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DARWIN REVIEW

Hybrid breeding in wheat: technologies to improve hybrid wheat seed production

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Abstract

Global food security demands the development and delivery of new technologies to increase and secure cereal production on finite arable land without increasing water and fertilizer use. There are several options for boosting wheat yields, but most offer only small yield increases. Wheat is an inbred plant, and hybrids hold the potential to deliver a major lift in yield and will open a wide range of new breeding opportunities. A series of technological advances are needed as a base for hybrid wheat programmes. These start with major changes in floral development and architecture to separate the sexes and force outcrossing. Male sterility provides the best method to block self-fertilization, and modifying the flower structure will enhance pollen access. The recent explosion in genomic resources and technologies provides new opportunities to overcome these limitations. This review outlines the problems with existing hybrid wheat breeding systems and explores molecular-based technologies that could improve the hybrid production system to reduce hybrid seed production costs, a prerequisite for a commercial hybrid wheat system.

Key words: Cereals, CHA, crop, fertility control, flower, heterosis, spike.

We should always keep in mind the obvious fact that the production of seed is the chief end of the act of fertilization; and that this end can be gained by hermaphroditic plants with incomparably greater certainty by self-fertilization than by the union of the sexual elements belonging to two distinct flowers or plants.

Charles Darwin (1876)

Introduction

The agricultural industries have shown spectacular improvements over the past 50 years. Food production remains dominated by the cereals, which make up around 50% of global food production (FAOSTAT, 2013). Since the introduction of the Green Revolution crops in the early 1960s, there has been a linear increase in total cereal production from less than 1 billion t to 2.6 billion t in 2011 (FAOSTAT, 2013).

Three factors have underpinned these rapid improvements: (i) improved varieties through the development and adoption of breeding technologies; (ii) expansion of the area under irrigation; and (iii) the widespread use of fertilizers, particularly nitrogen and phosphorus. Scope for expanding the area under irrigation or increasing fertilizer use are limited; therefore, future gains are most likely to come from efficient and accurate breeding and selection technologies. With the predicted growth in the world population to around 9 billion,

Abbreviations: CHA, chemical hybridizing agents; CMS, cytoplasmic male sterility; GM, genetic modification; GMS, genic male sterility; NCIII, North Carolina Design III; ORF, open reading frame; PPR, pentatricopeptide repeat; PTGMS, photo-thermo-sensitive genic male sterility; SI, self-incompatibility; TTC, triple testcross design.

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the World Food Summit on Food Security in 2009 set a target of 70 increased food production by 2050, which would require an annual rate of increase of 44 million t. This is an ambitious target for several reasons. Firstly, because of the increasing cost of fertilizers and their negative impact on the environment, there are serious concerns about the viability of existing production systems and the sustainability of the current growth rates. Secondly, the predicted environmental changes associated with climate change are expected to have an overall negative effect on agricultural production with significant crop declines in some countries; for example a 15–30% decline in production is predicted in Australia (PMSEIC, 2010).

In wheat, the rate of yield gain has declined over the past decade (FAOSTAT, 2013). This stands in contrast to the situation in rice and maize where yield improvements have continued unabated (FAOSTAT, 2013). Three main reasons have been proposed for the difference between maize and wheat, in particular: higher levels of investment in maize research through the involvement of the private sector, the early adoption and implementation of genetic engineering in maize improvement programmes, and the opportunities provided by hybrid technologies.

The Food and Agriculture Organization of the United Nations (FAO) predicts that major improvements in wheat yields will be critical in ensuring global food security. Consequently, there is renewed interest in technologies that offer yield advantages, particularly for low-yielding environments where wheat is widely grown (Tester and Langridge, 2010). One of the most promising options is to capture the yield benefits from heterosis in a hybrid wheat programme. Recent estimates of yield improvements associated with heterosis in wheat range from 3.5 to 15% (Longin *et al.*, 2012). The ability to exploit heterosis (hybrid vigour) in wheat has historically been difficult due to the strong inbreeding nature of wheat, a factor governed primarily by floral development and architecture, and the lack of practical fertility control systems.

Although hybrid wheat programmes have operated for several decades, hybrids account for a minor fraction of the total area sown. The discovery of male sterility and restoration systems in the 1960s triggered great interest in hybrid wheat from both the public and private sector. However, the hybrid systems that were explored were impractical and, consequently, difficult to use. Cytoplasmic male sterility has been difficult to use due to a lack of effective fertility-restoration genes. Genic male sterility have largely failed due to problems with fertility restoration, and chemical hybridizing agents suffer from problems of toxicity and selectivity. It has also been suggested that yield benefits in wheat are due to the combination of dispersed dominant alleles (Pickett, 1993) and that similar yield advantages could be achieved by conventional line breeding. Therefore, despite widespread interest, many companies have shut down their hybrid wheat programmes and today only a few still operate (Jordaan, 1996; Longin *et al.*, 2012). Currently, genetic engineering is being used to develop a range of innovative new hybrid breeding systems with several proposed or under development for wheat (reviewed by

Kempe and Gils, 2011). It is therefore time that ‘hybrid wheat’ is reassessed, especially in terms of modifying floral architecture to facilitate hybrid seed production based on new strategies, technologies and knowledge.

Heterosis in wheat

The main goal of hybrid breeding is to systematically exploit heterosis. Heterosis of a hybrid is expected to increase with the genetic divergence between its parents (Melchinger, 1999). Consequently, grouping of lines into genetically divergent heterotic pools is of paramount importance to make maximum use of heterosis (Reif *et al.*, 2005). Genetically divergent groups are not expected to exist in wheat elite germplasm adapted to a particular target environment, because of the intensive exchange of elite lines. Use of lines from different target environments has been suggested as a method to promote genetic diversity among pools (Koekemoer *et al.*, 2011). However this approach is complicated by the different requirements for vernalization, photoperiod, quality, and frost tolerance. Consequently, sophisticated solutions are required to develop genetically distinct groups with high heterotic combining ability for grain yield combined with high end-use quality (Longin *et al.*, 2012). An improved understanding of the underlying genetic mechanisms of heterosis represents a key step towards a systematic development of complementary groups of lines exhibiting high heterosis.

Heterosis can be explained genetically by: (i) the joint action of multiple loci with the favourable allele either partially or completely dominant (Bruce, 1910; Keeble and Pellew, 1910; Jones, 1917; Collins, 1921), (ii) overdominant gene action at many loci (East, 1936; Hull, 1945; Crow, 1948), and/or (iii) epistatic interactions between non-allelic genes (Richey, 1942; Schnell and Cockerham, 1992). Different classical quantitative genetic experiments have been conducted to elucidate the prevalent gene actions underlying heterosis (Hallauer and Miranda, 1988). The outcomes of these studies, however, are of limited use, because the estimated parameters reflect the net contribution of gene effects summed over all loci.

Quantitative trait loci mapping provides a means of determining the relative importance of these genetic mechanisms in heterosis (reviewed by Schnable and Springer 2013). To elucidate the genetic basis of heterosis, two prominent experimental designs have been applied: North Carolina Design III (NCIII) (Comstock and Robinson, 1952) and the triple testcross design (TTC) (Kearsley and Jinks, 1968) (Fig. 1). In NCIII, a segregating population derived from the cross between two inbred lines is backcrossed to its parents. The TTC is an extension of NCIII, where the segregating population is additionally backcrossed to the hybrid of the two parents. NCIII enables identification of loci contributing to heterosis. It is important to note, however, that the contribution of a particular gene to the genetic variation of heterosis is a function of its dominance effect and its cumulative interaction effects with all other loci in the genome (Melchinger *et al.*, 2007). While NCIII does not enable further partitioning of the heterotic effect into the main and interaction

