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Review

Grass flowers: An untapped resource for floral evo-devo

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Abstract The abrupt origin and rapid diversification of the flowering plants presents what Darwin called "an abominable mystery". Floral diversification was a key factor in the rise of the flowering plants, but the molecular underpinnings of floral diversity remain mysterious. To understand the molecular biology underlying floral morphological evolution, genetic model systems are essential for rigorously testing gene function and gene interactions. Most model plants are eudicots, while in the monocots genetic models are almost entirely restricted to the grass family. Likely because grass flowers are diminutive and specialized for wind pollination, grasses have not been a major focus in floral evo-devo research. However, while grass flowers do not exhibit any of the raucous morphological diversification characteristic of the orchids, there is abundant floral variation in the family. Here, we discuss grass flower diversity, and review what is known about the developmental genetics of this diversity. In particular, we focus on three aspects of grass flower evolution: (1) the evolution of a novel organ identity—the lodicule; (2) lemma awns and their diversity; and (3) the convergent evolution of sexual differentiation. The combination of morphological diversity in the grass family at large and genetic models spread across the family provides a powerful framework for attaining deep understanding of the molecular genetics of floral evolution.

Key words: awns, evolution of plant development, evolutionary developmental biology, floral sexuality, flower development, flower evolution.

1 Model Systems for Mechanistic Understanding

The evolution of the flower approximately 140 million years ago (Magallón et al., 2015) was a critical event in the history of terrestrial ecosystems. Flowers display fantastic diversity in form. Selection for pollination success—through intricate plant/insect interactions or optimized abiotic pollination—has likely been critical in shaping floral morphological diversity (Fenster et al., 2004; Friedman & Barrett, 2008). Probing floral diversity in an evo-devo framework allows one to ask questions about, for example, the evolution of novelty, morphological diversification, and the convergent evolution of plant form and function (Endress, 2011; Glover et al., 2015; Specht & Howarth, 2015). Thus, floral evolution represents an obvious arena for plant evo-devo.

Despite some significant inroads (reviewed in Glover et al., 2015; Specht & Howarth, 2015), mechanistic understanding of the molecular genetics of floral evolution remains elusive in most systems. Many of the systems in which these questions can be asked—families where floral morphological diversity is most spectacular (e.g., the orchids), or the taxa that are most informative in terms of phylogenetic placement (e.g., the sister to all other angiosperms—*Amborella trichopoda* Baill.), are currently intractable to most experimentation beyond candidate gene studies and comparative gene expression experiments, either on a large or small scale (Chanderbali et al., 2016). Although both candidate gene and comparative gene expression studies have been fruitful (e.g., Bharathan et al., 2002; Whipple et al., 2007; Yang et al., 2014), on their own, neither can reveal the precise mechanistic detail of the molecular evolution underlying morphological evolution. In addition, both candidate gene and comparative gene

Terminology

Anthesis, Time at which a flower is open and releasing pollen and/or pollen receptive.

Awn, A narrow appendage that is an extension of the vascular tissue; can be simple or branched.

Cleistogamous, Self-fertilization without the flower opening.

Diaspore, A 'unit of dispersal'; the seed and enclosing and attached structures.

Dicliny, Any breeding system that includes unisexual flowers. Dicliny includes, for example, monoecy, dioecy, gynodioecy, andromonoecy.

Floret, Reproductive structure in grasses homologous to a single flower in other plants. Contains pistil, stamens, lodicules, palea, and lemma.

Lemma, Outer-most whorl of a grass flower with unknown homology.

Lodicule, Scale-like structures in the grass flower, homologous to inner whorl tepals.

Spikelet, A branch of 1 to several flowers, subtended by a pair of glumes (bracts).

Staminode, Stamen that fails to produce pollen; sterile.

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expression studies depend on our understanding of molecular gene function obtained from model genetic systems. Vast evolutionary distances often separate the dominant model system, *Arabidopsis thaliana* (L.) Heynh. (arabidopsis), from those species most interesting to floral biologists. Conserved gene function cannot be assumed over 100+ million years of evolution, and fine-grained hypotheses about changes in gene function are very difficult to test rigorously without the molecular genetic tools available in model systems (Becker et al., 2011; Chanderbali et al., 2016). It is possible to test some aspects of gene function through heterologous transformation of arabidopsis, but it is very difficult to draw substantive conclusions using distantly related heterologous systems (Kramer, 2015). Identifying quantitative trait loci (QTL) within species can yield substantial insight into the genetic underpinnings of diversity, but QTL studies can only be used to study traits that vary at the species level (Mauricio, 2001). Macroevolutionary traits that are often of the most interest to floral biologists (e.g., the evolution of novel organs) are usually consistent within genera, and vary at deeper evolutionary levels (Endress, 2011). Thus, precisely connecting molecular evolution to floral morphological evolution remains challenging without access to the resources of an established model system.

Multiple model systems in single lineages (orders, families, genera) hold the most promise for detailed insight into the evolutionary molecular genetics of development above the species level (Chanderbali et al., 2016; Damerval & Becker, 2017). This has been clearly illustrated in the Brassicaceae, where functional comparisons between arabidopsis and *Cardamine hirsuta* L. have led to significant insights into the molecular evolution underlying leaf and fruit form (Hay & Tsiantis, 2006; Vlad et al., 2014; Hofhuis et al., 2016). In the eudicots, the Brassicaceae and Ranunculaceae each include multiple established or emerging systems that vary in key morphological traits (Kramer, 2009; Canales et al., 2010; Damerval & Becker, 2017). Studies in these systems allow for the precise dissection of gene function and for making strong connections between molecular and morphological evolution (e.g., Sharma & Kramer, 2013; Vlad et al., 2014).

The monocots represent ~24% of angiosperm diversity, and are characterized by a number of speciose, extremely morphologically diverse clades (e.g., the orchids, the Zingiberales, the Liliales, the palms, the grasses). However, the only family with multiple established genetic model systems in the monocots is the grass family (Poaceae). The grass family includes four more-or-less established model systems—*Zea mays* L. (maize), *Oryza sativa* L. (rice), *Brachypodium distachyon* (L.) P. Beauv., and *Setaria viridis* Beauv. (*Setaria*). Each of these systems is transformable, has a fully sequenced genome, and either a very well-established or growing collection of genetic resources and tools available (Goff et al., 2002; Strable & Scanlon, 2009; Brutnell et al., 2010; Brkljacic et al., 2011). CRISPR/Cas9 genome editing has been demonstrated in maize, rice and *B. distachyon* (Miao et al., 2013; Svitashev et al., 2015; Raissig et al., 2016), and *Setaria* is likely not far behind. In addition, there are a number of emerging systems that are being developed, and several cereal crops (e.g., barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* (L.) Moench)) have established research communities and a growing body of knowledge (reviewed in Chang et al., 2016). We argue that this powerful genetic framework can be fruitfully leveraged for mechanistic understanding of floral evo-devo.

2 A Primer of Grass Flower Morphology

Grass flowers are usually wind pollinated, although there are some reports of insect visitors (Soderstrom et al., 1971; Huang et al., 2002; Sajo et al., 2009; Ruiz-Sanchez et al., 2017) and they exhibit a number of features typical of wind-pollinated plants (Fig. 1). They are small, lack showy petals, usually exsert their anthers on long, thin filaments, and typically have feathery stigmas. Unisexuality, often associated with wind pollination, has evolved multiple times in the grasses, although unisexuality is still not common among the approximately 11,000 species in the family (Friedman & Barrett, 2008; Kinney et al., 2008).

The structure of grass flowers has been very recently and expertly reviewed (Kellogg, 2015), so we will only discuss aspects of the flower relevant to our discussion. A glossary of some specialized terminology is included on the first page. Grass flowers or florets develop in groups of 1–150, clustered on short branches called spikelets. Spikelets are typically subtended by two bracts called glumes and are arranged in inflorescences that resemble spikes, racemes, or panicles.

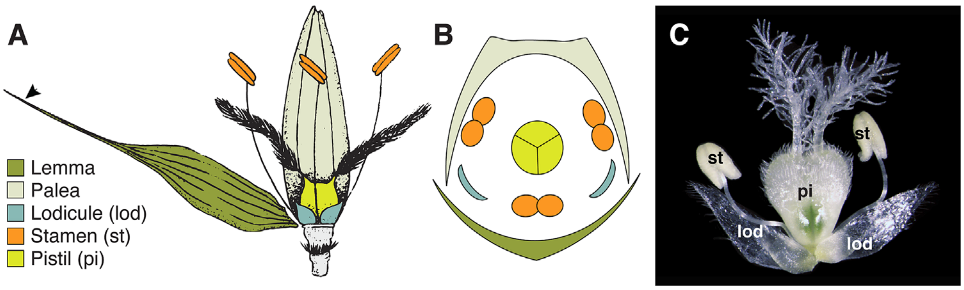

Fig. 1. Grass flower morphology. **A,** An idealized grass flower, showing the most common character states (two fluffy stigmas, three stamens, two lateral lodicules, and a two-keeled palea). The abaxial, awned lemma has been dissected away to reveal the inner whorls. Flower line drawing by Jennie Nguyen. **B,** Idealized grass floral diagram, color-coded as in A (adaxial side upwards). **C,** *Brachypodium distachyon* (Bd21-3) flower. *Brachypodium distachyon* usually has only two stamens. Palea and lemma removed.

