by the second harvest, more than either mutant at the third harvest and more leaf area than BW289 at the fourth harvest (Tukey's tests, p<0.05). However, Bowman had accumulated more dry leaf mass than BW289 only at the fourth harvest (Fig. 3.6C, Tukey's test p<0.05), and there were no significant differences between varieties for specific leaf area (Fig. 3.6D, Tukey's test, p>0.05). There was a significant interaction between variety and harvest date on relative water content (Fig. 3.6E, 2-way ANOVA, $F_{(2,53)}$=3.41, p=0.006), and while there were no differences between varieties or between harvest times for the first two harvests, by the third harvest at 43 DAS, Bowman had significantly greater RWC than BW289, the latter having fallen significantly since the second harvest date (Tukey's test, p<0.05). By the time of the fourth harvest date, all three cultivars had significantly lower RWC than at the prior harvest, and BW284 had significantly higher RWC than BW289 at that point (Tukey's test, p<0.05). It was noticeable that BW289's peak fresh leaf mass (Fig. 6A) and leaf area (Fig. 3.6B) occurred at 21 DAS, while that for Bowman and BW284 occurred at 32 DAS. Yet dry leaf mass (Fig. 3.6C) continued to rise for all three cultivars until 32 DAS.

3.3.3 Detailed assessment of developmental phenotypes

For a more-detailed examination of the variability of plant phenotypes between cultivars and over time, separate assessments were made of fresh mass across three organs – leaves, stems (and tillers) and ears for fresh (Fig. 3.7) and dry mass (Fig. 3.8), total area (Fig. 3.9) and relative water content (Fig. 3.10).

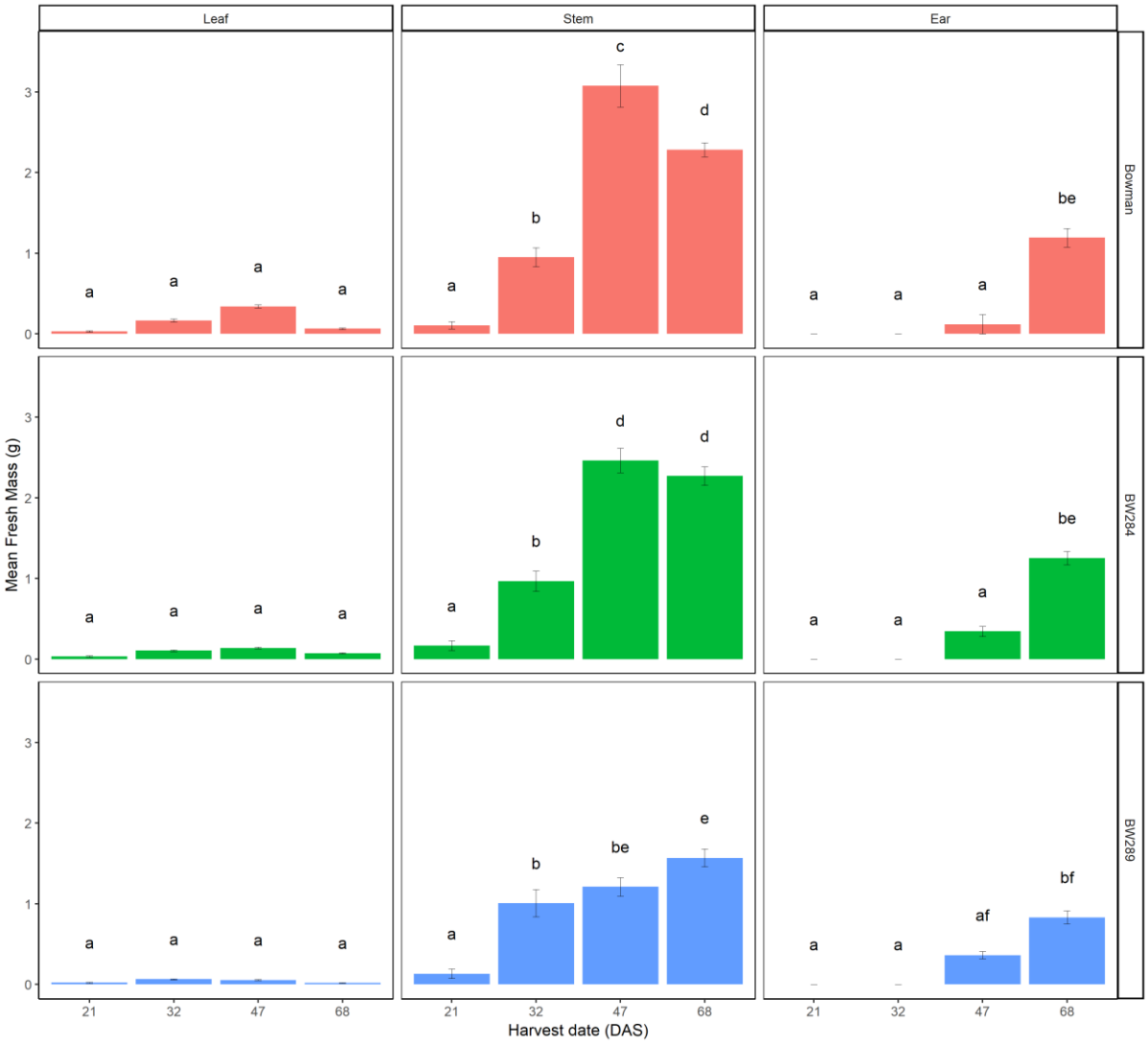

Figure 3.7: Mean fresh mass for the leaves (left), stem & first three tillers (middle) and ears (right) at four harvest dates. Means shown +/- SE at each point. N=6. N=6. Identical letters denote no significant differences found in pairwise comparisons using Tukey's test of model outputs.

From the detailed assessment of fresh mass (Fig. 3.7), mean leaf mass was broken down by variety and harvest date (Fig. 3.7, left panels), and while no differences were observed, it was clear that fresh mass peaked for Bowman and BW284, as previously noted (Fig. 3.6A). Bowman (Fig. 3.7, middle panels) had heavier stems / tillers than both mutants (3.5g vs. 2.5g and 1.2g for BW284 and BW289) at 47 DAS and they remained heavier than BW289's at 68 DAS by 46%, although they were not different from BW284's then (Tukey's tests, p_{crit}=0.05). No differences were seen between the cultivars in terms of fresh ear weight at any of the harvest points (Tukey's test, $p<0.05$), and Bowman (1.19 g) and BW284 (1.25 g) appeared somewhat heavier than BW289 (0.83 g) by 68 DAS

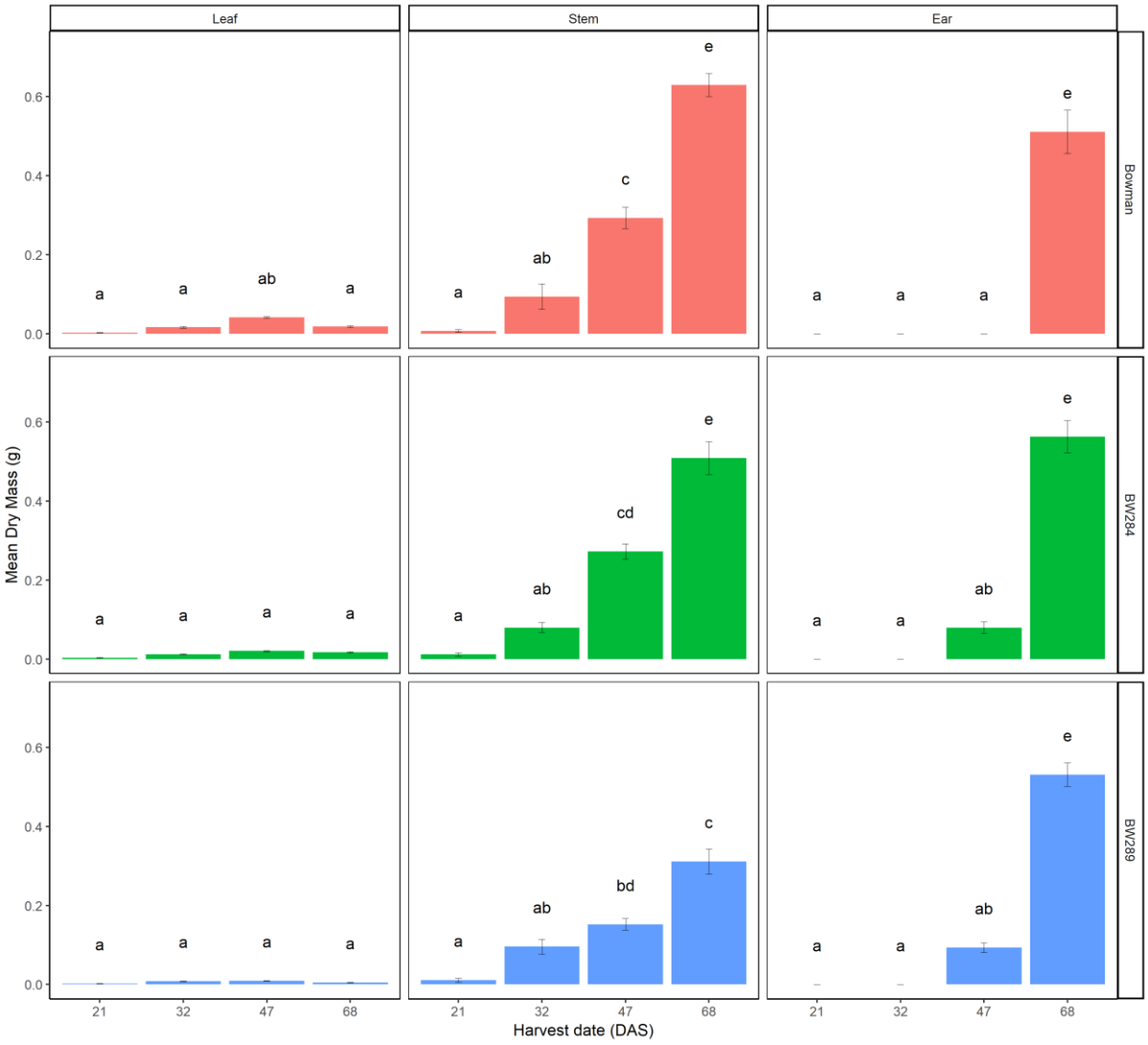

Figure 3.8: Mean dry mass for the leaves (left), stem & first three tillers (middle) and ears (right) at four harvest dates. Means shown +/- SE at each point. N=6. N=6. Identical letters denote no significant differences found in pairwise comparisons using Tukey's test of model outputs.

In common with the fresh mass data (Fig. 3.7), there were few obvious differences between leaves (Fig. 3.8, left panels) although peak dry mass was achieved by all three varieties at 47 DAS before reserves were remobilised and the rate of senescence increased. It was in the stems that the phenotypes were again clearer (Fig. 3.8, middle panels), where Bowman had significantly greater dry mass (0.29 g) by 47 DAS than BW289 (0.15 g, Tukey's test, $p<0.05$), but only modestly more than BW284 (0.27 g). There was a remarkable similarity in ear dry weight (Fig. 3.8, right panels) at 68 DAS, where BW284 had slightly heavier ears (0.56 g) than Bowman (0.51 g) and BW289 (0.53 g).

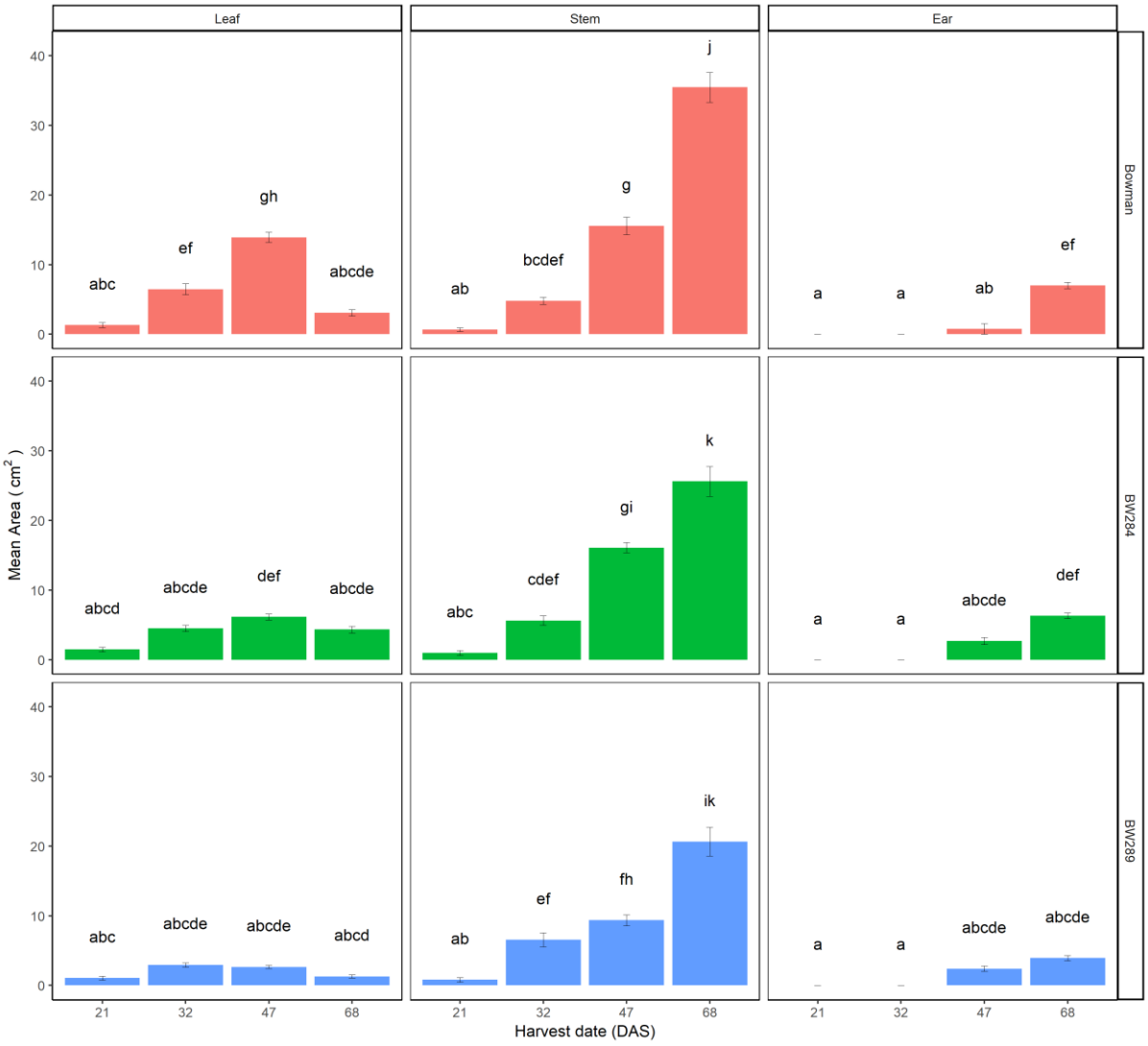

Figure 3.9: Mean area for the leaves (left), stem & first three tillers (middle) and ears (right) at four harvest dates. Means shown +/- SE at each point. N=6. N=6. Identical letters denote no significant differences found in pairwise comparisons using Tukey's test of model outputs.

One-sided surface area (Fig. 3.9) presented a more-complex picture with leaf area peaking at 47 DAS for Bowman and BW284, as seen previously (Fig. 3.6C), while stems continued to increase in area throughout the period (Fig. 3.9, middle panels), as did ears, all of which suggests a subtle remobilisation of reserves through time. Peak leaf area for Bowman (13.9 cm^2, Fig. 3.9, left panel) was more than double that of BW284 and over four times more than BW289's (Tukey's test, $p<0.05$). Yet by 68 DAS, these differences had disappeared, and BW284 now exhibited the greatest area (4.4cm^2), now 40% larger than Bowman and 227% more than BW289's, although all had experienced declines in area. Bowman's ability to increase stem area outlasted the clock mutants' (Fig. 3.9, middle panels), where Bowman at 47 DAS (15.6 cm^2) was

actually slightly slower than BW284's (16.1 cm^2), but both had by then exceed BW289 (9.4 cm^2, Tukey's test, $p<0.05$). Yet by 68 DAS, Bowman had the greatest surface area for stems & tillers (35.5 cm^2), 39% more than BW284 and 72% more than BW289 (Tukey's test, $p<0.05$). Bowman appeared to start filling its ears later (its ear area was just 0.8 cm^2 at 47 DAS, compared to 2.7 and 2.4 cm^2 for BW284 and BW289), while at 68 DAS, Bowman's ears had grown to 7.0 cm^2, a near ten-fold increase, compared to 6.3 cm^2 for BW284 and 3.9 cm^2 for BW289, which had not even doubled.

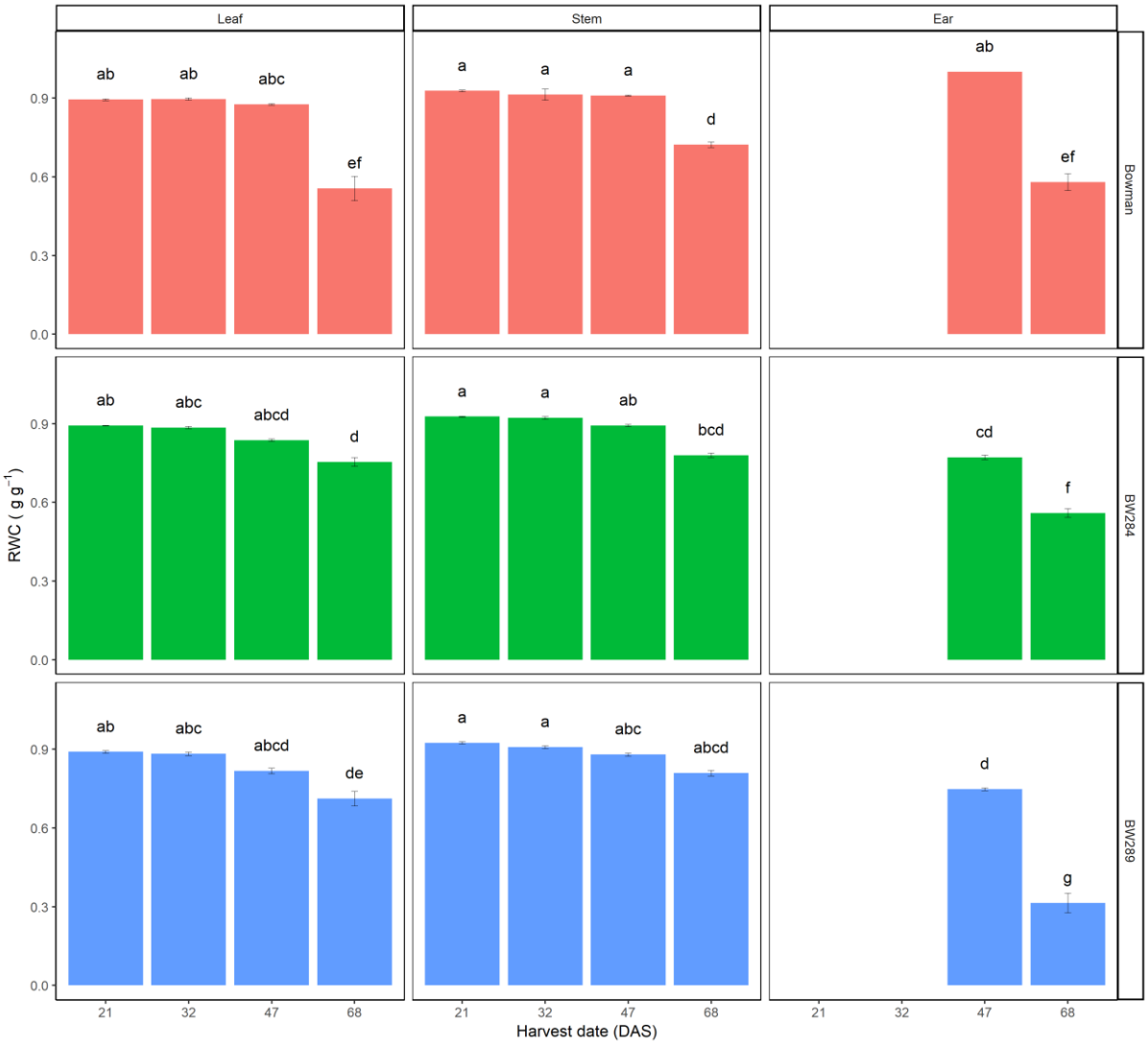

Figure 3.10: Mean Relative Water Content (RWC) for the leaves (left), stem & first three tillers (middle) and ears (right) at four harvest dates. Means shown +/- SE at each point. N=6. N=6. Identical letters denote no significant differences found in pairwise comparisons using Tukey's test of model outputs.

Relative water content declined significantly from the second to the fourth harvest date for leaves and stems, and between the third and fourth harvest date for ears (Fig. 3.10, Tukey's tests, $p<0.05$). At the leaf level, there were no differences between cultivars at 21, 32 and 47 DAS (Fig. 3.10, left panels), but, Bowman had significantly lower RWC than BW284 at 68 DAS (Tukey's test, $p<0.05$) There were no differences between any cultivar in terms of relative water content of stems & tillers (Tukey's test, $p<0.05$). The ears of Bowman had significantly higher RWC than either mutant at 47 DAS (Fig. 3.10, right panels), and remained higher than BW 289 at 68 DAS (Tukey's test, $p<0.05$).

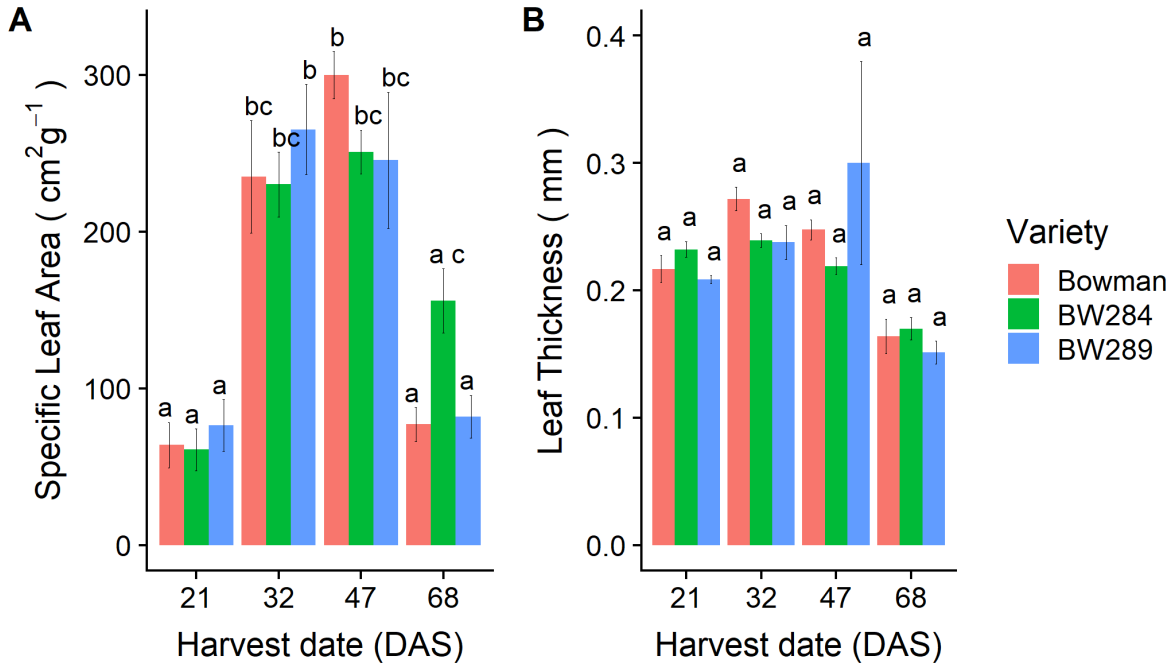

Figure 3.11: A) Mean Specific Leaf Area (SLA) and B) Mean leaf thickness (SLA x Leaf Dry Matter Content)$^{-1}$ at four harvest dates. Means shown +/- SE. N=6. Identical letters denote no significant differences found in pairwise comparisons using Tukey's test of model outputs.

Specific leaf area was calculated for each leaf, and then averaged over the plant (Fig. 3.11A); SLA rose between 21 DAS and 32 DAS, then fell again between 47 DAS and 68 DAS (Tukey's tests, $p<0.05$). There was a significant interaction between variety and harvest date on SLA (2-way ANOVA, $F_{(2,55)}=2.80$, $p=0.018$), but when leaf water content was corrected for (Fig. 3.11B), there were no differences between varieties at any harvest point, but leaf thickness overall had dropped significantly between 47 DAS and 68 DAS (Tukey's test, $p<0.05$).

The final harvest, at 149 DAS also showed consistent differences between the varieties (Fig. 3.12). There was a significant effect of variety on the number of ears (1-way ANOVA, $F_{(2,12)}$=6.15, p=0.012), where BW284 and BW284 had significantly more ears than Bowman (Fig. 3.12A, Tukey's test, p<0.05).Variety was also a significant predictor of grain number (1-way ANOVA, $F_{(2,12)}$=11.2, p=0.003), where Bowman and BW284 had significantly more grains per plant than BW289 (Fig. 3.12E, Tukey's test, p<0.05). Variety was not a significant predictor of dry mass (Fig. 3.12B), harvest index (Fig. 3.12C), grain mass (Fig. 3.12D) or thousand grain weight (Fig. 3.12F) (1-way ANOVAs, P>0.05).

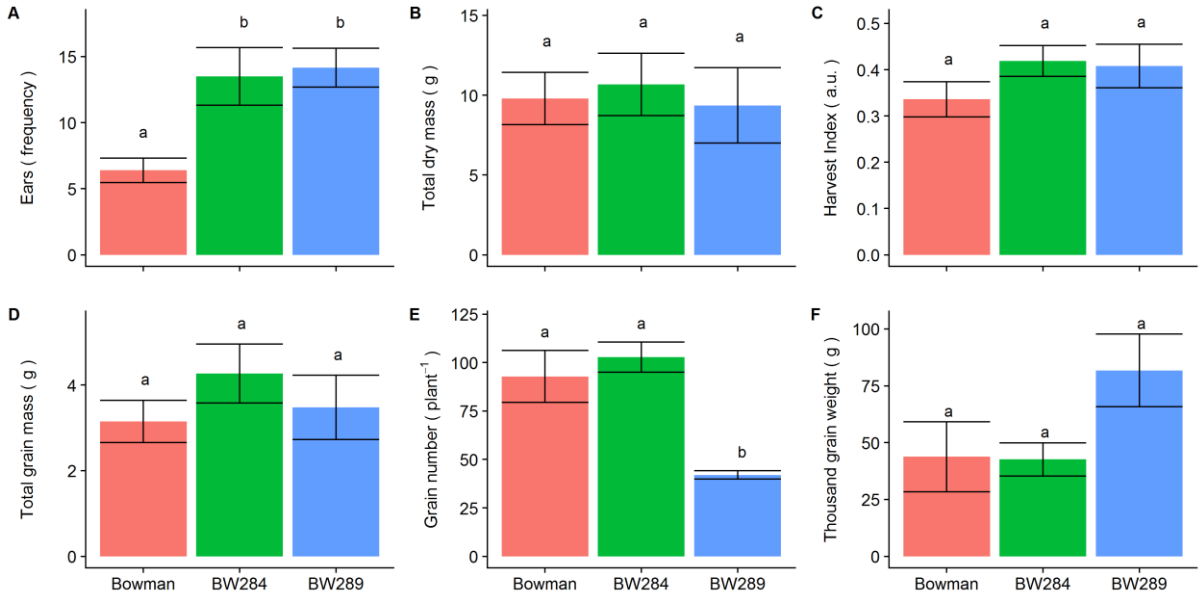

Figure 3.12: Final yield and yield components. A) Ear number, B) Total dry mass, C) Harvest index, D) Total grain mass, E) Grain number and F) Thousand Grain Weight at the final harvest at 122 DAS for Bowman and the two mutants BW284 and BW289. N=5-6, means shown +/- se.

3.3.4 Diurnal Responses of *A* and g_s to steady state illumination

To test whether the observed developmental differences could be accounted for by short-term differences in steady-state physiological performance, Bowman, BW284 and BW289 were dark adapted then a leaf exposed to constant light for ten hours in an IRGA (Fig. 3.13). The curves for assimilation show a consistent rise at the start of the period, with a steady decline during the course of the ten hours (Fig. 3.13A). The mean values for g_s also showed a rapid rise under high light followed by a decline during the rest of the day (Fig. 3.13B); but in the case of Bowman, and to a lesser extent BW284, there was a

pronounced peak in the afternoon. Combining these data into WUE_i, the consequence was that mean values for Bowman exceeded that for the mutants in the first hours, but then profoundly underperformed for a large proportion of the afternoon period (Fig. 3.13C); WUE_i for the clock mutants rose slowly during the course of the day and ended higher than Bowman. These data were parameterised and the statistical tests on those parameters are reported (Fig. 3.14)

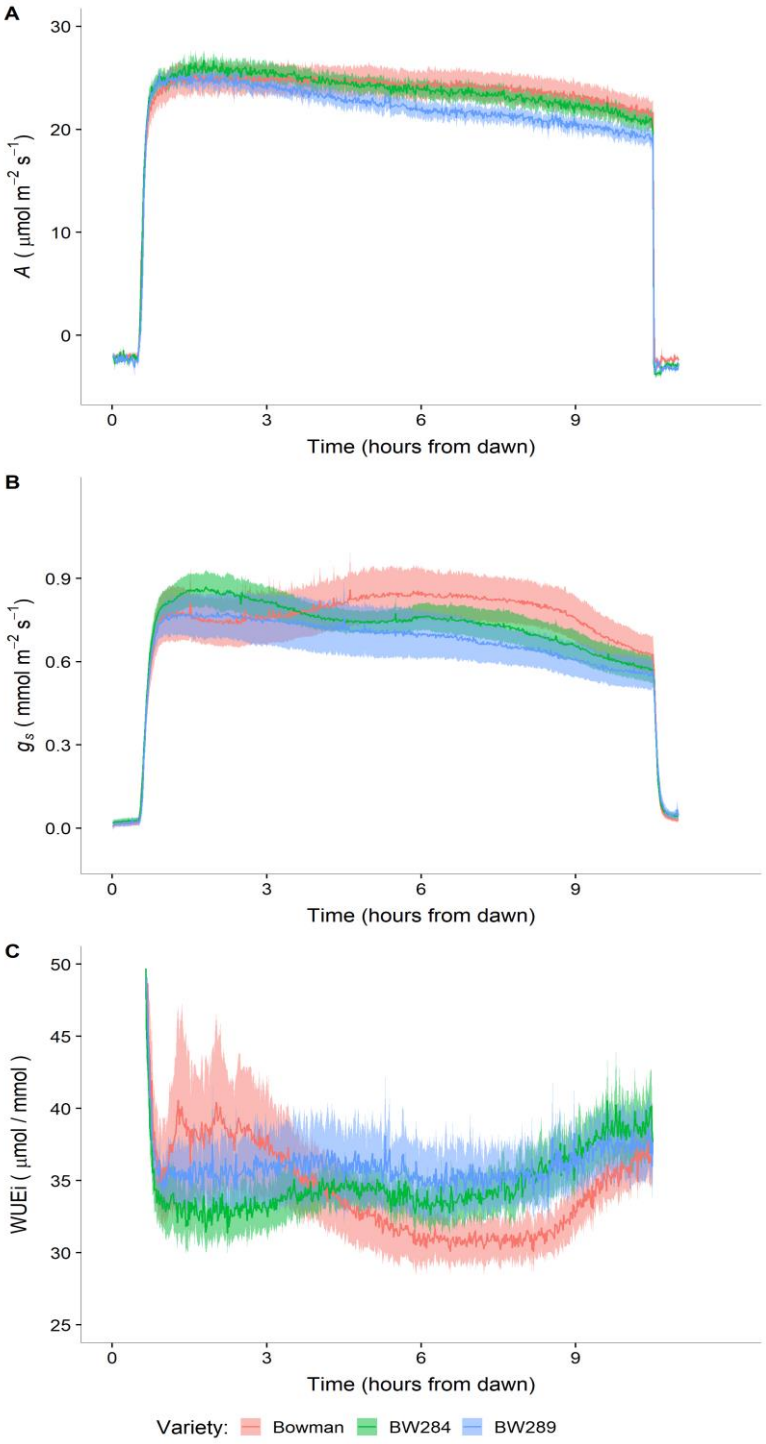

Figure 3.13: Diurnal curves for Bowman, BW284 and BW289. Plants were dark adapted before the experiment started. They were then given 30 minutes with no light, followed by 10 hours at 1000 µmol m^{-2} s^{-1} PAR then another thirty minutes with no light. Temp = 24°C in the cuvette, with atmospheric [CO_2] and [H_2O]. N=12 for each cultivar, means shown +/- se. A. Assimilation in µmol m^{-2} s^{-1} CO_2 shown for Bowman, BW284 and BW289. B. Stomatal conductance in mol m^{-2} s^{-1} H_2O for the three cultivars Bowman, BW284, BW289. C. Intrinsic Water Use Efficiency for Bowman, BW284 and BW289 in µmol mol^{-1}.

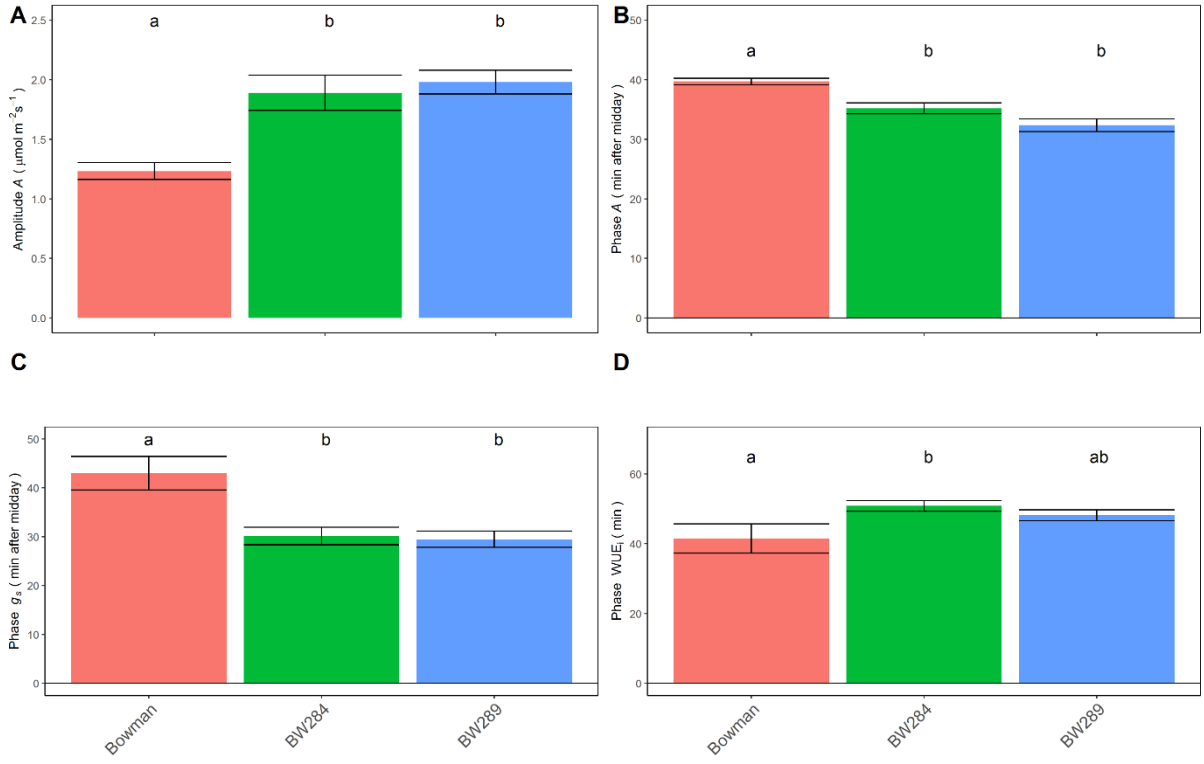

Figure 3.14: Parameterisation of steady state performance of Bowman, BW284 and BW289 from diurnal data (Figure 13). A) Amplitude of *A*. B) Phase of *A* C) Phase of g_s D) Phase of WUEi.. N=12 for each cultivar, means shown +/- se.

The diurnal curves (Fig. 3.13) for *A*, g_s and WUE_i were parameterised using the method described by Yin and Porporato (2017) as previously. Bowman had a significantly lower amplitude of assimilation over the course of the day than either clock mutant (Fig. 3.14A). Perhaps more interestingly, Bowman experienced an advance of its phase of assimilation by 40 minutes ahead of midday, compared to 35 and 32 minutes for BW284 and BW289 (Fig. 3.14B, Tukey's test, $p<0.05$). A more-extreme pattern emerged for the phase of g_s where Bowman's phase was advanced by 43 minutes, compared to 30 and 29 minutes for BW284 and BW289 (Fig. 3.14C, Tukey's test, $p<0.05$). Once again, the consequence of phase advance for g_s and *A* was a retardation of phase for WUE_i.

3.3.5 Diurnal responses of *A* and g_s to fluctuating light

While steady-state responses are one means of distinguishing between cultivars, an alternative means by which developmental differences in harvest outcomes could arise is by variability in the speed of a variety's response to changing environmental conditions, particularly light. In particular, given that photosynthetic responses should be more-or-less instantaneous to changing light levels, the mismatch

between responses of *A* and g_s ought to occur as stomatal aperture is at least in part determined by the clock. Furthermore, recent evidence has shown the impact of acclimation on responses, and in this context, an impaired clock could also be an important modulator of responses. This series of studies focused on two areas, whether there was any short-term acclimation to fluctuating light conditions and whether there was variability in response over the course of a day. Two separate studies were run (Fig. 3.15 A, C, E and B, D, F) varied by the acclimation process in the first seven hours of the day, otherwise the exposure to fluctuating light was identical.