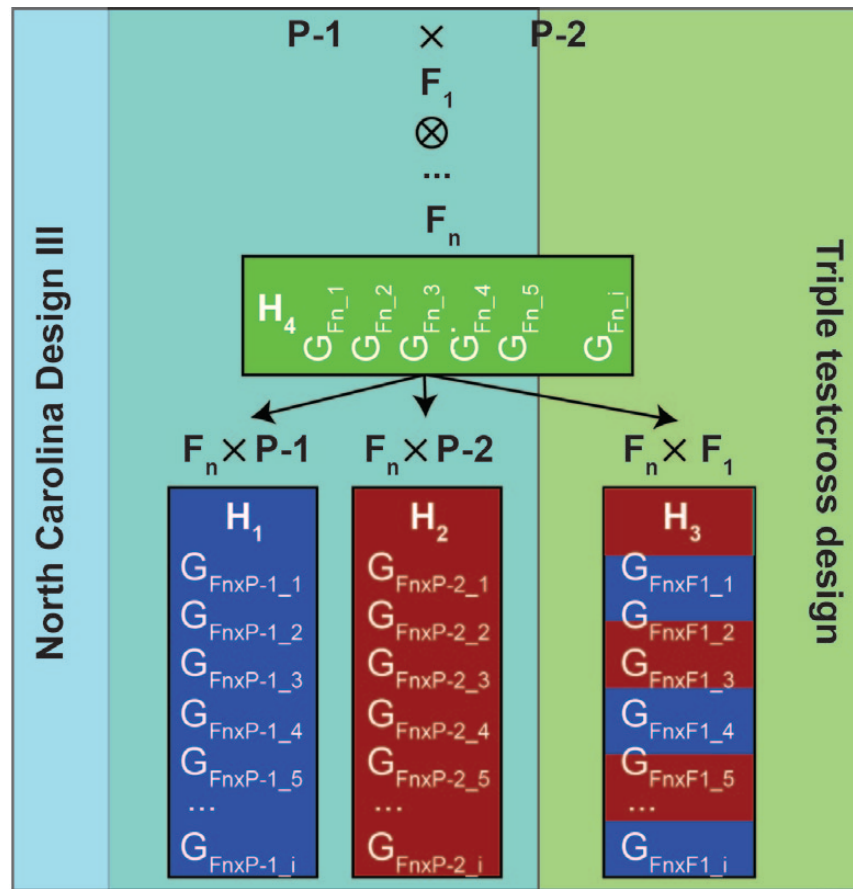


Fig. 1. Experimental designs for determining the genetic basis of heterosis. Both NCIII and TTC designs begin with an F₂ segregating population having i plant individuals, created from a cross between two parental inbred lines (P-1 and P-2) that differ in the trait of interest. Instead of selfing the F₂ to produce F₂:3 progeny, in the NCIII scheme all F₂ individuals are backcrossed as female parents with pollen from each parental line: P-1 and P-2. The individuals in the two resulting lines, denoted by G_{FnxP-1_j} and G_{FnxP-2_j} , are then scored for studied phenotypes. In the TTC scheme, the F₂ individuals are further backcrossed to F₁ to generate the third line G_{FnxF1_j} . The third line provides additional information to distinguish dominant effects.

components, TTC allows the presence of at least some types of interaction effects to be tested (Melchinger *et al.*, 2008).

Using the NCIII for mapping heterosis quantitative trait loci, García *et al.* (2008) showed that heterosis in maize was mainly due to dominant gene action, while dominance could not explain heterosis in rice, suggesting some epistatic effects. The difference between the mechanisms in maize and rice seemed to be related to the open versus the self-pollinated nature of the two species. The relevance of epistasis in self-pollinating species is further supported by a series of studies investigating the genetic basis of heterosis in the model species *Arabidopsis* (Kusterer *et al.*, 2007; Melchinger *et al.*, 2007; Reif *et al.*, 2009). The number of experimental studies in wheat on the genetic basis of heterosis is low (e.g. Yuan *et al.*, 2012). This is mainly due to the difficulty in producing the large quantity of experimental hybrids needed for precise phenotyping. Therefore, advances in promoting controlled cross-pollination in wheat represents a major step towards the study of the genetic basis of heterosis in this species.

Besides the surveys on the genetic basis of heterosis, there are multiple experiments on the molecular basis of heterosis (reviewed by Schnable and Springer, 2013). Molecular

explanations of heterosis include the complementation of allelic variation (e.g. Springer and Stupar, 2007) and variation in gene expression patterns (e.g. Guo *et al.*, 2006), as well as proteomic variation (e.g. Goff, 2011). Moreover, a potential role for epigenetic regulation in heterosis has also been proposed (e.g. He *et al.*, 2010).

Heterosis and hybrid performance of complex agronomic traits such as grain yield is very probably influenced by many loci. Genomic selection has been suggested to predict the phenotype for traits that are controlled by multiple genes with small effects. In this approach, a large number of markers distributed across the genome are used simultaneously to train a prediction model (Meuwissen *et al.*, 2001). Genomic selection has been used successfully to predict hybrid performance in wheat (Zhao *et al.*, 2013) and maize (Massman *et al.*, 2013). Moreover, genomic selection was successfully used to predict general combining ability effects in maize (Albrecht *et al.*, 2011; Riedelsheimer *et al.*, 2012; Zhao *et al.*, 2012a,b, 2013c). Genomics-based prediction has complemented data from gene expression and metabolomic profiling to yield promising accuracies for predicting hybrid performance (Frisch *et al.*, 2010; Fu *et al.*, 2010, 2012; Riedelsheimer *et al.*, 2012).

Summarizing, ‘omic-based prediction of hybrid performance is a promising avenue to significantly reduce the resources and time invested in hybrid breeding.

Floral architecture: capturing existing and novel variation

Using diversity in floral traits to breed ‘male’ and ‘female’ ideotypes

Redesigning the wheat flower will be important for efficient production of hybrid seed. While it is a complex procedure, this is an achievable target, as our understanding of the control of floral architecture has greatly improved over the past few years (see reviews by Barazesh and McSteen, 2008; Thompson and Hake, 2009).

Wheat flowers are composed of spikelets which are made up of bract-like organs, glumes, and florets (Fig. 2A–C). The lemma and palea envelop the male and female reproductive organs. At anthesis, rapid swelling of a small organ located at the base of the floret, called the lodicule (Fig. 2D), opens the floret and exposes the anthers and pistil for pollination, a state called chasmogamy. Wheat flowers are largely cleistogamous, and pollen is shed before or just after flowers start opening. Stiff glumes, lemmas, and paleas are often found in common wheat varieties, and are associated with traits that prevent flower opening and kernel shattering (Vogel, 1941; Zhang *et al.*, 2009). In barley, the physiological basis for chasmogamy is lodicule swelling; this separates the palea and lemma and allows anther extrusion through osmotically induced filament elongation (Heslop-Harrison and Heslop-Harrison, 1996).

