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Review

# The molecular and evolutionary basis of reproductive isolation in plants

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ABSTRACT

Reproductive isolation is defined as processes that prevent individuals of different populations from mating, survival or producing fertile offspring. Reproductive isolation is critical for driving speciation and maintaining species identity, which has been a fundamental concern in evolutionary biology. In plants, reproductive isolation can be divided into prezygotic and postzygotic reproductive barriers, according to its occurrence at different developmental stages. Postzygotic reproductive isolation caused by reduced fitness in hybrids is frequently observed in plants, which hinders gene flow between divergent populations and has substantial effects on genetic differentiation and speciation, and thus is a major obstacle for utilization of heterosis in hybrid crops. During the past decade, China has made tremendous progress in molecular and evolutionary basis of prezygotic and postzygotic reproductive barriers in plants. Present understandings in reproductive isolation especially with new data in the last several years well support three evolutionary genetic models, which represent a general mechanism underlying genomic differentiation and speciation. The updated understanding will offer new approaches for the development of wide-compatibility or neutral varieties, which facilitate breeding of hybrid rice as well as other hybrid crops.

## 1. Introduction

Reproductive isolation provides intrinsic driving force for speciation and maintenance of species identity by reducing gene flow between divergent groups. Reproductive isolation in plants occurs at different developmental stages, including prezygotic reproductive barriers preventing mating or formation of hybrid zygotes, and postzygotic reproductive barriers leading to reduction of fitness in offspring such as hybrid sterility, hybrid necrosis/weakness, and hybrid lethality (Fig. 1) (Ouyang and Zhang, 2013; Fishman and Sweigart, 2018).

The advent of molecular biology and genomic data in recent years has greatly advanced our understanding of the biological mechanisms regulating reproductive isolation. Here we summarize the recent progress on characterizing molecular mechanism and evolutionary driving force of reproductive barriers in plants, which is largely advanced by Chinese scientists in the past decade. In addition to the significance in advancing our understanding of these processes, these findings also have implications for crop genetic improvement.

## 2. Different types of reproductive isolation in plants

Prezygotic reproductive barriers are economic strategies for reproduction isolation among closely related plant populations, which may create distinct mating discrimination systems via variations in environmental adaptations, pollination patterns, or pollen recognition (Liu et al., 2004; Bomblies, 2010; Rieseberg and Blackman, 2010). Mechanisms of prezygotic reproductive isolation in plants were less investigated, and one observed case was the abnormalities of adherence and germination of the pollen grains in intersubspecific rice crosses (Xu, 1995; Liu et al., 2004; Niu et al., 2014). The number of pollen grains that adhered to the stigmas decreased significantly in intersubspecific crosses. In addition, various abnormalities of pollen tube growth were observed in intersubspecific crosses after pollination. The lower affinity between the pollens and stigma observed in intersubspecific

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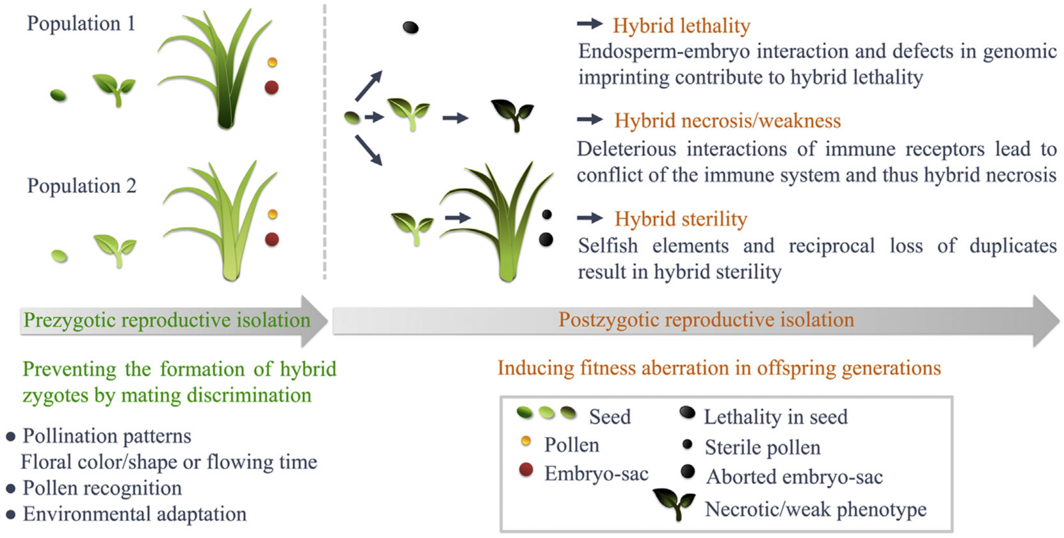

**Fig. 1.** Schematic drawing of prezygotic and postzygotic reproductive isolation at different developmental stages of plants.

hybridization resulted in reduced fertilization rate, which prevented the formation of hybrid zygotes and contributed to prezygotic reproductive isolation in rice.

