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REVIEW

Decades' progress and prospects on maize functional genomics and molecular breeding

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Maize (*Zea mays* L.) is not only an important cereal crop, but also a model plant species for genetic, cytologic, genomic, and molecular studies. Maize possesses tremendous phenotypic and genetic diversity. During the past few decades, researchers have made significant advances in multiple areas, including the genomic compositions and variations of maize and its ancestors, the genetic and genomic bases of maize domestication and evolution, the genetic architecture of various agronomic traits (yield, quality, biotic and abiotic stress responses, nutrient use efficiency, fertility and heterosis), and the development of novel molecular breeding technologies. In this review, we summarize these research achievements and provide a perspective for future maize research and breeding.

maize | functional genomics | molecular breeding | genome | yield | quality | abiotic stress | biotic stress | nutrient use efficiency | male sterility | heterosis

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Introduction

Maize is an important food and feed crop. After its initial domestication from the wild teosinte *Zea mays* ssp. *parviglumis* in the lowlands of the Balsas River basin, maize was introduced to the highlands of central Mexico, where it experienced an introgression from another wild teosinte *Zea mays* ssp. *mexicana*. This two-teosinte origin contributes to the propagation of maize across the world (Yang et al., 2023b). Maize is now widely planted worldwide due to its high-yielding potential and broad adaptability. The important role of maize in agriculture production prompts extensive investigation on its genomic composition, the underlying genetic and molecular basis of various agronomic traits, and the development of novel technologies for genetic improvement. Over the past decade, substantial progress has been made in these areas, all of which contribute towards a better understanding of maize biology and more efficient breeding of maize varieties with a higher yield, better quality, and higher resilience to various biotic and abiotic stresses for greener and sustainable agriculture.

Maize exhibits tremendous phenotypic diversity, which is largely attributed to the abundant genetic variations in the genome. Due to the rapid progress on sequencing technologies, tremendous progress has been made on elucidating the genomic compositions and variations of maize and its ancestors. Since the release of the first maize genome (B73) in 2009 (Schnable et al., 2009), dozens of high-quality genome assemblies have been published (Hufford et al., 2021; Springer et al., 2018; Wang et al., 2023a; Yang et al., 2019a), culminating in the assembly of a complete telomere-to-telomere maize genome recently (Chen et al., 2023d). With such information, new insights into maize domestication and genetic improvement have been unraveled (Chen et al., 2022d; Hufford et al., 2012; Wang et al., 2020a). Besides genomes, maize researchers have also engaged other omics, including transcriptomics (such as spatiotemporal transcriptomics), epigenomics, proteomics, metabolics, and netomics, to bridge genomics and phenomics (Han et al., 2023). Moreover, some studies have pushed the omics studies towards single-cell or population levels. These omics studies greatly deepened and broadened maize functional genomics.

Remarkable progress has also been made on dissecting and elucidating the genetic architecture regulating various important agronomic traits, including grain yield, plant architecture, flowering time, grain quality, abiotic and biotic stress resistance, fertility control, and heterosis of maize. Towards this goal, a variety of genetic populations have been developed, including an association panel (Yang et al., 2010b), artificial segregating populations such as nested association mapping (NAM) population and complete-diallel plus unbalanced breeding-derived intercross (CUBIC) population (Buckler et al., 2009; Liu et al., 2020d), and several genome-wide mutant libraries (Liang et al., 2019a; Lu et al., 2018; May et al., 2003). With these germplasm resources, dozens of new genes regulating various agronomic traits have been cloned and their regulatory mechanisms have been elucidated, providing valuable targets for molecular breeding of maize (Chen et al., 2022d; Huang et al., 2022b; Yan et al., 2023; Yu et al., 2025).

We have also witnessed major progress in molecular breeding technologies of maize. Firstly, the cloning of key regulatory genes for various traits and the identification of their natural variations aided in molecular marker-assisted breeding. Secondly, the cloning of haploid induction genes and the development of new doubled haploid (DH) technologies significantly boosted the efficiency of developing pure breeding lines (Gilles et al., 2017; Kelliher et al., 2017; Liu et al., 2017a). Thirdly, various genome-editing technologies have revolutionized both gene functional studies and the ways of creating novel alleles. In particular, the combinations of gene editing with DH technologies greatly enhanced the efficiency of targeted improvement of specific traits (Tian et al., 2024a). Fourthly, the advances in big data, machine learning, and artificial intelligence have allowed the development of new algorithms and models of genomic selection and prediction. All together, these new progresses in maize functional genomics and breeding technologies have set a solid foundation to bring maize biology and breeding into a new era.

Advances in maize omics studies

Maize genome and genomic variation

A high-quality maize reference genome is instrumental for various basic and applied studies. The first maize B73 genome was assembled in 2009 using Sanger sequencing technology (Schnable et al., 2009), which substantially improved our understanding of the structure and function of the maize genome. However, due to cost and read-length limitations, there were more than one hundred thousand gaps in the initial B73 genome. Afterwards, genomes of several inbred lines were assembled using the "next-generation" sequencing technologies, including PH207 (Hirsch et al., 2016), W22 (Springer et al., 2018), HuangZaoSi (Li et al., 2019a), and three European inbred lines F7, EP1, and DK105 (Haberer et al., 2020). Benefiting from the advance of long-read, single-molecule DNA sequencing technologies, more genomes of maize and its wild relative teosinte were assembled and released with substantially improved genome assembly quality, including the improved B73 genome (Jiao et al., 2017), and the genomes of Mo17 (a representative Lancaster inbred line) (Sun et al., 2018c), A188 (a genetic transformation competent line) (Lin et al., 2021), SK (a small kernel germplasm) (Yang et al., 2019a), K0326Y (a quality protein maize germplasm) (Li et al., 2020c), CIMBL55 (a prominent drought-resistant germplasm) (Tian et al., 2023), B73-Ab10 (Liu et al., 2020), NC358 (Ou et al., 2020), Ia453-*sh2* (Hu et al., 2021c), Dan340 (an excellent backbone inbred line of the LvDa Red Cob Group) (Zhao et al., 2022c), the 26 founders of the maize NAM population (Hufford et al., 2021), S37 and 11 temperate lines (Wang et al., 2023a) (Figure 1), as well as *Zea mays* ssp. *mexicana* (Yang et al., 2017a) and *Zea mays* subsp. *Parviglumis* (Huang et al., 2022b). These efforts culminated in a gapless telomere-to-telomere assembly for all chromosomes of the Mo17 genome by Jinsheng Lai's group (Chen et al., 2023d), which is the first complete assembly of the maize genome and represents a major engineering breakthrough in the field of complex genome assembly.

Maize was domesticated from lowland teosinte (*Z. mays* ssp. *parviglumis*) within the past 6,000 to 10,000 years (Doebley, 2004), and exhibits an extremely high level of genetic diversity among different lines (Buckler et al., 2006). Comparative genomics revealed that approximately 10% of genes were mutually nonsyntenic between the B73 and Mo17 genomes, and over a fifth had either large-effect mutations or large structural variations (Sun et al., 2018c). Therefore, besides the

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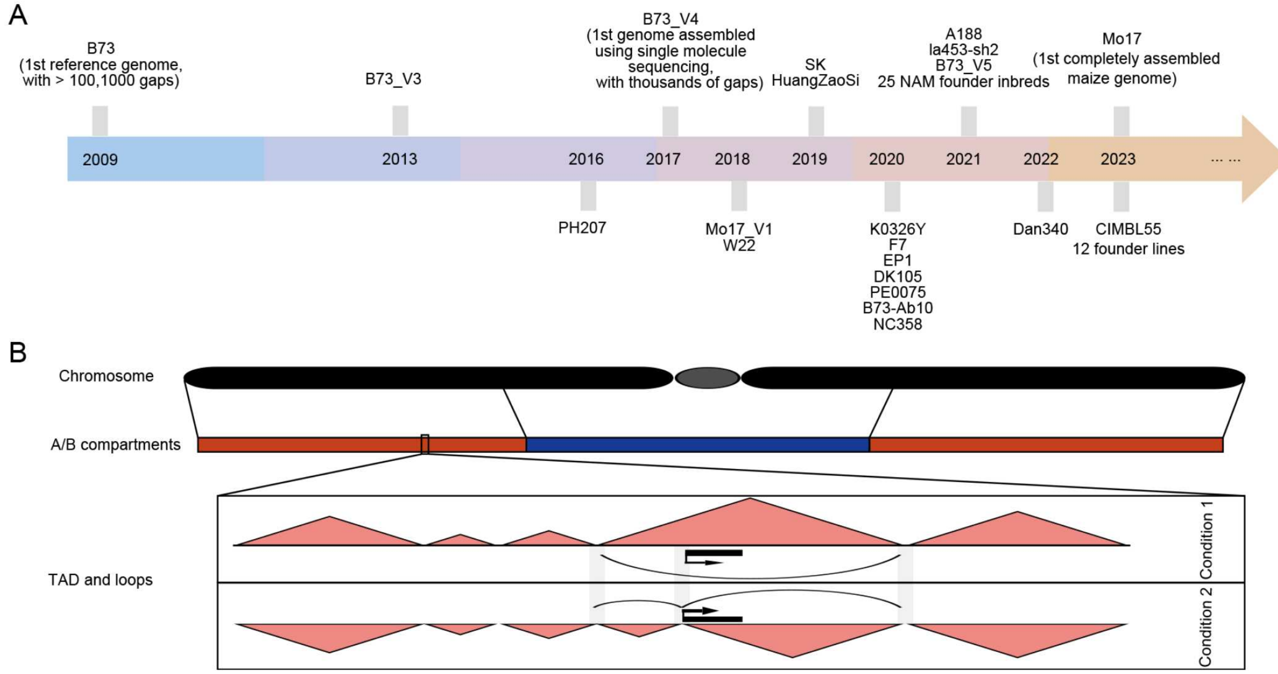

Figure 1. Maize genome assemblies and the 3D structure of the maize genome. A, Summary of the maize genomes assembled in the past quarter-century. The genomes of more than 50 maize inbred lines were assembled. B, 3D structure of the maize genome. In maize, the chromatin exhibits a bifurcated structure, with A and B compartments delineating the euchromatic regions typically localized to chromosome arms and the heterochromatic regions predominantly residing in the centromeric and pericentromeric regions, respectively. TAD-like domains were observed in maize. However, TADs in maize likely regulate gene expression mainly via chromatin loops at the TAD borders. Although TADs are highly consistent among different inbred lines and tissues, differential TADs are associated with altered gene expression. Stress treatment like low-Pi increased the isolation strength, leading to increased TADs in the maize genome.

strides made in reference genome assemblies, extensive efforts have been made to characterize the genetic variations among maize germplasm by population-scale whole genome resequencing. Using the high-throughput sequencing technology, Jinsheng Lai's group uncovered vast genetic variations among six elite maize inbred lines (Lai et al., 2010). The same group also analyzed the genomes of a large collection, including 278 temperate maize inbred lines from different stages of breeding history, and revealed highly dynamic genetic changes during modern breeding of maize, even within identity-by-descent regions (Jiao et al., 2012). Jianbing Yan's group identified more than 80,000 polymorphic structural variations across 521 diverse lines, and demonstrated that some variants affected gene expression and trait performance (Yang et al., 2019a). It is worth noting that based on large-scale population genetics studies, Jianbing Yan's group revealed that about 10% of the maize genome displayed evidence of introgression from the *mexicana* genome (Yang et al., 2017a) and that the origin of modern maize could be traced to an admixture between ancient maize and *Zea mays* ssp. *mexicana* (Yang et al., 2023b), providing critical evidence for the two-teosinte origin of modern maize. Moreover, Haiyang Wang's group identified more than 1,800 selective genomic regions for adapting to increased planting densities during modern breeding based on population studies of 350 elite lines from China and the United States (Wang et al., 2020a).

Maize pan-genome

Maize exhibits extensive genetic diversity, and pan-genome research enables the identification of numerous genes across different varieties. This provides a critical foundation for the exploitation of maize genetic resources, advancing breeding programs and enhancing our understanding of gene functions. The construction of the maize pan-genome experienced three stages (Shi et al., 2023a). The earliest approach was based on the "map-to-pan" strategy: mapping all short reads on the reference genome and assembling the unmapped reads from a group of individuals jointly to represent all DNA sequence in maize. To construct a maize pan-transcriptome as a proxy for the maize pan-genome, whole-seedling transcriptome sequencing (RNA-seq) was conducted on a panel of 503 diverse maize inbred lines representing the major U.S. grain heterotic groups. A total of 8,681 novel representative transcript assemblies (RTAs) including 1,425 core and 2,916 dispensable RTAs were identified using the "map-to-pan" strategy (Hirsch et al., 2014). The second strategy was genome assembly comparison: obtaining high-quality genome assemblies based on short-read or third-generation long-read sequencing data, calling structural variations (SVs) through whole-genome alignment (WGA). In maize, *de novo* genome assemblies of four European flint lines were generated based on Illumina short-read sequencing data, with scaffold N50 ranging from 6.1 to 10.4 Mb. These flint genomes largely complement the maize pan-genome and pan-genes. Further WGA analyses unveiled the dynamics of heterochromatic knobs and long terminal repeat retrotransposons in the maize genome, and a total of 34,352 core genes were first reported among dent and flint maize germplasms (Haberer et al., 2020). Moreover, *de novo* genome assemblies of 26 inbred lines from the maize NAM population were generated based on PacBio long-read sequencing data, with a mean scaffold N50 of 119.2 Mb (Hufford et al., 2021). A total of 103,000 pan-genes were released, including 32,052 core or near-core genes and 70,981 dispensable or private genes. A total of 27,228 transposable element (TE) families were annotated in the pan-genome TE library, among which 59.7% were core TEs and only 2.5% were private. Tropical and temperate germplasm are differentiated in different ways, including pan-gene composition, homeolog retention after polyploidy, abundance of TEs, and methylation profiles. Moreover, a panel of 791,101 SVs was identified in the NAM population. Beyond this, the latest strategy introduced a genome graph to provide a unified coordinate system for the sake of more effective pan-genome analysis. Recently, 721 lines consisting of 507 maize inbred lines, 31 landrace individuals, and 183 teosinte individuals were collected and were subject to Illumina short-read sequencing. A super graph pan-genome size of 6.71 Gb, including 4.57 Gb of the non-B73 reference sequence, was released (Gui et al., 2022). A total of 58,944 pan-*Zea* genes were found, among which 44.34% were dispensable genes, 3,452 genes were maize-specific, and 2,189 genes were teosinte-specific. Moreover, a total of 274,649 common SVs were detected in this pan-*Zea* genome, 60.03% of which were TE-related. Pan-genome provides complete genetic variations in the maize population, especially for underexplored SVs.

Although several maize pan-genomes have been published, there remains a trade-off between the diversity of genomic resources and assembly quality. To date, no pan-genome has achieved both comprehensive genomic resource coverage and high assembly quality. According to Moore's Law of long-read sequencing, a fully comprehensive and high-quality maize pan-genome is likely to be constructed in the future. Furthermore, the utilization of pan-genomes is still severely limited. For instance, although a significant number of SVs have been identified, their complexity has been greatly underestimated, and their functional implications remain poorly understood.

3D genome

Eukaryotic chromatin is organized into spatially constrained nuclear territories via chromatin looping. The non-random distribution of the genome in nuclei plays a critical role in gene regulation. In maize, the noncoding variants linked to agronomic traits often reside in distal regulatory elements that physically interact with target genes by chromatin loops. Deciphering the genome organization within nuclei provides insights into gene expression regulation and bridges the genotype-phenotype gap in maize. The three-dimensional genome (3D genome) represents the spatial organization of chromosomes via physical contact between distinct sites on the linear DNA within nuclei (Bouwman et al., 2022). Chromatin looping and folding caused by physical contacts between distinct sites can be identified through chromosome conformation capture (3C) techniques (Dekker et al., 2002; Lieberman-Aiden et al., 2009). These 3C methods can be coupled with high-throughput sequencing to detect DNA fragments that are spatially close together in the 3D structure of the genome (Dixon et al., 2012; Rao et al., 2014). The genome conformation plays a critical role in animal development, human diseases, and plant development (Corces and Corces, 2016; Dixon et al., 2012; Wen et al., 2023; Yu and Ren, 2017). In recent years, the applications of 3C-based technologies in maize have increased our understanding of the overall chromosomal organization and how it can modulate gene expression.

Like animal species, maize chromatin can be segmented into blocks, so-called local A/B compartments corresponding to euchromatin at chromosome arms and heterochromatin at centromeric and pericentromeric regions in general (Dong et al., 2017a) (Figure 1B). These local A/B compartments correlated with active gene regions and inactive TEs. On a sub-chromosomal scale, topologically associating domain (TAD)-like domains were observed in maize, although they possess different features from mammalian species (Dong et al., 2017a). TADs in maize showed asymmetric epigenomic features around the domain borders and overlapped with local A/B compartments. Chromatin loops spanning TADs were pervasive in maize, which is rare in mammalian species. TADs in maize and other large plant genomes showed little conservation among species (Dong et al., 2017a). More than 60% of TADs were variable between two maize lines, considerably higher than other large plant genomes like cotton (Tian et al., 2021c; Wang et al., 2018d). The unique features of TADs from maize and other large plant genomes indicate that different mechanisms from mammalian species could determine maize TADs or plant TADs in a broader scope (Dong et al., 2017a; Dong et al., 2020b).

The biological function of maize TADs was explored by comparing the chromatin conformations among mesophyll, bundle sheath, and endosperm tissues (Dong et al., 2020a). Around 80% of TADs in mesophyll were also observed in bundle sheath and endosperm tissues, suggesting stable chromatin organization among different tissues (Dong et al., 2020a; Sun et al., 2020b). TAD border changes were associated with tissue-specific gene expression. Genes associated with tissue-specific TAD borders showed higher expression levels. TADs created isolated chromatin environments in mammalian species, and genes within TADs tended to be coregulated (Le Dily et al., 2014; Zhan et al., 2017). However, gene expression patterns among three tissues showed that the expression of genes within maize TADs was not correlated (Dong et al., 2020a). TADs in maize likely regulate gene expression mainly via chromatin loops at the TAD borders. A recent study showed that low-phosphorus stress in maize leads to an increased number of TAD-like domains (Luo et al., 2024). It is worth noting that TAD identification highly relies on the resolution of 3C-based technologies and bioinformatic analysis. The biological function of TADs requires further investigations with more sophisticated technologies and bioinformatic algorithms in the future.

Maize is well-known for the long-distance regulation of genes, which were genetically identified in individual studies. Using high-resolution 3C-based technologies, the genome-wide interaction map of *cis*-regulatory elements (CREs) can be dissected (Li et al., 2019b; Peng et al., 2019; Sun et al., 2020b). These studies revealed a complex spatial organization of regulatory regions, which contains proximal-proximal interactions (interactions between promoters), proximal-distal interactions (interactions between promoters and distal regulatory regions away from genes), and distal-distal interactions (interactions between distal regulatory regions). A majority (>60%) of interactions were proximal-proximal interactions, but a significant portion (25% to 35%) of interactions were between promoters and distal regulatory regions (Li et al., 2019b; Sun et al., 2020b). Thousands of distal regulatory regions involved in chromatin interactions overlapped with metabolic quantitative trait loci (QTLs), emphasizing the power of 3C-based technologies in deciphering the regulatory mechanisms of agricultural traits (Peng et al., 2019; Sun et al., 2020b; Xu et al., 2020a).

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Chromatin interaction networks (ChINs) based on the chromatin contacts revealed gene clusters involved in tissue development and provided new clues to the regulatory mechanism of pleiotropic effects (Bonev and Cavalli, 2016; Concia et al., 2020; Li et al., 2019b).

