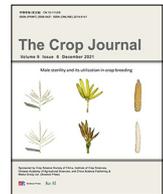




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Editorial

Male sterility in crops: Application of human intelligence to natural variation

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1. Introduction

Global food security faces a severe challenge, as world population is predicted to grow by 25% and reach 10 billion by the mid-21st century [1]. With limited agricultural land and fresh water, greater and more sustainable crop production needs to be achieved by use of modern agricultural technologies [2,3]. Among these are developing and exploiting more efficient heterosis utilization strategy with male sterile lines for hybrid breeding and seed production.

Plant male sterility involves a failure in the formation or release of viable pollen grains while the female organs remain normal. Male-sterility mutants contain morphologically altered sporophytic or gametophytic anther tissues. These may result from defects in transcriptional regulation, lipid metabolism, sugar metabolism, or other processes during plant anther and pollen development [4–6].

Identification and functional analysis of male sterile genes not only deepens our understanding of molecular mechanisms underlying anther and pollen development, but also facilitates developing and exploiting biotechnology-based male-sterility (BMS) systems for hybrid breeding and seed production [5]. Male sterility can be generated by either cytoplasmic or nuclear genes. Cytoplasmic male sterility (CMS) is controlled by both mitochondrial and nuclear genes and is used in commercial crop hybrid seed production in a three-line system consisting of male-sterile, maintainer, and restorer lines, although it often suffers from poor genetic diversity, increased disease susceptibility, and unstable restoration of CMS lines [5]. Nuclear-controlled male sterility is controlled by nuclear genes alone, including genetically stable genic male sterility (GMS) and environment-sensitive genic male sterility (EGMS). EGMS has long been used for efficient production of hybrid rice seeds with a two-line system consisting of male-sterile line and maintainer, while GMS has been used in BMS systems only recently, for example in seed production technology (SPT) and multi-control sterility (MCS) systems in maize [7,8]. As mentioned above, global food security requires new and effective agricultural technologies (such as BMS systems) to increase crop grain produc-

tion. Identifying more male-sterility genes and elucidating the molecular mechanisms of male sterility in crops are prerequisites for hybrid crop breeding and heterosis exploitation.

In this special focus “Male sterility and its utilization in crop breeding”, we collect ten articles on plant male sterility, including one review, three on transcriptomic and genomic analyses, two on CRISPR/Cas9 gene editing, three on map-based cloning of GMS genes, and one on a CMS gene. The aim of this special focus is to survey recent advances in elucidating mechanisms of male sterility and their potential applications in crop breeding.

2. Male-sterility genes cloned in crops

Nearly 200 male-sterility genes have been identified and characterized in plants, including the model plant *Arabidopsis* and crops such as rice, wheat, and maize [4–6]. Most of them are GMS genes and can be classified into four groups involved in transcription regulation, lipid metabolism, sugar metabolism, and other processes.

2.1. Transcription regulation

Plant anther development is a complex biological process that requires at least hundreds of genes precisely regulated by many transcription factors [9]. There is increasing evidence that several transcription factors and their homologs form a conserved regulatory pathway essential for anther development, such as the AtDYT1-AtTDF1-AtAMS-AtMYB80-AtMs1 pathway in *Arabidopsis* [10] and its corresponding OsUDT1-OsTDF1-OsTDR-OsMYB80-OsPTC1 pathway in rice [11]. However, such a regulatory pathway in maize remains to be found [5]. Most recently, the essential roles of ZmHHLH51 (orthologous to AtAMS and OsTDR) and ZmMYB84 (orthologous to AtMYB80 and OsMYB80) in maize male fertility have been confirmed by CRISPR/Cas9 gene editing [12]. In one of the studies described in this special focus, a new mutant allele of ZmHHLH51 gene was identified in maize inbred line Jing724 via EMS mutagenesis and bulked-segregant RNA-seq strategies, further confirming the key role of the ZmHHLH51 gene [13]. As

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reported in the same issue, a soybean male-sterile line was generated by CRISPR/Cas9 targeted editing of *GmAMS1*, a soybean ortholog of *AtAMS* in *Arabidopsis*, indicating that the conserved regulation pathway regulated by *GmAMS1* may also function in soybean anther development [14].