Postzygotic reproductive isolation is widely observed in crop plants, causing reduced fitness of hybrids between divergent varietal groups. These barriers are unfavorable traits for utilization of heterosis and are major obstacles for crop genetic improvement. Hybrid sterility represents the most common form of postzygotic reproductive barriers in plants, and a well-characterized example is the hybrid sterility between two subspecies of Asian cultivated rice (*Oryza sativa* L.) (Ouyang et al., 2009, 2010; Ouyang and Zhang, 2013; Ouyang, 2016). These two major groups of *O. sativa* subsp. *xian* and *O. sativa* subsp. *geng*, which were also referred to as *indica* and *japonica*, respectively (Kato et al., 1928), had been recorded as distinct rice types in the literature in China since the Han dynasty over 2000 years ago. Following the recommendation by Wang et al. (2018) and Wing et al. (2018), we will use *xian*/*geng* and *indica*/*japonica* naming systems interchangeably as the names of these two rice groups (and subspecies) in this paper according to the context. Such rice system provides a framework to understand the molecular basis of hybrid sterility. Complex interactions causing hybrid sterility in ten cases were characterized within *O. sativa* or between *O. sativa* and other rice species (Table 1) (Chen et al., 2008; Long et al., 2008; Mizuta et al., 2010; Yamagata et al., 2010; Yang et al., 2012; Kubo et al., 2016; Yu et al., 2016, 2018; Nguyen et al., 2017; Shen et al., 2017; Xie et al., 2017b; Koide et al., 2018). Recently, genetic architecture underlying hybrid sterility in rice has been systematically characterized using three pairwise crosses between three representative varieties of *geng* rice and two varietal groups of *xian* rice (Li et al., 2017). At the whole-genome level, a total of 43 quantitative trait loci (QTLs) and 223 digenic interactions for embryo-sac, pollen, spikelet fertility, and segregation distortion were detected; these results described a framework for reproductive isolation in rice. Ten new QTLs for fertility were detected both in inter and intrasubspecific crosses, suggesting that genetic basis leading to reproductive barriers differs substantially due to differentiation in rice germplasms. Loci responsible for fertility and segregation distortion in intrasubspecific crosses were observed, which provided implications to improve the fertility potential of *xian-geng* hybrids. The genetic components for different types of reproductive barriers also showed substantial differences in the light of evolution. Loci for hybrid female fertility were in common between different intersubspecific crosses and were likely to have arisen at the early stage of varietal group differentiation, whereas QTLs for pollen fertility were different among rice lineages and might evolve more rapidly. Epistasis contributed significantly to reproductive isolation in rice, as the effects of two-locus interactions on fertility were determined and quantified at the whole-genome level.

Extensive studies in *Arabidopsis thaliana* characterized another type of reproductive barriers, hybrid necrosis, featured by reduced vigor with autoimmune response in hybrids (Bomblies et al., 2007; Alcazar et al., 2009, 2014; Bomblies, 2010; Chae et al., 2016). In *Arabidopsis*, approximately 2% of intraspecific crosses produced $F_1$ hybrids with necrosis phenotypes (Bomblies et al., 2007). Species-wide genetic incompatibility was further investigated using thousands of *Arabidopsis* crosses, which identified that nucleotide-binding domain and leucine-rich repeat immune receptor genes were responsible for a large proportion of hybrid necrosis hot spots in the genome (Chae et al., 2014). Hybrid necrosis was also widely observed in other plants such as tomato (Kruger et al., 2002), lettuce (Jeuken et al., 2009), *Capsella* (Sicard et al., 2015), and rice (Yamamoto et al., 2010; Chen et al., 2014).

Hybrid lethality is also a common type of postzygotic reproductive barriers between closely related plant species, which occurs early after the formation of zygotes. Hybrid seed lethality widely observed in monkey flower was controlled by multiple QTLs (Garner et al., 2016; Oneal et al., 2016). Distinct patterns of phenotypic variations were observed in reciprocal crosses, suggesting that genetic loci with parent-of-origin effects contribute significantly to hybrid lethality. Such non-reciprocal postzygotic reproductive barrier was detected in *C. rubella* and *C. grandiflora* and was caused by negative interaction of multiple paternal *C. grandiflora* loci with at least one maternal *C. rubella* locus (Rebernig et al., 2015). Other studies in *Arabidopsis* (Kradolfer et al., 2013; Kirkbride et al., 2015; Wolff et al., 2015; Lafon-Placette et al., 2017), rice (Sekine et al., 2013), and tomato (Florez-Rueda et al., 2016) also provided evidence that such hybrid lethality was usually associated with defects in genomic imprinting, which was caused by genes with a parent-of-origin-specific manner. This implied the role of endosperm-embryo interaction in reproductive isolation (Lafon-Placette and Kohler, 2016).