In summary, recent advancements in the study of the 3D genome in maize have illuminated its intricate architecture, offering substantial promise for dissecting the genetic mechanisms of agronomic traits. However, the current studies on maize 3D genomes are limited by several factors. First, most 3C technologies applied in maize and other plant species are at low resolutions, like tens or even hundreds of kb, limiting the detection of fine-scale structures. A recent study successfully applied Micro-C technologies in *Arabidopsis* and achieved nucleosome resolution (200-bp) (Sun et al., 2024). New technologies with fine resolutions enabled accurate identification of chromatin loops and the boundaries of TAD-like domains, which help understand the relationship between the 3D structures and gene regulation. Second, the relationship between the 3D structure and diversity of maize genomes is less characterized. How pervasive variations such as structural variations in maize affect the 3D genome structure and whether the dynamics of 3D genome in maize contribute to the phenotypic diversity in maize populations are not clear. Large-scale 3D genome studies are required to answer these questions, although these kinds of studies are impeded by the high cost of high throughput 3D technologies. New technologies with improved resolution and significantly lower cost in the future will accelerate the 3D genome studies in maize.

Maize epigenomics

Epigenetics, the study of gene functions that are heritable but do not involve changes of genomic DNA sequence, has emerged as a critical field with profound implications for understanding plant development, evolution, and stress responses. Epigenetics is important because it allows plants to adapt to environmental changes and ensure proper development, which is crucial for plant survival and evolution and holds potential for crop improvement in times of global climate change (Kakoulidou et al., 2021). Factors influencing plant epigenetics include environmental factors (e.g., temperature, drought, and nutrient availability), developmental stages, as well as genetic factors like the presence of transposable elements and the activity of transposable elements-mediated chromosomal rearrangements (Abdulraheem et al., 2024). As a model plant, maize is suitable for studying epigenetic regulation in plants due to its complex genome composition and agricultural significance. Epigenetic regulation in maize is pivotal in understanding complex gene regulation and offers practical solutions for improving yield, resilience, and adaptability in varying environmental conditions. Continued research in this field would play a transformative role in advancing agricultural sustainability and meeting global food demands.

DNA methylation, one of the most important epigenetic marks, has been demonstrated to play various roles during development and abiotic stress responses (Zhang et al., 2018c). Loss of the function of DNA demethylase in maize leads to changes in methylation near genes, which affects transcription factor binding and further regulates gene expression (Xu et al., 2022a). ZmDDM1 is required for the formation of mCHH islands (regions with high CHH methylation, H=A, C, or T) and likely targets euchromatic regions through GC-rich motif binding, and thus remodeling chromatin to enable RNA-directed DNA methylation (RdDM) access in maize (Fu et al., 2018; Long et al., 2021; Long et al., 2019). RdDM enforces boundaries between heterochromatin and euchromatin in the maize genome (Li et al., 2015c). Male reproductive-specific and meiotic 24-nt phasiRNAs can contribute to increased CHH methylation levels of 24-PHAS loci *in cis* (Zhai et al., 2015; Zhang et al., 2021e), and ribosome binding upstream of 24-PHAS could accelerate the production of 24-nt phasiRNAs (Han et al., 2024). DNA methylation can affect starch synthesis by globally regulating the expression of starch synthesis genes and miRNAs (Hu et al., 2021d). Under drought stress, the methylation levels of drought-tolerant inbred lines were much more stable than those of drought-sensitive inbred lines (Wang et al., 2021c). During maize domestication and improvement, genome-wide methylation variation showed signs of weak natural selection, while regions exhibiting variation explained considerable phenotypic variations (Xu et al., 2020a). Moreover, taking advantage of biological methodologies, epigenomics has made significant strides in exploring gene expression and phenotypic variations at both the single-cell and large-scale population levels. BRIF-Seq, for instance, a novel single-cell DNA methylation sequencing technology capable of being applied across diverse species and tissues, has been developed (Li et al., 2019i). Large-scale studies involving 263 maize inbred lines integrated DNA methylation data with high-density SNP data, gene expression, and kernel metabolic data, revealing associations between variations in DNA methylation, gene expression, and phenotypes (Xu et al., 2019).

Genomic imprinting in maize, particularly in the endosperm, underscores the role of epigenetic mechanisms in parent-of-origin gene expression (Zhang et al., 2011). Genome-wide investigation of the epigenetic regulatory mechanism of imprinted gene expression uncovered distinct patterns between maternally expressed genes (MEGs) and paternally expressed genes (PEGs) (Chen et al., 2017a; Zhang et al., 2014b; Zhang et al., 2011). A subset of MEGs was associated with maternal-specific DNA demethylation, and MEGs were not directly related to allele-specific H3K27me3. In contrast, expressions of most PEGs were related to maternal-specific H3K27me3, with a subgroup of PEGs also associated with maternal-specific DNA demethylation (Zhang et al., 2014b; Zhang et al., 2011). Further, PEGs usually possess active H3K4me3 and H3K36me3, as well as repressive H3K27me3, while endosperm-specific MEGs were only associated with maternally preferred H3K4me3 and H3K36me3 (Dong et al., 2017b). Moreover, nucleosome positioning is involved in modulating the plasticity of gene transcriptional status (Chen et al., 2017a; Dong et al., 2018b). A recent study elucidated the role of the maize DNA demethylase in regulating the expression of imprinted genes, thereby enhancing our understanding of the mechanisms underlying imprinting in maize (Xu et al., 2022a). Besides, imprinted long non-coding RNAs were detected, and they were shown to display a similar epigenetic regulatory mechanism as protein-coding genes. Additionally, it has been shown that transposons can create potentially novel imprinted genes (Li et al., 2023d). Interestingly, imprinted genes were also found in the maize embryo at the early stage (Meng et al., 2018). Altogether, these studies systematically revealed the multiple facets of the epigenetic regulatory mechanism of genomic imprinting in maize.

Epi-transcriptome has been emerging as a key player in the regulation of gene expression. A galaxy platform—deepEA for interactive analysis of epitranscriptome sequencing data has been developed (Zhai et al., 2021). An analysis showed that N^6-methyladenosine (m^6A) sites were widely distributed in the 3′ untranslated regions (UTR) of protein-coding genes, and m^6A modifications played a role in regulating the alternative polyadenylation site choice and influencing translational status (Luo et al., 2020b). m^6A modification was also shown to interplay with gene duplication (Miao et al., 2020). Additionally, a study revealed evolutionary conservation and divergence of the m^6A methylome in plants (Miao et al., 2022).

Epigenetic modifications can introduce a new layer of genetic diversity that may complement conventional breeding strategies. For example, genes such as *ZmROS1a* and *ZmROS1b*, involved in DNA demethylation, regulate kernel weight (Xu et al., 2022a). In addition, epigenetic modifications highlight the potential to improve resilience to abiotic stress, a growing concern due to climate change. For instance, DNA methylation patterns have been shown to affect maize's response to heat stress (Guo et al., 2021a). While spontaneous epigenetic variations can sometimes be unstable, they can be stabilized through epigenome editing via clustered regularly interspaced short palindromic repeats (CRISPR) system, providing a means to introduce beneficial traits more rapidly (Tang et al., 2022). Despite the significant potential of epigenetic approaches to improving yields, stress resilience, and overall agronomic performance, challenges remain in fully understanding the complex interactions among epigenetic marks, environmental factors, and agronomic traits, as well as in translating laboratory findings into field applications. Future research should focus on the long-term stability and inheritance of epigenetic modifications in maize, as well as the development of more refined tools for manipulating epigenetic marks.

Maize transcriptomics

Transcriptome profiling is essential for understanding gene activities and functions. Over the last decade, lots of transcriptome studies have been performed to detect genes and cellular processes underlying the development of tissues in maize. A comprehensive transcriptome study of the maize embryo, endosperm, and intact seed, sampled at 2-day intervals from 0 to 38 days after pollination (DAP), provided an extensive view of transcriptome dynamics over seed development (Chen et al., 2014). Considering highly dynamic and complex developmental process underlying early maize seed development, the same research group subsequently constructed a high temporal-resolution transcriptome landscape for nucellus (embryo sacs included) at an interval of 4 or 6 hours within 6 DAP (Yi et al., 2019) and for embryo sacs and ovules (embryo sacs not included) precisely collected within the first four days of seed development (Li et al., 2023f), which uncovered a lot of crucial seed-specific genes involved in early seed development. Several other studies explored the starch biosynthesis (Xiao et al., 2016a; Zhang et al., 2019f), grain filling (Shen et al., 2022), and differential abscisic acid accumulation (Niu et al., 2022) during maize seed maturation via transcriptome analysis. Transcriptome profiling studies were also performed for various non-seed tissues. Analyses of dynamic transcriptomes of 10 key stages across the entirety of anther development in maize uncovered 751 key phase-specific genes for anther development (Han et al., 2022). Through the RNA-seq method, differentially expressed genes associated with fertility instability of S-type cytoplasmic male sterility were also explored in maize (Su et al., 2016). The transcriptome landscapes of roots (Li et al., 2011), stalk (Chen et al., 2021a; Le et al., 2022; Xie et al., 2022b) were also explored. The RNA-seq method was also widely used to explore the dynamics of the transcriptome under abiotic stresses (Yu et al., 2018b), biotic stresses (Wang et al., 2017c), and heterosis (Ma et al., 2018b; Zhan et al., 2023) in maize.

Typical RNA-seq was performed using tissues or cell type samples. In order to achieve a high resolution and to investigate cellular heterogeneity, single-cell RNA-seq methods have been developed (Jovic et al., 2022), which analyze the transcriptome of thousands of cells, respectively, in a single experiment. Via the single-nucleus RNA sequencing technology, signaling networks governing the movement and development of grass stomata were described in maize (Sun et al., 2022). In addition, single-cell transcriptomics analysis has decoded the gene regulatory network of endosperm differentiation, revealing the temporal dynamics of early endosperm development. This approach captured the sequential specification of distinct cell types and provided unprecedented insights into molecular mechanisms of tissue differentiation that were previously undetectable in bulk tissue analysis (Yuan et al., 2024). However, the spatial patterns of gene expression can not be directly resolved by single-cell transcriptomics. Spatial transcriptomics represents a technology that enables the sequencing of total mRNA in fresh frozen tissues while simultaneously retaining the spatial positional information of these transcripts. To overcome the limitation of single-cell RNA-seq, spatial transcriptomics analysis was performed during the grain filling stage of maize seeds recently, which identified 11 cell populations and 332 molecular marker genes (Fu et al., 2023). Notably, only hundreds to thousands of expressed genes can be detected using single-cell and spatial transcriptomics analysis at present, far fewer than those of typical RNA-seq.

Besides exploring the genes and cellular processes associated with development, the RNA-seq method was also useful for detecting expression quantitative trait loci (eQTLs). Based on population-level transcriptome analyses, 16,408 eQTLs (Fu et al., 2013a) and 19,554 splicing quantitative trait loci (sQTLs) (Chen et al., 2018) were identified in maize seeds. However, the application of typical RNA-seq in large-scale experiments is constrained by cost and labor. Recently, a massively parallel 3′ end RNA sequencing (MP3RNA-seq) method was developed for high-throughput transcriptome profiling (Chen et al., 2021a). Utilizing MP3RNA-seq, a total of 25,797 eQTLs regulating expressions of 15,335 genes in maize stem were identified (Chen et al., 2021a).

Netomics enhances our understanding of the maize functional genome

Omics methodologies not only provide insights into the gene abundance across various genetic layers but also offer valuable information regarding the functional aspects of genomic elements. Network analysis serves as an effective approach to elucidating gene functions. Over the past decade, significant advancements in the field of netomics have been made in maize, specifically in the areas of co-expression, regulatome of tran-

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scription factors, interactome, and multi-omics integration. A maize gene co-expression network was constructed utilizing the graphical Gaussian model, leveraging extensive RNA-seq data (Ma et al., 2017). This network successfully predicted gene modules associated with crucial aspects of maize development, including nutrient utilization, metabolism, and stress response. Another notable contribution of gene co-expression networking involved the development of the maize conditional co-expression network (MCENet) platform, which integrates 701 transcriptomic and 108 epigenomic datasets, allowing for the exploration of diverse conditional networks (Tian et al., 2018). At the regulatome level, the maize leaf regulatory network was constructed by employing ChIP-seq data from 104 transcription factors, revealing intricate and redundant characteristics of the plant transcription regulatory network (Tu et al., 2020). More recently, an extensive analysis utilizing ampDAP-seq data from 161 transcription factors has further expanded our understanding of the maize regulatory landscape, providing deeper insights into the complex transcriptional control mechanisms governing maize endosperm development (Yuan et al., 2024). Furthermore, extensive spatio-temporal translatome and transcriptome data from 33 maize tissues or developmental stages were collected, enabling the construction of an intra- and inter-omics regulatome encompassing genome-wide detectable transcription factors (Zhu et al., 2023). Regarding the interactome, the Protein-Protein Interaction Database for Maize (PPIM) was developed, which encompasses 2,762,560 interactions among 14,000 proteins (Zhu et al., 2016). Additionally, a large-scale wet-experimental Y2H assay was conducted, identifying over 360,000 protein-protein interactions. Furthermore, a multi-omics network integrating genomic 3D interactions, transcriptomic co-expression, translatomic co-expression, and interactomic networks was assembled (Han et al., 2023). This integrated multi-omics network facilitated the systematic elucidation of the molecular networks governing maize plant architecture, yield, and flowering time. The construction, analysis, and utilization of single or multi-omics networks significantly contribute to our understanding of functional genomics and establish a robust foundation for future crop breeding endeavors.

Quantitative trait genetic studies in maize

Most agriculturally important traits are inherited quantitatively, following the polygenic and omnigenic hypotheses due to multiple genes or QTLs and the interaction effect between genes, as well as between genes and environment (Boyle et al., 2017). To unravel the genetic basis of the quantitative traits, linkage mapping based on statistically linear regression and maximum likelihood estimate methods is a well-established approach to discover the relevant QTLs (Lander and Botstein, 1989; Zeng, 1994). Linkage mapping generally detects few QTLs per experiment as it is based on a bi-parental segregating population such as recombinant inbred line (RIL). The fine mapping and map-based cloning of detected QTLs are necessarily performed in a large-scale near isogenic line (NIL) population for achieving sufficient mapping resolution (Dinka et al., 2007). Genome-wide association study (GWAS) based on diverse germplasm collections is another solution to efficiently identify QTLs due to historical recombination events and rapid linkage disequilibrium decay (Flint-Garcia et al., 2003).

In the past 15 years, GWAS has been a routine tool to decode the genetics of maize quantitative traits. The fruitful accomplishments of GWAS on diverse important traits have been made, mostly due to the rapid progress of analytic toolkits and mapping populations with high-resolution genotype data.

GWAS tools

To improve GWAS power for complex traits, the statistical and analytic toolkits for plant species have been explosively advanced in the past few years (Xiao et al., 2017). The mixed linear model (MLM) was first proposed by Zhang et al. (2005) for GWAS in animals with accounting for pedigree-based genetic related matrix (GRM). Albeit highly efficient, it is not suitable for an analytic framework in crops, as the breeding pedigree information is often not recorded completely. A unified mixed linear model that uses marker-based GRM instead of pedigree-based GRM provided an alternative strategy for plant GWAS (Yu et al., 2006). Since then, the MLM method has become the routine GWAS tool.

The researchers kept improving GWAS power by enhancing computational and analytic efficiency. While increasing sample size and marker number can improve statistical power, it also significantly increases the computational burden. An efficient-mixed model association (EMMA) method was proposed to improve GWAS efficiency through optimizing matrix operations via spectral decomposition algorithm (Kang et al., 2008). However, this method solves the model equations iteratively per marker, termed as "exact method", resulting in a big computational challenge in analyzing millions of markers on large-scale individuals. To further enhance the analytic efficiency, two algorithms—the factored spectrally transformed linear mixed model (FaST-LMM) and the genome-wide efficient mixed-model association (GEMMA)—were independently proposed based on a similar strategy on the mixed-model equation optimization (Lippert et al., 2011; Zhou and Stephens, 2012). They further rewrote the model equations that refactor the traditional likelihood function of the mixed model to a form analogous to the likelihood of a linear regression model, which transformed the multiple-parameter estimation into a single-parameter optimization problem. Given the hypothesis that "quantitative traits are generally polygenically inherited", it was proposed to estimate model parameters once and constantly test markers iteratively, as another efficient solution to speed up GWAS computation for large-scale datasets. The most prevalent methods were termed as the P3D (population parameters previously determined) and the EMMA expedited (EMMAX) algorithms for large-scale GWAS research (Kang et al., 2010; Zhang et al., 2010).

Many GWAS methods are based on the hypothesis that the common disease is caused by common variants in human genetics (Reich and Lander, 2001). The experimental and empirical evidence demonstrated that rare variants are key sources of complex traits in plants (Chen et al., 2022b; Xiao et al., 2016b). However, the rare variants are often filtered out in GWAS because of low statistical power (Eichler et al., 2010). Moreover, the close linkage of multiple rare functional variants could trigger indirect association or synthetic association (Dickson et al., 2010), which may be one source of the missing heritability. To address this GWAS limitation related to rare variants, three GWAS approaches are available. When two or three independent causal variants, with weak effects, are located closely in an LD block, it is statistically hard to detect any single variant, but it would be possible to detect them as a group, termed as a multi-variant set test (Casale et al., 2015). The variant-set test is generally workable and statistically efficient for most situations when high-density genome-wide variants are available. Another solution for multiple local variants is the haplotype-based association analysis, which focuses on the identification of haplotypes using multiple variants. The haplotype-based methods are widely used by geneticists as the identified haplotypes are always biologically meaningful (Zhang et al., 2012). It was proven to be helpful for dealing with synthetic associations in maize (Lin et al., 2012), tomato (Lin et al., 2014), and rice (Yano et al., 2016). Compared with the set test method, the haplotype method probably has better power and interpretable ability as there is better prior knowledge on the population history and trait architecture. Typically, the mixed-model method, known as the parametric method, often assumes that the phenotype or marker effect follows the normal distribution. Given the concern that few rare variants with a large effect probably result in an untypical phenotypic distribution, a nonparametric GWAS method, ADGWAS, which tests median differences between allelic groups rather than the mean, was proposed. ADGWAS demonstrated a good balance between false positives and statistical power for those traits with abnormal distribution and large-effect rare variants (Yang et al., 2014). The mentioned GWAS methods are proven to be efficient in identifying rare functional variants, and the choice of which method should be flexible due to the distinct experimental and hypothetical scenarios. The complementary use of other analytic methods provides the potential to systematically unravel the biological implications of complex quantitative traits.

GWAS populations

In recent decades, association mapping in plants is often performed in natural populations with high genetic and phenotypic diversity. Natural populations have the advantage that allows GWAS signals to be inferred at high resolution, but the complex population structure and rare variants in natural populations may be a big barrier for GWAS power (Liang et al., 2021b). It is necessary to develop new population designs that can remove the confounding variables and increase the frequency of rare variants (Scott et al., 2020).

In maize, the first large-scale and multi-parent population, the NAM population, comprising 25 RIL populations, was developed by crossing the maize inbred line B73 with another 25 genetically diverse inbred lines (Yu et al., 2008). The NAM population, with clear population structure, can use historical and recent recombination events simultaneously, demonstrating a powerful capability to thoroughly unravel the genetic architecture of agriculturally important traits (McMullen et al., 2009). However, the NAM design lacked intercrossing among non-B73 parents, which may lose the power to detect the QTLs between families. The imbalanced parental composition may reduce the GWAS efficiency for rare multi-allelic variants.

In contrast to NAM, the multi-parent advanced generation intercross (MAGIC) population provides a better solution of thorough intercross and balanced founder composition. The maize MAGIC population was created by intercrossing eight diverse parental lines over multiple rounds, resulting in a balanced parent composition and abundant recombination events. It provides a useful resource for maize genetic research and breeding (Dell'Acqua et al., 2015).