Another transcription factor, ZmBHLH16, encoded by *Rf4* gene in a C-type CMS maize line [15], is associated with a gain-of-function mutation in the *ZmMs23* gene, and the null allele of *ZmMs23* is responsible for male sterility (*ms23*) in maize [16]. *ZmMs23* interacts with other bHLH transcription factors (such as ZmBHLH51 and ZmBHLH122), and may function cooperatively in a sequential manner or as a complex to coordinate transcription in anther development [16,17]. As described in this special focus, single-cell RNA sequencing of meiocytes and microspores indicates that the *Rf4* gene is involved in redox homeostasis of the C-type CMS maize line, suggesting that some transcription factors (such as *ZmMs23/Rf4*) act in both GMS and CMS in maize [18].

2.2. Lipid metabolism

Fatty acids and their derivatives are essential building blocks for the formation of anther cuticle and pollen wall. Disruption of lipid metabolism during anther and pollen development often leads to male sterility. More than 60 lipid metabolism-related GMS genes have been identified and characterized in plants, and are involved in the formation of anther cuticle, pollen wall, and subcellular organelle membranes in anther wall layers [4]. Comprehensive understanding of lipid metabolism, its associated genes, and their roles in plant reproductive development will facilitate the application of these genes in molecular breeding and hybrid seed production [4].

Lipid metabolism, including lipid biosynthesis and transport, plays a critical role in plant male gametophyte development, especially in the formation of anther cuticle and pollen exine [9]. Sporopollenin precursors and cuticular lipid monomers are produced mainly in the endoplasmic reticulum (ER) of tapetal cells and then transported to the surface of microspores and anther epidermis by lipid transport proteins (LTPs) and ATP-binding cassette G (ABCG) transporters to form pollen wall and anther cuticle, respectively. In this special focus, identification of three kinds of lipid metabolic GMS genes in maize, rice, wheat and foxtail millet is reported. For example, orthologs of wheat *TaMs1* gene in rice (*OsLTPg29*) and maize (*ZmLTPg11* and *ZmLTPx2*) which encode Type-G nLTPs required for male fertility have been isolated [19]. The *osltpg29* mutant created by gene editing displayed male sterility like the *tams1* mutant, but was different from the double mutant of *zmltpg11/zmltpx2*, suggesting that *TaMs1* and its orthologous genes have diverged in their biological function during plant evolution [19]. By map-based cloning, maize *ZmMs2* gene was found to encode an ABCG transporter, which acts in lipid transport for the formation of anther cuticle and pollen exine [20]. With the discovery of a foxtail millet male sterile EMS mutant, *no pollen 1 (snp1)*, *SNP1* was found to encode an ER-localized glucose-methanol-choline oxidoreductase [21], which is orthologous to rice *OsNP1* [22] and maize *ZmLPE1/ZmMs20* [23,24], suggesting a mechanism of pollen development conserved across plant species and identifying a male-sterility gene potentially useful for exploitation of heterosis in foxtail millet.

2.3. Sugar metabolism

Sugars, such as sucrose, glucose, fructose, trehalose and their derivatives, serve as the basic source of energy and as structural constituents of plant cells [9]. Sugar metabolism affects plant male reproduction, including callose wall and primexine formation, intine development, pollen maturation and starch accumulation, anther dehiscence, and pollen germination and tube growth.