Grass flowers contain (moving from the center of the flower) a terminal pistil or gynoecium that develops into a single grain when fertilized, stamens, lodicules, a palea, and a lemma (Fig. 1). Grass pistil and stamen structure is similar to stamen and pistil structure in other wind-pollinated angiosperms. Peripheral to the stamens are two (sometimes 3 or, rarely, 0) lodicules. Lodicules are scale-like organs that, in many grasses, swell at the time of anthesis (flower opening) and push apart the outer sterile organs of the flower. Peripheral to the lodicules is the palea, a bract-like organ. The palea is typically 2-keeled (2 veins) and often so thin that it is translucent. The lemma, another bract-like organ, is positioned opposite and below the palea. The lemma is typically green, vascularized, and resembles a modified leaf or bract. In many grasses, the lemma is elaborated with an awn—an extension of the lemma midvein. Awns may manifest as simple projections on the lemma, as long and needle-like, or as branched and highly elaborated structures (Fig. 2).

In contrast to grass stamens and carpels, which are easily homologized to stamens and carpels in other species, the homologies of lodicules, the lemma, and palea have been less clear. Lodicules have been proposed to be homologous to nectaries, staminodes, or inner whorl tepals. Although lodicules are in the correct position to be derived from inner whorl tepals, their homology is uncertain because they are morphologically distinct from monocot tepals, and serve a mechanical rather than an attractive function in grass flowers (reviewed in Clifford, 1987; Kellogg, 2015).

Results from classical genetics and comparative gene expression studies in the grasses and close grass outgroups have largely resolved the lodicule homology debate (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2007; Bartlett et al., 2015). The sister lineage to all other grasses—the Anomochlooideae—includes two genera —*Streptochaeta* Schrad. ex Nees (1 species) and *Anomochloa* Brongn. (3 species)—neither of which have lodicules (Sajo et al., 2008, 2012). The stamens and carpels in *Streptochaeta* flowers are immediately surrounded by two trimerous whorls of bracts, followed by six additional spirally-arranged bracts (Fig. 2). In *Streptochaeta*, the two whorls of bracts are distinct from one another, and only the innermost whorl expresses the B-class MADS-box genes (Whipple et al., 2007). The B-class MADS-box genes are petal organ identity genes in the eudicots, and play a role in specifying tepal identity in the monocots *Tricyrtis* Wall. and in the orchids *Oncidium* Sw. and *Phalaenopsis* Blume (Mondragon-Palomino & Theissen, 2011; Hsu et al., 2015; Otani et al., 2016). B-class genes are also expressed in second whorl tepals of close relatives of grasses, *Joinvillea ascendens* Gaudich. ex Brongn. & Gris and *Elegia* L. (Whipple et al., 2007), as well as in the lodicules of the grasses maize and rice (Yadav et al., 2007; Bartlett et al., 2015). Critically, B-class genes are essential for lodicule identity. Lodicules in B-class gene mutants are homeotically replaced with palea/lemma-like organs in both maize and rice (Ambrose et al., 2000; Nagasawa et al., 2003; Bartlett et al., 2015). These data support the hypothesis that lodicules and inner whorl tepals

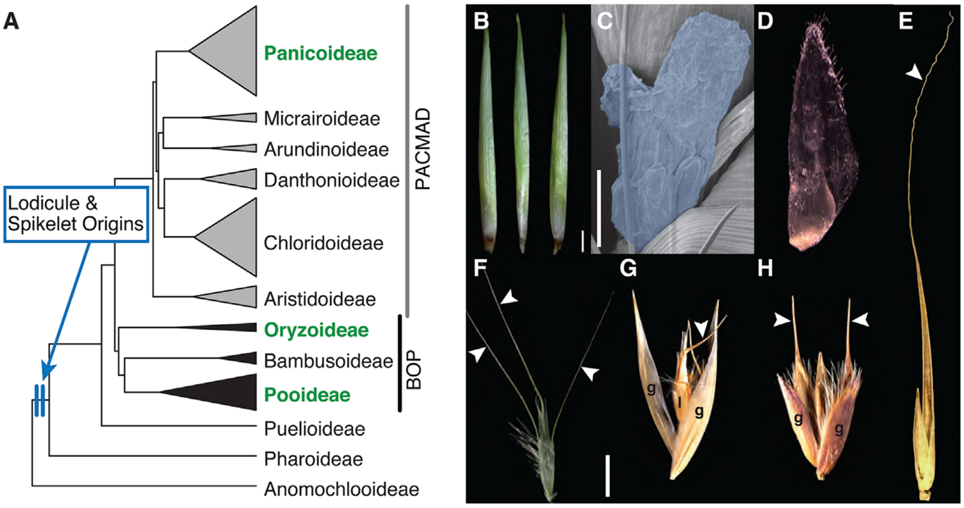

Fig. 2. Grass phylogeny overview and grass floral organ morphology. **A,** Grass phylogeny adapted from GPWG II (2012). The bulk of grass diversity is in the BOP (Bambusoideae, Oryzoideae, Pooideae) and PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae) clades, included within the spikelet clade. Triangles approximately proportional to clade size (species number). Clades that include model systems are the Panicoideae (maize, sorghum, *Setaria*), Oryzoideae (rice), and Pooideae (*Brachypodium distachyon*, barley, wheat). Blue tick marks indicate the likely origins of the true grass spikelet, and of lodicules. **B,** Bracts X–XII from *Streptochaeta angustifolia* Soderstr. (Anomochlooideae), likely homologous to inner whorl tepals of flowers in close outgroups, and lodicules in BOP/PACMAD flowers. **C,** One of a pair of lodicules from *Triraphis mollis* R. Br. (Chloridoideae). Lodicule false-colored blue. **D,** One of a pair of lodicules from *B. distachyon* (Pooideae). **E,** Bracts VI–VIII from a spikelet equivalent of *S. angustifolia*. The long, twisted awn on bract VI is indicated with an arrowhead. **F,** Lemma from *T. mollis* with 3 awns. **G,** *Centropodia glauca* (Nees) Cope (Chloridoideae, Columbus, 1998) spikelet showing bent lemma awn. **H,** *Eriachne pallescens* var. *pallescens* R. Br. (Micrairoideae, Columbus, 2000) spikelet showing simple, short awns. Scale bars in **A**–**D** = 5 mm, in **E** = 1 mm. g, glume; l, lemma. Arrowheads indicate awns in all panels.

share homology (Whipple et al., 2007). Thus, inner whorl monocot tepals, the innermost whorl of bracts in *Streptochaeta*, and lodicules can be considered both positionally homologous, and homologous because of shared developmental patterning mechanisms.

The lemma and palea have both been interpreted as strictly floral structures, homologous to the outer tepal whorl of other monocots; or as vegetative structures, homologous to bracts (lemmas) or prophylls (paleae) (reviewed in Kellogg, 2015; Lombardo & Yoshida, 2015). Molecular evidence and mutational analysis, while instructive, has not resolved lemma or palea homology conclusively. Some gene expression patterns and genetic mutants support the lemma and palea as floral, likely homologous to outer-whorl tepals. However, the lemma and palea are inserted at different levels on the floral apex (not in a single whorl), often differ in form within a flower, and are strongly offset in their development in many taxa, supporting distinct lemma and palea identities (Kellogg, 2015; Lombardo & Yoshida, 2015). In addition, in some rice mutants the lemma and palea are affected in different ways by the same gene mutation (e.g., Ohmori et al., 2009; Yuan et al., 2009; Jin et al., 2011), supporting the interpretation that the lemma and palea don't share a single organ identity or, in turn, a single origin. Kellogg's interpretation, which we favor, is that the palea may be derived from a pair of fused outer tepals. The lemma exhibits features and gene expression patterns characteristic of both an outer tepal (sepal) and a bract, and may represent a novel organ type (Kellogg, 2015). While these aspects of grass floral evolution are certainly interesting, they are not the focus of our review.

