The Genetic Control of Stomatal Development in Barley: New Solutions for Enhanced Water-Use Efficiency in Drought-Prone Environments

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Abstract: Increased drought frequency due to climate change is limiting the agronomic performance of cereal crops globally, where cultivars often experience negative impacts on yield. Stomata are the living interface responsible for >90% of plant water loss through transpiration. Thus, stomata are a prospective target for improving drought tolerance by enhancing water-use efficiency (WUE) in economically important cereals. Reducing stomatal density through molecular approaches has been shown to improve WUE in many plant species, including the commercial cereals barley, rice, wheat and maize. Rice with reduced stomatal density exhibit yields 27% higher than controls under drought conditions, reflecting the amenability of grasses to stomatal density modification. This review presents a comprehensive overview of stomatal development, with a specific emphasis on the genetic improvement of WUE in the grass lineage. Improved understanding of the genetic regulation of stomatal development in the grasses, provides significant promise to improve cereal adaptivity in drought-prone environments whilst maximising yield potential. Rapid advances in gene-editing and ‘omics’ technologies may allow for accelerated adaption of future commercial varieties to water restriction. This may be achieved through a combination of genomic sequencing data and CRISPR-Cas9-directed genetic modification approaches.

Keywords: water-use efficiency; cereal crops; gene-editing; stomatal density; yield; drought tolerance

1. Introduction

Shifting hydrologic patterns as a consequence of climate change are evident, and droughts are becoming more frequent globally [1]. In historically arid lands like Australia, advanced modelling algorithms have revealed several alarming predictions that imply a future with significantly longer and more severe drought events. This is especially true for the southern areas, which include the grain belt regions reserved for extensive crop production [2]. The impacts of water scarcity are even manifesting in more hydrologically stable climate regions. In Europe, the increased incidence of warm season droughts has caused a rise in agronomic management costs associated with irrigation [3,4]. For example, some countries in the Mediterranean Basin have experienced multiple impacts on public water resources. This is due to a need to allocate significant water reserves to the prevention of agricultural losses during low rainfall periods [4].

Heavy consumption of fossil fuels since the industrial revolution has brought a surge in atmospheric CO2, serving as a significant force behind our evolving climate [5]. The climate change associated impacts of rising CO2 levels have polarising effects on plant
physiology. On the one hand, increased atmospheric CO\textsubscript{2} levels allow for greater photosynthetic efficiency (CO\textsubscript{2} fertilisation effect) with reduced transpirational water loss [6]. On the other hand, rising CO\textsubscript{2} concentration may lead to a warmer global climate. According to a number of models, this rise in temperature substantially negates CO\textsubscript{2} fertilisation through water loss caused by evaporative cooling in response to heat stress [7]. An additional impact of CO\textsubscript{2} induced temperature increases is the enhanced probability of drought, posing substantial risks to plant survival and thus agricultural productivity [8].

As a consequence of adverse climate impacts, net agricultural losses are a growing economic burden globally, with an estimated loss of $4.2 trillion AUD by 2100 in Australia alone if adaptive strategies are not implemented [9]. Based on the latest climate data, the southern Wheat Belt regions of Australia are expected to be especially impacted. Southern Australia is predicted with high confidence to experience continued rainfall declines and increased agricultural droughts under all possible climate scenarios [10]. Of particular concern is the climate derived impact on the agricultural industry in developing and least developed countries around the world. Recent modelling using historical climate data in West Africa indicates that an average temperature increase of 1 °C from the decade of 2000–2009 resulted in a loss of $6.19 billion USD. This was due to the combined sorghum and wheat yield reductions caused by climate effects [11]. In addition, the impacts of climate change on Africa are expected to cause future substantial drying of the continent with increased agricultural drought indicated with high confidence [10]. In terms of food security, over half of the total world population (52%) are at risk of malnutrition if no adaptation of current agricultural practices is employed when climate change is taken into account [12].

As a result of impending environmental challenges, a number of mitigation measures have been developed to help predict and manage future rainfall deficits. For example, the use of simulated crop responses to water deficit have been employed in soybean to assist in timing of water supplementation [13]. In turn, this has assisted to safeguard yields during drought events. It should be noted that although agronomic strategies are effective at reducing yield losses under drought, many crop growing communities do not have access to various mitigation approaches and thus have an increased risk of future yield losses. Agricultural strategies have thus become focused on breeding commercial crop varieties with improved water-use efficiency (WUE) in environments where water availability is limited [14]. WUE can be defined as the ratio of CO\textsubscript{2} fixed in photosynthetic processes (A) versus water vapour lost to the atmosphere via stomata [15]. Stomata are specialised cellular units on the leaf epidermis, acting to maximise CO\textsubscript{2} diffusion for carbon assimilation by photosynthesis, whilst minimising transpirational water loss. Greater levels of water loss are characterised by a high plant stomatal conductance (g\textsubscript{s}), which is a measure of net diffusion of CO\textsubscript{2} entering or water exiting the pore [16]. Stomatal morphologies vary among plant families—However, the guard cell pair surrounding the pore are a ubiquitous feature in all species, adjusting turgor pressure through osmotic processes to alter the size of the pore opening (stomatal aperture) in response to environmental signals [17,18]. Stomata serve as the channel via which water escapes plant tissues into the atmosphere, accounting for >90% of plant water loss [19]. In turn, these structures are also key to optimising water retention and hence are a potential focus for improving WUE in crop species growing in drought-susceptible locales [20]. As previously mentioned, increased temperatures brought on by climate change, in combination with reduced rainfall, are predicted to increase the frequency that crops experience drought and heat stress events. High temperature environments with accompanying water scarcity are especially detrimental to crop survival. This is due to the induction of evaporative cooling measures to reduce heat stress effects by increasing transpiration through the stomatal pore. The result is decreased plant temperature to the cost of increased water loss. Under drought, the extent to which heat stress is negated as a result of increased stomatal apertures was shown to depreciate significantly. For example, in poplar, evaporative cooling reduced leaf temperature by 9 °C under well-watered conditions—however, this dropped to a 1 °C
reduction in leaf temperature under drought stress [21]. Thus, it is evident that combined high heat and drought conditions may impact heat stress tolerance processes in crops whilst simultaneously reducing WUE. As a result, mitigation strategies may need to be adapted to target plant stomatal responses under high heat and drought to achieve a balance between optimum cooling capacity and plant water retention.

Stomatal density has been identified to influence WUE. However, the trait itself is controlled by a variety of environmental factors, including atmospheric CO$_2$ levels, temperature, prolonged water stress, and light intensity [21–23]. Previous studies using model plants (e.g., Arabidopsis thaliana) have revealed that a suite of genes are involved in controlling stomatal density [24,25]. Thus, there is a possibility for selective breeding to improve traits attributed to stomatal adaptation to improve WUE in crops. However, knowledge remains limited regarding gene networks implicated in cereal stomatal development. Hence, further research is needed on the molecular mechanism influencing stomatal density in these economically prominent crop species.