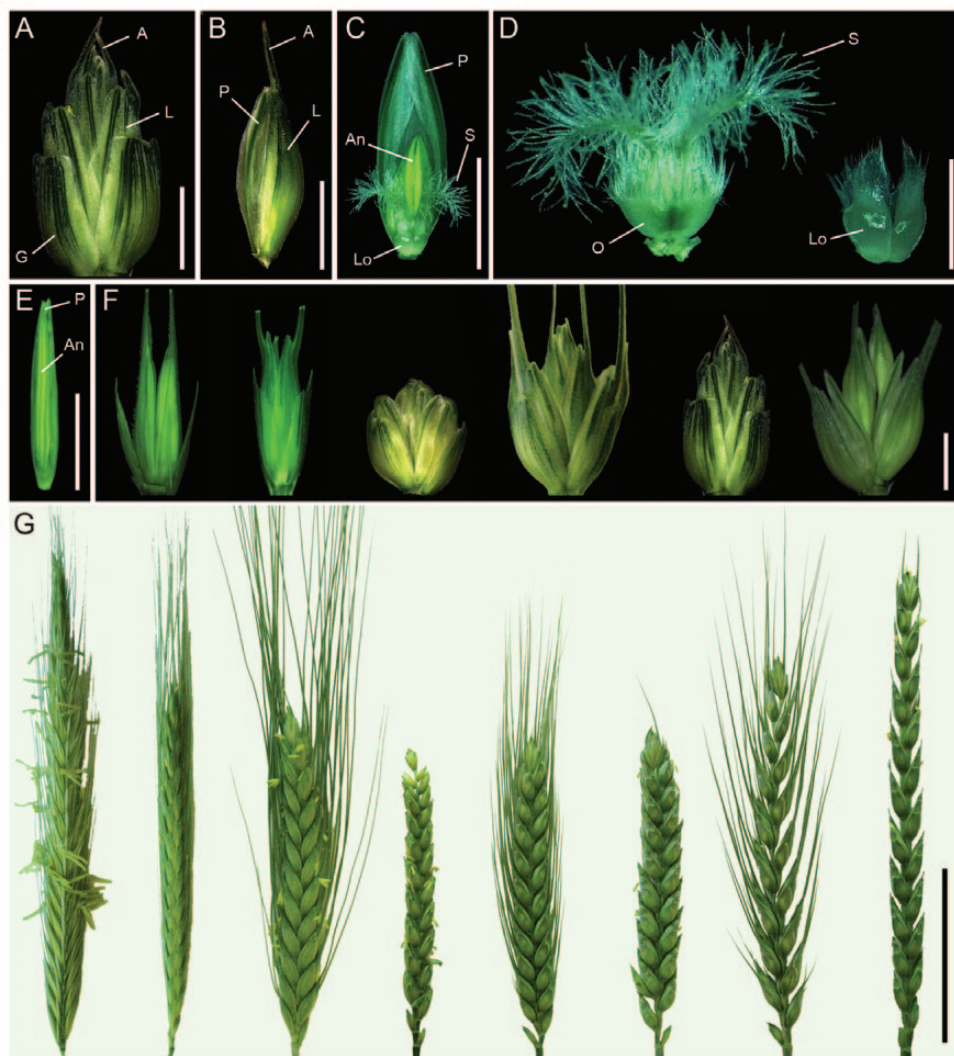


Fig. 2. Structure of wheat flowers and spikes. (A) Wheat spikelet. (B) floret. (C) Palea and reproductive tissues. (D) Lodicule and female reproductive tissues. (E) *Secale cereale* (rye) floret. (F) Spikelets of various Triticeae species, from left to right: rye, *T. monococcum* ssp. *boeoticum*, three *T. aestivum* varieties (Chinese spring, Magenta, and Kite) and *T. aestivum* landrace. (G) Spikes of various Triticeae species, from left to right: rye, *T. monococcum* ssp. *boeoticum*, *T. turgidum* ssp. *Durum*, and five *T. aestivum* (bread wheat) varieties (Chinese Spring, Cadoux, Ghurka, and Sentinel, Kite). Bars, 5 mm (A–C, E, F); 2 mm (D); 5 cm (G). A, awn; An, anther; G, glume; L, lemma; Lo, lodicule; O, ovary; P, palea; S, stigma.

Ideally, both male and female parental plants for hybrid seed production would possess open flowering spikelets and the following desirable traits to achieve cross-pollination. Large lodicules, a soft lemma, and palea in well-spaced spikelets along long spikes (Murai *et al.*, 2002) (Fig. 2G, see the two *laxatum*-like spikes on right) would also enable each floret to open widely. The male ideotype plant would be tall with long extruded anthers producing large quantities of long-life pollen able to disperse metres away. In comparison, the female ideotype would be a shorter plant with multiple chasmogamous florets to maximize pollen reception. Stigmatic hairs would be long, fully extruded and receptive for extended periods. Most importantly, the female ideotype should be male sterile and/or self-incompatible (Fig. 3), therefore preventing self-pollination and ensuring cross-fertilization for commercial hybrid seed production (see section on ‘Fertility control systems’). This also allows row interplanting or mixed planting of male and female parental lines. Finally, the flowering time of male and female plants should be synchronized.

Variation and heritability estimates for most of these traits in cultivated wheat are often moderate to high (see reviews by Virmani and Edwards, 1983; Pickett, 1993), suggesting that much progress can be made in improving the cross-pollinating ability of inbred parents derived from breeding populations. For several of these traits, the genetic control appears to be simple, implying that single or few genes are responsible for the phenotype. The diversity among grass inflorescences is a result of variation in the identity and determinacy of floral meristems produced throughout inflorescence development (Fig. 2F, G). A range of synthetic wheats, where variation in floral morphology has been reported, is available (e.g. Chhabra and Sethi, 1991; Murai *et al.*, 2002; Yang, 2010). Mutants and associated genomic and genetic resources in barley, widely seen as a model for wheat, have provided powerful new tools for the isolation and characterization of genes controlling developmental processes of flower formation (Druka *et al.*, 2011) (Table 1). Some close relatives of wheat, such as rye (*Secale cereale* L.), are obligate outcrossers and

possess a floral architecture that enhances cross-pollination. Rye contains large anthers that are fully extruded from the floret (Fig. 2E, G), and a self-incompatibility system to prevent selfing.

Changes in floral meristems have also been important in crop domestication. For example, the domestication gene *Q* is a major regulator of floral architecture and has resulted in the introduction of free-threshing characteristics and a compact spike in cultivated wheat (Simons *et al.*, 2006). Such floral characters have inadvertently reduced cultivated wheat’s ability to cross-pollinate.

Genes controlling floral architecture in wheat and barley

Floral development of monocotyledonous and dicotyledonous species can be explained by an ABCDE model whereby organ identities are determined by a specific class or a combination of classes of genes (Coen and Meyerowitz, 1991; Theissen, 2001). Some of these regulatory genes are well conserved across species. It is anticipated that this model could be partially translated to wheat floral development (Ciaffi *et al.*, 2011) and be exploited for the purpose of redesigning the floral architecture of male and female plants (Table 2). There are a number of cereal genes, such as various MADS box genes, involved in floral determinacy and differentiation of the glume, lemma, and lodicule (Table 2; Sreenivasulu and Schnurbusch, 2012). For example, *OsMFO1* regulates palea and lodicule identity in rice (Ohmori *et al.*, 2009), and *TaQ* is involved in the determination of glume shape, lodicule size, and other floral traits in wheat (Simons *et al.*, 2006). These genes are potential targets for manipulating wheat’s floral architecture.

One of the most promising targets is the microRNA *miR172*. MicroRNAs are regulatory small RNAs that repress gene expression by targeting a cognate mRNA for cleavage or translational repression. *miR172* is conserved in higher plants and regulates A-class APETALA2 (AP2) and

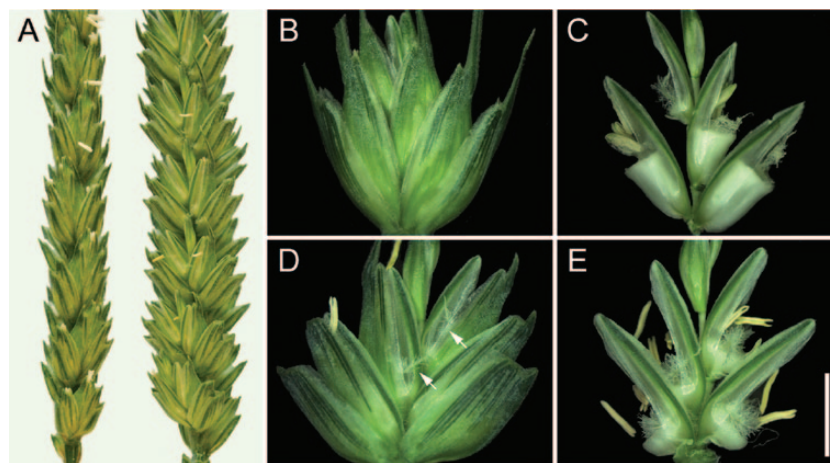


Fig. 3. Differences between fertile and sterile wheat flowers and spikes. (A) Fertile (left) and sterile (right) spikes of wheat. (B) Fertile spikelet. (C) Glumes and lemmas removed from the fertile spikelet in (B). (D) Sterile spikelet. Arrows indicate stigmas extruded from the floret. (E) Glumes and lemmas removed from the sterile spikelet in (D). The unfertilized ovary expands horizontally opening the floret. Bar, 5mm.

Table 1. Barley mutants showing altered floral development, which could be exploited for the molecular identification of wheat floral genes

Manipulation of these genes in wheat could help either enhance cross-pollination or increase hybrid seed set per spike (Takahashi, 1972; Larsson, 1985; Forster et al., 2007; Shahinnia et al., 2012).