The random-open-parent association mapping (ROAM) population is a flexible multi-parent design in maize. The maize ROAM population is derived from 14 diverse parental lines that were randomly intercrossed with each other to develop 10 interconnected RIL populations. Compared with the NAM and MAGIC designs, the ROAM design does not rely on intercrossing between specific parents, hence enabling flexible integration of new populations into currently existing resources whenever necessary (Xiao et al., 2016b).

Jianbing Yan's group recently reported a new population design in maize for thoroughly exploring breeding resources and identifying functional genes and favorable alleles directly for breeding (Liu et al., 2020d). This population was termed as the CUBIC population, which was derived from 24 elite Chinese maize inbred lines. The 24 founders were crossed under the complete diallel cross (CDC) mating design. Thirty F_1s with excellent agronomic performance were selected for further crossing in CDC for favorable allele enrichment. Another set of random F_1s was selected for open pollination in isolation to maintain the genetic diversity. After 6 generations of intercrossings with open pollination in isolation and followed by 6 generations of self-pollinations by single-seed descent, the CUBIC population consisted of 1,404 recombinant inbred lines was constructed. This large-scale design provides new potential for gene identification of agronomic traits (Xiao et al., 2021) and genomic breeding (Cheng et al., 2022; Yan et al., 2021a; Yang et al., 2022d).

Sequence-indexed insertional libraries in maize are important toolkits for identifying genes based on phenotypes (forward genetics) or assigning phenotypes to genes (reverse genetics). Many critical genes controlling plant development, signaling response, and tolerance to biotic/abiotic stresses have been isolated and characterized using mutants. In the last five years, with the help of next-generation sequencing technologies, multiple maize mutant sequence-indexed resources have been developed using various mutagenesis methods, including EMS mutant library (MEMD) (Lu et al., 2018), Mu insertion mutant library (ChinaMu Project) (Liang et al., 2024a; Liang et al., 2019a), and Dissociation insertional mutant library (MEILAM database) (Lyu et al., 2021). The MEMD gene-index library currently contains 746,607 point mutations that are tagged to 36,015 genes, approximately 91% of the annotated maize protein-encoding genes (http://elabcaas.cn/memd/public/index.html#/). The ChinaMu sequence-indexed library contains 108,994 high-quality germinal *Mu* insertions that are tagged to 27,957 genes, covering about 63% of the annotated maize genes (http://chinamu.jaas.ac.cn/). Compared with UniformMu (16,090 genes) (McCarty et al., 2013; McCarty et al., 2005) and BonnMu (16,392 genes) (Marcon et al., 2020), the number of genes tagged by ChinaMu was about twice that of the mutant library of the counterparts. The MEILAM Mutant database contains 70,600 *Ds* insertion sites that cover 16,048 annotated genes, accounting for about 36% of the total genes in the maize genome (http://www.maizetepolymorphism.com/AcDs/). These libraries have almost saturated coverage of annotated maize genes, offering essential resources for further genetic, biochemical, and molecular analysis of genes influencing critical agronomic traits.

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Perspectives on GWAS in maize

The ultimate purpose of GWAS is the identification of functional genes and favorable alleles for maize breeding. The innovations of statistical methods and mapping populations can surely promote our understanding of genetic basis of breeding-relevant complex traits. The deeper insight for these important traits will positively feed back and accelerate the breeding process.

In the era of omics, all layers of omics data can be rapidly collected. The transcriptomic, metabolic data has been proven valuable for not only linking the genomic variants to phenotype but also identifying the causative genes and biological regulatory networks responsible for agriculturally important traits (Xie et al., 2024; Zhang et al., 2024b). With the increasing scale and diversity of crop resequencing data, rare variants will become the vital obstacle for future GWAS research in crops. The hybrid of artificial intelligence (AI) algorithm and traditional statistical methods will be the possible direction to further boost the GWAS power (Yin et al., 2020). The GWAS study should also focus on the understanding of the genetics of phenotypic plasticity and genotype-by-environment interaction (GXE) of agriculturally important traits (Jin et al., 2023; Moore et al., 2019). The identification of key genes and the systematic dissection of regulatory networks in genetic and post-genomic layers will be beneficial for designing and breeding the future-suitable varieties in maize.

Yield-related traits in maize

Maize yield is predominantly influenced by three key factors: the number of ears per unit area, the number of kernels per ear, and kernel weight. The number of ears per unit area is mainly determined by planting density, which is largely impacted by the plant architecture traits, including leaf angle, plant height, ear height, tassel size, and root system architecture. Factors such as the number of kernel rows, the number of kernels per row, and ear length determine the total number of kernels per ear. The weight of a kernel is primarily determined by its size and nutritional content. Beyond these key components of maize yield, flowering time is also a pivotal trait that significantly impacts yield performance. This is because flowering time can influence plant architecture through multiple ways, such as modulating leaf number, leaf distribution, plant height, and ear height (Li et al., 2016; Peiffer et al., 2014). Furthermore, flowering time can influence the duration of the grain-filling stage and modulate the impact of various stresses on yield, such as heat and drought (Tardieu et al., 2018; Trachsel et al., 2017; Verrico and Preston, 2025). Beyond these aspects, flowering time also plays an important role in the assimilate accumulation during the vegetative stage, the sink capacity, and the allocation of photosynthates to kernels during the reproductive stage (Wingler and Soualiou, 2025)

Maize ear traits

Ear architecture is one of the key elements affecting grain yield. As classical quantitative traits, ear traits are controlled by numerous genetic loci and are determined during the ear developmental stages (Vollbrecht and Schmidt, 2009). In the past three decades, hundreds of inflorescence-related genes have been characterized mainly from the ear mutant analysis, which showed that these genes could regulate ear development by controlling meristem size and determinacy (Richardson and Hake, 2022). In the recent ten years, the complex quantitative variations of ear traits have started to be systematically and comprehensively studied, resulting in a series of significant achievements from gene analysis to regulatory network unveiling (Du et al., 2022).

Control of ear development: knowledge from the inflorescence mutant analysis

Ear development starts from the proliferation of inflorescence meristem (IM), which is critical for generating the lateral organ primordium, spikelets, and floral meristems, and it is controlled by CLAVATA (CLV)-WUSCHEL (WUS) feedback signaling pathway first discovered in *Arabidopsis* (Wu et al., 2018). The CLV-WUS signaling pathway is proven functionally conserved across plant species, with major regulators cloned from *fasciated ear* mutants in maize (Brand et al., 2000; Clark et al., 1997; Fletcher et al., 1999; Wu et al., 2018). *Bif3*, the maize *WUS* ortholog, encodes a homeodomain transcription factor and is expressed in the meristem organizing center to coordinate meristem activity (Chen et al., 2021d). *ZmCLAVATA3/EMBRYO SURROUNDING REGION-RELATED7* (*ZmCLE7*) and *ZmFON2-LIKE CLE Protein1* (*ZmFCP1*), the maize *CLV3* orthologs, are activated by Bif3 and perceived by their receptors to repress *Bif3* expression (Je et al., 2016; Rodriguez-Leal et al., 2019). Several major *CLV3* receptors, including the *CLV1* ortholog THICK TASSEL DWARF1 (TD1) (Bommert et al., 2005), the *CLV2* ortholog FASCIATED EAR2 (FEA2) (Bommert et al., 2013b; Taguchi-Shiobara et al., 2001), and a LRR receptor-like protein FASCIATED EAR3 (FEA3) (Je et al., 2016), as well as the downstream effectors, such as COMPACT PLANT2 (CT2) (Bommert et al., 2013a), ZmGB1 (Wu et al., 2020) and CORYNE (Je et al., 2018), were characterized in maize.

In addition to the CLV-WUS pathway, Zhang et al. found that GRF-interacting factor1 (GIF1) could regulate IM determinacy by interacting with GROWTH REGULATING FACTORs (Li et al., 2022g; Zhang et al., 2018a). Yang et al. (2021b) revealed that a set of maize CC-type glutaredoxins (GRX) acted redundantly to modify the redox state of FASCIATED EAR4 (FEA4), a bZIP transcription factor, and moderate FEA4 activity in controlling IM development through a putative auxin signaling pathway. Meanwhile, auxin signaling was confirmed to regulate the early steps required for inflorescence formation by controlling boundary domains during axillary meristem formation (Galli et al., 2015). Furthermore, studies using mutants (i.e., *Silky3* and *Tasselseed5*) with defects in floral sex determination revealed an essential role of jasmonic acid in floral development (Lunde et al., 2019; Luo et al., 2020a; Wang et al., 2020a). These mutants typically produce a disorganized and small ear, making them hard to be used directly in breeding. However, these studies deepen our knowledge of maize inflorescence development and ear trait formation.

Kernel number determination: from QTL to gene regulatory networks

The genetic dissection of kernel-number-related traits began by identifying QTLs that underlie the natural variations of ear traits, such as kernel row number (KRN) and kernel number per row (KNR) (An et al., 2020b; Liu et al., 2020d; Liu et al., 2015b; Liu et al., 2015c; Xiao et al., 2016b). In the past decade, facilitated by high-quality reference genomes and advancements in genotyping technologies, several kernel-number-related QTLs, such as *KRN1* (Wang et al., 2019c), *KRN2* (Chen et al., 2022d), *KRN4* (Du et al., 2017; Du et al., 2020; Liu et al., 2015c), *KRN5* (Shen et al., 2019), *KRN8* (Han et al., 2020b), *KNR6* (Jia et al., 2020; Li et al., 2021b; Li et al., 2023e), *YIGE1* (Luo et al., 2022), *qEL7* (Ning et al., 2021), and *EAD1* (Pei et al., 2022), have been cloned by QTL fine mapping and GWAS, and deeply characterized for their regulatory mechanisms in maize (Figure 2A). The first cloned KRN QTL, *KRN4*, is located in a 3-kb intergenic region and functions as a distal enhancer of the *Unbranched3* (*UB3*) gene (Du et al., 2017; Du et al., 2020; Liu et al., 2015c). *KRN4* can recruit UB2-centered transcription complexes and fine-tune *UB3* expression by chromatin interactions with the *UB3* promoter (Du et al., 2020). GIF1/GRFs complex was also found to bind the *UB3* promoter and regulate its expression (Li et al., 2022g; Zhang et al., 2018a). *UB2* and *UB3* are duplicate SBP-box transcription factor genes that redundantly control KRN formation by limiting cell differentiation to the lateral domains of IM through cytokinin biosynthesis and signaling pathways (Chuck et al., 2014; Du et al., 2017; Du et al., 2020). Moreover, *UB2*/*UB3* and their close homolog *Tasselsheath4* (*TSH4*) control transcriptional networks of maize inflorescence development under simulated shade and the domestication of maize ears (Dong et al., 2024; Kong et al., 2023). *KRN5* encodes an inositol polyphosphate 5-phosphatase and moderates kernel row number formation through a novel phosphoinositide signaling pathway (Shen et al., 2024). The serine/threonine protein kinase encoding gene *KNR6*, the first cloned QTL controlling ear length and KNR, was found to interact with and phosphorylate an Arf GTPase-activating protein (AGAP), thereby regulating auxin flux in IM (Jia et al., 2020; Li et al., 2021b; Li et al., 2023e). *YIGE1*, another ear-length QTL encoding an unknown protein, is predicted to be involved in sugar and auxin signal pathways to regulate ear elongation (Luo et al., 2022). *qEL7* functions as an ethylene biosynthesis enzyme and serves as a key signal to control spikelet formation and floral fertility, which reveals a novel ethylene signaling that affects kernel number (Ning et al., 2021). An aluminum-activated malate transporter, EAD1, plays an essential role in regulating maize ear development by altering malate delivery to developing ear tips (Pei et al., 2022). Together, the characterization of these ear traits QTLs addresses how kernel number is genetically fine-controlled by complex gene regulatory networks.

Ear trait improvement: from selection to knowledge-driven engineering

Hundreds of QTLs that control complex ear traits have been identified (An et al., 2020b; Liu et al., 2020d; Liu et al., 2015b; Xiao et al., 2016b). Most of these QTLs have a minor effect, and some show a considerable effect but only in certain genetic backgrounds. These QTLs can be identified because their beneficial alleles arise naturally and are maintained in the modern maize population during domestication and improvement. For example, *KRN2* was found as a key domestication gene controlling maize kernel row number, and it experienced convergent selection in both maize and rice (Chen et al., 2022d). These convergently selected genes provide an excellent target for future crop improvement (Chen et al., 2022d). Moreover, some core regulators of ear development, such as *ZmCLE7* and *ZmFCP1*, are not linked to ear trait QTLs, suggesting that some potential yield genes may be difficult to identify in natural populations due to the absence of beneficial natural alleles (Liu et al., 2021c). A knowledge-driven genome editing study targeted these potential yield genes and created novel beneficial alleles with significant effects on kernel number, which suggested that knowledge-driven breeding by design is an important alteration for ear trait manipulation (Liu et al., 2021c). Therefore, with a rapid accumulation of knowledge about ear traits, from gene cloning to a developmental regulation atlas, knowledge-driven engineering promises a new era of ear trait improvement practice.

Kernel traits

Maize seed development initiates from double fertilization, where a sperm cell fertilizes the egg to form a diploid zygote, and the other sperm cell fertilizes two fused polar central cells to form a triploid cell. The zygote undertakes a complex network of developmental programs to form a mature embryo, whereas the triploid central cell develops into an endosperm. Understanding the complex genetic network governing seed development is a challenging task, as it involves numerous genes in many biological processes. Genetic analysis prioritizes the genes that give rise to visible seed phenotypes when mutagenized. These genes are defined as key genes for seed development. It was estimated that there are ~800 key seed development genes in maize (McCarty et al., 2005). Major classes of seed mutants are described as *defective kernels* (*dek*), *empty pericarp* (*emp*), *embryo defective* (*emb*), *small kernels* (*smk*), *rough endosperm* (*rgh*), *shrunken*/*sugary*, and *opaque* (McCarty et al., 2005). Researchers used other names to highlight features of the mutants. Nonetheless, this reflects the impact on the development of the embryo, endosperm, and other specific tissues. Facilitated by the generation of several mutagenesis populations in maize (Liang et al., 2019a; Lu et al., 2018; Marcon et al., 2020; McCarty et al., 2005), scientists have made tremendous progress in dissecting these seed genes during the last decade (Figure 2B).

Organellar RNA editing

Mitochondria and plastids are derived from endosymbiosis; hence, their genome structure and gene expression are similar to those in bacteria. One unique feature of their gene expression in higher plants is RNA editing, which is largely C-to-U editing. Higher plant mitochondria contain over 500 editing sites, while chloroplasts contain approximately 30 editing sites. Editing deficiency at key sites affects the function of the encoded proteins, thereby impacting plant growth, development, and environmental adaptation and even causing embryonic lethality (Barkan and Small, 2014; Dai et al., 2021).

Many of the genes affecting kernel development have been found to be involved in RNA editing, including PPR. PPR proteins are classified as P-class and PLS-class; the latter is further divided into PPR-E, PPR-E+, and PPR-DYW subclasses based on their C-terminus domain. Over the last decade, the functions of several dozen PPR proteins have been identified in maize (Tables S1 and S2). In general, it has been found that P-type PPR proteins are mostly involved in intron splicing and RNA maturation. PLS-type PPR proteins are generally involved in RNA C-to-U editing, whereas atypical PPR proteins tend to have a more important function. Consistent with the number of editing sites, many PPR-PLS proteins are targeted to mitochondria. Some function on the editing at one or a few sites (Li et al., 2014; Li et al., 2019j; Liu et

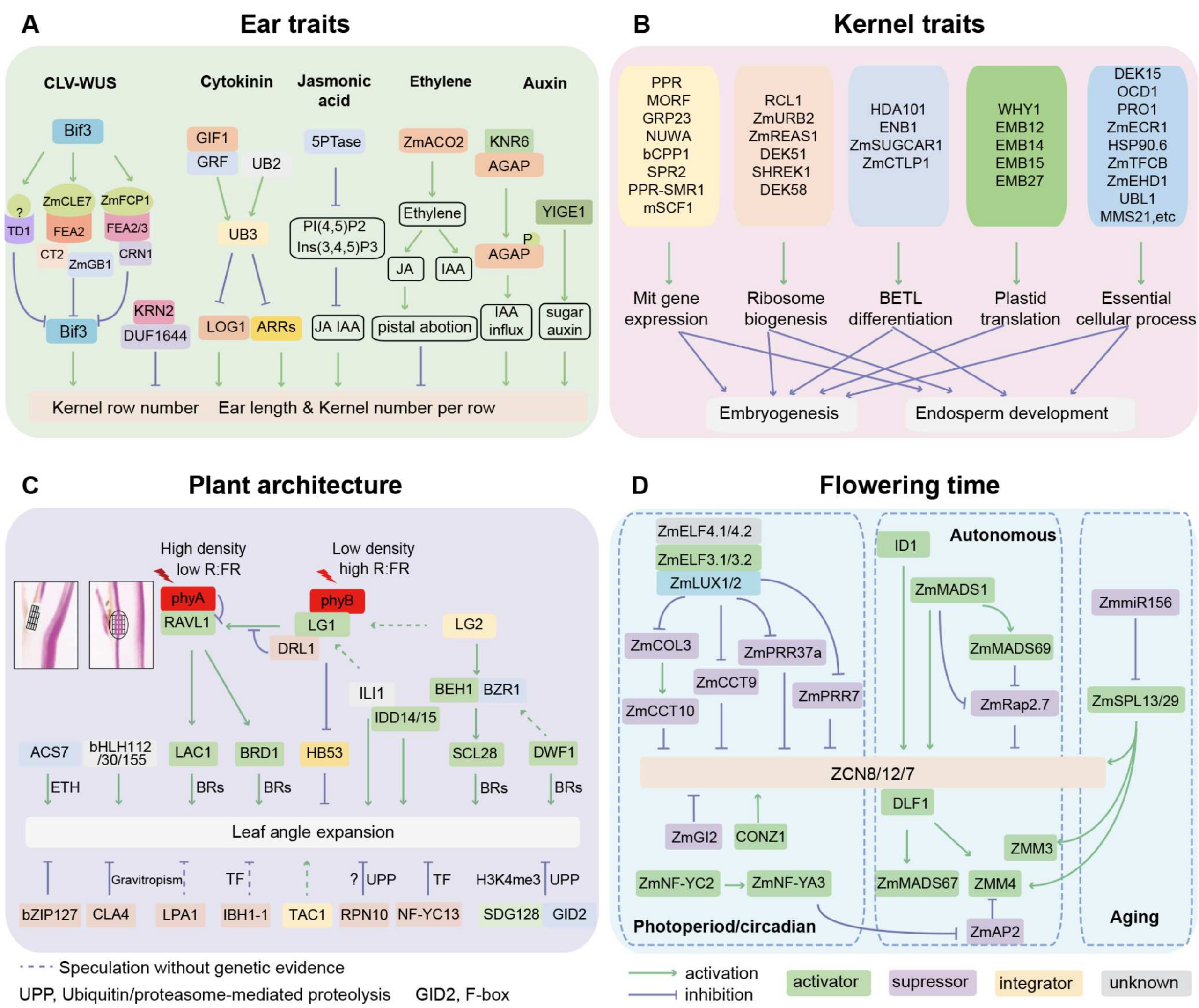

Figure 2. The regulatory models of maize yield-related traits. A, Gene regulatory networks controlling ear traits in maize. B, Gene regulatory networks controlling kernel traits in maize. C, Ideal plant architecture for high-density planting in maize and the underlying genes that have been identified. D, Gene regulatory networks of flowering time in maize. The maize florigen ZCN8 acts as an integrator of the regulatory network and is regulated by multiple flowering genes. Protein interactions are presented as stacked rectangles. The regulatory relationships between genes are indicated by different types of arrows.

al., 2013b; Sun et al., 2015; Sun et al., 2018a; Wang et al., 2017b; Xiu et al., 2016; Yang et al., 2017c; Zang et al., 2024), while a few function on the editing at many sites (Wang et al., 2023i; Wang et al., 2019e; Yang et al., 2022f). Based on the impact on the encoded protein, editing deficiency at mitochondrial transcript sites produces phenotypes ranging from very minor to embryo lethality, reflecting the impact on the mitochondrial function and the requirement of mitochondria for embryogenesis and endosperm development.