Grass flowers do not exhibit the wild morphological diversification characteristic of the orchids, or the tropical gingers, but there is interesting and informative floral variation in the family. Multiple established and emerging model systems in the grasses allows for the dissection of this variation in a molecular genetic framework. We argue that there are at least three aspects of grass floral morphology that can be studied for significant insight into the mechanistic basis of floral evolution. Those three aspects are: (1) the evolution of a novel organ identity—the lodicule, (2) lemma awns and their diversity, and (3) the convergent evolution of sexual differentiation. Here, we review what is known about the genetic control of these traits, and discuss their evolution in an evo-devo framework. Each grass model system has a different gene naming system, so for the sake of clarity, we will use the rice nomenclature (McCouch, 2008).

3 A Novel Organ Identity: The Lodicule

3.1 The evolution of the lodicule

Lodicules have the potential to reveal the molecular underpinnings of how novel organ identities arise in flowers. Lodicule identity likely arose once in the ancestor of the spikelet clade, which includes the bulk of grass diversity (Fig. 2). Lodicules likely play an important role in promoting out-crossing. When the pollen is mature, the lodicules swell and push the spikelet or flower open. This allows the anthers to emerge from the flower and release pollen away from the stigmas (Kellogg, 2015). The lodicule's role in out-crossing is potently illustrated by cleistogamous barley mutants, where the failure of lodicules to swell and force the flower open results in self-pollination (Nair et al., 2010).

Lodicules vary in terms of number and organization in grass flowers. Grass flowers usually have 0, 1, 2, or 3 lodicules, with 2 lodicules being most common (Clifford, 1961). These two lodicules are almost always positioned laterally, towards the side of the flower closest to the lemma (Fig. 1). When a third, adaxial lodicule is present, it is often offset from the whorl in which the lateral lodicules are found, and is often reduced (reviewed in Clifford, 1987; Kellogg, 2015). When there is a single lodicule in the flower, it is the likely product of fusion of the two lateral lodicules into a single abaxial lodicule. Flowers with a single adaxial lodicule have only been observed in some male *Pharus virescens* Döll, which are quite variable (Clifford, 1961; Judziewicz, 1987). The primarily abaxial and lateral arrangement of lodicules in grass flowers may be the result of a 'palea interaction zone' on the adaxial side of developing flowers (Cocucci & Anton, 1988). The developing palea may act as an auxin sink, thus inhibiting the development of floral organs on the adaxial side of the flower (Bartlett et al., 2015).

There is variation in lodicule morphology at both large and small evolutionary distances (Jirásek & Jozífová, 1968). In the Pharoideae, lodicules are generally absent (Clark & Judziewicz, 1996), but male flowers of some *Pharus* P. Browne species have 1–3 minute, rounded lodicules (Judziewicz et al., 1999). The ancestor of the BOP+PACMAD clade is hypothesized to have lost the adaxial lodicule (GPWG, 2001). In the BOP clade, Oryzoideae (including rice) have two lodicules (0 in Luziola, Kellogg, 2015). Pooideae also usually have two lodicules that are membranous and lack veins (Fig 2., Stebbins, 1956; Jirásek & Jozífová, 1968), but lodicules have been lost completely in 7 pooid genera. Additional variation in Pooideae is found in Phaenospermateae (14 spp., some previously thought to be bamboos) and Stipeae (530 spp.), which have 2 or 3 lodicules (Clifford, 1987; Kellogg, 2015), suggesting at least two independent reversals to the plesiomorphic state of 3 lodicules in the subfamily (Hochbach et al., 2015). In the pooid *Nephelochloa* Boiss. (1 sp.) and most Meliceae (ca. 160 spp.), the two lodicules are fused along their length (Kellogg, 2015). Bambusoideae have 3 lodicules, with the exception of male flowers in the herbaceous bamboos (Olyreae), which can lack lodicules. The three lodicules in the woody bamboos tend to be long, with marginal trichomes and many veins, whereas herbaceous bamboo lodicules are shorter, glabrous, and have few veins (Soderstrom & Ellis, 1987; Judziewicz et al., 1999). PACMAD grasses consistently have 2 lodicules (rarely absent). Chloridoideae lodicules are fleshy and often distinctively lobed (Fig. 2). Some Danthonioideae species have lodicules with microhairs, whereas others are glabrous. In the Panicoideae, lodicules are particularly fleshy, even when flowers are not at anthesis. Occasionally panicoid lodicules are fused, or reduced to a simple fringe of hairs (Kellogg, 2015). At smaller evolutionary distances variation in lodicule form is understudied, but in the tribe Aveneae there is considerable, systematically-relevant variation in lodicule morphology (Wölk & Röser, 2014). Further fine-scaled study of lodicule diversity is likely to reveal more variation in shape and form.

Lodicules offer an opportunity to explore not only how a novel organ identity arises, but also the genetics of variation in organ form. Comparisons between taxa with lodicules (e.g., maize, rice) and without lodicules (e.g., *Streptochaeta)* allow for uncovering gene expression patterns correlated with the evolution of the lodicule. Critically, once candidate genes have been identified, multiple genetic model systems in the family allow for the detailed dissection of candidate gene functions in closely-related systems. Variation in lodicule morphology within the family, especially between model taxa (e.g., between *B. distachyon* and maize), allows for determining the evolutionary genetics of variation in organ form, as for leaf and fruit morphology in the Brassicaceae (Vlad et al., 2014; Hofhuis et al., 2016). Here, we review what is already known about the genetics of lodicule development and function.

3.2 The genetics of lodicule development

Based on experimental evidence in arabidopsis and *Antirrhinum majus* L., the ABC model was proposed to explain how the four floral whorls of sepals, petals, stamens, and carpels of flowers are specified (Coen & Meyerowitz, 1991). This model now includes five classes of homeotic genes, termed A–E, with A-class alone specifying the sepals, A-class and B-class together specifying petals, B-class and C-class together specifying stamens, C-class alone specifying carpels and termination of the floral meristem, D-class specifying ovules, and E-class required in all four floral whorls (Theissen, 2001; Ditta et al., 2004). With the exception of the A-class *APETALA2* (*AP2)* homologs, all the core genes of the ABC(D)E model are type II MADS-box transcription factors from the MIKC^C group (Becker et al., 2000). Recent evidence indicates that parts of the ABC(D)E model can be extended to the grasses, particularly for B- and C-class function (e.g., Nagasawa et al., 2003; Nair et al., 2010; Dreni et al., 2011; Bartlett et al., 2015). The homology of the core ABC(D)E model genes and other floral development regulators between multiple model grasses and eudicots provides an opportunity to study the evolution of a novel organ identity.

B-class function is determined by two gene lineages, the *AP3*-like and *PI*-like MADS-box genes. Rice and maize each have a single ortholog of *AP3: SUPERWOMAN (SPW1)* and *SILKY1 (SI1)*, respectively. Both maize *si1* and rice *spw1* mutants display complete homeotic transformation of the lodicules into palea/lemma-like structures (Nagasawa et al., 2003; Bartlett et al., 2015). While *AP3* homologs have been maintained as single-copy in the grasses, *PI* homologs have undergone gene duplication. All grasses that have been sampled have at least two *PI* homologs (Whipple et al., 2004; Bartlett et al., 2016). The rice *PI* homologs, *OsMADS2* and *OsMADS4,* play partially redundant roles in lodicule development. *OsMADS2* preferentially controls lodicule development, while *OsMADS4* controls both lodicule and stamen development (Yao et al., 2008). Maize has three *PI* homologs*;* an ortholog of *OsMADS2, STERILE TASSLE SILKY EAR 1 (STS1/ Zmm16),* and two homologs of *OsMADS4, Zmm18* and *Zmm29* (Munster et al., 2001). As in rice, loss-of-function *sts1* mutants display homeotic transformation of the lodicules into palea/ lemma organs, as predicted by the ABC(D)E model (Bartlett et al., 2015), consistent with lodicules' likely homology with inner whorl tepals (Ambrose et al., 2000; Whipple et al., 2007; Bartlett et al., 2015).

STAMENLESS1 (*SL1,* also described as *OPEN BEAK* (Horigome et al., 2009)) regulates B-class function in rice. *SL1* is a C_2H_2 zinc finger protein that specifies lodicule development though the regulation of *SPW1* in whorl two. Like B-class mutants, lodicule primordia initiate in *sl1* mutants, but are homeotically transformed into palea/lemma-like organs. *SPW1* gene expression is lost in the developing inflorescences of *sl1* mutants, indicating that *SL1* upregulates *SPW1* (Xiao et al., 2009). *SL1* and its arabidopsis ortholog *JAGGED (JAG)* show diversified functions in flower and leaf development despite similar gene expression patterns. While *SL1* regulates floral development, *JAG* regulates leaf and floral organ shape, as well as mediating interactions between the meristem and floral organ primordia, not floral organ identity (Dinneny et al., 2004; Schiessl et al., 2012).