Domestic Barley (Hordeum vulgare L.) has been favoured for millennia for its extraordinary capacity to survive environmental challenges [26,27]. Considering the array of environmentally resilient traits associated with the wild progenitor H. vulgare subsp. spontaneum, including the possession of mutable gene regions with high mutation rates to promote climate adaptability, there is no surprise that barley was one of the first crops to be domesticated during the agricultural revolution over 10,000 years ago [26,28,29]. Barley remains one of the most versatile, economically critical crops in global agriculture, ranking fourth in total quantity produced versus other cereal grains [30]. Within Australia, barley ranks second in total production by volume, placing emphasis on a need to refine and improve cultivars to reduce yield losses from environmental challenges [31,32]. In addition, Australia monopolises a large majority of barley exportation, comprising 30–40% of global malting barley exports and approximately 20% of animal feed and human consumption exports [33]. Australian barley varieties are of international interest as a result of their high malting quality for use in the beer industry and production of exceptional grade, clean stock feed [33].

Relative to other major cereal grains, such as maize and wheat, barley demonstrates a superior resilience to environmental constraints. Thus, barley yield is generally more stable with changing conditions [34]. Resilience-associated traits include drought tolerance and adaptation to a range of soil conditions including phytotoxic acidic soils containing high levels of aluminium ions [35,36]. These factors in turn make barley the preferred choice for cultivation when considering unstable external conditions that may limit the yield of other crops. This likely explains barley cultivation by agrarian communities in agriculturally restrictive regions, including areas of high altitude such as Nepal, or Mediterranean regions with low seasonal rainfall [34,37].

Despite the suite of environmentally resistant traits possessed by barley, yield and survival capacity remain thoroughly challenged by the impending effects of climate change. Major abiotic stresses imposed on barley include the advent of increased salinity and drought, which are exacerbated by rises in global temperature [38]. Regardless of these negative climatic impacts on yield, barley remains one of the most protean and amenable cereals to such circumstances. This stands testament to the extensive use of barley in regions such as Western Australia, where dry climates encourage saline soils and prolonged drought periods [39,40]. Hence, barley can be considered a good candidate for agronomic improvement in a world where the negative impacts of climate change are likely to force adaptation of current agricultural practices [41].

In the following sections, we will assess the current understanding of stomatal density control, with particular emphasis on the genetic potential in barley for enhanced WUE in the scope of current climate change. Recent genomic advances have led to the characterisation of the whole barley genome. As a result, quantitative trait loci (QTL) have been identified for a variety of agronomic traits through genome-wide association analysis (GWAS) and population fine-mapping [42]. Finally, the potential of existing genomic data will be
evaluated in assisting characterisation of genes predicted as involved in barley stomatal density variation.

2. The Genetic Control of Stomatal Density Improves WUE

Stomata are ancient morphological structures, serving as prime regulators of water retention even prior to the divergence of the primitive, non-vascular hornworts and mosses from early land plants over 400 million years ago [43,44]. Throughout the lineages of land plants, stomata exhibit a high degree of variance in both morphology and epidermal distribution among species [45]. Drought tolerance mechanisms linked to stomatal mechanics can be traced to archaic lineages. Paleozoic land flora have been found to possess stomatal systems sensitive to the drought-associated hormone abscisic acid (ABA), for refined maintenance of water-loss under a limited root system physiology [46]. Despite a temporally consistent relationship between stomata and drought tolerance, we must question to what extent stomata may aid modern agriculture in producing yield-effective, climate adapted crops.

It is widely known that stomatal patterning, density and mechanical response influence WUE, and a variety of recent studies have been conducted to assess the yield impacts of altered stomatal characteristics on crop WUE [47]. Table 1 provides the results of previous studies examining the effect of various stomatal modifications on traits including CO$_2$ assimilation capacity, photosynthetic efficiency, WUE and yield. Overall, the outcomes of current studies indicate that reducing stomatal density and aperture enhances WUE across multiple plant families, with minimal impact on the efficiency of both photosynthetic and carbon assimilation processes. In turn, the aforementioned stomatal modifications either improve crop yields under drought conditions or stabilize yields in commercial crop species (Table 1). In Asian rice (Oryza sativa), a water intensive crop with high sensitivity to drought, overexpression of rice EPIDERMAL PATTERNING FACTOR1 (OsEPF1) was reported to increase resistance to reduced water availability [48]. OsEPF1 is a known negative regulator of stomatal development. Thus, the effect of OsEPF1 overexpression was a concordant reduction in stomatal density. This was to the extent where overexpression lines produced higher yields than controls under some environments, despite reduced rates of photosynthesis [48,49]. Overexpression lines in the study showed significant improvements to WUE, where seedlings utilised 60% of the normal water intake during germination [48]. Those authors also reported survival capacity in OsEPF1 overexpression lines under conditions of high atmospheric CO$_2$, drought conditions and elevated temperature (40 °C), of which all factors represented projected future climate [50]. Although the survival of low stomatal density lines was marked under conditions with climatic stress, it should be noted that stomata are dynamic structures, and they respond to a variety of external signals—including air composition, heat, and light [51,52]. Elevated CO$_2$ is reported to reduce stomatal apertures by 20–40% in some plant species, and this may also have caused significant enhancements in WUE for OsEPF1 overexpression lines under elevated CO$_2$ [53]. However, it is likely that low stomatal density could contribute substantially to WUE of cultivars exposed to drought conditions. In rice, only lines with marked reduction (88%) in stomatal densities relative to controls demonstrated statistically significant decreases in the amount of CO$_2$ assimilated and stomatal conductance, whilst groups with milder reductions exhibited almost identical rates of CO$_2$ assimilation and stomatal conductance as the control [48].

A related study using Arabidopsis thaliana reported a strong negative correlation between stomatal density and the size of stomatal complexes, leading to the conclusion that genetic mechanisms may co-regulate both characteristics [54]. In contrast, Caine et al. (2018) reported a positive correlation between stomatal complex size and density in rice, observing a 12% reduction in the size of stomatal complexes in overexpression lines versus controls, but only for the group with the most significant reduction in stomatal density (88%) versus the control [48]. This may suggest that rice, as a monocot in contrast to A. thaliana as a dicot, possesses different molecular responses to alterations in stomatal density,
and that the size of rice stomatal complexes may be less sensitive to alterations in density relative to *A. thaliana*. These findings also suggest that stomatal complex size may have additional impacts on WUE that are currently unknown [55].

Finally, the secondary impacts of reduced stomatal densities should be considered across multiple climatic effects. Caine et al. (2018) demonstrated the high survival of rice *OsEPF1* overexpression lines under high heat and CO$_2$ independently, yet did not sufficiently examine functionality under combined conditions, as this is suspected to occur in future climate change impacted environments [48]. For example, stomatal density reductions caused decreases in stomatal conductance under high CO$_2$. However, under high temperatures (35–40 °C), *OsEPF1* overexpression lines had poor survivability with the cost of reduced WUE as a result of greatly enlarged stomatal apertures, which were predicted to compensate for substantially lowered stomatal density [48,56]. Since high CO$_2$ is noted to cause reductions in stomatal aperture, the enhancement of WUE under combined high heat (>35 °C) and CO$_2$ stress on such genotypes may be a possibility. However, whether this may be of detriment to survivability is yet to be determined in *OsEPF1* overexpression lines [48,57].

