Stomatal development: focusing on the grasses
Christopher Hepworth¹, Robert S Caine², Emily L Harrison², Jennifer Sloan¹,² and Julie E Gray²

The development and patterning of stomata in the plant epidermis has emerged as an ideal system for studying fundamental plant developmental processes. Over the past twenty years most studies of stomata have used the model dicotyledonous plant Arabidopsis thaliana. However, cultivated monocotyledonous grass (or Gramineae) varieties provide the majority of human nutrition, and future research into grass stomata could be of critical importance for improving food security. Recent studies using Brachypodium distachyon, Hordeum vulgare (barley) and Oryza sativa (rice) have led to the identification of the core transcriptional regulators essential for stomatal initiation and progression in grasses, and begun to unravel the role of secretory signaling peptides in controlling stomatal developmental. This review revisits how stomatal developmental unfolds in grasses, and identifies key ontogenetic steps for which knowledge of the underpinning molecular mechanisms remains outstanding.

Introduction
Stomata function as the interface between plants and atmosphere, exerting control over gaseous diffusion and balancing the uptake of carbon dioxide with the loss of water vapour [1]. Regulation of stomatal development is of critical importance in allowing plants to adjust their gaseous exchange to suit the prevailing environmental conditions [2–4]. Stomatal development has been extensively studied, and has emerged as an excellent system for investigating cell-fate specification and cellular differentiation [5,6**]. The distribution of stomata on the leaf surface is a highly regulated process with a level of plasticity, and components regulating stomatal development continue to be identified [7,8]. Much of our current understanding stems from work conducted on the model dicot Arabidopsis thaliana and many comprehensive reviews are available [9,10].

Although cereal grasses provide the majority of human nutrition we still know surprisingly little about their stomata. As scientific focus moves towards the engineering of ‘climate ready crops’ that will be better suited to predicted warmer, drier, higher carbon dioxide environments, understanding the regulatory mechanisms of grass stomatal development and patterning could prove key to future success. In this review, we outline recent advances emerging from studies of grasses and discuss the outstanding questions.

The grass stomatal lineage
The development of stomatal complexes in grasses differs to that of the dicots in a number of ways. Most notably, grass stomata are formed from dumbbell-shaped guard cells (GCs) that are flanked by subsidiary cells (SC) which develop in parallel rows within defined and specific epidermal cell files. In contrast, the GCs of dicots are kidney-shaped and form stomata that are scattered throughout the epidermis in a less orderly pattern. In nascent leaves, grass stomatal development occurs along a spatiotemporal gradient with the earliest stages occurring basally, and proceeding as cells move upwards as the leaf expands [11,12]. This developmental pathway can be broken down into 6 stages and is illustrated using barley (Hordeum vulgare) in Figure 1. Initially, close to the leaf base, prior to stomatal-lineage cell specification, potential precursor cells proliferate in particular files (Stage 1). As undifferentiated cells are pushed further up the leaf blade alternate cells enter the stomatal development pathway via an asymmetric ‘entry’ division leading to a smaller guard mother cell (GMC) and a larger sister cell (Stage 2). Cells from files on either side of a newly formed GMC then also divide asymmetrically to form subsidiary mother cells (SMCs) (Stage 3). After the cells have increased in size, mature GMCs are flanked by two nascent SCs (Stage 4), a final symmetric division of the GMC leads to the formation of two immature GCs (Stage 5). The stomatal complex matures and expands to form a pair of dumbbell-shaped GCs, which separate to form the stomatal pore (Stage 6). Thus, each mature grass stomatal complex includes a central pore, a dumbbell-shaped GC pair and two flanking SCs. Each complex overlies an airspace,
or ‘sub-stomatal cavity’, which forms between the mesophyll cells of the underlying layer, to facilitate efficient gaseous diffusion in and out of the leaf. Several recent studies provide insights into the transcriptional and regulatory mechanisms underpinning grass stomatal development. These make use of grass genome sequences and build on knowledge gained from Arabidopsis.