Defects in sugar metabolism-associated genes critical for anther and pollen development often result in GMS. In one article in this special focus, 62 sugar-metabolic GMS genes and their essential roles in plant anther and pollen development are summarized, 112 putative sugar-metabolic GMS genes in maize are predicted based on bioinformatics and RNA-seq, and their potential applications in crop hybrid breeding and seed production are outlined [6]. This review will shed light on the role of sugar metabolic pathways in controlling pollen development and male fertility in plants, especially in maize, once the predicted GMS genes are confirmed via reverse-genetic approaches such as CRISPR/Cas9 technology. In the same issue, rice *OsGPT1* is reported [25] to be required for tapetum function and pollen development. The *osgpt1* mutant exhibits delayed tapetum degeneration, decreased Ubisch body formation and thinner pollen exine, suggesting that *OsGPT1* coordinates the development of anther sporophytic tissues and male gametophyte by integrating carbohydrate and fatty acid metabolism in plastids. A similar report [26] indicated that deficiency in *ZmMs33/ZmGPAT6* disrupts structure and function of chloroplasts in anther endothecium, resulting in maize male sterility. A mitochondrial gene *orf346* was also reported [27] to cause pollen abortion in oilseed rape CMS line, largely as a result of inadequate energy supply and excessive ROS accumulation in anthers. These reports highlight the importance of sugar and lipid metabolism in plastids for regulating tapetal PCD and anther development in both GMS and CMS lines.

2.4. Other processes

Except for the three groups of male sterile genes described above, other gene types have also been reported to regulate anther and pollen development in plants, among them microRNAs and hormone-associated GMS genes. In this special focus, genome-wide analysis of transcription factors and their potential microRNA regulators was conducted for maize male fertility with 118 microRNAs and 99 microRNA-transcription factor gene pairs identified to be associated with maize anther development [28]. Among them, both microRNAs (*zma-miR319* and *zma-miR159*) and transcription factor genes (*ZmMs7* and *ZmLOB30*) were found to regulate or influence the expression of *ZmGAMYB* (encoding a GA-responsive MYB transcription factor), which was validated by CRISPR/Cas9 knockout as being critical for maize male fertility. These results expand our understanding of the functions of transcription factors and their microRNAs regulators of plant male fertility.

3. Male-sterility genes discovered by bioinformatic and reverse genetics strategies

Most reported male-sterility genes in plants have been identified via forward-genetics approaches [29], which are dependent on natural or artificial male-sterility mutants. As GMS genes often show anther-specific or -preferential expression patterns, candidate GMS genes can be predicted using bioinformatic and anther transcriptomic analyses [4,5,30,31]. Considering the functional conservation of GMS genes during plant evolution, reverse-genetics strategies (such as CRISPR/Cas9 and RNAi) have been used to identify homologs of cloned GMS genes in other plants, especially in species with very few GMS mutants or that are not amenable to map-based cloning by forward-genetics approaches. Examples are soybean *GmAMS1* [14], wheat *TaMs26* and *TaMs45* [32,33], barley *HvMs1* [34], and tomato *SIMYB33* [35].

An integrated bioinformatics and reverse genetics strategy is also effective for identifying GMS genes with functional redundancy, as multiple mutation sites or fragments can be simultane-

ously generated in paralogous genes whose single or double mutants may display normal male fertility [12]. For natural or EMS mutants, however, it is relatively difficult to do so using forward-genetics strategies, given that multi-gene mutations rarely occur naturally.

4. Strategies for using male sterility in crop breeding

Many commercial grain crops are bred as hybrids because such plants are healthier, larger, and more productive than parental inbreds, owing to heterosis or hybrid vigor. Hybrid seed production requires cross-pollination of genetically diverse parental lines, and thus for self-pollinating plant species, male sterile lines are used as female parents in hybrid seed production. CMS three-line and EGMS two-line systems for seed production have been established and used in many crops such as maize, rice, barley, and rapeseed, contributing to large yield increases [36]. However, the two systems often suffer from several intrinsic problems, including poor genetic diversity between CMS and restoration lines, increased disease susceptibility and unreliable restoration of some CMS lines, and unstable sterility of EGMS lines in abnormal environments [36]. Elucidating the molecular mechanisms underlying male sterility will be helpful for improving crop hybrid breeding systems.