**Table 1**
QTLs and locus pairs causing postzygotic reproductive isolation in rice.

| Cross | Locus | Allele | Protein | Phenotype in hybrids | Mechanism | Model | Reference |
|---|---|---|---|---|---|---|---|
| *O. sativa* subsp. *xian* × subsp. *geng* | *DPL1*<br>*DPL2* | *DPL1-N*$^{+}$/*DPL1-K*$^{-}$<br>*DPL2-K*$^{+}$/*DPL2-N*$^{-}$ | Duplicated genes encoding plant-specific small proteins | Pollen germination failure | Pollen grains carrying the loss-of-function alleles at two loci are abortive | Parallel divergence model | Mizuta et al., 2010 |
| *O. sativa* × *O. glumaepatula* | *S27*<br>*S28* | *S27-T65*$^{+}$/*S27-glum*$^{s}$<br>*S28-glum*$^{+}$/*S28-T65*$^{s}$ | Duplicated genes encoding mitochondrial ribosomal protein L27 | Pollen sterility | Pollen grains carrying the loss-of-function alleles at two loci are sterile | Parallel divergence model | Yamagata et al., 2010 |
| *O. sativa* × *O. nivara* | *DGS1*<br>*DGS2* | *DGS1-nivara*$^{s}$/*DGS1-T65*$^{+}$<br>*DGS2-nivara*$^{+}$/*DGS2-T65*$^{s}$ | Duplicated genes encoding a protein homologous to DNA-dependent RNA polymerase III subunit C4 | Pollen sterility | Pollen grains carrying the loss-of-function alleles at two loci are sterile | Parallel divergence model | Nguyen et al., 2017 |
| *O. sativa* × *O. rufipogon* | *Hwi1*<br>*25L1*<br>*25L2*<br>*Hwi2* | <br>*None/25L1*<br>*25L2-t/25L2*<br>*Hwi2/hwi2* | *Hwi1* encodes a leucine-rich repeat receptor-like kinase protein<br>*Hwi2* encodes a secreted putative subtilisin-like protease | Weakness syndrome in the hybrids | *Hwi1* and *Hwi2* cause interspecific hybrid weakness by activating the autoimmune response in the hybrids | Parallel divergence model | Chen et al., 2014 |
| *O. sativa* subsp. *xian* × subsp. *geng* | *hsa1*<br>*HSA1a*<br>*hsa1*<br>*HSA1b* | <br>*HSA1a-i*$^{s}$/*HSA1a-j*<br><br>*HSA1b-i*$^{s}$/*HSA1b-j* | *HSA1a* encodes a plant-specific domain of unknown function protein<br>HSA1b shows similarity to a nucleotide-binding protein | Embryo-sac abortion | *HSA1a-i*$^{s}$/*HSA1a-j* causes semi-sterility due to selective abortion of the *HSA1a-i*$^{s}$ embryo-sac in *HSA1b-i*$^{s}$ homozygous plants;<br>the genotype of *HSA1a-i*$^{s}$-*HSA1b-i*$^{s}$/*HSA1a-j-HSA1b-j* causes F$_2$ female sterility | Parallel mutations occurring in the *xian* and *geng* rice groups | Kubo et al., 2016 |
| *O. sativa* subsp. *xian* × subsp. *geng* | *Sa SaF*<br>*Sa SaM* | *SaF*$^{+}$/*SaF*$^{-}$<br>*SaM*$^{+}$/*SaM*$^{-}$ | F-box protein<br>Small ubiquitin-like modifier E3 ligase-like protein | Pollen sterility and preferential abortion of pollen grains with *SaM*$^{-}$ | *SaM*$^{+}$ and *SaF*$^{+}$ can selectively kill the pollen grains with *SaM*$^{-}$ | Sequential divergence model | Long et al., 2008 |
| *O. sativa* × *O. meridionalis* | *qHMS7ORF2*<br>*qHMS7ORF3* | *ORF2*$^{D}$/*ORF2*$^{M}$<br>*ORF3/none* | A RIP domain containing protein<br>Grass family specific protein | Pollen sterility and preferential elimination of pollen grains lacking *ORF3* | *ORF2*$^{D}$ in *O. sativa* has a pollen-killing function, which kills Mer-type pollen grains without *ORF3* in hybrids | Sequential divergence model | Yu et al., 2018 |
| *O. sativa* subsp. *xian* × subsp. *geng* | *S5 ORF3*<br>*S5 ORF4*<br>*S5 ORF5* | *ORF3*+/*ORF3*−<br>*ORF4*+/*ORF4*−<br>*ORF5*+/*ORF5*−/*n* | Hsp70<br>Membrane protein<br>Aspartic protease | Embryo-sac sterility and preferential abortion of embryo-sac with *ORF3*− | *ORF5*+ in combination with *ORF4*+ can selectively eliminate the embryo-sac without *ORF3*+ | Parallel-sequential divergence model | Chen et al., 2008; Yang et al., 2012; Zhu et al., 2017 |
| *O. sativa* × *O. glaberrima* | *S1 TPR1*<br>*S1 SSP* | *OgTPR1/OsTP1*<br>*OgSSP/none* | Trypsin-like peptidase and RRS domains<br>Peptidase domain-containing protein | Both male and female sterility in F$_1$ hybrids | The *O. glaberrima*-derived alleles cause preferential abortion of gametophytes with *O. sativa*-derived components in F$_1$ hybrids | Parallel-sequential divergence model | Xie et al., 2017b; Koide et al., 2018 |
| *O. sativa* subsp. *xian* × subsp. *geng* | *Sc* | *Sc-i*<br>*Sc-j* | A DUF1618-domain protein | Pollen sterility and preferential abortion of pollen grains with *Sc-j* | The high expression of *Sc-i* in sporophytic cells causes suppression of *Sc-j* expression in pollen, resulting in selective abortion of pollen with *Sc-j* and segregation distortion in hybrids | Unknown | Shen et al., 2017 |
| *O. sativa* *Aus* × subsp. *xian* or subsp. *geng* | *S7 ORF3* | *S7*$^{ai}$/*S7*$^{i}$/*S7*$^{cp}$ | A tetratricopeptide repeat domain containing protein | Embryo-sac sterility and preferential abortion of embryo-sac with *S7*$^{cp}$ in *S7*$^{ai}$/*S7*$^{cp}$ and *S7*$^{i}$ in *S7*$^{ai}$/*S7*$^{i}$ backgrounds | Downregulation of *ORF3* restores fertility and segregation distortion in hybrids | Unknown | Yu et al., 2016 |