RNA editing in chloroplasts is mostly studied in chloroplast mutants such as the yellow or albino leaf mutants. Some mutants also display seed phenotypes. The maize QTL locus, *qKW9*, encodes a PPR-DYW protein that is required for the C-to-U editing at the *ndhB* transcript. Thus, the mutation affects photosynthesis and grain filling (Huang et al., 2020). ZmNUWA, ZmDYW2A, and ZmDYW2B are involved in the editing at several sites in plastids, and loss of function in these genes causes a severe *emp* phenotype in maize (Wang et al., 2024d).

Mitochondrial intron splicing

Intron splicing in plant mitochondria requires a large number of nuclear coding splicing factors (de Longevialle et al., 2010). The mitochondrial genome contains a total of 23 group II introns in maize. Among these, there are 17 introns requiring *cis*-splicing and 6 introns requiring *trans*-splicing (Clifton et al., 2004). Most of these introns reside in the transcripts coding for components of mitochondrial complex I, such as *nad1*, *nad2*, *nad4*, and *nad7*, while a few are in the *ccmFc*, *rps3*, and *cox2* transcripts.

P-type PPR proteins were found to mediate the *cis*- and *trans*-splicing of introns. Loss of function of these PPR genes impairs intron splicing, hence the expression of a functional protein, causing mitochondrial dysfunction. The mutant often exhibits a defective kernel phenotype, including impaired embryogenesis and endosperm development.

Some PPR proteins are involved in the splicing of one intron, whereas other PPR proteins are required for the splicing of multiple introns in maize mitochondria (Table S2). PPR-SMR1 and small PPR protein 2 (SPR2) have been found to participate in the splicing of most introns (Cao et al., 2022a; Chen et al., 2019b). PPR-SMR1 is a special PPR protein containing a small MutS-related (SMR) domain, which is required for the splicing of nearly 75% of the mitochondrial group II introns. PPR-SMR1 interacts with the CRM-like RNA-binding protein mCSF1, jointly participating in intron splicing (Chen et al., 2019b). SPR2, composed of only four PPR motifs, mediates the splicing of 15 introns in *nad1*, *nad2*, *nad4*, *nad5*, and *nad7* (Cao et al., 2022a). These studies suggest that SPR2 and PPR-SMR1 are general factors in mitochondrial intron splicing.

Plastid translation

Analysis of the embryo-defective mutants in maize uncovered that plastid translation is essential to maize embryogenesis. However, the molecular mechanism remains obscure. The *whirly* 1 (*why1*), *emb12*, *emb14*, and *emb27* are all impaired in plastid translation. Why1, EMB14, and EMB15 function in plastid ribosome biogenesis (Li et al., 2015b; Xu et al., 2021a; Zhang et al., 2013). *Emb12* encodes plastid translation initiation factor 3 (Shen et al., 2013). *Emb27* encodes the plastid ribosomal protein S13 (Liu et al., 2024c). All these mutants show genetic background dependence. In the W22 genetic background, loss of function of these genes arrests embryogenesis, whereas in other genetic backgrounds such as B73 or Mo17, seeds develop normally, but the seedlings show *albino* leaves (Li et al., 2015b; Shen et al., 2013; Xu et al., 2021a; Zhang et al., 2013). This implies the presence of a suppressor controlling the requirement of plastid translation for embryogenesis in different genomes of maize inbreds. Analysis of *emb27* revealed that defects in plastid translation also affect the splicing of several introns in *atpF*, *rpl2*, *rps12-i2*, and *ycf3-i2* (Liu et al., 2024c).

Ribosome biogenesis

Recent studies identified the functions of several ribosomal biogenesis factors (RBFs) and their critical role in maize seed development. ZmReas1, a homolog of the yeast AAA-ATPase protein Reas1, functions in the maturation and export of the maize ribosomal 60S subunit. Loss of ZmReas1 arrests maize seed development, causing embryo lethality (Qi et al., 2016b). Impaired ribosome biogenesis triggers changes in differentially transcribed genes and translated RNAs, including translational suppression of cell division and growth-related genes. *ZmUrb2* encodes a Urb2 domain-containing protein and is required for ribosome biogenesis by affecting pre-rRNA processing (Wang et al., 2018a). Mutations in *ZmURB2* cause small viable kernels. Interestingly, natural variations in *ZmURB2* were found to be associated with maize kernel length (Wang et al., 2018a). Maize mutant kernels deficient in RCL1, an RNA 3′-terminal phosphate cyclase-like protein that functions in the maturation of 18S rRNA, exhibited a small and shrunken appearance along with abnormal development of embryo and endosperm (Wang et al., 2022e). *DEK58* encodes a protein homologous to yeast Rrp15 and is necessary for the accurate and efficient processing of pre-rRNA at early stages. Loss of *DEK58* produces an aberrant processing product, P-25S*, and mainly influences 60S ribosomal subunit synthesis. Null mutation of *DEK58* arrests embryogenesis and endosperm development, causing embryo lethality (Ma et al., 2024). In addition to cytoplasmic RBFs, mitochondrial ribosomal proteins (mRPs) are also critical for maize seed development. Both *DEK44* and *MN** encode mitochondrial ribosomal proteins (mRPL9 and mRPL10, respectively). Loss of either gene disrupts mitochondrial protein expression and further impairs mitochondrial function, ultimately leading to embryo-lethal phenotypes with small kernels (Feng et al., 2022c; Qi et al., 2019)

Basal endosperm transfer layer development

The basal endosperm transfer layer (BETL) functions as a nutrient transporter, anti-microbial barrier, and signal mediator between filial and maternal tissues (Offler et al., 2003). As such, the development of BETL is essential for seed development.

ENB1 encodes a cellulose synthase 5 that is predominantly expressed in BETL (Wang et al., 2022c). Loss of *ENB1* reduces flange cell wall ingrowths of the BETL and impairs seed development, particularly endosperm development. While overexpression of *ENB1* increases BETL cell wall ingrowth, suggesting an important role of ENB1 in BETL cell wall ingrowth (Wang et al., 2022c). ZmSUGCAR1 plays an important role in sugar transport from maternal tissues to filial seed tissues in the BETL (Yang et al., 2022a). ZmSUGCAR1 is specifically expressed in the BETL and is a major sugar transporter in BETL. Loss of *ZmSUGCAR1* causes a defective kernel phenotype (Yang et al., 2022a). *ZmCTLP1* encodes the choline transporter-like protein 1 located in the *trans*-Golgi network. A point mutation of *ZmCTLP1* renders impaired BETL cell wall ingrowth and small kernels (Hu et al., 2021a), suggesting that lipid homeostasis affects BETL and seed development. HDA101, a maize histone deacetylase that removes the acetyl group from lysine residues in histone, regulates the expression of BETL-specific genes (Yang et al., 2016a). The *hda101* mutant displays a small kernel phenotype with defects in the BETL region and down-regulated BETL-specific gene expression. This study shows that BETL development is regulated at the chromatin level.

Other mechanisms

Besides the above, many other mechanisms involving diverse biological processes affect seed development. This includes genes involved in cell division (He et al., 2019; Huang et al., 2019), protein synthesis (Huang et al., 2024b), protein degradation (Chen et al., 2021b; Wang et al., 2019b; Xu et al., 2023), cell redox homeostasis (Zhang et al., 2021d), tubulin folding (Zhou et al., 2023a), riboflavin biosynthesis (Dai et al., 2019), alternative pre-mRNA splicing (Bai et al., 2019; Zuo et al., 2019), RNA m^6A modification (Luo et al., 2023a), auxin homeostasis (Wang et al., 2020e), metal transport (He et al., 2021; Zang et al., 2020), vitamin B6 biosynthesis (Yang et al., 2017d), biogenesis of small noncoding RNAs (Li et al., 2017c; Zhang et al., 2022c; Zhao et al., 2020), post-translational addition of SUMO to target proteins (Zhang et al., 2021c), and other processes (Chen et al., 2023c; Li et al., 2022j). The identification of additional genes in maize, combined with comparative genomic insights from other crops (Bai et al., 2024; Zhou et al., 2024), will shed light on the regulatory networks underlying maize kernel development.

Plant architecture

Ideal plant architecture for high-density planting

Over the past few decades, maize yields have continuously increased, and much of the increase is achieved by increased plant densities. In the 2030s, a 52% yield improvement is estimated through dense planting and soil improvement (Luo et

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al., 2023b). Currently, in the northwest maize growing region of China, the maximum planting densities for some maize varieties (e.g., MC670, Denghai 1769, MY73) can be reached ~1.35×10^5–1.5×10^5 ha^{-1}. Plant architecture is an important agronomic trait affecting maize planting density and grain yield. Ideal plant architecture is also known as smart canopy architecture, which is genetically selected or designed for high-density planting. Although the concept of maize ideal plant architecture was originally proposed in 1975 and widely used in modern maize breeding and production, the underlying mechanism remains largely unknown. In recent years, it is widely accepted that an ideal plant architecture suitable for high-density planting of maize should at least include: (i) more upright leaves above the primary ear (LAE), larger leaf angles (LA) at the primary ear position and below, and less tassel branch number, thus to allow better light capture and higher canopy photosynthetic capacity; (ii) optimal plant and ear heights, and a strong culm, thus to increase stalk lodging resistance and facilitate mechanical harvest; (iii) improved root system to enhance root lodging resistance and increase the uptake of nutrient and water (Jafari et al., 2024; Tian et al., 2024a; Wang et al., 2020a; Wang et al., 2022f; Wei et al., 2018a). Identifying and fine-tuning the expression of genes related to ideal plant architecture will foster the advancement of smart breeding and smart agriculture (Zhou and Li, 2025).

Narrowing leaf angle tailored for high-density planting

Leaf angle (LA), an important component of maize plant architecture, directly governs compactness and planting densities, making it an important target for genetic improvement in high-density planting (Cao et al., 2022b; Tian et al., 2019). The variation of maize LA is mainly affected by the development of the ligular regions, which are composed of the ligule, auricles, and the central connecting tissue (ligular median regions). Recent studies revealed that the variation in lignin deposition, asymmetric cell division, and elongation at the adaxial side of the ligular region play critical roles in regulating leaf angle formation (Cao et al., 2022b; Kong et al., 2017; Wang et al., 2024b).

In recent years, multiple key regulators of maize LA have been identified by GWAS, QTL cloning, and reverse genetics analyses, including *ZmILI1* (*qLA2*), *ZmIBH1-1* (*qLA2-1*), *ZmbHLH30*, *ZmbHLH155*, *ZmCLA4* (*qLA4-1*), *ZmTAC1*, *ZmACS7*, *ZmbZIP127*, *ZmBEH1*, *ZmBZR1*, *ZmSCL28*, *Upright Plant Architecture1* (*UPA1*), *UPA2*, *lac1*, *ZmHB53*, *ZmIDD14* and *ZmIDD15* (Figure 2C) (Chen et al., 2024; Dou et al., 2021; Duan et al., 2022b; Kong et al., 2017; Liu et al., 2024a; Shi et al., 2019; Shi et al., 2024; Tian et al., 2011; Tian et al., 2024a; Tian et al., 2019; Wang et al., 2024b; Zhang et al., 2014a). The *ZmILI1* (*Increased Leaf Inclination1*), *ZmbHLH30*, *ZmbHLH155*, and *ZmIBH1-1* encode atypical and typical bHLH transcription factors, respectively, regulating leaf angle by directly affecting the transcription of diverse targets mainly related to the cell expansion (e.g., *EXPs*), cell wall biogenesis, and hormone pathways (Cao et al., 2020a; Ren et al., 2020; Wang et al., 2024b). The *ZmCLA4* is an ortholog of *LAZY1* in rice and has been identified as a transcriptional repressor regulating LA through affecting multiple hormones (auxin, BR, ABA, JA, and ethylene) signaling pathways (Dou et al., 2021; Zhang et al., 2014a). The ZmACS7 (ACC synthase 7) catalyzes ethylene biosynthesis, and its increased expression or protein stability results in a greater leaf angle and reduced plant height (Li et al., 2020d). The bZIP transcription factor ZmbZIP27 negatively regulates nitrogen-mediated leaf angle size by regulating the expression of ZmmiR528 and modulating lignin deposition in ligular regions (Chen et al., 2024). *ZmBZR1* (*Brassinazole Resistant1*) and *ZmBEH1* (*BZR1/BES homolog gene1*) are targeted by LG2 and subsequently directly activate the expression of *ZmSCL28* (*Scarecrow-Like 28*) to positively regulate leaf angle. *UPA1* and *UPA2* are two LA QTLs identified from a maize-teosinte recombinant inbred lines population. *UPA1* encodes a brassinosteroid C-6 oxidase (*brd1*) that directly catalyzes the final steps in brassinosteroid synthesis and promotes leaf angle (Tian et al., 2019). *UPA2* has been identified as a 2-bp sequence polymorphism in the distant regulatory region of *ZmRAVL1* that directly activates the transcription of *brd1*, thus affecting leaf angle. Interestingly, *UPA2* affects differential binding by DRL1 (DROOPING LEAF1, a YABBY transcription factor), which physically interacts with LG1 and subsequently represses its transcriptional activation of *ZmRAVL1* (Tian et al., 2019). Importantly, introgressing the wild *UPA2* allele into modern hybrids or directly editing *ZmRAVL1* significantly increased maize yield under high-density conditions, indicating that these genes are the potential targets for genetic improvement desired for high-density planting (Tian et al., 2019). Notably, ZmRAVL1 directly regulates the expression of *lac1*, which encodes a cytochrome P450 that catalyzes C-22 hydroxylation and predominantly regulates upper leaf angle through affecting the brassinosteroid levels (Tian et al., 2024a). ZmHB53, a homeodomain-leucine zipper II (HD-ZIP II) transcription factor, acts as a target of LG1 to regulate leaf angle by directly controlling the elongation and division of ligular adaxial and abaxial cells (Shi et al., 2024). Moreover, two paralogous INDETERMINATE DOMAIN (IDD) genes, *ZmIDD14* and *ZmIDD15*, function redundantly to promote auricle development and leaf angle by interacting with ZmILI1 (Liu et al., 2024a).

Reducing lodging under high-density conditions

Increasing planting density is one of the most effective strategies to increase maize yield, while lodging is the major factor limiting yield increase under high-density planting conditions (Xue et al., 2017). Recent studies have revealed that increasing the strength of stalks, altering the angle of brace roots, and reducing the plant and ear heights can all significantly enhance lodging resistance (Shah et al., 2021; Sun et al., 2018b; Wang et al., 2020c; Wu et al., 2022; Xue et al., 2017; Zhang et al., 2020f; Zheng et al., 2023). The *stiff1* gene encodes an F-box domain protein and is a major QTL regulating stalk strength. Editing *stiff1* resulted in stronger stalks and reduced stalk lodging (Zhang et al., 2020f). MicroRNA528 is a monocot-specific miRNA induced by N luxury and affects lodging resistance by negatively regulating the transcript abundance of laccases (*ZmLAC3* and *ZmLAC5*) (Sun et al., 2018b). A recent study revealed that loss of function of *ZmYUC2* and *ZmYUC4*, two YUCCA encoding genes that are preferentially expressed in the root tip, caused enlarged angles of brace root and increased root lodging resistance under high-density planting conditions (Zheng et al., 2023).

For the regulation of plant and ear heights, multiple key regulators involved in auxin, GA, BR, JA, and ethylene biosynthesis or signaling pathways have been identified in the past decades. Among them, *Brachytic2* (*Br2*, also known as *qph1*, *qpa1*) is a major QTL for plant height and has great potential for maize high-density planting (Wei et al., 2018b; Xing et al., 2015;

Zhang et al., 2019e). *Br2* encodes an ATP-binding cassette type B (ABCB) and functions in auxin efflux out of the meristematic region in the shoot, thus affecting plant and ear heights (Knöller et al., 2010; Landoni et al., 2022; Li et al., 2020b; Wei et al., 2018b; Xing et al., 2015; Zhang et al., 2019e; Zhao et al., 2023a). It is well known that GA plays an essential role in plant height regulation, therefore, disrupting or suppressing genes involved in GA biosynthesis (e.g., *GA3ox2*, *GA20ox3*, *GA20ox5*) or signaling pathways by using CRISPR/Cas9 generated semi-dwarf maize plants with significantly reduced plant and ear heights (Paciorek et al., 2022; Zhang et al., 2020d). Meanwhile, the overexpression of transcription factor *ZmSPL12* further represses the transcription of *GA3ox2*, thus leading to decreased plant height and enhanced lodging resistance (Zhao et al., 2022a). A recent study suggests that JA levels affect plant height in maize (Li et al., 2024a). Although *GRF-interacting factor1* (*GIF1*) has been identified as a key regulator of shoot architecture and meristem determinacy, its null alleles exhibit very short internodes and fascinating inflorescences and thus failed to be directly used in maize breeding (Zhang et al., 2018a). *ZmRPH1* (*Reducing Plant Height 1*) encodes a microtubule-associated protein (MAP) and its overexpression lines exhibit lowered plant and ear heights and increased lodging resistance, indicating that modulation of microtubules to repress cell elongation and plant height is a potential approach in breeding cultivars for high-density planting without reducing maize yield (Li et al., 2020g). In addition, a recent study integrating high-throughput phenotyping and GWAS revealed 13 hub genes (e.g., *ZmVATE*, *ZmTBL10*, *ZmPHD1*) that might play crucial roles during rapid growth and might be used for desired plant height for high-density planting (Wang et al., 2023g).

Increasing tolerance to high-density planting

It is worth noting that under high-density planting conditions, a reduced red to far-red light ratio (R:FR) or actual shading signals trigger a series of morphological and physiological adaptive responses, including promoted stem/stalk elongation, decreased mechanical stalk strength, reduced branching and decreased LA, collectively known as shade avoidance syndrome (SAS) (Shi et al., 2019; Wang et al., 2016b). Recent studies have revealed that multiple maize photoreceptors, including phytochrome A (phyA1/2), phyB1/2, phyC1/2, cryptochrome 1b, phytochrome-interacting factors (PIFs, including PIF3s, PIF4s, PIF5s), and other plant architecture-regulating genes (*ZmLAC1*, *ZmHB53*, etc.) play a crucial role in maize density tolerance by regulating shade avoidance responses. ZmPhyA accumulates in high-density shading conditions and interacts with a B3 domain transcription factor ZmRAVL1 to promote its degradation, thereby inhibiting its activation on *lac1* and *brd1*/*UPA1* and consequently decreasing leaf angle (Tian et al., 2024a; Tian et al., 2019). Disrupting the expression of maize *PIFs* or continuous expression of hyperactive variants of *phyB* efficiently attenuated shade avoidance responses and had greater potential to increase plant tolerance to high-density planting (Dubois et al., 2010; Li et al., 2020f; Sheehan et al., 2007; Shi et al., 2017b; Wu et al., 2019a; Zhao et al., 2022d). Maize PhyB physically interacts with the LG1 and significantly enhances its stability under high R:FR light (low-density), while promoting its degradation under low R:FR light (high-density) conditions. *ZmHB53* is one of the direct targets of LG1 involved in the regulation of leaf angle, plant height, and density tolerance (Shi et al., 2024). Beyond phyA and phyB, CRY1 has also been shown to regulate density tolerance as the overexpression of maize *CYR1b* significantly reduced plant height and increased the resistance of lodging under high-density conditions (Chen et al., 2023e). In addition, three SBP-box transcription factors UB2/UB3/TSH4 have been identified as crucial regulators mediating tillering, early tassel branching, and grain yield traits in response to developmental signals and simulated shade (Chuck et al., 2014; Kong et al., 2023; Liu et al., 2021e; Xu et al., 2017a). Thus, integrating these useful molecular and genetic resources related to shade responses will be a powerful tool to accelerate the breeding of maize varieties with ideal plant architecture and enhanced tolerance to high-density planting.