Caine et al. (2018) claimed that the production of reduced stomatal density rice lines overexpressing *OsEPF1* outcompeted controls under some climate conditions in terms of overall yield. These conditions involved a three-day period of drought post-flower emergence in 88-day old individuals, where lines with an intermediate reduction in stomatal density maintained a 27% higher grain yield [48]. Overexpression of *EPF1* gene orthologs in *Triticum aestivum* (bread wheat) produced similar phenotypic effects. However, wheat lines with the greater observed reductions in stomatal density (>50% decrease) were highly susceptible to yield losses relative to controls despite enhanced WUE, yet those lines with moderate reductions (<50% decrease) maintained stable yields comparable to controls, even under drought stress, with the added benefit of enhanced WUE [58]. It is clear that maintenance of yield stability during drought is possible in climate susceptible cereals such as wheat and rice. Thus, potentials exist for the selection of water-use efficient cultivars. These results are promising when considering the economic viability of cereals in a future where droughts are predicted to become frequent [59]. Considering the combined results of Caine et al. (2018), intermediate reductions in stomatal density appear to have the most optimal impact on crop viability, where a balance between stable yield and improved WUE may be achieved [48].

The suitability of reducing nocturnal transpiration (a mechanism identified to contribute to lower WUE) through genetic selection has been recently assessed in grapevine [60]. Reductions in CO$_2$ assimilation are known to be a secondary effect associated with decreases in stomatal density and are hence a factor that can potentially limit yield due to reduced carbon intake. Thus, minimising water loss at night (stemming from improper stomatal closure) may provide an additional avenue to enhance WUE without a reduction in CO$_2$ assimilation to improve yield. In *A. thaliana*, it has been shown that substantial reductions in stomatal density did not significantly impact carbon assimilation under conditions of normal light intensity, and that carbon assimilation was comparable to controls with the benefit of improved WUE [61]. Compensatory mechanisms of increased stomatal aperture were observed in reduced stomatal density phenotypes, which has been attributed to the stability of CO$_2$ assimilation despite alterations to stomatal distribution [61]. Despite these positive effects, it should be noted that carbon assimilation and corresponding photosynthetic rate was reduced versus controls in low stomatal density *A. thaliana* phenotypes under conditions of elevated CO$_2$ and high light intensity [61]. Thus, yield impacts may need to be considered for low stomatal density crops grown in high light intensity regions, which in general is a defining characteristic of drought susceptible agronomic lands.

In another recent study, overexpression of an *EPF* ortholog (*HvEPF1*) in barley lines with stomatal density reductions approximately half of the control and concordantly a smaller size of the stomatal complex exhibited superior WUE under severe drought in greenhouse experiments [62]. *HvEPF1* overexpression barley substantially outperformed
controls under drought stress in a number of aspects, including light-adapted quantum yield (a measure of physiological stress) and leaf relative water content. Thus, barley demonstrates a strong potential for receiving stomatal-based enhancements for improved drought tolerance [62]. In an additional experiment, HvEPF1 overexpression lines with substantial reductions in stomatal density (24% and 12% of the control stomatal density) displayed enhanced WUE under well-watered conditions with greatly reduced stomatal conductance and minimal loss of CO₂ assimilation capacity [62]. Even more remarkably, following biomass analysis of substantial stomatal reduction lines versus controls, it was found that HvEPF1 overexpression barley possessing 12% of the control stomatal density, did not exhibit significant impacts on yield associated traits including seed number and harvest index under both well-watered and water-restricted conditions [62]. These results for barley, where yield impacts were negligible even under extreme stomatal density reductions, greatly contrast that of bread wheat, which displayed reduced yield under stomatal density reductions greater than 50% [58,62]. Such amenability of barley to stomatal density modification highlights the relevance of the crop as an excellent candidate for sustainable farming in a future predicted to be heavily based on restricted water use. Finally, these data provide insight into the variability of tolerance to alterations in stomatal distribution between commercial cereals, and ultimately suggests that effective enhancement of WUE will require a tailored approach specific to species physiology for yield maintenance and economic viability.

Overall, the combined results of recent studies illustrate a deeply rooted interconnection between stomatal morphology and distribution with plant WUE, thus highlighting the relevance of improving our understanding of stomatal development for sustainable agriculture. This is especially important in the context of commercial crop species, where the current understanding regarding molecular control of stomatal formation is limited [63]. Hence, a detailed examination of stomatal development in the grasses is needed for the genetic improvement of cereal grains and enhanced adaptability to climate change.

Table 1. Summary of studies investigating the impact of stomatal modification on plant WUE, stomatal conductance (gs), carbon assimilation, photosynthetic efficiency and grain yield.

| Type of Stomatal Modification | Plant Response | Study Species | Citation |
|-------------------------------|----------------|---------------|----------|
| Increased density             | Enhanced CO₂ assimilation capacity. | Mosses | [64] |
|                               | Increase in gs, no impact on photosynthetic efficiency or CO₂ assimilation rate. | Arabidopsis | [65] |
|                               | No impact on photosynthetic efficiency. | Rice | [66] |
| Clustered stomata             | Impaired CO₂ diffusion, no impact on photosynthetic efficiency. | Arabidopsis | [65] |
| Reduced aperture              | Increased WUE, decrease in gs. | Arabidopsis | [67] |
| Reduced density               | Increased WUE, decrease in gs, reduced decrease in grain yield under water restriction (29.68%) versus higher stomatal density varieties (33.57%). | Wheat | [68] |
|                               | Increased WUE, enhanced survivorship and evaporative cooling in drought and high temperature (40 °C) versus controls, 27% increase in yield versus controls under drought conditions. | Rice | [48] |
|                               | Increased WUE, stable yield under water-restriction (<50% reduction in stomatal density only). | Wheat | [58] |
Table 1. Cont.

| Type of Stomatal Modification | Plant Response                                                                                           | Study Species | Citation |
|-------------------------------|----------------------------------------------------------------------------------------------------------|---------------|----------|
| Reduced density               | Increased WUE, Decrease in $g_s$, minimal reduction in CO$_2$ assimilation, barley lines with 88% reductions in stomatal density produce yields comparable to controls grown under well-watered conditions even under water-restricted conditions. | Barley        | [62]     |
|                               | Increased WUE, 80% higher survival rate under drought (versus control plants), reduced leaf water loss, no impact to photosynthetic efficiency under well-watered conditions, higher photosynthetic rate than controls under drought conditions. | Maize         | [69]     |
|                               | Increased WUE, no impact on photosynthetic efficiency, no difference in fruit yield versus controls under both well-watered and water restricted conditions, enhanced dehydration tolerance. | Tomato        | [70,71]  |