While B-class function in patterning both eudicot petals and grass lodicules appears deeply conserved, the function of A-class homologs in grass flower patterning is less clear. Homologs of *AP1* are clearly present and expressed in grass flowers, however grass *AP1*-like gene expression patterns suggest that these genes do not fit with classical A-class function of arabidopsis *AP1* (Preston & Kellogg, 2006, 2007). Grass *AP1* homologs are involved in the transition to flowering in both wheat *(AP1* homolog *VRN1)* (Yan et al., 2003; Chen & Dubcovsky, 2012) and rice (Kobayashi et al., 2012), contributing to innovation in grass reproduction (reviewed in Zhang & Yuan, 2014). Although recent loss-of-function analysis of the *OsAP1/FUL* gene family demonstrates mutant phenotypes consistent with classical A-class function in rice (Wu et al., 2016), other explanations may also fit these data. Recent work dissecting the cis-regulatory elements of *AtAP1* also clearly indicates that the classical A-class function of *AP1* in patterning petals likely evolved within the Brassicaceae, and A-class organ identity function is not necessarily conserved across the angiosperms (Ye et al., 2016).

Although *AP1* function as a canonical A-class organ identity gene remains unclear outside of the Brassicaceae, other A-class genes do have clear roles in lodicule development. In rice, *SHATTERING ABORTION 1 (SHAT1)* is an *AP2*-like transcription factor. *shat1* mutants have lodicule defects that include enlargement and/or an increase in lodicule number and can have lodicules that are transformed into palea/lemma like organs. Seed shattering, a major trait in domestication, is lost in *shat1* mutants. *SHAT1* is expressed throughout the plant including in the abscission zone of developing spikelets (Zhou et al., 2012). In barley, *CLEISTOGAMY 1 (CLY1,* later named *HvAP2* (Nair et al., 2010)) is an ortholog of *SHAT1*. Both *CLY1*/*HvAP2* and *SHAT1* are regulated through miR172. *cly1* mutants have a single nucleotide polymorphism (SNP) in the miRNA-binding domain that prevents miR172 from degrading the *cly1* transcript. This change in transcript regulation results in lodicules that are much smaller than those seen in wild type, and in cleistogamy, in which flowers are unable to open, enabling self-pollination rather than outcrossing (Kuusk et al., 2002). A *CLY1* epiallele with altered transcript level not associated with sequence variation at the miR172 binding site resulted in lodicules that were able to swell, although not enough to push the flower open (Wang et al., 2015). *AP2*-like genes in all three genomes of hexaploid wheat, the *TaAP2*/*WAP2* homeologs, are each also regulated by miR172. Unlike barley, where lodicules are either large or small (and unable to swell), variation in wheat lodicule size is continuous from small to large. This variation in wheat lodicule size is associated with the sequence at the miR172 binding site of the *TaAP2/WAP2* locus, as well as ploidy level (Ning et al., 2013). The maize co-orthologs of *TaAP2/WAP2* are called *INDETERMINATE SPIKELET 1 (IDS1)* and *SISTER OF INDETERMINATE SPIKELET 1 (SID1)* (Chuck et al., 1998, 2008; Nair et al., 2010). *IDS1* and *SID1* are also regulated through miR172, and have roles primarily in meristem identity and determinacy (Chuck et al., 1998, 2008). Misregulation of *IDS1* through altered miR172 binding results in defects in sexual differentiation (Chuck et al., 2007, 2008) (See section on sexual differentiation, below).

In contrast to the cleistogamous *cly* mutants, barley *hvlaxatum-a (hvlax-a)* mutants have flowers that are able to open in spite of the homeotic transformation of their lodicules into stamenoid structures. *hvlax-a* mutant flowers show pleiotropic defects including reduced marginal growth of the palea and lemma. These palea/lemma defects are likely what allow *hvlax-a* flowers to open, in spite of lodicule defects. *HvLAX-A* and its paralog *HvUNICULME4 (HvCUL4)* are orthologs of arabidopsis *BLADE-ON-PETIOLE 1 (BOP1)* and *BOP2,* which are expressed in lateral organ boundaries and control both leaf and flower morphogenesis (Hepworth et al., 2005; Jost et al., 2016). Unlike their arabidopsis orthologs, *HvLAX-A* and *HvCUL4* are at least partially divergent in function. *hvlax-a* mutants have strong inflorescence phenotypes, but no leaf phenotypes, whereas *hvcul4* mutants display leaf patterning and reduced branch number, while not altering inflorescence traits. Consistent with their different roles, the gene expression patterns of *HvLAX-A* and *HvCUL4* are not identical, suggesting subfunctionalization in the lineage leading to barley and/or the grasses (Tavakol et al., 2015; Jost et al., 2016).

Grass C- and E-class genes function largely as predicted by the ABC(D)E model of floral development. The E-class genes are not restricted to specific whorls, but rather are required throughout all four floral whorls for proper floral development and floral meristem determinacy (reviewed in Krizek & Fletcher, 2005). Grass *SEPALLATA*-like and sister subfamily *AGL6*-like genes have been shown to function across the floral meristem (Malcomber & Kellogg, 2004; Ohmori et al., 2009; Thompson et al., 2009; Cui et al., 2010; Dreni & Zhang, 2016). The C-class genes do not directly pattern grass lodicules or eudicot petals. Instead, the absence of C-class expression is essential for second whorl organ development (reviewed in Krizek & Fletcher, 2005). In rice, loss-of-function C-class mutants *osmads3* and *osmads58* result in loss of reproductive organ identity and ectopic lodicule formation, as predicted by the ABC (D)E model (Kyozuka & Shimamoto, 2002; Dreni et al., 2011). *ABERRANT PANICLE ORGANIZATION 1* (*APO1*), a putative F-box protein and homolog of arabidopsis *UNUSUAL FLORAL ORGANS 1 (UFO1),* is also important for C-class function in rice. *apo1* mutants produce extra lodicules and additional floral abnormalities that together with expression suggest that *APO1* is a positive regulator of C-class gene *OsMADS3* (Ikeda et al., 2007). This is an interesting contrast to arabidopsis, where *UFO* positively regulates *AP3,* a B-class gene (Chae et al., 2008), representing possible evolutionary divergence in gene function.

The model system framework for studying the evolution of a novel organ is especially powerful because while all the grass floral regulators identified thus far have arabidopsis homologs, there are often key functional differences (e.g., *SL1* vs. *JAG; APO* vs. *UFO*). This mix of conserved and divergent gene functions allows for studies of subfunctionalization, neofunctionalization, or of recruitment of genes and gene networks into new roles in the development and evolution of novel organ identities.

4 Organ Diversification: Lemma Awns

4.1 Lemma awns are diverse in form and function

Awns are organ elaborations that occur on the lemmas (occasionally glumes or paleae) of grass flowers. In *Streptochaeta*, one of the outer bracts surrounding the flower has a long, twisted awn (bract VI, Fig. 2). Awns typically extend from the apex of the lemma midvein, but may also diverge from the abaxial surface of the lemma below the apex. In *Bromus* L., the awn extends from between two teeth at the lemma apex. Awns are common but not ubiquitous in the grass family (Kellogg, 2015), with a likely complex evolutionary history. In the Danthonioideae, where awn presence and absence has been carefully mapped onto a well-resolved phylogeny, awns have likely been lost numerous times, and also occasionally regained (Humphreys et al., 2011). Awns may be long or short, branched or unbranched (*Aristida* L., *Pappophorum* Schreb.); with or without trichomes; barbed or smooth. Awn morphology is diagnostic for some genera. For example, *Aristida* is characterized by three awns, whereas *Stipa* L. has a large, twisted awn (Kellogg, 2015). Studying the evo-devo of awns has the potential to inform us about the genetics of the diversification of organ form.