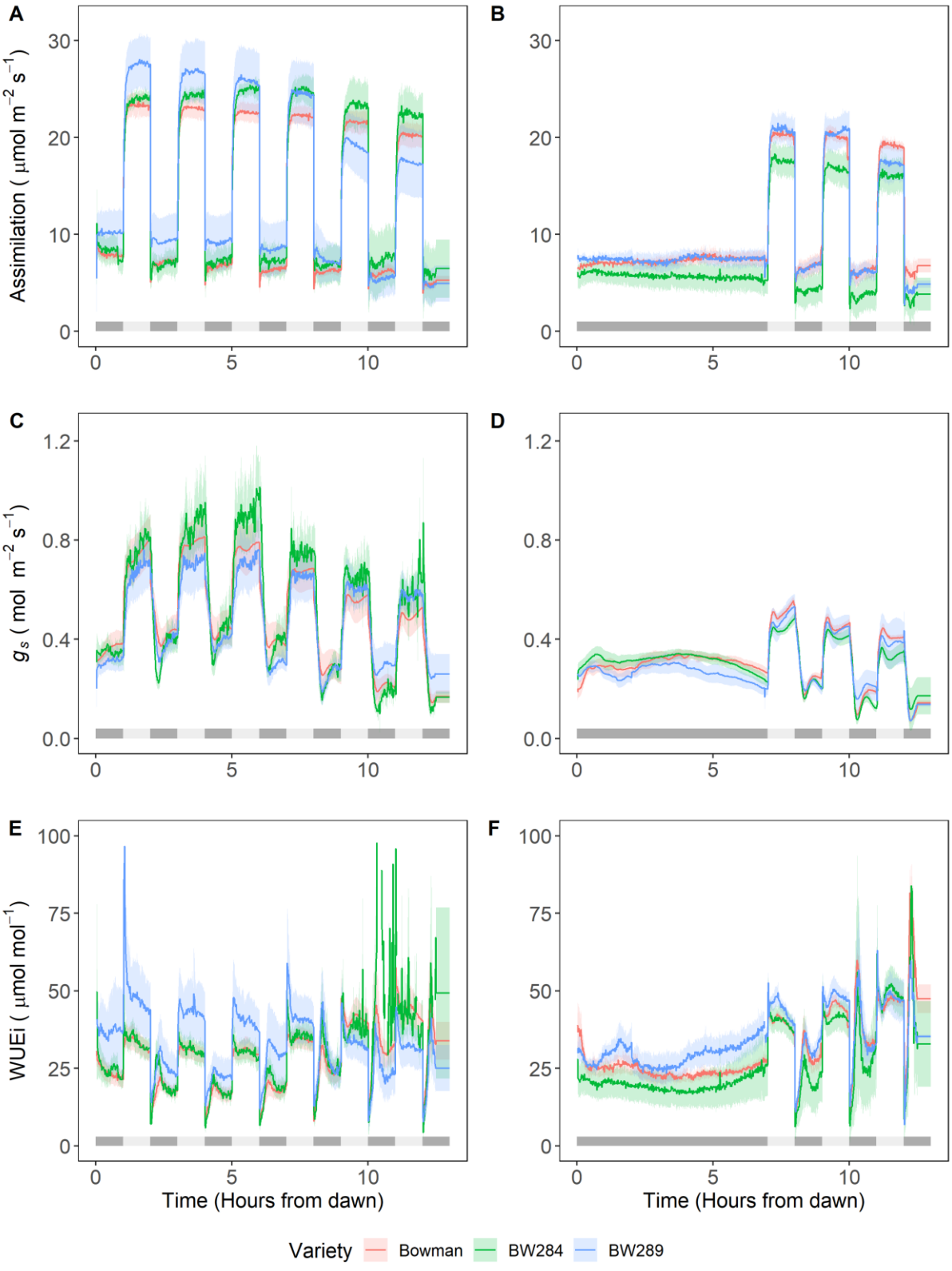

Figure 3.15: Time series of kinetics experiments, A, C, E 6 hourly step change repetitions from low (100 µmol m^2 s^{-1}) to high (1000 µmol m^2 s^{-1}) light; B, D, F 3 step change repetitions, pm only from low (100 µmol m^2 s^{-1}) to high (1000 µmol m^2 s^{-1}) light, following an initial 7 hours at low light. A, D Assimilation. B, E stomatal conductance. C, F WUE_i. Data shown are means at each time point +/- se. N = 6.

The outputs of the fluctuating light studies, reported above (Fig. 3.15), were parameterised into the slope parameters *tau* (τ, the time constant for the model), the maximum slope, Sl_{max} and the time taken to reach 95% of maximal values ($\tau_{0.95}$). Comparisons of the parameters for τ, Sl_{max} $\tau_{0.95}$ were used as dependent variables in two-way repeated measures ANOVAs considering variety and experiment as the treatments, where 'experiment' meant the first or second study in the series in which the light regime changed (Fig. 3.15). Bowman had a significantly shorter τ for assimilation than BW284 (Fig. 3.16D) under increasing light intensity; there was no difference between the mutants (2-way ANOVA, $F_{(2, 74)}$=4.41 p=0.015). Further, there was an interaction between variety and acclimation for $\tau_{0.95}$ (2-way ANOVA, $F_{(2, 74)}$=5.11, p=0.008) on assimilation (Fig. 3.16G) under increasing light intensity. There was also an interaction between acclimation and variety on the slope of g_s (Fig. 3.16B; 2-way ANOVA, $F_{(2,61)}$=4.79, p=0.011) under increasing light intensity. There were no other significant differences detected in the analysis of kinetic responses.

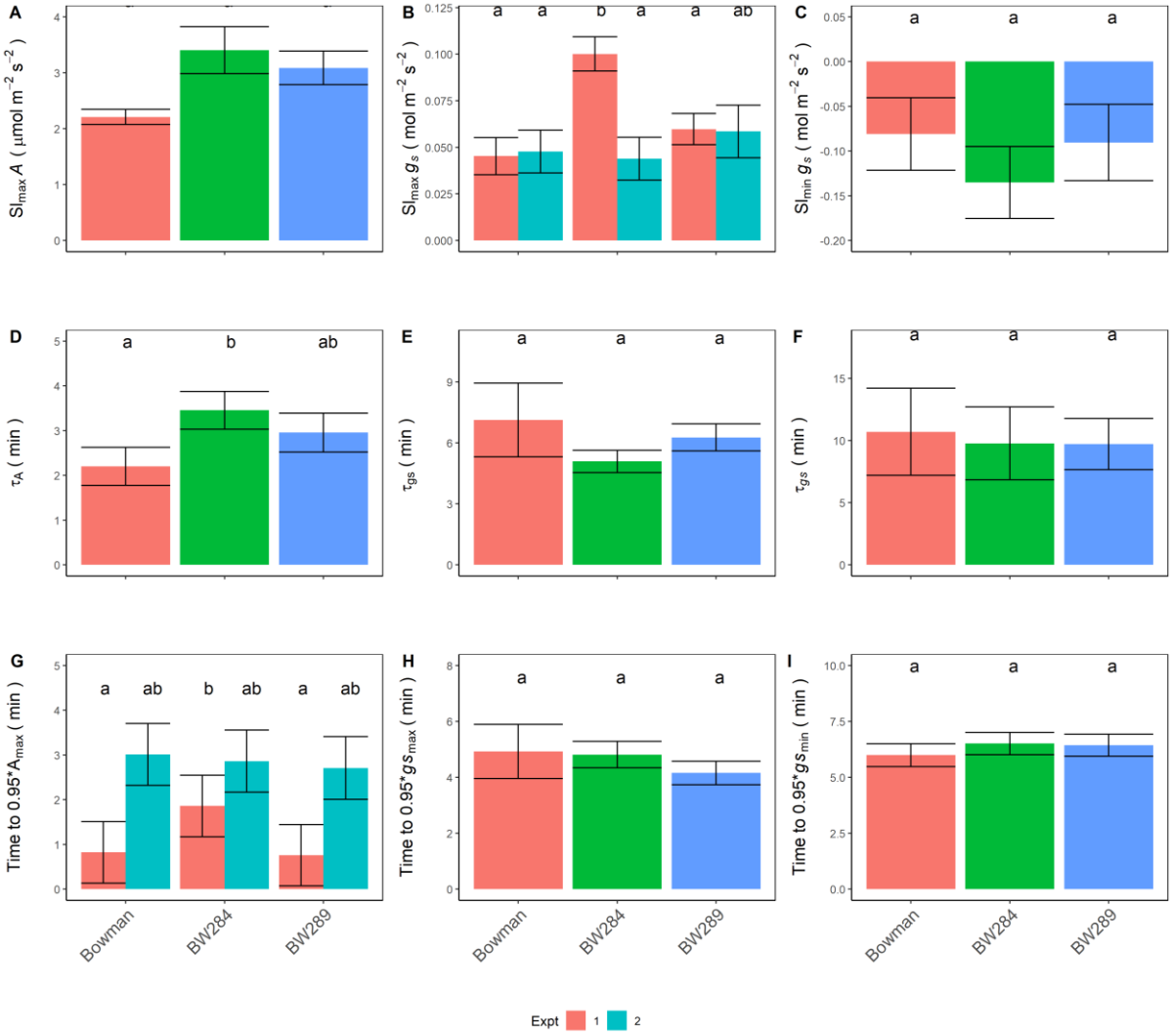

Figure 3.16: Parameterisation of kinetics of Figure 3.15, for pm only. For each parameter, the effect of experiment (with or without the 7 hour low light period) and variety were considered as factors. Effects for the interaction, and main effects are noted as letters above the bars. A) Maximum slope of assimilation C) Time constant of increase E) Predicted maximal value of assimilation B) Maximum slope of stomatal conductance D) Time constant of increase of conductance F) Predicted maximal value of stomatal conductance. G) Time taken to reach 0.95 of maximal assimilation H) Time taken to reach 09.5 of maximal stomatal conductance I) Time taken to reach 0.95 of minimal stomatal conductance. N=12, means shown +/- se. Different letters denote significant differences on Tukey's test between pairs measurements.

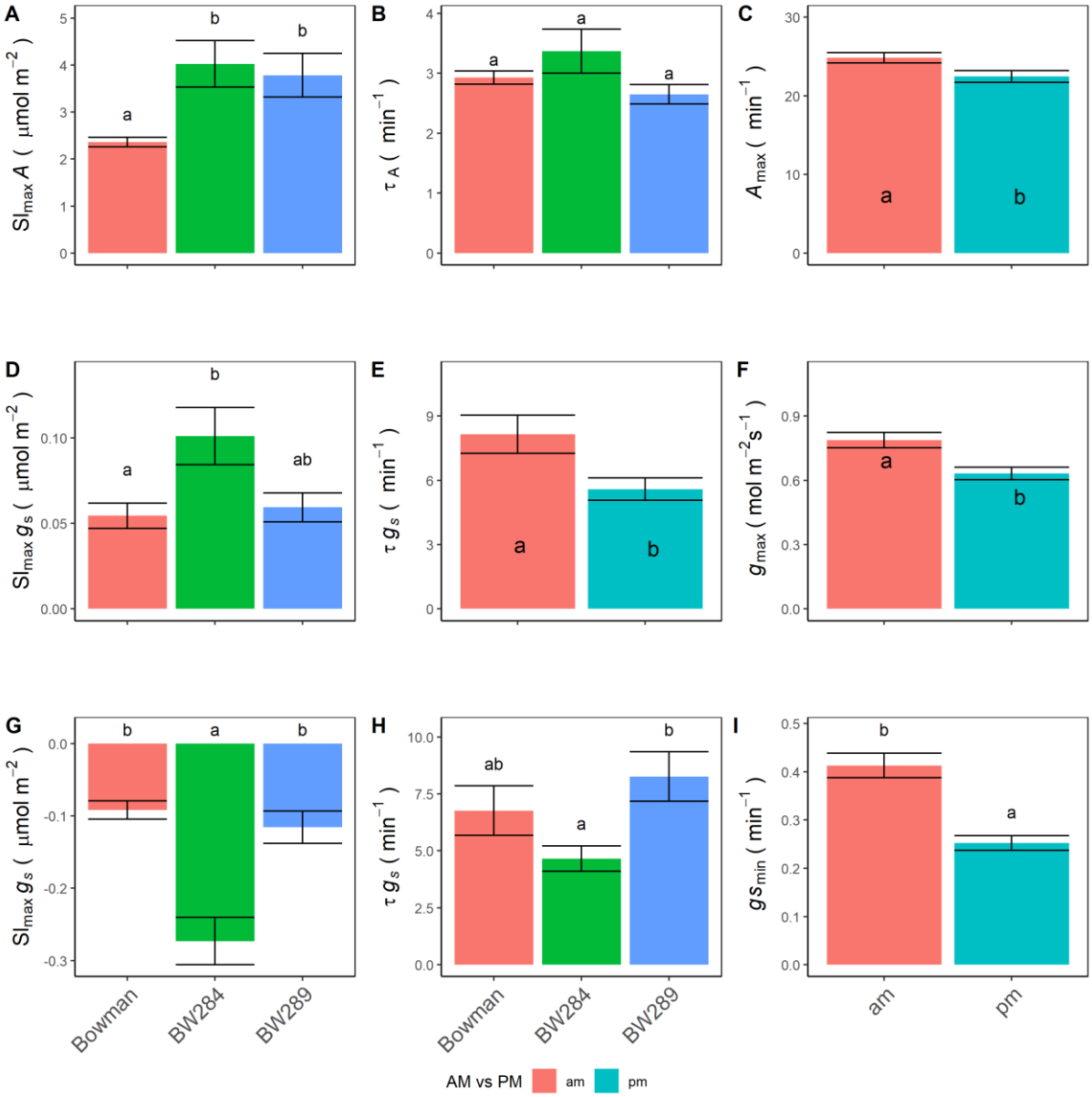

Figure 3.17: Parameterisation of kinetics of full diurnal of alternating high and low light for 12 hours (Figure 3.15 A, C & E). A) Maximum slope of increasing assimilation. B) Time constant of change in increasing assimilation. C) Maximal predicted value of increasing assimilation. D) Maximum slope of increasing stomatal conductance. E) Time constant of increasing stomatal conductance. F) Maximum predicted value of increasing stomatal conductance. G) Maximum slope of declining stomatal conductance. H) Time constant of decline in stomatal conductance I) Minimal predicted value of declining stomatal conductance. Data shown are means +/- se, N=6 in each case. Minimal models shown from initial 3-way ANOVAs of dv ~ Variety x am or pm x hour. Different letters indicate significant differences between pairwise contrasts on Tukey's test from model outputs.

The kinetics of the full diurnal of alternating high – low light (Fig. 3.15A, C, E) were parameterised according to the exponential model described above to understand the extent to which cultivar was affected in performance over the course of the day. The maximum slope of *A* was shallower for Bowman compared to the two mutants (Fig. 3.17A, Tukey's test, $p<0.05$) and shallower than BW284 for slope of increasing and decreasing g_s (Fig. 3.17.D &G, Tukey's tests, $p<0.05$). Whether the fluctuating light was

experienced in the morning or afternoon affected maximal predicted values of A and gs under rising and falling light intensity (Fig. 3.17 C, F and I), with the first six hours giving rise to higher maximum values then the second six hours. The impact of variety or time-of-day on measurements of tau were mixed – there was no Impact of variety on τ_A (Fig. 3.17B, $p>0.05$). Measurements of τ_{gs} for increasing light were higher in the morning than in the afternoon (Fig. 3.17E, 1-way ANOVA, $F_{(2,85)}=6.31$, $p=0.014$). Under declining light levels, τ_{gs} was shorter for BW284 than BW289, although there were no differences with Bowman (Fig. 3.17H, 1-way ANOVA, $F_{(2,78)}=3.66$, $p=0.030$.

3.3.6 Assessment of stomatal Anatomical characteristics relating to leaf number and developmental stage

Anatomical data were analysed in two ways: to consider the impact of leaf number on anatomy, and of developmental time on anatomy. Data from the constant high light diurnal study reported above were analysed to consider the interaction between variety with the stomatal anatomy of third or flag leaves. On the third leaf of the main stem (Fig. 3.18A), Bowman had significantly higher stomatal density than both BW284 and BW289 (Tukey's test, $p<0.05$). At the leaf level, the third leaf had significantly more stomata per mm^2 than the flag leaf (2-way ANOVA, $F_{(1,32)}=4.79$, $p = 0.036$).

There was also no interaction between variety and leaf number on guard cell length (2-way ANOVA, $F(2, 30)=2.91$, $p=0.070$). BW284 and BW289 had significantly longer guard cells than Bowman (Fig. 3.18B), but not compared to each other on the third leaf (2-way ANOVA, $F_{(2,30)}=8.71$, $p =0.0010$). The third leaf was significantly shorter than the flag leaf (2-way ANOVA, $F(1,30)=4.74$, $p=0.037$). When the density of epithelial cells were taken into account by calculating stomatal index (Fig 18C), no differences were observed between cultivars or between leaves (2-way ANOVA, $p>0.05$). There was no correlation of guard cell length and stomatal density (Fig. 3.18D, 2-way ANOVA, $p<0.05$).

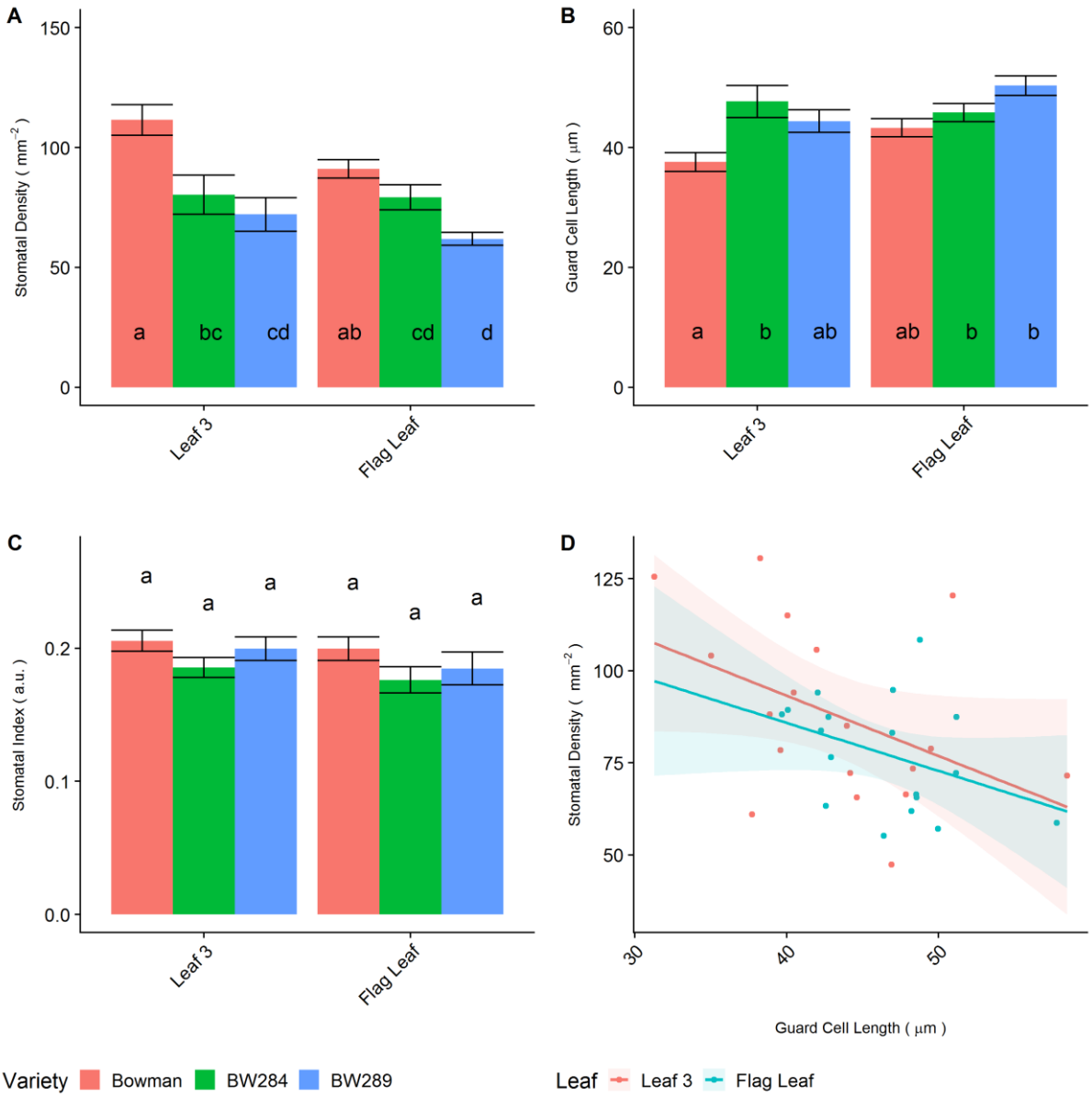

Figure 3.18: Comparisons of stomatal anatomy by variety and leaf number. The third and flag leaf are compared in each case. A Stomatal density. B Guard cell length. C Stomatal index. D Correlation of stomatal density with guard cell length. Means are shown +/- se for A-C. Different letters indicate significantly different means based on Tukey's test on ANOVA models. D Linear regression shown +/- 95% CI.

Developmental progress of stomatal density and size on the second and third leaves (as they became fully expanded) were tracked for the first three harvests at 11, 21 and 32 DAS (Fig. 3.19). There was a significant interaction between variety and harvest date on stomatal density (Fig. 3.19A, 2-way ANOVA, $F_{(6,127)}$=23.0, $p<2.2x10^{-6}$). Although there were no observable differences between cultivars at the first harvest after 11 days (Tukey's test, $p<0.05$), by 21 days, Bowman had a significantly lower stomatal density than BW284 (Tukey's test, $p<0.05$), but this difference did not persist to the third harvest after 32 days (Tukey's test,

$p<0.05$). At 48 DAS, Bowman had a significantly higher stomatal density than BW289 by the third harvest (Tukey's test, $p<0.05$). BW284 also had significantly greater stomatal density than BW289 at the second harvest (Tukey's test, $p<0.05$). When stomatal size was considered (Fig. 3.19B), there was a clear tendency for the guard cell length to get smaller at successive harvests. There were once again no differences between cultivars after 11 days. However, by 21 days after sowing, Bowman had significantly longer guard cells than BW284. There were no differences between varieties by 32 DAS (2-way ANOVA, $F_{(8,39)}=22.7$, $p=2.07\text{x}10^{-12}$). Stomatal density and guard cell length were inversely linearly related (Fig. 3.19C), with a main effect for variety, and an interaction between harvest time and stomatal density in predicting guard cell length (3-way ANOVA, $F_{(7,40)}=27.9$, $=1.78\text{x}10^{-13}$).

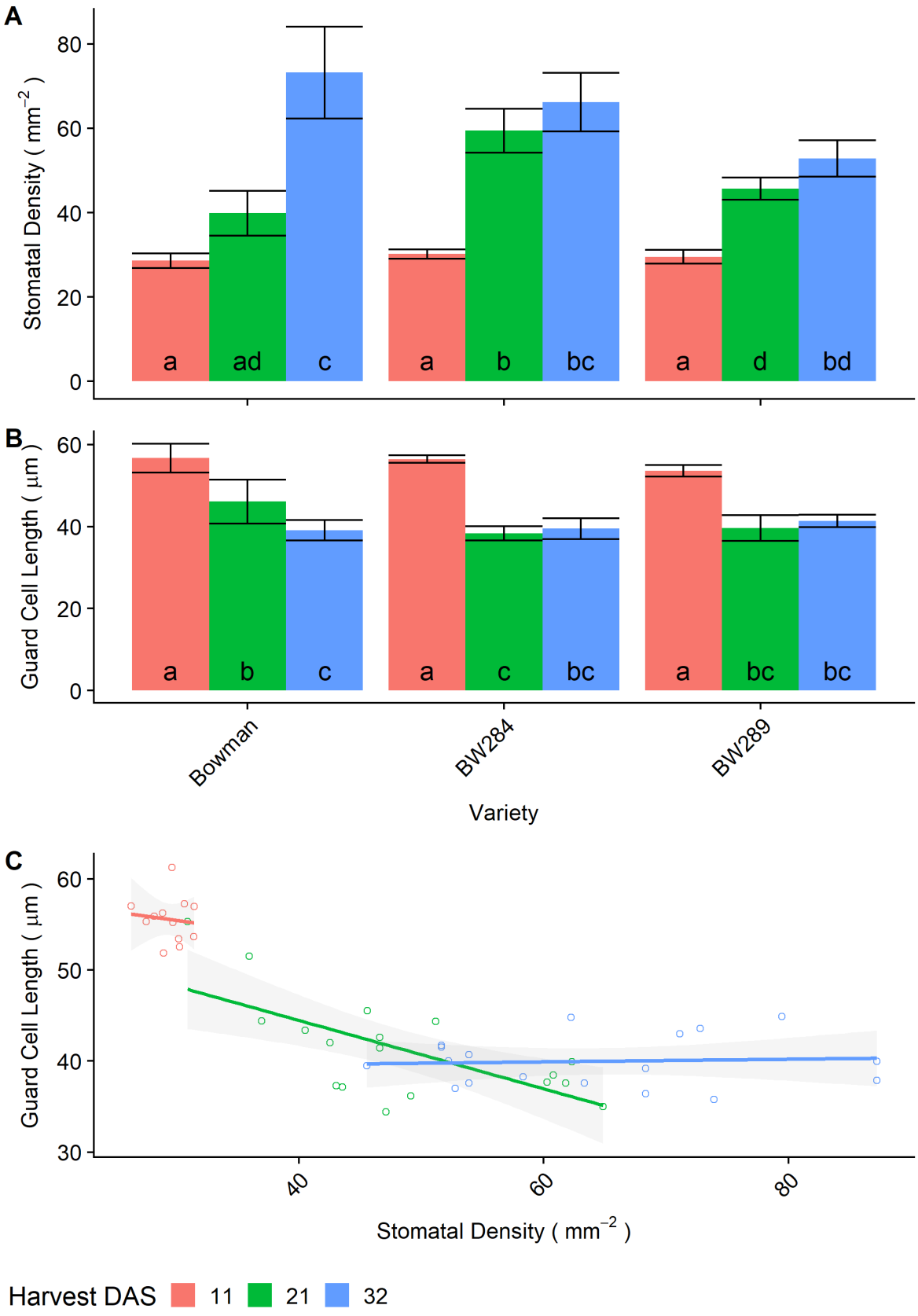

Figure 3.19: Comparison of stomatal anatomy of abaxial third leaf over time. A Stomatal density. B Guard cell length. C Correlation of stomatal density with guard cell length for each harvest. A, B Data are means +/- se, N=6. C Data shown are pairwise points, linear relationships modelled at each harvest point based on linear regression; error bars are 95% CIs. Different letters in A & B indicate significant differences of pairwise comparisons based on Tukey's test of model outputs.

3.4. Discussion

Previously, studies have shown that mutations at the *eam8* and *eam10* loci (Druka et al., 2011) prevent function of circadian clocks in barley, and that the *HvLUX1* and *HvELF3* genes were the ones affected by the mutations (Zakhrabekova et al., 2012, Campoli et al., 2013, Faure et al., 2012).

3.4.1 Clock mutants retain some circadian characteristics

The studies described above (Figs. 3.1 & 3.2) illustrated that the circadian clock mutants BW284 and BW289, retained some physiological output in their circadian clocks (Figs. 3.1 & 3.2), with a clear reduction in the amplitude of g_s under constant light conditions in the mutants in line with previous data from Faure et al. (2012) and Campoli et al. (2013) on gene expression in the *eam-8* and *eam-10* mutants, although the phase appears to shift to later rather than earlier in the subjective day as opposed to fdata found from gene expression There remains some question over whether the clock signals observed in Figures 3.1 & 3.2 would persist as experimental conditions became less constrained. In 12h dark-12h light day night periods (Fig. 3.3) evidence emerged that although the mutants exhibited some clock functionality when environmental cues were available, physiological differences with Bowman remained. Parameterisation of the data (Fig. 3.4) showed that at low light levels, BW284 had lower assimilation than BW289. More importantly, a difference in the phase of stomatal conductance was observed (Fig. 3.4B), suggesting that the diurnal timing of stomatal aperture in steady-state conditions was different between Bowman and the clock mutants.

3.4.2 Steady state physiological parameters highlight mutants' impairment to the clock

Parameterisation of steady-state diurnal measurements (Fig. 3.14) highlighted a greater amplitude of assimilation for the mutants compared to Bowman under higher light intensity, which did not exist at lower light levels suggesting that the clock mutants had greater variability in responses and less control over *A* as light intensity increased. Importantly, the shift in phase (Fig. 3.4B) was confirmed and extended at higher light levels (Fig. 3.14B-D), with that shift clear in *A*, g_s and WUE_i data. The afternoon 'hump', that

is more-noticeable in g_s and WUE_i than *A* for diurnals at both low and high light has been noted in *Arabidopsis* (Matthews et al., 2018). We show that clock mutations in barley affect the timing of peak *A*, g_s and WUE_i, showing a later phase for *A* and g_s and an earlier phase for WUEi, the phase shift having been hinted at previously (Dodd et al., 2004, Dodd et al., 2005). Here we show that for some reason related to the clock mutation, Bowman reduces photosynthate accumulation later in the day relative to the clock mutants, at least in part through coordination with stomatal aperture (Mott, Sibbernsen & Shope, 2008). Perhaps the reduced assimilation is modulated by starch accumulation, but it could equally arise owing to reduced coordination between mesophyll and stomata in the absence of the relevant EC gene control since it is clear that different tissues in the leaf have clocks with different periods (Hassidim et al., 2017, Yakir et al., 2010, Graf et al., 2010).

It is noteworthy that higher g_s is experienced later in the day without significant increases in assimilation for Bowman in particular. As there was no obvious difference in assimilation rate, these data suggest that this additional thermodynamic capacity to do work enabled by the transpiration flux could be used for translocation of sugars (Kang et al., 2007) as recently modelled (Pokhilko and Ebenhoh, 2015), to aid replenishment of calcium pools (MacRobbie, 1998) or to assist in the repair of damaged tissues (Zufferey et al., 2011). Bowman also had a lower WUE_i compared to the clock mutants towards the end of the diurnal period and a higher RWC overall. These facts also suggest that Bowman uses higher rates of transpiration in the afternoons compared to the mutants, perhaps in anticipation of high pm temperatures in bright conditions. An alternative view would point to the relatively lower g_s in Bowman at the start of the day, when temperatures are typically lower and photosynthetic machinery expected to be in good repair. This is underlined by the phase shift in WUE_i (Fig. 3.14D) far towards the start of the day in Bowman relative to the clock mutants; in either event, the clock mutations appear to alter behaviour and possibly fitness (Dodd et al., 2005). Another alternative might be that under 'square wave' light, the fitness advantage of circadian control is of stomatal opening in response to blue light at dawn is reduced, since no peak of temperature or light in the afternoon period need be anticipated Matthews &

Lawson, 2019, Vialet-Chabrand et al., 2017c, Dodd et al., 2005). In the fluctuating experiment, *A* for BW289 appeared to fall during the course of the measurements, while g_s remained steady, while for Bowman the reverse was the case (Fig. 3.15). The lack of coordination between mesophyll demands for high C_i and stomatal supply were clear for the mutant where water was lost wastefully; this may arise from the impaired clocks failing to communicate effectively across tissues (Hassidim et al., 2017, Yakir et al., 2011, Mott, Sibbernsen & Shope, 2008).

3.4.3 Little evidence that the clock has an impact on kinetic physiological performance while the link with stomatal anatomy is weak

It was possible to make a number of predictions in respect of the clock mutants in the expectation that a failure to anticipate time-of-day would have a negative impact on the clock in such activities as stomatal responses (Hassidim et al., 2017, Yakir et al., 2011). Therefore, we could suppose that acclimation to intra-day environmental signals would vary between varieties and by fluctuating light history. However, there was limited evidence for acclimation affecting the cultivars differently, with BW284 notable for having a steeper slope of g_s with acclimation rather than without and paradoxically a longer time to 95% maximal *A* with acclimation (Fig. 3.16B, G). Overall, this suggests that there is minimal control by the clock over physiological acclimation in the short term; other evidence suggests the clock is important over longer timescales (Dodd et al., 2005).

Clock mutants' kinetic responses to changing light intensity over the course of the day should differ from those seen in Bowman, again in part owing to control of stomatal aperture (Hassidim et al., 2017). Parameterisation of the full day's kinetic responses gave mixed support to these expectations. Bowman was slower to respond to changing light than BW284 (Figs 3.17A, D & G). Results for the time constant of response to changing light were also inconsistent – there was a distinction between both am and pm periods for the time constant of stomatal conductance under increasing light (Fig. 3.17E), but only variety was important when light levels declined (Fig. 3.17H). Maximal values of *A* and g_s were affected by time of day, but not by variety. Overall, it appears that BW284 and Bowman show the greatest heterogeneity

of kinetic responses, and this could go some way to explaining how BW284's final harvest performance remained in line with Bowman's: effective stomatal kinetics offer the potential to improve WUE and thereby yield for a given volume of water (McAusland et al., 2016, Lawson and Blatt, 2014), although why an impaired clock should improve stomatal functioning is not clear from these data, although the presence of multiple clocks in the leaf is a relevant consideration (Hassidim et al., 2017).

BW289 had kinetic responses that were in line with Bowman's, and we must look elsewhere for differences in yield; the very early flowering of BW289 is the most likely candidate. Given the supposed impact of the clock mutations on the plant's perception of time (Dodd et al., 2005), the lack of interaction between stomatal kinetics and cultivar was surprising, particularly as time of day had a significant impact on predicted maximal and minimal values of A and g_s. This is further evidence that the impact of the clock in the short term can be difficult to determine, even if longer term effects like time to heading remain more-clearly defined (Druka et al., 2011). Further work is needed in this area to understand fully the interaction of diurnal time and cultivar on kinetic responses.

One possibility is that the difference in kinetics could have arisen as a result of heterogeneity either in the size of the stomatal pore, or in their density. In general, smaller pores might have permitted faster kinetic responses (Raven, 2014), while higher density might lead to increased maximal conductance or assimilation rates owing to shorter path lengths for gaseous diffusion (Bertolino et al., 2019). These results show no trade-off between guard cell length and stomatal density, irrespective of leaf position in barley, although work in barley EPF mutants showed a positive correlation (Hughes et al., 2017) while others have found a negative one over geological timeframes (Franks and Beerling, 2009a). Bowman's consistent higher density of stomata should lead to higher maximal rates of photosynthesis (Lawson and Blatt, 2014), but there is little evidence of that occurring in these data.

The impact of variety on guard cell length (and by extension, stomatal size) was mixed, with Bowman's smaller guard cells at the third leaf stage not being a feature at the flag leaf stage; these were not backed

up by consistently improved stomatal dynamics in these data, as would be expected from empirical and theoretical calculations (Lawson and Blatt, 2014, Lawson and Vialet-Chabrand, 2019).

3.4.4 The early flowering phenotype remains

Regardless of the degree of impairment in clock functioning, it was important to confirm the early flowering phenotype in the mutants, which is where their commercial importance lies (Zakhrabekova et al., 2012). Evidence for early flowering behaviour (Fig. 3.5) was clear from growth stage assessments, and possibly from tiller number, although the etiolated phenotype was harder to infer from plant height. The fact that BW284 did not progress as rapidly through its growth stages as BW289 was to be expected as previous work has indicated that BW284 is approximately 4 days later to flower than BW289 under either long or short days (Faure et al., 2012, Zakhrabekova et al., 2012, Campoli et al., 2013), and both mutants flowered earlier than Bowman in this study. Another interesting factor is that it was typically some time – 34 days in the case of the *Hvlux* mutant – before phenotypic differences at the level of stem extension or early flowering were apparent, underlining the longer term impact of the clock over the shorter term effects (Faure et al., 2012, Campoli et al., 2013)

3.4.5 Clock mutants may be source-limited while Bowman appears sink-limited

Detailed analysis of organ development over time underlined the findings on the early-flowering phenotype described above. Surprisingly, mean fresh and dry leaf mass did not vary significantly over time (Fig. 3.7-8) perhaps as old leaves senesced while new ones expanded, but stems, as major sinks for photosynthates (Paul and Foyer, 2001), did increase in fresh and dry mass, presumably as stored photosynthates drew in water by osmosis. Clock impairments have been linked to the reduced ability to store and time the release of starch (Graf et al., 2010), and this may explain the difference in outcomes between BW289, with the most-attenuated clock function over the long term, and BW284, which appears to retain more of its circadian functionality – notwithstanding the fact that over diel cycles, little difference between the two cultivars could be found. Remobilisation of photosynthate is unlikely to occur from the

ears, and they saw no differences between varieties over time for fresh or dry mass. Barley is commonly sink-limited (AHDB, 2018), so storage in the stem could buffer problems of low grain number. Some evidence can be seen in the area measurements for the stems (Fig. 3.9), which could be seen as a proxy for volume, and which follow a similar pattern of developmental differences to those seen for fresh and dry mass.

Although all plants were well-watered throughout the study, relative water content (RWC) declined for all organs between the start and the end (Fig. 3.10). Surprisingly, the mutants had lower RWC at the ears compared to the leaves by 68 DAS, while Bowman maintained similar RWC between those organs. Although early maturity is a known characteristic of the mutants, it is perhaps surprising that leaf water status remained significantly higher than that at the ears. There were no differences in RWC of stems & tillers between all three cultivars and again, RWC was higher than that at the ears. The variability in RWC may reflect differences in the timing of senescence (Distelfeld et al., 2014), particularly as the clock mutants would be expected to show this feature earlier under conditions of abundant water and sunlight.

In combination these data define three broad strategies adopted by the different cultivars. Bowman has the greatest total leaf area, but many fewer ears compared to the mutants (which is critical in barley compared to wheat in defining yield (AHDB, 2018)), but with relatively many grains per ear. BW284 has significantly lower total leaf area, but many more ears and high grain number per plant. Finally BW289 has an even lower total leaf area, but relatively many ears although each ear had low grain number. Overall, Bowman appears to be sink limited, as outlined in the AHDB Barley Growth Guide (2018), and as expected for a barley cultivar (Bingham et al., 2007a). Conversely, the mutants appear to be source limited, with their relatively lower leaf areas filling either fewer grains to higher mass / grain (BW289) or more grains at lower mass (BW284). Source limitation might be expected as a function of early maturity, with less investment in leaf matter.

It was important to consider some of the physiological parameters driving the differences in phenotype at the leaf level. Managing total leaf area is part of the optimisation process that all plants carry out to

manage the size of source vs sink (Bingham et al., 2007), although there remain many other factors not considered here: root vs shoot, time to heading etc. Clearly a substantial fraction of source material will be derived from leaf level photosynthesis (Serrago et al., 2013), which could in principle offset leaf area in determining total leaf photosynthate.

3.4.6 Overall plant health and robustness may affect general conclusions

Specimens of BW289 were notably poor – being visually etiolated with relatively small leaves - despite there being no difference in steady state assimilation per unit area over the course of the day. Thus it was possible that there were other consequences to the plants of these mutations above and beyond the photoperiod / early flowering phenotype. It was also possible that the leaves were not as viable for as long in the mutants compared to Bowman (Distelfeld et al., 2014), and therefore the period during which the extra photosynthates were available was shorter.

A further unknown was the impact of water stress on the performance of the mutants compared to Bowman. If the conductance differences arose because Bowman was better at anticipating afternoon temperature or PAR increases, then Bowman might be expected to outperform the mutants on g_s at higher temperatures and saturating light levels, particularly when stressed. Habte et al (2014) contended that in shoots, it was osmotic potential that drove expression of clock genes, with the latter having only minor effects on gas exchange. However the data presented here suggested that differences in gas exchange did exist, and that those differences vary during the course of the day between individuals with and without fully-functioning circadian clocks. Additional work is needed to understand clock genes expression levels during the course of vegetative development, and the point at which they begin to have an impact on seedlings' gas exchange. However, there ought to be consequences of diurnal variation in physiological performance, and these were seen to some extent in the biomass data, with Bowman failing to generate significantly more biomass than BW284, probably as the latter had more-responsive stomata. Furthermore, there were suggestions that variation in stomatal density and pore size was linked to observed physiological variation. As such, the circadian mutants may have desirable anatomical and

physiological features that would be of interest to breeders attempting to manage yield stability under climate variability. That these phenotypes existed unobserved in existing commercial barleys is also of interest, underlines the value of interrogating the existing barley germplasm for desirable traits (Wang et al., 2017, Druka et al., 2011).

3.5. Conclusions

- Previous data in LL for impaired diel gene expression levels and over developmental time on early maturation confirmed in BW284 and BW289 in this study.
- Impaired coordination between or arrhythmia at the guard cell clock and mesophyll clock may affect g_s and A, and by extension WUE_i in mutants over diurnal timeframes.
- Bowman conforms to sink-limited barley stereotype; clock mutants may be source limited under prevailing conditions.
- Steady-state diurnal responses showed clear phase differences in Bowman compared to clock mutants, with the latter having earlier peaks in A and g_s, and later peak in WUE_i.
- The clock did not appear to have a significant impact on kinetic performance, either in terms of acclimation or the impact of time of day.
- Stomatal data do not support expectations derived from kinetic or steady-state data; Bowman had higher SD but not higher maximal A or g_s. Bowman had generally lower SS, but not faster-responding stomata.