3. Complex Gene Networks Direct Stomatal Development

Investigation into control of stomatal mechanics for the improvement of WUE originated in the mid-20th century, with trials heavily focused on the use of chemical-based manipulation of stomatal signalling. Such treatments involved the use of antitranspirants, which inhibit transpirational water loss. A caveat of antitranspirants is that they also produce negative secondary artifacts, including reduced photosynthesis from limited CO$_2$ diffusion [72]. Metabolic inhibitors, including the drought associated hormone ABA, are known to safeguard water vapour escape. However, their use also generates the caveat of impaired CO$_2$ diffusion due to reducing the size of stomatal apertures [73]. The extent of photosynthetic reduction imposed by ABA treatment has been challenged in recent studies, which have found that short term ABA hormone treatment improves WUE in model plants without adverse effects on photosynthetic efficiency [74]. ABA is tightly linked to stomatal mechanics and development, where its roles include the modulation of guard cell behaviour during drought and the initiation of leaf senescence under temporally extended stress conditions [75,76]. ABA receptor genes (such as PYRABACTIN RESISTANCE/PYRI-LIKE) and associated products implicated in ABA signal transduction, have been characterised in Arabidopsis as potential molecular targets for WUE improvement [77,78]. Some investigation has been completed on barley lines overexpressing the HvNAC005 gene encoding a ‘no apical meristem’ (NAC) transcription factor with ABA responsive promoter elements, where overexpression lines exhibited increased senescence in the presence of ABA [79]. This suggests that HvNAC005 may be a possible target for the control of stress-directed nutrient remobilisation and improved crop yields (as senescence events adversely impact yield). Hence, rather than increasing ABA inputs on crops to increase WUE, the manipulation of ABA responsive gene network sensitivity to plant native ABA levels may provide the key to minimising transpirational water loss. However, further investigation is required for the ABA pathway implicated in guard cell responses under drought stress in barley, which could reveal new methodologies for the genetic improvement of WUE.

As previously highlighted in our assessment of ABA signalling, a shift in focus on genetic rather than chemical/hormonal modification of stomatal development and mechanical response holds significant potential for the improvement of sustainable agriculture. A genome-based approach for cereal climate adaption can be considered economically effective on a multifaceted scale. First, the production of high WUE cultivars by genetic
selection, effectively eliminates chemical/hormonal treatment costs to reduce transpiration. Second, hydrologic inputs are substantially minimised as a result of growing yield-stable cultivars possessing an environmentally tailored genetic response [80]. At present, the majority of genetic knowledge underpinning stomatal development is derived from the model organism *Arabidopsis thaliana*. The results of genetic studies in *A. thaliana* have revealed that stomatal formation and distribution is underpinned by a highly complex signal transduction pathway, involving the combined interaction of various receptor kinases, transcription factors, and signalling peptides [81]. These signalling networks exist to modulate cell-fate transitions of protodermal cells (PDCs) in the epidermis and subsequent stomatal leaf surface architecture [82]. Three major genes, *SPEECHLESS (SPCH)*, *MUTE* and *FAMA*, are implicated in stomatal differentiation—all encode basic helix-turn-helix (bHLH) transcription factors and are critical in directing the progression of stomatal progenitors into mature guard cells in a pathway known as the stomatal lineage [83]. It should also be noted that the stomatal lineage does not exhibit a linearity of PDC fate, and that intermediates may branch into alternative developmental pathways. For example, as shown in Figure 1, after SPCH-directed transition of PDCs into meristemoid mother cells (MMC) and formation of cell pairs comprising a meristemoid and stomatal lineage ground cell (SLGC), this complex has four potential fates. Cell pairs may undergo MUTE-directed guard mother cell (GMC) formation, generate more SLGCs by amplifying division of self-renewing meristemoids, participate in spacing division forming meristemoid-pavement cell complexes, or in rare cases, cells may exit the lineage [83]. SPCH is a critical element in stomatal development—its action serves as an entry checkpoint into the stomatal lineage [83]. The crucial role of the transcription factor is indicated by the observation that SPCH gene knockout plants are incapable of stomata formation [84]. With this knowledge, SPCH modulation has been identified as a primary target for stomatal density regulation. As such, additional elements of the stomatal development pathway include the widely examined peptide signalling cascade involved in SPCH phosphorylation and subsequent inactivation (Figure 1). Pathway members include various mitogen activated protein kinases (MAPKs) that participate in the phosphorylation cascade and the signalling peptides EPF1/2 and STOMAGEN, which act antagonistically on ERECTA family receptor kinases (ERf) and their TOO MANY MOUTHS (TMM) coreceptor to regulate stomatal development [83,86].

An additional coreceptor, from the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family, has been suggested as important for the stomatal lineage. SERK genes have ancient origins traced to algae and exist in multiple copies (SERK1-5) in *Arabidopsis*. Studies have shown SERK gene redundancy in that knockout mutants for individual SERK genes are not observed to affect stomatal development, yet complete knockout mutants produce abnormal stomatal clustering [87]. SERKs are unusual in that these receptors have a wide range of implicated roles in plant physiology, including modulation of apoptosis and plant immunity—they remain an enigmatic aspect of the stomatal pathway in need of increased research [83]. SERKs are documented to behave as coreceptors in the BRASSINOSTEROID (BR) pathway, which negatively regulates stomatal development by phosphorylation/inactivation of BRASSINOSTEROID INSENSITIVE 2 (BIN2), preventing BIN2’s phosphorylation/inactivation of YODA in the MAPK cascade [88]. Hence, negative regulation occurs through continued activity of YODA, leading to SPCH inhibition. However, as shown by Figure 1, the BR pathway is also found to positively modulate stomatal development by preventing BIN2-mediated phosphorylation of SPCH, leading to increased SPCH activity [89]. Due to the conflicting effects of the BR pathway, there is likely additional regulatory effects in action—it has been proposed that discrepancies in BR regulation are due to differential function of the BR pathway in the cotyledon and hypocotyl, however this inference requires further analysis [90].
Figure 1. Pathway underlying stomatal development based on combined experimental data collected for the model plant Arabidopsis thaliana. In the peptide signalling pathway, negative regulators EPF1/2 compete with STOMAGEN for binding sites on TMM/ERf/SERK complexes to activate the MAPK cascade and inhibit activity of SPCH downstream by MPK3/6-mediated phosphorylation of serine and threonine residues in the N-terminus and MAPK target domain of the SPCH transcription factor. Through action of brassinosteroids (BR) on BRASSINOSTEROID INSENSITIVE 1 (BRI1)/SERK receptor complexes, both positive and negative regulation of stomatal development can occur. By BR negative regulation, the activity of BIN2 can be suppressed, preventing YODA phosphorylation (leading to the continued inhibition of SPCH activity). By BR positive regulation, BIN2 suppression prevents phosphorylation of SPCH, hence maintaining its activity. Active SPCH stimulates the progression of PDCs into MMCs, and MMC transformation into SLGCs and meristems by asymmetric (entry) division. MUTE activity modulates GMC formation and FAMA activity modulates symmetric divisions as GMCs transition into mature guard cells. ICE1/2 associates with SPCH, MUTE and FAMA to ensure appropriate functionality of the bHTH transcription factors in the regulation of cell fate transitions. Additionally included are the cell fate transition possibilities for the various cells of the stomatal lineage. MKK4/5/7/9 and MPK3/6 refer to various mitogen-activated protein kinases. HTH refers to the helix-turn-helix motif of the SPCH transcription factor. BSUs refer to BRI1 suppressors in the BR regulated pathway (acting upstream of BIN2).
A well-known member of the stomatal development pathway is the signalling peptide EPF1, which has been covered previously in our examination of gene-directed stomatal density control in the cereals. EPF1 competes with STOMAGEN for binding sites located on TMM/ERf receptor complexes. EPF1 binding serves to block the activity of SPCH, triggering autophosphorylation of the ERf receptor complex and subsequent activation of the MAPK cascade, ultimately leading to phosphorylation of serine and threonine residues in the MAPK domain and the N-terminus of the SPCH protein [83]. As STOMAGEN is a positive regulator of stomatal development, its binding to the receptor complex inhibits downstream activation of the MAPK cascade. The potential of controlling EPF1 expression as a negative regulator of stomatal density has been widely demonstrated in recent trials [48,58,62]. However, further work is needed to determine the phenotypic effects of manipulating associated members of the peptide cascade in cereal species. For example, experimental work has been completed in *Arabidopsis* for a sister peptide, EPF2, which also competes with STOMAGEN for binding sites on TMM/ERf receptor complexes. EPF2 has been identified as an early pathway peptide, determining the quantity of protodermal cells that will enter the lineage, whereas EPF1 is critical later in the lineage during the GMC differentiation stage despite having the same inhibitory effect on SPCH [91]. As with EPF1, overexpression studies on EPF2 have demonstrated greatly improved WUE in model plants—hence, further analysis on the manipulation of EPF2 orthologs in cereals would be beneficial to determine whether EPF2 can serve as an additional pathway regulator in the production of climate adapted cultivars [63].