Flowering time

Overall genetic architecture of maize flowering time

Flowering time is an important agronomic and adaptive trait that affects plant adaptation to different geographical regions and cropping systems. Maize landraces exhibit wide natural variation in days to flowering, ranging from 30 days to more than four months (Colasanti and Muszynski, 2009). To dissect the genetic architecture of maize flowering time, numerous QTL mapping studies have been conducted using various bi-parental linkage populations (Chardon et al., 2004; Xu et al., 2012). Due to the limited mapping resolution and allelic diversity of bi-parental linkage populations, natural populations that harbor multiple alleles at a given locus and have accumulated abundant historical recombinations were widely employed to dissect trait variation in plants. GWAS for flowering time has been performed using natural populations containing different sources of maize accessions (Bouchet et al., 2013; Romero Navarro et al., 2017; Yang et al., 2014). However, natural populations are underpowered in detecting low-frequency causal alleles and are less effective for traits that are highly correlated with population structure (e.g., flowering time) (Auer and Lettre, 2015; Liang et al., 2021b). To exploit the complementary advantages of linkage populations and association panels, a variety of multi-parent linkage populations have been constructed in maize, and joint linkage association mapping for flowering time has been conducted using these populations. Using the NAM population, Buckler et al. (2009) performed a large-scale genetic mapping for flowering time and found that maize flowering time was dominated by numerous small additive QTLs with few genetic or environmental interactions. Subsequently, the MAGIC (Dell'Acqua et al., 2015) and CUBIC (Liu et al., 2020d) populations were developed and used to explore the genetic basis of maize flowering time. Similar to the conclusions from the NAM population, most flowering time loci identified in MAGIC and CUBIC populations exhibit small allelic effects and little epistatic interactions (Dell'Acqua et al., 2015; Liu et al., 2020d).

Increasing evidence shows that maize, a typical open-pollinated monoecious crop, appears to possess distinct genetic architecture features in flowering time variation compared with the self-fertilization crops, such as barley, rice, and soybean (Liang et al., 2021b). Genetic mapping studies in barley, rice, and soybean have shown that the flowering time variation in these crops tends to be controlled by several major loci plus many minor loci with widespread epistasis and genotype by environment interactions (Beche et al., 2020; Hori et al., 2015; Huang et al., 2012; Kong et al., 2018; Li et al., 2019g; Li et al., 2017e; Lu et al., 2016; Mao et al., 2017; Zhang et al., 2015a). In general, the flowering time QTLs mapped in these selfing crop species tend to have larger additive effects than those detected in maize (Liang et al., 2021b). For example, *ZmCCT10* is the most significant flowering time QTL mapped in the maize NAM population, which confers only ~3 days difference in flowering time (Buckler et al., 2009; Ducrocq et al., 2009; Hung et al., 2012), while the additive allelic effects of its rice homolog *Ghd7* and wheat homolog *VRN2* are over 20 days (Dubcovsky et al., 1998; Xue et al., 2008). Due to the much smaller additive effects, until now, only several maize flowering time QTLs have been cloned, including the flowering time repressors *ZmRap2.7* (Salvi et al., 2007), *ZmCCT10* (Hung et al., 2012; Yang et al., 2013), *ZmCCT9* (Huang et al., 2018), and flowering activators *ZmMADS69* (Liang et al., 2019b), *Zea mays CENTRORADIALIS 8* (*ZCN8*) (Guo et al., 2018), *delayed flowering1* (*dlf1*) (Sun et al., 2020a), *ZmNF-YC2* (Su et al., 2021), *constans of Zea mays1* (*conz1*) (Wu et al., 2023), *ZmCOL3* (Jin et al., 2018), and *ZmELF3.1* (Zhao et al., 2023b).

Regulatory mechanisms of maize flowering time

Besides QTL cloning, many other flowering time genes have been identified via mutant cloning, comparative genomic analyses, and other reverse genetics methods, such as *ZMM4* (Danilevskaya et al., 2008) and *ZmMADS1* (Alter et al., 2016). Among the cloned flowering time genes, the maize florigen gene *ZCN8* functions as a hub in the maize flowering time regulatory network (Lazakis et al., 2011; Meng et al., 2011). *ZCN8* is transcribed and translated in mature leaves and then moves to the shoot apical meristem, where it interacts with the bZIP transcription factor DLF1 to form the florigen activation complex (FAC) that directly activates the inflorescence identity genes *ZMM4*/*ZmMADS4* and *ZmMADS67* to promote inflorescence development (Muszynski et al., 2006; Sun et al., 2020a). Notably, most of the currently cloned maize flowering time genes regulate flowering through controlling *ZCN8* expression (Figure 2D).

ZmCCT10, *ZmCCT9*, *ZmCOL3*, *conz1*, *ZmNF-YC2*, and *ZmNF-YA3* play important roles in mediating maize photoperiodic flowering (Huang et al., 2018; Hung et al., 2012; Jin et al., 2018; Su et al., 2018; Su et al., 2021; Wu et al., 2023; Yang et al., 2013). *ZmCCT10* and *ZmCCT9* are homologs of the rice photoperiod response regulator *Ghd7* and encode CMF-type CCT transcription factors (Li and Xu, 2017). *ZmCCT10* and *ZmCCT9* function as long-day (LD)-dependent flowering repressors by down-regulating *ZCN8* expression to delay flowering under LD conditions (Huang et al., 2018; Hung et al., 2012; Yang et al., 2013). Interestingly, a LINE/L1 TE located within *ZmCCT10* intron has been identified to repress *ZmCCT10* expression, thereby promoting flowering specifically under short-day (SD) conditions (Zhong et al., 2021). Candidate gene association analysis identified another LD-specific flowering repressor *ZmCOL3*, which encodes a COL-type CCT transcription factor and inhibits flowering by activating *ZmCCT10* expression under LD conditions (Jin et al., 2018). *Zea mays* GIGANTEA2 (ZmGI2) is an ortholog of the circadian clock gene GI in *Arabidopsis* and binds to the *ZCN8* promoter to suppress its expression, thereby delaying flowering under LD (Li et al., 2023h). *ZmNF-YC2* and *ZmNF-YA3* are LD-specific flowering promoters. ZmNF-YC2 binds to the *ZmNF-YA3* promoter to increase *ZmNF-YA3* expression, which in turn enhances the expression of flowering activator *ZCN26* and reduces the expression of flowering repressor *ZmAP2*, consequently up-regulating *ZMM4* expression to promote flowering (Su et al., 2018; Su et al., 2021). The circadian genes *ZmELF3.1/3.2* and *ZmLUX1/2*, components of the evening complex, promote maize flowering time under both LD and SD conditions (Zhao et al., 2023b). ZmELF3.1/3.2 can up-regulate *ZCN8* expression by interacting with ZmELF4.1/4.2 and ZmLUX1/2 to reduce the expression of several flowering repressors, such as *ZmCCT10*, *ZmCCT9*, *ZmCOL3*, *ZmPRR37a*, and *ZmPRR7/73*, thus relieving their suppression on *ZCN8* transcription. The *CONSTANS* (*CO*)-*FLOWERING TIME LOCUS T* (*FT*) module plays a conserved and important role in regulating photoperiodic flowering in plants (Song et al., 2015). The recent study showed that CONZ1, a maize homolog of CO, bound to the *ZCN8* promoter to activate its expression under SD conditions (Wu et al., 2023).

In addition to photoperiod signals, *ZCN8* also receives signals from non-photoperiod pathways (Figure 2D). *id1* encodes a monocotyledon-specific C2H2 transcription factor and acts upstream of *ZCN8* to promote flowering (Colasanti et al., 1998; Meng et al., 2011). The MADS-box transcription factor *ZmMADS69* promotes maize flowering through down-regulating *ZmRap2.7* expression, thereby relieving the repression of *ZmRap2.7* on *ZCN8* (Liang et al., 2019b). *ZmMADS1* directly binds to the promoter of *ZCN8* to activate its expression and promote flowering (Guo et al., 2018). In addtion, the recent study showed that *ZmMADS1* can also regulate *ZCN8* expresion through the *ZmMADS69*-*ZmRap2.7*-*ZCN8* module (Han et al., 2025). Two SPL transcription factors, *ZmSPL13* and *ZmSPL29*, have recently been identified as positive regulators of both juvenile-to-adult vegetative transition and floral transition in maize, which promote flowering through directly activating the expression of *ZCN8* in leaves and *ZMM4* and *ZMM3* in shoot apexes (Yang et al., 2023a). In addition to *ZCN8*, other members of phosphatidylethanolamine binding protein (PEBP) family are also involved in flowering time control. *ZCN7* and *ZCN12* are considered alternative florigens in maize, whose expressions are negatively correlated with flowering time and can be regulated by *ZmCCT10*, *ZmMADS69*, *id1*, and the evening complex (Castelletti et al., 2020; Liang et al., 2019b; Mascheretti et al., 2015; Minow et al., 2018; Zhao et al., 2023b; Zhong et al., 2021). The overexpression of *TFL1*-like genes *ZCN1*, *-2*, *-4*, and *-5* leads to very late flowering. Among them, only ZCN2 could interact with DLF1 and hence ZCN2 might compete with ZCN8 to antagonistically regulate flowering time (Danilevskaya et al., 2010).

Although many genes associated with flowering time in maize have been successfully cloned, our understanding of their regulatory mechanisms is primarily confined to the transcriptional level. There is a pressing need to delve deeper into the post-transcriptional, translational, and post-translational regulatory mechanisms that govern maize flowering time. Furthermore, the influence of nutrients, hormones, temperature, and various biotic and abiotic stress factors on the timing of flowering requires more extensive investigation.

Evolutionary mechanisms of maize flowering time

Maize was domesticated from teosinte (*Zea mays* ssp. *parviglumis*) in southwestern Mexico about 9,000 years ago (Matsuoka et al., 2002). Teosinte is mainly distributed in tropical regions and is very sensitive to photoperiod. When grown in temperate regions, teosinte exhibits severely delayed flowering or even fails to flower. In contrast, modern maize has adapted to a wide range of geographical and ecological regions (Kuleshov, 1933). The functional variants of cloned flowering time QTLs provide us with valuable clues to explore how maize adapted to vastly diverse environments from its tropical origin. A CACTA-like TE insertion in the *ZmCCT10* promoter was identified as the causal variant of a maize photoperiod QTL located on chromosome 10 (Hung et al., 2012; Yang et al., 2013). Another Harbinger-like TE insertion positioned ~57-kb upstream of *ZmCCT9* underlies a LD-specific flowering time QTL on chromosome 9 (Huang et al., 2018). Both the CACTA-like TE insertion at *ZmCCT10* and the Harbinger-like TE insertion at *ZmCCT9* were newly occurring mutations after initial maize domestication, and they were targeted by selection as maize spread into temperate regions, resulting in both TEs being predominantly distributed at higher latitudes. However, the CACTA-like TE insertion at *ZmCCT10* appeared to arise earlier than the Harbinger-like TE insertion at *ZmCCT9* (Huang et al., 2018). SNP-1245 and Indel-2339, located in 1.2-kb and 2.3-kb upstream of *ZCN8*, respectively, were verified as causal sequence variants that affect flowering time (Guo et al., 2018). SNP-1245 co-segregated with a common flowering time QTL mapped in six maize-teosinte mapping populations, with the teosinte parents carrying the late flowering allele SNP-1245G and the maize parent harboring the early flowering allele SNP-1245A. SNP-1245 was a target of selection during early maize domestication, which drove the pre-existing early flowering allele SNP-1245A present in *Zea mays* ssp. *parviglumis* to near fixation in maize. Interestingly, the other early flowering allele at Indel-2339 (Indel-2339Del, a 3-bp deletion) that originated from highland teosinte *Zea mays* ssp. *mexicana* introgressed into the SNP-1245A haplotype and contributed to maize adaptation to northern high latitudes (Guo et al., 2018). Recently, Zhao et al. (2023b) reported that two tightly linked retrotransposon insertions in the *ZmELF3.1* promoter were associated with early flowering and were selected to help maize latitudinal adaptation. All variants described above are located in gene promoters or intergenic regions, implying that regulatory variation might play important roles in maize flowering time adaptation (Liang et al., 2021b). Recently, Barnes et al. (2022) and Wu et al. (2023) reported that amino acid mutations also contributed to maize flowering time adaptation. A nonsynonymous mutation in the flap-lid domain of *High PhosphatidylCholine1* (*HPC1*), encoding a phospholipase A1 enzyme, altered phosphatidylcholine levels to promote low temperature adaptation and earlier flowering in highland maize (Barnes et al., 2022). Interestingly, the highland *HPC1* allele was introgressed from the wild highland teosinte *Zea mays* ssp. *mexicana* and has been maintained in the Northern United States and European Flint lines (Barnes et al., 2022). A G to A transition (SNP166) in the B-box domain of *conz1* conferred a photoperiod flowering QTL identified in a maize-teosinte BC_2S_3 population. The SNP166A allele that reduced photoperiod response occurred after initial domestication, and its selection enhanced maize adaptation to higher latitudes in temperate regions (Figure 2D) (Wu et al., 2023). A recent study showed that a 178-bp copy number variation in the *ZmMADS1* promoter was significantly associated with flowering time and played an important role during maize spread into higher altitudes (Han et al., 2025). *ZmMADS69* and *dlf1* were also targeted by selection (Liang et al., 2019b; Sun et al., 2020a), but their specific roles in maize spread remain unknown. Joint analyses of the latitudinal distribution of the early flowering alleles of *ZmCCT9*, *ZmCCT10*, *Vgt1*, *conz1*, and *ZCN8* reveal that these early flowering alleles were targeted in a stepwise manner to promote maize gradual spread into high latitudes (Liang et al., 2021b).

The identification of causal variants of flowering time QTLs provides valuable breeding targets for diverse maturation requirements across different cropping systems or the utilization of elite tropical germplasms. It has been reported that an optimal flowering time can maximize maize yield (Parent et al., 2018). However, it is important to recognize that the majority of flowering time genes exhibit pleiotropy. Typically, plants with earlier flowering times have fewer leaves, reduced plant and ear heights, and a slight decrease in yield per plant. To address this challenge, one strategy is to integrate major effect alleles from other target traits or to edit the regulatory regions of flowering time genes and select alleles that either minimize pleiotropy or even enhance multiple traits synergistically. Furthermore, the smaller stature of early flowering plants presents an opportunity for high-density planting, which could potentially enhance population-level yields. Another strategy is to select maize lines that exhibit early flowering time but still maintain a long grain-filling stage, coupled with high photosynthetic capacity and delayed senescence. This combination ensures adequate source and sink capacity, thereby contributing to optimal yield performance under both ideal and stress conditions (Trachsel et al., 2017; Wingler and Soualiou, 2025).

Kernel quality

Maize kernels represent a rich reservoir of essential stored materials and metabolites, including starch, protein, oil, and vitamins. This nutritional composition renders maize a valuable food source for both humans and animals (De Lumen, 1990). Seed storability is also an important component of kernel quality. The vigor of the seed plays vital roles in plant growth, resistance to environmental stresses, grain quality, and crop productivity in maize. To improve the quality of maize kernels, it is crucial to delve into the genetic and molecular foundations underlying the synthesis and regulation of these nutritional components and seed vigor. This understanding will pave the way for targeted breeding efforts aimed at enhancing the overall quality of maize kernels.

Starch

Starch is mainly deposited in maize endosperm, and generally constitutes ~70% of seed dry weight (Hannah and Boehlein, 2017). It is the major storage carbohydrate in maize kernels, and is highly associated with grain yield. Raising starch content has great significance for breeding high-yield maize varieties, which provide calories for humans and animals. In addition, starch has also been used in various industrial applications such as biofuels, paper manufacturing, high-fructose corn syrup, and pharmaceuticals (Egharevba, 2020). Therefore, it is urgent to improve starch quantity and quality in maize to meet the needs of a growing human population.

Over the past three decades, much is now known about the starch biosynthesis pathway via mutant and biochemical analysis. The cloned key genes included those encoding starch synthases (e.g., GBSS, SSI, SSII, SSIII, and SSIV), branching

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enzymes (e.g., BEI, BEIIa, and BEIIb) and debranching enzymes (e.g., ISA and PUL) (Hannah and Boehlein, 2017). Starch biosynthesis and accumulation are unexpectedly complex, as many carbohydrate metabolic processes including sucrose metabolism, sugar metabolism, and the trehalose pathway, directly or indirectly influence starch synthesis (Fichtner and Lunn, 2021; Li et al., 2019k).

GWAS has been a powerful tool for dissecting the genetic architecture of starch content and composition in maize kernels during the past decade. A GWAS identified 27 loci significantly associated with amylose content (Li et al., 2018a). These loci were resolved to 39 candidate genes that include transcription factors, glycosyltransferases, glycosidases, as well as hydrolases. Most of these genes were located in the upstream pathway of amylose synthesis, aiding in molecular manipulation for changing amylose content in maize kernels. In addition, Hu et al. (2021b) used a multi-parent population, which contains six RIL populations and exhibits abundant diversity in starch content, to dissect the genetic basis of starch synthesis and accumulation in maize kernels. Three statistical methods, including single linkage mapping (SLM), joint linkage mapping (JLM), and RIL-based GWAS, systematically identified 50 unique QTLs with 18 new QTLs and limited pairs of epistatic QTLs for starch content. Subsequently, the pathway-driven analysis identified *ZmTPS9* as the causal gene for the QTL *qSTA4−2*, which was detected by all three statistical analyses. *ZmTPS9* encodes a trehalose-6-phosphate synthase in the trehalose pathway. Knockout of *ZmTPS9* increased kernel starch content and, in turn, kernel weight in maize. These findings indicate that the genetic variance components for starch content are largely determined by additive effects, providing insights into the genetic basis of this trait and offering molecular breeding strategies for starch enhancement.

The synthesis and accumulation of starch and protein-like zeins are coordinated in maize kernels. Zhang et al. (2016c) found that two endosperm-specific transcription factors (TFs), Opaque 2 (O2) and prolamin-box binding factor 1 (PBF1), regulating zein protein genes, also controlled starch synthesis (Figure 3). The starch content of *PbfRNAi* and *o2* single mutant was decreased by ~5% and 11%, respectively, and that of the double mutant was reduced by 25%. The protein and RNA levels of starch biosynthetic genes like *PPDKs*, *SSIII*, *SSIIa*, and *SBEI* were reduced in *o2* and *PbfRNAi* single mutant, and were further reduced in the double mutant. These findings provided new strategies for breeding high-yield and high-quality maize varieties. Qing et al. (2025) found an additional endosperm-specific TF, ZmEREB167, which functions as a negative regulator in maize endosperm development by directly targeting and repressing the expressions of *O2*, *ZmNRT1.1*, *ZmIAA12*, *ZmIAA19*, and *ZmbZIP20*. Knockout of *ZmEREB164* resulted in increased kernel size, starch content, and protein content. Recently, Chen et al. (2023b) found that the TFs ZmNAC128 and ZmNAC130 directly regulated all *γ-zein* genes and six important starch metabolism genes (*Brittle2*, *pullulanase-type starch debranching enzyme*, *granule-bound starch synthase 1*, *starch synthase 1*, *starch synthase IIa*, and *sucrose synthase 1*). The aforementioned cloned genes in starch pathways facilitate developing of special maize. Using a CRISPR/Cas9 technology, Dong et al. (2019) knocked out *SH2* and *WX* to develop supersweet-waxy maize. They generated a series of gene-editing lines and obtained single or double mutant lines. *sh2*::*wx* double mutants have higher soluble sugar content as well as amylopectin contents than wild-type plants. This gene editing-based approach opens the door for modifying many other genes that could also be integrated into the scheme for breeding. ZmICE1a coordinates the defense-storage trade-off in maize endosperm by promoting starch synthesis while suppressing defense pathways (aleurone layer defenses and JA/IAA synthesis). This regulatory mechanism prioritizes energy storage over defense during kernel development, representing an adaptation to balance growth and protection in cereal crops (Wang et al., 2024a).