Class	Name	Developmental effect
<i>com1.a</i>	<i>Compositum</i>	Branched spikelet
<i>dub1</i>	<i>Double seeds 1</i>	Fasciation of the floret (wide lemma trait) resulting in the formation of double (<i>dub1</i>) and triple-kernel mutants
<i>Flo</i>	<i>Extra floret</i>	A single adventitious floral bud (spikelet) occasionally arise below the central bud and form an extra floret
<i>Int</i>	<i>Intermedium spike</i>	Extra spikelet
<i>Lax</i>	<i>Laxatum</i>	Rachis internodes; conversion of the lodicules into anthers in <i>lax-a</i> but the extra anthers are deficient
<i>Dsp</i>	<i>Dense spike</i>	Dense or compact spike; rachis internode length
<i>mov1/mov2</i>	<i>Multi-ovary</i>	The lodicules of the <i>mov1</i> mutant become somewhat leafy or sepal-like, stamens are partially or completely converted into pistils
<i>mul1/vrs4</i>	<i>Multiflorus1/supernumerary florets</i>	Number of floral buds increase, 2 or more florets are produced within a spikelet, the alternating florets face each other and the multi-floreted structure is contained within a pair of glumes

Table 2. Transcription factors that alter flower morphology in wheat and barley as potential molecular targets for allele mining

Class	Wheat and barley genes	Orthologue gene	Orthologues and developmental role	References
APETALA2	<i>HvCly1</i> , <i>TaQ</i>	<i>indeterminant spikelet1 (ids1</i> , maize)	Lodicule size; spike morphology; number of florets per spikelet; sex determination in the tassel and branching in inflorescences.	Nair et al. (2010); Chuck et al. (2007)
APETALA3	<i>TaAP3</i> , <i>HvAP3</i>	<i>SUPERWOMAN1</i> <i>OzSPW1</i>	Identity of stamens and lodicules	Nagasawa et al. (2003)
AGAMOUS-LIKE6-like MADS box	<i>TaMADS16</i> , <i>HvAGL6</i>	<i>MOSAIC FLORAL ORGANS1</i> (<i>OzMFO1/MADS6</i>)	Identity of lodicules and ovules; lodicule size	Ohmori et al. (2009)
AGAMOUS	<i>TaWAG</i>		Stamen development	Meguro et al. (2003)
SHORT INTERNODES	<i>HvLks2</i>		Awn elongation; pistil morphology	Yuo et al. (2012)
KN1-like homeobox	<i>TaWKNOX1</i> , <i>HvBKN3</i>		Meristem identity; number of flower in the spikelet	Takumi et al. (2000); Osnato et al. (2010)

AP2-like transcription factors in maize (Lauter et al., 2005; Chuck et al., 2007, 2008), rice (Zhu et al., 2009), and barley (Nair et al., 2010). *miR172* functions in regulating the transition between developmental stages of flower formation and in specifying floral organ identity (Zhu and Helliwell, 2011).

In barley, a critical balance between AP2 and *miR172* is required for normal lodicule development. In cleistogamous florets, the lodicule is atrophied, due to an imperfect homeotic conversion controlled by an AP2 transcription factor CLEISTOGAMY1 (CLY1) (Nair et al., 2010). Mutation at the *miR172* target site of *CLY1* causes a loss of conductive tissue in the lodicules and a failure of the lemma and palea to open at anthesis. The wheat domestication gene *Q* encodes an AP2-like transcription factor, which is also a possible *miR172* target. The *Q* gene influences the number of florets per spikelet and might regulate lodicule development as a possible

orthologue of barley *Cly1*. The recessive *q* allele present in diploid wheat, hexaploid wheat mutants, and natural variants results in an elongated rachis and reduced number of florets per spikelet (Zhang et al., 2011), in contrast to cultivated wheat with the dominant *Q* allele. However, it is currently not clear whether *Q* controls determinacy of the spikelet meristem or heterochronic development of the floral meristem (Shitsukawa et al., 2009).

miR172 is also implicated in maize sex determination, which occurs through abortion of female carpels in the tassel and arrest of male stamens in the ear (Chuck et al., 2007). *tasselseed6 (ts6)* and *tasselseed4 (ts4)* mutations permit carpel development in the tassel while increasing meristem branching, showing that sex determination and acquisition of meristem fate share a common pathway. The *ts4* phenotype has been shown to be the consequence of a loss of *miR172*

expression, while *ts6* possesses a mutation in the *miRNA miR172* binding site of the *AP2*-like transcription factor *indeterminant spikelet1* (Chuck *et al.*, 2007). The role of *AP2*-like transcription factors in sex determination, floral architecture, and ultimately fecundity implicates *miR172* as a master floral development regulator.

Although some interesting targets could be modified for improving hybrid seed production in wheat, this survey also raises several issues. Firstly, most studies to date have determined that strong alleles of major genes regulate floral organ identity, a trait that should not be modified in male and female ideotypes unless it could be reverted in the F1 plants. We need to identify alleles associated with quantitative differences in organ size to manipulate floral architecture. Secondly, how can we modify floral organs without losing valuable domestication traits such as free-threshing (Simons *et al.* 2006)? Pleiotropic effects of floral genes on phenology and other plant structures are well described. For example, a single amino acid substitution in the *Q* gene changes properties of the transcription factor, which in turn affects the expression of many downstream genes, explaining its pleiotropic nature (Simons *et al.*, 2006). We need to identify new genes and alleles that regulate floral development without altering the ultimate fate of meristematic cells.

Fertility control systems

An effective hybrid seed production system requires a reliable and cheap system for forcing outcrossing. This depends on blocking self-pollination by inducing male sterility or self-incompatibility. Several options have been explored in wheat.

Chemical hybridizing agents (CHAs)

The term CHA describes this class of chemicals in hybrid seed production that cause male sterility (Fig. 4A) and, depending on mode of action and dosage, can sometimes lead to female sterility (McRae, 1985). An advantage inherent to CHA use is that male sterility can be induced in the female inbred parent by simply spraying a chemical, therefore significantly reducing production costs. The use of CHAs allows the production of a high number of parental combinations for estimating germplasm combining ability.

A CHA is only useful for commercial hybrid seed production if it selectively induces male and not female sterility, is genotype independent, and has systemic activity and persistence to allow for different stages of maturity among the treated plants. Because rain, wind, and heat can reduce the efficacy of CHA application in the field, it is important that the period of application is broad enough to overcome these negative environmental conditions. The CHA must be non-phytotoxic and non-mutagenic, environmentally safe, economic to synthesize, practical to apply, and flexible in the dosage to permit a secure margin for application. Finally, CHAs must not affect F1 seed quality and seedling or plant vigour. Because of such stringent prerequisites, few CHAs have been taken up by commercial

seed companies (Virmani and Edwards, 1983; Pickett, 1993; Cisar and Cooper, 2002).

The earliest report of CHA use in wheat was maleic hydrazide (Hoagland *et al.*, 1953) followed by anti-lodging and height-reducing agents like ethephon (Ethrel) (Rowell and Miller, 1971), gibberellins (Porter and Wiese, 1961), and RH531 and RH532 (Jan *et al.*, 1974, 1976). All these chemicals showed strong phytotoxic effects and inadequate male sterility across a range of environments and their commercial use was considered too risky. This led to the development of next-generation CHAs such as fenridazon-potassium (RH-0007, HYBEX[®]) (Mizelle *et al.*, 1989), the sogital compound SC2053 (Orsan) (Wong *et al.*, 1995), azetidine-3-carboxylic acid (WL 84811) from Shell, clofencet (Genesis[®]) from Monsanto, and sintofen (Croisor[®]100) from Saaten Union Recherche. RH-007 was used for commercial production in the USA and Europe for a limited time, because it only worked in select genotypes and in a narrow application window and was therefore deemed commercially high risk (Cisar and Cooper, 2002). WL84811 was used in Europe, the USA, South Africa, China, Australia and New Zealand until it was discontinued because toxic residues were detected in F1 seed produced on treated plants (Pickett, 1993). Genesis[®] was used in wheat for commercial hybrid seed production in the USA and Europe until 2007 (Cisar and Cooper, 2002; Parodi and de los Angeles Gaju, 2009). Croisor[®]100, a plant growth regulator (EFSA, 2010), is the only CHA currently being used in Europe for commercial production of hybrid wheat. Although the modern CHAs are effective across a broad range of genotypes and have reduced phytotoxicity, their commercial deployment is still hindered by a narrow window for application, which is subject to the prevailing environmental conditions.