Although SPCH serves as a molecular entry gate into the stomatal lineage, SPCH overexpression does not increase stomatal number, yet does cause ectopic epidermal cell differentiation [92]. This indicates that SPCH does not directly influence terminal differentiation of cellular intermediates into guard cells, but rather acts on the divergence of cellular intermediates in the amplifying and spacing pathways of asymmetric division. SPCH is noted to exhibit indirect control of stomatal formation, where SPCH levels determine the path of progression of meristemoid cells through the stomatal lineage. This was demonstrated in a study by Pillitteri and Torii (2007), where meristemoids with extended SPCH activity were suppressed from exiting a stem cell-like state of asymmetric division [93]. The antagonistic relationship between MUTE and SPCH has also been identified, with increased intracellular MUTE levels serving as a critical switch for meristemoid escape from asymmetric division and differentiation into GMCs [94,95]. Expression of FAMA is induced post-differentiation of stomatal cell precursors into GMCs, with the primary function to arrest further symmetric division and ensure termination of proliferative meristematic activity to preserve guard cell morphology and functionality. Without the inhibitory regulation of FAMA, stomatal development is hijacked by incessant symmetric division of GMCs, forming tumours that ultimately disrupt epidermal physiology [96]. Despite the activities and expression patterns of SPCH, MUTE and FAMA being clearly illustrated to be co-integrated, at present little is known about interactions between these critical master regulators of stomatal development [97]. For instance, specific mechanisms pertaining to how SPCH and MUTE may modulate each other’s expression are currently unknown [97]. Studies have shown that SPCH binds to the MUTE promoter; however, further investigation is required to determine the molecular factors involved in the initiation of MUTE promoter binding and subsequent domination of MUTE in late meristemoids [97,98].

Beyond the peptide induced signalling cascade, the heterodimeric partners INDUCER OF CBP EXPRESSION 1/SCREAM (ICE1/SCRM) or ICE2/SCREAM2 (SCRM2) associate with SPCH, MUTE and FAMA and are critical for ensuring appropriate cell-fate transitions within the stomatal lineage [81]. Together, SPCH and the transcriptionally self-activated ICE1/2 contribute to a negative feedback loop through the targeted enhancement of TMM and EPF2 expression, therefore reducing SPCH activity to prevent any further protodermal cells from entering the stomatal lineage [99,100]. It was found that MUTE exhibits binding capacity to TMM and ICE1/2 promoters and is critical for FAMA initiation and thus stomatal maturation through FAMA promoter binding [95]. Both MUTE and SPCH are capable of
binding to the EPF2 promoter with conflicting effects on expression, such that MUTE acts to repress the original SPCH signal by reducing EPF2 transcript levels, whilst maintaining the TMM/ERf complex for binding to EPF1 [95]. EPF1 is critical in the maintenance of stomatal patterning, predicted to suppress cells adjacent to GMCs from becoming guard cells by inhibiting their progression through the stomatal lineage [99].

Through a detailed examination of genetic pathways involved in stomatal development, it is clear that gene product interactions are highly dynamic. Such sheer complexity of pathway interactions highlights a need to focus on key regulatory switches, such as the bHTH transcription factors MUTE, SPCH and FAMA to guide our ability to alter stomatal variation. With greater resolution regarding the extent of stomatal control of these regulatory switches, we may gain a greater capacity to fine-tune stomatal distribution characteristics in commercial plants for enhanced climate adaptation.

4. Stomatal Development in the Dicotyledons versus the Grasses

The genes regulating stomatal development possess a remarkable level of conservation across plant families. A recent study illustrated the ancient origins of the TMM/EPF/ERECTA signalling pathway, where insertion of the moss Physcomitrella patens derived TMM and EPF1 genes into Arabidopsis thaliana EPF knockout mutants was capable of partially restoring stomatal density phenotypes [101]. The functional extension of the TMM/EPF module from basal bryophytes to A. thaliana provides significant evidence that the TMM/EPF/ERECTA module is also highly likely to be functionally conserved in the grass stomatal lineage. The evident conservation of gene products in the stomatal development pathway is further highlighted in studies examining cereals, where master regulator SPCH, MUTE and FAMA orthologs were identified in rice and maize [102,103]. In addition, SPCH and FAMA orthologs have been identified as required for correct stomatal distribution and formation in rice cultivars [103,104]. Despite inferred conservation of stomatal development genes across plant families, the extent to which gene products are under parallel mechanisms of control is highly questionable, as evidenced by a combination of major morphological differences in stomata and their patterns of development between dicotyledons and the grasses.