## 3. Evolutionary genetic models for postzygotic reproductive isolation

Understanding the origin and mechanisms of reproductive isolation would explain how and why genetic incompatibilities evolve, and this is of fundamental importance in evolutionary biology. Based on previous results from rice studies, three evolutionary genetic models have been proposed to explain the processes for installing the hybrid sterility systems, the parallel divergence, sequential divergence and parallel-sequential divergence models (Fig. 2 and Table 1) (Ouyang and Zhang, 2013). These models well summarize the evolutionary genetic courses underlying the instances of postzygotic reproductive isolation so far investigated in all organisms, including plants and animals. Recent studies in plants have provided fresh data for validation of these models.

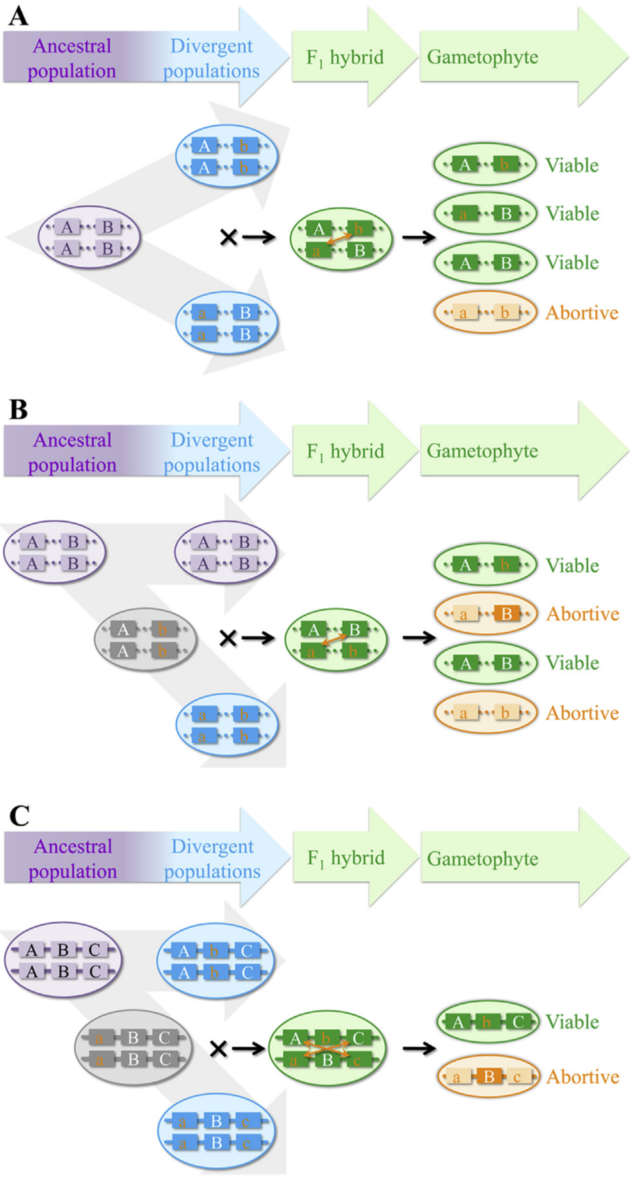

**Fig. 2.** Updated models for evolutionary and genetic architecture of postzygotic reproductive isolation. **A**: The parallel divergence model for postzygotic reproductive isolation caused by two parallelly diverged genes in respective populations. The double-headed orange arrow shows the possible deleterious interaction between different alleles. The dashed line indicates either linked or unlinked genes. **B**: The sequential divergence model for postzygotic reproductive isolation caused by two sequentially mutated genes in one lineage. **C**: The parallel-sequential divergence model for postzygotic reproductive isolation caused by more than two genes. The full line indicates linked genes.

### *3.1. Parallel divergence model explains postzygotic reproductive isolation in a wide range of species*

In the parallel divergence model, an ancestral population splits into two allopatric populations, in which two duplicated loci generated by genome evolution accumulate genetic differences in their respective species (Fig. 2A). The gametophytes abort when two parallelly diverged copies of loss-of-function mutants are combined in hybrids. Therefore, duplicated genes that experience divergent evolution lead to a double-null homozygote due to reciprocal loss of the genes in the offspring, which may result in postzygotic reproductive isolation.

Two locus pairs *S27/S28* (Yamagata et al., 2010) and *DPL1/DPL2* (Mizuta et al., 2010) appeared to adopt such a strategy in producing reproductive barriers by genome duplication of essential genes in gametophyte development, followed by reciprocal loss of unlinked duplicated genes in two allopatric populations. The progeny of crosses between divergent populations produced sterile gametophytes when they lacked both of the functional alleles at duplicated loci after meiosis. Recently, another duplicated locus pair of *DGS1/DGS2* could also be placed into the perspective of the parallel divergence model (Nguyen et al., 2017). Hybrid male sterility derived from an interspecific cross between Asian cultivated rice *O. sativa* and wild rice *O. nivara* was caused by lacking both functional alleles at two duplicated loci.