With more GMS mutants and causal genes recently identified and cloned in crops, GMS genes in combination with new technologies (such as CRISPR/Cas9 gene editing) have been used to develop more efficient BMS systems in maize and other crops [5]. Recessive genic male-sterility (RGMS) systems such as SPT, MCS and Manipulated GMS Maintainer (MGM), have been established in maize using *ZmMs45* [7], *ZmMs7* [8], *ZmMs26* [37], and *ZmMs30* [38], SPT-like systems have been established in rice with *OsNP1* [39] and *OsCYP703A3* [40], and a dominant genic male sterility (DGMS) system has been established in maize and other plant species based on *ZmMs7* [41] and *Zmms44* [42]. These BMS systems can be extended to other crops such as wheat, soybean, and oilseed rape to increase the effectiveness of heterosis use and grain yield.

RGMS systems have been used to produce transgenic maintainer lines by which non-transgenic RGMS lines can be propagated as female parents more efficiently than can be achieved by manual or mechanical emasculation in hybrid seed production [7,8,37,38]. For cross-pollinated crops, DGMS lines have special value in heterosis utilization, because mixing their planting with that of normal fertile plants may increase commodity grain production, owing to the partitioning of more energy and assimilates into female organs and increased nitrogen use efficiency under stress conditions [43–45]. Moreover, it is feasible to integrate multiple desirable traits into elite DGMS lines using modern breeding technologies such as marker-assisted backcrossing and recurrent selection [1,3].

5. Problems and challenges in the application of male-sterility systems in crop breeding

Although many plant male-sterility genes have been identified, and several crop BMS systems have been developed [5,36], most of the systems have not been used in crop hybrid breeding and seed production. Before their successful application, several key issues should be addressed, among them the security, effectiveness, and feasibility of use of male-sterility genes and their BMS systems.

First, most male-sterility genes have been cloned in *Arabidopsis* and rice. Whether these genes play similar roles in other plants awaits investigation. Second, the male-sterility lines used as parents must be genetically stable and incur no yield penalty in various genetic backgrounds and environments, as unstable male

sterility will lead to low purity of hybrid seeds and thus greatly reduce grain production. Male-sterile lines should be evaluated for genetic stability and field performance before hybrid breeding and seed production [38]. Third, although RGMS systems can be used to produce non-transgenic hybrid seeds [7,8,37,38], they are subject to genetic modification (GM) regulation in many countries. Finally, both hybrid seed and grain productivity should be increased by use of male-sterility systems (such as DGMS systems in maize) together with other modern agricultural technologies, such as marker-assisted breeding, genetic engineering, synthetic biology, and genome editing [46].

For successful commercialization of a hybrid crop, three requirements need to be met: (1) the level of heterosis or hybrid performance should be high enough to offset the additional cost in hybrid seed production, (2) seed production yield should be high enough so that the hybrid seeds are affordable to farmers, and (3) seeding rate should be much lower than that of non-hybrids so that much less seed will be needed for planting hybrids. These three factors determine the final economic feasibility of a hybrid crop: seed producers can make profits by producing and selling hybrid seeds, while farmers can make profits despite the higher cost of hybrid seeds, by exploiting their superior performance. Ideal male-sterility systems should thus meet the above requirements. Their wide adoption may lead to novel crop production models and ultimately help ensure global food security and sustainable agriculture.

6. Summary and perspective

To meet the food demands of the growing world population with limited agricultural land and fresh water, more effective agricultural technologies should be developed to increase food production. Hybrid breeding in maize and rice as well as other crops has contributed greatly to increases in crop yields. Identification of more male-sterility genes and their mutants in crops is a prerequisite for crop hybrid breeding and seed production. As shown in the special focus of this issue, the integrated utilization of bioinformatic and transcriptomic analyses [6,13,18], CRISPR/Cas9 gene editing technology [14,19,21], and EMS mutagenesis and map-based cloning [13,21], has led to feasible strategies for discovering more male-sterility genes and creating more male-sterility mutants. This approach will advance our understanding of molecular mechanisms underlying plant anther and pollen development and improve hybrid breeding and seed production systems in crops.

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