As shown by Figure 2, observational studies in the grasses versus the model dicotyledon Arabidopsis thaliana reveals a stark divergence in stomatal complex development. Stomatal development under an A. thaliana model involves the formation of kidney shaped guard cells by a mesogenous pathway, where cells of the stomatal complex and their neighbours each descend from progenitors in the stomatal lineage [105]. Members of the grass family produce guard cells of a dumbbell morphology, flanked by subsidiary cells (SCs) that are formed by perigenous development, such that SCs of the stomatal complex are not descended from progenitors in the guard cell stomatal lineage [106]. Unlike the pavement cells (PCs) of A. thaliana, PCs surrounding grass stomatal complexes originate from the asymmetric divisions of subsidiary mother cells (SMCs) and are thus also of perigenous origin (Figure 2). The molecular basis behind such developmental variation in grasses has been recently assessed using a Brachypodium model and has revealed a number of discrepancies between the dicot and monocot stomatal lineage. First, SPCH has undergone a duplication event in grasses, leading to a paralogous pair with partial redundancy directing early stomatal development [103,104]. The SPCH pair holds potential in cereal stomatal density control, as SPCH2 knockouts show greater reduction in density than SPCH1 knockout mutants [107].
Figure 2. Illustration of pathway variability in stomatal development in *Arabidopsis thaliana* versus the grasses. In grasses, GMCs and subsidiary mother cells (SMCs) formed within two separate lineages associate to form mature stomatal complexes consisting of a guard cell pair flanked by subsidiary cells (SC) of perigenous origin (A, D). In *Arabidopsis*, a series of cell state transitions of the MMC leads to the formation of a guard cell complex with surrounding pavement cells (PC) of mesogenous origin (B). (C) shows the stages of mature stomatal complex formation in the grasses. ICE1/2 and the SPCHs have roles in stomatal file specification and selection of precursors for GMC formation. As in *Arabidopsis*, MUTE (in tandem with ICE1/2) modulates grass GMC formation. However, MUTE is also translocated from GMCs (light green cells) to adjacent cells to promote SMC formation and polarisation (large purple cells). PAN2 has roles in SMC asymmetric division by inducing function of PAN1 downstream, forming PCs (white with purple nuclei) and SCs (small purple cells). FLP acts to ensure appropriate orientation during symmetric division of GMCs into GdCs. FAMA and ICE1/2 activity is required for appropriate maturation of the stomatal complex. FAMA has additional roles in subsidiary cell formation. SCR and SHR operate in concert to regulate cell entry into the stomatal lineage, subsidiary cell formation, and stomatal complex maturation. EPF1 is predicted to negatively regulate ICE1 through a putative MAPK cascade; however, this requires experimental verification.

Contrasting *A. thaliana*, ICE1 and ICE2 do not exhibit functional redundancy in some grasses, where ICE1 acts during asymmetric entry division, while ICE2 contributes to the modulation of guard cell morphogenesis and differentiation under a *Bradypodium* model [107]. Studies in rice have shown that both ICE1 and ICE2 interact with SPCH, MUTE and FAMA during all cellular transitions of the stomatal lineage [108]. However, knockout mutants for ICE1 could not form stomata, versus ICE2 knockout mutants, which exhibited no stomatal defects [108]. This result indicates that in rice, ICE1 and ICE2 have partially divergent roles, such that ICE1 is critical for cellular entry into the stomatal lineage in a similar manner to *Bradypodium*. Furthermore, some divergence of functionality has been characterised for MUTE and SPCH in the monocot lineage [103]. Deviation of MUTE...
functionality in grasses is illustrated in recent studies unveiling that *MUTE* is critical for forming the grass exclusive SCs by promoting SC recruitment [109]. The prominence of *MUTE* as a major regulator of stomatal development in grasses has excellent potential for future cereal improvement. It has been extensively demonstrated that SC mechanics allow for more effective regulation of stomatal aperture and that *MUTE* knockouts are incapable of SC formation [104,109]. Thus, using *MUTE* as a molecular lever to modulate SC development provides an additional target for cereal climate adaption and enhanced WUE by genetic improvement. Raissig et al. (2017) revealed that *MUTE* migrates from GMCs into SMCs, indicating that *MUTE* activity in SC development is still derived from the stomatal lineage and translocated to progenitor SCs [109]. Future studies focusing on the regulatory mechanisms directing *MUTE* transport to progenitor SCs, and *MUTE* targets of the SC development pathway may be beneficial to determine how best to regulate SC formation for cereal climate adaptability.

*FAMA* orthologs have been inferred to maintain similar functionality between dicots and the grasses, yet studies indicate that *FAMA* is not required in the suppression of excessive symmetric division of GMCs in grasses and instead is predicted to work in concert with ICE1/2 in morphogenesis of the stomatal complex to full maturity [105,108]. The results of a recent study focused on rice also unveiled the role of *FAMA* in subsidiary cell formation [108]. An ortholog of the *FOUR LIPS (FLP)* transcription factor gene was recently identified in rice to share functionality with the *FLP* gene in *Arabidopsis*, acting during the transition phase from GMC to GdC [108]. Novel SCR (SCARECROW) and SHR (SHORTROOT) genes have additionally been characterised in rice, with the genes observed to participate in multiple stages of stomatal development. These include the regulation of stomatal lineage initiation, subsidiary cell formation and final morphogenesis of the stomatal complex [66,108]. The prominent role of SCR and SHR in the rice stomatal lineage further highlights divergent characteristics of the grass stomatal development pathway in relation to eudicot species.

There exist similarities in peptide signalling for stomatal development between *A. thaliana* and the grasses. For instance, *EPF1* overexpression reduces stomatal density in barley as documented in *A. thaliana* [99]. Further interrogation of barley *EPF1* is required to determine whether *HvEPF1* functions to regulate the stomatal lineage through a similar pathway to *A. thaliana* or via an alternate system. Strangely in grasses, ICE1 appears more detrimental to stomatal development than SPCH, as hinted by changes to MAPK target domains in *Brachypodium SPCH* versus *A. thaliana*. ICE1 knockouts are stomatalless in *Brachypodium* even when ICE2 and ICE1 prevent stomata formation in *Arabidopsis* [107]. Thus, it is predicted that putative MAPK cascades exist in grasses that regulate cell entry into the stomatal lineage primarily by *EPF1*-induced ICE1 targeting via MAPK mediated phosphorylation, leading to down-regulation of ICE1 rather than SPCH *(Figure 2)*; however, further experimentation is needed to confirm pathway mechanics [105]. In wheat, orthologs have been identified for a related peptide, *EPIDERMAL PATTERNING FACTOR*-like protein 9 (*EPFL9*), that is a known positive regulator of stomatal development in *A. thaliana* [104]. In rice, *EPFL9* knockouts exhibit 8-fold reduction in stomatal density relative to controls, indicating the presence of both positive and negative regulatory mechanisms governing the grass stomatal development pathway in relation to eudicot species.

A combined assessment of dicot and monocot pathways of stomatal development reveals multiple points of contrast. Three major differences exist between the grasses and *A. thaliana*, as shown by *Figure 2*. First, no meristemoid stage exists in grasses, suggesting stomatal precursors directly form GMCs by asymmetric division and thus do not exhibit self-renewal capacity [111]. Second, the formation of SCs in grasses by pathway mechanics that are currently not well characterised. Finally, the formation of filed stomata parallel to the leaf vein, leading to ordered stomatal distribution in grasses versus scattered distribution in *A. thaliana* [104]. Since high levels of conservation have been revealed to exist in genes that regulate stomatal development in plant families, such variation in stomatal development may be attributed to alternative wiring of gene networks, such as
the modification of ICE1, ICE2 and MUTE roles observed in grasses. Hence, although our understanding of stomatal development is extensive in *A. thaliana*, there is substantial evidence that the use of an *A. thaliana* model for genetic improvement is limiting our ability to harness the true molecular potential of stomatal control in cereal crops. It is thus essential that a greater understanding of the grass stomatal lineage and its regulatory mechanisms are manifested to ensure the increased productivity of cereal crop molecular selection and breeding strategies.