Besides, known duplicated genes in other plants, such as *HPA1/HPA2* for recessive embryo lethality (Bikard et al., 2009), *HISN6A/HISN6B* for hybrid lethality (Blevins et al., 2017), and *AtFOLT* paralogs for sterility in *Arabidopsis* (Durand et al., 2012), and *pTAC14* homologs for hybrid lethality in *Mimulus* (Zuellig and Sweigart, 2018), also fitted well with the parallel divergence model. In addition, whole-genome duplication events associated with reciprocal gene loss contributed to multiple rounds of speciation in yeast and thus led to the rapid emergence of new yeast species (Scannell et al., 2006). Therefore, the parallel divergence model resulting from genomic duplication followed by fixation of one copy via genetic drift and nonfunctionalization/subfunctionalization of the other copy provides common source of reproductive isolation and eventually speciation.

This parallel divergence model can be further extended to deleterious interaction between non-duplicated genes that under parallel evolution in two allopatric populations. Parallel divergence was detected at the *hsa1* locus that regulated hybrid female fertility in *xian-geng* cross (Kubo et al., 2016). The ancestral population contained the *HSA1a-j* and *HSA1b-i*$^{s}$ at the *hsa1* locus. During the evolution, parallel mutation events occurred in the *xian* and *geng* rice groups, generating *HSA1a-i*$^{s}$-*HSA1b-i*$^{s}$ and *HSA1a-j-HSA1b-j* haplotypes, respectively. In the progeny, the *HSA1a-i*$^{s}$/*HSA1a-j* caused semi-sterility due to selective abortion of the *HSA1a-i*$^{s}$ embryo-sac in *HSA1b-i*$^{s}$ homozygous plants, while the genotype of *xian/geng* haplotype caused F$_2$ female sterility, although the molecular mechanism remained to be elucidated. The parallel divergence model can also explain hybrid necrosis/weakness caused by interactions among genes involved in autoimmune response (Fig. 2A). Deleterious interactions were detected between parallelly diverged immune genes, which led to conflict of the immune system and hybrid weakness in plants. In rice, two incompatible dominant loci of *Hwi1* and *Hwi2* caused interspecific hybrid weakness by activating the autoimmune response in the hybrids (Chen et al., 2014). The origin of *Hwi1* was possibly induced by transposable elements in the wild rice, whereas the *Hwi2* locus was generated by a missense mutation event in another cultivated rice lineage. The evolutionary mechanism of these two loci was in principle congruent with the parallel divergence model, which caused hybrid weakness syndrome by incompatible interaction between diverged functional loci from respective populations.