Cytoplasmic male sterility (CMS)

CMS in plants is based on rearrangements of mitochondrial DNA, which lead to chimaeric genes and can result in the inability to produce fertile pollen (Hanson and Bentolila, 2004; Horn, 2006). For example, recent sequencing of the first CMS-derived mitochondrial genome (K-type) from wheat revealed novel fusions between open reading frames (CMS-ORFs) of low sequence homology with genuine protein-coding genes (Hanson and Bentolila, 2004; Liu *et al.*, 2011). Exactly how these CMS-ORFs induce male sterility is still unclear, although they have been identified as disrupting tapetal cell or microspore function by invoking oxidative stress responses (Karpova *et al.*, 2002; Pring *et al.*, 2006; Fujii and Toriyama, 2008, 2009). CMS can arise both spontaneously and following mutagenesis, or be the result of interspecific, intraspecific, and intergeneric crosses (Kaul, 1988). In cultivated wheats, CMS lines can be created by initially crossing common wheat as the pollen donor to wild wheat (e.g. *Triticum timopheevii* Zhuk.) or related species such as *Aegilops*, *Hordeum*, and *Secale*, and then backcrossing to common wheat (Wilson and Ross, 1962; Mukai and Tsunewaki, 1979; Martin *et al.*, 2008). According to Adugna *et al.* (2004), cytoplasm from as many as 35 species can be transferred to common wheat,

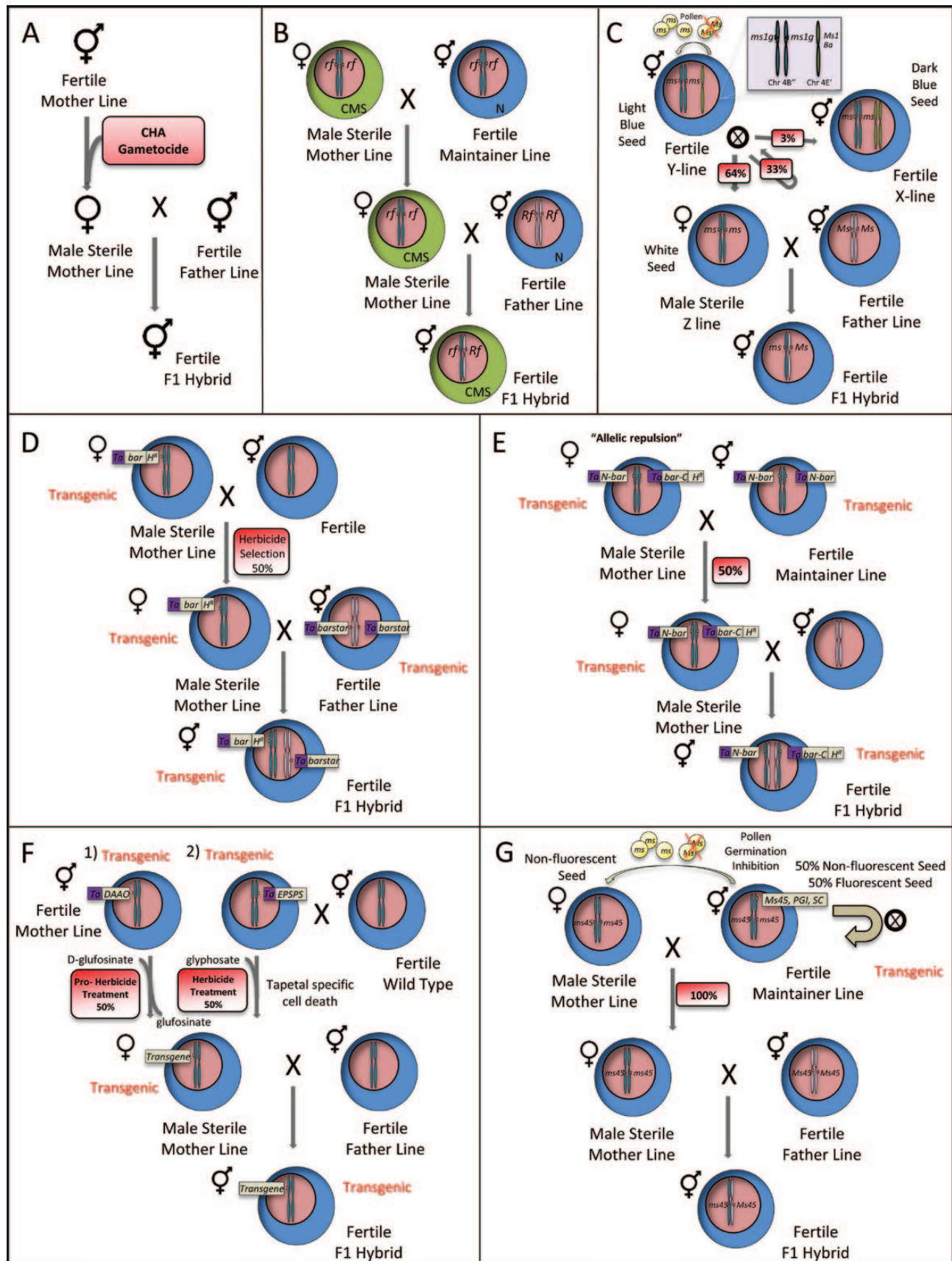


Fig. 4. Hybrid breeding systems that utilize pollination control. (A) Chemical hybridizing agents (CHAs). (B) CMS-based hybrid breeding system. Individuals independently inherit cytoplasmic male sterility (CMS) or native (N) cytoplasm as well as nuclear-encoded restorer loci (*Rf*, *rf*). (C) XYZ-like hybrid wheat breeding system 4E-*ms* (Zhou *et al.*, 2006), based on the non-conditional recessive male-sterile mutant *ms1* located on chromosome 4BS (e.g. *ms1g* mutant allele). Fertility can be restored to the homozygous male-sterile mutant

and among these complete to partial sterility has only been observed for 20 species.

The effect of male sterility-inducing cytoplasm in wheat can be counteracted by nuclear-encoded fertility-restorer (*Rf*) genes. *Rf* genes are classified as either sporophytic or gametophytic in action depending on the affected tissues. Sporophytic *Rf*s are more practical for hybrid breeding because heterozygotes (*Rf/rf*) produce 100% viable pollen grains whereas only 50% of pollen grains are viable in gametophytic heterozygotes. This can, in the F1, have undesired yield penalties. Molecular cloning of *Rf*s from a range of species has revealed that they often encode proteins containing a common degenerate motif called a pentatricopeptide repeat (PPR) (Bentolila *et al.*, 2002; Brown *et al.*, 2003; Desloire *et al.*, 2003; Kazama and Toriyama, 2003; Koizuka *et al.*, 2003; Akagi *et al.*, 2004; Komori *et al.*, 2004). These are sequence-specific RNA-binding proteins, which in some cases can directly bind CMS-ORFs, typically suppressing their transcription and translation (Gillman *et al.*, 2007; Kazama *et al.*, 2008; Uyttewaal *et al.*, 2008). The recent identification of disrupted untranslated region structures flanking wheat K-type CMS mitochondrial gene sequences (Choi *et al.*, 2012) is suggestive of the corresponding wheat *Rf* gene sequences encoding PPR proteins. Growing bioinformatics capabilities are now supporting the prediction of RNA recognition specificities derived from knowledge of PPR tracts (Barkan *et al.*, 2012). Coupled with the complete K-type CMS mitochondrial genome sequence, this information may now help elucidate exactly which PPR gene sequence(s) can act as *Rf*s. This is an important step towards being able to identify, develop, and deploy molecular markers for tracking key genes responsible for CMS expression and full fertility restoration within hybrid breeding programmes.