Chapter 4: Landraces and wild barley relatives exhibit a range of *A*, g_s and WUE_i characteristics in the growth chamber and field.

4.1. Introduction

Agricultural consumption remains the most significant use of water, with up to 70% of freshwater withdrawals worldwide devoted to this purpose (FAO, 2015). Under pressure from increasing population and changing diets, this contribution is likely to increase in the coming years (IPCC, 2014, McGuire, 2013). One of the key determinants of overall crop performance under increased constraint is likely to be water use efficiency (WUE), which is defined as water used per unit of biomass gained (Lawson and Blatt, 2014), and which links the requirement to use water directly to yield.

4.1.1 Genetic diversity in landraces

Against the backdrop of the pressure to do more with less, that is to produce more crop per drop (Davies and Bennett, 2015), the need to identify genetic targets for improved WUE increases. It is perhaps surprising how few GM varieties are in use globally, notwithstanding regional disquiet over their use, given the prodigious efforts devoted to their development (Lawlor, 2013). While a single GM trial has been approved in the UK for improved photosynthesis in wheat (McGrath, 2017), and a handful are in place globally, other research groups are also studying pre-existing germplasm. Landraces, which can be considered as older, regionally-adapted varieties, fell out of favour as they did not meet Green Revolution requirements for high input tolerance, and thus could offer an excellent source of genetic diversity (Brozynska et al., 2016, Baranger et al., 2004). Elsewhere, other researchers have turned to wild relatives, either undomesticated examples of extant commercial species or in closely-related species (Zaharieva et al., 2001, Baranger et al., 2004, Brozynska et al., 2016).

4.1.2 Improved WUE_i in steady-state and kinetic responses of A and g_s

Both steady-state g_s and *A* as well as dynamic performance are potential routes to improving yield (Lawson and Blatt, 2014, McAusland et al., 2016, Wong et al., 1979, Fischer et al., 1998). Faster stomata

are likely to reduce the mismatch between CO_2 demand at the mesophyll and water loss (Lawson 2014), particularly in the context of rapid closure as light levels decline e.g. under shade flecks, thereby saving water which would otherwise be wasted.

The aim of this study was to characterise the natural variation in physiology and anatomy of wild barleys and landraces in comparison to elite varieties in both the field and growth chamber. The primary objective was to understand the extent of variation in g_s, A and WUE_i characteristics in non-elite cultivars compared to commercial varieties, and to understand whether, in a diverse range of evolutionary solutions to the problem of water management versus yield, interesting solutions had been reached which do not exist in current commercial germplasm. The second objective was to test both steady-state and kinetic responses to changing light conditions to understand the variety and variability in these metrics. The third objective was to link the physiological steady state / kinetic data to anatomical data to understand the coordination between the two, and under what circumstances those solutions might lead to improved yield potential.

4.2. Methods

4.2.1 Field Trial

Plants were grown in the Polytunnel at KWS UK Ltd.'s trials site at Thriplow, Cambridgeshire as described in Chapter 2 including table 1 for a full list of varieties tested.

Periodically, step change measurements were taken using a Li-COR 6400 (Li-COR Inc., Lincoln, Nebraska, USA) IRGA on a subset of the plants including the wild barleys B3733 and B3745, the landraces Hatif de Grignon and Golden Archer, and the elite cultivars KWS Sassy and KWS Orwell. The youngest fully expanded leaf was placed in the cuvette (rather than targeting a particular succession, to account for developmental differences). Plants were settled at 100 µmol m^{-2} s^{-1} PAR before being given 1000 µmol m^{-2} s^{-1} PAR for 45 minutes. At the same time, Fv/Fm and Fq'/Fm' measurements were also taken on the Li-COR. The plants were measured both in the morning and afternoon to consider the impact of time-of-

day on physiological processes. Up to 5 measurements were collected simultaneously in the morning and afternoon periods.

4.2.2 Stomatal anatomy

Additionally, stomatal information was taken from the 6 selected varieties at the end of the period using the method of Weyers and Johansen (Weyers and Johansen, 1985) as described in Chapter 2. Furthermore, anatomical gs_{max} was calculated from maximal calculated *A*, stomatal pore length and guard cell length and some physical constants as described by Franks and Farquhar (2001) in Chapter 2.

4.2.3 Growth Chamber

Two trials were carried out on landraces as described in Chapter 2 above in a Sanyo Fitotron in 12h: 12h light-dark cycles at 840 µmol m^{-2} s^{-1}.

4.2.4 Fluorescence & Gas exchange

Fluorescence measurements were taken with a Hansatech FMS portable fluorescence meter (Hansatech, Burwell, UK) on the youngest fully expanded leaf. Light response curves of Fq'/Fm' and *A* were run at PAR values of 50, 100, 200, 400, 600, 800, 1000, 1250 and 1500 µmol m^{-2} s^{-1}, and A-C_i and A-Q curves developed and parameterised using Sharkey's method (Sharkey et al., 2007) as described in 2.4.1.3 above. Assimilation and stomatal conductance responses to changing light levels were modelled as exponential functions (Whitehead and Teskey, 1995) as described in 2.4.1.3 above.

4.2.5 Statistics

All data were processed in R, and statistical analyses undertaken in that environment using Rstudio (R Core Team, 2018). All tests were run as generalised linear models, taking genotype as a fixed effect and block and day that measurements were taken as fixed effects where sufficient degrees of freedom allowed. ANOVAs were run to test predictions and where models were significant overall, Tukey's test of pairwise comparisons used to understand the effect of particular genotypes.

4.3. Results

Polytunnel 'field trial' results are presented before those from the growth chamber. Three lines of evidence will be considered: those relating to physiology, those relating to anatomy, and those relating to biomass and yield. Generalised linear models presented below take Replicate|Block as random effects where possible, in addition, Replicate|Day was also considered as a random effect as measurements were made over multiple days.

4.3.1 Field trial chlorophyll fluorescence parameters

Chlorophyll fluorescence is a technique that allows the researcher to distinguish (in this instance) the performance of Photosystem II separate from other photosynthetic activities was measured on a subset of the varieties present (Fig. 4.1).

B3733 had significantly lower maximal efficiency of photosystem II in the dark (*Fv/Fm*, Fig. 4.1A) compared to all other cv's, while there were no differences between B3745, Golden Archer, Hatif de Grignon and KWS Orwell (Tukey's test, $p<0.05$). B3733 had significantly lower maximal efficiency at a light level of 840 µmol m^{-2} s^{-1} PAR (*Fv'/Fm'*, Fig. 4.1B) compared to B3745; there were no differences between these two and any other cultivar (Tukey's test, $p<0.05$). The operating efficiency of Photosystem II (*Fq' / Fm'*) for B3733 was significantly lower than B3754 (Fig. 4.1C), but neither were different from the other cultivars (Tukey's test, $p>0.05$). Possibly as a result of its capacity impairment seen in Figure 4.1A, B3733 had a significantly higher value for non-photochemical quenching (Fig 1D) than B3745, but neither were different from any of the other cultivars (Tukey's tests, $p<0.05$).

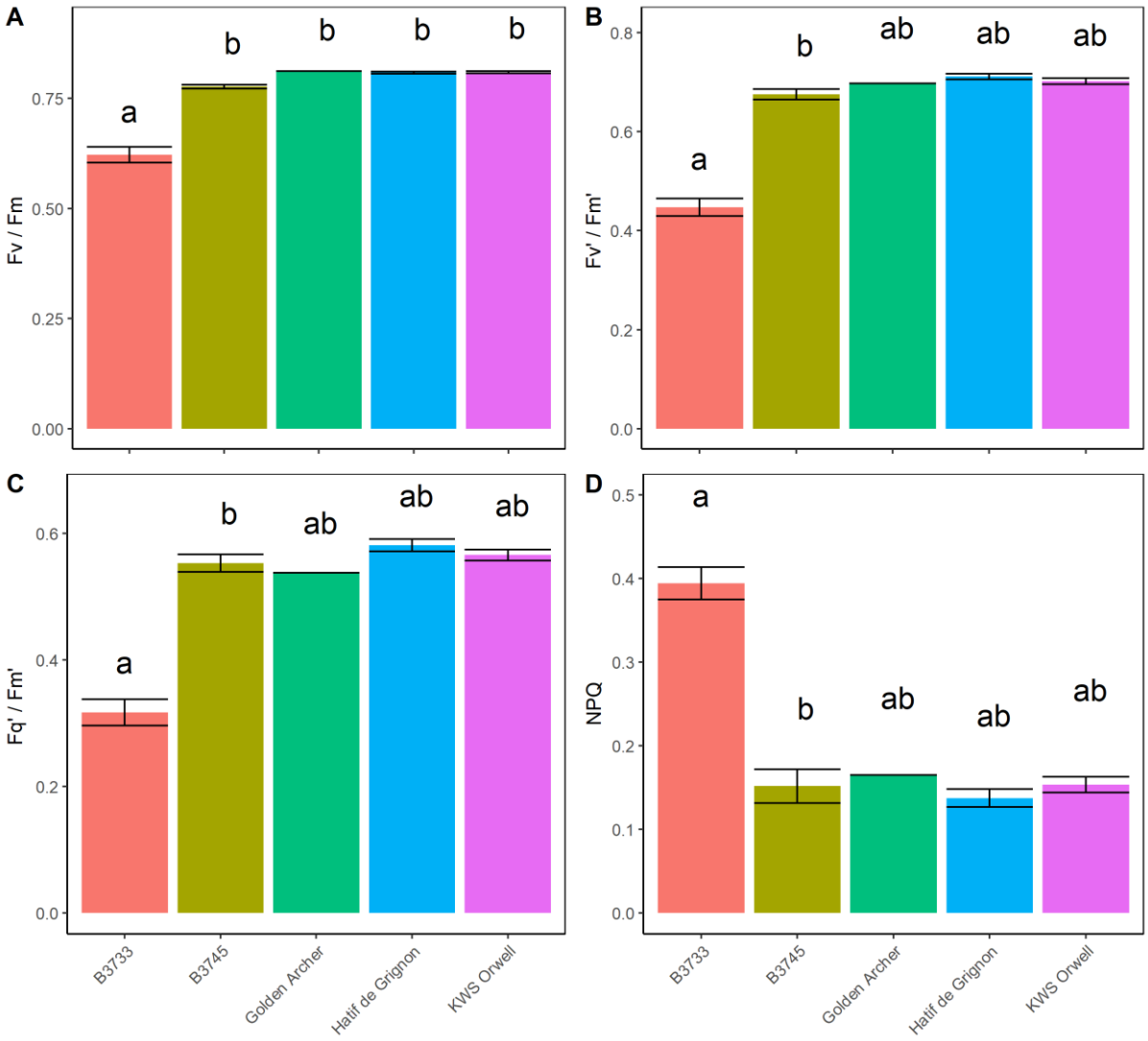

Figure 4.1: Fluorescence outputs for the wild barleys B3733 and B3745, the landraces Golden Archer and Hatif, and the elite variety, KWS Orwell. A) Maximum efficiency of photochemistry (*Fv/Fm*). B) Maximum efficiency of photochemistry at 840 µmol m^{-2} s^{-1} par (*Fv'/Fm'*). C) Operating efficiency (*Fq' / Fm'*) at 840 µmol m^{-2} s^{-1} light. D) Non-Photochemical Quenching (NPQ, *Fm/Fm'*-1). Means are shown +/- se. No significant differences were found in cultivars sharing the same letters at p_{crit}=0.05 on Tukey's test of pairwise comparisons.

4.3.2 Field trial analysis of steady-state and kinetics of g_s and *A*

Dark-adapted plants were given single step changes of light from 0 to 1000umol m^{-1} s^{-1} PAR for 40 minutes (Fig. 4.2), which were parameterised (Fig. 4.3) using a first-order exponential model (Whitehead and Teskey, 1995).

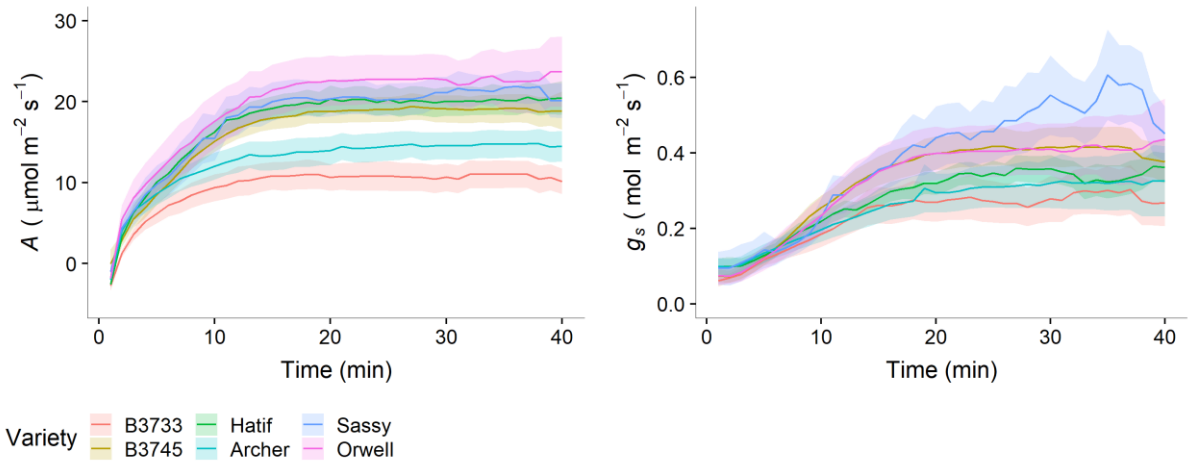

Figure 4.2. Step changes from 0 to 1000 µmol m^{-2} s^{-1} PAR for a selection of 6 barley varieties, with time change starting at t=2 min, to remove noise. A) Assimilation and B) Stomatal conductance. Means shown +/- se, N=5-6 for each variety.

The lowest predicted A_{max} (for B3733, Fig. 4.3A) was 37% lower than the highest value (for KWS Orwell), and was significantly different (Tukey's test, $p<0.05$). KWS Orwell had the longest mean τ_A, at 9 minutes, compared to 4.6 minutes for B3733 (Fig. 4.3C) although they were not significantly different (Tukey's test, $p>0.05$). The slope of *A* which takes into account Amax, A_0 (the start point) and τ_A also showed some variability, but here, B3733 had the steepest slope, 1.6x greater than that of Golden Archer (Fig. 4.3B).

The difference between the highest predicted g_s (B3745, 0.52 µmol m^{-2} s^{-1}) and lowest (Golden Archer, 0.37 µmol m^{-2} s^{-1}) was significant (Fig 3D, Tukey's test, $p<0.05$). The results for the parameterisation of g_s followed a similar pattern to those of *A*; there were no pairwise differences between varieties in terms of the slope of g_s or time constant (Figs. 4.3E & F, Tukey's tests, $p>0.05$). Nevertheless, the steepest slope for g_s (B3733) was 2.5x that of the shallowest (Golden Archer), while the longest time constant (KWS Archer, 18.1 minutes) was 7.3 minutes longer than that for B3733.

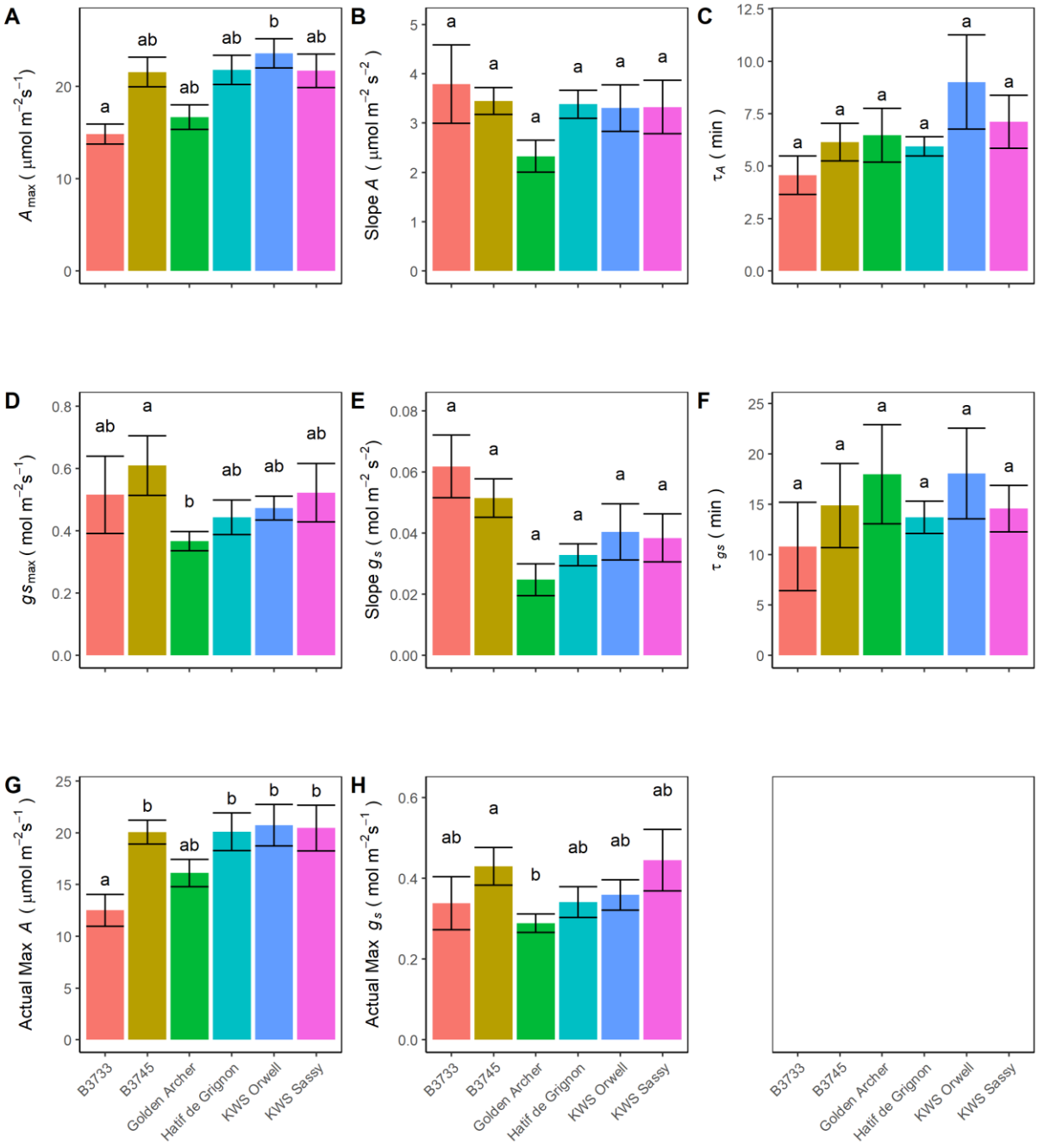

Figure 4.3: Parameterisation of kinetics in Figure 4.2 using a first-order exponential model. Top row, parameterisation of assimilation: A) Maximal predicted value B) Maximal slope and C) time constant (tau, τ) of increasing *A*. Middle Row, parameterisation of stomatal conductance D) maximal predicted value E) maximal slope and F) time constant of increasing g_s. Bottom row, maximum values achieved in steady-state after 40 minutes for G) assimilation and E) stomatal conductance. In each case, N=5-6, means shown +/- se. Identical letters show no significant differences between means based on Tukey's test of model outputs at p=0.05.

It was also possible to calculate the actual maximum g_s and *A* achieved. Once again, B3733 had the lowest maximum value of *A* (Fig. 4.3G, 12.5 µmol m^{-2} s^{-1}) compared to KWS Orwell, which had the highest (20.7 µmol m^{-2} s^{-1}), but B3745, Hatif de Grignon and KWS Sassy also generated significantly higher maximal values compared to B3733 (Tukey's tests, $p<0.05$). The rank for actual compared to

predicted maximal values for g_s was also similar (Figs. 4.3D & H), although achieved values were consistently lower. B3745 achieved a significantly higher maximal rate of stomatal conductance (48% higher) than Golden Archer (Fig. 4.3H).

4.3.3 Field trial stomatal anatomy

Stomatal anatomy is expected to link to speed of response via stomatal size as well as maximal responses based on stomatal density (Lawson and Blatt, 2014).

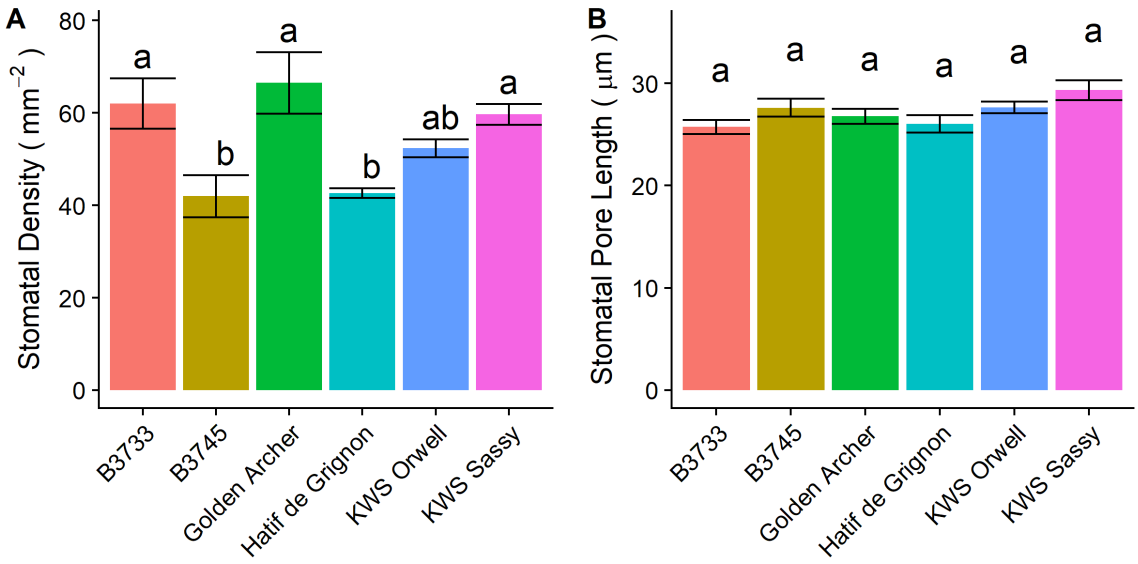

Figure 4.4: Stomatal anatomy of 6 select barley cultivars A) Stomatal density and B) Stomatal Pore length. Means shown +/- se, N=5-6. Identical letters denote no significant differences between means based on Tukey's test of model outputs at p=0.05.

The wild barley variety B3745 had the lowest stomatal density (41.9 mm^{-2}, Fig 4A), and along with Hatif de Grignon (42.6 stomata mm^{-2}) had significantly lower density than B3733 (62.0 mm^{-2}), Golden Archer (66.5 mm^{-2}) and KWS Sassy (59.7 mm^{-2}, Tukey's tests, $p<0.05$). Meanwhile, there were no pairwise differences between varieties in stomatal pore length (a proxy for stomatal size, Tukey's tests, $p>0.05$), with KWS Sassy having the longest pores (29.3 µm, Fig. 4.4B) which were 14% longer than the shortest pores (B3733, 25.7 µm).

Although there is some expectation of a trade-off between stomatal size and density (Franks and Beerling, 2009a), none was found in this case. Similarly, it is supposed that stomatal density links more closely to maximal values for photosynthesis and stomatal conductance than speed of response (Franks et al., 2015, Aasamaa et al., 2001). However, we found no significant interactions between stomatal density and predicted or actual A_{max}, or with predicted or actual gs_{max} by variety (2-way ANOVA, p>0.05). Similarly, there was no evidence that stomatal size was linked to speed-of-response (2-way ANOVAs, p>0,05) for the model including any of τ_A, τ_{gs}, $slope_A$ or $slope_{gs}$.

4.3.4 Field trial harvest measurements at maturity

At the completion of the field trial, a range of data relating to harvest were recorded. These data (Fig. 4.5) were grouped by the similarity of yield characteristic they were measuring. The first shows critical markers of overall yield (Fig. 5 A-C), the second row, three indicators of potential yield (Fig. 4.5D-F) and the third, ear quality (Fig. 4.5G-H).

Maritime (56.7g) and KWS Irina (44.1g) had significantly higher grain mass per plant (Fig. 4.5A) than the wild barleys B3733 (20.3g) and B3745 (17.0g), as well as the landraces Bere (14.7g), Dea (9.6g), Golden Archer (14.7g) and Hatif de Grignon (10.0g) (Tukey's test, p<0.05). In addition, Alpha (18.8g), B3736 (29.9g), KWS Kosmos (29.0g) and KWS Orwell (22.3g) had significantly lower grain mass than Maritime (Tukey's test, p<0.05). The wild barleys Alpha, B3733, B3745 and the landraces Bere, Dea, Golden Archer and Hatif de Grignon all had between 45% and 56% less biomass (Fig. 4.5B) than the elite cultivar Maritime (Tukey's test, p<0.05); there were no other differences between cultivars (Tukey's test, p>0.05). The landraces Dea (0.17 g g^{-1}) and Hatif de Grignon (0.16 g g^{-1}) had a significantly lower HI (Fig. 4.5C, HI = Grain Mass·Biomass^{-1}) than the elite varieties KWS Irina (0.50 g g^{-1}), KWS Kosmos (0.41 g g^{-1}), KWS Sassy (0.48 g g^{-1}) and Maritime (0.48 g g^{-1}) and the wild barleys B3733 (0.41 g g^{-1}) and B3736 (0.39 g g^{-1}) (Tukey's test, p<0.05). Furthermore, the wild barley Alpha (0.25 g g^{-1}) had a significantly lower HI than KWS Irina (0.50 g g^{-1}) (Tukey's test, p<0.05).

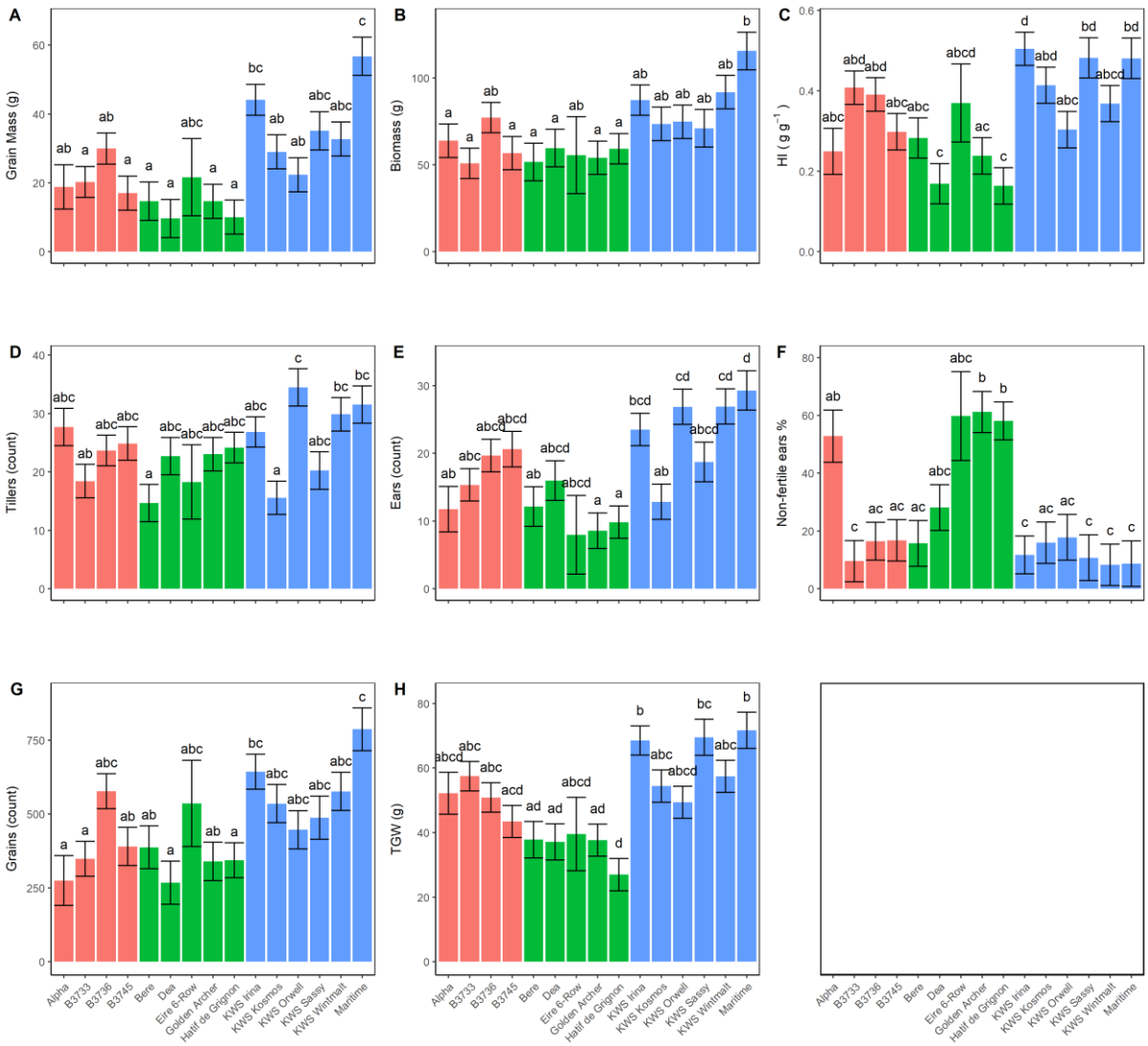

Figure 4.5: Harvest at maturity for 15 barley cultivars; wild barleys in red, landraces in green and elite cultivars in blue. A) Total above-ground biomass B) Tiller number C) Number of ears D) Number of grains per ear E) Thousand grain weight F) Grain mass per plant G) Harvest Index (=Grain mass / Total biomass). Data shown are means +/- se, N=5-6. Identical letters indicate no significant differences between means based on Tukey's test of model outputs at p=0.05.

Potential yield in barley is closely tied to tiller number (AHDB, 2018). The first indicator of potential yield in this study was the number of tillers (Fig. 4.5D). The landrace Bere (14.7 tillers) and the elite variety KWS Kosmos (15.6 tillers) had significantly fewer tillers on average than KWS Orwell (34.4 tillers), KWS Wintmalt 29.9 tillers) and Maritime (31.5 tillers) (Tukey's test, $p<0.05$). The wild barley B3733 (18.4 tillers) also had fewer tillers than KWS Orwell (Tukey's test, $p<0.05$). The number of ears per plant differed from the number of tillers as the latter were not always fertile (Fig. 4.5E). The landraces Golden Archer (8.6 ears) and Hatif de Grignon (9.8 ears) had significantly fewer ears than the elite cultivars KWS Irina (23.5 ears), KWS Orwell (26.9 ears), KWS Wintmalt (26.9 ears) and Maritime (29.3 ears) (Tukey's

test, $p<0.05$). In addition, the wild barley Alpha (11.7 ears), the landrace Bere (12.1 ears) and the elite cultivar KWS Kosmos (12.8 ears) had significantly fewer ears than KWS Orwell, KWS Wintmalt and Maritime (Tukey's test, $p<0.05$). B3733 (15.3 ears) also had fewer ears than Maritime (Tukey's test, $p<0.05$). An estimate of non-fertile ears per plant can be made from these data (Non-fertile ears = 1-(Ears/Tillers), Fig. 4.5F). B3733, KWS Irina, KWS Sassy, KWS Wintmalt and Maritime all had significantly fewer infertile ears (between 41.1 pp and 52.9 pp difference) compared to Alpha, Golden Archer and Hatif de Grignon (Tukey's test, $p<0.05$). The wild barleys B3736 (16.5% infertile) and B3745 (16.8%), the landrace Bere (15.7%) and the elite cultivars KWS Kosmos (16.0%) and KWS Orwell (17.8%) all had significantly more fertile ears than Golden Archer (61.2%) and Hatif de Grignon (58.1%) (Tukey's test, $p<0.05$).

Two measurements of ear quality were considered, the number of grains per plant (Fig. 4.5G) and thousand grain weight (Fig. 4.5H). Alpha (275 grains $plant^{-1}$), B3733 (349 grains $plant^{-1}$), Dea (268 grains $plant^{-1}$) and Hatif de Grignon (343 grains $plant^{-1}$) all had significantly fewer grains per plant than KWS Irina (643 grains $plant^{-1}$) and Maritime (787 grains $plant^{-1}$) (Fig. 4.5G, Tukey's test, $p<0.05$). Furthermore, B3745 (390 grains $plant^{-1}$), Bere (387 grains $plant^{-1}$) and Golden Archer (339 grains $plant^{-1}$) all had significantly lower grain number than Maritime (Tukey's test, $p<0.05$). Thousand grain weight is a useful measure of yield quality that is known to have a genetic component (Bezant et al., 1997). B3736, B3745, KWS Irina, KWS Kosmos, KWS Sassy, KWS Wintmalt and Maritime had a significantly higher TGW than Hatif de Grignon of between 1.9x and 2.7x (Fig. 4.5H, Tukey's test, $p<0.05$). Bere (37.8g), Dea (37.1g) and Golden Archer (37.6g) also had lower TGW than KWS Irina (68.5g), KWS Sassy (69.5g) and Maritime (71.7g) (Tukey's test, $p<0.05$).

The field trial results described above demonstrate a wide variety of responses, from fluorescence and changes in response to step changes in light, to anatomical differences and finally, to achieved harvest outcomes. In order to test some of these variables with greater control over environmental conditions,

it was decided to study plant performance on a similar range of measures in a controlled growth chamber, as detailed in the coming sections.

4.3.5 Chlorophyll fluorescence measurements in the growth chamber

The first test of plant responses under controlled conditions in the growth chamber consisted of chlorophyll fluorescence measurements. In the growth chamber, a subset of plants was used for reasons of space, in this case the landraces Eire 6-Row, Golden Archer, Dea, Hatif de Grignon and the elite cultivar KWS Irina. Fluorescence responses were recorded (Fig. 4.6). While it might be expected that there were no differences in Fv / Fm values, there was an effect of variety overall on Fq' / Fm' although no pairwise differences between cultivars; there were also no differences between cultivars in their NPQ responses.

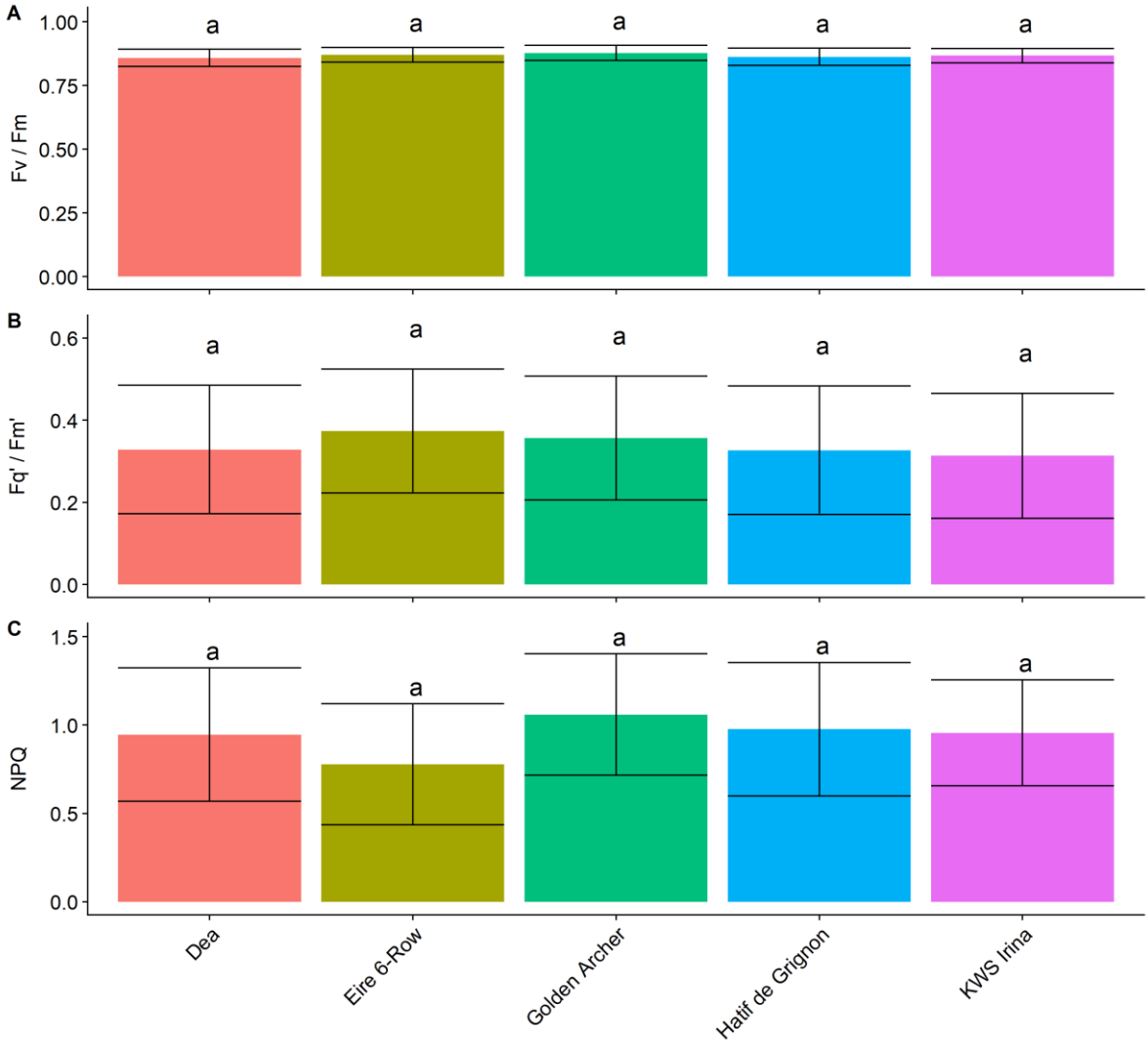

Figure 4.6. Fluorescence measurements of 4 landraces and 1 elite (KWS Irina) barley variety grown in a controlled environment. A) Maximal efficiency of photosynthesis (Fv / Fm). B) Efficiency of photosystem II at PAR = 1000 µmol m^{-2} s^{-1} (Fq' / Fm') C) Non photochemical quenching at PAR = 1000 µmol m^{-2} s^{-1} (NPQ). Means shown +/- se, N=6. Identical letters indicate no significant differences between means at p=0.05.

Using the Technologica fluorescence imager, a light curve of PAR against Fq'/Fm' was generated for the four landraces Dea, Eire 6-Row, Golden Archer and Hatif de Grignon as well as the elite cultivar KWS Irina (Fig. 4.7).