Awn function is best described in terms of fruit dispersal, seed germination, and seedling establishment. Grass fruits are usually dispersed still associated with remnants of the flower or spikelet. Thus, we (and others) use the term 'diaspore' to describe the generalized grass dispersal unit. Awns can aid in diaspore dispersal by attaching to passing animals, and may also play a role in wind dispersal. In a grassland ecosystem, diaspores with long awns disperse more effectively over short distances (less than 1m) than diaspores with short or no awns (Diacon-Bolli et al., 2013). In *Aristida* and *Microlaena* R. Br., awns may serve to ensure the diaspore lands on the soil in an orientation that positively affects both germination and seedling establishment (Peart, 1981). Hygroscopic awns bend and straighten as humidity levels change, propelling the diaspore and potentially helping seeds travel to suitable microsites for germination, or helping to bury the seeds (Peart, 1979; Elbaum et al., 2007; Molano-Flores, 2012). Directional diaspore movement may be aided by angled barbs on the awns, which act like ratchets, allowing for smooth movement in one direction, but catching in the opposite direction (Elbaum et al., 2007; Kulić et al., 2009; Wolgemuth, 2009). Diaspores with longer awns may be better able to survive high-intensity fires because they can bury themselves deeper. Fire intensity is not always predictable or reliable, which may be why awn-length diversity is maintained (Garnier & Dajoz, 2001). In *Stipa tenacissima* L., awns help prevent predation by ants. The awns themselves are too big for the ants to cut off in a timely manner, and make the diaspores cumbersome and heavy for the ants. Once buried, they can be excavated by teams of ants, but this is prevented once the awns are broken off at a preformed dehiscence zone at the base of the awn—the ants can no longer find the diaspores (Schöning et al., 2004). Thus, awn function in fruit and seed biology is complex, highly context-dependent, and driven in large part by awn morphology.

Awns have also been intensely studied in cultivated rice and its wild relatives because awn loss was under selection during rice domestication. Awns were likely selected against because they make harvesting, processing, and storing grain challenging, or because of an association between awn loss and other desirable yield traits (Luo et al., 2013; Hua et al., 2015). Although most cultivated rice is awnless, experiments with awned rice accessions have demonstrated that awns can have roles in both pollination and dispersal. Rice flowers with long awns inhibit outcrossing in the closed panicles typical of domesticated rice by preventing the free exposure of anthers and stigmas (Ishii et al., 2013). Awns can also have an indirect effect on diaspore dispersal. Flowers near the base of the inflorescence with long awns help retain diaspores higher up on the inflorescence, even though the higher diaspores have already become detached from the inflorescence (Ishii et al., 2013). These results demonstrate that although awns have critical functions in dispersal, they may also have more subtle roles in pollination and fruit biology.

In barley and wheat, awns have not been lost during domestication, likely because they are a significant source of photosynthate during grain development, and awn loss under domestication would likely have had a negative effect on yield (Grundbacher, 1963). There is potentially functional diversity in awn length in barley. Barley cultivars and landraces with shorter awns have persisted in regions with high precipitation, perhaps because spikelets with shorter awns collect less water in them, resulting in less lodging (Yuo et al., 2012). Although this is an intriguing idea, the connection between awn length and water retention in spikelets has not been explicitly tested.

This diversity in awn structure and function provides an exciting evolutionary framework in which to explore the genetics of awn development. The existence of both awned (*B. distachyon, Oryza rufipogon* Griff., barley) and unawned (maize, *Setaria, O. sativa*) model systems in the grass family allows for the fruitful dissection of the developmental genetics of awns. Here, we review what is already known about the genetics of awn development and elaboration.

4.2 Molecular genetics of awn development

One gene with a clear role in awn development in rice is *AWN-1* (*An*-1). In rice, long awns on the lemma are characteristic of wild rice species, while the two species of cultivated rice—*Oryza sativa* (Asian rice) and *Oryza glaberrima* Steud. (African rice)—usually lack awns. Within Asian rice, many cultivars and landraces of *O. sativa* subsp. *indica* S. Kato have awns, while most cultivars of *O. sativa* subsp. *japonica* S. Kato do not (Toriba & Hirano, 2014). In studying this diversity, *An-1* was found underneath a major QTL for awn length that may have been under selection during domestication. The long-awn allele of *An-1* acts as a dominant gene with pleiotropic effects: *An-1* positively regulates awn length and grain length, but negatively affects grain number per panicle. *An-1* encodes a bHLH transcription factor that regulates cell division at the lemma apex (Luo et al., 2013).

A second regulator of awn development, *AWN-2 (An-2)/ LONG AND BARBED AWN1 (LABA1),* was also the likely target of selection under rice domestication. *An*-2 regulates awn length and awn barbing and encodes LONELY GUY LIKE 6 (OsLOGL6), an enzyme that catalyzes the first step of cytokinin synthesis (Luo et al., 2013; Hua et al., 2015). Most rice cultivars have a single nucleotide deletion in *An*-2 that results in a likely non-functional protein. Awned lines with the non-functional *an*-2 allele are never barbed, while awns are barbed in lines with the functional *O. rufipogon An*-2 allele. Each barb comes from a single epidermal cell that is swollen at first, becoming sharp over time. Interestingly, *An*-2 transcripts are found in epidermal cells, but not in swollen barb initial cells or the barbs themselves later on in development (Hua et al., 2015), suggesting that *An*-2 acts non-cell autonomously. Although selection under domestication may have been for shorter awns, *an*-2 is also associated with yield traits. The wild progenitor (*O. rufipogon)* allele of *An*-2 increases awn length, but also reduces grains per panicle and tillers per plant. Thus *an*-2 might have been selected under domestication not only because of awn traits, but also because it positively affected yield (Hua et al., 2015).

A third regulator of awn length putatively under selection during rice domestication is *REGULATOR OF AWN ELONGATION 2 (RAE2)* or *GRAIN NUMBER, GRAIN LENGTH AND AWN DEVELOPMENT 1 (GAD1). RAE2/GAD1* encodes a cysteine-rich peptide in the EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) family (Bessho-Uehara et al., 2016; Jin et al., 2016). *RAE2/GAD1* causes the proliferation of vasculature in the awn, which may allow for awn elongation (Bessho-Uehara et al., 2016).

Interestingly, *O. sativa* subsp. *japonica,* although it has likely non-functional alleles of *an-1*, *an*-2, and *rae2/gad1*, can still form awns. There are a number of monogenic mutants in *O. sativa* subsp. *japonica* that develop awns (Itoh et al., 2000; Liu et al., 2007; Toriba et al., 2007; Itoh et al., 2008; Song et al., 2012; Tanaka et al., 2012). Two of these are genes involved in post-transcriptional gene silencing—*SHOOTLESS2 (SHL2)* and *Os-DICER-LIKE4 (Oryza sativa DCL4)* (Liu et al., 2007; Toriba et al., 2010). *SHL2* encodes an RNA-dependent RNA polymerase with likely roles in the ta-siRNA pathway (Toriba et al., 2010). Weak *shl2* alleles produce abaxialized lemmas with very long awns, suggesting that *SHL2* plays a role in suppressing awn elongation (Toriba et al., 2010; Toriba & Hirano, 2014). *SHL2* may act by silencing the auxin response gene *OsETTIN2 (OsETT2)*. Silenced *OsETT2* in *shl2* mutants reduces awn length in awnless cultivars. Silencing *OsETT2* in an awned cultivar also reduces awn length, or eliminates awns completely (Toriba & Hirano, 2014). These results suggest that in addition to non-functionalization of *an-1*, *an-2,* and *rae2*, awn regulators are actively silenced in unawned rice cultivars.

Asian and African rice were domesticated independently (Vaughan et al., 2008), and awn loss is likely due to changes at separate loci. QTL analyses show that awn loss in African rice is under the control of an independent locus to *An-1*, as African rice has functional copies of both *An-1* and *RAE2/GAD1* (Furuta et al., 2015; Bessho-Uehara et al., 2016). There is also evidence for independent loci controlling the development of barbed rice awns. In awned Asian rice accessions, a single mutation in *an-2* is perfectly correlated with barbless awns. However, some accessions with a putatively functional *An-2* gene had barbless awns, and a major QTL for barbed awns in these accessions is on a separate chromosome to *An-2* (Hua et al., 2015). In contrast, alleles of the barley gene *short awn 2* (*lks2* for *length2*) appear to have been repeatedly selected in the evolution of awn development. While Asian barley cultivars with short awns all share a single mutation in a critical LKS2 protein domain, SNPs surrounding the critical mutation differ depending on geographic origin of the cultivar. This result indicates that the critical mutation may have arisen independently in China and in the Himalayas (Yuo et al., 2012). Similarly, *RAE2*/*GAD1* has likely been repeatedly inactivated in Asian rice cultivars (Bessho-Uehara et al., 2016). These results demonstrate that awn development and evolution can have a complex history, even within a single genus.