In wheat, two or three major restorer loci are required for complete fertility restoration (Bahl and Maan, 1973). According to Ma and Sorrells (1995), the universal expression of as many *Rf* genes as possible seems to be beneficial for obtaining stable and high fertility restoration. In order to maintain a male sterile line, it must be crossed to a sister line (called the maintainer line), which has the identical nuclear genotype but a fertile

cytoplasm derived from an elite adapted line. The maintainer line carries recessive restorer allele (*rf*); therefore, when this male-fertile line is crossed to a sterile CMS plant, it creates sterile progeny (Fig. 4B). For commercial hybrid seed production, a male-sterile line must be crossed to a line carrying dominant restorer alleles with excellent pollinator qualities. This is necessary for producing fertile F1 seed. Here, it is unimportant if the pollen donor exhibits alien or native cytoplasm. The only prerequisite is that the genotype is homozygous for *Rf* gene(s).

In general, CMS is a relatively inflexible system that is only feasible for hybrid seed production when CMS mutants and effective fertility restorers are available in a given crop and if the CMS mutation is not associated with yield penalties or other undesirable phenotypic effects. Moreover, CMS systems are frequently sensitive to environmental factors, particularly temperature and photoperiod (Kaul, 1988). To date, only *T. timopheevii* Zhuk.-derived male-sterile cytoplasm have been used for commercial production of wheat hybrids (Longin *et al.*, 2012). However, this cytoplasm has undesirable side effects that are environment dependent (Baier *et al.*, 1978). These include incomplete fertility restoration and shrivelled F1 seed, which taken together can compromise hybrid yield (Adugna *et al.*, 2004). Commercially, this compromised heterotic advantage must still be sufficient to compensate for increased production and marketing costs that is inherent to such a complicated breeding strategy.

The possibility of either introducing CMS mutations via somatic hybridization or engineering CMS *de novo* could circumvent some of the issues that have hindered the uptake of CMS on a commercial scale. Somatic hybridization has been demonstrated in rice, where asymmetric protoplast fusion transferred BT- and LI-type CMS cytoplasm into the *Oryza japonica* cultivar Sasanashiki (Akagi *et al.*, 1995). Engineering CMS *de novo* is likely to be more challenging because it requires either stable organelle transformation or nuclear transformation, in which CMS-invoking proteins need to be retargeted to the appropriate organelle. Encouragingly, stable plastid genome transformation has recently been demonstrated in wheat (Cui *et al.*, 2011) whereas mitochondrial transformation

(Z-line) through the action of *Ms1* on alien chromosome 4E (*Agropyron elongatum* ssp. *ruthenicum* Beldie). Seed monosomic for 4E (*Ms1*) (Y line) is identifiable by light blue aleurone (*Ba*), whereas disomic seed (X line) is identifiable by a dark blue coloration. 4E is poorly transmitted through the male germline when plants monosomic for 4E are selfed, resulting in 64% white-seeded progeny. (D) Dual-component Barnase/Barstar transgenic system in which tapetal cell-specific expression (*Ta*) of Barnase (*bar*) induces male sterility. Linking the *bar* with herbicide resistance gene (*H^R*) allows in-field positive selection of male-sterile individuals by herbicide spraying. Barnase can be inactivated by the Barstar inhibitor (*barstar*), resulting in restored fertility. (E) Split barnase in allelic repulsion transgenic system of inducing male sterility. Tapetal cell-specific expression (*Ta*) of complementary barnase (*N-bar*, *bar-C*) gene fragments are located at allelic positions. Protein fragment ligation via intein-mediated protein *trans*-splicing induces Barnase activity and male sterility. Allelic positioning of the complementary *bar* fragments leads to completely segregation during meiosis resulting in male fertility and seed set in the F1 hybrid progeny. (F) Pro-herbicide (1) and herbicide (2) transgenic pollination control systems. (1) Tapetal cell-specific expression of D-amino acid oxidase (DAAO) converts D-glufosinate into the cytotoxic glufosinate, resulting in male sterility. (2) A specific promoter-intron combination drives expression of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) in all tissues except tapetal cells and microspores, inducing sterility upon the application of the herbicide glyphosate. (G) SPT uses a transgenic maintainer line for the propagation of a homozygous male-sterile mutant mother line (*ms45*). The maintainer transgene contains a dominant fertility restorer (*Ms45*) and a seed colour marker (*SC*), and is biologically contained to the maintainer line through the action of a pollen germination inhibitor (*PGI*). *SC* allows the visual separation of transgenic maintainer seed from non-transgenic male-sterile seed. F1 hybrids produced from this process are non-transgenic.

is yet to be established. The inability to transform the mitochondrial genome would normally preclude engineering CMS; however, a recent study showed that the plastid genome can act as a surrogate (Ruiz and Daniell, 2005). In this study, tobacco plastids were engineered to express *phaA*, a β -ketothiolase from *Acinetobacter* sp. This induced a male-sterile phenotype that was partially reversible by increasing the photoperiod.

Despite the many challenges, engineering CMS could significantly reduce the labour-intensive exercise of incorporating sterility-inducing cytoplasm(s) into breeding materials, a significant cost-prohibitive step to hybrid seed production.

Self-incompatibility

Self-incompatibility (SI) is a biological mechanism that prevents self-pollination in open-pollinated species. Although wheat is fully self-fertile, SI is widespread in the grasses, and cereal rye (*S. cereale* L.), a close relative of wheat, is an obligate outbreeder. In all grass systems studied, gametophytic SI is controlled by two multiallelic loci, *S* and *Z* (reviewed by Langridge and Baumann, 2008). The interaction of two genes means that SI in the grasses has several features that differentiate it from the more common, single-locus systems. Of particular importance are differences in reciprocal crosses and the varying levels of compatibility (namely, the percentage of compatible pollen) between two plants. Compatibility can range from 0 to 50, 75, or 100%, depending on the genotypes. For example, if a cross is made between plants with the genotype *SI.1 ZI.2* as female and *SI.2 ZI.3* as the pollen donor, 75% compatible pollen grains will be scored (as four pollen genotypes are produced, three compatible *SI.1Z.3*, *SI.2Z.1*, and *SI.2Z.3*, and *SI.1Z.1*, which is incompatible), whereas the reciprocal cross will show 50% of the pollen as compatible (two pollen genotypes: *SI.1Z.1*, which is incompatible and *SI.1Z.2*, which is compatible).

Could the grass SI system be activated in wheat to provide the basis for a hybrid system? A difficulty is that neither *S* nor *Z* have been cloned from any of the grass systems being studied, although both loci have been mapped at high resolution in *S. cereale* (Hackauf and Wehling, 2005), *Lolium perenne* (Shinozuka *et al.*, 2010), and *Phalaris corulescens* (Bian *et al.*, 2004). Indeed, despite considerable effort in several different SI systems, no system has been fully explained at the molecular level (Franklin-Tong, 2008). However, it may be possible to bring SI into wheat via a diploid progenitor or close relative. Interestingly, polyploid grasses can show the same level of SI as their diploid relatives and parents. The SI reactions can occur even if only one *S-Z* pair of alleles in the diploid pollen of tetraploid grasses is matched in recipient pistil. This process has been demonstrated in *S. cereale*, *Festuca pratense*, and *Dactylis glomerata* (Lundqvist, 1957, 1962, 1969), and in *L. perenne* (Fearon *et al.*, 1984a,b). Therefore, it may be possible to generate a SI wheat if the requisite genes can be identified in a close relative and introgressed into the wheat genome.

Genic male sterility systems

The utilization of mutations in nuclear-encoded genes, known as nuclear (NMS) or genic (GMS) male sterility can

greatly broaden the choice of parental lines when compared with CMS systems. They also avoid negative alloplasmic and cytoplasmic effects on yield, as well as problems associated with complete fertility restoration.