5. Climate Resilient Cereal Crops through Genetic Improvement Using Barley as an Example

The barley genome is expansive and one of the largest sequenced to-date, consisting of 5.1 Gb (Giga base pairs) and over c.39,000 coding genes in a set of seven chromosomes [112,113]. Its diploid nature makes barley a flexible species for experimental analysis in assessing molecular mechanisms underlying agronomically favourable traits [114]. A defining feature of the barley genome is its large composition (80%) of mobile genetic elements, serving as building blocks for epigenetic modulation [113,115]. Over the past decade, the barley draft sequence has served as a critical tool for identifying genes associated with important agronomic traits pertaining to yield and environmental resilience [116–118]. Further advances have led to the completion of the barley reference genome, which opens additional avenues for genetic interrogation [112,119].

With access to the complete barley genome, novel suites of genes are rapidly being characterised that are suspected to underlie phenotypes attributed to increased yield, improved grain quality, and tolerance to biotic and abiotic stress. For instance, a recent study has unveiled a series of QTLs in barley suspected to influence variation in grain size and weight, which led to the isolation of 45 associated genes [120]. Grain size and weight are major determinants of cultivar productivity and can ultimately bolster the economic viability of a selected genotype. Using complete genome data, Wang et al. (2019) were able to gain increased resolution to identify genes controlling grain size, and also discover the extension of function of a smaller subset of genes in other cereals by identifying orthologous sequences in rice, maize and wheat [120]. This discovery in turn suggested the presence of similar molecular control mechanisms of grain size and weight as barley in other cereals, which enhances the potential of manipulating these candidate genes to bolster yield characteristics across a range of crop species.

In addition to yield associated traits, reference genome assisted studies have expanded to identify critical genes for barley environmental tolerance, including those implicated in salinity, heat and drought tolerance [121–123]. With the advent of whole genome sequence accessibility, QTL mapping for agricultural traits has become highly efficient and thus an increase in QTL based studies has occurred in recent years for genomic characterisation [124]. A study investigating the genetic basis of the black grain trait in barley, identified a suite of 21 candidate genes isolated through QTL mapping and fine mapping of a doubled haploid population derived from a cross of Tibetan landrace W1 and commercially relevant Hindmarsh genetic lines [125]. Resolution of the statistically significant marker containing region was made possible with the aid of complete reference genome data, which also served critical for the design of new primers for novel markers used in fine-mapping [112,125]. Future functional analysis of critical genes within the QTL mapping region would be considerably beneficial to gain further insight into the genetic basis of barley environmental resistance, as the black grain trait is noted to be associated with a variety of biotic and abiotic stress tolerance mechanisms in wild populations, to the extent where only pigmented grain phenotypes are prevalent at altitudes beyond 4000 m above sea level [126].

Indeed, with advancements in bioinformatics and genetic databases, there exists a plethora of community-accessible sequence information and database alignment tools. The Barleymap tool combines marker information from the combination of Barley Physical Map and the Morex reference genome data [112,116]. In addition, the ENSEMBL (https://plants.ensembl.org/index.html: accessed on 10 May 2021) and GrainGenes
(https://wheat.pw.usda.gov/: accessed on 10 May 2021) genome browsers, which use the IBSC_v2 and MorexV2 assemblies, respectively, serve as valuable resources for the identification of novel candidate genes underlying agronomic traits of interest. Both databases provide a combination of easily accessible information pertaining to sequence data, gene structure and domain architecture to assist in bioinformatics based functional prediction. Such technology can aid in further understanding the underlying gene regulation of agronomic traits in barley. Post-gene identification, the presence of CRISPR/Cas9 gene-editing technology grants the potential to build gene networks in concert with transcriptomics, to further enhance our understanding of gene functionality to produce superior barley cultivars genetically suited for specified agricultural purposes. Such work has recently aided the construction of regulatory networks in barley under drought stress [127,128].

Current research has provided significant knowledge regarding the genetic basis of yield and stress tolerance related phenotypes in barley species. In particular, a substantial level of resolution has been gained regarding the identification of genes implicated in barley drought tolerance. This has been achieved by QTL mapping studies focusing on populations generated from crosses of agronomically relevant cultivars and wild barley lines. Resistance to drought in barley is a complex quantitative trait—a notion that is consolidated by the relatively low individual impact of single QTLs on drought tolerance phenotypes observed across multiple studies [129,130]. There exists a generous level of data underlying the genetic basis of drought tolerance. However, a substantial investigation is yet to be conducted regarding the molecular characterisation of barley stomatal development, despite stomatal density and morphology being repeatedly documented as major contenders for the genetic improvement of WUE, in addition to possessing relatively high values of heritability [62,131,132]. This situation is made further urgent by the fact that no data currently exists on the identification of critical genes in barley stomatal development. Any barley related studies that have investigated stomatal density, all use gene orthologs that have been characterised in an A. thaliana model [62]. As discussed earlier, striking differences are observed in both stomatal morphology and underlying developmental pathways between A. thaliana and the grasses. This hinders the effectiveness of genetic analysis for stomatal control in cereal crop species based on an A. thaliana model. To discover prominent genes influencing stomatal characteristics in barley, molecular strategies can be improved for breeding cereal species with high WUE. Access to community genome data allows for accelerated characterisation of candidate genes underlying key agronomic traits by QTL identification.

In recent years, genome-wide association studies (GWAS) have become an increasingly powerful tool in agricultural genetics, with the potential to reveal suites of genes previously unknown to be associated with complex quantitative traits, or otherwise confirmed conserved functions of genes previously identified in closely related plant species [133]. The emergence of GWAS as a powerful tool in the investigation of quantitative traits and the genetic mechanisms underlying their variability, can be attributed to rapid advancements in next-generation sequencing technologies. These technologies allow for greater resolution of complex genetic signatures in time frames previously unachievable with past methodologies due to technical limitations [134]. The effectiveness of GWAS has been repeatedly demonstrated as a multitude of publications continue to report novel QTLs for agronomic improvement, including those associated with morphological traits impacting yield. For instance, a GWAS on drought tolerance recently revealed a number of candidate genes positively associated with tiller number, which is a morphological phenotype critically linked to yield [135].