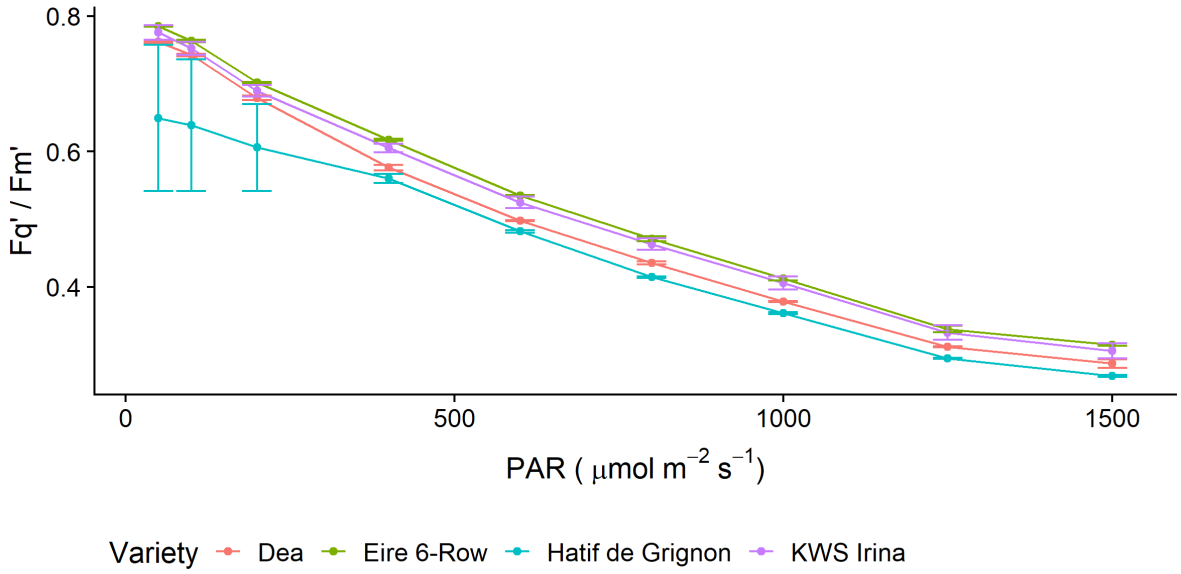

Figure 4.7. Fq' / Fm' response curve for a range of light levels between 50 µmol m^{-2} s^{-1} and 1500 µmol m^{-2} s^{-1} for 3 landraces and the elite cultivar KWS Irina. Means shown +/- se, N=2-3.

Hatif de Grignon had the lowest mean *Fq'/Fm'* (0.48) averaged over all light levels compared to all the other cultivars (0.52-0.55), and was significantly lower than the others at every light level (Tukey's tests, $p<0.05$); meanwhile Eire 6-Row's average *Fq'/Fm'* (0.55) overall remained higher than that of Dea (0.52, Tukey's test, $p<0.05$).

4.3.6 Photosynthetic capacity under changing light (A-Q) or internal [CO_2] (A-C_i)

One way to understand plant responses to the key environmental variables of light level and [CO_2] is to measure the assimilation response of plants to changing internal [CO_2] and light intensity or PAR (Sharkey et al., 2007), which allow some understanding of the biochemistry underlying photosynthetic activity. The curves can be modelled and key parameters extracted as described in section 4.2.4 above. Data were collected in two separate experiments, and values calculated relative to KWS Irina in each study, then combined.

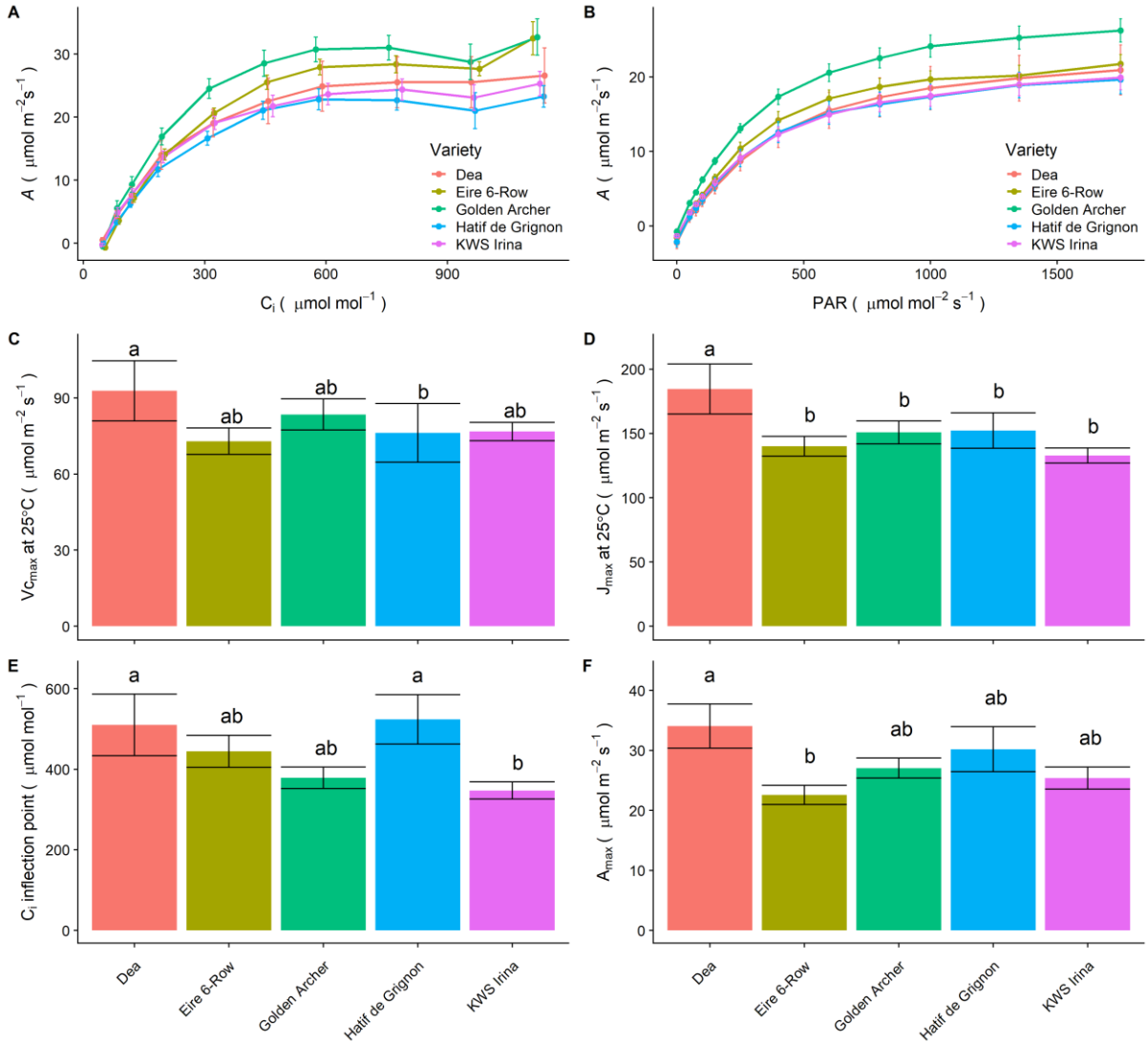

Figure 4.8. Response curves of 4 landraces and KWS Irina assimilation rates to changes in [CO_2] and light intensity over two experiments, and parameterisation of those curves. Parameterised values were normalised to the mean values for KWS Irina across both studies. A) Response curve of assimilation to changes in C_i. B) Response curve of assimilation to changes in light intensity. C) Vc_{max} at 25°C. D) J at 25°C. E) Inflection point of Rubisco activation to RuBP regeneration. F) Maximum value of assimilation at saturating light intensity. Means shown +/- se, N=6-8. Identical letters indicate no significant difference between a pair of comparisons on Tukey's test at p=0.05.

The results of two separate studies on the assimilation response of barley to changes in C_i (Fig. 4.8A) and PAR (Fig. 4.8B) permitted a deeper understanding of the natural variation in rubisco activation, RuBP regeneration, the inflection point between the two and the maximal rate of assimilation under saturating light. Data were recalculated to normalise the parameterisation to the overall mean values for KWS Irina across both studies. The trend was for Dea to have the highest Vc_{max} at 25°C (Fig. 4.8C) compared to Hatif de Grignon (Tukey's test, $p<0.05$). Dea also had the highest value of J at 25°C

compared to all other varieties (Fig. 4.8D, Tukey's test, $p<0.05$). Along with Hatif de Grignon, the inflection point between Rubisco activation limitation on *A* and RuBP regeneration being limiting (Fig. 4.8E) was also higher in Dea than in KWS Irina (Tukey's test, $p<0.05$). Finally, Dea produced the highest maximal assimilation rate of CO_2 at saturating PAR than Eire 6-Row (Tukey's test, $p<0.05$).

4.3.7 Growth chamber stomatal morphology

Since stomata are the main interface between the bulk atmosphere and the internal structures of the plant, stomatal morphology can link physiological observations to final harvest outcomes. Therefore, stomatal morphology was assessed by comparing density and guard cell length (Fig. 4.9).

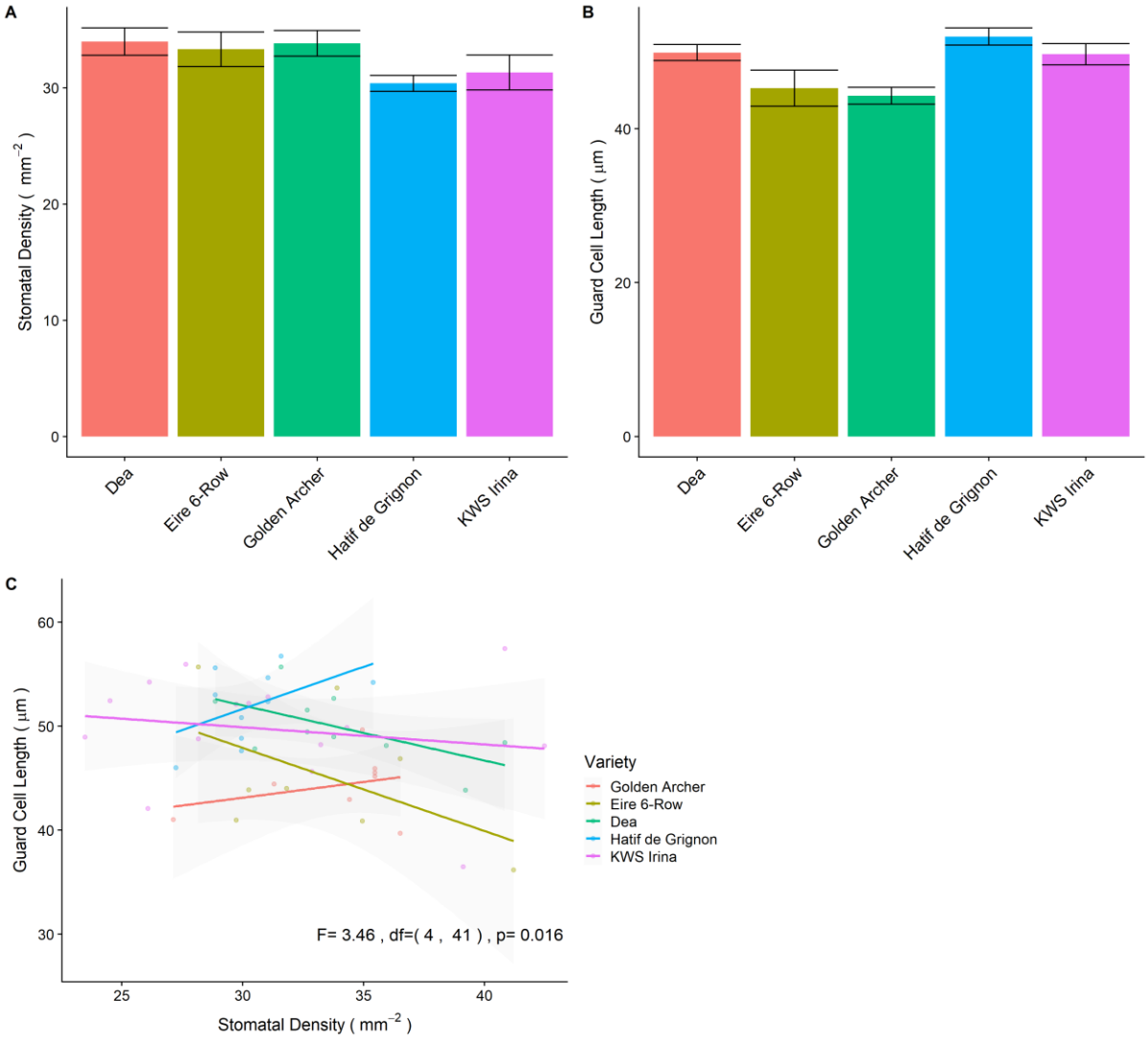

Figure 4.9. Analysis of stomatal morphology for 4 barley landraces and KWS Irina. A) Stomatal density. B) Guard cell length. Means shown +/- se, N=7-10. C) Scatterplot of stomatal density vs. guard cell length. Individual variety and overall regressions shown +/- se.

The range of stomatal densities appeared fairly constrained (Fig. 4.9A), with Golden Archer having the highest density on the abaxial surface of the third leaf (33.8mm^{-2}) while Hatif de Grignon had the least (30.4 mm^{-2}), and there were no pairwise differences between varieties (Tukey's test, $p>0.05$). Meanwhile, guard cell length also varied over a narrow range, with Hatif de Grignon having the longest guard cells (52.0 µm) and Golden Archer the smallest (44.3 µm). While there was a interaction between stomatal density and variety in predicting guard cell length (Fig. 4.9C, 2-way ANOVA, $F_{(4, 41)}=3.46$, $p=0.016$) some varieties had a negative slope coefficient (e.g. Dea, Eire 6-Row and KWS Irina) while others (Hatif de Grignon and Golden Archer) showed positive slope coefficients.

4.3.8 Growth chamber yield analysis

The plants in the growth chamber study were harvested before final maturity. Therefore only above-ground biomass and tiller data were acquired (Fig 10).

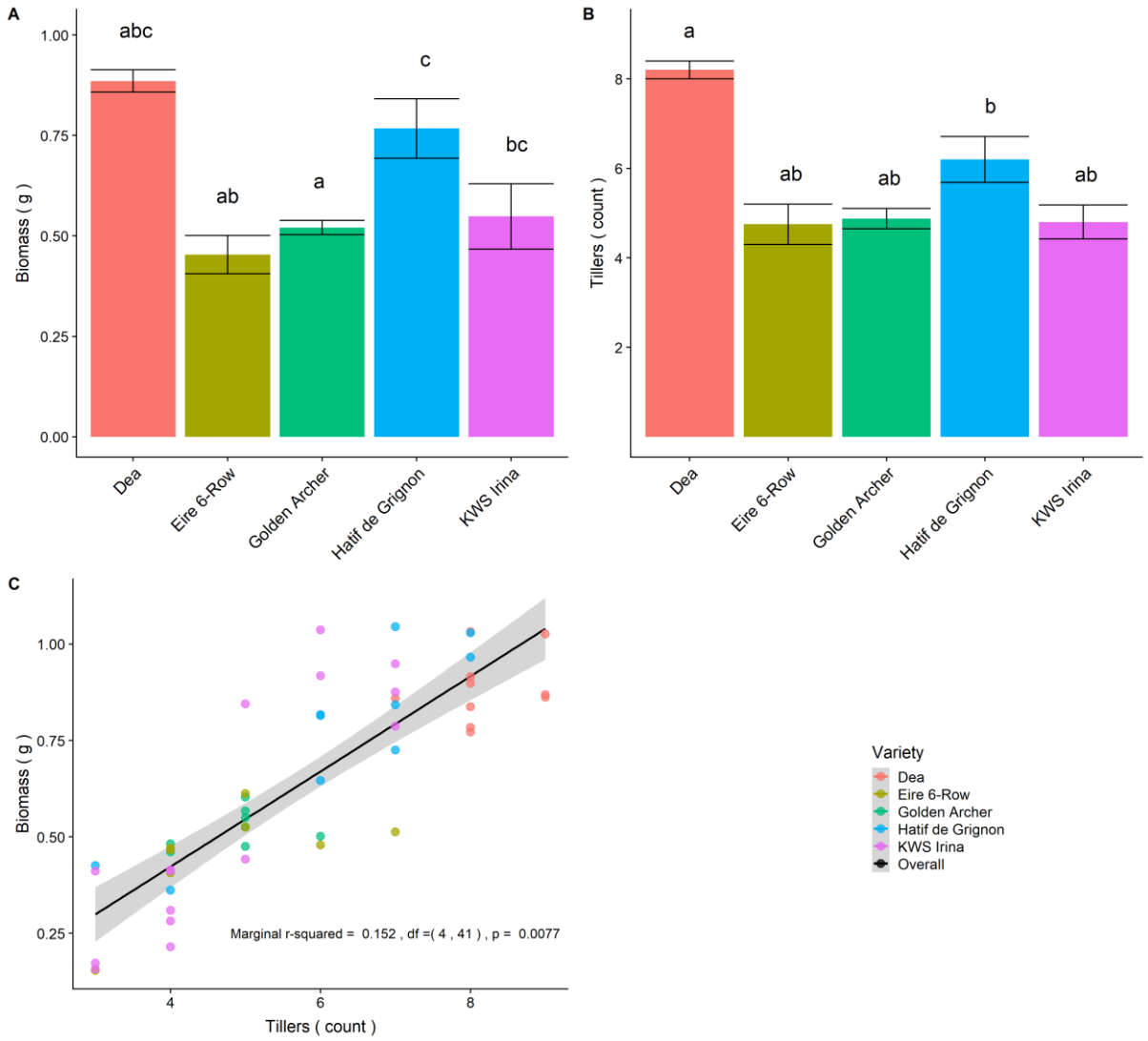

Fig 4.10. Harvest data for 4 landraces and the elite variety KWS Irina grown in controlled conditions. A) Above-ground biomass. B) Number of tillers. Means shown for A) and B) +/- se, n=7-15; identical letters indicate no significant differences between pairs of comparisons on Tukey's test at p=0.05. C) Scatterplot of tiller number against biomass with line of best fit shown +/-se, N=7-15.

Although Dea had the highest biomass on average (Fig. 4.10A, 0.89g), it was not significantly different from any other variety's biomass (Tukey's test, p>0.05). However, Hatif de Grignon did have greater biomass (0.77g) compared to Eire 6-Row (0.45g) and Golden Archer (0.52g). In addition, KWS Irina

(0.55g) also had greater biomass than Golden Archer. Tiller number is a key determinant of grain yield in barley, and in this instance (Fig. 4.10B) Dea again had the greatest number of tillers on average (8.2) and significantly more than Hatif de Grignon (6.2). There was a significant positive correlation overall between tiller number and biomass (Fig. 4.10C) although the multiple R^2 was relatively low (R^2=0.152, 2-way ANOVA, $F_{(1,34)}$=4.0, p=0.0077).

4.4. Discussion

The aim of this study was to characterise the natural variation in anatomical and physiological differences among a diverse range of barleys. After many decades of breeding for yield, it was predicted that the elite varieties (KWS Irina, KWS Kosmos, Maritime, KWS Orwell, KWS Sassy and KWS Wintmalt) would have superior performance in terms of physiological outputs and harvest metrics, in particular key yield metrics such as grain number and mass, as well as harvest index. The physiological responses and yield outcomes for the landraces were expected to be modestly more disappointing, and the wild barleys the worst of all. However, a caveat with these data include the range of temperatures experienced in the Polytunnel, which peaked at 40^{o}C on warm days, and may have affected responses by the different genotypes.

4.4.1 Field trials suggest a diversity of physiological, anatomical and yield capacities

In the field, with six representative varieties selected for detailed analysis, it was striking that the wild barley, B3733, had a significantly lower *Fv/Fm* value compared to the other cultivars, which typically indicates elevated stress in the plant, and this was underlined by the high NPQ value seen (Baker, 2008). Elsewhere, chlorophyll fluorescence did not appear to discriminate between varieties. It is possible that differences observed in B3733 were linked to changes in Calvin Cycle kinetics / capacities (such as chlorophyll content) or to anatomical differences, for instance in stomatal size or density; that B3733 was stressed was initially probed by a step change in light intensity to understand speed and extent of response in *A* (McAusland et al., 2016). The low values achieved by B3733 in predicted and actual A_{max}

confirmed the outputs of the fluorescence and underlined the stress being experienced by the plant, although the source of the stress was not identified – it could relate to adaptations arising from its origins in Central Asia, but as the same applies to the other wild barleys, this criticism may not be useful. Step changes in light had more success in distinguishing between responses in the other varieties. Golden Archer was notable for relatively slow response rates to changing light, and relatively low maximum g_s compared to KWS Sassy which was in line with prior predictions, and with B3745, which was not as the wild varieties were not expected to have superior kinetics of responses to changes in light compared to either landraces or elite cultivars given the extent of breeding over time (inadvertently) targeting stomata conductance as a proxy for yield (Fischer et al., 1998).

Under the expectation that stomatal density correlates positively with high maximal values of g_s and A (Franks and Farquhar, 2007, Doheny-Adams et al., 2012), and negatively with pore size (Buessis et al., 2006), stomatal anatomy was examined. Golden Archer, which had relatively low maximal values for g_s and A (see Fig. 4.3) had the highest stomatal density of the cultivars examined. Conversely, B3745 had relatively low stomatal density, but high maximal values of g_s and A. In neither event was the hypothesis of a size-density trade-off confirmed (Doheny-Adams et al., 2012), nor that of high density-high g_s (Dow et al., 2014, Franks and Farquhar, 2001). Only KWS Sassy conformed to expectations. Perhaps barley is unusual in its links between anatomy and physiology, perhaps the links only exist for short periods of time (see Chapter 3, for instance, for the variability in the trade-off between stomatal density and size over developmental time, and Hetherington and Woodward (2003)) or at specific combinations of growth stage and leaf maturity which were not examined in this study.

Harvest Index (HI) was targeted in the Green Revolution to favour grain over straw production (Evenson and Gollin, 2003), and therefore it would be expected that elite cultivars would have a higher HI compared to the others. It seems probable that higher grain mass in the elite varieties was the result of high HI relative to wild barleys and landraces, but no elite cultivars outperformed *all* their non-elite peers. This may be due to the lack of additional N application in this study, which in modern varieties

appears deleterious (Sylvester-Bradley and Kindred, 2009, Bingham et al., 2012). One of the key ways in which barley yield can be affected is in tiller number. Barley, unlike wheat, is relatively fixed in florets / spikelet (Sadras and Slafer, 2012, AHDB, 2018), while grain size is generally believed to be highly heritable and thus rather invariant (Sadras and Slafer, 2012). Here we noted that there was a wide diversity in the rate of tillering among all cultivar types (for instance, the elite varieties were among the highest and lowest tillering cultivars. Nevertheless, not all tillers were fertile, and in particular, the landraces were very susceptible to producing non-fertile tillers compared to either the elite varieties or, somewhat surprisingly, the wild barleys with the exception of Alpha. Grain number is relatively fixed in barley relative to tiller number (having one floret per spikelet), and although there was a wide range of outcomes across the cultivars on this measure, once again, varieties as diverse as B3736 and Eire 6-Row showed no penalty relative to the top-performing elite varieties such as Maritime. Grain number and grain weight combine in the Thousand Grain Weight (TGW) measure, and here the elite cultivars appeared to outperform the landraces fairly consistently, but not so much the wild barleys. The wild barleys studied here appear to offer the breeder interesting avenues to explore in the search for new germplasm (Wang et al., 2017, Russell et al., 1997).

No fertilisers were used in these studies, and the lack of additional nitrogen could have affected the performance of the elite cultivars (Sylvester-Bradley and Kindred, 2009). Meanwhile, daytime temperatures in the polytunnel did rise periodically past 40^{o}C during the course of collecting the field study data, and such stress is known to affect yield negatively in cereals (Bidinger et al., 1977) with negative effects of high temperatures not just during stem elongation, but also during booting and heading (Ugarte et al., 2007). All cultivars would be affected by extreme temperatures, but we would predict that landraces bred for cool, moist climates like Bere (Orkneys Islands) and Eire 6-Row (Ireland) would be worst affected (GRU, 2019). The evidence for such outcomes was mixed – Bere had low tiller number but few non-fertile ears and adequate TGW for instance. On the other hand, Maritime was a consistently strong performer under the prevailing conditions, as might be expected for a variety bred

for use in Australia (Wheeler, 2013). Nevertheless, it was possible to show that there was wide variation in harvest outcomes both between the three groups – elite cultivars, landraces and wild barleys, as well as within them. Therefore additional work was undertaken in the growth chamber in an attempt to shed further light on these differences at the vegetative stage of growth.

4.4.2 Growth chamber studies underline the importance of Genome x Environment interactions

In the growth chamber, attention was focused on the landraces Eire, Golden Archer, Hatif Dea and Hatif de Grignon. Here, they were compared with KWS Irina, a strong-performing spring cultivar bred for the British market and which remains on the UK Recommended List (AHDB, 2019). Here, *Fv/Fm* values were consistent across cultivars, and in line with expectations (Baker, 2008), settling around 0.8, suggesting that all plants were in good health. As in the field trial, it was not possible to distinguish between varieties on *Fq'/Fm'* nor NPQ, and this insight was underlined by a lack of interaction between light level and cultivar in an *Fq'/Fm'*-PAR light curve. Better discrimination between cultivars was possible using gas exchange to assess elements of Calvin cycle activity. In particular, landraces tended to higher Vc_{max}, J and C_i inflection point values, most notably the variety Dea. The fact that the elite variety KWS Irina had the lowest value of J and the lowest C_i inflection point (the moment at which RuBP regeneration rather than Rubisco activation becomes limiting (Sharkey et al., 2007)), suggests an opportunity for further exploration of the physiology of cultivars such as Dea. The links between physiology and anatomy were tenuous and inconsistent when cultivars were taken into account, while the links between physiology and yield were equally rare. One interesting observation to emerge from the stomatal data was the lack of a consistent association between stomatal size (i.e. guard cell length) and stomatal density, as predicted from previous work (Doheny-Adams et al., 2012), when variation at the cultivar level was taken into account. A finding which was replicated in the field for Golden Archer and Hatif de Grignon.

There appeared to be an unequivocal link between tiller number and total vegetative biomass at harvest in the growth chamber studies, and that association was significantly determined by variety, underlining

the results of the polytunnel harvest at final maturity, although it proved impossible to directly replicate the polytunnel harvest results in the growth chamber, probably as a result of heterogeneous conditions in the former. As was previously noted, tiller number is crucial in barley when considering final grain yield (Sadras and Slafer, 2012), and these results overall suggest cultivars such as Dea or Hatif de Grignon could be attractive in relation to breeding for higher tiller number in modern elite cultivars if the number of aborted tillers could be effectively controlled.

The take home message of this work is that even in relatively heterogeneous conditions, it is possible to discern great diversity in outcomes in physiology, anatomy and harvest in a range of barley genotypes. Although generally the elite varieties performed as expected in terms of harvest outcomes, it was difficult to link those harvest metrics to the kinetic or anatomical data presented here. Furthermore, there is clearly great heterogeneity in the landrace and wild barley performance across multiple dimensions of physiology such as response rates to changing light and maximal stomatal conductance.

Wild barleys appear particularly interesting as they represent absolutely unexplored opportunities to incorporate novel genetic traits into the existing elite germplasm. In the growth chamber, there was less variability, and again it is interesting to note that in the absence of additional fertilisation, elite cultivars do not always produce the greatest biomass or tiller number. From the growth chamber results, it appears that landraces have something to offer the breeder. Dea had high rates of Vc_{max} and *J* and C_i inflection compared to KWS Irina, and produced the largest harvest, greater than that seen for KWS Irina. Furthermore, Dea exhibited strong tillering both in the field and growth chamber.

4.5. Conclusions

- The link between stomatal density and size turns out to be less strong in barley than is suggested in the literature.
- Physiological, anatomical and harvest measurements in a panel of wild, landrace and elite cultivars in polytunnel conditions resulted in wide diversity of outcomes.

- Elite varieties did not always outperform non-elites, including in some surprising areas such as harvest index.
- The physiology of plants in the growth chamber could be linked to desirable harvest outcomes at the end of the studies.
- The single replication of the field trial was not sufficient to understand the effect of GxE interaction on the results.

Chapter 5: The impact of drought on plant performance through WUE variability and the circadian clock; physiology, anatomy and mechanisms.

5.1. Introduction

Climate change is driving the need to discover new phenotypes in our plant germplasm, particularly in relation to crop productivity (van Ittersum et al., 2013). Meanwhile, rising population and changing dietary preferences plus biofuel production underpins the requirement to increase yields by around 50% by 2050 (Ray et al., 2013), with little additional land likely to become available. Furthermore, climate change predictions suggest that not only will [CO_2] be higher, but also global mean temperatures (IPCC, 2014). This implies that there will also be greater evaporative demand from the atmosphere, and plants themselves are likely to 'run hotter' as increasing [CO_2] means stomata will have smaller apertures and therefore less transpiration will occur (Long et al., 2006). Under climate change, there is also predicted to be more frequent, severe and persistent episodes of drought (IPCC, 2014). While there is no agreed definition of water stress terms, we consider mild to moderate drought to mean a reduction in soil water content to a level at which a recovery of plant function remains possible. In Arabidopsis, this may be around 30% of field or pot capacity (Harb et al., 2010), but it ought to be lower in barley, which is known to be a drought-tolerant plant.

5.1.1 Plant responses to drought

In the context of drought, we observe two alternatives commonly used by plants to manage the problem of water availability: conservatism / non-conservatism (Valladares et al., 2000, Chapin, 1980). In the former case, the plant may *escape* drought by flowering early to complete the life cycle before the water stress becomes too acute, but at a significant cost to yield (Shavrukov et al., 2017). The plant may also *avoid* drought by closing stomata in response to water stress in order to maintain cell turgor and hydraulic conductivity (Shavrukov et al., 2017); this, too comes with a significant yield penalty. The non-conservative plant will *tolerate* drought by maintaining a relatively high rate of transpiration (Harb

et al., 2010). If the drought episode proves transient, then the non-conservative plant will maintain relatively high yield in the face of the period of water stress (Shavrukov et al., 2017). If the water stress exceeds the capacity of the plant to tolerate the drought in terms of length, duration or severity, then it risks a significant and potentially fatal outcome with the prospect of no yield at all. It is thought that barley, like wheat, is non-conservative under drought (Munns et al., 2010) although there is evidence that it is able to switch tactics under severe drought depending on genotype and environmental cues (Wiegmann et al., 2019).

In *Arabidopsis* it has been shown that there is an interaction between drought and the circadian clock, where a number of pathways are known to mediate plant responses to stress in *Arabidopsis* (Schroeder et al., 2001, Estavillo et al., 2011). And there is emerging evidence that drought responses and the clock are linked in barley (Cantalapiedra et al., 2017, Monteagudo et al., 2019). In the late 1980s, NILs were created in a Bowman background, then an elite spring line (Druka et al., 2011). Two of those lines are of interest here, as they have impairments to the circadian clock, although work presented earlier (see Chapter 3) suggests that these impairments may have more to do with seasonal initiation of flowering time than affecting the day-to-day performance of the plants or their response to changing environmental conditions, particularly light. The *eam10* mutant, BW284 exhibits the escape characteristic by going to flowering many days earlier than Bowman (Lundqvist, 2014). The *eam8* mutant, BW289 also flowers somewhat early, but maintains a relatively high yield compared to Bowman (Lundqvist, 2014). It is possible that it presents avoidance or tolerance behaviour in the context of water stress. Bowman itself is a modern high-performing variety (Franckowiak et al., 1985) which is thought to be non-conservative, and which is expected to maintain high stomatal conductance even under drought conditions.

5.1.2 Stress signalling in plants

Of the several pathways known to be involved in stress signalling by the plant, one is of particular interest to this study. Root-to-shoot signalling may be effected by direct sensing of water potential

through the plant (Bertolino et al., 2019). In addition, ABA production by the plant also plays a part, as does some activity by guard cells themselves such as Ca^{2+} signalling and others (Schroeder et al., 2001). But in recent years it has become evident that 3'-PhosphoAdenosine-5'-Phosphate (PAP) is another stress-signalling molecule (Estavillo et al., 2011).

PAP is produced in the chloroplasts and is present in low levels in plant cells, where it is a by-product of the assimilation of sulphates (Bohrer et al., 2015), and is broken down by SAL1 (SALt1) into AMP and inorganic phosphate (Litthauer et al., 2018, Estavillo et al., 2011). SAL1 is inactivated by oxidative stress, such as occurs during drought or high light intensity, meaning [PAP] increases under stress (Estavillo et al., 2011). In this context, PAP has been found to be a key in retrograde signalling from the chloroplast to the nucleus (Phua et al., 2018), and may turn out to integrate a wide range of retrograde signals (Phua et al., 2018). A test for PAP has been in use for a number of years now, and is highly sensitive to changes in concentration in the (leaf) tissues (Estavillo et al., 2011). Exogenous PAP alone is sufficient to extend the circadian period (Phua et al., 2018, Litthauer et al., 2018), but it is not known if the reverse is also true – that the clock influences PAP concentration. This oxidative stress-PAP pathway is widely conserved across plant kingdoms (Chan et al., 2016). It is expected that differences in PAP concentration levels will occur dependent on genotype. In *Arabidopsis*, plants that lack a circadian clock lack fitness (Dodd et al., 2005), and so an increase in [PAP] may be expected, due to clock mutants experiencing greater stress. Meanwhile plants experiencing water stress are expected to exhibit higher [PAP] in line with previous findings in Arabidopsis (Estavillo et al., 2011).

This study aimed to understand the types of responses of Bowman and the two clock mutants to:

- Changes in soil moisture content, and explain them as functional (i.e. physiological) or anatomical differences, with physiological differences interpreted as resulting from the clock mutations as the latter are expected to have an impact on the behaviour according to the time of day.

- Link soil moisture status to leaf moisture levels and ultimately, identify a mechanism for stress communication at the leaf level. Again, this is based on the expectation that clock mutations will have a negative impact on the plant's ability to communicate water stress.
- The objectives were to explore the clock mutations reduced stomatal responses to drought against a background of identical stomatal anatomy.

5.2. Methods

5.2.1 Experiment 1: Physiological and anatomical consequences of mild drought (20% FC)

Plants were germinated in 0.25l pots in Levington's F2+S compost with each pot's mass approximated to +/-5g on a balance, and watered to full capacity. After germination in the greenhouse the pots were randomised into 15 blocks with 1 replicate per block for each treatment. The pots were placed in the University of Essex plant phenotyping platform (PSI, Prague, Czech Republic) with the temperature controlled to 20^{o}C, and humidity controlled to 40%. Valoya lights (Valoya Oy, Melkonkatu, Helsinki, Finland) were set to 400 µmol m^{-2} s^{-1} on 12h:12h day:night cycles. The platform included daily automatic weighing and rewatering. Two treatments were used: the Control or Well-watered treatment targeted 50% field capacity (as previously measured for the pot) and the Drought or Water-Stress treatment targeted 20% of field capacity.

At regular intervals, stomatal conductance was measured using an AP-2 porometer (Delta-T Devices Ltd, Burwell, Cambs, UK), the light in the phenotyping platform being set to either 400 µmol m^{-2} s^{-1} (low light) or 1000 µmol m^{-2} s^{-1} (high light) to check the progress of the drought and the plant responses to light and moisture availability. In addition, thermal image measurements were taken on a FLIR thermal camera (FLIR Systems, Wilsonville, Oregon, USA), and analysed on FLIR's software. Two light levels were used, 400 µmol m^{-2} s^{-1} (low light) and 700 µmol m^{-2} s^{-1} (high light, the maximum available in the imaging area on the phenotyping platform).

Stomatal impressions were taken using the method of Weyers and Johansen (Weyers and Johansen, 1985). Analyses were undertaken on an Olympus BX60 microscope (Olympus Corp., Shinjuku, Tokyo, Japan). At the end of the measurement period, the above-ground portions of the plant were harvested and weighed, and height measured.

5.2.2 Experiment 2: Physiological and anatomical consequences of moderate (10% FC) and severe (5% FC) drought

Ninety-six plants were germinated in Levington F2+S pots, and randomised into 3 treatments with 3 genotypes. The treatments were Control (Well-watered, 50% of previously determined field capacity), moderate drought (10% FC) and severe drought (5% FC). The genotypes used were Bowman, BW284 and BW289. The plants were placed in the phenotyping platform at the University of Essex (PSI Systems, Prague, Czech Republic), at 20^{o}C, 40% relative humidity and at 400 µmol m^{-2} s^{-1} of light on 12h:12h day-night cycle. Plants were watered by hand, with rate of drying limited to the rate achieved by either of the slowest-drying plant or the rate achieved for pots drying by evaporation only, whichever value was the highest. When pots had reached their target weight, they were rewatered daily to their maintenance target. Weights were measured on a Fisherbrand DB352 balance (Fisher Scientific, Loughborough, England).

The concentration of phospho-adenosine phosphate (PAP) was estimated using the methods of Litthauer et al. (2018), adapted for barley (plants were hand-ground in liquid nitrogen, not sonicated with beads). Prepared samples were analysed in an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, California, US) as described in Chapter 2, section 2.7 above.

At the end of the measurement period, the above-ground portion of the plants was harvested, and fresh mass measured on a Fisherbrand DB352 balance (Fisher Scientific, Loughborough, England). The plants were then dried in an oven for 2 days at 80^{o}C, and weighed again for dry mass.

5.2.3 Experiment 3: Physiological and anatomical consequences of moderate (10% FC) drought

Plants of the genotypes Bowman, BW284 and BW289 were grown as per the method for experiment 2, except that there were only two treatments, Well-watered at 50% FC and moderate drought at 10% FC. 48 plants were randomly allotted to 8 blocks placed in the phenotyping platform at the University of Essex (PSI Systems, Prague, Czech Republic). Plants were watered by hand using a Fisherbrand DB352 balance (Fisher Scientific, Loughborough, England) as per the method for experiment 2.

A-C_i and A-Q response curves were measured on the plants using a LiCOR 6400 IRGAs (LiCOR Inc., Lincoln, Nebraska, USA) as described in Chapter 2 above. The concentration of phospho-adenosine phosphate (PAP) was estimated as described in Chapter 2 above. Stomatal impressions were taken using the method described previously in section 2.5. Anatomical g_s was derived from stomatal data (Dow and Bergmann, 2014) on stomatal pore and guard cell length, along with maximal assimilation parameters derived from the A-C_i response curves using the Franks & Farquhar (2001) model.

5.3. Results

5.3.1 Experiment 1: Physiological and anatomical consequences of mild drought (20% FC)

The overall purpose of the first drought study was to examine the impact of moderate (20% FC) drought on the different varieties used – Bowman, BW284 and BW289 under automatic rewatering on the phenotyping platform.