### 3.2. Updated sequential divergence model supported by new examples

The sequential divergence model suggests that postzygotic reproductive isolation might be caused by negative interaction between two linked ancestral and nascent alleles (Ouyang and Zhang, 2013). The ancestral genotype is retained in one lineage, whereas the nascent genotype arises in another lineage by sequentially occurred mutations (Fig. 2B). This model was proposed to explain the evolutionary history of hybrid sterility locus *Sa* in rice, which conferred male sterility and transmission ratio distortion in hybrids with two adjacent genes *SaF* and *SaM* (Long et al., 2008). The *xian* varieties carried the ancestral haplotype of $SaM^{+}SaF^{+}$, while in *geng* subspecies, the derived $SaM^{-}SaF^{-}$ haplotype constituted the predominant one. In *Sa* heterozygotes, negative interaction between *xian* type $SaF^{+}$ and $SaM^{+}$ and *geng* type $SaM^{-}$ led to selective elimination of pollen carrying $SaM^{-}$ and thus reduced male fertility in the progeny.

Another major QTL *qHMS7* identified recently in rice hybrid sterility also conformed to the sequential divergence model (Fig. 2B), which regulated male fertility in hybrids between the wild rice and Asian cultivated rice with two tightly linked genes *ORF2* and *ORF3* (Yu et al., 2018). The $ORF2^{D}$ in *O. sativa* had a pollen-killing function in a sporophytic manner, which selectively eliminated pollen grains without *ORF3* in hybrids. The ancestral genotypes with $ORF2^{N}$ or $ORF2^{M}$ showed deleterious interaction with the derived haplotype $ORF2^{D}ORF3$ that originated sequentially in the same lineage, leading to segregation distortion by preferential transmission of selected pollen grains, which drove genetic differentiation and reproductive barriers.

A recent study showed that structural changes and copy number variations at the *Sc* locus led to incompatible interaction between *xian* alleles and *geng* promoter, thus conferring *xian-geng* hybrid male sterility and segregation distortion (Shen et al., 2017). In this system, the *geng* allele *Sc-j* encoding a DUF1618-domain protein was essential for pollen development, whereas in *xian* varieties, two or three tandem-duplicated *xian Sc-i* copies evolved distinct promoters different from that in *geng* subspecies. *Sc-j* was expressed specifically in anthers in *geng* varieties, while *Sc-i* copies showed a high-level and constitutive expression pattern compared with that of *Sc-j*. Thus, in the heterozygous genetic background, the expression of *Sc-j* was largely suppressed in early-bicellular pollen by allelic suppression from the high expression of *Sc-i* in sporophytic cells, which induced selective abortion of *Sc-j*-pollen reproductive isolation. Another hybrid sterility locus *S7* was identified regulating *Aus-geng*/*xian* female fertility in hybrids (Yu et al., 2016). Female gametophytes with *xian* or *geng* genotype were aborted in heterozygous genetic background. Downregulation of *S7-ORF3* restored spikelet fertility in hybrids, while transformation of *Aus S7-ORF3* allele in *geng* resulted in sterility. Although based on present understanding each of these two cases comprised only one involved gene, we believe more genes will be found participating in the related pathways, possibly following the sequential divergence model.

The sequential divergence model was also extensively supported by studies in animals such as the *zeel-1*/*peel-1* combination in *Caenorhabditis elegans* (Seidel et al., 2008), the *t-complex distorters* and *responder* in mouse (Herrmann et al., 1999; Bauer et al., 2005, 2007), the *Lhr*/*Hmr* pairs in *Drosophila* (Brideau et al., 2006), as well as the *Aep2*/*OLI1* (Lee et al., 2008) and *MRS1*/*COX1* (Chou et al., 2010) cases in *Saccharomyces*. Although all the cases identified in the sequential divergence model were genes located in adjacent regions, this model might be extended to unlinked loci if we learn more in the future (Fig. 2B).

Based on understandings from these examples especially with new data in the last several years, the sequential divergence model may comprehensively explain the strategy adopted by a wide range of organisms during the establishment of postzygotic reproductive barriers, representing a general mechanism to maintain the driving force of reproductive isolation and eventually speciation.