Mutations in nuclear-encoded genes that cause male sterility can occur spontaneously or be induced. Historically, spontaneous mutants have been observed and retained by wheat breeders; examples include Pugsley's, Langzhou, BNY-S, and Taigu, which affect *Ms1* (4BS) *Wtms1* (2B), and *Ms2* (4DS) fertility loci (Pugsley and Oram, 1959; Deng and Huang, 1993; Xing *et al.*, 2003; Zhou *et al.*, 2008). Mutations can also be induced through exposure to physical (e.g. γ -rays and X-rays) or chemical (e.g. ethylmethane sulfonate) mutagens. Examples of ionizing radiation-induced male-sterile wheats are Probus (*ms1b*) and Cornerstone (*ms1c*), whereas ethylmethane sulfonate-induced male-sterile wheats include FS2 (*ms1d*), FS20 (*ms5*, 3AL), and KS87UP9 (*ms3*, 5AS) (Fossati and Ingold, 1970; Driscoll and Barlow, 1976; Sasakuma *et al.*, 1978; Maan *et al.*, 1987). Depending on the mutated locus, they are either dominant (*Ms2*, *Ms3*) or recessive (*ms1*, *ms5*), and can be classified as being conditional or non-conditional, depending on whether environmental factors revert fertility.

Comparable to CMS, conditional GMS can be temperature and/or photoperiod dependent, with mutations classified as being either thermo-, photoperiod- or photo-thermo-sensitive GMS (PTGMS). An example of the utility of PTGMS is the two-line hybrid rice system, which takes advantage of a mutation in *O. japonica* cv. Nongken 58S. This system has been used successfully for grain production in China since 1995 (Mei *et al.*, 1999). However, the deployment of a PTGMS two-line hybrid wheat system (BS20, C49S) in China has been limited by two factors. Firstly, effective fertility-restoring germplasm seems to be restrictive (Zhang, 2005), and secondly, certain climatic regions are not conducive to sterility expression. For example, reports have highlighted the failure of Chongqing PTGMS line C49S in the plains of Jiang Han (Chen *et al.*, 2005).

Limitations inherent to conditional GMS can be overcome by the use of non-conditional GMS mutants. However difficulties arise in the maintenance, multiplication, and selection of pure male-sterile populations, a necessity in large-scale production of hybrid seed. One way to overcome the problem of large-scale production of male steriles is by breeding lines in which the male-sterile mutant locus is tightly linked to a visual marker. An example of this is the dominant GMS mutant *Ms2* locus, which is linked by 0.19 cM to the *Rht10* dwarfing locus (Bing-Hua and Jing-Yang, 1986; Yang *et al.*, 2009). The dwarfing locus facilitates the identification of tall male fertiles from dwarf male steriles. However, large-scale hybrid production is limited by the need to manually remove tall male fertiles from the female stand.

Problems associated with propagating pure stands of male steriles can also be circumvented through the use of cytogenetic chromosomal manipulation coupled with recessive non-conditional GMS mutants such as XYZ-like three-line systems (Driscoll, 1972, 1985; Zhou *et al.*, 2006; Zhou and Wang, 2007) (Fig. 4C). The original XYZ system, proposed by Driscoll (1972, 1985), relied on the

addition of a fertility-restoring chromosome to the male-sterile Cornerstone (*ms1c*) mutant (Driscoll, 1977); in this case, the additional chromosome was to be derived from the related species *S. cereale* L. 5R was proposed as the fertility-restoring chromosome because it carries a dominant visual marker (hairy peduncle, *hp*). To avoid the limitation of using a mature plant character like *hp*, the *blue aleurone* (*Ba*) seed colour marker from chromosome 4 of *Agropyron elongatum* ssp. *ruthenicum* Beldie (4E) was introduced into the spontaneous Lanzhou (*ms1g*) mutant background (Zhou *et al.*, 2006; Zhou and Wang, 2007). The blue seed colour marker, when coupled with a high-speed seed sorter, allows easy separation of genotypes. This system, termed 4E-*ms*, relies on a maintainer line (line Y) for propagating plants homozygous for the recessive Lanzhou (*ms1g*) mutation (line Z). It also takes advantage of the poor transmissibility of addition chromosomes through the male germline, allowing only pollen containing *ms1g* mutant alleles to fertilize the homozygous recessive male sterile. This effectively generates 100% homozygous male-sterile mutant progeny. For this system to be effective, the recessive male-sterile mutant must have 100% phenotypic penetrance, otherwise rouging of selfed female inbred progenies would be required in the F1 generation, an additional cost to production.

Islam and Driscoll (1984) observed the expression of a normally latent fertility gene when using the *ms1c* mutant allele. A similar phenomenon may affect the *ms1g* mutant used in the 4E-*ms* system. However, only three of ten independent Y lines, when selfed, generated Z lines that were completely male sterile (Zhou *et al.*, 2006; Zhou and Wang, 2007).

The usefulness of a seed selectable marker like *Ba* has now found application in the dominant *Ms2* system where blue kernel colour was introgressed into both bread and durum wheats via chromosome 4E from *Elytrigia elongata* (Host) Nevski (Tian and Liu, 2001). The inheritance ratio for blue-seeded short male steriles versus white-seeded tall male fertiles was 19.7 and 80.3%, respectively. Such a low proportion of male steriles is considered commercially limiting and is probably due to the reduced inheritance of alien chromatin. Another limiting factor to commercial deployment is that, when blue-seeded male-sterile lines are crossed to normal varieties, the dominant blue-seeded male-sterile phenotype transmits to 20% of F1 progeny, requiring an additional seed selection step. It should be noted that such issues do not limit the utility of this system for recurrent selection and wheat population improvement.

Genetic modification (GM) systems for hybrid breeding

Despite the development of different CHA, CMS, and GMS systems in wheat over the last 60 years, each has serious drawbacks in either F1 fertility restoration or in providing complete male sterility in the female inbred parent under a range of environmental conditions. The first description of the application of recombinant DNA technologies for engineering a wheat fertility control system was in 1997. De Block *et al.* (1997) created a dominant GMS system that relied on

tapetal cell ablation induced through the targeted expression of a cytotoxic bacterial ribonuclease (Fig. 4D). This RNase is encoded by the *barnase* gene, an integral component of Bayer's Seedlink® system for commercial hybrid canola production. Seedlink® couples glufosinate resistance (LibertyLink®) with the sterility-inducing properties of the *barnase* gene allowing in-field selection of male-sterile female parents. F1 fertility restoration is achieved through the highly specific inactivation of RNase activity by the Barstar protein (Fig. 4D), introduced via the male inbred parent (Mariani *et al.*, 1992; Hartley, 1989).

This type of dual-component dominant system is limited by the requirement for transgenes in each crossing partner, which results in extra breeding time. Recent fine-tuning of *barnase*-mediated sterility has led to the development of a recessive split system for wheat, where the *barnase* gene is encoded in two non-overlapping fragments at complementary loci (Fig. 4E) (Gils *et al.*, 2008; Kempe *et al.*, 2009). Termed allelic repulsion, RNAase is expressed when complementary *barnase* gene fragments are co-expressed in the same tissues. Targeted expression in tapetal cells induces cell death and subsequent male sterility. Introducing complementary *barnase* 'isoloci' derivatives into the same individual creates male-sterile females, which can easily be propagated by crossing to a homozygous single 'isolocus' maintainer line. Linking the 'isoloci' not present in the maintainer line to herbicide resistance allows easy selection of male-sterile progeny. Although this split-gene system harnesses many advantages found in classic GMS systems, herbicide selection is disadvantageous because it requires overplanting and eliminating half the sown plants in order to attain a pure stand of male-sterile female inbreds. Extra seed handling adds to hybrid seed production costs.

Chemically induced GM systems

In recent decades, the search for an ideal CHA has contributed to the development of many inducible molecular systems where chemical application can control fertility through the action of a transgene (Fig. 4F). Conditional chemical fertility control systems have been developed around both synthetic and naturally occurring phytotoxic agents. Examples include herbicides as well as naturally occurring plant hormones like abscisic acid, jasmonic acid, ethylene, and cytokinins. For example, the herbicide-catalysing properties of a cytochrome P450 induces sterility in tobacco through the action of a tapetal cell-specific promoter. Tapetal cell death results in male sterility (O'Keefe *et al.*, 1994). A limitation to its commercial deployment has been the finding that CYP105A1 expression can at times generate unwanted pleiotropic affects due to the disruption of brassinosteroid signalling and homeostasis (Dasgupta *et al.*, 2011). Despite these issues, progress has been made in the targeted modification of P450s for altered specificity and activity (Hawkes and Vernooij, 2012).