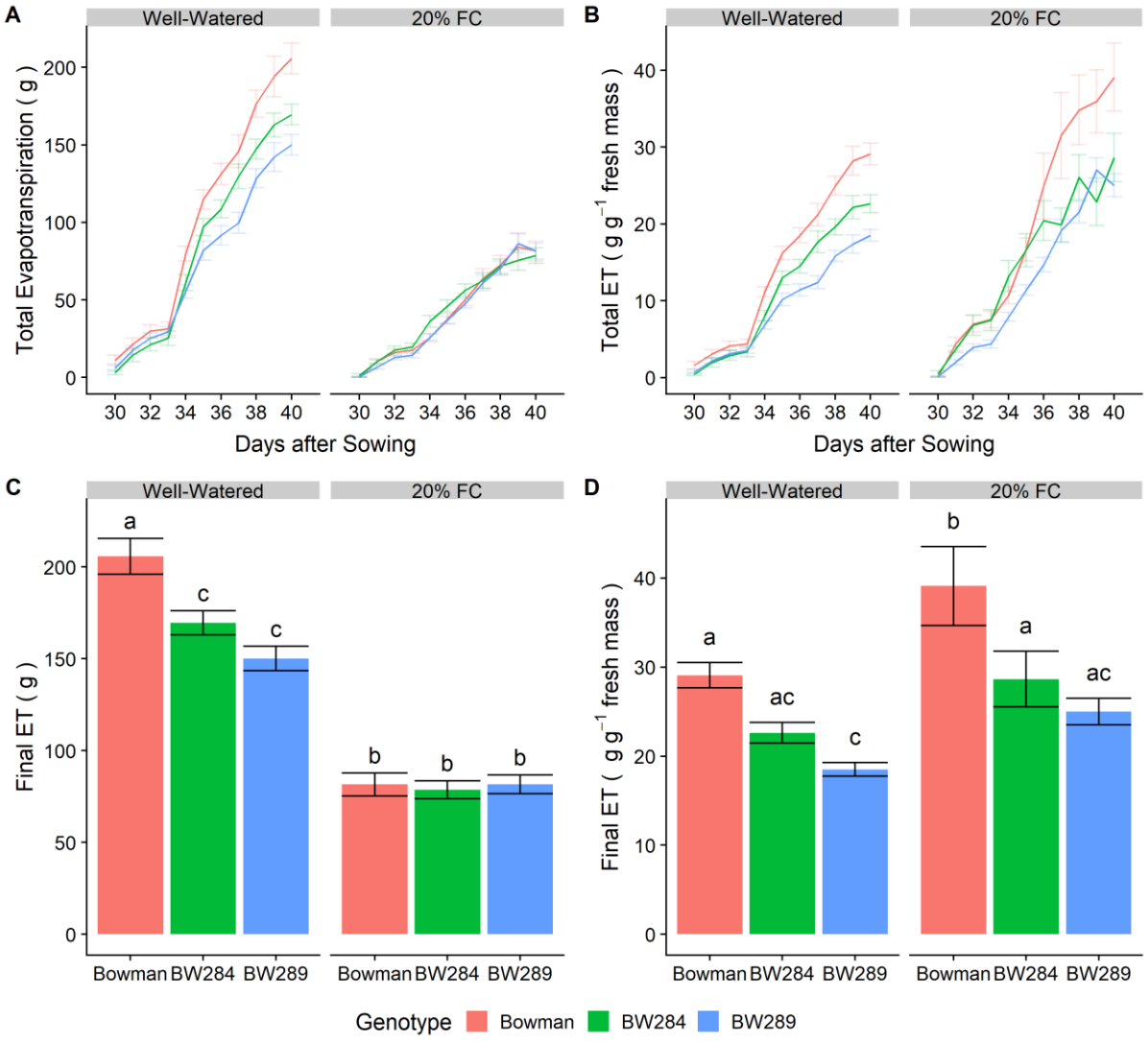

Figure 5.1. Cumulative water evapotranspiration by Bowman, BW284 and BW289 under drought (20% FC) or well-watered conditions during droughting process. Pots were allowed to dry at their own rate until the target mass was reached. A) Cumulative total evapotranspiration. B) Cumulative evapotranspiration per g dry mass. C) Total ET at final time point. D) Total ET per g dry mass at final time point. Means shown +/- se, N=18-23. Different letters denote significant differences between pairwise comparisons on Tukey's test at p=0.05.

Bowman had consistently the highest rate of evapotranspiration (ET) compared to either mutant during the course of the study (Fig. 5.1A) when well-watered, but under water stress, all genotypes followed a similar pattern of ET. At 40 DAS (the final day of the experiment, Fig. 5.1C)), cumulative ET for Bowman was significantly higher (206g) than that for either mutant (170g and 150g respectively) under well-watered conditions (Tukey's test, $p<0.05$). However, under drought conditions, no differences were seen (Fig. 5.1C). It was possible that plant size was affecting the results, as plants that were larger at the start would have greater water loss, hence higher ET. Therefore, total ET was divided by the fresh mass of

each plant at the end of the study (no interim values were available). Bowman continued to exhibit the highest rate of ET per gram biomass during the course of the study under well-watered conditions (Fig. 5.1B), while under drought conditions, Bowman had greater ET per unit biomass than the mutants from 36 DAS. Analysis at 40 DAS (Fig. 5.1D) showed that Bowman had higher ET g^{-1} fresh matter under well-watered conditions (29.1g) compared to BW289 (18.5g). Under water-stressed conditions, Bowman also had the highest ET g^{-1} fresh weight (39.1g g^{-1}) compared to either BW284 (28.6g g^{-1}) or BW289 (25.0g g^{-1}, Tukey's tests, $p<0.05$).

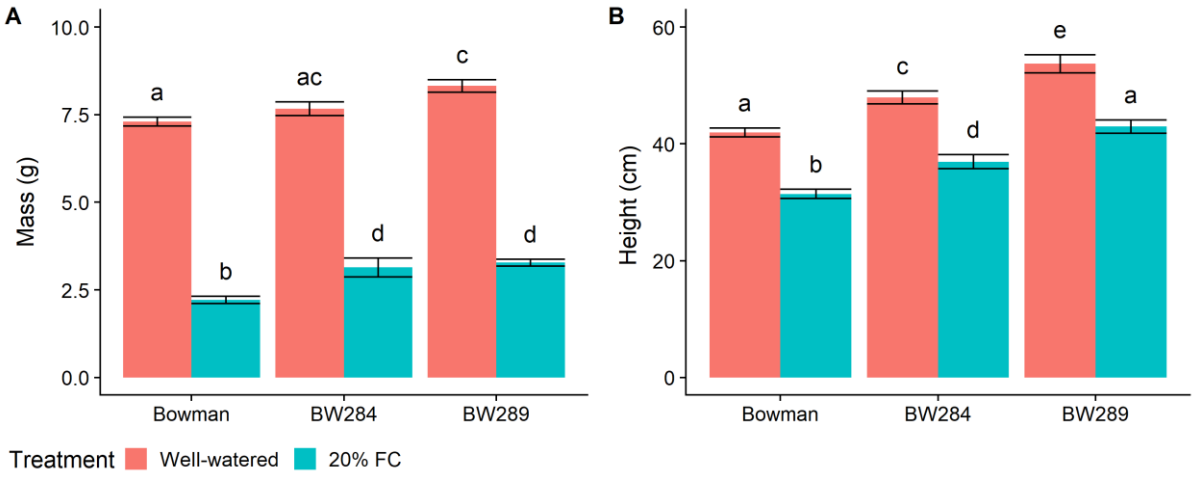

Figure 5.2. Harvest data for Bowman, BW284 and BW289. A) Total fresh above-ground biomass and B) Height above soil level. Means shown +/- se, N=6-8. Identical letters indicate non-significant differences between pairs of means from Tukey's test at p=0.05.

The data on water use were underlined by the harvest data (Fig. 5.2), which were taken at 40 DAS. Bowman achieved lower fresh mass (7.3g) than BW289 (8.3g) under well-watered conditions, and at 20% FC, had lower biomass (2.2g) than either BW284 (3.1g) or BW289 (3.3g, Tukey's tests, $p<0.05$)). These data on biomass were backed up by data for plant height (Fig. 5.2B), where the BW289 (54cm) was significantly taller than BW284 (48cm), the latter in turn being significantly taller than Bowman (42cm) under well-water conditions (Tukey's tests, $p<0.05$). Under drought conditions, all varieties were significantly shorter than their equivalents under control conditions (Fig. 5.2B), and again, BW289 (43cm) was significantly taller than BW284 (37cm) and both were taller than Bowman (31cm, Tukey's tests, $p<0.05$)).

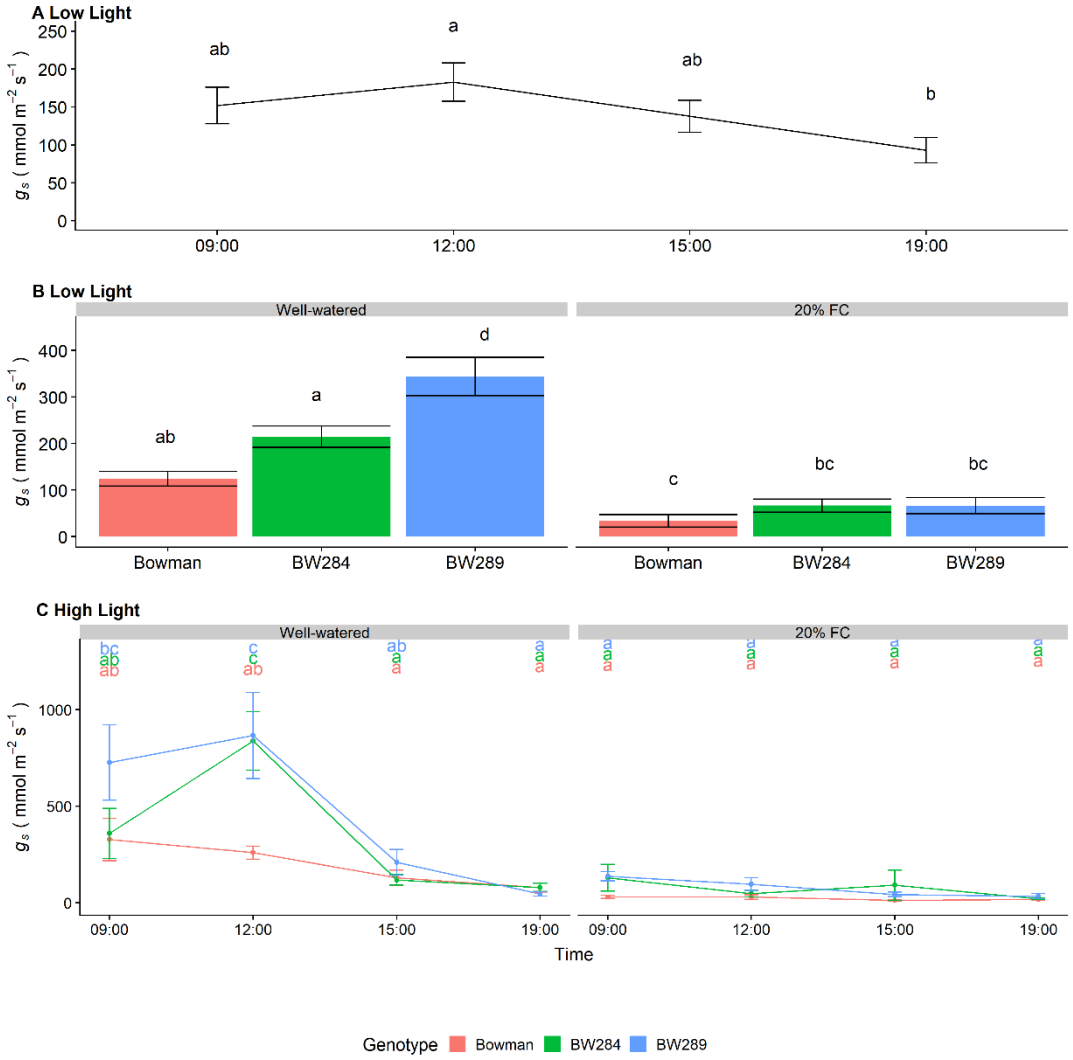

Figure 5.3. Time series of stomatal conductance measured by porometry A) By time of day under low light (100 µmol m^{-2} s^{-1}) B) Under low light (100 µmol m^{-2} s^{-1}) by variety and treatment. C) Under high light (1000 µmol m^{-2} s^{-1}) by time of day by treatment. Means shown +/- se, N=10. Different letters denote significant pairwise differences on Tukey's test at p=0.05.

Porometry was used to determine steady-state g_s under high or (separately) low light at four time points throughout the course of a day (Fig. 5.3). At low light (Fig. 5.3A) g_s rose during the morning, and declined after noon, with stomatal conductance significantly higher at 12:00hrs than at 19:00hrs. There were also clear differences between varieties depending on treatments at both low (Fig. 5.3B) and high light (Fig. 5.3C). While there were no differences between cultivars at low light under drought conditions (Fig. 5.3B, right panel), with all lying between 34.1 and 66.5 µmol m^{-2} s^{-1}, under control conditions (Fig. 5.3B, left panel), BW289 had the highest g_s (344 µmol m^{-2} s^{-1}) compared to either BW284 (215 µmol m^{-2} s^{-1}) or Bowman (124 µmol m^{-2} s^{-1}, Tukey's tests at $p<0.05$). Under high light (Fig. 5.3C), the situation was

more-complex. There were no differences between any varieties at any time point under water-stressed conditions, with g_s varying between 12 and 137 µmol m^{-2} s^{-1} (Tukey's test, $p>0.05$). Yet when water was available, BW289 responded particularly strongly compared to the other varieties. At 09:00, BW289 achieved 726 µmol m^{-2} s^{-1} compared to 359 µmol m^{-2} s^{-1} for BW284 and 327 µmol m^{-2} s^{-1} for Bowman, while by 12:00, BW289 was averaging 866 µmol m^{-2} s^{-1} compared to an increase to 838 µmol m^{-2} s^{-1} for BW284 and both were significantly greater than Bowman, which had declined to 260 µmol m^{-2} s^{-1} (Tukey's test, $p<0.05$). By the afternoon, there were also no differences between the varieties.

A proxy for stomatal conductance is thermal imaging (Fig. 5.4), which can be directly related to g_s if a wet and dry reference are available (Leinonen et al., 2006) although in this case a dry but not a wet reference were used, so all values are relative not absolute. Two measurements were taken, the extent of cooling compared to the dry reference (Fig. 5.4A) reported as a positive value, where higher values mean greater cooling, and temperature heterogeneity across the leaf surface, reported as the standard deviation of temperature (Fig. 5.4B).

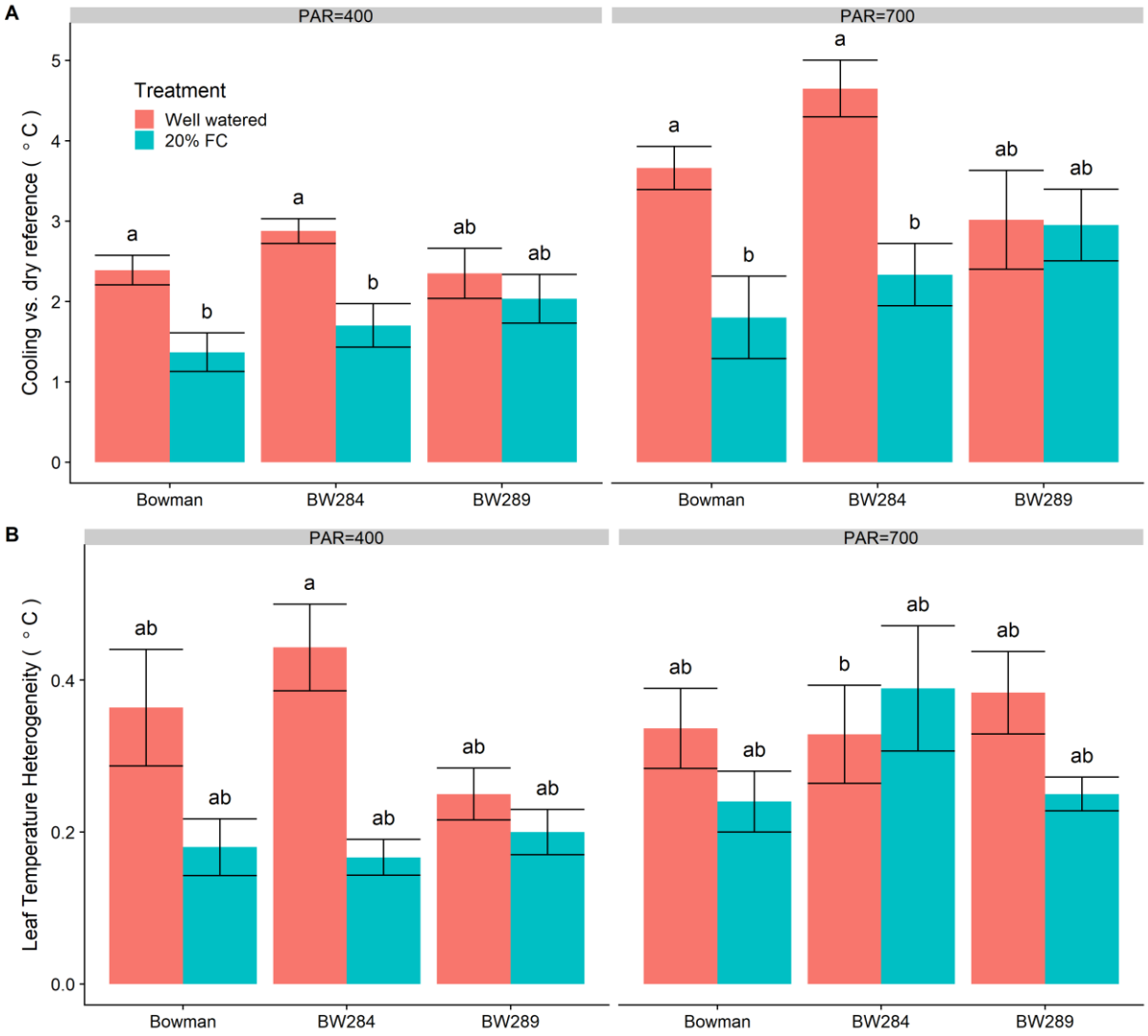

Figure 5.4. Cooling parameters from thermal images of Bowman, BW284 and BW289 leaves under well-watered and drought conditions under low light (PAR = 400 µmol m^{-2} s^{-1} and high light (PAR = 700 µmol m^{-2} s^{-1}). A) Cooling relative to ambient temperature calibration and B) Variability in temperature across the leaf measured as standard deviation. Means

More cooling was seen at high light (Fig. 5.4A, right panel) compared to low light (Fig. 5.4A, left panel) under well-watered conditions. Under high light, Bowman increased cooling significantly, from 1.8 °C to 3.7°C while BW284 also increased cooling significantly, by 129% to 4.7 °C (Fig. 5.4A, Tukey's test, $p<0.05$). At low light leaf temperature heterogeneity reduced most under drought in BW284, with a standard deviation across the leaf of 0.44 °C for control compared to just 0.17 °C for droughted plants (Fig. 5.4B, Tukey's test, $p<0.05$). Leaf temperature heterogeneity rose for BW284 under high light between the control and drought conditions, in contrast to the other two cultivars, where it fell.

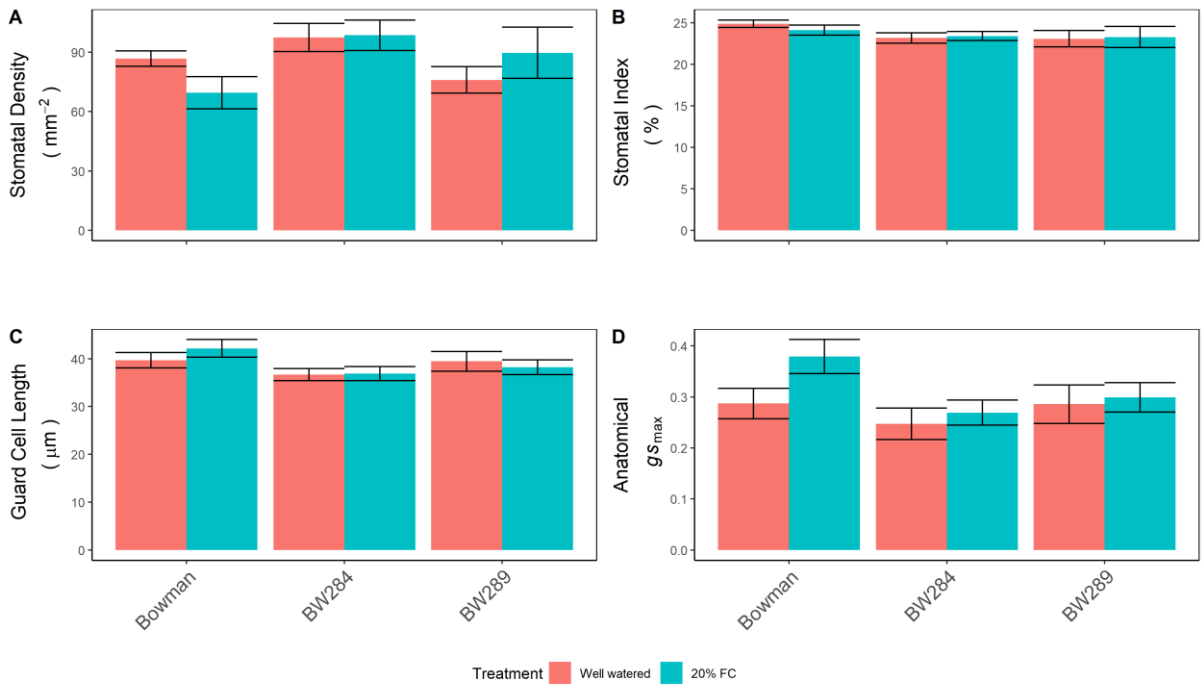

Figure 5.5. Stomatal anatomy for Bowman, BW284 and BW 289 under well-watered and drought conditions. Means shown +/- se, N=8-11. No differences at either the model or pairwise level were observed for any of these measurements at p_{crit}=0.05.

From the abaxial stomatal impressions (Fig. 5.5) captured for the same (i.e. third) leaf as used for porometry and thermal imaging, there was a surprising lack differences among varieties and treatments on stomatal anatomical features. Stomatal density was higher in Bowman under control conditions (87 mm^{-2}) compared to drought (70 mm^{-2}), while for BW284 and BW289 it was higher under drought conditions, in line with expectations (Fig. 5.5A). Stomatal index appeared invariant across varieties and treatments (Fig. 5.5B). Elsewhere, guard cell length was higher under control conditions for BW289 (39.5µm) compared to water-stressed conditions (38.2 µm), while for Bowman, the reverse was the case (39.7 µm vs. 42.2 µm, Fig. 5.5C). Bowman leaves under drought had the highest anatomical gs_{max} (0.38 µmol m^{-2} s^{-1}), and in all cases, anatomical gs_{max} was higher under drought rather than well-watered conditions.

5.3.2 Experiment 2: Physiological and anatomical consequences of moderate (10% FC) and severe (5% FC) drought

In light of the results of the first study described above, further studies were undertaken in which the level was water stress was increased from 20% FC to 10% and 5%, thereby expecting to highlight physiological and anatomical differences between varieties. In this study, the rate of drying was limited to the rate of the slowest replicate to control for larger plants drying more-rapidly.

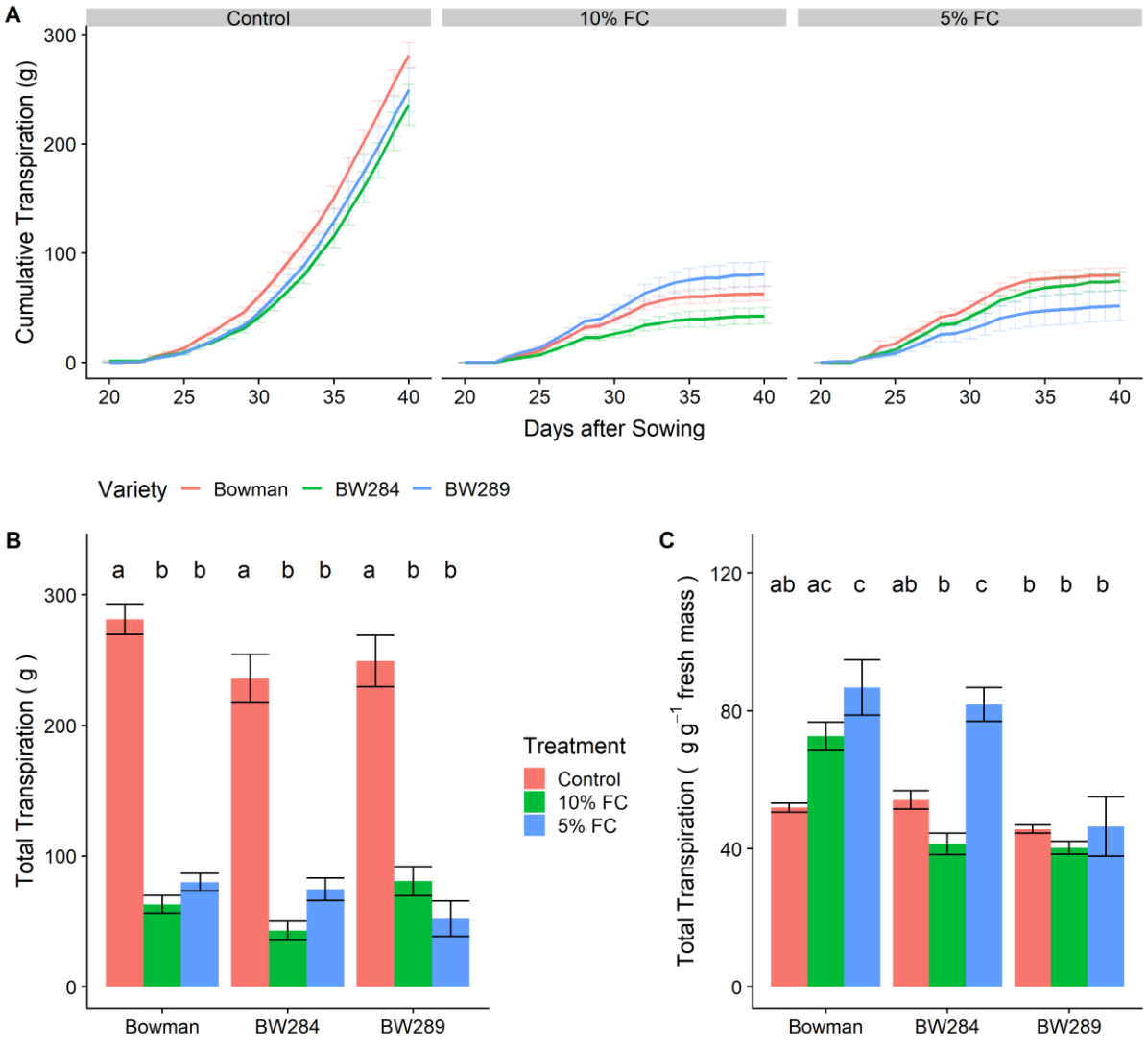

Figure 5.6. Transpiration for Bowman, BW284 and BW289 under control and two drought conditions. A) Cumulative transpiration from the onset of water stress at 20 DAS. B) Total transpiration at 40 DAS. C) Total transpiration at 40 Das per g fresh mass at harvest. Pots without plants were used to estimate daily soil evaporation. Bars shown +/- se, N=6-8. Identical letters indicate no significant differences on pairwise Tukey's test of estimated means at p=0.05.

There was a degree of variability of response to drought among the cultivars Bowman, BW284 and BW289 (Fig. 5.6A). Bowman transpired more water (281g) than BW284 (236g) and BW289 (249g, Fig. 5.6B). However, at 10% of field capacity BW289 transpired the most water and BW284 the least, while at 5% of field capacity, BW289 transpired the least and BW284 almost as much as Bowman (Fig. 5.6B). Control plants did indeed transpire greater volumes of water than drought plants (Fig. 5.6B, Tukey's test, $p<0.05$), but there were no statistical differences considering drought intensity or cultivar. As was the case in the first study, Bowman transpired significantly more water per unit of fresh mass (Fig. 5.6C) than BW284 and BW289 under moderate (10% FC) drought conditions by 43% and 44% respectively (Tukey's test, $p<0.05$). Under rather more severe water stress (5% FC), the differences were less acute, but transpiration for Bowman (87g g^{-1}) remained significantly higher than that for BW289 (47g g^{-1}, Tukey's test, $p<0.05$), and higher than BW284's (82g g^{-1}). There were no differences between varieties under control conditions (Tukey's test, $p>0.05$).

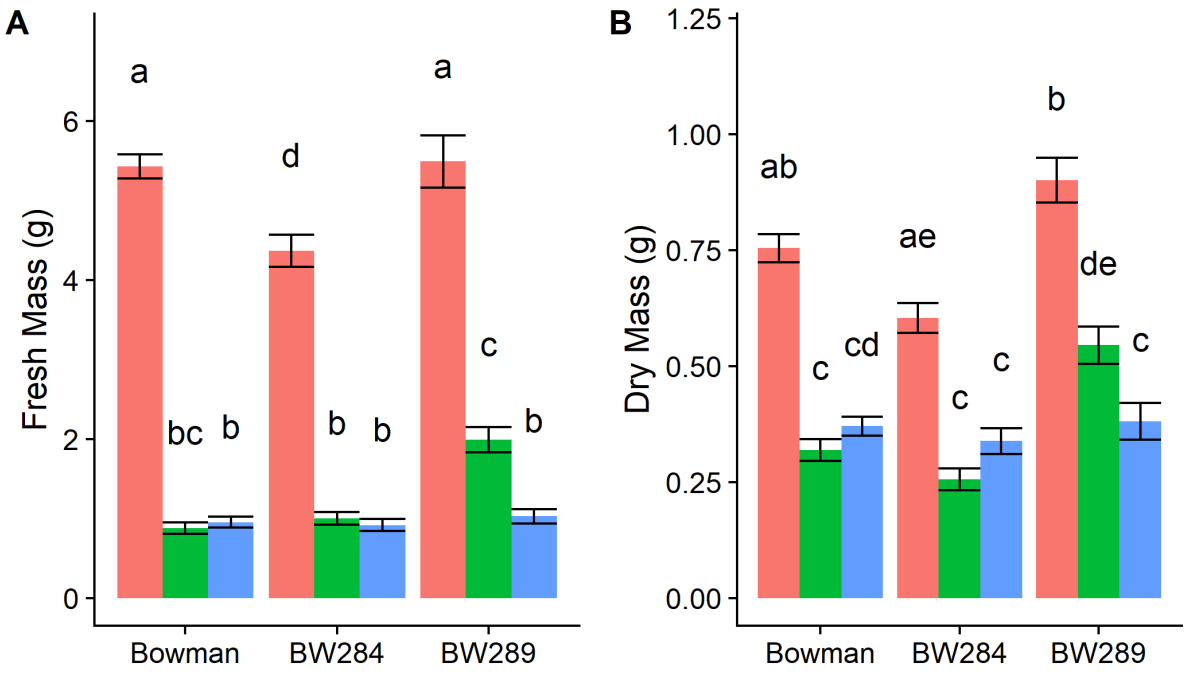

Figure 5.7. Harvest biomass of Bowman and clock mutants under control, 10% of field capacity and 5% of field capacity drought conditions from the second and third experiments in the series. A) Fresh biomass B) Dry biomass. Means shown +/- se, N=6. Identical letters indicate no significant differences of pairwise Tukey's tests of model outputs at p=0.05.

Combining the second and third studies in this series allowed a detailed examination of harvest outcome to take place as there were no differences in fresh or dry mass between experiments (1-way ANOVAs, $p>0.05$). The mutant BW284 had significantly less fresh mass (4.4g) than either of the other two cultivars when well-watered (Bowman=25% more, BW289=26% more, Tukey's test, $p<0.05$, Fig. 5.7A). Under drought stress, BW289 maintained a higher fresh mass at 10% FC (2.0g) than either of the other cultivars (Bowman=56% less, BW284=50% less), and significantly more than BW284 (Tukey's test, $p<0.05$, Fig. 5.7A). BW284 also had 33% lower dry mass (Fig. 5.7B) under control conditions than BW289 (Tukey's test, $p<0.05$) and 20% less than Bowman, while at 10% FC, BW289 (0.54g) maintained significantly more dry mass than Bowman (41% less) and BW284 (53% less, Tukey's tests, $p<0.05$).

The concentration of phospho-adenosine phosphate (PAP) was expected be higher in the clock mutants under drought since their lack of fitness in the prevailing environment was hypothesised to lead to a greater degree of stress.

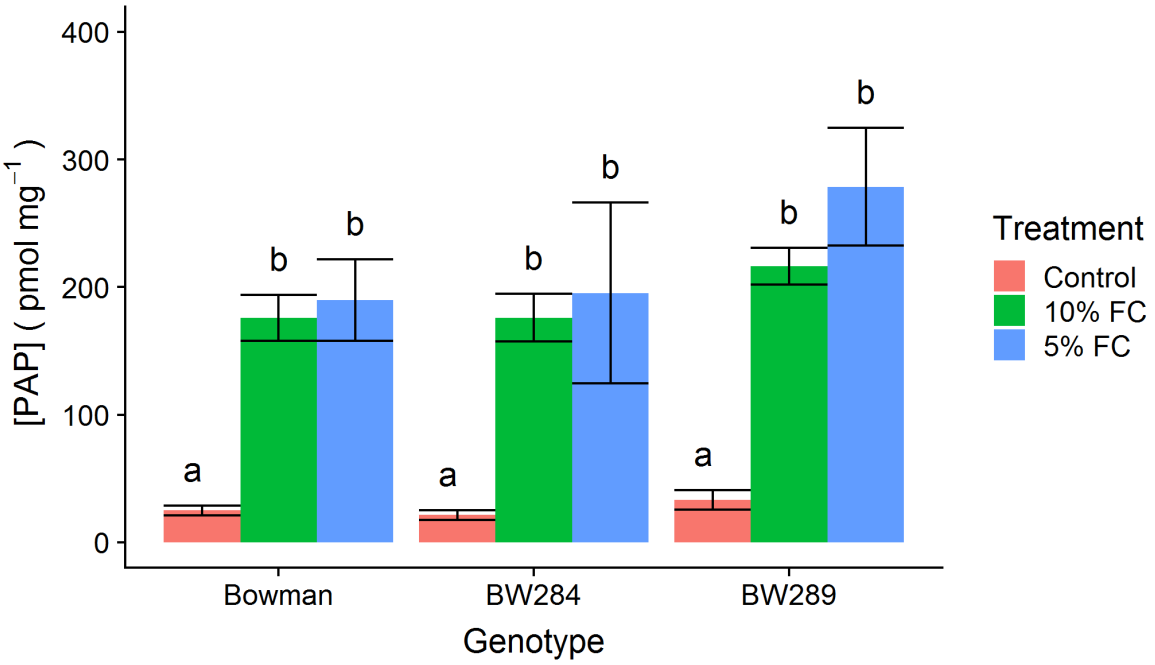

Fig 5.8. PAP concentration derived from HPLC measurement for the second and third studies in the series for Bowman and the clock mutants BW284 and BW289. Means shown +/- se, N=25-26. Identical letters indicate no significant differences between pairwise Tukey's test of model outputs at p=0.05.

The results of [PAP] testing were combined for the second and third studies (Fig. 5.8). PAP concentration was significantly higher for the severest drought (5% FC) condition (218 pmol mg^{-1}) compared to 10% FC (189 pmol mg^{-1}) and both were greater than control (26 pmol mg^{-1}, 2-way ANOVA, F(2,80), $p<2x10^{-16}$, Fig. 5.8). Although the results did not reach the threshold of significance, it was notable in Figure 5.8 that BW289 had the highest [PAP] at 5% drought (279 pmol mg^{-1}) and at 10% drought (216 pmol m^{-1}), while BW284 had the next highest [PAP] at 5% drought 195 pmol mg^{-1}), also higher than Bowman (190 pmol mg^{-1}).

5.3.3 Experiment 3: Physiological and anatomical consequences of moderate (10% FC) drought - additional results

With the results for 10% and 5% FC water stress conditions being relatively similar in the second study, the third study used only 10% FC as the drought stress condition. The stress was applied for 14 days with

daily transpiration limited to that of the slowest-drying pot.

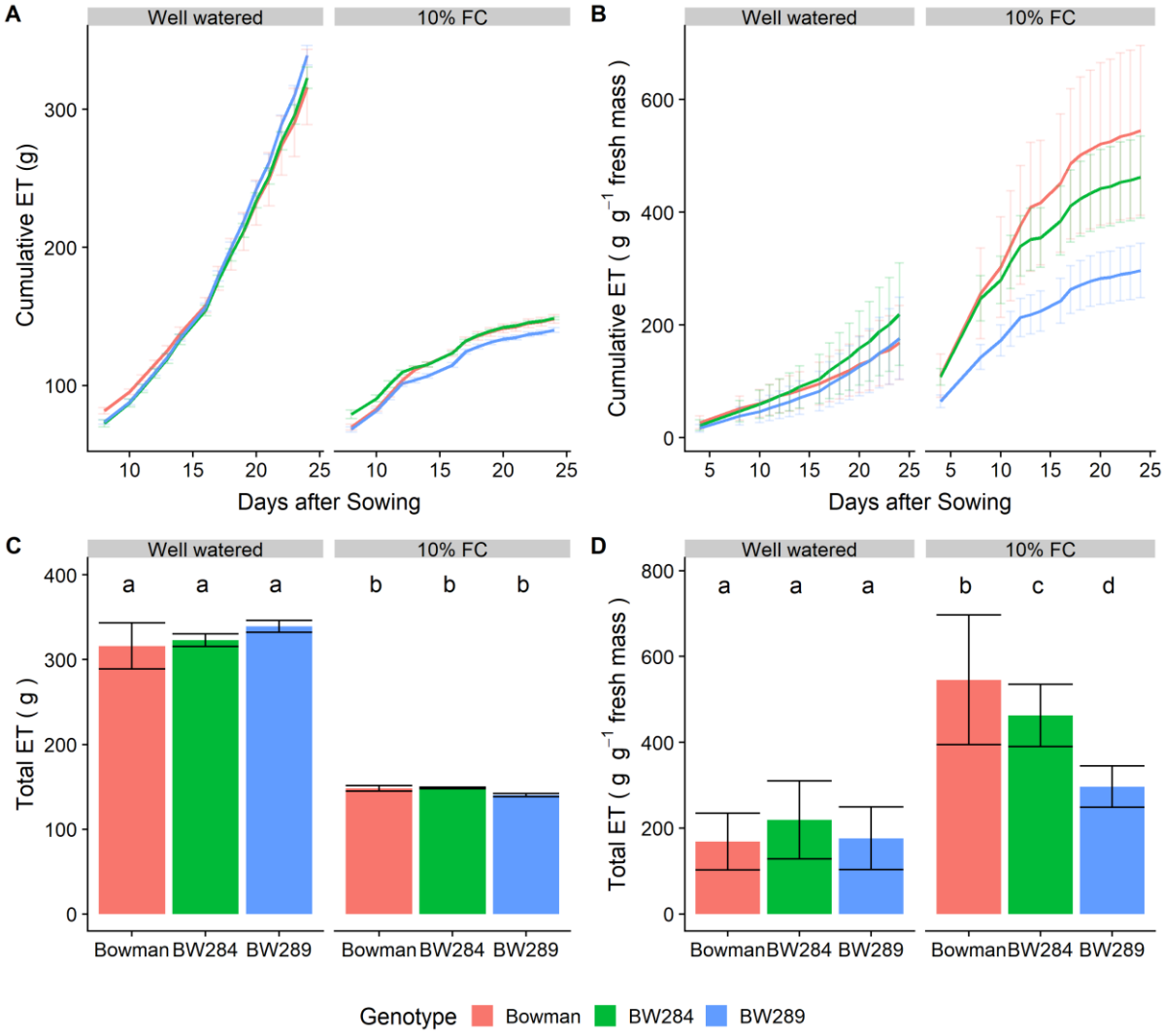

Figure 5.9. Transpiration for Bowman and 2 clock mutants under well-watered (control) and 10 % field capacity conditions. A) Cumulative transpiration over the period of progressive drought. B) Cumulative Transpiration over the period of progressive drought per gram fresh mass. C) Total transpiration at 24 DAS. D) Total transpiration per gram fresh mass at 24 DAS. Bars / points shown +/- se, N=7-8. Identical letters indicate no significant differences of pairwise Tukey's test of model outputs at p=0.05.

While unadjusted transpiration was similar between cultivars across treatments (Fig. 5.9A), the well-watered treatment had greater total transpiration (326g) than the drought condition (145g, Fig. 5.9C, Tukey's test, $p<0.05$). Yet when the fresh mass of the plant was taken into account (Fig. 5.9B), BW284 (462 g g^{-1}) and to an even greater extent BW289 (296 g g^{-1}) had consistently lower transpiration than Bowman (545 g g^{-1}) under drought conditions (Fig. 5.9D, Tukey's tests, $p<0.05$). The performance under drought confirms data reported earlier for the first experiment (see Fig. 5.1 above).