### 3.3. Parallel-sequential divergence model explains complex systems with more than two components

If more than two members were involved in postzygotic reproductive isolation, such as the killer-protector system of *S5* (Yang et al., 2012), incompatible interactions arose from the genes that had undergone both parallel and sequential divergence (Fig. 2C) (Ouyang and Zhang, 2013). The *S5* system expanded the understanding in reproductive isolation by involving three tightly linked genes, which regulated embryo-sac fertility in *xian-geng* cross (Chen et al., 2008; Yang et al., 2012). *ORF3* was predicted to code for a heat shock protein (Hsp70), *ORF4* for a membrane protein, and *ORF5* for an aspartic protease, all of which had different alleles in *xian* and *geng* rice. The typical *xian* and *geng* varieties contained the combination of *ORF3*+*ORF4*−*ORF5*+ and *ORF3*−*ORF4*+*ORF5*−, respectively. In the hybrid, the extracellular ORF5+ damaged cell wall integrity and produced signals that were sensed by the plasma membrane-localized ORF4+, which triggered stress response and consequently endoplasmic reticulum (ER) stress in ovaries due to the accumulation of increased misfolded proteins in ER (Yang et al., 2012; Zhu et al., 2017). The unresolved cell wall damage, stress response, and ER stress selectively induced premature programmed cell death (PCD) in the developing megaspores without *ORF3*+, resulting in embryo-sac abortion and segregation distortion in the offspring. The chaperone activity of the protector ORF3+ helped protein folding in ER to resolve ER stress and thus preventing premature PCD, which led to preferential transmission of embryo-sacs with *ORF3*+ and rescued the fertility of the *xian-geng* hybrids. These results indicated the role of egoism in reproductive barriers conducted by selfish elements that acted for their own favor.

The *S5* system originated by gene duplication after the split of the Oryzeae tribe from other grasses, most likely through *Helitron* transposition (Du et al., 2011; Ouyang et al., 2016). The *S5* complex had undergone neofunctionalization after duplication and generated incompatible *xian* and *geng* genotypes in pre-differentiated rice groups by a combination of mutational steps, giving rise to the trigenic reproductive isolation system. Natural selection contributed to the spread of *ORF3*+*ORF4*−*ORF5*+ in *xian* rice, whereas founder effect associated with domestication led to the increased frequency of *ORF3*−*ORF4*+*ORF5*− in *geng* population. Once the frequencies of incompatible genotypes had increased in the respective populations, typical *xian* and *geng* rice groups would generate deleterious interactions upon hybridization eventually leading to reproductive isolation and restructuring of rice genetic composition.

Another case provided new data for the parallel-sequential divergence model (Fig. 2C). The *S1* locus caused reduced fertility and biased transmission ratio in hybrids of two rice species *O. sativa* and *O. glaberrima* with the involvement of lineage-specific genome fragment in different populations (Xie et al., 2017b; Koide et al., 2018). Based on understandings from two independent studies, the preferential abortion of gametophytes from *O. sativa* was likely caused by a combined effect of an *O. glaberrima*-specific peptidase-coding gene *SSP* and an *O. glaberrima*-divergent gene *OgTPR1* with trypsin-like peptidase and ribosome biogenesis regulatory domains. Obviously both *SSP* and *OgTPR1* were necessary but not sufficient for the *S1* function. In the interspecific hybrids, these specific or divergent genes from *O. glaberrima* may show deleterious interactions with other components in *O. sativa* or *O. glaberrima* genome, which selectively killed *O. sativa* gametophytes during male and female gametogenesis. One might infer that the involved components in *O. glaberrima* genome emerged sequentially in the African rice, whereas the additional component in *O. sativa* evolved parallelly in the Asian cultivated rice lineage.

Dobzhansky (1937) gave a first glimpse in the isolation mechanism of species 80 years ago by proposing deleterious interactions between diverged genes in hybridizing populations. The three evolutionary genetic models further explained why the unfit trait in hybrids was fixed due to neutral or adaptive evolutionary changes in different lineages at the molecular and evolutionary levels. The primary functions of the genes involved in reproductive isolation systems were always highly diverse. They may play an essential role in the development of the organism in respective populations, as featured by the parallel divergence model, although their biological functions were distinct. These hybrid sterility genes were beneficial to the individuals within the populations, and were maintained in the populations by evolutionary driving force. However, in other cases such as in sequential or parallel-sequential divergence model, the primary functions of the hybrid sterility genes may or may not be essential for the plants. Knockout mutants of these genes in homozygous background may not affect the development of the organisms. But a selective advantage existed in one of the alleles in the hybrid, which led to segregation distortion in the offspring, causing hybrid sterility and reproductive isolation as a by-product. Thus, the three evolutionary genetic models represent a general mechanism underlying reproductive isolation and speciation, and such accumulation of genetic divergence and functional differentiation would ultimately lead to the installation of reproductive barriers and restructuring the genetic compositions of different lineages.