There is a connection between awn development and carpel development in rice, barley, and *B. distachyon*. Both stigmatic hairs and awns are affected in *1ks2* barley mutants, as in other (uncloned) smooth-awned barley mutants (Lundqvist & Franckowiak, 2003; Yuo et al., 2012). *LKS2* is in the *SHORT INTERNODES* (*SHI*)-like transcription factor family, and variation in *lks2* results in short awns and short stigmatic hairs in barley flowers (Yuo et al., 2012). A second barley gene in the *SHI* gene family, *SIX-ROWED SPIKE 2 (Vrs2)*, also has a role in lemma growth and awn elongation early in spikelet development. Early in development, *vrs2* mutants have very extended awns at the base of the inflorescence, but it's not clear whether this awn elongation phenotype persists to anthesis (Youssef et al., 2016). The *SHI* gene family includes the *STYLISH* genes, which regulate gynoecium development in arabidopsis (Kuusk et al., 2002, 2006). In rice, the YABBY transcription factor *DROOPING LEAF* (*DL*) has clear roles in the development of carpels and the leaf midrib (Nagasawa et al., 2003). Interestingly, *DL* also regulates the initiation of awns in an awned rice cultivar. Introgression of the mutant *dl* allele into awned rice results in a drastic reduction in the number of spikelets with awns in a dose-dependent manner (Toriba & Hirano, 2014). An awnless mutant in *B. distachyon* also has a carpel development phenotype (Derbyshire & Byrne, 2013). Thus, there is an interesting connection between awn and carpel development, suggesting that carpel genes might have been recruited to novel functions in the evolution of awn development.

The barley *Hooded* mutant has revealed that the awn/lemma boundary domain from which awns are initiated has a distinct identity. *Hooded* mutants have no awns because a secondary floral meristem with reverse polarity, rather than an awn, is initiated on *Hooded* mutant lemmas. The *Hooded* mutant phenotype is because of the ectopic expression of the *KNOTTED1-like Homeobox* (*KNOX*) gene, *BKN3*, in developing lemmas, from which *BKN3* is normally excluded (Müller et al., 1995; Richardson et al., 2016). The *Hooded* phenotype has been interpreted as the result of either an inflorescence or floral meristem forming on the awn, induced by ectopic *BKN3* gene expression (Williams-Carrier et al., 1997; Richardson et al., 2016). Intriguingly, overexpression of maize *KNOTTED1* (*KN1)* under the ubiquitin promoter in barley also results in a *Hooded* phenotype. Although the ubiquitin promoter drives *KN1* expression throughout these plants, only awns produce ectopic meristems (Williams-Carrier et al., 1997). These results indicate that either awn identity or awn/lemma boundary domain identity is critical in the development of the *Hooded* phenotype: lemma awns are competent to respond to the *KN1* signal in ways that monocot leaves are not (Williams-Carrier et al., 1997; Richardson et al., 2016). *lks2*, which regulates awn length, is epistatic to *Hooded* – supporting the hypothesis that a particular awn identity is necessary for the *Hooded* phenotype to develop (Williams-Carrier et al., 1997; Roig et al., 2004; Richardson et al., 2016).

The results of these investigations lead to a number of questions about the development and diversification of lemma awns. What differentiates lemmas in grasses with and without awns? Is there evidence for the repeated evolution of a novel 'awn identity', or is 'awn identity' suppressed in unawned grasses? How do awn initiation and awn elaboration genes interact? The awn/carpel pleiotropy in barley, rice, and *B distachyon* suggests a link between awns and stigmatic hairs. What are these awn/carpel genes doing in unawned grasses? Through a mix of forward and reverse genetics, guided in part by candidate gene studies in non-model grasses, the model systems available in the grasses provide the experimental framework to answer these questions.

5 Sexual Differentiation in Grass Flowers

5.1 The convergent evolution of sexual differentiation in grass flowers

Most angiosperm flowers are hermaphroditic and have bisexual flowers. However, other sexual systems have evolved repeatedly, including separate male and female flowers on the same individual (monoecy) and individuals of separate sexes (dioecy). The repeated evolution of unisexuality in the angiosperms is a classic example of convergent evolution. Studying the development of floral sexuality across angiosperms has revealed that there are likely many different genetic pathways to unisexuality (Diggle et al., 2011). However, this hypothesis cannot be explicitly tested at the molecular level using arabidopsis and its relatives: flowers in the Brassicaceae are almost entirely hermaphroditic (Al-Shehbaz, 2011; Soza & Di Stilio, 2014). In contrast, sexual systems in the grasses are extremely diverse.

In the early diverging grasses, both *Anomochloa* and *Streptochaeta* (Anomochlooideae) are hermaphroditic (Judziewicz & Soderstrom, 1989), while all members of the Pharoideae are monoecious (Clark & Judziewicz, 1996). Most BOP and PACMAD grasses are hermaphroditic, but monoecy is found in the herbaceous bamboos, Chloridoideae, Panicoideae, and Oryzoioideae (Kellogg, 2015). A few grass clades, such as *Bouteloua* Lag. (Columbus et al., 2000; Kinney et al., 2007) and *Poa* supersection *Homalopoa* (Dumort.) Soreng & L.J. Gillespie (Giussani et al., 2016), are highly variable in their expression of floral sexuality. In *Bouteloua*, fifteen species (out of ca. 60) have unisexual flowers that range from andromonoecy (individuals with hermaphroditic and male flowers), gynodioecy (individuals with hermaphroditic and female flowers), or trimonoecy (individuals with male, female, and hermaphroditic flowers). The phylogeny of the group (Columbus et al., 1998; Columbus et al., 2000) suggests unisexuality has arisen 2–7 times and that monoecy can lead to dioecy (Kinney et al., 2007). Similarly, in *Poa* supersection *Homalopoa*, the phylogeny suggests that other sexual systems are stepping stones in the evolution of dioecy. In addition, reversions to hermaphroditism in section *Homalopoa* underscore the flexibility of sexual systems in some grass clades (Giussani et al., 2016).

Wind pollination coupled with unisexuality may allow for the independent optimization of male vs. female function in flowers (Charlesworth, 1993; Freeman et al., 1997). Perhaps because of this release of constraint on floral form, morphologically distinct male and female flowers and inflorescences have evolved numerous times in the grass family (e.g., maize, herbaceous bamboos, *Jouvea* E. Fourn., *Phyllorachis* Trimen) (Kellogg, 2015). This differentiation is most familiar in the male tassels and female ears of maize, where male and female spikelets differ not only in floral sexuality, but also in adult flower number, glume morphology, and position on the plant (Cheng et al., 1983). Highly specialized sexual dimorphism is a synapomorphy for *Poa* section *Dioicopoa* E. Desv. Male and female plants in *Dioicopoa* differ in plant size, flower size (bigger female flowers and plants), flower number (fewer pistillate flowers), and in floral pubescence (female flowers hairy) (Giussani et al., 2016). Thus, the grass family not only offers the opportunity to explore the genetics of floral sexuality, but also the genetics of sexual dimorphism in plants.

There are grass model systems that are strictly hermaphroditic (*Oryza, B. distachyon)* and grass systems that have unisexual flowers (barley, maize, *Setaria,* sorghum). Critically, unisexuality has arisen independently in barley (Pooideae) and the Panicoid model grasses (maize, *Setaria,* sorghum). As for lodicules and awns, this diversity allows both for the precise dissection of gene function in the development of floral sexuality, and for elucidating the pathways that lead to the independent evolution of unisexuality. Here, we review what is known about the genetics of sexual system development (what we term sexual differentiation, reserving 'sex determination' for the development of dioecy (after Diggle et al., 2011).

5.2 The molecular genetics of sexual differentiation

The molecular control of sexual differentiation in grasses has been investigated most thoroughly in maize and barley. Unlike rice, maize and barley are not completely hermaphroditic and mutants in both species offer an opportunity to explore the molecular biology of sexual differentiation. Maize is monoecious, with separate male (tassel) and female (ear) inflorescences that initiate as hermaphroditic, but undergo differential suppression of reproductive organs (Cheng et al., 1983). In the male tassel, carpel growth is suppressed post-initiation by programmed cell death and in the female ear, stamens are suppressed by cell cycle arrest (Calderon-Urrea & Dellaporta, 1999; Kim et al., 2007). In addition, maize has paired spikelets on both the tassel and ear, and each spikelet contains two flowers (upper and lower). In the ear, growth is suppressed in the stamens of the upper flower and in the entire lower flower of each spikelet, leading to pairs of spikelets that each have single female flowers (Cheng et al., 1983).

The barley inflorescence contains two rows of three spikelets at each node. The central spikelet contains a single hermaphroditic flower, while each lateral spikelet is either completely neuter or contains a single male flower (Åberg & Wiebe, 1946; Wiebe & Reid, 1961). The lateral spikelets do not produce grain in wild *Hordeum* species and some accessions of cultivated barley (two-rowed barley), and are therefore usually described as 'sterile', even when they contain fertile stamens. In some barley accessions – termed six-rowed barley, the lateral spikelets contain a bisexual flower and are fully fertile. Thus, barley cultivars range from being andromonoecious (when lateral spikelets are male) to hermaphroditic (when lateral spikelets are completely neuter or bisexual) (Åberg & Wiebe, 1946).