There was some evidence that there was an impact of drought on stomatal anatomy (Fig. 5.10), although where an association existed between drought and anatomy, there was no interaction between variety and treatment.

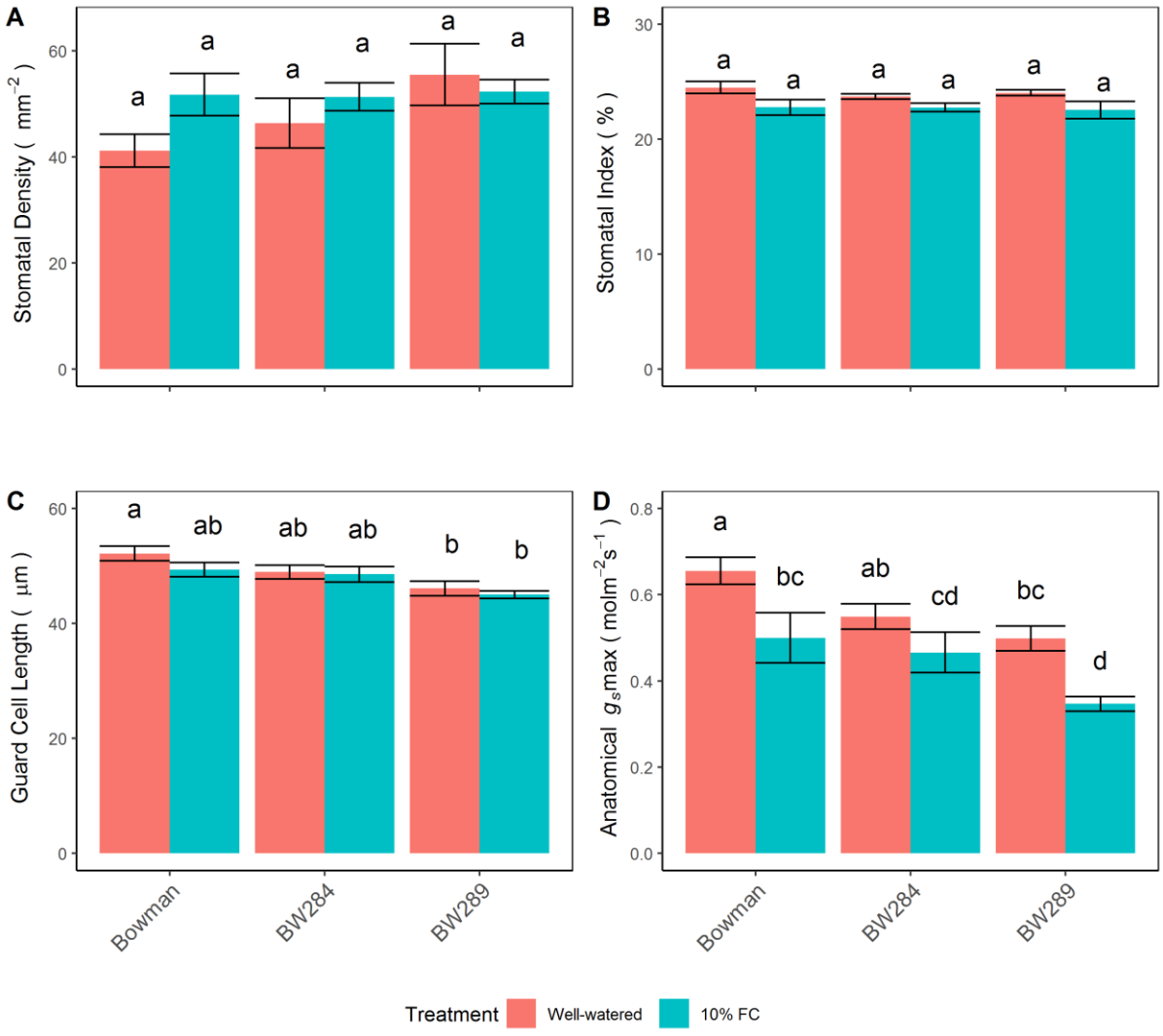

Figure 5.10. Actual and calculated stomatal anatomy for Bowman and clock mutants under drought (10% FC) and well-watered (Control) conditions. A) Stomata Density B) Guard cell length C) Pore length D) Calculated anatomical gs_{max}. Means shown +/- se, N=8. Different letters denote significant differences on Tukey's test at p_{crit}=0.05.

As was the case in the second study, there was no relationship between treatment or variety on stomatal density (Fig. 5.10A, 2-way ANOVA, $p>0.05$) or stomatal index (Fig. 5.10B, 2-way ANOVA, $p>0.05$). BW289 had significantly smaller guard cells (46.1µm) than Bowman (52.1µm, Fig. 5.10A, Tukey's test, $p<0.05$) under well-watered conditions. Droughted plants had significantly (24%) lower

anatomical gs_{max} than controls (Fig. 5.10D), while BW289 also had significantly lower anatomical gs_{max} (0.42 µmol m^{-2} s^{-1}) compared to Bowman (0.58 µmol m^{-2} s^{-1}) irrespective of treatment.

One way in which physiological responses to changing environmental conditions were understood was to measure assimilation responses of plants under conditions of changing internal [CO_2] (A-C_i responses, Fig. 5.11) and changing light intensity, known as A-Q responses (Fig. 5.12). The data from these dose-response curves were modelled and key parameters extracted which spoke to critical physiological processes. Furthermore, drought was expected to affect these responses, as was the circadian clock.

Assimilation rates were measured in response to changing C_i (Fig. 5.11A), where BW284 consistently had the lowest assimilation compared to Bowman and BW289 under control conditions, but not under drought conditions. Two principal parameterisations using the method developed by Sharkey et al. (2007) were considered (Figs. 5.11B-C), that is Vcmax, which relates to the rate at which Rubisco is activated, and *J*, which relates to RuBP regeneration, both critical parts of the Calvin Cycle. BW289 had the highest Vc_{max} under both control (72.1 µmol m^{-2} s^{-1}, Fig. 5.11B) and water-stress (62.6 µmol m^{-2} s^{-1}) treatments. Bowman had the lowest (36.5 µmol m^{-2} s^{-1}) under water stress. Meanwhile, BW289 had the highest value for J under well-watered conditions (Fig. 5.11C, 142 µmol m^{-2} s^{-1}) and BW284 under water-stress conditions (141 µmol m^{-2} s^{-1}). Once again, Bowman had the lowest value for J (81 µmol m^{-2} s^{-1}) . In general, the clock mutants managed to maintain output under drought better than Bowman for both Vc_{max} and *J*, echoing the findings from the porometry and thermal imaging.

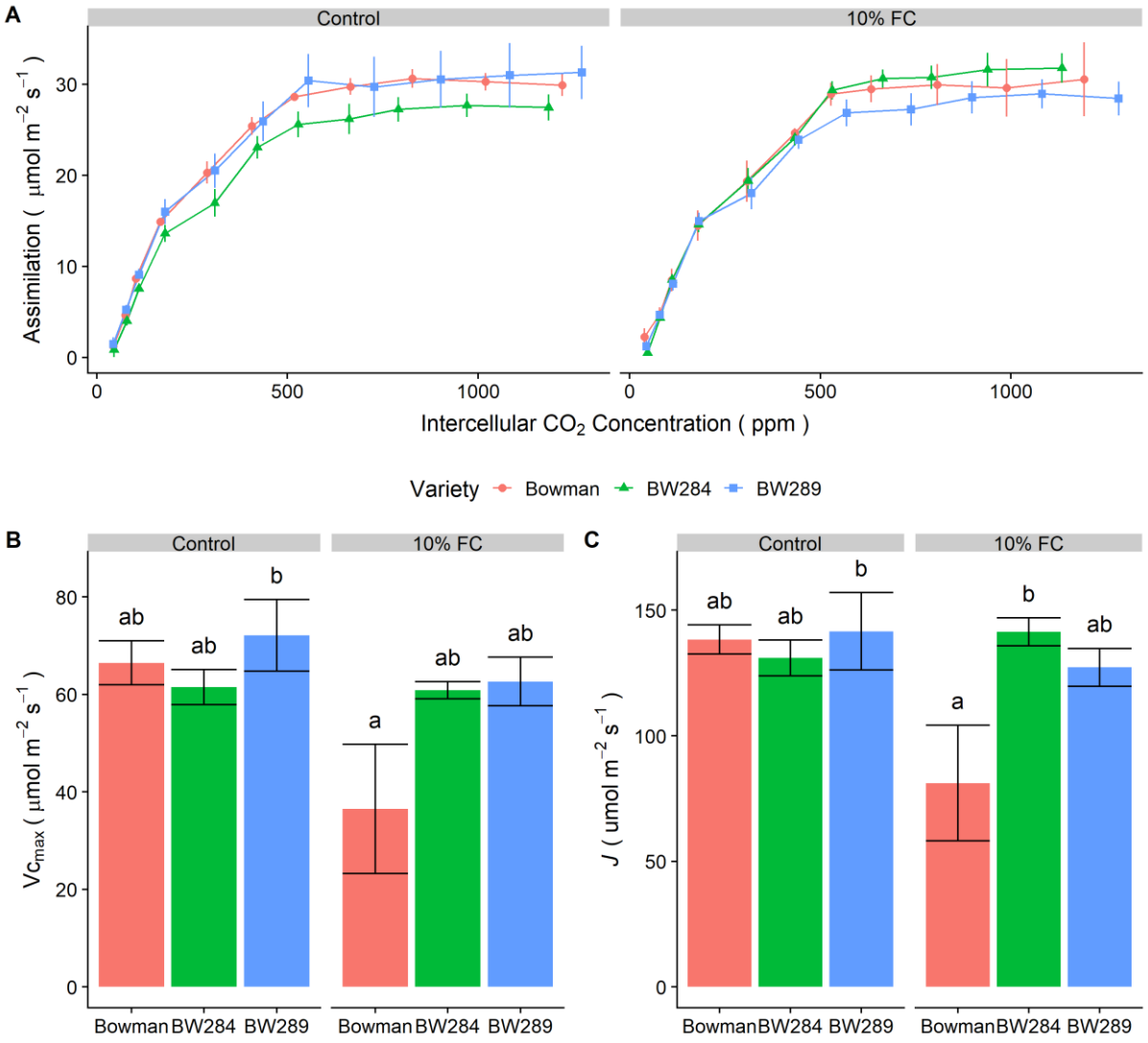

Figure 5.11. A) Response curve of assimilation to changing levels of intercellular [CO_2] (C_i) at saturating light for Bowman and clock mutants under well-watered (Control, lhs) and drought (10% FC, rhs) conditions. B) Vc_{max} from parameterisation of A-C_i response curve. C) J from parameterisation of A-Ci response curve. Means shown at each point +/- se. N=3-5.

In a similar vein, response curves of assimilation to changing light intensity were measured (Fig. 5.12A), modelled, and key parameters extracted (Figs. 5.12B & C). Under control conditions, the cultivars exhibited similar assimilation responses. Under water-stressed conditions, Bowman appeared to be more-affected than the clock mutants (Fig. 5.12A). The light response curves were modelled and parameters extracted using using Buckley and Diaz-Espejo's model (2015) and data are presented for the initial slope, Φ and the curvature of that slope, Θ. No statistical differences were found between varieties or treatments, but Bowman had the lowest values of Φ (0.29, Fig. 5.12B) and Θ (0.35, Fig. 5.12C) respectively under drought conditions.

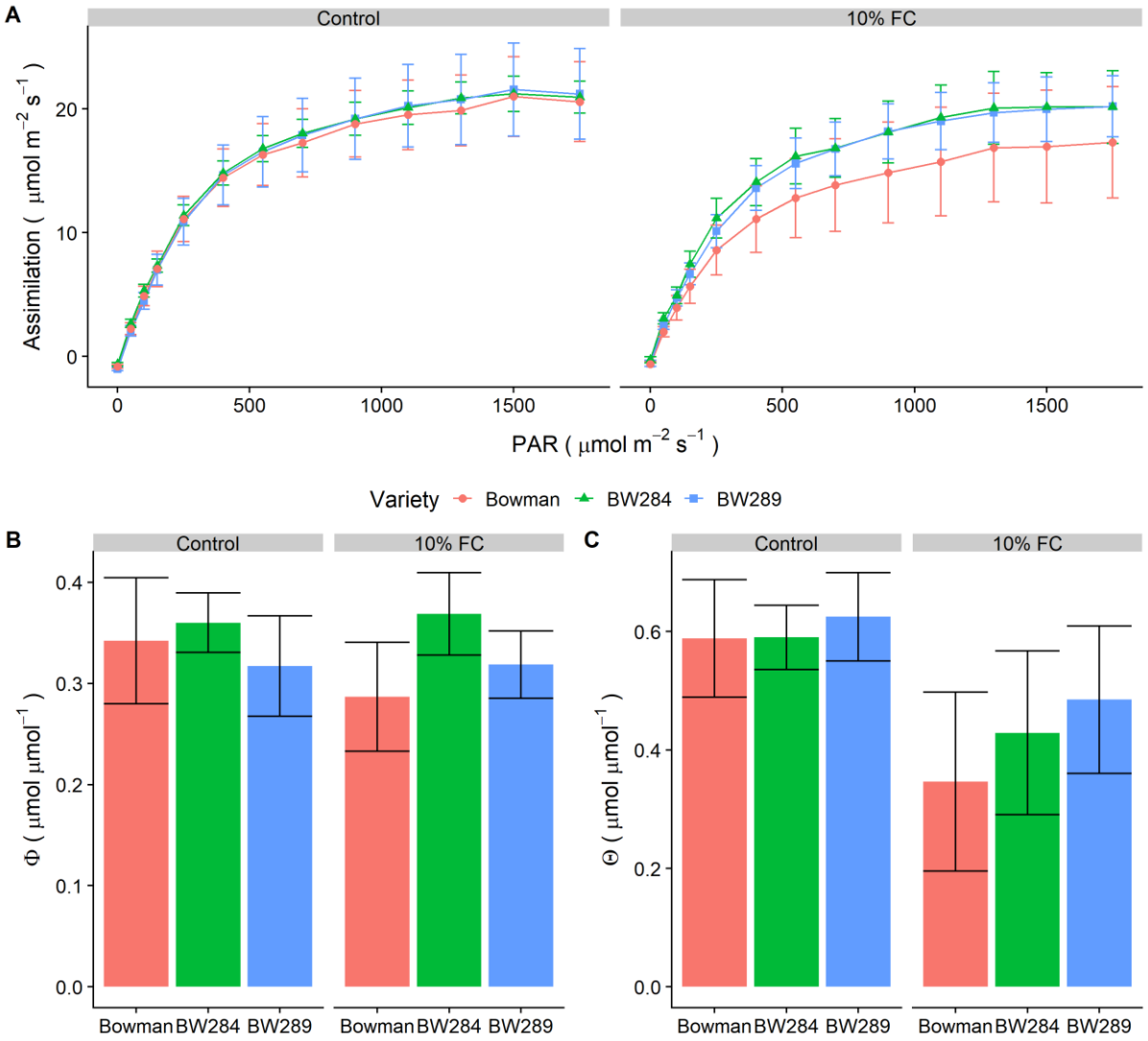

Fig 5.12. Assimilation responses of Bowman and 2 clock mutants to changing light intensity from 0 to 1750 µmol m^{-2} s^{-1} PAR at constant [CO_2] and temperature for Bowman and clock mutants under well-watered (Control, lhs) and drought (10% FC, rhs) conditions. Means shown at each point +/- se, N=4-5.

5.4. Discussion

This series of studies aimed to understand the physiological and anatomical impacts of circadian clock mutations on drought responses in barley. As the circadian clock has been linked to fitness in *Arabidopsis* (Dodd et al., 2005), clock mutants were expected to exhibit greater signs of stress, including impaired physiological performance under drought. Furthermore, although it is not clear whether the clock itself affects [PAP], the reverse is known to be true (Litthauer et al., 2018), and given the predicted lack of fitness in clock mutants, [PAP] was expected to be higher in those plants during drought stress.

5.4.1 Plant behaviour and physiology in response to drought

There was little difference in ET between plants, but when differences in size were accounted for (Lawlor, 2013), a much greater variability in water use emerged; BW289 could readily be distinguished from Bowman. Having already established that BW289 had the greater impairment to its clock (see Chapter 3) than BW284, it appears that the mutations result in a situation in which lower ET per unit biomass is consistently produced under drought and in which BW289 uses less water than BW284, which in turn uses less water than Bowman, and these results were confirmed in each study. Bowman itself was developed specifically for use in water-stressed regions of the US (Mahalingam and Bregitzer, 2019), so to discover that the clock mutants were even more parsimonious of water than Bowman was surprising. It appears that BW289 has a conservative phenotype under severe drought whereas BW284 and Bowman are non-conservative, while under moderate drought, both mutants appear to have a conservative phenotype when Bowman remains non-conservative. These results appear to confirm the notion that barley can change behaviour under increasing drought stress in a manner that is genetically determined, pleiotropic and closely linked to control of flowering time (Wiegmann et al., 2019, Munns et al., 2010). Bowman does not appear to be able change phenotype under drought, remaining non-conservative throughout. The *Hvelf3* clock mutant BW289 is known to exhibit an early maturing phenotype flowering up to nine days earlier than Bowman (Lundqvist, 2014), and here also showed a switch from non-conservative behaviour at 10% FC to conservative at 5% FC, suggesting that an escape or avoidance pathway had been invoked. Meanwhile, BW284 is expected to be less 'early' by 4-10 days (Campoli et al., 2013), and remained relatively non-conservative even under severe drought (5% FC).

Having low transpiration per gram of material could result in lower biomass overall for the mutants compared to Bowman. Here we show that BW289 in particular continued to show enhanced biomass production under moderate drought compared to Bowman, while BW284 sometimes did so. While it is clear that in the course of a growing season, Bowman is likely to produce the greatest biomass (see

Chapter 3 and Lundqvist (2014)) compared to the clock mutants; during vegetative growth under water-stressed conditions, these differences were reversed.

It is possible to explain the divergence in harvest outcomes in terms of stomatal conductance under well-watered conditions and low light regardless of time of day, as BW289 consistently produced higher g_s compared to BW284 or Bowman. Under high light and well-watered conditions, BW289 had higher g_s than either BW284 or Bowman only at the mid-day point. But this is regarded as a critical element in overall yield, as maintaining the mid-day canopy temperature depression through high transpiration can enhance yield (Fischer et al., 1998, Balota et al., 2008). Naturally, there are other developmental strategies associated with responses to drought, such as control of leaf size and thickness. Balota et al. (2008) also found that drought-resistant plants had smaller leaves, another characteristic previously associated with BW289 (see Chapter 3). Furthermore, other areas of water flow through the plant, such as leaf, stem or root hydraulics and root patterning may also prove to be relevant, although they were not part of this study.

These data were not entirely confirmed by thermal imaging, where under high light, BW284 appeared to be most successful and BW289 the least successful at leaf cooling under well watered conditions. There did appear to be a greater amount of temperature heterogeneity for BW289 under high light, well-watered conditions, suggesting the position on the leaf where gas exchange measurements were made could be important. Meanwhile, under drought conditions, the clock mutants did appear to deliver modestly more cooling than Bowman irrespective of light level.

5.4.2 Anatomical and physiological causes of enhanced performance under drought

The question then arises of whether there are anatomical differences between stomata that can account for this ability to produce greater cooling, particularly under drought on the part of the clock mutants, as higher stomatal density is typically associated with greater g_s (Franks and Beerling, 2009a, Lawson and Blatt, 2014). In the first and third studies described above, the link between stomatal

density and maximal g_s remained weak. In the third study, Bowman had the highest transpiration per gram biomass and BW289 the least under drought, suggesting BW289 had the highest efficiency of water use. Furthermore Bowman had the highest and BW289 the lowest anatomical gs_{max}, linked to BW289 having the highest SD and smallest guard cells (Franks and Farquhar, 2001). Taken together, the suggestion is that BW289 had a high potential stomatal conductance, and stomata that could move rapidly (Lawson and Blatt, 2014). Once again, other anatomical differences were modest, with no obvious trade-off between stomatal density and size despite evidence that such a negative correlation is widespread (Lawson and Blatt, 2014, Hetherington and Woodward, 2003, Franks and Beerling, 2009b). Predictions that plants with smaller stomata should be better able to tolerate drought (Doheny-Adams et al., 2012) and that conservative plants would be preferred in an environment of severe droughts (Bertolino et al., 2019) found some favour in these studies, with both BW284 and especially BW289 regularly producing as much if not more biomass than Bowman, a plant known to tolerate water stress well (Franckowiak et al., 1985).

Drought did not appear to have a profound impact on the cultivars' responses to light intensity, except perhaps in the convexity (i.e. $\delta A/\delta Q$), where drought appeared to reduce responsiveness as light intensity increased, particularly for Bowman (Buckley and Diaz-Espejo, 2015). Data for responses to changing C_i gave a clearer impression of impact on the physiological capabilities of these cultivars. Bowman under drought conditions appeared to exhibit lower values of Vc_{max} and J than under control conditions, and in particular than BW284 under drought. Vc_{max} values are indicative of the rate of Rubisco activation (Sharkey et al., 2007, Farquhar et al., 1980). *J* models the regeneration of RuBP at the end of the Calvin Cycle, and either Vc_{max} or *J* can be limiting (along with triose phosphate utilisation) (Sharkey et al., 2007). When either Vc_{max} or *J* were limiting, the clock mutants experienced less impact from drought on Calvin cycle activities, while in Bowman both Rubisco activation and RuBP regeneration appeared impaired (Farquhar et al., 1980). Overall, drought appeared to limit Bowman's physiological responsiveness more than the clock mutants', not only to changing light intensity in the first instance,

but also the rapidity with which Bowman can initiate and maintain the Calvin Cycle. Decreased assimilation is be expected under stress (Anjum et al., 2011), yet Bowman's water use efficiency appears to decline (higher ET relative to mutants) owing to its non-conservative phenotype,and possibly also relating to the extent of stress experienced as a result of that non-conservative behaviour (Lawlor and Cornic, 2002). On the other hand, the clock mutants appear to be desensitised to stress (Knight et al., 1998), perhaps owing to higher background levels of PAP expressed as a consequence of lack of fitness (Dodd et al., 2005). In addition, they appeared to be able to switch between conservative and non-conservative strategies, thus maintaining physiological responses. In particular, they appeared to avoid the worst declines in *A* associated with moderate drought (Lawlor and Cornic, 2002), and continued to transpire more efficiently, both relatively in terms of biomass and absolutely, giving greater water use efficiency as a result.

5.4.3 Mechanisms of drought responses

The concentration of phospho-adenosine phosphate is known to increase under drought or high light stress (Estavillo et al., 2011) and PAP can extend the period of the circadian clock (Litthauer et al., 2018), and therefore clock mutants, with their hypothesised reduced fitness (Dodd et al., 2005) were predicted to experience higher levels of stress in general and therefore expected to accumumlate greater [PAP] compared to a cultivar with a functioning clock such as Bowman under either well-watered or water-stressed treatments. In the absence of drought, evidence for constitutively higher [PAP] in the clock mutants was mixed, although it was a third higher in BW289 than Bowman. Yet as hypothesised, [PAP] rose in response drought across the board, and the more severe the drought, the greater the extent of that rise. Furthermore, the cultivar with the earliest maturing phenotype, BW289, also exhibited the greatest concentrations of PAP under moderate and severe drought. The other clock mutant, BW284, which exhibits less 'earliness', had [PAP] closer to that of Bowman, but concentrations were always the same or greater for the former compared to the latter. Overall, these results offer tentative evidence that the clock does affect [PAP] as well as the reverse being the case (Litthauer et al., 2018), and that in

cultivars with an impaired clock, higher [PAP] arise, probably as a result of impaired fitness. The same evidence also suggests that one element of the PAP-SAL1 pathway is conserved across a wide range of species, including, for the first time, barley (Chan et al., 2016). Downstream of PAP, BW289 was able to change its behaviour from non-conservative to conservative as the intensity of drought rose, and this phenotype ties in with the observed rise in [PAP] from moderate to severe drought. BW289 was able to moderate water use, becoming more efficient under drought compared to peers, and delivered equivalent or greater biomass at the vegetative stage. Meanwhile, BW284 remained relatively non-conservative irrespective of drought intensity, had water use much closer to that of Bowman, and generated no additional yield advantage.

Taken together, these results suggest that the clock mutants, BW284 and BW289 have phenotypes under drought that are informed by but not limited to earliness. The varieties are able to maintain high levels of responsiveness to light and Calvin Cycle activity under drought while using no more water in the case of BW284 and less in the case of BW289, particularly under severe drought. As a result, the clock mutants are more-efficient users of water, and manage to eke out biomass gains that are as good as if not better than Bowman, a variety specifically intended for water-stressed environments. A useful extension of this work would be to test the sensitivity of circadian rhythms to drought to see if the mutants retained their impaired cycles or were totally arrhythmic, and to test the extent of earliness relative to Bowman given that stress tends to delay heading in early flowering lines although stress timing appears important (Ogrodowicz et al., 2017). Mechanistically, PAP concentrations appear to underline the mutant's performance under drought, particularly the ability to switch from non-conservative to conservative behaviour by BW289. These characteristics should be of great interest to breeders looking for novel germplasm to breed for improved drought responses, particularly as the material already exists, with known genetic underpinnings in the guise of earliness in NILs of the elite cultivar, Bowman. Improved yield under drought is a critical outcome for breeders to achieve in the

context of climate change, even in the UK as well as more widely, and cultivars such as these give rise to opportunities to manage even under very severe droughts indeed.

5.5. Conclusions

- Clock mutants transpire more-efficiently under drought per unit biomass, and one of them could even switch from non-conservative to conservative behaviour under severe drought.
- The mutants used water more-efficiently, maintaining light responsiveness and Calvin Cycle activity better than Bowman under drought. As a result, they are able to maintain or even improve yields compared to Bowman when water-stressed.
- PAP concentrations are higher in BW289, and to a lesser extent BW284, under drought compared to controls, suggesting that the clock has an impact on [PAP], and potentially extending the range of species in which the PAP-SAL1 pathway exists to barley.
- These data offer a route for breeders to select for characteristics which may be advantageous under future climate scenarios.

Chapter 6: Wild barley shows a range of anatomical and physiological responses to drought that have consequences for yield

6.1. Introduction

6.1.1 Wild barley relatives are potential sources of novel phenotypes

In the context of maintaining or improving yield under an increasingly variable climate, it is possible, even likely, that wild crop relatives possess phenotypic diversity which might be exploited by breeders (Brozynska et al., 2016). Barley is a major crop of arid regions (Dawson et al., 2015), having been domesticated in the Middle East near the Fertile Crescent around 8-10 thousand years ago (kya), with a later, second area of domestication believed to have occurred in Central Asia (probably around modern day Turkmenistan, Kazakhstan, Tajikistan or Uzbekistan) (Badr et al., 2000, Morrell and Clegg, 2007), a region of cold, arid steppe (Rubel and Kottek, 2010). Research on wild barley genotypes and phenotypes is generally in its infancy (Brozynska et al., 2016). From the perspective of European, Near Eastern and African crop breeding, Central Asian wild barleys have received surprisingly little attention in terms of genetic resources and physiological characteristics, despite offering haplotypes otherwise under-represented in regional elite barley germplasm (Morrell and Clegg, 2007, Dawson et al., 2015).

6.1.2 Drought elicits a range of physiological responses dependent on phenotype

Moderate drought will be defined here as the reduction in soil water content to a level from which full recovery of plant function remains possible, which is in line with the definition used in Harb et al (2010), and was empirically determined to be elicited by a soil moisture content of 10% of field capacity (FC) in barley. Plants under water stress exhibit two contrasting behaviours associated with particular phenotypes. First, conservative plants respond to drought by either escaping stress altogether (i.e. flowering early) or by avoiding it through maintenance of high cell turgor pressure and low tissue water potential, primarily and initially through the medium of stomatal closure when drought is detected at the roots (Valladares et al., 2000). The cost of this behaviour is lower yield, although the chance of

survival is enhanced (Valladares et al., 2000). The non-conservative phenotype, on the other hand, is apt to maintain wider stomatal apertures, but also detoxify ROS, and produce LEA proteins and osmolytes / osmoprotectants such as proline (Harb et al., 2010, Claeys and Inze, 2013). In essence, non-conservative plants attempt to outlive the episode of drought, and focus on reproduction over survival. This is the phenotype that has been bred for historically, as under most circumstances it generates a superior yield performance relative to the conservative phenotype (Araus et al., 2002), although in general non-conservative phenotypes are a species-wide response in many cereals (Munns et al., 2010). However, as the water availability consequences of climate change worsen, and dryland productivity decreases in many regions (IPCC, 2019), it may be that human adaptation includes a preference for lower yields with low variability (favouring a conservative phenotype) over high yield with high variability (Araus et al., 2002, Bertolino et al., 2019).

There is a genetic bottleneck in many agriculturally important species, resulting from the repeated crossing of closely-related individuals targeting a small subset of possible phenotypes (Brozynska et al., 2016, Lopes et al., 2015), although a meta-analysis of global diversity in a range of crops has challenged this assertion, with diversity falling in the 1960s, but rising into the 1990s as crop wild relatives and synthetic varieties began to be explored (van de Wouw et al., 2010). In the context of drought, searching in existing-but-ignored germplasm would appear to offer many benefits – it is no slower to get traditionally-bred varieties to market than it is for GM ones, it has the benefit of uncontroversial public acceptance and the DNA will already be optimised for the species (Qu et al., 2016, Wang et al., 2017, Lopes et al., 2015). In any event, it is clear that wild barleys (*H. vulgare* spp. *spontaneum*) offer potential genetic resources which are largely untapped (Brozynska et al., 2016) and in some cases, ecotypes have already been identified that represent opportunities for breeders to improve drought responsiveness of elite barley (Bedada et al., 2014).

6.1.3 Stomatal responses to drought

Stomatal responses to drought have been demonstrated to be a crucial element in overall plant behaviour under water stress (Valladares et al., 2000, Harb et al., 2010), with increasing intensity of drought linked to smaller apertures (Farquhar and Sharkey, 1982). There is a tension between assimilation of carbon dioxide through photosynthesis and the exit of water from the plant as transpiration, of which stomatal conductance is a key component (Farquhar and Sharkey, 1982). There is a degree of variability in stomatal morphology between and within species (Willmer and Fricker, 1996), with the combination of pore aperture, stomatal size and density jointly determining g_s (Lawson and Blatt, 2014). Meanwhile, stomatal conductance has been directly correlated to assimilation and, by extension, yield (Fischer et al., 1998, Wong et al., 1979), with a trade-off existing between stomatal size and density, where lower densities drove greater dry weight in *Arabidopsis* (Doheny-Adams et al., 2012) or led to drought tolerance (with no yield cost) in barley (Hughes et al., 2017). Therefore, stomatal conductance is known to be closely linked to yield, can be delivered through a wide range of combinations of aperture, size and density, while lower density appears to be a desirable trait in the context of water stress.

One way in which stomatal response to water stress could link to stomatal size might be in the rate of response to changing environmental conditions. That is, the rapidity with which stomata can open and close (Lawson et al., 2010), since stomatal conductance response may be up to an order of magnitude slower than the response of assimilation (Lawson and Blatt, 2014).

When an environmental variable (typically light) is modulated during experimentation, the rate of response of stomatal conductance can be determined and can be readily modelled (McAusland et al., 2016, Gerardin et al., 2018, Vialet-Chabrand et al., 2017b). The key parameters relate to the time constant of the exponential expression, and the maximum slope which relates that time constant to the start and end points of a step change in light intensity. Furthermore, it is possible to estimate both the

steady-state start and end points of such a step change, and to supply initial values into an iterative function by Bayesian methods (Vialet-Chabrand et al., 2016).

This study aimed to understand the effect of water stress on the physiology and anatomy of two wild barleys acquired from the John Innes Centre Germplasm Resources Unit (GRU, 2019). These accessions (B3733 and B3745) were originally collected in Turkmenistan and represent some of the genotypic diversity of Central Asian barley germplasm (GRU, 2019, Morrell and Clegg, 2007). This study aimed to link the anatomical and physiological features of barley to the transpirational water loss required to achieve harvest outcomes. Earlier work had identified the wild barley lines B3733 and B3745 as having very different kinetic responses of A and g_s to changes in light along with a divergence in stomatal anatomy (see Chapter 4). The objectives were to characterise the drought responses of B3733 and B3745 in comparison to Bowman, a formerly-popular elite variety introduced in the 1980s, which is known to have desirable yield characteristics under drought (Franckowiak et al., 1985). Further objectives were to understand the impact on leaf physiology, stomatal anatomy and harvest of the impact of transient moderate drought imposed from tillering (Growth Stage 2, GS2), and the impact of moderate drought from germination (GS1) onwards.

6.2. Methods

The study comprised two separate drought experiments conducted in the growth chamber at the University of Essex, Wivenhoe Park, UK between 17/1/2018 and 24/9/2018. In each case, the wild barleys B3733and B3745 were grown alongside the elite variety Bowman, with the intention of comparing the impact of transient, moderate drought on plants in the tillering (ie vegetative stage) of development (GS 2) with the impact of moderate drought on plants that had been subjected to water stress *ab initio* (from Growth Stage 0).

6.2.1 Experiment 1: The physiology & anatomy of barley under transient moderate drought

Sixty plants in two equal-sized batches of Bowman, B3733 and B3745 were stratified at 4°C for days before being placed in the phenotyping platform at the University of Essex under the usual conditions as described in Chapter 2. Drought was applied from 19 DAS until the end of the experiment at 47 DAS, with a target of 10% of field capacity in the drought condition and 'Well-watered' being 50% of field capacity. Field capacity had previously been determined by saturating 6 pots containing modular compost in water and allowing them to drain under gravity overnight to determine the maximum field capacity and then oven drying to determine the dry mass of compost at 0% moisture content. Capacity targets were then determined as:

$$Target\ Mass = Dry\ Mass + \%\ Target \cdot (Field\ Capacity - Dry\ Mass)$$

6.2.1.1 Additional methods used in the first experiment

To gain a better understanding of both steady-state and kinetic responses of g_s and *A* to changing light levels, diurnal light response curves were measured using a LiCOR 6400 IRGA as described in section 2.3 above. In addition, stomatal impressions were taken using the method of Weyers and Johansen (1985) as described in section 2.5 above from the site where gas exchange measurements were taken, with stomatal index calculated as SI = Stomatal density / (Stomatal density + Epithelial density). Finally, harvest data were recorded directly at the end of the drought period at 47 DAS, including above-ground fresh biomass, total leaf area, tiller number and growth stage on Zadok's scale.

6.2.2 Experiment 2: The physiology and anatomy of barley under developmental drought

Thirty seeds of Bowman, B3733, B3736 and B3745 were planted into damp Levington F2+S modular compost at approximately 70% FC, placed into the phenotyping platform at the University of Essex under conditions as described in Chapter 2, and were allowed to dry to toward target immediately. Plants in the well-watered condition were maintained at 50% of field capacity, and under drought, at 10% of field capacity, that capacity having been determined in the first study above.

Assimilation responses to changing light intensity and internal [CO_2] were measured using the method described in section 2.4.1. Stomatal impressions were taken using the method described by Weyers and Johansen (1985) at the end of the drought period using the same leaves that had been measured in the assimilation response curves, as described in section 2.5. Harvest measurements were made directly following the end of the drought period (38 DAS), with above-ground fresh and dry biomass (after drying at 80^{o}C for 24 hours), water content ((Fresh mass-Dry Mass)/Fresh Mass), number of tillers (excluding the main stem), growth stage on Zadok's scale and total leaf area per plant as measured on a LiCOR LI3100C (LiCOR Inc., Lincoln Nebraska, USA) leaf area meter.

6.3. Results

The results of two experiments are summarised below. In the first, a period of moderate drought was initiated during tillering and applied transiently to mimic early to mid-season drought in the field. In the second experiment, the same target level of drought was applied from sowing, so water stress was present from germination and increased thereafter for the duration of the study.

6.3.1 Experiment 1: Transient moderate drought affects barley physiology & anatomy

To examine the effect of soil drying, the cumulative mass of water lost through evapotranspiration was increased progressively at the rate of the slowest-drying pot, with all plants consequently reaching the target level of drought simultaneously.

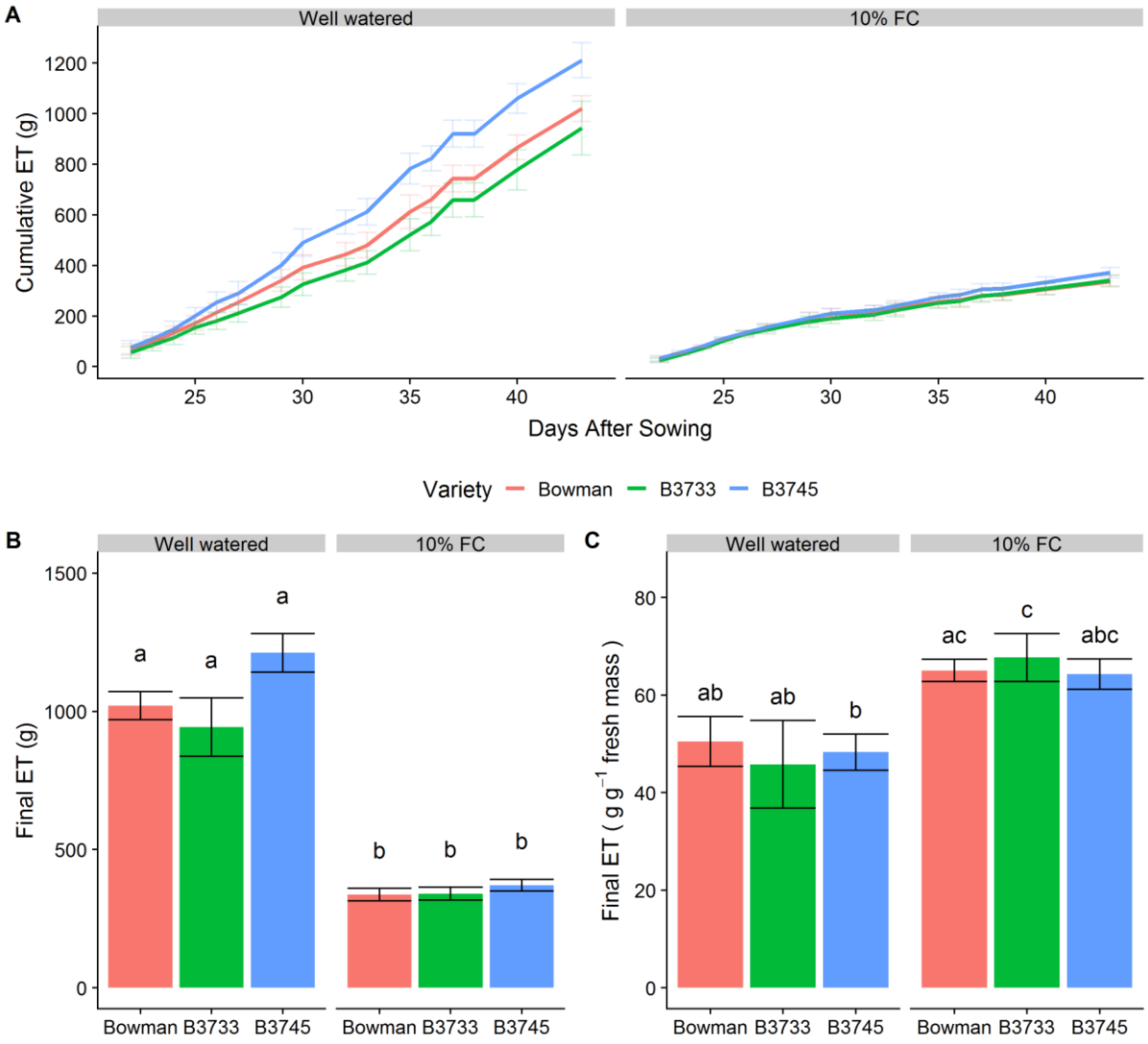

Figure 6.1. Progress of drought of Bowman and two wild barleys, B3733 and B3745. A) Time series of cumulative evapotranspiration during period of water stress. B) Cumulative evapotranspiration at the final measurement point at 43 DAS. C) Cumulative evapotranspiration at 43 DAS per gram fresh mass at that point. Means shown +/- SE. N=9. No significant difference exists between pairs of identical letters on Tukey's test at p_{crit}=0.5.