**Brachypodium: A model for recent discovery**

Despite differences in morphology and patterning, the basic helix-loop-helix transcription factors underpinning stomatal fate in Arabidopsis, SPEECHLESS (SPCH), MUTE and FAMA together with heterodimeric partners INDUCER OF CBF EXPRESSION1 (ICE1) and SCREAM2 (SCRM2) are highly conserved, with origins which predate the divergence of the mosses and hornworts from ancestral land plants \[5,6^{**},13,14^{**}\]. The discovery of functionally orthologous grass genes \[15–18\] has shed light on the mechanisms responsible for stomatal development and patterning in grasses. Liu et al. \[12\] investigated putative orthologues of *SPCH*, *MUTE* and *FAMA* in both rice and maize (*Zea mays*) and revealed at least one *SPCH* and a *FAMA* gene that are required for stomatal development in rice. More recently, Raissig et al. \[14^{**}\] used the wheat relative *Brachypodium distachyon* (Brachypodium), to dissect the roles of grass *SPCH* and
ICE/SCRM orthologues. They found that although Brachypodium uses SPCH and ICE/SCRM gene products to regulate stomatal formation, the grass pathway is ‘alternatively wired’ to achieve correctly patterned stomata. Specifically, a SPCH duplication event has occurred in grasses leading to two functional but partially redundant paralogues: BdSPCH1 and BdSPCH2 which both act early during stomatal development. For ICE/SCRM family members, a divergence of function has occurred in comparison to Arabidopsis orthologues. Rather than being functionally redundant, BdICE1 and BdSCRM2 control overlapping stages of stomatal development; BdICE1 primarily functions during the initial asymmetric entry division, BdSCRM2 acts later during the differentiation of GMCs prior to the formation of SMCs [14**]. The observation that the expression of the BdSPCH1/2 and BdICE1/SCRM2 genes is limited to stomatal cell files suggests that the regulation of these genes or proteins across the leaf blade is critical for the correct patterning of stomata across the leaf. How such spatial regulation is achieved is a key next line of enquiry.

The presence of flanking SCs is common to all grass stomatal complexes and these cells have long been believed to assist in altering aperture size in a timely and energy efficient manner [19]. However, despite their important role, little has been known about how SCs are developmentally programmed. Again, recent studies in Brachypodium are beginning to shed light on the area. The discovery that BdMUTE moves from GMCs, via plastomesmata, into neighboring SMCs where it acts to establish SMC identity has advanced our understanding of monocot stomatal development considerably [6**]. Mutants lacking BdMUTE function known as subsidiary cell identity defective (sid) plants, produce GCs without flanking SCs. These plants have allowed researchers to test the importance of SCs in grass stomatal behaviour, for the first time. The finding that sid/ plants have reduced stomatal gas exchange and impaired growth, confirm the important role of SCs and suggests opportunities for the enhancement of stomatal aperture control and plant productivity via the targeted manipulation of SC development.

**Signaling peptides regulate grass stomatal development**

In concert with the bHLH transcription factors, a family of cysteine-rich cell-to-cell signaling peptides regulates the cellular divisions and cell fate transitions required for stomatal development. These epidermal patterning factors (EPFs) and their associated receptor components are well-characterised in Arabidopsis with EPF2 primarily regulating asymmetric entry divisions and EPF1 primarily overseeing the differentiation of GMCs and stomatal spacing. The EPF-like peptide known as EPFL9 or STOMAGEN positively regulates stomatal development by competing with EPF2 during early stomatal development to promote stomatal lineage cell fate [20–25].

Recently, Hughes et al. [26] characterised the role of HvEPF1; a barley orthologue of AtEPF1/2 (Figure 2), which when ectopically expressed inhibits stomatal development. Analysis of HvEPF1 over-expressing barley leaf epidermis revealed that many GMCs do not progress to form stomatal complexes. Moreover, high levels of expression of HvEPF1 inhibit the asymmetric ‘entry’ division that produces GMCs, the maturation of GMCs, the production of SMCs and sub-stomatal cavity formation. Thus a grass signaling peptide similar in sequence to Arabidopsis EPF1 and 2 is able to prevent GMC formation and cause the arrest of GMC development prior to SMC generation but how HvEPF1 functions at normal endogenous levels remains to be investigated. One potential function given the large number of arrested GMCs devoid of SCs, is that HvEPF1 primarily downregulates HvSPCH protein levels thereby preventing GMCs from proceeding further through the stomatal lineage. Whether HvEPF1 activity directly or indirectly regulates the HvMUTE gene or protein or other targets downstream of HvSPCH is intriguing area for future study.

The severe reductions in stomatal frequency and gas exchange brought about by increasing HvEPF1 levels led to improved barley drought tolerance and water use efficiency. Any reduced capacity for photosynthesis did not impact on grain production under either well-watered or drought conditions. These results suggest promising routes for cereal crop improvement through stomatal density manipulation. Our knowledge of grass EPF/L function is further extended by a study describing the use of gene editing techniques to knock-out a rice orthologue of Arabidopsis EPFL9, OsEPFL9a (Figure 2) causing up to 8-fold reductions in stomatal density [27**]. These barley and rice EPF/L studies confirm that, as in Arabidopsis, both positive and negative stomatal development regulators are active in grasses.