In barley and maize, both transcriptional regulators and hormone pathways play critical roles in sexual differentiation, but the full pathway leading to dicliny has not been elucidated in either species (DeLong et al., 1993; Bensen et al., 1995; Doebley et al., 1995; Komatsuda et al., 2007; Whipple et al., 2007; Ramsay et al., 2011; Youssef et al., 2016). Here, we discuss what is known about the genetics of sexual differentiation in maize and barley.

5.3 Hormones and sexual differentiation in maize

Jasmonic acid (JA) is essential for suppressing carpels in the tassel and lower flowers of the ear early in maize inflorescence development (Fig. 3). Two genes in the JA pathway, *TASSELSEED 1* and *TASSELSEED 2* (*TS1* and *TS2*), have been cloned thus far (DeLong et al., 1993; Acosta et al., 2009). *TS1* encodes a 13-lipoxygenase that catalyzes the production of (13S)-hydroperoxyoctadecatrienoic acid early in JA biosynthesis, and *TS2* encodes a short-chain alcohol dehydrogenase that is likely also involved in JA biosynthesis (DeLong et al., 1993; Acosta et al., 2009). *ts1* and *ts2* mutant plants have fully feminized tassels and derepressed lower flowers in the ear (Irish et al., 1994). *TS2* is expressed in carpels of the tassel just before cell death occurs, implying that *TS2* mediates the signal for cell death through JA (DeLong et al., 1993). Thus, genes involved in the JA pathway, *TS1* and *TS2,* act early in maize inflorescence development to suppress growth in the carpels of the tassel and in the lower flowers of the ear.

Because JA is essential for carpel suppression in maize, other studies have closely examined homologs of *TS2* in other grass species. The panicoid grass *Tripsacum dactyloides* (L.) L. contains a homoeolog of *TS2*, *GYNOMONOECIOUS SEX FORM 1* (*GSF1*), that has a similar expression pattern to *TS2. gsf1* mutants also have a feminized inflorescence (Li et al., 1997), providing support for the hypothesis that *TS2*-like genes have conserved functions in sexual differentiation within the Panicoideae. In *Bouteloua*, a genus with extreme sexual system diversity, a *TS2* homolog exhibited non-neutral evolution in hermaphroditic *B. hirsuta* Lag., and neutral evolution in monoecious and dioecious *B. dimorpha* Columbus (Kinney et al., 2003). This evidence suggests that *TS2*-like genes are under purifying selection in *B. hirsuta* and may be associated with sexual differentiation in *B. dimorpha,* but further functional characterization is needed. However, in rice and sorghum, *TS2* homologs are expressed in other organs besides the inflorescence, and in the stamens and carpels of hermaphroditic rice flowers (Malcomber & Kellogg, 2006). This indicates that *TS2* homologs are not acting to suppress sex organ growth in rice. Furthermore, *TS2* homolog amino acid sequences are conserved across many grasses, and amino acid changes do not correlate with the emergence of unisexual flowers (Malcomber & Kellogg, 2006). Given these results, as well as the wide range of JA functions in plant development (Gundlach et al., 1992; Creelman & Mullet, 1995), it is possible that *TS2* homologs have more general roles in development in grasses outside the Panicoideae and there are

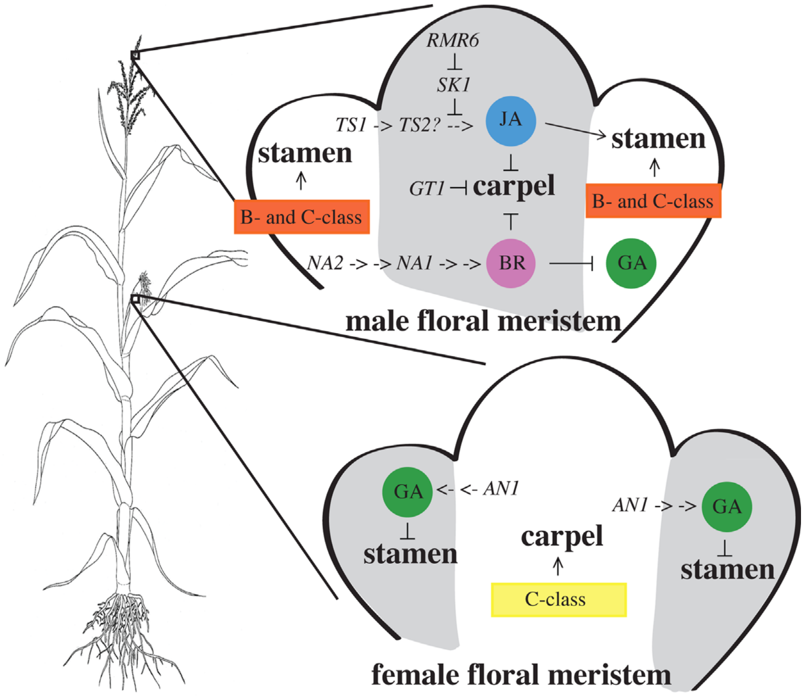

Fig. 3. A model of genetic and hormone interaction in sex differentiation in maize. In the male floral meristem, the carpel aborts while the stamens develop. B- and C- class MADS box transcription factors and jasmonic acid (JA) promote stamen development. Brasinosteriods (BR), JA, and transcription factor *GT1* repress the carpel. In the female floral, the carpels develop while stamen growth is arrested. C- class MADS box transcription factors promote carpel development while gibberellins (GA) repress stamen development. Maize plant drawing reproduced with the permission of D. G. Mackean and Ian Mackean.

alternative genetic controls of unisexuality in other grasses (Malcomber & Kellogg, 2006).

SILKLESS 1 (*SK1*) is a classic maize gene in the sex differentiation pathway recently shown to encode a UDP-glycosyltransferase (Hayward et al., 2016). *sk1* mutants have aborted upper flowers and no silks, indicating that SK1 protects upper flowers in the ear from abortion (Calderon-Urrea & Dellaporta, 1999). *TS2* is epistatic to *SK1* (Irish et al., 1994; Calderon-Urrea & Dellaporta, 1999), and *TS2* is ectopically expressed in the upper ear flowers of *sk1* mutants, suggesting that *TS2* is likely acting to abort the upper flower (Dellaporta & Calderon-Urrea, 1994). SK1 localizes to the peroxisome of tobacco BY-2 cells where, coincidentally, bioactive JA-Ile is synthesized (Hayward et al., 2016). This localization supports the hypothesis that *SK1* disrupts JA-mediated carpel abortion in upper ear flowers (Irish et al., 1994; Calderon-Urrea & Dellaporta, 1999). Overexpression of *SK1* results in fully feminized tassels, demonstrating that high expression levels of *SK1* can act to protect carpel abortion in the tassel (Hayward et al., 2016). Further molecular studies of *SK1* are needed to determine how *SK1* is protecting against carpel abortion, and how it is interacting with the JA pathway.

Another gene that may act with *SK1* to regulate carpel development in the tassel is *REQUIRED TO MAINTAIN REPRESSION 6* (*RMR6*). *rmr6* mutants have derepressed lower flowers in the ear and feminized tassels (Parkinson et al., 2007). However, in contrast to *tasselseed* mutants, stamens in *rmr6* tassels are functional (Parkinson et al., 2007). *RMR6* encodes a large subunit of RNA polymerase IV and is predicted to control miRNA biosynthesis (Erhard et al., 2009). *SK1* is epistatic to *RMR6*, suggesting that they operate in the same pathway to control sexual differentiation in the tassel (Parkinson et al., 2007). A tantalizing possibility would be that *RMR6* acts to regulate *SK1* expression.

Gibberellin (GA) regulation of sexual differentiation is critical in both the ear and the tassel. *ANTHER EAR 1* encodes a cyclase that catalyzes the production of *ent*-kaurene in the GA biosynthetic pathway (Bensen et al., 1995). *ANTHER EAR 1* mutants are semi-dwarfed, have shorter internodes, and have derepressed stamens in the ear (Bensen et al., 1995). Other dwarfed GA mutants also have derepressed stamens in the ear (Emerson, 1912; Evans & Poethig, 1995). In the tassel, exogenous GA application results in complete feminization, similar to *tasselseed* mutants (Hansen et al., 1976). Thus, GA biosynthesis suppresses stamen development in ears and needs to be downregulated in the tassel to achieve exclusively male flowers.