There was a clear effect of treatment on the progress of ET, with a total of 1050 g of water used under well-watered conditions, while just 350g was used under drought conditions (Figs. 6.1A & B). The wild barley B3745 had the highest ET under both well-watered (1212 g) and water-stressed (371 g) conditions (Fig. 6.1B). When the fresh mass of the plant was taken into account (Fig. 6.1C), B3733 had the lowest ET per gram fresh mass under control conditions (46 g g^{-1}) and the highest under drought conditions (68 g g^{-1}), a substantial switch in behaviour. Bowman had the highest ET per gram fresh mass under control conditions (51 g g^{-1}) but not under drought (65 g g^{-1}).

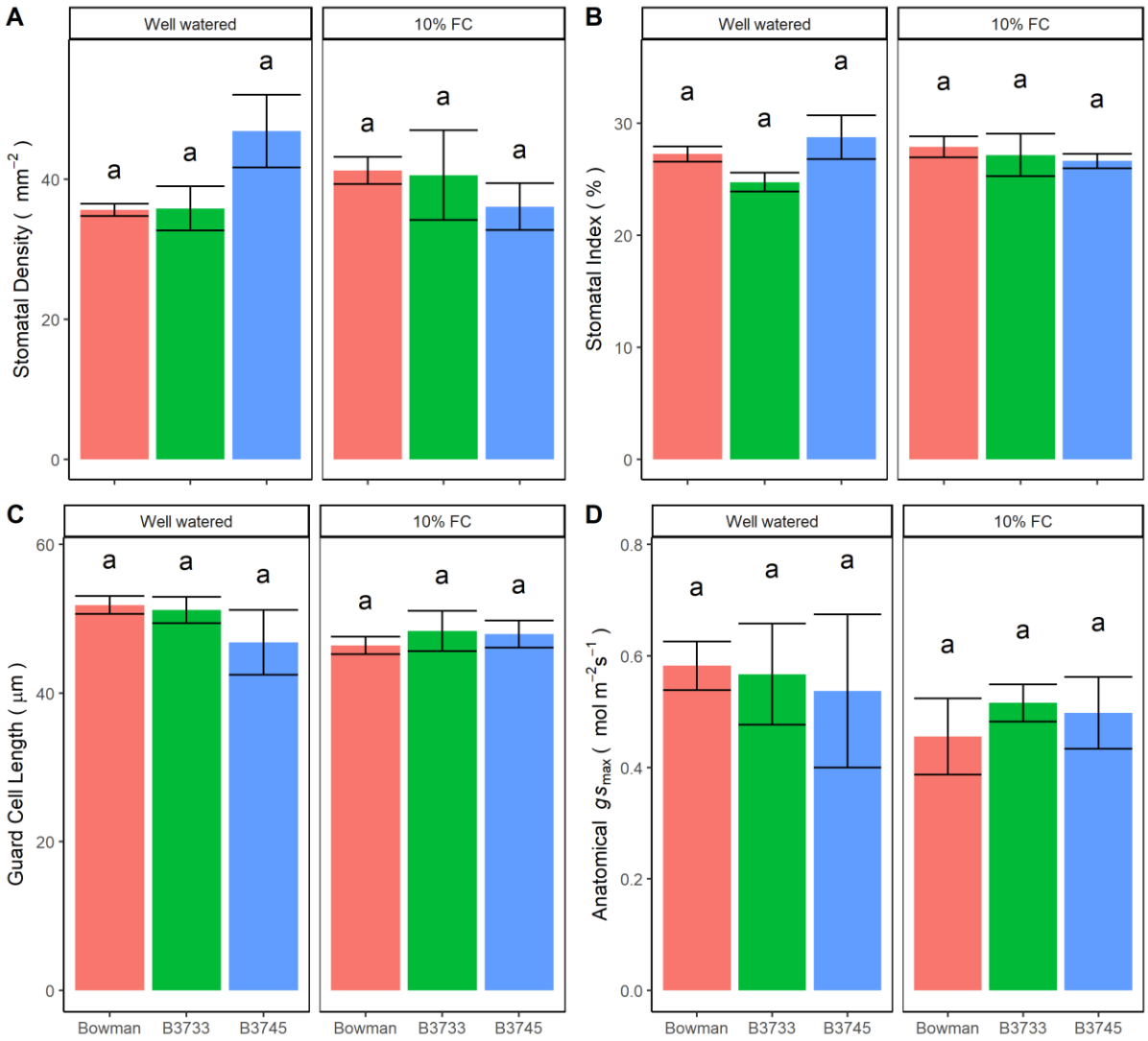

Figure 6.2. Stomatal anatomical features of Bowman and two wild barleys, B3733 and B3745. A) Stomatal density. B) Stomatal index (=Stomatal density / (stomatal density + epithelial density). C) Guard cell length. D) Anatomical gs_{max}. Means shown +/- SE. N=4-5. Significant differences between pairs of means on Tukey's test at p_{crit}=0.05 denoted by different letters.

Stomatal anatomy and density are largely developmentally determined, and no significant differences were found in stomatal density (Fig. 6.2A) and guard cell length (Fig. 6.2C) across treatments. Furthermore, there were no differences in stomatal index (Fig. 6.2B) or anatomical gs_{max} (Fig. 6.2D). However, B3745 had the highest stomatal density (Fig. 6.2A) under control conditions (46.9 mm^{-2}), and the lowest under drought (36.1 mm^{-2}). Overall, differences in stomatal anatomy were modest, although B3745, with the highest total ET under well-watered conditions also had the highest SD.

Stomatal anatomy (Fig. 6.2) could have gone some way to explaining the ET data (Fig. 6.1). An alternative explanation was that physiological activity rather than anatomy underlined differences in ET. To that end, varieties were subjected to periods of fluctuating and steady state light over the course of a day (Fig. 6.3) to gain a better understanding of plant responses.

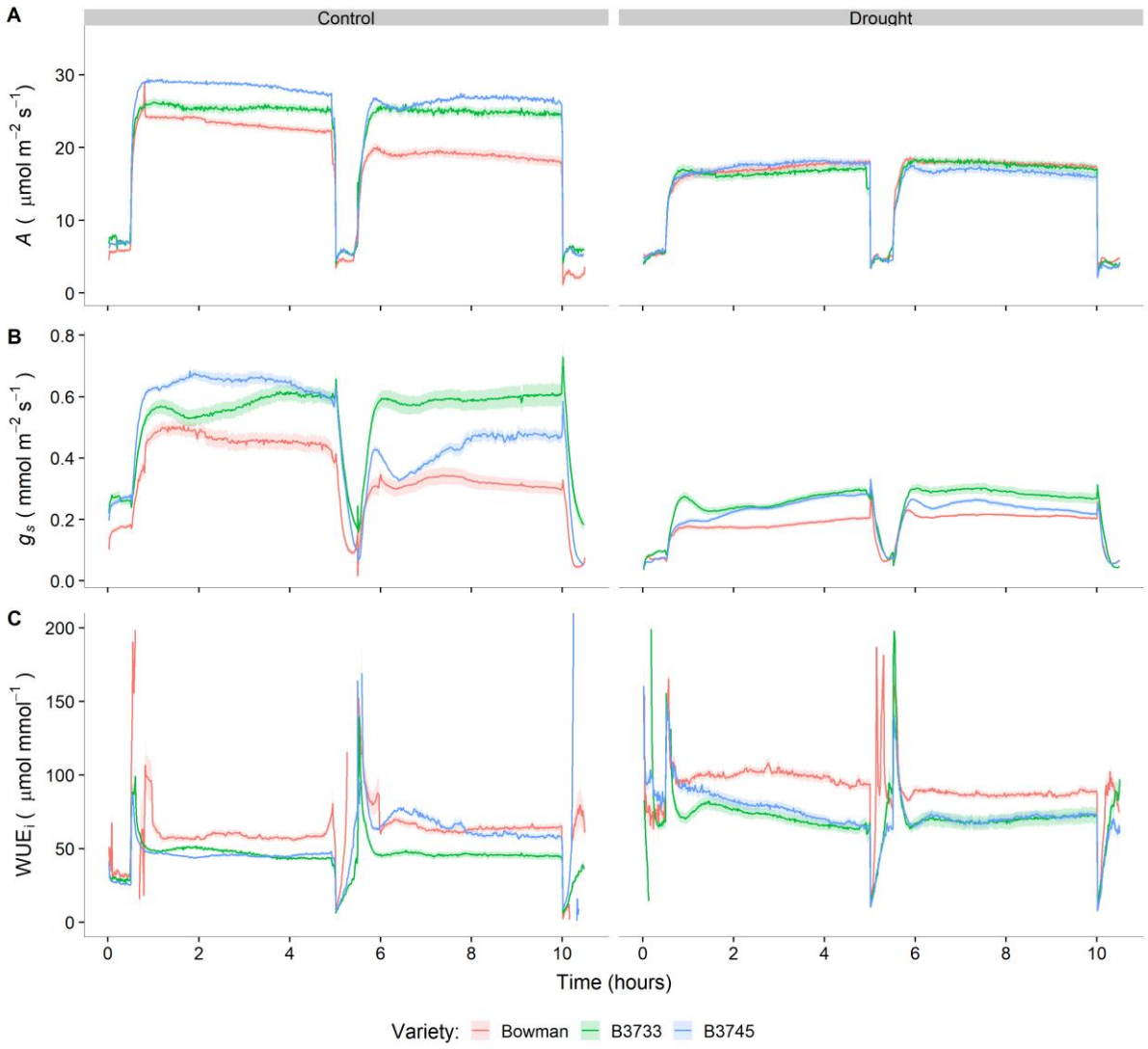

Figure 6.3. Diurnal physiological responses of Bowman and two wild barleys (B3733 ad B3745) to drought for 10.5 hours from dawn. The plants were first settled for 0.5 hr at 100 µmol m^{-2} s^{-1} PAR before light intensity was increased to 1000 µmol m^{-2} s^{-1} for 4.5 hours. The light intensity was then decreased to 100 µmol m^{-2} s^{-1} before the cycle started again. A) Assimilation (*A*). B) Stomatal conductance (g_s). C) Intrinsic Water Use Efficiency (WUE_i). Means shown +/- SE. N=8-10.

Under well-watered conditions, B3745 had consistently the highest assimilation rates throughout the measurement period, while that for B3733 was generally somewhat lower during the first period up to 5 hours after dawn, and Bowman the lowest of all (Fig. 6.3A). All varieties had very similar responses over time under drought (Fig. 6.3A). Stomatal conductance followed a similar pattern under well-watered conditions (Fig. 6.3B). Under water-stressed conditions, Bowman consistently had the lowest g_s compared to the wild varieties and B3733 the highest (Fig. 6.3B). Bowman had the highest WUE_i under well-watered conditions, in the first period, but not consistently so after 5.5 hours had passed (Fig. 6.3C). Under water-stressed conditions, Bowman had generally the highest WUE_i while that for the wild varieties was similar across most of the day (Fig. 6.3C). The wild varieties matched high *A* with high g_s under control conditions, but Bowman exhibited the higher WUE_i. Under drought, differences in *A* were constrained, but Bowman maintained lower g_s, giving the latter better WUE_i overall.

Kinetic responses were modelled from the first 30 minutes after the change in light intensity reported in Fig. 6.3, and reflect the time taken to respond to those changes in light intensity, the associated rate of change of responses, and the maximal and minimal values reached at the start and finish.

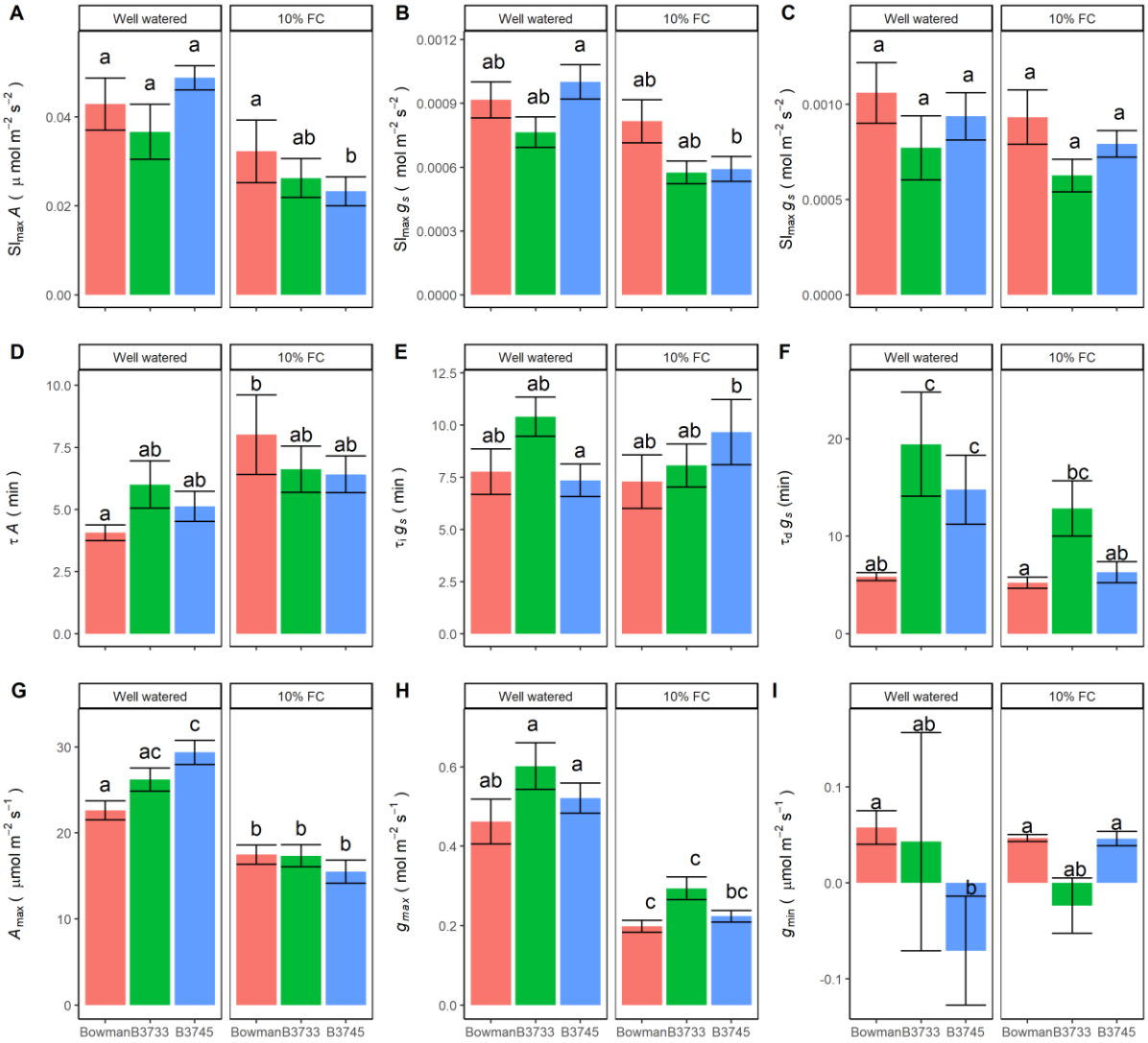

Figure 6.4. The time series of *A*, g_s and WUE_i under fluctuating light conditions (Fig. 6.3) were parameterised (into kinetic components) following a change in light intensity after 0.5 hours, 5 hours, 5.5 hours and 10 hours for Bowman and the wild barleys B3733 and B3745. A) Slope of increasing *A*. B) Slope of increasing g_s. C) Slope of decreasing g_s. D) Time constant for increasing *A*. E) Time constant for increasing g_s. F) Time constant of decreasing g_s. G) Maximum predicted *A* at 1000 µmol m^{-2} s^{-1}. H) Maximum predicted g_s at 1000 µmol m^{-2} s^{-1}. I) Minimum predicted g_s at 100 µmol m^{-2} s^{-1}. Means shown +/- SE. N=8-10. No significant differences on Tukey's test at p_{crit}=0.05 were observed between identical letters.

Compared to water-stressed plants, well-watered ones had significantly more-rapid responses of *A* and g_s to increasing light intensity but not of g_s under decreasing light intensity (Fig. 6.4A-C). B3745 had a significantly higher slope of *A* in response to increasing light (Fig. 6.4A) when well-watered compared to watered-stressed conditions (0.049 µmol m^{-2} s^{-2} vs 0.023 µmol m^{-2} s^{-2}, Tukey's test, $p<0.05$), with a concomitant decline in the rate of response of g_s by 69% from 0.001 mol m^{-2} s^{-2} (Tukey's test, $p<0.05$). In general, there was little difference in the rate of response to changing light for Bowman or B3733 under

either well-watered or drought conditions, while for B3745 showed much greater sensitivity to water stress.

The time constant, τ_A, for Bowman was significantly shorter under well-watered conditions than under drought (4.1 min vs 7.5 min, Tukey's test, $p<0.05$, Fig. 6.4D). T_{gs} for the response to increasing light (Fig. 6.4E) was 31% higher for B3745 (Tukey's test, $p<0.05$) and 30% lower for B3733, although τ_{gs} did not decline significantly in the latter case. There was greater heterogeneity in g_s responses to decreasing light intensity (Fig. 6.4F). The response for Bowman was virtually unchanged, while that for B3745 declined by 54% (Tukey's test, $p<0.05$, Fig. 6.4F). Time constants appeared to increase modestly under drought for assimilation when light increased, were on average unchanged for g_s when light intensity increased and saw a decrease in g_s when light intensity decreased.

Overall, there was a significant decline in A_{max} under drought (Tukey's test, $p<0.05$, Fig. 6.4G). The highest predicted maximal value of A was for B3745 under control conditions (27.4 µmol m^{-2} s^{-1}) which was significantly higher than for Bowman (22.6 µmol m^{-2} s^{-1}, Tukey's test, Fig. 6.4G). There were no differences between varieties under water-stressed conditions. Once again, the g_{max} responses (Fig. 6.4H) followed a similar pattern to that seen for A_{max}, with significant declines under well-watered conditions. For minimum g_s under declining light intensity, there was considerable heterogeneity, and large estimation errors (Fig. 6.4I). A consistent fall in maximal rates for both g_s and A under drought occurred under increasing light, with the greatest impact on the assimilation rates of wild varieties.

Data from the first 30 minutes following a change in light intensity were discarded and data for the remaining 4 hours until the next change in light level were used to estimate steady-state responses (Fig. 6.5), and separated into mean, phase and amplitude parameters.

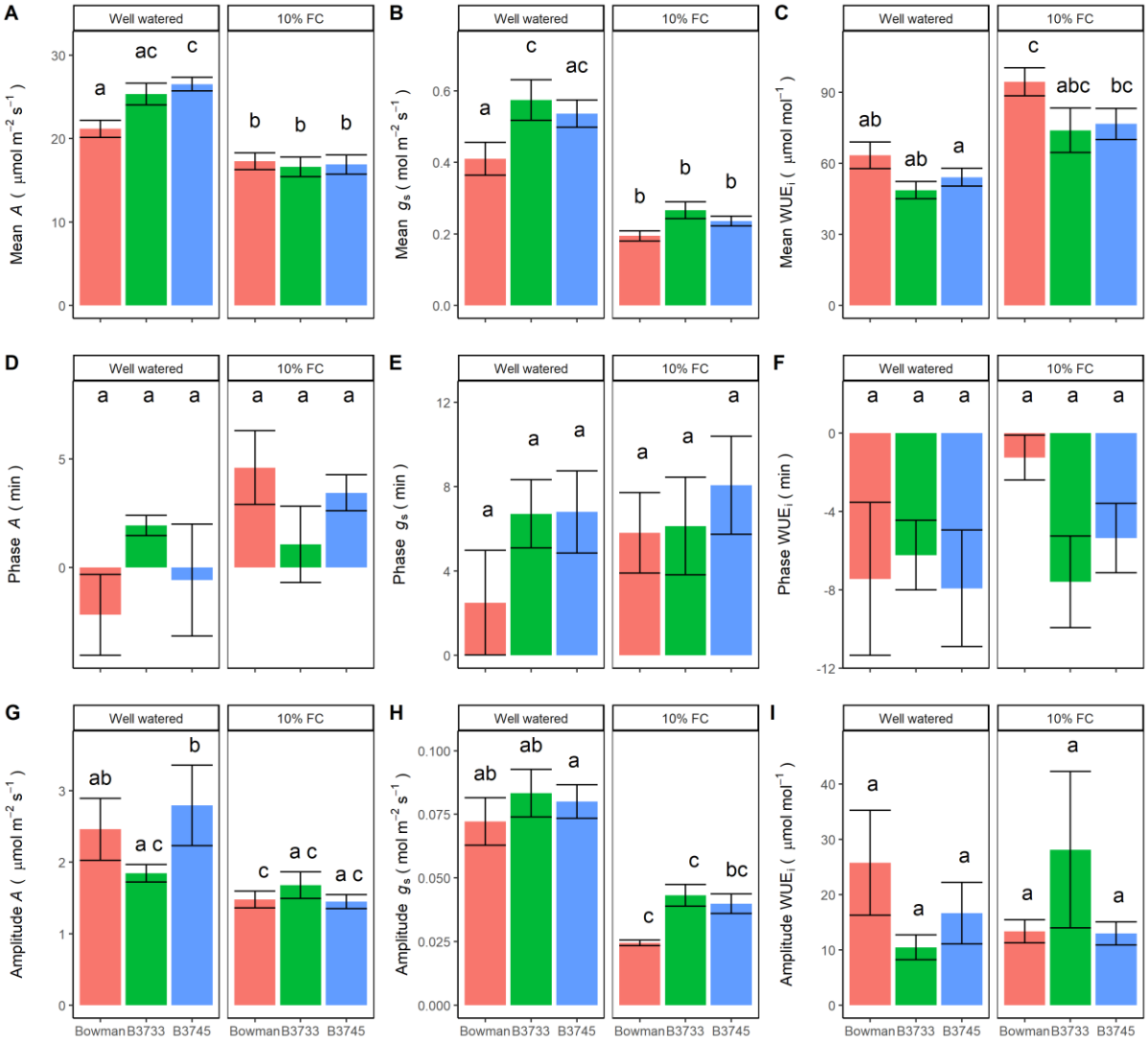

Figure 6.5. Parameterisation of steady-state responses to constant light intensity for Bowman, and the wild barleys B3733 and B3745. Three parameters were extracted for each of *A*, g_s and WUE_i. The mean was a simple average over the period from 0.5 hrs to 4.5 hours after a change in light level. The phase was the centroid of the data over the same time period less 2.5 hours (the midpoint of each steady-state period). The amplitude was given by the standard deviation over the whole time period for each replicate. A) Mean *A*. B) Mean g_s. C) Mean WUE_i. D) Phase of *A*. E) Phase of g_s. F) Phase of WUE_i. G) Amplitude of *A*. H) Amplitude of g_s. I) Amplitude of WUE_i. Means shown +/- SE. N=6-9. No significant differences were detected by Tukey's test at $p_{crit} = 0.05$ where letters are identical.

Significantly lower mean *A* (Fig. 6.5A) and g_s (Fig. 6.5B) occurred under drought, but the decline in g_s was greater than that for *A*, leading to a significant increase in WUE_i (2-way ANOVAs, $F_{(2,16)}$, $p<0.05$,Fig. 6.5C). B3745 had a significantly higher mean *A* (27.6 µmol m^{-2} s^{-1}) than Bowman (21.2 µmol m^{-2} s^{-1}, Fig. 6.5A, Tukey's test, $p<0.05$) under well-watered conditions, and compared to B3745 under drought (15.7 µmol m^{-2} s^{-1}, Tukey's test, $p<0.05$). The pattern was broadly repeated for mean g_s. B3733 had significantly

higher g_s under well-watered conditions (0.57 g_s mol m^{-2} s^{-1}, Fig. 6.5B), than Bowman did under control conditions (0.41 mol m^{-2} s^{-1}) and compared to B3733 under drought (0.27 mol m^{-2} s^{-1}).

The phase of the steady-state time series was used to estimate how the data were distributed about the mid-point of each steady-state period. No significant differences were observed between treatments.

Higher amplitude (i.e. standard deviation) was noted for assimilation responses (Fig. 6.5G) under well-watered compared to water-stressed conditions, with B3745 falling furthest (from 2.8 to 1.4 µmol m^{-2} s^{-1}, Tukey's test, $p<0.05$), while the difference for Bowman under drought vs. control was a 40% decline in amplitude (Tukey's test, $p<0.05$). There was a significant decline in the amplitude of g_s under drought as well (2-way ANOVA, $F_{(2,16)}$, $p<0.05$), with Bowman having the lowest of g_s all (0.024 mol m^{-2} s^{-1}, Tukey's tests, $p<0.05$, Fig. 6.5H). There was a high degree of variability in WUE_i responses (Fig. 6.5I), but no significant differences between treatments. The effect of drought on amplitude of steady state responses was broadly as expected, with declining A and g_s, and a variable impact on WUE_i.

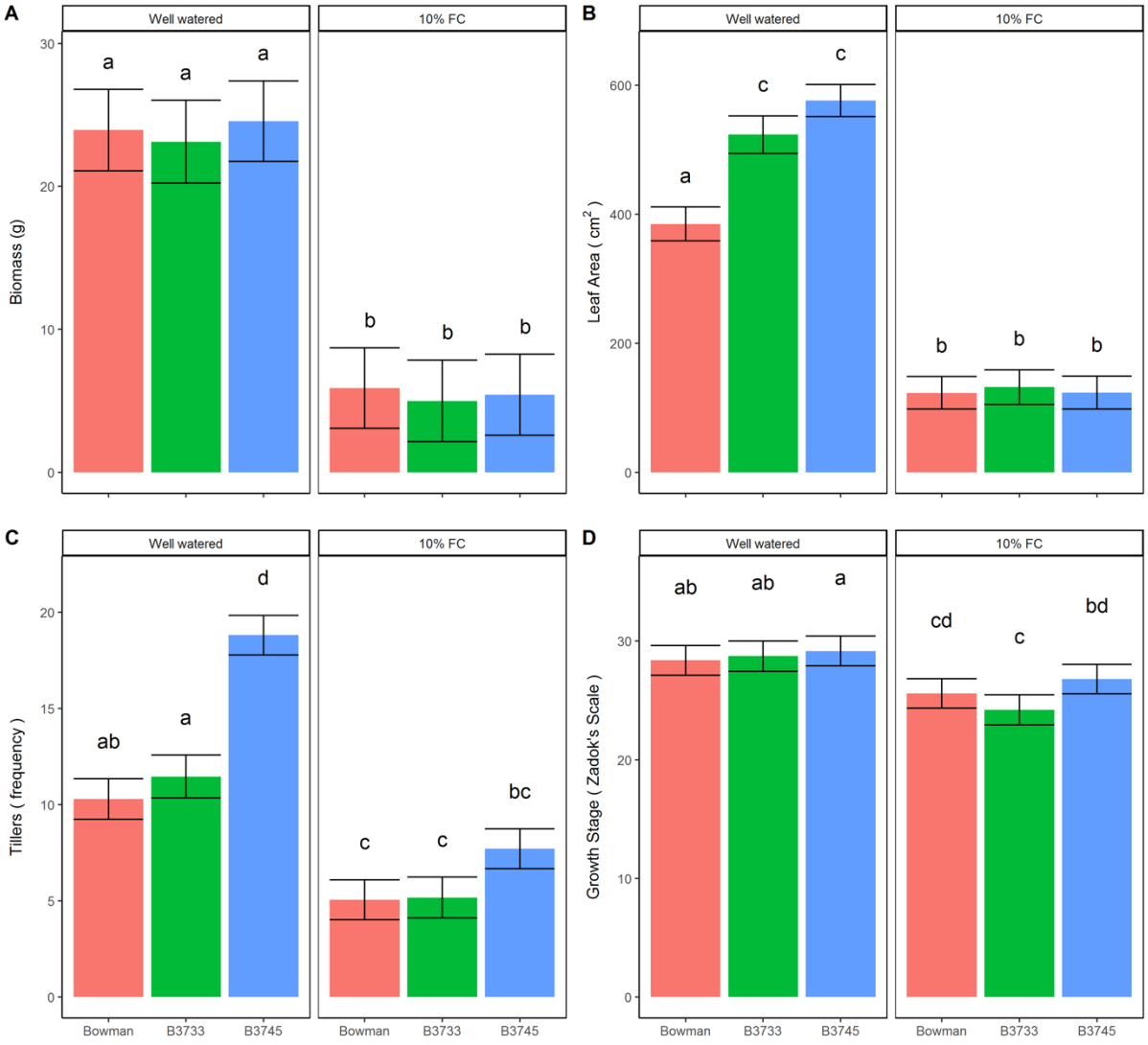

Figure 6.6. Harvest of Bowman and two wild barleys, B3733 and B3745 at the end of the water-stress period at 38 DAS. A) Above-ground biomass. B) Total leaf area. C) Number of tillers. D) Growth stage (Zadok's scale). Means shown +/- SE, N=9-10. Bars with identical letters showed no significant differences in pairwise testing on Tukey's test at p_{crit}=0.05.

Drought has multiple potential impacts on harvest outcomes (Fig. 6.6). As expected, drought led to significant declines across all measures of yield (2-way ANOVAs, $F_{(2,16)}$, $p<0.05$, Fig. 6.6A-D), including above-ground biomass (Fig. 6.6A). B3745 had the greatest leaf area (Fig. 6.11B) under well-watered conditions (576 cm^2), and it was significantly greater than Bowman's (385 cm^2), as was B3733's (505 cm^2, Fig. 6.6B). There were no significant differences in leaf area between cultivars under drought, with B3733 (132 cm^2) only 7% larger than Bowman (123 cm^2). Tillers (Fig. 6.6C) are a known determinant of final yield in barley, and there was a clear interaction between varieties and treatments.B3745 had the greatest number of tillers, this time under both control (18.6 tillers) and drought (7.7 tillers) conditions

(Fig. 6.6C, Tukey's test, $p<0.05$). B3745 under well-watered conditions also had significantly more tillers than either B3733 (11.6 tillers) or Bowman (10.3 tillers, Fig. 6.6C, Tukey's test, $p<0.05$). Differences in growth stage did emerge even in the short period during which drought was applied (Fig. 6.6D). B3745 had the most advanced growth stage by the point of harvest under both well-watered (GS = 29.2) and water-stressed (GS = 26.8) conditions, and significantly more than B3733 under drought conditions (GS = 24.2, Fig. 6.6D, Tukey's test, $p<0.05$). With the exception of above-ground biomass, harvest data show the effect of GxE interactions in tillers, leaf area and growth stage.

6.3.2 Experiment 2: Developmental drought affects the physiology and anatomy of barley

It is known that the impact of drought can vary depending on the point at which it is applied, so rather than introduce drought after plants were established, here stress was introduced *ab initio* to determine the effect on physiology, stomatal development and harvest.

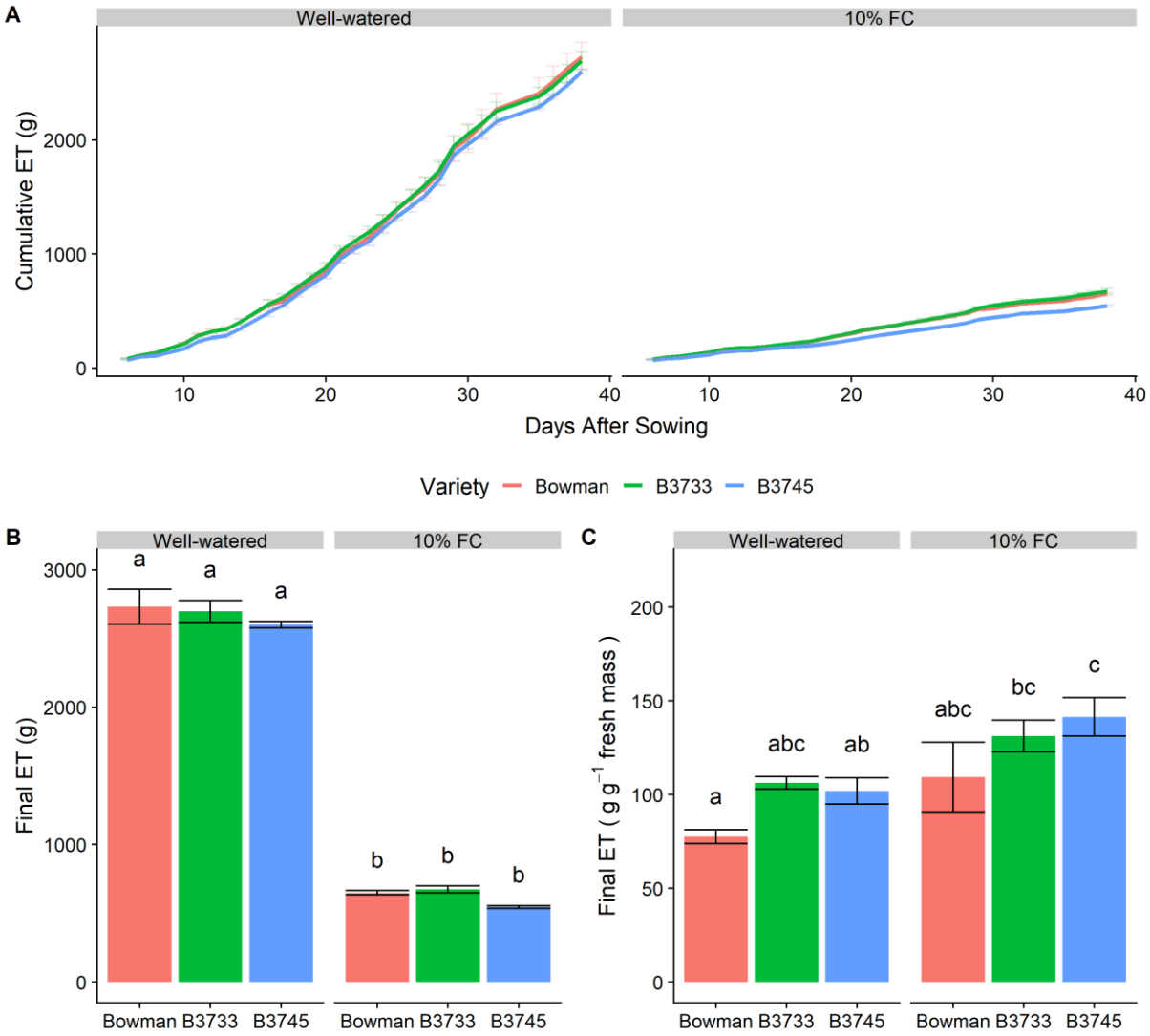

Figure 6.7. Progress of drought through time for Bowman and the wild barleys B3733, B3736 and B3745. A) Cumulative evapotranspiration (ET) from initiation of drought. B) Cumulative ET on the final day of the drought. C) Cumulative ET on the final day of the drought relative to plant fresh mass at that point. Means shown +/- SE. No significant differences on Tukey's test of pairwise comparisons were found between bars with identical letters at p_{crit}=0.05.

As in the previous experiment, water loss was replaced daily back to the volume lost by the slowest drying plant. The cumulative mass of water used in ET (Fig. 6.7B) was significantly higher for the well-watered compared to water-stressed conditions (2-way ANOVA, $F_{(1,30)}$, $p=2.0x10^{-16}$). When accumulated above-ground biomass at harvest was taken into account, B3745 now had the highest not lowest ET under drought (Fig. 6.7C).

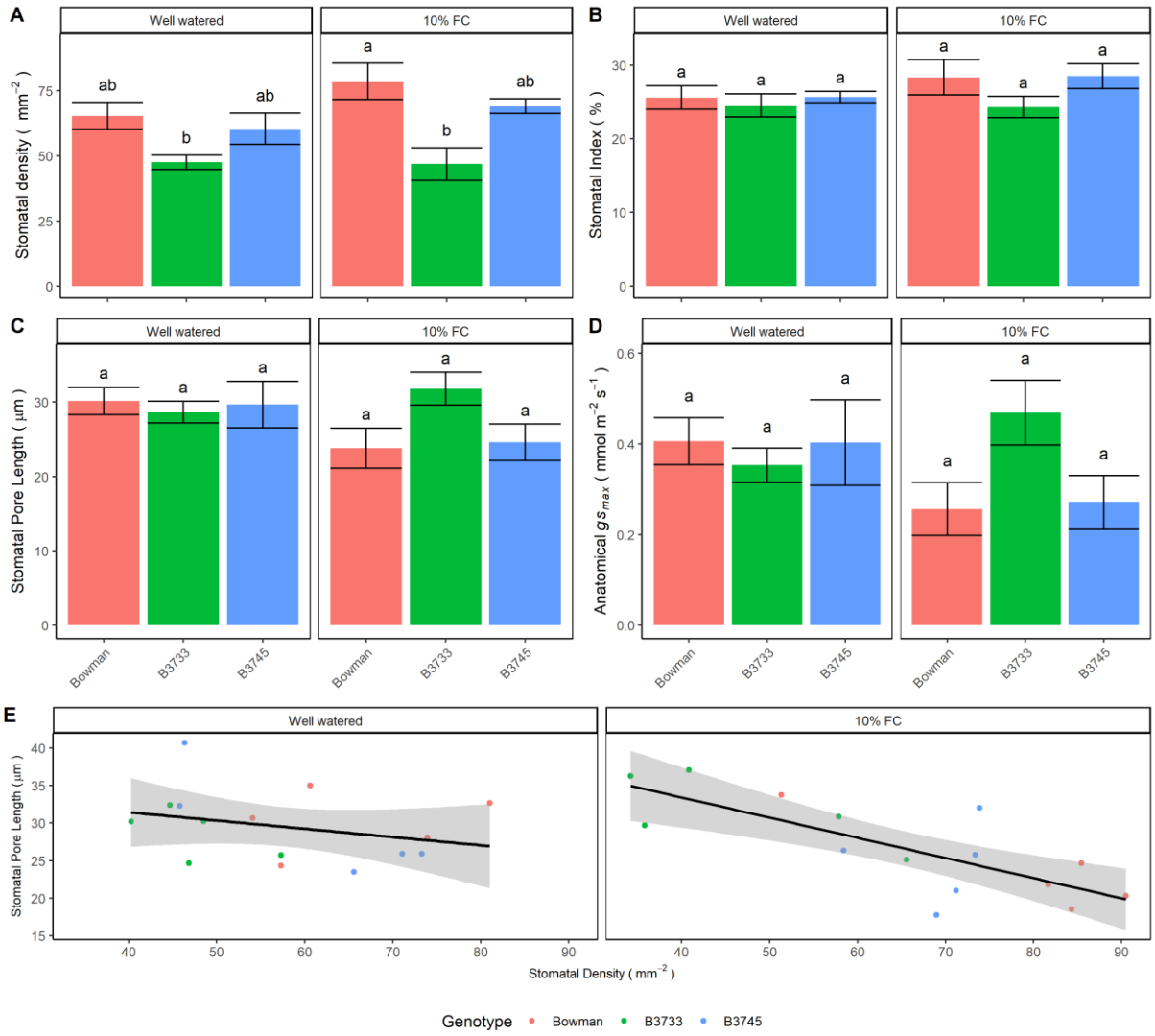

Figure 6.8. Exploration of the stomatal anatomy of Bowman and the wild barleys B3733 and B3745. A) Stomatal density. B) Stomatal Index (= 100 x SD / (SD+ED)). C) Stomatal pore length. D) Anatomical gs_{max}. E) Scatterplot of Stomatal pore length on stomatal density. Means shown +/- SE. N=5. Identical letters indicate no significant pairwise differences between means on Tukey's test at p_{crit} = 0.05.