## 4. Improvement of fertility in intersubspecific or interspecific crosses in rice breeding process

Intersubspecific *xian-geng* hybrids show strong heterosis but reduced fertility, which hinders further increasing of rice yield potential. Previous studies identified that in addition to typical *xian* and *geng* varieties, there existed a special group of rice germplasm, referred to as wide-compatibility varieties, that produced fertile hybrids when crossed to either *xian* or *geng* varieties (Ikehashi and Araki, 1984). Such wide-compatibility varieties contained specific combination of alleles (referred to as wide-compatibility alleles or neutral alleles) at the hybrid sterility loci, which enabled hybridization and gene flow between the two subspecies, thus maintaining species coherence and providing an opposing force for reproductive isolation. Reduced fertility in hybrids can be thus rescued by manipulating of hybrid sterility loci, providing a promising approach in utilization of intersubspecific heterosis.

Molecular marker-assisted backcross breeding is an effective way for construction of wide-compatibility varieties by the transfer of multiple neutral alleles or specific genotypes into elite *xian* or *geng* varieties. One of the approaches for utilizing the intersubspecific heterosis of rice is breeding of *xian*-compatible *geng* lines, which produce high-fertility *xian-geng* hybrids. By integration of the wide-compatibility allele *S5-n* and three *xian* alleles *S7-i*, *S8-i* and *S9-i* at hybrid embryo-sac sterility loci into the photoperiod-sensitive male sterile plant, the improved line produced fertile hybrids with a significant degree of heterosis (Chen et al., 2011). Pyramiding of wide-compatibility alleles into an elite variety has been well applied for exploiting the intersubspecific heterosis, as double-neutral genes could improve the fertility of intersubspecific crosses (Shahid et al., 2013). Three neural alleles, *S5-n*, *S7-n* and *S17-n*, were used for fertility restoration through their incorporation into an elite *geng* restorer line W107 (Wan, 2010). Using an integrated backcross breeding strategy, the compatibility of an elite *xian* restorer line 9311 to a broad spectrum of *geng* varieties was largely improved by introducing two wide-compatibility alleles, *S5-n* and *f5-n* (Mi et al., 2016). The pyramiding line showed 33.6%–46.7% increase of spikelet fertility when crossed to *geng* varieties, with constant agronomic performance of the original 9311. These breeding materials would facilitate the utilization of intersubspecific heterosis from *xian* and *geng* crosses in rice breeding.

The recently developed technology of CRISPR/Cas9 system also speeds up the breeding of hybrid-compatible lines (Ma et al., 2015). Such genome editing-based approach can rapidly generate non-functional alleles at the hybrid sterility loci, based on understandings in related genetic models and molecular mechanisms. By knocking out one or two of the three *Sc-i* copies by CRISPR/Cas9 system, the gene dosage of *Sc-i* was precisely reduced, which rescued the expression level and thus the function of *Sc-j* in pollen development, providing an effective approach to break down the reproductive barrier by overcoming the male sterility in hybrids (Shen et al., 2017). In a similar way, silencing of *SaF* or *SaM* by RNA interference or artificial disruption of *SaF* and *SaM* alleles using the CRISPR/Cas9 approach at the *Sa* locus successfully created “neutral” *Sa* alleles, which facilitated intersubspecific hybridization with restored male fertility in *xian-geng* crosses (Xie et al., 2017a). Hybrid-compatible lines with *S1-n* allele were also created using the CRISPR/Cas9 system, either by knocking out *SSP* or *OgTPR1* in *O. glaberrima* varieties (Xie et al., 2017b; Koide et al., 2018). This enabled interspecific hybrid breeding and utilization of interspecific heterosis.

## 5. Conclusions and perspectives

The reproductive isolation and speciation field is probably one of the most rapidly developing areas in plant biology in the past decade, as we knew nothing about the regulatory genes and related mechanisms of such barriers a decade ago. While map-based cloning represents an efficient traditional method for cloning hybrid sterility genes in these years, rapid advance in genomic technology such as CRISPR/Cas9 will bring new opportunities to expand the molecular and evolutionary understanding of incompatibility loci that are involved in reproductive barriers across a wide range of plant species. In the next decade, dissection of biochemical pathways and interaction networks underlying more cloned genes will provide new insights into the underlying mechanisms that drive reproductive isolation and species divergence. In addition, compared to the advanced progress in postzygotic reproductive isolation in plants, the explorations in mechanistic understanding of prezygotic reproductive isolation have been largely ignored. Future work in both genetics and molecular analyses of prezygotic reproductive barriers across a range of plants especially crops will promise new insights in isolation mechanism and speciation.

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