Syngenta and Monsanto have also turned to herbicides with alternative modes of action for the development of various fertility control systems. For example, Syngenta has adapted D-glufosinate, the non-phytotoxic enantiomer

component of the commercial herbicide glufosinate (marketed by Bayer as Liberty™, Ignite™, or Basta™) for use as a CHA (Fig. 4F) (Hawkes *et al.*, 2011a,b). In their method, D-glufosinate is applied to plants expressing a modified form of D-amino acid oxidase (DAAO) in tapetal cells, just prior to sporogenesis. Engineered DAAO oxidizes D-glufosinate to 2-oxo-4-(methylphosphinyl)-butanoic acid, a short-lived intermediate that is rapidly converted to phytotoxic L-glufosinate through the activity of endogenous L-glutamate transaminase (Lea *et al.*, 1984; Dröge-Laser *et al.*, 1994). Phytotoxic L-glufosinate causes tapetal cell death, which in turn induces male sterility.

As an alternative to the catalytic conversion of proherbicides to phytotoxic compounds, Monsanto proposed several systems whereby either specific promoter–intron combinations, microRNA-mediated translational, or transcriptional repression could be used to specifically unprotect tapetal or microspore cells from the phytotoxic effects of glyphosate (Fig. 4F) (Brown and Santino, 1997; Conner *et al.*, 2002; Allen *et al.*, 2007). These systems are based around transgenes expressing glyphosate-insensitive activity of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) derived from the *Agrobacterium* sp. strain CP4 (marketed by Monsanto as Roundup™).

As each of these systems requires chemical spraying, fertility control is still subject to short biological windows for application and environmental factors such as wind and rain, which at times can compromise efficacy. Conditional male fertility is therefore preferred over conditional male sterility, thus ensuring male-sterile female inbred parents are inherently 100% sterile. Compromised male fertility restoration is indeed acceptable when it is only required for propagating the female inbred parent. Although this would be an ideal chemical-based system, one has yet to be commercially developed.

Transgenic construct driven non-GM systems

The use of transgenes to control fertility is clearly advantageous in reducing hybrid seed production costs. However, all of the technologies described above generate hybrid seed that is transgenic. Burdensome regulatory requirements for commercial release and restrictions to world trade of GM crops have spurred the development of new breeding approaches that use transgenic systems but generate non-transgenic seed. These approaches are being developed within both the public and private sectors and are finding application to both fertility control and heterosis breeding (Lusser *et al.*, 2012; Waltz, 2012). The techniques used encompass genome editing nucleases, oligonucleotide-directed mutagenesis, and RNA-dependent DNA methylation.

Genome editing nuclease-based technologies harness a cell's endogenous mechanism to repair induced DNA double-stranded breaks by homologous recombination and non-homologous end joining. These site-specific lesions can be induced in a host cell either transiently or stably through the design, synthesis, and expression of artificial zinc finger nucleases, transcription activator-like effector nucleases, or meganucleases (Curtin *et al.*, 2012). Synthetic nucleases are

finding application in targeted gene inactivation, addition of genes of interest, gene replacement, and trait stacking. Companies including Dow Agrosiences (Indianapolis, IN, USA), Sangamo Biosciences (Richmond, CA, USA), and DuPont/Pioneer (Johnston, IA, USA) (Lusser *et al.*, 2012) are currently leading the commercial development of 'genome-edited' non-transgenic hybrids for crops including maize and oilseed rape.

Similar to nuclease-based technologies, oligonucleotide-directed mutagenesis uses chemically synthesized oligonucleotides to invoke a plant's DNA-repair machinery at a specific target site within the genome. Oligonucleotides are designed to share homology with the target sequence, with the exception of a few nucleotides. Successful *in vivo* gene modification has been demonstrated in maize, rice, tobacco, and wheat to create plants insensitive to the action of acetolactate synthase inhibitor-based herbicides (Zhu *et al.*, 2000; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Iida and Terada, 2005; Dong *et al.*, 2006). Oligonucleotide-directed mutagenesis-induced herbicide tolerance in wheat is likely to find application in roguing during hybrid seed production process.

In these techniques, the genetic information encoding the desired trait is either transiently present in the plants or stably integrated in intermediate plants. The gene in the heterozygous condition segregates following selfing, and some progeny do not have the transgene.

RNA-dependent DNA methylation-based technologies induce transcriptional gene silencing by methylation of promoter sequences. Here, inverted repeat sequences encoding RNAs homologous to promoter regions of target genes are introduced into plant cells, forming small double-stranded RNAs, which can direct DNA methylation and silencing of the homologous sequences. Instead of stably introducing a foreign DNA sequence, this technique takes advantage of a plant's epigenetic machinery. An example of transcriptional gene silencing to hybrid breeding is the targeted *trans*-inactivation of male fertility genes in maize (Cigan *et al.*, 2005, 2012). Constitutively expressed inverted repeats of the *Ms45* promoter were used to induce transcriptional silencing of the *Ms45* gene. Importantly, fertility was completely restored by either expressing the *Ms45* coding region with a non-target promoter (Cigan *et al.*, 2005) or in the presence of *mop2-1* (*mediator or paramutation 2*) homozygous recessive alleles, which encode a gene similar to *Arabidopsis* NRPD2/E2, the second-largest subunit of plant-specific RNA polymerases IV and V (Sidorenko *et al.*, 2009).

A good example of a transgenic construct-driven non-GM system is the recently deregulated proprietary process developed by DuPont Pioneer (Pioneer Hi-Bred International, 2011) for the production of maize hybrids. This system overcomes many of the problems with existing fertility control systems and facilitates large-scale production of male-sterile lines that are used as female inbred parents. The system, termed 'seed production technology' (SPT) (Fig. 4G) is similar in principle to 4E-*ms*, but instead of a fertility-restoring addition chromosome, it uses a fertility-restoring transgene in the maintainer line (line Y). The SPT maintainer line contains three transgenic

components linked in a single construct and a single, homozygous recessive locus for male sterility: a single dominant wild-type allele complementary to the recessive male sterility allele, a pollen germination inhibition transgene, and a fluorescent seed colour marker gene. In this system, the dominant male fertility transgene (*Ms45*) in maize can completely restore fertility to the recessive male-sterile mutant *ms45*. This system is designed to restrict the transgenic event to the maintainer line, preventing the transgene entering the female inbred parent and subsequent F1 hybrid seed. The containment works on two levels: firstly, through the action of a pollen germination inhibition transgene. The SPT maintainer plants produce equal amounts of transgenic and non-transgenic pollen (1:1). All transgenic pollen is rendered infertile because of the lack of pollen germination, with the only pollen capable of fertilizing the female being non-transgenic. The second level of containment is through the action of a fluorescent seed colour marker (*dsRED*). This marker, when used in combination with high-speed Sataki seed sorters, allows physical separation of transgenic SPT maintainer seed from non-transgenic male-sterile female inbred seed. The SPT maintainer line is used as a pollinator only in male-sterile female inbred bulking fields. The resulting 100% non-transgenic male-sterile female inbred seed is then used in the hybrid seed production phase, in which the F1 hybrid seed produced is non-transgenic.

Future perspectives

Hybrid wheats are seen to have higher agronomic potential than line varieties due to improved grain and straw productivity and yield stability under harsh environmental conditions (Longin *et al.*, 2012). Rapid developments in wheat genomics, understanding of gene function, and the targeted modification of plant phenotypes using GM technologies is likely to increase the efficiency of hybridization and therefore aid in the development of more cost-effective hybrid seed production systems. Deployment of novel transgenic constructs to drive non-GM hybrid breeding systems may be a step towards alleviating public concern over GM crops. Hybrid wheat is likely to be advantageous to the economic, agronomic, technological, and environmental aspects of wheat cultivation and production. This will play a crucial role in improving global food security and helping to meet the ambitious production targets for 2050.

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