Stomata are major determinants of transpiration rates, and while there were no significant differences between treatment, Bowman had the highest stomatal density (SD) under well-watered conditions (65.4 mm^{-2}, while it was significantly higher under drought for Bowman (78.6 mm^{-2}) than for B3733 (46.8 mm^{-2}) (Tukey's test, $p<0.05$). There were no significant differences between treatments for stomatal index (Fig. 6.8B). There were also no significant differences in stomatal pore length between treatments (Fig. 6.8C), nor in anatomical gs_{max} (Fig. 6.8D). Stomatal pore length tended to increase in B3733 when plants were water-stressed, but decreased in Bowman and B3745. There was a close association between stomatal pore length and density, with a steeper slope observed under drought conditions than under

well-watered ones (2-way ANOVA, $F_{(2,16)}$, $p<0.05$). Compared to the first experiment described earlier, there was a greater interaction between varieties and drought in stomatal development.

Drought is known to affect the extent and rate of stomatal opening, thus to gain a better understanding of the physiological impact of the drought process the assimilation responses of the different barleys to changing light (A/Q) and internal [CO_2] (C_i) (A/C_i) were measured (Fig. 6.9) to gain an understanding of the balance of Calvin Cycle activities (Rubisco activation vs. RuBP regeneration) as well as the rate at which *A* increased with increasing light intensity, and the curvature of that light response curve.

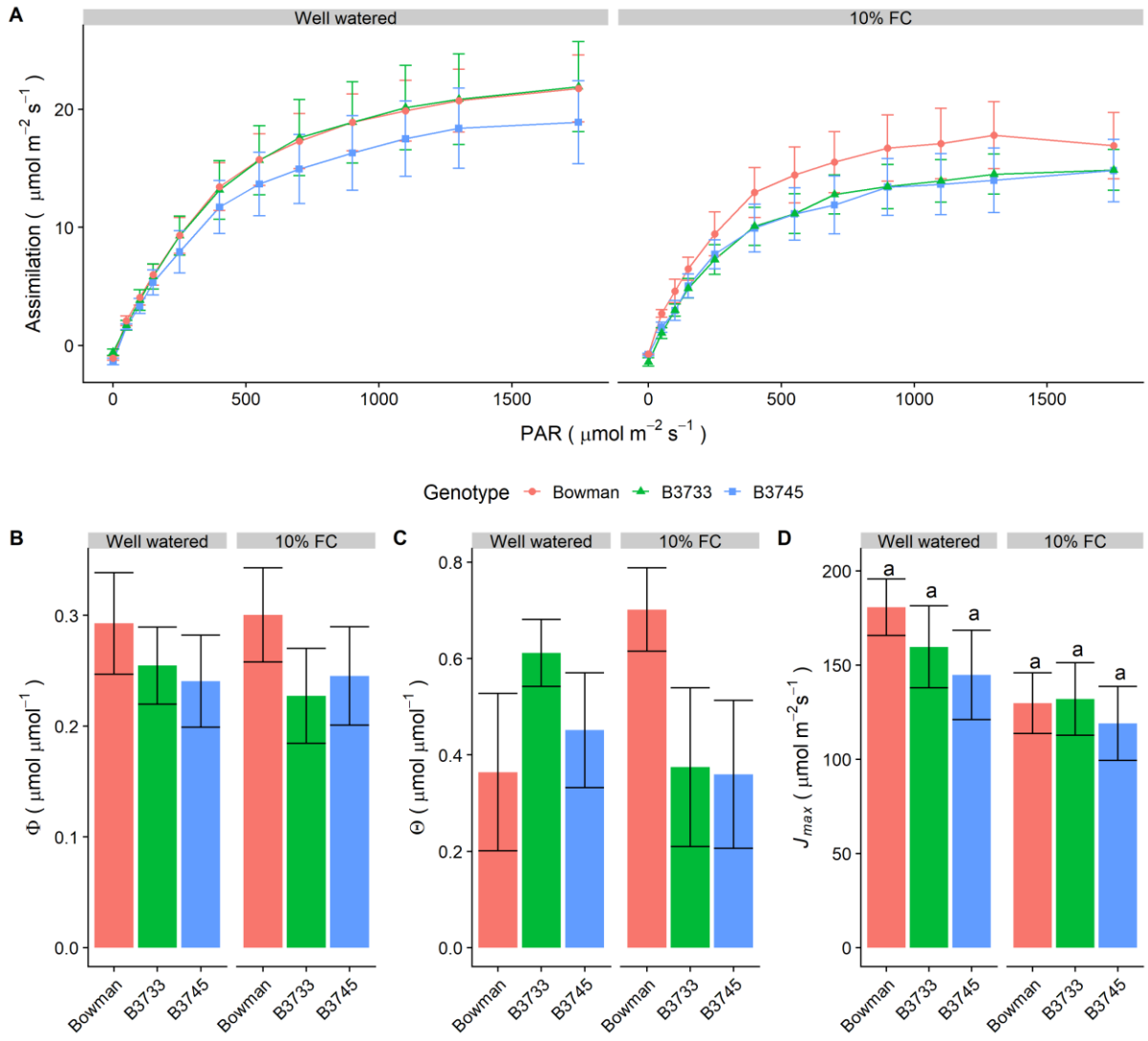

Figure 6.9. Assimilation response of 2 wild barleys and 1 elite barley (Bowman) to changes in light intensity (Q, PAR) at constant [CO_2], VPD and temperature for well-watered and water-stressed conditions. A) A/Q response curves. B) Φ, the initial slope of the electron transport rate, *J*, under changing light intensity. C) Θ, the curvature of the response curve of *J* to changing light intensity. D) J_{max}, the maximum predicted rate of electron transport. Each point represents the mean observation at that point +/- se. N=5. Identical letters indicate no significant difference in pairwise comparisons of means on Tukey's test at p_{crit}=0.05.

Drought was expected to affect the initial slope of the electron transport rate as light intensity increased, but no significant differences were observed (Fig. 6.9B). The same was true for the convexity of the response curve (Fig. 6.9C). There was a significant effect of water stress on the maximal rate of electron transport, J_{max} although there was no interaction with variety (2-way ANOVA, $F_{(5,22)}$, $p<0.05$, Fig. 6.9D).

Assimilation responses to C_i were recorded at constant saturating light as previously established (Fig. 6.13), while [CO_2] was varied (Fig. 6.9).

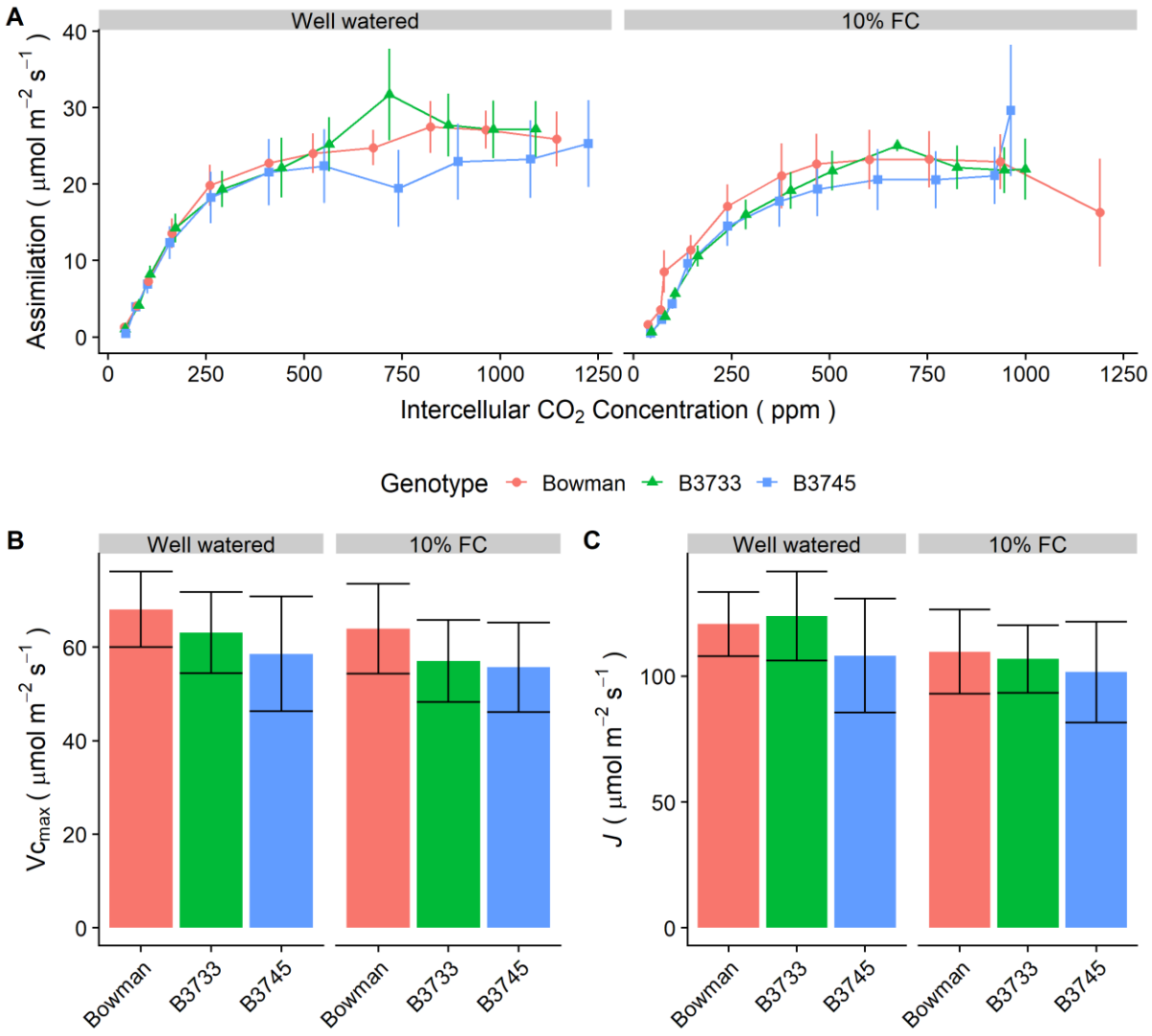

Fig 6.10. Assimilation response curves of 2 wild barleys (B3733 and B3745) and 1 elite (Bowman) to changes in C_i under well-watered and water-stressed conditions at constant, saturating light. A) Left panel shows responses of well-watered plants at 60% field capacity and right panel responses of droughted plants at 10% field capacity. Each point represents the mean of observations for that Ci +/- se. N=5. B) Vc_{max} representing assimilation responses limited by the level of Rubisco activation. C) *J*, representing assimilation responses limited by the rate of RuBP regeneration. Means shown +/- se. N=5.

No significant differences were observed between treatments in the assimilation responses to changing C_i (Fig. 6.10A-C), either in terms of the initial slope of the response curve, Vc_{max} (Fig. 6.10B) or the maximal electron transport rate, *J* (Fig. 6.9C). Yet under well-watered conditions, wild barleys were able to operate at higher C_i of over 1000 ppm compared to peak C_i of under 1000 ppm under drought conditions for the same level of external [CO_2] of 1500 ppm.

The plants were harvested directly after the end of the drought period (Fig. 6.11). Data are shown for above-ground fresh and dry biomass as well as water content. Tiller number, a key determinant of final yield in barley, was recorded as was growth stage on Zadok's scale, and finally leaf area, which plants often reduce in response to drought.

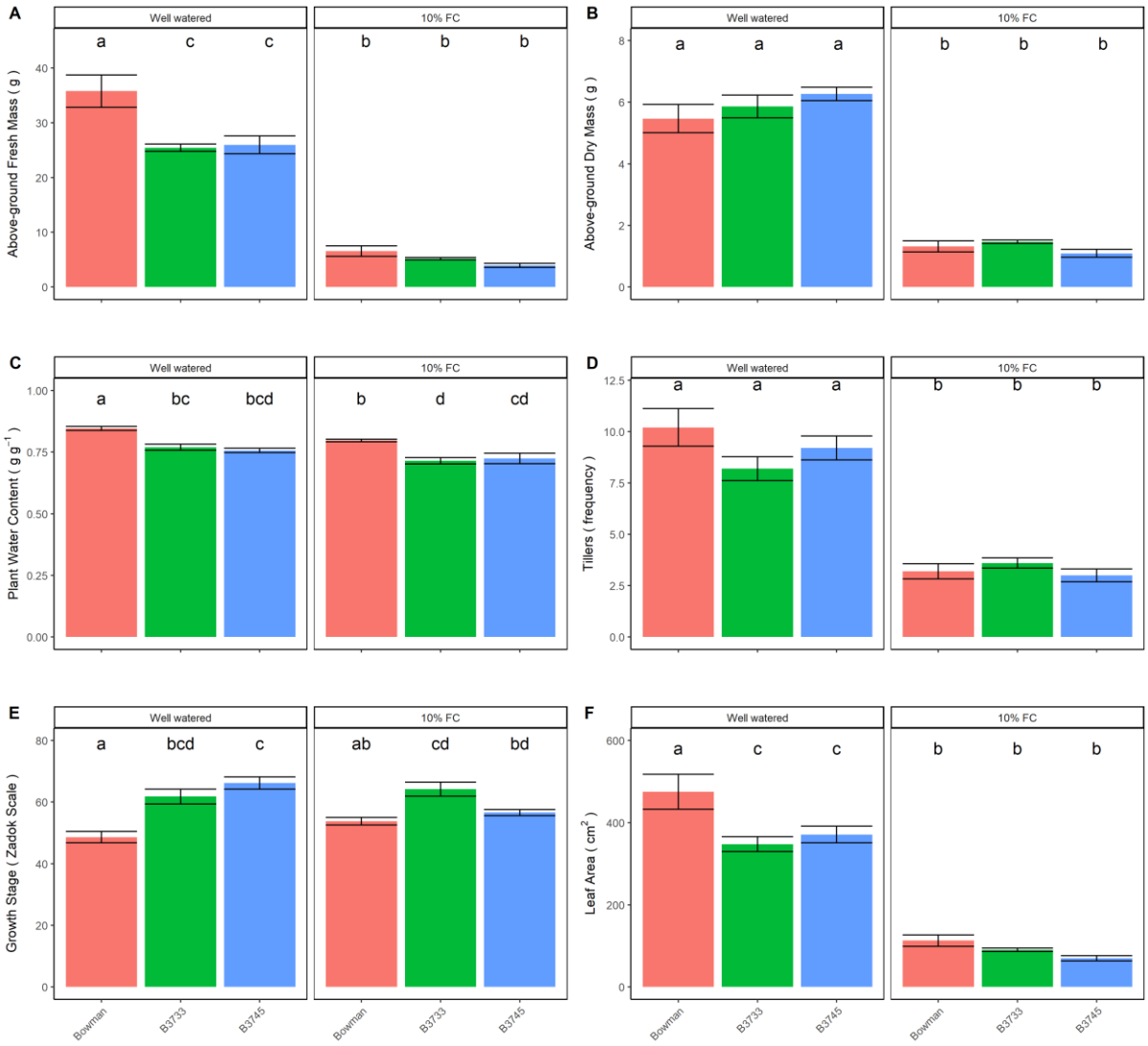

Figure 6.11. Harvest performance of Bowman and 2 wild barleys under well-watered and water-stressed conditions. A) Above-ground biomass. B) Dry biomass. C) Water content. D) Number of tillers. E) Growth stage (Zadok's scale). F) Total leaf area. Means shown +/- SE. N=5. Identical letters indicate no significant pairwise differences between means on Tukey's test at p_{crit} = 0.05.

Bowman achieved the greatest above-ground fresh mass at harvest (Fig. 6.10A) under both control and drought conditions (35.8 g and 6.6 g respectively), with its fresh mass under control conditions significantly greater than any of the other cultivars (Tukey's tests, $p<0.05$). B3745 had the lowest fresh mass under drought (4.0 g). There was a clear impact of treatment on above-ground biomass when

plants were dried (Tukey's test, $p<0.05$, Fig. 6.10B), but Bowman tended to have the lowest dry mass (5.5g) under well-watered conditions, and B3745 the highest (6.3 g) while B3745 continued to have the lowest dry mass under drought conditions (1.1 g). Plant water content was significantly lower under water-stress compared to control conditions (2-way ANOVA, $F_{(5,22)}$, $p<0.05$) and Bowman had the highest water content (Fig. 6.10C) under well-watered conditions (0.85 g g^{-1}) and under drought conditions (0.8 g g^{-1}).

Tillers are a major determinant of productivity in barley, and each variety saw a significant fall in tiller number under drought (Tukey's test, $p<0.05$), although there was no interaction between variety and treatment (Fig 6.10D).

Plants may escape, avoid or tolerate drought by accelerating progress through growth stages, or avoid it by slowing progress and directing more resources towards growth (for instance, tillering or leaves). B3745 moved more-swiftly through the growth stages under control conditions (Fig. 6.10E) reaching GS 66 by harvest time, significantly more advanced than Bowman (GS 49, Tukey's test, $p<0.05$). Under water stress, B3733 was the most-advanced (GS 64) while Bowman remained the least-advanced (GS 54). It is worth noting that B3745's growth stage was further advanced under control compared to water-stressed conditions, while the other two varieties were more advanced in maturity under drought.

Leaf area is a growth parameter that is readily varied in response to drought (Fig. 6.10F) either by reducing the rate of production or increasing the rate of senescence. Bowman had the greatest leaf area under control conditions (475 cm^2), significantly more than both B3733 (by 27%) and more than B3745 (by 40%, Tukey's tests, $p<0.05$, Fig. 6.10F). Leaf area was reduced on average by 77% under drought compared to well-watered conditions, where Bowman again achieved the highest leaf area (113 cm^2) as much as 61% more than that achieved by B3745.

6.4. Discussion

The aims of this study were to explore the influence of drought on the physiological and anatomical responses of wild barleys in comparison with a modern elite variety, and to understand the impact on yield. Since drought episodes increasingly start during establishment as well as late spring (IPCC, 2019, AHDB, 2019), both late-spring water-stress and drought initiated at sowing were studied. Drought is the biggest abiotic threat to yield for farmers, particularly in arid regions, but will have wider ramifications under climate change. Thus under climate change, characterising physiological responses in regionally important crops such as barley is potentially valuable to breeders (Araus et al., 2002, Tambussi et al., 2007, Aprile et al., 2013).

6.4.1 Barley's responses to drought initiated at tillering are clear from physiological but not anatomical outcomes

Control of stomatal aperture to modulate g_s and hence water loss is a critical activity for plants under drought and is an early response to water stress (Valladares et al., 2000), which is effected by a combination of stomatal anatomy and behaviour (Bartlett et al., 2016). In the first experiment, moderate 'late-spring' drought was used to probe plant responses from anatomy to physiology. Stomatal anatomy is a developmentally-determined trait that is influenced by environmental variables, with signalling from older leaves affecting stomatal density and size as well as leaf thickness or leaf area (Lake et al., 2001, Bergmann and Sack, 2007). Furthermore, there is a well-established trade-off between stomatal density and size (Franks and Farquhar, 2001).

As transient water stress was only initiated during the tillering phase in the first experiment, it was expected that stomatal density and size would largely be unaffected because these features are already determined at that point owing to the reduction in plasticity during cell differentiation (Croxdale, 2000, Dow and Bergmann, 2014). No differences were in fact observed since all plants had access to abundant water during development. Reduced stomatal density (with larger stomata) is associated with lower g_s yet a maintenance of biomass (Doheny-Adams et al., 2012), which has important implications for the

maintenance of yield under drought and is a potentially attractive breeding target. B3745 had a tendency to exhibit relatively high stomatal density under well-watered conditions, and relatively low density under drought, suggesting an attempt to support yield by reducing the rate of water loss while limiting the consequences for yield. Different species and genotypes may exhibit a range of responses to water stress, but here the barley accessionsnwere judged by their conservative / non-conservative characteristics (Chapin, 1980, Valladares et al., 2000). Barley is expected to be non-conservative under drought (Munns et al., 2010), prioritising growth over maintenance of water potential, such as by adjusting stomatal anatomical features or stomatal activity.

While it was not possible to explain variations in evapotranspiration in the first experiment from stomatal anatomy alone, adjustment of stomatal aperture in response to water stress is a distinguishing phenotype of conservatism, with greater transpiration associated with higher biomass under moderate drought (Harb et al., 2010), and greater steady-state g_s and A has been closely linked to higher final yield (Fischer et al., 1998). B3733 exhibited a strong non-conservative response to drought by increasing ET which could have resulted from increasing stomatal aperture, a factor which increases yield potential. The findings from the first experiment show all varieties had similar levels of reduction in steady-state responses under water stress, although there was a trend towards higher mean g_s for B3733. There was also no evidence of a phase shift based on slower stomatal responses, unless those responses were symmetrical for opening in the morning and closing in the evening. There was a general trend to lower amplitude under drought which probably reflected stomatal limitation, and greater sluggishness of response (Durand et al., 2018, Gerardin et al., 2018), reflecting lower water potential in the leaf (Aasamaa et al., 2001). In the quest for exploitable traits, there is little to suggest that steady-state physiology will be a fruitful area of research except to increase mean g_s and thereby reduce stomatal limitation on A, but this factor is already a well-established breeding target.

The wild varieties had stronger kinetic responses to drought than did Bowman, with the former maintaining stomatal aperture for longer when light levels changed. Slower response to changing light

under water stress is regarded as a non-conservative trait (Qu et al., 2016), where g_s responses in particular are expected to speed up in order to minimise the mismatch with the response of *A* (Durand et al., 2018, Gerardin et al., 2018, Lawson et al., 2010). These slow-changing g_s behaviours offer compelling breeding targets which have not been explicitly exploited to date, and offer new opportunities to improve water stress responses to maintain yield even in cultivars such as Bowman that have already been bred for a degree of drought tolerance.

There appears to be greater transient stomatal limitation of *A* under changing light conditions for B3745 compared to Bowman when drought is applied. Stomatal limitation is an adaptive response suggestive of conservatism under drought (Lipiec et al., 2013, Farooq et al., 2009), in which restricted CO_2 assimilation into the leaf reduces the actual assimilation rate below its potential (Farquhar and Sharkey, 1982), and is the consequence of lower stomatal conductance. However, slower stomatal closing and opening under drought is also indicative of non-conservatism where maintenance of biomass is more important than maintenance of water potential (Qu et al., 2016). These data therefore suggest that Bowman is more conservative in its responses to drought than B3745 based on speed of stomatal movement.

Yield is the sum of a wide range of responses to environmental stimuli over the course of a season (Lawson and Blatt, 2014). The leaf area of wild varieties under drought declined substantially, a well-known conservative response (Lawlor, 2013). Tiller number is also affected by drought in barley (Samarah, 2005), and B3745 had a non-conservative tillering response, thus maintaining potential yield (AHDB, 2019). Meanwhile, B3745's advanced growth stage under drought compared to B3733 could be a conservative (escape) or a non-conservative (tolerant) (Harb et al., 2010) phenotype, although a slowing of passage through growth stages must be conservative (Valladares et al., 2000). The distinction between conservatism and non-conservatism is implicitly expected to be consistent across such phenotypes as leaf size or rapidity of stomatal closing (Araus et al., 2002). Plants' responses to moderate 'late-spring' water stress are much more complex than a simple conservative-non-conservative

dichotomy, probably reflecting the broad variety of genes, proteins and metabolites known to be evoked under abiotic stress (Bechtold, 2018).

6.4.2 The consequences of drought on barley's anatomy, physiology and yield are complex

Wild barleys' biomass-adjusted ET when water stress was applied from sowing was suggestive of relatively non-conservative behaviours from B3745 and B3733, particularly under drought conditions. Water stress tended to increase stomatal density, indicating an increase in maximal *A* and g_s, notably for Bowman and B3745 in contrast to B3733 (Franks and Beerling, 2009b), which would indicate greater conservatism in the latter (Valladares et al., 2000). Higher stomatal density was clearly offset by decreasing stomatal size under drought, indicative of improved dynamic responses to changing light (Hetherington and Woodward, 2003, Franks and Farquhar, 2001), again, most notably for Bowman and B3745. It is probable that, given slower responses are expected under drought (Qu et al., 2016), a decrease in stomatal size is a conservative trait. Stomatal index (SI) was consistent across varieties, but did not decline under drought as expected (Casson and Gray, 2008). Higher SD at constant SI is consistent with reduced cell size and by extension leaf expansion, an adaptation that often occurs before photosynthetic capacity is affected (Hsiao, 1973). Bowman and B3745 under drought were generally conservative for leaf expansion. The confirmation of a close association between SD and pore length (Doheny-Adams et al., 2012), is also a developmental response heavily influenced by water stress (Hamanishi et al., 2012). The trade-off appeared closer under drought, and should have influenced water use (Caine et al., 2019), but this was only confirmed in the case of B3745. Alternative explanations are necessary in the case of B3733 and Bowman.

Drought reduced J_{max}, as expected (Farooq et al., 2009) for sound anatomical reasons resulting from stomatal limitation via lower anatomical gs_{max} for Bowman and B3745, but not for B3733. Otherwise, physiological capacity appeared surprisingly unaffected by drought, despite the lengthy period of stress (Hsiao, 1973), with the impact of drought on assimilation responses to changing C_i suggesting that key Calvin Cycle activities were largely unaffected, with little impact on either Rubisco activation or RuBP

regeneration based on the measured responses (Farquhar et al., 1980). The implication of the A-Ci results was that these plants maintained photosynthetic potential in the face of drought, a non-conservative trait.

Early application of drought stress had an obvious impact on above-ground biomass, both fresh and dried, and the number of tillers present, but did little to distinguish between varieties. Bowman's higher water content than the wild varieties under both treatments, suggest the former prioritises maintenance of water potential, a conservative characteristic (Harb et al., 2010). Meanwhile, the somewhat slower passage of Bowman (and B3745) than B3733 through their growth stages under drought was suggestive of an avoidant conservative strategy (Harb et al., 2010). The impact of growth stage was largely offset by leaf area, another trait that is very sensitive to drought (Harb et al., 2010, Hsiao, 1973). Bowman tended to develop a larger canopy (a non-conservative response) than the wild varieties under both treatments through maintenance of higher turgor giving rise to greater fresh but not dry biomass. Conversely, the wild barleys maintained rather less turgor and perhaps went through their developmental stages more rapidly.

Plant development is a complex trait that integrates a wide variety of signals such as light intensity or soil moisture availability, with plant genotypic and phenotypic traits (Araus et al., 2002). This series of experiments witnessed behaviours in which there was not always a clear or consistent conservative / non-conservative dichotomy. Plants have a number of options when faced with the choice of growth or survival, and these choices are not 'all-or-nothing' under water-stress (Claeys and Inze, 2013). Bowman, a cultivar known to be tolerant of drought (Franckowiak et al., 1985) was generally conservative compared to the wild barleys when water was limited either from germination or from tillering. Even the larger leaf canopy did not result in higher dry biomass. Conversely, B3745 exhibited a competing range of conservative and non-conservative phenotypes, while B3733 appeared non-conservative in most situations regardless of the point in development at which drought was introduced. Plant breeders face a wide range of choices about varieties fit for multiple environments in the face of rapid climate-

driven change (Araus et al., 2002). The range of behaviours described above offer opportunities to 'fine-tune' drought phenotypes to meet specific demand for novel, regional products.

6.5. Conclusions

- Water stress in barley initiates a range of responses encompassing developmental changes to anatomy and physiology, and leading to alterations to yield and yield potential which differ when initiated from germination or tillering.
- The effects of water stress can elicit conservative or non-conservative responses in barley, but given the complexity of integrating responses from molecules to canopies, responses lie on a continuum and are not either/or.
- Drought affects kinetic and steady-state physiological responses more than the capacity to produce them, while anatomical responses take longer to appear.
- Against expectations, the wild varieties exhibited a number of non-conservative behaviours more strongly than Bowman, with B3733 appearing the most focused on growth over survival, and B3745 somewhere in between.
- Tiller number, leaf area and growth stage were better indicators of GxE differences at harvest compared to biomass.

Chapter 7: Final Discussion & Conclusions

7.1. General Discussion

The impact of climate change is being felt already and will be felt for decades, even centuries, to come (IPCC, 2014) with greater [CO_2] driving higher global mean surface temperatures along with periods of drought (as well as heavy precipitation) expected to occur more frequently with greater duration and severity (IPCC, 2014), putting pressure on water resources. Meanwhile, world population is expected to rise to 9.6bn by 2050 (McGuire, 2013), and along with changing diets plus biofuel use, will drive up the demand for crops (Amin et al., 2006). Yet current advances in yield appear to have plateaued against a requirement for them to nearly double by 2050 (Ray et al., 2013).

Stomata are major mediators between rising [CO_2], which drives higher temperatures and affects moisture availability, and plant behaviour, via the control of CO_2 entry into the plant offset by H_2O exit as transpiration, and captured as WUE based on the size, density and responsiveness of stomata (Hetherington and Woodward, 2003, Keenan et al., 2013). Manipulation of stomatal characteristics such as density has led to historic gains in productivity in crops such as wheat (Fischer et al., 1998) through the Green Revolution and into more recent times (Evenson and Gollin, 2003). Water use in agricultural production remains an issue both where water availability appears satisfactory, and where water stress exists (Condon et al., 2002). Finding novel phenotypes that blend high yield and parsimonious water use could help water-efficient production (Dawson et al., 2015, Wiegmann et al., 2019), and two approaches were considered for the major cereal crop, barley: mutant lines with impairments to the functioning of the circadian clock, and wild / landrace barley varieties that predate crop improvements resulting from the Green Revolution (Lundqvist, 2014, Monteagudo et al., 2019, Brozynska et al., 2016).

7.1.1 Circadian clock mutants offer a rapid route to improving WUE_i in barley

Circadian clock mutants introgressed into the elite variety Bowman allowed researchers to understand the impact of the impairments on barley habit and growth (Druka et al., 2011), with a QTL at *eam-10* (subsequently identified as the *HvLUX* clock gene) bred into Mari, which rapidly became popular with

farmers in Sweden for its early-flowering phenotype (Lundqvist, 2014, Campoli et al., 2013). Stomatal anatomical and physiological performance in relation to water use was examined in two cultivars, BW284 and BW289, which had mutations in proteins forming the Evening Complex of the clock in Arabidopsis (Nusinow et al., 2011). Dynamic responses of A, g_s and WUE_i to changing light intensity in those mutants were little different from the wild type, but as the measurement period lengthened from minutes to hours to an entire diurnal period, the activity of the clock became more evident. It seems that over the shortest timescales, the influence of the clock is minimal, being overridden by other environmental information such as light intensity, which is known to have a major impact on stomatal behaviour (Blatt, 2000). Yet as the timescale increased to a full daylight period, circadian influences on stomatal aperture became apparent, in line with previous results showing a clear diurnal pattern (Dodd et al., 2005).

Steady-state responses of g_s did not arise from anatomical causes as there were no differences in stomatal size and density between cultivars. Lack of differences between stomata also suggested that these anatomical features, which are developmentally determined (Lake et al., 2001), are not all influenced by the clock. As short-term stomatal dynamics were also unaffected by the clock, other causes for longer-term behaviour must be sought, perhaps mediated by altered sensitivity to biochemical signals such as those evoked under stress.

Bowman was expected to be sink limited, in line with elite barleys (Bingham et al., 2007b), with reduced capacity to accumulate photosynthate at the ears given the extent of investment in leaf area (Bingham et al., 2007b). The consequence was a low priority placed on late-afternoon assimilation as stem and ear carbohydrate stores filled. On the other hand, clock mutants were probably source-limited, having smaller leaves (Bingham et al., 2007a). Consequently, the plants had a strong theoretical incentive to maintain photosynthate supply for as long as was congruent with improved yield.

Phospho-adenosine phosphate, is a retrograde chloroplast-to-nucleus signal that is invoked under drought (and light stress) in *Arabidopsis* (Estavillo et al., 2011). When the mutants were subjected to reduced water availability, PAP concentration tended to rise in the clock mutants compared to Bowman, potentially extending recent results which showed that PAP itself can influence the period of the clock (Litthauer et al., 2018), by suggesting that in the absence of a clock, plants suffer greater stress and increase PAP production.

The clock mutants had better WUE_i under drought than Bowman, extending their attractive assimilation characteristics at steady state, and are more responsive to fluctuations in light, in contrast to their behaviour when well-watered. The consequence is improved yield relative to Bowman under drought, perhaps because their source-limitation through smaller leaves is more-attractive under water stress (Anjum et al., 2011).

7.1.2 There is significant genetic potential to be tapped in wild and landrace barleys

Wild barleys and landraces are potential unexploited sources of novel phenotypes and genetic resources (Dawson et al., 2015, Mahon et al., 2016, Newton et al., 2010). The wild barleys selected for the work presented here originated in Central Asia, and therefore were not well-represented in the European / Middle Eastern or North African elite germplasm (Morrell and Clegg, 2007). The landraces selected represented a broad cross-section of Northern and Southern European types (GRU, 2019) with a range of habits and end uses. Water-use efficiency of the selected cultivars was characterised relative to current elite varieties, and a wide diversity of outcomes for stomatal anatomy as well as g_s, A and WUE_i were noted. The landrace, Golden Archer, had slow stomatal kinetic responses, while the wild barley, B3745, had much more rapid responses to a step increase in PAR in line with the elite variety KWS Sassy, in which stomatal responses were presumed to be optimised through breeding (Fischer et al., 1998). In contrast to most published evidence (Franks and Beerling, 2009a, Doheny-Adams et al., 2012, Lawson and Blatt, 2014) was the existence of low maximal values of g_s in high SD plants and vice versa, and no offsetting changes in stomatal size, although speed of response may be influenced by other factors than

size, such as active shuttling of osmotica between subsidiary and guard cells (Franks and Farquhar, 2007). Wild barleys and landraces have been shown to achieve better outcomes in critical yield components (AHDB, 2018), despite elite varieties being subject to intensive breeding improvement programmes over many decades (Evenson and Gollin, 2003, Bezant et al., 1997, Wang et al., 2016, Samarah, 2005, Newton et al., 2011), which suggests that further improvement in yield can be achieved by the incorporation of these wild barley phenotypes into breeding lines.

Bowman is considered to be a drought-tolerant variety (Francowiak, 2014), so its responses to water stress were compared to the wild barleys at two key growth stages: germination and tillering. A reduction in stomatal aperture is usually one of the initial responses to drought (Valladares et al., 2000), while over the longer term a reduction in water availability may affect stomatal density and size (Lake et al., 2001, Bergmann and Sack, 2007). Therefore, the reduction in SD under drought from germination was expected, although plants exhibited similar capacities to respond to changing light and C_i at that growth stage. When drought was initiated later on, at tillering, no differences were seen in stomatal anatomy, as expected. There was also little impact on steady-state physiology although the wild barley B3733 had less stomatal limitation of *A*, a desirable phenotype. The wild barley B3745 exhibited rapid kinetic responses to drought, which suggested it would be able to maintain yield under moderate water stress compared with Bowman, another desirable trait. In general, it is not possible to make unambiguous statements of conservatism or non-conservatism in these plants, as each exhibited an array of behaviours in the face of water stress. Since responses to drought are a complex interaction between the push of source and the pull of sink (Anjum et al., 2011, Passioura, 1996), it is perhaps not surprising that no definitive categorisation can be given. In the context of UK farming, where climate change is expected to bring a warmer, wetter climate on average, but with less summer rainfall expected in the South and East of England (Knox et al., 2010, IPCC, 2019), breeders are likely to favour non-conservative traits where rainfall is relatively higher and conservative ones where it is likely to be lower. A range of plant anatomical and physiological phenotypes have been identified here which offer

breeders a toolkit that could be used to increase yield stability under more-variable climactic conditions through the use of cultivars with offsetting conservative and non-conservative traits.

Water stress initiated a range of conservative and non-conservative responses in wild barleys that were seldom clear-cut in their overall phenotype. Drought affects stomatal density and size when initiated early in development. Physiological responses were more-important at tillering. Tiller number, leaf area and growth stage effectively differentiated between varieties, and all are valuable yield components that could be targeted by breeders.

7.2. Final conclusions and possible future work

Barley remains the fourth most traded crop globally (USDA, 2018), primarily for animal feed (80%+ of production) and for human food / alcohol. In the UK, it is also a critical ingredient in Scotch whisky distilling, a major export industry which uses only British barley (Mahon et al., 2016). Hence support for barley production is important both nationally and internationally, and this task is complicated by the ramifications of climate change. In the UK, the CO_2 fertilisation effect is primarily offset by higher temperatures during the growing season and increased risk of drought (Cai et al., 2016, Fitzgerald et al., 2016, Macabuhay et al., 2018), which leads to the South and East growing drier while the North and West will be wetter; all regions will be hotter (IPCC, 2019, Rubel and Kottek, 2010, Hatfield et al., 2011, Knox et al., 2010).

The circadian clock mutants described above are attractive as sources of genetic material for two primary reasons: the mutated genes underlying the phenotypic changes are now known ((Campoli et al., 2013, Faure et al., 2012), and they exist as NILs in a Bowman background. As a result, incorporating the mutated genes into elite germplasm ought to be straightforward. Introgressing these genes into modern elite varieties should give rise to improved varieties with better WUE_i characteristics, which may also be attractive for breeders seeking to produce varieties that are conservative under drought, perhaps in the most arid regions such as the Mediterranean basin or Near East above and beyond the established phenotypes of earlier flowering.

Wild and landrace barleys have already been used as genetic resources for crop improvement as seen in increased scores for diversity (van de Wouw et al., 2010). However, the wild barleys described here possess alleles that are probably not extant in elite European cultivars (Morrell and Clegg, 2007), and hence offer a rich diversity of phenotypes with underlying genetics that are unavailable from other sources.

One of the ways in which these wild and landrace barleys may prove useful is in enhancing yield stability under abiotic stress (Newton et al., 2010). Yield stability is likely to become an increasingly important factor in UK agriculture, where business-as-usual is under threat from climate change (Knox et al., 2010). The use of heterogeneous landrace populations and genetic material from wild barleys holds out the prospect that UK farming can become much more resilient to a range of threats, both biotic (such as increased pest abundance) and abiotic (such as water and heat stress).

While breeders have historically focused on optimising a single target outcome – high yield – future scenarios call for a more-rounded approach with changes likely to species and varieties grown, farm management practices and consumer preferences and expectations. In these scenarios, a return to pre-Green Revolution practices such as encouraging heterogeneity between individuals in a varietal population as well as diversity within each individual's genotype would go some way to assuaging fears around agriculture in relation to climate change and lead to greater stability in most seasons. But it would be culturally costly for the farming and food industries, and require a different mind-set in such areas as recommended lists or quality assurance programmes. As with many climate change risks, the evidence already exists in the public domain, but stakeholders are unwilling to accept the implications for fear of the costs. What has been described above is a series of inexpensive resources which can be applied to crop improvement for the benefit of all.

Chapter 8: References

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