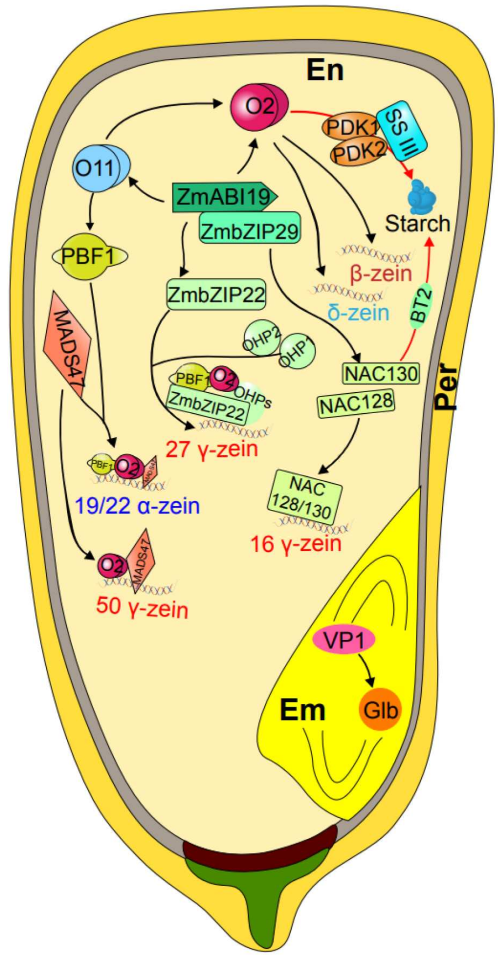

Figure 3. Transcriptional regulatory networks of maize seed storage protein genes. Zeins are the main storage proteins in maize endosperm. The expression of *zein* genes is regulated by various transcription factors, which either act individually or jointly with other transcription factors. It is worth noting that some of these factors also regulate the expression of genes related to starch synthesis. Em, embryo; En, endosperm; Per, pericarp.

Protein

Maize kernels accumulate several types of storage proteins as sources of amino acids and carbon skeletons for the seedling. About 60%–70% of maize endosperm storage proteins are prolamins, including 19- and 22-kD α-zeins, 15-kD β-zeins, 50-, 27-, and 16-kD γ-zeins, 18-kD and 10-kD δ-zeins (Boston and Larkins, 2009). Zeins are specifically synthesized in the endosperm about 10 days after pollination (DAP) on polyribosomes of the rough endoplasmic reticulum (RER), and the proteins are subsequently translocated into the lumen of the RER, where they form insoluble protein bodies with a diameter of 1–2 μm. Zein-encoding genes account for nearly 50% of total transcripts in the endosperm, with α- and γ-zein transcripts representing up to 30% and 15%, respectively (Chen et al., 2014; Hunter et al., 2002).

The regulatory network of *zein* gene expression by TFs has greatly advanced in the last few decades (Yang et al., 2023c) (Figure 3). The endosperm-specific bZIP transcription factor O2 was originally reported to regulate the *15-kD β-zein* and *22-kD α-zein* (Schmidt et al., 1992). Further, it was reported that O2 targets directly all *zein* promoters with the exception of the *16-kD γ-zein* (Li et al., 2015a; Zhan et al., 2018). OHPs (O2-heterodimerizing proteins) interact with PBF1 to regulate the expression of *27-kD γ-zein*; PBF1 can promote O2 binding, resulting in the synergistic transactivation of *α-zein* genes (Yang et al., 2016b; Zhang et al., 2015c). ZmbZIP22, together with PBF1, OHP1, and OHP2, controls the expression of *27-kD γ-zein* (Li et al., 2018b). ZmMADS47, a MADS-box protein interacting with O2, regulates *α-zein* and *50-kD γ-zein* (Qiao et al., 2016). Two endosperm-specific NAC TFs, ZmNAC128 and ZmNAC130, specifically transactivate the expression of *16-*, *27-*, and *50-kD γ-zein* (Chen et al., 2023b; Song et al., 2024; Zhang et al., 2019g). ChIP-seq analysis revealed that O11 (Opaque11, a bHLH protein) directly regulated O2 and PBF1 expressions during endosperm development (Feng et al., 2018b; Feng and Song, 2018). Recently, a B3 domain-containing TF, ZmABI19, was identified as an "a hub of hubs" in maize filling by directly regulating multiple key endosperm-filling factors including *O2*, *Pbf1*, *ZmbZIP22*, *NAC130*, and *O11* (Yang et al., 2021c; Zhan, 2021). Furthermore, ZmbZIP29 interacts with ZmABI19 to synergistically transactivate O2 expression and regulates *zein* gene expression (Yang et al., 2022c). Another B3 family TF, VP1 (VIVIPAROUS-1), was found to play essential roles in scutellum development and protein reallocation from the endosperm to embryo (Zheng et al., 2019). Understanding the expression pattern and regulatory mechanism of storage protein synthesis will facilitate genetic improvement of kernel nutritional composition. The separated intranuclear droplets play an important role in gene regulation. Specific proteins and nucleic acid molecules enriched within these droplets can participate in gene transcription and post-transcriptional regulation, thereby influencing gene expression levels. The maize storage protein gene family is regulated by multiple transcription factors. It is worthwhile to investigate whether such as transcription factors and RNA-binding proteins undergo liquid-liquid phase separation (LLPS) during seed and endosperm development. Understanding how LLPS regulates gene expression and storage compound biosynthesis would provide valuable mechanistic insights into maize nutritional quality improvement.

The synthesis of storage proteins in maize kernels is regulated multidimensionally by sugar signaling, nitrogen levels, and hormonal signals. Studies have shown that sucrose not only serves as a carbon source and energy substrate, but also as a signal dynamically regulating the synthesis of zein through the SnRK1-ZmRFWD3-O2 signaling axis (Li et al., 2020a). Sucrose can also enhance the DNA-binding capacity and transcriptional activation activity of the O2 protein via SnRK1a1-mediated direct phosphorylation of its Thr387 residue (Yang et al., 2024a). Nitrogen (N) availability also significantly influences storage protein content. Under sufficient nitrogen conditions, the transcription factor PBF1 suppresses starch biosynthesis genes (e.g., *Su1* and *Sbe2b*), redirecting carbon skeletons toward zein synthesis. Conversely, under nitrogen deficiency, PBF1 dissociates from *zein* gene promoters, reducing nitrogen-dependent zein synthesis while reallocating carbon resources to other metabolic pathways (Ning et al., 2023). Additionally, hormonal signals, such as abscisic acid (ABA), precisely regulate zein synthesis through the ZmABI19-ZmbZIP29-O2 module. Phosphorylation of ZmABI19 (Thr57), ZmbZIP29 (Thr75), and O2 (Thr387) by SnRK2.2 kinase significantly enhances their transcriptional activity, thereby maximizing the accumulation of storage reserves (Yang et al., 2022c). These pathways elucidate a molecular network in which sugar signaling, nitrogen availability, and hormonal signals collaboratively regulate storage protein synthesis through transcription factors and their post-translational modifications. This network provides critical targets for directed improvement of kernel nutritional quality (e.g., high-protein breeding) and nitrogen fertilizer utilization efficiency.

Zein proteins, which constitute a major portion of maize endosperm proteins, are considered of poor quality because they are deficient in lysine, an essential amino acid. The *o2* mutant, characterized by significantly reduced zein content, exhibits a twofold increase in lysine content. However, the *o2* mutant also has a soft endosperm, making the kernels vulnerable to damage during harvesting. Genetic suppressors of *o2* (known as *o2* modifiers or *Mo2s*) can restore a normal kernel phenotype with vitreous endosperm, resulting in a variant of the *o2* maize with high-lysine content known as "Quality Protein Maize" (QPM). In China, some cultivated QPM materials may have lost certain factors responsible for the vitreous endosperm modification, resulting in a semi-hard endosperm phenotype (Wu and Messing, 2011). Studies have shown that increasing the content of γ-zein proteins in QPM is essential for restoring the vitreous endosperm phenotype by promoting the production of numerous small protein bodies (Wu and Messing, 2010). The duplication of the *27-kD γ-zein* gene has been identified as the major QTL for endosperm modification, and three copies of the *qγ27* allele were found to be more effective in endosperm modification for QPM (Liu et al., 2019a; Liu et al., 2016b). More *Mo2* loci have been identified on chromosomes 1, 6, 7, and 9 using an F_2 population derived from the K0326Y (a QPM line from South Africa) and W64A*o2* varieties (Li et al., 2020c). While QPM varieties have demonstrated improved protein quality, they often suffer from reduced yield due to the direct regulation of starch synthesis by the O2 gene (Zhang et al., 2016c). One potential solution is the editing of the *zein* gene family to develop non-*o2*-based QPM, thereby addressing both the protein quality and yield concerns (Langer et al., 2023).

Over the course of long-term domestication and artificial selection, the overall protein content in maize seeds has gradually declined from approximately 30% to 10% (Flint-Garcia et al., 2009a). The University of Illinois has undertaken a breeding program spanning over a century to investigate the impact of artificial selection on seed composition. This endeavor has resulted in the development of genetic material encompassing four protein content levels: 4%, 7%, 15%, and 30% (Moose et al., 2004). The control of seed protein content is influenced by multiple genetic loci (Karn et al., 2017; Renk et al., 2021). In a recent study, researchers analyzed QTLs related to seed protein content in teosinte and established a population of near-isogenic lines. They successfully identified and cloned the first major high

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protein gene, *teosinte high protein 9* (*THP9*), from teosinte. *THP9* encodes asparagine synthase 4 (ASN4), an important factor of nitrogen metabolism essential for asparagine synthesis. The introgression of *Thp9-T* into the B73 inbred resulted in notable increases in seed protein content by about 35%, root nitrogen content by about 54%, stem nitrogen content by about 94%, and leaf nitrogen content by about 18%. Furthermore, *Thp9-T* also exhibited the capacity to improve seed protein content in a hybrid Zhengdan 958, indicating its valuable potential for high protein breeding improvements (Huang et al., 2022b). Cloning additional high-protein loci will contribute to a comprehensive understanding of the genetic mechanisms involved in seed protein content and facilitate the development of high-protein maize varieties to address the protein shortage in feed production in China (Yang et al., 2023c).

Oil

Maize oil, primarily deposited in the embryo as triacylglycerol, is chemically characterized by a fatty acid profile of approximately 11% palmitic acid (C16:0), 2% stearic acid (C18:0), 24% oleic acid (C18:1), 62% linoleic acid (C18:2), and 1% linolenic acid (C18:3) (Yang and Li, 2018). It exhibits high caloric value and is rich in polyunsaturated fatty acids and antioxidants like vitamin E, making it a valuable source of animal feed and edible oil for humans. Enhancing oil content and composition in maize kernels holds paramount importance in securing the provision of vegetable oil and high-energy feed.

As quantitative traits, maize oil content and composition are governed by many loci or genes. Over the past decades, numerous QTLs for oil content and compositions have been identified in multiple populations, including bi-parent maize populations, multi-parent maize populations, maize-teosinte populations, and diverse natural maize populations (Fang et al., 2020; Yang and Li, 2018). These results indicate that the heritable variations in oil content and composition were primarily attributed to additive effects. Notably, in a maize-teosinte population, the increasing alleles of more than 60% of the QTLs originate from teosinte, indicating the widespread existence of favorable alleles in teosinte, which has great potential in enhancing both the content and fatty acid compositions of maize kernel oil (Fang et al., 2020). In comparison with oil content, the genetic basis of oil composition is relatively simple. For instance, a total of 74 loci were found to be significantly associated with oil content and composition by using a genome-wide association study in a panel with diverse maize inbred lines (Li et al., 2013). Of these, 26 significant loci could explain up to 83% of the phenotypic variation. In contrast, the number of significant loci associated with the oil component trait is small, ranging from 1 to 7. Based on the favorable alleles of these associated loci, Li et al. (2013) found that high-oil maize accumulated more beneficial alleles compared with regular maize, indicating that pyramiding favorable alleles is one effective strategy for elevating oil content during the selection of high-oil lines. Additionally, a variance-heterogeneity GWAS, which is an effective complement for traditional GWAS, identified 77 candidate variance-heterogeneity genes for 21 oil-related traits in maize kernels with additive and epistatic interaction effects contributing to the phenotypic variance (Li et al., 2020e). The proposed favorable allele combinations for oil improvement and low phenotypic variability will provide opportunities to stabilize efficient breeding and selection of high-oil maize. A complementary pathway analysis revealed that in addition to fatty acid biosynthesis, biosynthesis of wax esters, sphingolipids, phospholipids, and flavonoids also contributed to oil and fatty acid accumulation in maize kernels (Li et al., 2019d). The identification of significant pathways and related genes in this study has provided opportunities for the efficient improvement of high-oil maize varieties.

Although much progress has been made in dissecting the genetic basis of oil content and composition, our knowledge of the molecular mechanisms underlying oil biosynthesis and accumulation remains poorly understood. To date, only nine genes associated with oil traits have been isolated by either homology-based method (*ZmSAD1*, *ZmLEC1*, *ZmWRI1*, *ZmGE2*), positional cloning (*DGAT1-2*, *ZmFatB*), or association mapping (*FAD2*, *ACP*, *LACS*) (Yang, 2018). Among these genes, two (*ZmLEC1*, *ZmWRI1*) are TFs, one (*ZmGE2*) is a regulator of embryo size, and the remaining six genes function in oil biosynthesis. The functional sites of all genes except *ZmLEC1* are mined in diverse maize inbred lines, and provide promising targets for improving oil content and composition in maize. Interestingly, the favorable allele (a 3-bp insertion in the last exon, named F469) of *DGAT1-2* enhances oil content in maize kernel without altering embryo proportion or yield production (Chai et al., 2012; Zheng et al., 2008). Consequently, the introgression of the favorable F469 allele from high-oil inbred line By804 into Zhengdan958 by marker-assisted backcross elevates its oil content to 4.5%, representing an absolute and relative increase of 0.7% and 18.0%, respectively (Hao et al., 2014). Notably, the grain yield of the improved hybrid is not compromised compared with the original hybrid. These findings provide empirical evidence of applying a marker-assisted backcrossing strategy to improve quantitative traits.

With the development of metabolic engineering technologies, these advances in high-oil maize genomics and genetics provide new opportunities for high-oil maize breeding. In the future, we could extend the use of kernels to vegetative tissues, utilizing them as factories to produce triacylglycerol, eicosapentaenoic acid, and docosahexaenoic acid, and hence develop maize as an oilseed crop, a nutrient biofactory, and an energy crop (Li et al., 2023b).

Vitamin

Maize kernels are rich in nutritious vitamins that are critical for human health and plant development, such as carotenoids and tocochromanols. During the last ten years, great progress has been made in elucidating the genetic basis of vitamin variation via linkage mapping, association mapping, and positional cloning.

Carotenoids represent a structurally diverse group of isoprenoid pigments that function both as vitamin A precursors (e.g., β-carotene) and non-provitamin A bioactive compounds (e.g., lutein). Although the carotenoid biosynthetic pathway is well characterized in plants, limited knowledge is known in maize. In the past two decades, the pathway-driven association analysis has mined a series of functional sites of key enzymes in the carotenoid biosynthesis pathway, such as *PSY1*, *LCYE*, *crtRB1*, and *crtRB3* (Fu et al., 2013b; Harjes et al., 2008; Yan et al., 2010; Zhou et al., 2012). Analysis in the NAM panel reveals that eleven biosynthetic genes explain the majority of natural variations in carotenoid levels in maize grain (Diepenbrock et al., 2021). The genetic basis of carotenoid variation in maize kernel was also explored through linkage and association mapping of eight carotenoid traits in six RIL populations (Yin et al., 2024). A total of 77 unique additive QTLs and 104 epistatic QTL pairs were identified, revealing 22 overlapping hotspots. A genome-wide association study uncovered 244 candidate genes, including 23 linked to the carotenoid pathway, with *ZmPTOX* highlighted as a key gene affecting carotenoid levels. *ZmPTOX* encodes a putative plastid terminal oxidase that produces plastoquinone-9 used by two enzymes in the carotenoid pathway. In addition, two key enzymes, *SCD* and *DXS2*, in the plastid methylerythritol phosphate (MEP) pathway, which is the upstream pathway of carotenoids and tocopherols, were cloned by positional cloning (Fang et al., 2020; Zhang et al., 2019b). *SCD* encodes a 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP) synthase, which converts 2C-methyl-D-erytrithol 2,4-cyclodiphosphate to HMBPP in the penultimate step of the MEP pathway. Loss of function of *SCD* reduced the levels of carotenoids and tocopherols in both leaves and seeds (Zhang et al., 2019b). *DXS2* encodes a 1-deoxy-D-xylulose-5-phosphate synthase 2, which catalyzes glyceraldehyde 3-phosphate and pyruvate to produce 1-deoxy-D-xylulose-5-phosphate in the first step of the MEP pathway (Fang et al., 2020). Knockout of *DXS2* reduced the levels of carotenoids. Moreover, a TE in the first intron of *DXS2* was shown to up-regulate gene expression and boost kernel carotenoid content. This TE variant, originating from teosinte, exhibits strong signatures of selection during maize domestication and modern breeding, and is almost fixed in yellow maize. These studies extended the contribution of the MEP pathway to carotenoid biosynthesis in maize kernels. In addition, carotenoids modulate kernel texture. *Ven1* (encoding a β-carotene hydroxylase 3) was found as a major QTL, which controls the vitreous and opaque endosperm formation by regulating the composition of polar carotenoids and non-polar carotenoids (Wang et al., 2020b).

Tocochromanols, including tocopherols and tocotrienols (collectively vitamin E), are a class of lipid-soluble, plastid-synthesized antioxidants synthesized by all plants. Their dietary intake, primarily from seed oil, provides vitamin E and other health benefits. In the past decades, the genetic basis underlying the natural variations in tocochromanol levels has been decoded via linkage mapping and GWAS. GWAS using diverse maize inbred lines identified γ-tocopherol methyltransferase (VTE4) as a major locus controlling α-tocopherol concentrations, while tocopherol cyclase (VTE1), homogentisate geranylgeranyltransferase (HGGT1), and a perphenate/arogenate dehydratase were shown to have modest effects on tocotrienol traits in maize kernel (Li et al., 2012; Lipka et al., 2013). To leverage the statistical power and mapping resolution, two statistical methods including JLM and GWAS were employed, and 52 QTLs for individual and total tocochromanols in the US NAM populations were identified (Diepenbrock et al., 2017). Of these QTLs, 14 were fine-mapped to candidate genes, and six encoded novel regulators of tocochromanols in plants. Notably, two chlorophyll biosynthetic enzymes that are major genetic regulators of tocopherol variation were identified. Simultaneously, using six RIL populations, SLM and RIL-GWAS identified 41 unique QTLs and 32 significantly associated SNPs for individual and total tocopherol traits, respectively (Wang et al., 2018b). Deep analysis unveiled novel genes involved in fatty acid, chlorophyll, and plastid function metabolic pathways, suggesting a critical role of non-tocopherol pathway genes in influencing tocopherol content in maize kernels. Subsequently, *qVE5*, a major QTL affecting tocopherol accumulation in maize kernels, was cloned as *ZmPORB2* via a positional cloning approach (Zhan et al., 2019). *ZmPORB2* encodes a protochlorophyllide oxidoreductase, and positively regulates tocopherol content in both leaves and kernels. A 5/8-bp insertion/deletion (InDel058) in the 5′ UTR was identified as the causal variant, which affected *ZmPORB2* expression, and hence tocopherol content. The homologous gene *ZmPORB1* is the functional gene of QTL-*qVE1*, which positively regulates the content of tocopherols and chlorophyll in maize kernels. The double mutant showed severe developmental defects with no kernels (Liu et al., 2024b). Furthermore, studies on mutants confirmed that *ZmSPS2* played a crucial role in regulating the α/γ-tocopherol ratio, thereby enhancing α-tocopherol content in maize. Overexpression of *ZmSPS2* resulted in an increase in α-tocopherol content and a high α/γ-tocopherol ratio (Feng et al., 2025).