Brassinosteroids (BR) also have a role in suppressing carpels in the tassel. *NANA PLANT 1* (*NA1*) is an enzyme in the BR biosynthetic pathway that synthesizes a BR intermediate (Hartwig et al., 2011). *na1* mutant plants have mostly feminized tassels, short stature, and misshapen leaf epidermal cells. *NA1* is expressed in stamens throughout their development and in the outer cells of carpel primordia in the tassel right before cell death. *NANA PLANT 2 (NA2)* is the ortholog of arabidopsis *DWARF1*, which encodes another enzyme involved in BR biosynthesis (Best et al., 2016). Because GA and BR interact in rice and arabidopsis (Bai et al., 2012; Tong et al., 2014), genetic interactions between GA and BR were assessed in double mutants. Best et al. demonstrate GA is needed to produce feminized tassels in *na2* plants and BR is not involved in producing stamens in ears of GA mutants (Best et al., 2016). These observations suggest that BR is suppressing levels of GA in the tassel and have no functional role in mediating GA sexual differentiation in the ear.

5.4 Transcriptional regulation of sexual differentiation

Several transcription factors in maize and barley are crucial for sexual differentiation. In maize, *GRASSY TILLERS 1 (GT1)* encodes a Class 1 HD-ZIP transcription factor that partially controls carpel suppression in the tassel (Whipple et al., 2011). *gt1* mutant plants have partially derepressed carpels in the tassel, increased vegetative branching, and elongated leaves on the ear (Whipple et al., 2011). Under normal growing conditions, *GT1* mRNA is present in axillary buds and the adaxial side of leaf primordia, but is not present in the shoot apical meristem (SAM) (Whipple et al., 2011). In flowers of developing tassels, *GT1* is highly expressed in carpels that are destined to abort (Whipple et al., 2011). Because carpels are derepressed in the tassel and not in lower flowers of the ear, *GT1* likely has a specific role in suppressing carpel growth in the tassel. Another transcription factor working with *GT1* to suppress vegetative growth is *TEOSINTE BRANCHED 1* (*TB1*). This TCP transcription factor suppresses vegetative axillary branches and male organs in the apical region of the ear (Doebley et al., 1995). *GT1* expression is reduced in the axillary buds of *tb1* mutants, suggesting that *TB1* is epistatic to *GT1* (Whipple et al., 2011). In barley, the *TB1* ortholog, *HvTB1*, partially suppresses both vegetative growth, as well as stamen and carpel development in lateral spikelets (Ramsay et al., 2011). Thus, growth suppression, regulated in part by *GT1*-like and/or *TB1*-like genes, likely has an important role to play in sexual differentiation both in maize and in barley.

There are five barley loci, named *VRS1-5* (*SIX-ROWED SPIKE1-5*) that suppress floral organ development in lateral spikelets. Each monogenic *vrs* mutant develops hermaphroditic, fertile lateral spikelets, and has a higher grain yield per inflorescence (2-rowed vs. 6-rowed barley). *VRS1* is a *GT1*-like gene that was a target of selection under domestication (Komatsuda et al., 2007). *vrs1* mutants (some six-rowed barley cultivars) have derepressed, bisexual lateral spikelets that produce fertile seed (Komatsuda et al., 2007). *VRS1* is highly expressed in the carpels of male flowers in the lateral spikelets, presumably acting in carpels to suppress growth in the lateral male flowers of 2-rowed barley (Komatsuda et al., 2007; Sakuma et al., 2013). The *vrs1* phenotype is modified by the genotype at *HvTB1*, indicating a genetic interaction between *GT1*-like genes and *TB1*-like genes in both barley and maize (Ramsay et al., 2011; Whipple et al., 2011).

VRS2 encodes a *SHORT INTERNODES (SHI)* transcription factor that likely regulates hormone levels in the barley inflorescence (Youssef et al., 2016). In 'Bowman' barley, stamens initiate in lateral spikelets, but carpel growth is repressed. *vrs2* mutants in the Bowman background have partially derepressed growth in lateral spikelets at the base and tip of the inflorescence, and fully bisexual lateral spikelets in the center (Youssef et al., 2016). *VRS2* may influence sexual differentiation in lateral spikelets by regulating hormone levels. In *vrs2* mutant inflorescences, hormone levels are disrupted and many hormone-associated genes were found to be differentially expressed through transcriptome analysis. These results suggest that regulation of hormone pathways by *VRS2* may be instrumental in regulating lateral spikelet development (Youssef et al., 2016).

There are interesting connections between meristem determinacy genes and sexual differentiation in both barley and maize. *VRS4* encodes a *LATERAL ORGAN BOUNDARY* domain transcription factor and is an ortholog of maize *RAMOSA2 (RA2)*, which suppresses branching in the tassel and ear (Bortiri et al., 2006; Koppolu et al., 2013). Unlike other *vrs* mutants, *vrs4* mutants have multiple bisexual lateral flowers in which carpel growth is derepressed. (Koppolu et al., 2013). In maize, the spikelet determinacy gene *IDS1* also affects sexual differentiation. *IDS1* encodes an AP2-like transcription factor that promotes spikelet meristem determinacy and floral meristem identity (Chuck et al., 1998). *ids1* mutants have extra flowers in spikelets of the tassel and ear, but normal sexual differentiation is maintained (Chuck et al., 1998). Curiously, ectopic *IDS1* expression results in a *tasselseed* phenotype, as demonstrated in *tasselseed4* (*ts4*) and *Tasselseed 6* (*Ts6*) mutants (Chuck et al., 2007). *TS4* encodes a microRNA (miR172e) that targets *IDS1,* and *Ts6* is a dominant allele of *IDS1* that has a mutation in its miR172e binding site, resulting in ectopic *IDS1* expression in *ts4* and *Ts6* mutant tassels (Chuck et al., 2007, 2008). These results highlight a link between meristem determinacy and sexual differentiation in the grasses.

The grasses offer an excellent opportunity to investigate the links between sexual differentiation pathways and organ identity pathways in floral development. Although much remains to be discovered, recent work suggests that carpel abortion in the maize tassel is organ-identity specific rather than whorl-specific (Bartlett et al., 2015). Double and single genetic mutant analyses indicate that in maize B-class mutants, the stamens are homeotically transformed into carpels, but then undergo organ abortion, regardless of their position in the flower (Bartlett et al., 2015). This is in contrast to cucumber, where organ abortion is whorl specific rather than organ identity specific. In male cucumber flowers, carpel abortion only happens in the central carpels, and not in stamens transformed into ectopic carpels in B-class mutants (Kater et al., 2001). This distinction between maize and cucumber highlights the diversity of developmental pathways to unisexuality in the angiosperms (Diggle et al., 2011), and underlines the importance of investigating the molecular linkages between organ identity and sexual differentiation in the grasses.

Developmental pathways to unisexual flowers are diverse, both across the angiosperms (Diggle et al., 2011), and within the grasses themselves (Reinheimer et al., 2010). This diversity has been interpreted to mean that the molecular mechanisms underlying the development of unisexuality are likewise diverse (Diggle et al., 2011). Although the grasses offer an excellent system to test this hypothesis, there is not enough yet known about the hormonal regulation of unisexuality outside of maize to draw substantive conclusions. What is known supports the hypothesis that hormonal regulation of unisexuality is not conserved in the grasses: *TS2*/JA-mediated carpel abortion may be specific to maize and its close relatives

(Li et al., 1997; Malcomber & Kellogg, 2006). In contrast, transcriptional regulators that have been identified in both barley and maize are almost all unified by the property of negatively regulating growth. *GT1, TB1, VRS1, VRS4* (and its maize ortholog, *RA2*) all act to repress growth in various ways during development—in flowers, but also in vegetative (*GT1, TB1)* and inflorescence branches *(VRS4, RA2)*. Thus, understanding the transcriptional control of growth repression in maize and barley flowers, and how growth repression interacts with organ identity pathways, may be key in understanding the convergent evolution of floral sexuality more broadly.

6 Conclusions

Grass floral diversity, although modest in comparison to some other clades, offers an opportunity to dissect the molecular underpinnings of the evolution of novelty, the evolution of morphological diversification, and the convergent evolution of floral sexuality. In each case, the combination of model systems that are morphologically distinct, and diversity in the family at large, provides a framework to identify and functionally characterize the genes and gene networks that drive the development and evolution of floral form. Comparisons between very well-studied genes in the eudicots and their homologs in the grasses also allows for understanding gene and gene family evolution at a nuanced level. Which classes of genes are more likely to be recruited to a new function? Which aspects of gene function are more likely to be conserved, and which diverge over time? More detailed understanding of gene function in the grasses will also inform candidate gene and comparative gene expression studies in lineages, particularly in the monocots, that might display fantastic diversity, but will likely never be developed as genetic model systems. Thus, the time is ripe to leverage grass diversity for fundamental understanding of flower evolution and development.

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