Through using the barley reference genome, it becomes possible to identify the candidate genes implicated in stomatal density through GWAS and/or QTL mapping using a range of bioinformatic tools and techniques. Candidate gene identification may assist in the future design of molecular markers specific to candidate genes controlling stomatal density, for cultivar improvement through molecular marker-assisted selection [136]. Further database assisted interrogation of candidate genes may aid in functional prediction for
future analysis. Moreover, through the use of transcriptomic data collected from future CRISPR/Cas9-induced gene knockouts or overexpression mutants for the identified candidate gene(s), this can potentially direct the construction of gene networks modulating stomatal density control in economically important cereal species. Rapid advancements in molecular technologies open new pathways for the acceleration of cereal improvement of WUE. In recent years, omics technologies have emerged as effective tools for the improvement of cereal drought tolerance. Transcriptomic studies can provide an intricate snapshot of the gene expression landscape during drought stress events. This can assist in the capture of not only novel gene sequences for drought tolerance regulation, but also the pathway interactions between genes expressed under water restriction. One such study in barley has revealed a series of genes involved in environmental sensing, including genes encoding osmotic biosensors, which likely interact with hormonal signalling pathways [137].

The transcriptomic environment of the stomatal lineage has previously been established in Arabidopsis [138]. It is recommended that future transcriptomic studies be performed on the stomatal lineage in barley and other cereals, given the power of such analysis in elucidating stomatal gene network composition and future targets for enhancing WUE. Proteomic and metabolomic studies have served as additional outputs for building an enhanced understanding of barley drought tolerance. In barley, hundreds of drought-induced proteins and metabolites have been characterised under drought stress. Such proteins and metabolites were linked to a number of affected pathways, including photosynthesis and nitrogen metabolism [139]. Increased understanding of protein and metabolite profiles in barley and other cereals, can also assist in the identification of novel gene networks that are up- or down-regulated under drought stress response. Epigenetic regulation in cereals serves as another major target in future crop breeding. An increased capacity to modify crop epigenetic profiles may allow for alteration of the transcriptomic environment, allowing for the production of new phenotypes without alteration of gene sequences [140].

Finally, breeding through genomic selection is now becoming increasingly more effective as full genome profiles increase in detail and coverage for multiple crop species. Using combined marker information across the entire genome, a number of studies have shown that genomic selection models are capable of predicting varieties with the greatest genetic potential with high accuracy for wheat, barley, maize and oat [141].

A small suite of genes have been functionally characterised for the regulation of stomatal development in a number of cereal crops, as listed in Table 2. As a result of prior functional validation, such genes may serve as potential molecular targets for future CRISPR-Cas9 directed gene-editing. For example, current commercial crop varieties may be edited to target negative regulators of stomatal density in order to generate over-expression lines with reduced stomatal density and enhanced WUE. Conventional breeding methods have historically taken 10–15 years or more for the improvement of commercial plant varieties [142]. Thus, with the advent of gene-editing technologies, time frames from genetic modification to commercial production may be effectively reduced. In addition, targeted gene-editing means that secondary traits will not be incorporated in addition to the trait of interest, which is a common caveat of conventional breeding methods [143].
### Table 2. List of predominant genes experimentally verified to regulate stomatal development in various cereal crops.

| Species | Gene Name | Function | Citation |
|---------|-----------|----------|----------|
| Rice    | RSD1 (RICE STOMATAL DEVELOPMENT DEFECT 1) | Inhibits ectopic asymmetric division and clustering, stomatal file specification, regulator of stomatal development, required for normal expression of related genes in the stomatal development pathway. | [144] |
| Rice, Maize | SDD1 (STOMATAL DENSITY AND DISTRIBUTION 1) | Inhibits ectopic asymmetric division and clustering, negative regulator of stomatal density. | [69,144] |
| Rice    | SCR1/2 (SCARECROW 1/2) | Controls cell entry into the stomatal lineage (meristemoid mother cells to meristemoids), regulates subsidiary cell formation, expression regulated by OsSPCH and OsMUTE (Rice). | [108,145] |
| Rice, Maize | SHR1/2 (SHORTROOT 1/2) | Stomatal file positioning, controls cell entry into the stomatal lineage, regulates subsidiary cell formation, regulator of stomatal density. | [66] |
| Rice, Maize, Bradypodium | MUTE | Guard mother cell formation, migrates to subsidiary mother cells to induce subsidiary cell formation, regulates expression of multiple genes in the stomatal development pathway. | [108,109,146] |
| Rice, Bradypodium | SPCH1/2 (SPEECHLESS) | Controls cell entry into the stomatal lineage, stomatal file development, SPCH2 knockout mutants exhibit a greater decrease in stomatal density versus SPCH1 knockout mutants. | [103,107,108] |
| Rice    | FLP (FOUR LIPS) | Negative regulator of guard mother cell symmetric division, maintains orientation of symmetric division in guard mother cells. | [108] |
| Rice    | FAMA | Regulation of subsidiary mother cell asymmetric division, ensures correct formation or mature guard cell complexes. | [107,108] |
| Rice, Bradypodium | ICE1/2 (INDUCER OF CBF EXPRESSION 1/2) aka SCREAM1/2 | Heterodimeric partner of SPCH, MUTE and FAMA, regulation of cell entry into the stomatal lineage, guard mother cell formation, guard cell maturation. | [107,108] |
| Rice, Wheat, Barley | EPF1/2 (EPIDERMAL PATTERNING FACTOR1/2) | Negative regulator of stomatal development, inhibits SPCH activity. | [48,58,62] |
| Bradypodium | YODA (YDA) | Negative regulator of stomatal development, regulates asymmetric cell divisions, inhibits stomatal clustering, maintains normal stomatal spacing/patterning, inhibits SPCH activity. | [147] |
6. Conclusions and Future Directions

We are now living in an era where the agricultural impacts of drought are becoming increasingly evident, as reflected in observed yield losses and reduced survivability of essential crops globally. Previous studies have demonstrated that reducing stomatal density enhances WUE, thus improving drought tolerance in cereals with minimal impacts on yield, photosynthetic efficiency and CO₂ assimilation. Its combined economic importance, excellent adaptive capacity and general amenability to extreme reductions in stomatal density, make barley a desirable candidate for stomatal modifications to produce yield-stable, drought-tolerant cultivars. Effective improvement of barley WUE requires a detailed understanding of the genetic mechanisms underlying stomatal density control, to which data is expansive in the model eudicot Arabidopsis, but virtually absent for the monocotyledonous grasses. A variety of molecular techniques now exist which can allow for rapid identification of genes implicated in stomatal formation in cereals. Future studies should focus on building a greater understanding of the genetic interactions underpinning stomatal development in grasses with a reduced reliance on model eudicots such as Arabidopsis. To build a transcriptomic profile of the specific genes expressed in a developing stomatal complex would serve as an excellent starting point for establishing the grass stomatal gene network. GWAS and QTL mapping may serve as additional methods for the identification of genes governing the stomatal lineage in grasses. An improved understanding of the molecular mechanisms governing grass stomatal development may provide novel genetic avenues for fine-tuning stomatal density characteristics. Direct targeting of genes with CRISPR-Cas9 technology will allow for effective production of reduced stomatal density varieties, in a timeframe exceedingly more rapid than a conventional breeding program. This may, in turn, fast-track our production of cultivars for introduction into global markets where drought is a limiting factor on agricultural production.

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