In summary, these studies made a comprehensive assessment of natural variations in the levels of carotenoids and tocochromanols in maize kernels, establishing the foundation for improving carotenoid and tocochromanol content in seeds of maize and other major cereal crops.

Seed storability

Seed storability or longevity governs the regeneration cycle of seeds, which refers to the overall capacity of the seed to germinate and emerge after sowing and retain this potential during postharvest storage. It is a complex quantitative trait that is determined by the interactions between environmental conditions and the genetic factor. The major loci for seed storability, such as germination potential, germination index, and seedling percentage, were detected using a linkage and association mapping approach in maize (Guo et al., 2021b; Han et al., 2018; Han et al., 2014; Ku et al., 2014; Liu et al., 2019c; Ma et al., 2022b; Wu et al., 2019b). The omics methods such as transcriptomics and proteomics were used to explore the molecular mechanisms of maize seed storability. Tens of thousands of differentially expressed genes were mined by transcriptomic analysis, and candidate genes related to seed viability were identified by GWAS (Ma et al., 2022b). Proteomic identification showed that small heat shock proteins (sHSPs), late embryogenesis abundant proteins (LEA), and antioxidant enzymes were highly upregulated in the seeds with high vigor. The differentially expressed miRNAs and their target genes were enriched in glycine degradation and galactose metabolism (Jin et al., 2022), and exerted their effects on seed aging resistance through ethylene activation signaling pathways, hormone synthesis and signal transduction, and plant organ morphogenesis (Song et al., 2022).

Some of the genes have been cloned and elucidated for seed vigor in maize. Hormonal signals, especially those of gibberellin (GA), play a dominant role in determining whether a seed germinates or not. The six GA metabolization-related genes (*ZmCPS1*, *ZmCPS2*, *ZmKS1*, *ZmKS4*, *ZmKO1*, and *ZmGA20ox4*) influence seed storability by converting stored GA precursors into active GA during early germination (Song et al., 2011). During the maturation and drying process of many plant species, raffinose family oligosaccharides (RFOs) accumulated in seeds,

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and a raffinose synthase-related gene *ZmRS* was identified in maize. The complete loss of raffinose had adverse effects on seed viability of maize, and the increase of galactinol could not compensate for the loss of raffinose, suggesting that RS might maintain inositol content in seeds through a non-productive cycle of galactinol synthesis/hydrolysis (Li et al., 2017f). Recently, it was identified that ZmDREB2A directly bound to the DRE motif of promoters of both *ZmGH3.2* (encoding indole-3-acetic acid (IAA) deactivating enzyme) and *ZmRAFS* (encoding raffinose synthase), and regulated the lifespan of maize seeds by stimulating the production of raffinose while limiting auxin-mediated cell expansion (Han et al., 2020a). ZmPIMT2 (PROTEIN L-ISOASPARTYL O-METHYLTRANSFERASE), regulated by ZmVIVIPAROUS1 (ZmVP1), binds to *ZmMCCα* in mitochondria. This interaction plays an important role in isoAsp damage repair and positively affects maize seed vigor (Zhang et al., 2023g).

Overall, few genes regulating seed vigor have been identified, and the relationships between seed vigor, seed storage compounds, and related phenomena (e.g., pre-harvest sprouting) (Liu and Tian, 2023) remain poorly understood, warranting further investigation.

Abiotic stress tolerance and nutrient use efficiency

With global climate change, environmental stresses such as drought, waterlogging, and extreme temperatures frequently threaten crop production and cause substantial yield loss. Soil salinization and alkalization often occur with underground water mining and pumping, which also negatively affects maize yield. Excessive use of fertilizers not only brings about environmental deterioration, but also increases agricultural input. Therefore, it is an urgent demand to decipher the mechanisms of how plants respond and adapt to these stresses and breed maize varieties with improved stress tolerance and nutrient use efficiency.

Water stress resistance

Drought is a central natural disaster that causes great crop yield loss worldwide. With global warming, 50% of the Earth's regions are expected to suffer from water scarcity in the middle of this century (Gupta et al., 2020). Great efforts have been made to study the genetic control of maize drought responses. Roots absorb and transport water and nutrients to support the growth of the whole plant; therefore, they play a key role in plant drought tolerance. The root cortical aerenchyma could play significant roles in maize drought adaptation (Jaramillo et al., 2013). Interestingly, the root cortical cell size is positively correlated, while the root cortical cell file number is negatively correlated to maize drought tolerance (Chimungu et al., 2014). Lateral root density also plays a negative role in maize drought tolerance (Zhan et al., 2015). These studies demonstrated the important roles of root architecture in maize drought response. Recently, a study has dissected the genetic loci underlying root architecture in response to drought based on 373 maize inbred lines (Li et al., 2023a). Moreover, maize drought tolerance was proposed to be regulated by the lactate levels and histone lactylation modifications in the root (Shi et al., 2023b).

Besides the underground roots, the aerial parts also play pivotal roles in plant drought responses. By using a high-throughput multiple optical phenotyping system, more than 10,000 image-based traits (i-traits) were collected from leaves of 368 maize inbred lines, representing the largest phenotypic responses of maize to drought so far (Wu et al., 2021b). The expression variations of molecules in a population could also be treated as "traits" for GWAS in genetic dissection of drought responses. For instance, more than 70,000 eQTLs have been identified to control the gene expression variation in drought responses of 224 maize accessions (Liu et al., 2020g). Up to one thousand drought-responsive metabolites have been detected in 385 maize inbred lines. mGWAS have identified 3,415 mQTLs and dozens of environment-specific hub genes (Zhang et al., 2021b). Besides, the natural antisense transcripts, circular RNAs, and translational regulation events also play important roles in maize drought responses (Lei et al., 2015; Xu et al., 2017b; Zhang et al., 2019d). During the past century, the maize yield has been significantly increased, but that is accompanied with increased drought susceptibility (Lobell et al., 2014); the mechanisms underlying this phenomenon remain largely unknown. A recent study reveals that the genome-wide inverted repeats, particularly transposon-mediated inverted repeats (TE-IRs), underpin a key genetic mechanism balancing maize drought tolerance and yield (Sun et al., 2023a). All of these studies, as well as the release of the reference genome of CIMBL55, a drought-tolerant maize germplasm (Tian et al., 2023), not only deepen our knowledge of the genetic control of maize drought response but also are beneficial for breeding drought-tolerant maize cultivars and contribute to world food security (Mu and Dai, 2023).

Drought tolerance is a complex trait that is controlled by multiple genes. Much effort has been given to clone key genes regulating maize drought tolerance (Figure 4A). ABA is a plant stress hormone and plays pivotal roles in stress responses. A couple of genes regulating ABA signaling have been functionally studied in maize drought response. The PYR/PYL/RCAR family (PYLs) is known as ABA receptors. In the presence of ABA or drought, PYLs interact with and inhibit clade A protein phosphatase 2Cs (PP2Cs), which leads to the activation of the SnRK2 family protein kinases. The activated SnRK2s phosphorylate and activate the downstream ABA response pathways. There are 13 *ZmPYL* genes in the maize genome, and *ZmPYL8*, *9*, *12* play positive roles in drought tolerance (He et al., 2018). ZmPP2C-A10 negatively regulates maize drought tolerance, but it has a functional allele, InDel-338, which mediates the ABA and ER stress signaling to regulate drought tolerance (Xiang et al., 2017). PTPN, a PTP-like nucleotidase, plays conserved roles in controlling drought tolerance of maize and *Arabidopsis* through mediating the crosstalk between ABA signaling and vitamin C biosynthesis (Zhang et al., 2020a). The mitogen-activated protein kinase ZmMPKL1 negatively regulates drought-induced ABA production, thus it is harmful for maize drought tolerance (Zhu et al., 2020a).

TFs could regulate the expression of multiple genes, thus playing important roles in controlling plant development and stress responses. There are many TFs that have been identified to regulate maize drought tolerance. For instance, *ZmNAC111* positively regulates maize drought tolerance, but an insertion of 82-bp MITE transposon represses the expression of *ZmNAC111* and negatively regulates maize drought tolerance (Mao et al., 2015). Two other NAC TFs, ZmNAC48 and ZmNAC49, also play positive roles in drought tolerance. ZmNAC48 is regulated by its *cis*-natural antisense transcripts in drought response, and

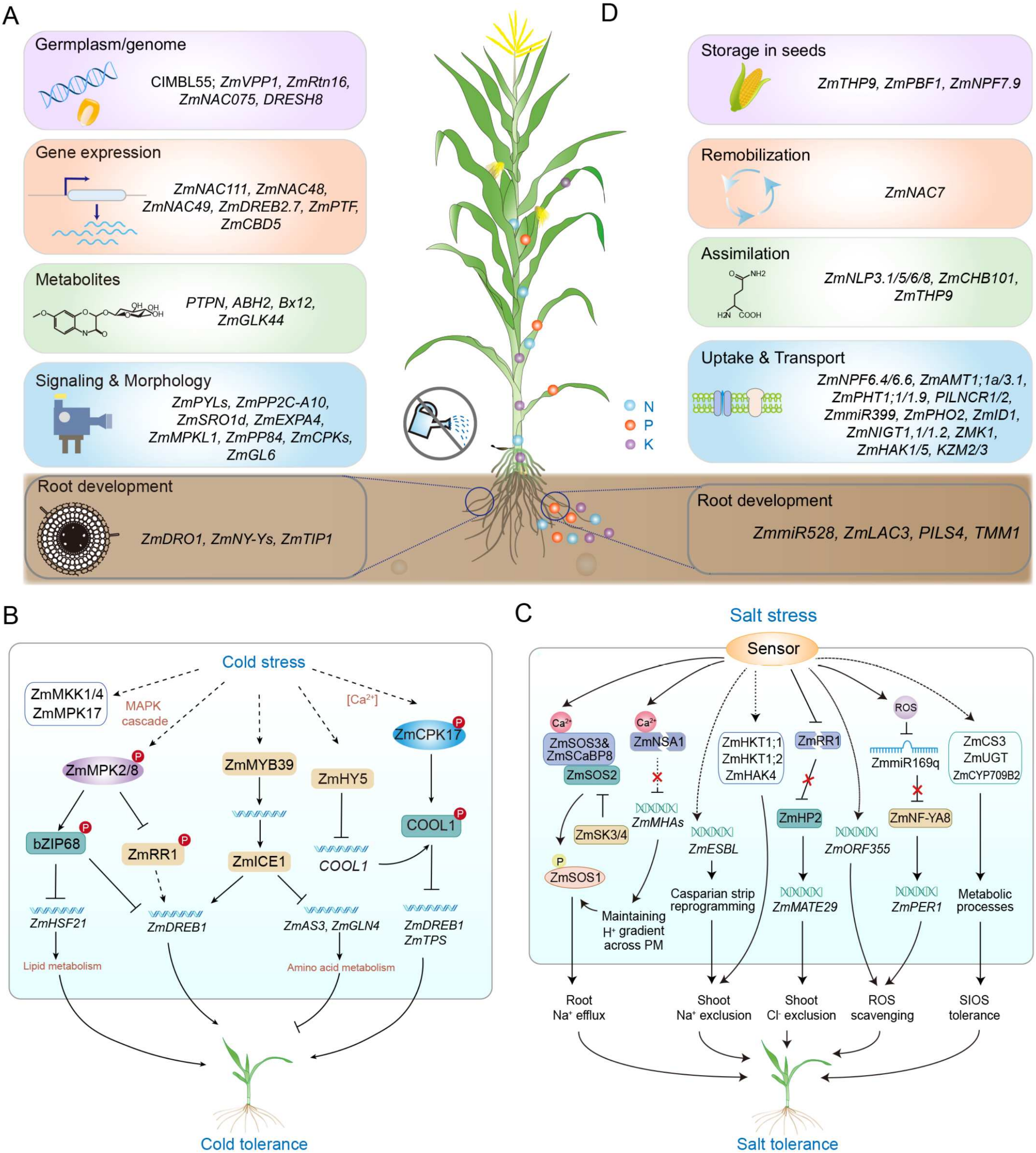

Figure 4. Advances in genetic dissection of maize abiotic stress response and resistance, and nutrient use efficiency. A, The genetic contributors of maize drought resistance are dissected from different aspects, including germplasm resources, genomics, gene expression regulation, metabolic alterations, signaling transduction, morphological changes, and root development. The major identified and cloned genes were shown. B, Cold stress responses in maize. *ZmDREB1* transcription factors, which promote maize cold tolerance, are regulated by ZmICE1, ZmMYBs, COOL1, and bZIP68. ZmMPK2/8 phosphorylates bZIP68 and ZmRR1 to mediate their stability, while ZmCPK17 enhances COOL1 stability through phosphorylation. Furthermore, maintaining amino acid and lipid metabolism homeostasis is crucial for maize cold tolerance. C, Current progress in understanding the mechanisms of maize salt tolerance. SIOS, salt-induced osmotic stress. D, Molecular mechanisms of nutrient (N, P, and K) use efficiency in maize. The genes that have been functionally characterized are classified into different categories.

ZmNAC49 functions to reduce stomatal density, thus enhancing maize drought tolerance (Mao et al., 2021; Xiang et al., 2021). Via candidate gene association analysis, the natural variations in the promoter of ZmDREB2.7 have been detected to control gene

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expression and plant drought tolerance (Liu et al., 2013a). ZmPTF1, a bHLH TF, positively regulates ABA synthesis and root development, thus enhancing maize drought tolerance (Li et al., 2019e). ZmLBD5 negatively regulates maize drought tolerance through regulating the TPS-KS-GA2ox module in GA and ABA biosynthesis (Feng et al., 2022b). Nuclear factors Y (NF-Ys) are important TFs. It has been reported that ZmNF-YA1 interacts with ZmNF-YB16-YC17 to promote root development and enhance maize drought tolerance (Yang et al., 2022e).

Besides the regulatory factors of ABA signaling and the TFs, there are some other regulators in maize drought tolerance. *ZmVPP1*, a vacuolar-type H^+-pyrophosphatase encoding gene, promotes drought tolerance of maize seedlings. A 366-bp insertion in the promoter enhances the expression of *ZmVPP1* in drought-tolerant maize genotypes (Wang et al., 2016d). *ZmPP84*, encoding a clade F PP2C, represses ZmMEK1-ZmSIMK1 signaling pathway, thus negatively regulating maize drought tolerance (Guo et al., 2023b). The S-acyltransferase ZmTIP1 mediates the palmitoylation of ZmCPK9 and positively regulates the length of root hairs and maize drought tolerance (Zhang et al., 2020e). ZmCPK35 and ZmCPK37 could also enhance maize drought tolerance (Li et al., 2022h). Cuticular waxes, long-chain hydrocarbon compounds, help plants cope with drought stress by protecting plants from water loss. The maize glossy 6 (*GL6*) gene plays a positive role in drought tolerance via regulating the intracellular trafficking of cuticular waxes (Li et al., 2019e). It has been reported that the phosphorylation regulatory mode ZmBSK1-ZmCCaMK and the SRO protein ZmSRO1d-R play positive roles in maize drought tolerance (Gao et al., 2022; Liu et al., 2021a). A key reason that drought causes maize yield loss is due to its effects on the anthesis and silking interval (ASI). Ectopic expression of maize α-expansin4 (*ZmEXPA4*) in ears reduces ASI under drought conditions (Liu et al., 2021a). All these genes and functional alleles could be potential targets in maize drought-tolerance breeding.

Although most of the studies on maize drought responses have been done with seedlings, some studies have been conducted with yield performance as a drought-tolerance index, suggesting a higher likelihood of the usage of the related genes and genetic manipulating strategies in breeding. Researchers from Syngenta ectopically express a rice trehalose-6-phosphate in maize ears to improve yields from 9%–123% under non-drought to mild and severe drought conditions, relative to yields from nontransgenic controls (Nuccio et al., 2015). By using an ABA-inducible strategy, the gene DEEPER ROOTING 1 has been shown to increase maize yield by 40% under a water-limited environment (Feng et al., 2022a). Maize ARGOS8 negatively regulates ethylene responses. A recent study has used the CRISPR/Cas9 system to generate new variants of ARGOS8, and overexpressing these variants in maize increases yield under drought conditions with no yield penalty under normal conditions (Shi et al., 2017a). One of the most important goals of the fundamental research on maize drought responses is breeding. The drought-tolerant genes and functional alleles could be valuable targets for the breeding of drought-tolerant maize cultivars with advanced gene manipulation approaches.

Waterlogging caused by flooding is also a kind of water stress that threatens maize growth and yield. Unlike drought, flooding leads to hypoxic stress. Both stresses cause reduced photosynthetic efficiency, disrupted respiration, and damage to cell membrane integrity. However, flooding stress leads to oxygen deficiency, and plants adapt to the low-oxygen environment by forming adventitious roots and developing aerenchyma. Through the GWAS approach, a study has dissected the genetic architecture of waterlogging responses with 368 inbred maize lines and detected 16 QTLs and 33 candidate genes (Yu et al., 2018a). It has been reported that the group VII ethylene response factor ZmEREB180 functions positively in waterlogging resistance (Yu et al., 2019). ZmCAO1, another positive regulator of waterlogging resistance, has been reported recently, although it has some other roles in photosynthesis (Li et al., 2021c). Ectopically expressing some genes from other species, for instance, HaHB11 and HaOXR2 from sunflowers (Raineri et al., 2022; Torti et al., 2020), could also improve maize waterlogging resistance. As a wild relative to *Zea mays*, *Zea nicaraguensis* is highly tolerant to waterlogging. Researchers have used this germplasm to study the physiological and genetic mechanisms of waterlogging resistance. By using *Zea nicaraguensis*, researchers have found that constitutive aerenchyma formation and radial oxygen loss barrier in roots could be beneficial for waterlogging resistance (Gong et al., 2019; Watanabe et al., 2017). These studies are helpful for our understanding of how maize resists waterlogging, but more genes remain to be cloned for breeding waterlogging-resistant maize in future.

Cold stress response in maize

Cold stress significantly affects maize survival, yield stability, and geographical distribution. Originating from tropical regions, maize is naturally sensitive to low temperatures during early growth stages, particularly in temperate cultivation regions. When temperatures drop below 10°C, maize seedling growth slows considerably, and growth ceases completely between 6°C and 8°C. At these low temperatures, cellular and tissue damages may become irreversible, leading to reductions in plant height, root length, nutrient uptake, chlorophyll content, and photosynthetic efficiency (Allen and Ort, 2001). These effects not only reduce maize yield and quality but also limit its cultivation in high-latitude and high-altitude regions. Therefore, enhancing maize cold tolerance is crucial for enabling early sowing and ensuring stable productivity in the face of accelerated global climate change.

Cold sensing in plants begins with physical changes in plasma membrane (PM) rigidity, which are detected by potential membrane sensors. The structural alterations of sensor proteins could trigger a cascade of secondary messengers, such as reactive oxygen species (ROS) burst and Ca^{2+} flux, which activate the downstream cold signaling pathway (Kerbler and Wigge, 2023). Membrane proteins involved in the perception of cold stress signal trigger Ca^{2+} transients. In rice, the endoplasmic reticulum- and PM-localized Gα subunit interacting protein CHILLING TOLERANCE DIVERGENCE 1 (COLD1) triggers cold-induced Ca^{2+} influx and confers cold tolerance (Ma et al., 2015). Similarly, the COLD1 homolog ZmCOLD1 in maize enhances low-temperature tolerance by regulating Ca^{2+} signaling and hormone responses. However, its precise molecular mechanism remains unclear (Zhou et al., 2023b).