Phylogenetic and functional analyses suggest that in addition to the stomatal bHLH transcription factors and the epidermal patterning factors, their cognate receptor components TMM and ERECTA family, are almost certainly also conserved throughout land plants [28,29]. This provides a strong indication that a conserved functional stomatal development module exists in the grasses. However, whilst the evidence is clear that a number of EPF/L peptides are conserved between dicots and monocots [13,26], the specifics relating to how each function in grasses is not clear. As several grass genome sequences are now accessible, we revisit the EPF/L story in grasses (Figure 2). Almost all of the grass genomes that we surveyed encode two peptides which cluster closely with Arabidopsis EPF1 and EPF2 stomatal regulators. The exception is wheat (Triticum aestivum) which being hexaploid has multiple orthologues of both EPF1 and EPF2. Strikingly, our analysis reveals that for EPFL9, a
gene duplication event has occurred in the grasses leading to at least two distinct EPFL9-like genes in all species surveyed.

Having identified at least two EPF1/EPF2 genes and two EPFL9 equivalents (Figure 2), the next question is 'Do all of the identified EPFL genes encode peptides that regulate stomatal development, and if so, how do they facilitate communication between developing stomatal lineage cells?' It is clear that grass stomatal complexes are formed by two distinct types of asymmetric divisions (which form the GMC and SMCs) and that the bHLH transcription factors regulating these divisions have to some degree functionally diversified from Arabidopsis [6**,14**]. It remains unknown whether EPF signaling peptides evolved in parallel to bHLH transcription factors to regulate SC development in grasses. Clearly, further functional studies of the potential regulators of grass stomatal formation identified here (Figure 2) and elsewhere [28,34,35] are required to further decipher stomatal development and patterning in grasses.

Complexity of stomatal patterning in grasses

Whilst in Arabidopsis the development of stomata is possible in most parts of nascent leaves this is not the case in grasses. As grass leaves grow and increase in width
more stomatal and non-stomatal files must form. How these are specified remains unknown. Our observations of developing leaves in rice and barley seedlings suggest that in the earlier forming leaves this process is dynamic with files containing stomatal lineage cells at differing developmental stages occurring in close proximity (Figure 3a and b). It is not uncommon to concurrently observe GMCs flanked by nascent SCs in one stomatal file and more recently formed GMCs without SCs in an adjacent file (white arrows, Figure 3a), or symmetrically dividing GMCs with flanking in close proximity to nascent GMCs where SCs have yet to form (white arrows, Figure 3b). With EPF/L peptides known to be important in regulating stomatal lineage cell placement in Arabidopsis [25], it will be interesting to learn to what extent their control extends both within and between stomatal files in grasses.

Our observations also revealed that the linearity of grass stomatal development within files of stomata is not always continuous and that earlier staged cells can occasionally form further from the leaf base than more advanced stomatal structures (Figure 3c and d). This suggests that the stomatal development module in grasses must not only be fluid between cell files but also within a file. We are yet to determine the importance of EPF/L peptide function in enabling such patterning.

**Next steps**

We have begun to gain insights into how grasses regulate the production of stomata. However, a number of fundamental questions remain unanswered. Most notably, ‘What are the regulatory switch(es) that specify which epidermal files will produce stomata during early leaf development?’ and ‘How is the regular spacing of stomata...
within files achieved whilst also maintaining the development and spacing of SCs? Learning the answers to such questions could facilitate the generation of more refined cereal crop cultivars that are better suited to the predicted future climate, or increased frequency of severe weather events. For example, by increasing the number of stomata in grasses and or altering stomatal performance, we may be able to increase the photosynthetic potential of plants [35]. Moreover, increases in stomatal number could lead to transpirational water flux that may be beneficial in aiding root development, and nutrient uptake [36–39]. Conversely, by reducing stomatal number we should be able to improve soil water retention, drought tolerance and water use efficiency [26].

Acknowledgements
We apologize to authors not cited due to space limitations, and thank the BBSRC Newton Rice Research Fund for funding.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
- of special interest
- of outstanding interest

Transcription factor MUTE (an ortholog of Arabidopsis MUTE) was identified as being essential for the development of subsidiary cells in grasses. Interestingly, MUTE was able to move from guard mother cells, via the plasmodesmata, to flowering cells before specifying subsidiary cell formation.


Experiments demonstrating that the same core transcriptional regulators control stomatal initiation in both Arabidopsis and grasses. However, the specific structure, function and regulation of the gene families have diverged.

A functional barley orthologue of Arabidopsis EPF1 (hEPF1) is described. Overexpression of HvEPF1 led to reduced stomatal formation, predominantly by the prevention of guard mother cell maturation. Subsequent physiological analysis revealed reducing stomatal density could improve drought tolerance without a deleterious effect on yield.

Through the use of CRISPR-Cas9 technology the authors mutated the stomatal regulatory gene OsEPFL9 which is related to Arabidopsis EPFL9/STOMAGEN. This led to an 8-fold reduction in stomatal density. This finding suggests a role for positive signaling peptides in grass stomatal development.