Phosphorylation events mediated by Ca^{2+} signaling are critical to the cold stress response (Ding et al., 2022; Peng et al., 2024; Wang et al., 2021b). In *Arabidopsis*, calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1), a PM-associated serine/threonine kinase, plays a vital role in cold stress response by suppressing the cold-induced activation of MPK3/6 or activating the MEKK1-MKK2-MPK4 signaling cascade (Yang et al., 2010a; Zhao et al., 2017b). Similarly, PM-localized CPK28 senses and decodes cold-induced Ca^{2+} signals to enhance cold tolerance in *Arabidopsis* (Ding et al., 2022). In rice, OsCPK24 positively regulates chilling stress response (Liu et al., 2018b). Interestingly, maize CPKs exhibit negative regulation in cold tolerance. For example, ZmCPK1 directly phosphorylates the ERF3 protein, inactivating it and thereby impairing the cold stress response (Weckwerth et al., 2015). Under cold stress, ZmCPK17 translocates to the nucleus, where it phosphorylates and stabilizes the COLD-RESPONSIVE OPERATION LOCUS 1 (COOL1) transcription factor, ultimately reducing maize cold tolerance (Zeng et al., 2025). The mechanisms by which Ca^{2+} signals are decoded during cold response and how Ca^{2+}-mediated signaling contributes to cold tolerance remain to be determined.

Upon cold stress, mitogen-activated protein kinase (MAPK) cascade plays a critical role in activating and transducing cold signals to regulate cold tolerance in plants (Chen et al., 2021c). In *Arabidopsis*, MEKK1-MEK2-MPK4 positively regulates *DREB1* gene expression and freezing tolerance by antagonizing the MKK4/5-MPK3/6 pathway (Li et al., 2017b; Zhao et al., 2017b). In maize, ZmMKK1, ZmMKK4, and ZmMPK17 confer tolerance to cold stress in transgenic *Arabidopsis* or tobacco plants (Cai et al., 2014; Kong et al., 2011; Pan et al., 2012) (Figure 4B). A recent study identified two clade C MPK subfamily members, ZmMPK2 and ZmMPK8, which negatively regulate maize cold tolerance (Li et al., 2022k; Zeng et al., 2021). The ZmMPK8 activity is activated by cold treatment, phosphorylating and promoting the degradation of ZmRR1 (Zeng et al., 2021). Interestingly, the absence of a 15-residue region containing Ser15 in ZmRR1 inhibits its phosphorylation by ZmMPK8, thereby preventing its degradation via the 26S proteasome pathway (Zeng et al., 2021). Additionally, ZmMPK8 interacts with ZmbZIP68 and phosphorylates Ser250 of ZmbZIP68 to increase its protein stability (Li et al., 2022k). These findings suggest an important role for MPK signaling in the phosphorylation network-mediated transcriptional regulation of maize cold stress response.

Plant responses to temperature changes are primarily regulated by transcriptional signal transduction. It is well established that DREB1 transcription factors act as key regulators in cold stress responses by positively modulating the expression of *cold-responsive gene*s (*COR*s), which are crucial for cold tolerance across various plant species (Shi et al., 2018; Thomashow, 1999). INDUCER OF CBF EXPRESSION 1 (ICE1) has been found to directly promote the expression of *DREB1* genes, enhancing their cold-induced activation in several plant species (Jiang et al., 2022b; Tang et al., 2020; Zhang et al., 2017). In maize, ZmICE1 not only activates *DREB1* genes, but also suppresses key glutamate/asparagine biosynthesis genes, such as *ZmASs*, modulating mitochondrial ROS production and contributing to cold tolerance. Natural variation in the *ZmICE1* promoter at nucleotide position −465 affects its binding affinity to ZmMYB39, which in turn modulates *ZmICE1* expression, further influencing cold stress tolerance (Jiang et al., 2022b). This finding revealed the role of ZmMYB39-ZmICE1-ZmDREB1 transcriptional cascade in both metabolic reprogramming and *COR* gene expression to regulate maize cold tolerance. In addition to ICE1, the transcription of *DREB1* genes is regulated by other transcriptional regulators. For example, ZmRR1 (type-A response regulator 1) enhances cold tolerance by promoting *ZmDREB1* and *ZmCesA* (*Cellulose synthase*) expression (Zeng et al., 2021). Overexpression of *ZmMYB31*, *ZmMYB-IF35* in *Arabidopsis*, was found to upregulate the expression of *DREB1* genes, thereby enhancing the tolerance of transgenic *Arabidopsis* plants to cold and oxidative stresses (Li et al., 2019f; Meng and Sui, 2019). In contrast, ZmbZIP68 acts as a negative regulator by inhibiting *ZmDREB1* expression, with its locus targeted during maize's early domestication (Li et al., 2022k). Additionally, ZmbZIP68 has been shown to directly target and suppress the expression of *ZmHSF21*, a heat shock transcription factor. Intriguingly, ZmHSF21 enhances cold tolerance during both the seedling and germination stages by regulating lipid metabolism homeostasis, independent of the ZmDREB1-mediated cold response pathway (Gao et al., 2024). This finding suggests the importance of accuracy in regulating cold-responsive gene expression for optimal cold tolerance in maize.

Natural variations represent valuable genetic resources for improving maize cold tolerance. Recent advances in GWAS and QTL mapping approaches have identified numerous SNP loci associated with cold tolerance traits such as germination rate, seedling growth, and photosynthetic efficiency (Yi et al., 2021; Zhou et al., 2022c). For instance, integrating multi-omics analysis has identified candidate genes involved in MAPK signal transduction and fatty acid metabolism that are associated with maize germination (Zhang et al., 2020b). Furthermore, QTL-seq approaches have isolated valuable cold tolerance genes (*ZmTSAH1* and *ZmbZIP113*) from the maize wild relatives (*Tripsacum dactyloides* and *Zea perennis*) (He et al., 2023). However, the functional validation of these candidates remains limited. This gap has been addressed by a recent breakthrough study on *COOL1*—a locus encoding bHLH transcription factor in maize by GWAS. The promoter polymorphisms in *COOL1* showed a strong association with cold tolerance variation. The superior $COOL1^{HapA}$ allele contains an A-box motif that is specifically recognized by the cold-inducible bZIP transcription factor ZmHY5, leading to the repression of *COOL1* by cold stress. As a negative regulator of maize cold tolerance, COOL1 could directly suppress the expression of *ZmDREB1* and trehalose biosynthesis genes (*TPS*). Furthermore, cold stress triggers nuclear localization of ZmCPK17 kinase, which phosphorylates and stabilizes COOL1 protein to brake *COR* gene expression. In addition, the evolutionary evidence has shown that the cold-tolerant *COOL1* variant is predominantly distributed in northern high latitudes, providing the possibilities for developing maize varieties to better adapt to cooler climate regions (Zeng et al., 2025). Further studies will employ multi-omics approaches to identifying cold-associated alleles, facilitating the development of "super cold-resilient" maize varieties.

Heat stress response in maize

High temperature is becoming a major threat to maize production (Zhao et al., 2017a). Heat stress (HS) negatively impacts maize by reducing its photosynthetic rate, weakening pollen viability, hindering pollen dispersal, shortening the grain filling duration, and so on, ultimately diminishing yield and quality. The mechanisms of HS response could be related to plant cell wall remodeling, ER stress response, hormone accumulation and oxidative stress, and accelerated transcription and translation of HSPs in maize (Li et al., 2024c; Li et al., 2020i; Liu et al., 2019b; Zhao et al., 2021). Using genetic approaches to improving maize heat tolerance could be a good strategy under the global warming trend.

Cellular stress response

Heat stress creates ER stress in maize and sets off the unfolded protein response (UPR). The transcript levels of *ZmbZIP60* are related to heat tolerance in maize. This gene has undergone selection during maize domestication (Li et al., 2018c). As an important marker for the initiation of UPR, bZIP60 activates the expression of type A HSF-HSFTF13 under high temperature, thereby upregulating the expression levels of HSP genes and enhancing the thermotolerance of maize. Additionally, ZmIRE1 is associated with the splicing of *ZmbZIP60* transcripts (Li et al., 2020i). Recently, it has been demonstrated that ZmbZIP60 is involved in a thermo-protective mechanism by activating the expression of HEAT UP-REGULATED GENE 1 (*ZmHUG1*), a gene encoding ER chaperone protein to prevent protein aggregation (Xie et al., 2022a). Calcium-dependent protein kinases (CDPKs)/CPKs play critical roles in thermotolerance. For example, heat-induced ZmCDPK7 affects the expression of *RBOH*s, *APX1*, *CAT1*, and *HSP*s, interacts with the small heat shock protein sHSP17.4, and phosphorylates sHSP17.4 and RBOHB (Zhao et al., 2021). Heterologous expression of *Arabidopsis thaliana AtGRXS17* in maize improves thermotolerance through increased chaperone function and regulation of heat-responsive genes (Sprague et al., 2022). QTL mapping reveals that HSP101, a member of the HSP100 family, contributes to the thermotolerance of male meiosis in maize (Li et al., 2022i). By negatively regulating stomatal opening under heat stress, the ZmMKK9-ZmMPK20-ZmRIN2 cascade balances transpiration and leaf cooling, thereby enhancing plant thermotolerance (Cheng et al., 2023). The genetic or pharmacological interference with cellulose biosynthesis triggers many different types of responses (Wolf, 2022). The plant cell wall remodeling mediated by *Cellulose synthase A2* (*ZmCesA2*) also contributes to heat stress responses in maize (Li et al., 2024c). Additionally, four genes with a role in calcium signaling are candidates for thermotolerance of seed-set in maize (Gao et al., 2019a).

Hormone accumulation and oxidative stress tolerance

Heat stress can impact maize growth, yield, and productivity by disrupting hormonal balance and inducing oxidative damage. ZmNF-YA3 improves high temperature tolerance through the ABA-related pathway (Su et al., 2018). The mutants of the *NEEDLE1* gene display temperature-sensitive reproductive defects, and this gene was found to affect ROS levels and auxin homeostasis (Liu et al., 2019b). It was shown that ZmCDPK7 participated in thermotolerance by phosphorylating and activating ZmRBOHB, an ROS-producing enzyme (Zhao et al., 2021). Additionally, *ZmHsf11*, a member of class B heat-shock transcription factors (HSFs), decreases heat stress tolerance by down-regulating oxidative stress-associated genes, increasing ROS levels, and decreasing proline content (Qin et al., 2022).

Other transcriptional regulation

The HSFs are critical regulators of heat stress response and thermotolerance (Ohama et al., 2017). ZmHSF20 and ZmHSF4, two B2a heat shock factors, can enhance maize heat tolerance via direct or indirect regulation of the plant cell wall development (Li et al., 2024c). In a recent study, alternative splicing of ZmHSFA2B, a typical A2 class HSF protein, generates a self-regulatory loop that fine-tunes heat stress response in maize. ZmHSFA2B-II interacts with ZmHSFA2B-I, which positively regulates maize heat stress (Song et al., 2025).

Overall, HS-related research in maize is still in its infancy, and many key questions are to be investigated. For example, maize is susceptible to HS, particularly at the reproductive stage, and the impact of HS on maize is closely regulated by associated environmental covariables such as humidity, soil moisture content, and other factors. Nevertheless, so far, there are almost no essential HS stress-related natural alleles or genes detected or potentially to be used to maintain maize yield, particularly at the reproductive stage.

Salt and alkaline stress response

Salt stress represents a major abiotic constraint on maize production. High concentrations of soil salts lead to osmotic stress, ion (Na^+ and Cl^-) toxicity, high pH stress, oxidative stress, and further adverse effects on maize (Cao et al., 2023; Munns et al., 2020; Yang et al., 2023d). Through both forward and reverse genetics approaches, numerous genes involved in maize salt tolerance have been identified (Figure 4C), significantly advancing our understanding of the physiological, metabolic, and genetic mechanisms underlying this trait.

Osmotic stress tolerance

Salt stress decreases the osmotic potential of the rhizosphere, thereby hindering root water absorption. Maize counters salt-induced osmotic stress (SIOS) by increasing its cytosolic osmoticum and reducing leaf transpiration. A recent metabolomics-based assay reveals that ZmCS3 (a citrate synthase involved in TCA cycle), ZmUGT (a glucosyltransferase participating in flavonoid metabolism), and ZmCYP709B2 (a cytochrome P450 enzyme) are associated with SIOS tolerance in maize. Although the exact mechanisms are unclear, favorable alleles of these genes can improve SIOS tolerance in an additive manner (Liang et al., 2021a). ABA-mediated stomatal closure is a major mechanism for transpiration reduction. Several studies have demonstrated the involvement of ABA in the salt tolerance of maize. For instance, the expression of *AtLOS5*, an *Arabidopsis* gene involved in ABA biosynthesis in maize, enhances salt tolerance by boosting root hydraulic conductivity (Zhang et al., 2016a). The transcription factor ZmbZIP4 regulates the expression of several ABA biosynthesis genes and contributes to increased salt tolerance in maize seedlings (Ma et al., 2018a). Additionally, *ZmSTG1* modulates the lipid composition of the thylakoid membrane via the ABA signaling pathway, thereby maintaining photosystem activity and improving salt tolerance (Mei et al., 2023).

Salt ion exclusion

Salt-grown maize plants must transport approximately 98% of the Na^+ that enters the roots back into the surrounding soil, thereby preventing tissue Na^+ from reaching a toxic level. This root-to-soil Na^+ efflux process is mainly regulated by the salt overly sensitive (SOS) pathway (Liang et al., 2024b; Yang and Guo, 2018). Under salt stress, ZmSOS3 senses increased cytoplasmic Ca^{2+} levels, and interacts with and activates ZmSOS2. Following activation, the ZmSOS3-ZmSOS2 complex activates ZmSOS1 through phosphorylation, thereby increasing

Na^+ transport and promoting Na^+ exclusion in roots. In the maize inbred line LH65, a 4-bp frameshift deletion within *ZmSOS1* disrupts its phosphorylation by the ZmSOS3-ZmSOS2 complex, thereby compromising Na^+ efflux and leading to increased salt sensitivity (Zhou et al., 2022b). Additionally, ZmSOS3-like calcium-binding protein 8 (ZmSCaBP8) activates ZmSOS2, enhancing salt tolerance in maize. In contrast, the shaggy/glycogen synthase kinase 3 (GSK3)-like kinases, ZmSK3 and ZmSK4, inhibit salt tolerance by suppressing ZmSOS2 activity (Li et al., 2023c). The Na^+/H^+ antiporter, ZmSOS1, mediates the root-to-soil Na^+ efflux, which relies on the H^+ established by the PM H^+-ATPase (Yang and Guo, 2018). Cao et al. (2020b) showed that under saline-alkaline stress conditions, Ca^{2+} bound to ZmNSA1 (an EF-hand domain-containing protein), triggering its degradation via the 26S proteasome and leading to increased expression of two PM H^+-ATPase genes (*ZmMHA2* and *ZmMHA4*), thereby facilitating Na^+/H^+ antiporter-driven root-to-soil Na^+ efflux. A 4-bp deletion in the 3′ UTR of *ZmNSA1* reduces its translation efficiency and protein abundance, thus promoting maize tolerance to saline-alkaline stress.

The process of retrieving Na^+ from the root xylem vessels serves as a key mechanism for excluding Na^+ from the shoot and enhancing salt tolerance. Three QTLs regulating xylem Na^+ retrieval have been identified: *ZmNC1* (*Na^+ Content 1*) and *ZmNC3* encode the HKT1 family Na^+ transporters ZmHKT1;1 and ZmHKT1;2 (Zhang et al., 2018e; Zhang et al., 2023c); whereas *ZmNC2* encodes the HAK family Na^+ transporter ZmHAK4 (Zhang et al., 2019c). These three QTLs contribute to shoot Na^+ exclusion by retrieving Na^+ from the root xylem vessels. An insertion of an LTR/gypsy retrotransposon that causes a frameshift mutation in ZmHKT1;1 is associated with increased Na^+ accumulation in the shoot. Conversely, a nonsynonymous single-nucleotide polymorphism (SNP) that enhances the Na^+ transport activity of ZmHKT1;2 correlates with reduced shoot Na^+ accumulation (Zhang et al., 2018e; Zhang et al., 2023c). In addition, a 12,586-bp insertion in the first intron of *ZmHAK4* is linked to decreased transcription of *ZmHAK4* and increased Na^+ translocation from roots to shoots (Zhang et al., 2019c). The beneficial alleles of these Na^+-transport genes decrease shoot Na^+ content by up to 70% (Zhang et al., 2023c), suggesting great potential for their application in the breeding of salt-tolerant maize.

Preventing the loading of Na^+ and Cl^- into the stele provides an alternative route for promoting shoot Na^+ exclusion. The Casparian strip (CS) is a specialized structure in the cell wall of root endodermis cells and serves as a barrier that limits the apoplastic movement of water and solutes (Barbosa et al., 2019). Recent findings reveal that the ZmESBL protein plays a crucial role in preventing stele Na^+ loading by mediating reprogramming of the CS barrier under salt conditions (Wang et al., 2022g). In the absence of functional ZmESBL, CS barriers were disrupted, allowing increased transport of Na^+ from roots to shoots, resulting in a salt-hypersensitive phenotype (Wang et al., 2022g). A type-A response regulator, ZmRR1 (response regulator 1), negatively regulates maize salt tolerance by mediating the regulation of Cl^- transport. Under salt stress, ZmRR1 undergoes degradation and releases ZmHP2 (a key mediator of cytokinin signaling) upon ZmRR1 inhibition. This, in turn, activates downstream cytokinin signaling, leading to elevated expression of *ZmMATE29*, which encodes a tonoplast-localized Cl^- transporter. The increased *ZmMATE29* expression facilitates Cl^- sequestration into vacuoles within root cortex cells, thereby reducing Cl^- delivery to the root stele. Notably, a nonsynonymous SNP strengthens the interaction between ZmRR1 and ZmHP2, attenuating the salt-induced transcriptional upregulation of *ZmMATE29* and consequently resulting in heightened salt sensitivity (Yin et al., 2023).

Oxidative stress tolerance

Salt stress frequently leads to the excessive accumulation of ROS, thereby causing oxidative damage in plants. Maintenance of ROS homeostasis is crucial for plants to effectively acclimate to saline conditions (van Zelm et al., 2020). Maize plants carrying the mitochondrial chimeric gene *ORF355* (associated with S-type cytoplasmic male sterility, CMS-S) show enhanced antioxidant enzyme activity and improved salt tolerance (Xiao et al., 2023). Another study revealed that under salt stress conditions, ROS accumulation reduced ZmmiR169q levels, leading to increased transcript levels of *ZmNF-YA8*. Subsequently, ZmNF-YA8 enhances the transcript levels of *ZmPER1* (a gene encoding an antioxidant enzyme) and promotes ROS elimination and salt tolerance (Xing et al., 2022). Additionally, salt-induced ROS accumulation downregulates miR408 levels and increases the transcript levels of *ZmLAC9* and *ZmLAC18*, which enhance salt tolerance by modulating lignin deposition in the cell wall (Qin et al., 2023). A recent study identified *SbAT1*, a gene encoding an atypical G protein γ subunit, as a negative regulator of alkaline tolerance in multiple crops, including sorghum, maize, rice, and millet. Under alkaline stress, SbAT1 interacts with the G protein β subunit, leading to reduced phosphorylation of plasma membrane intrinsic protein 2 (PIP2) aquaporins. This reduction compromises the H_2O_2-exporting activity of PIP2s, resulting in an elevated intracellular H_2O_2 accumulation and increased sensitivity to alkaline stress (Zhang et al., 2023a).

Other salt tolerance mechanisms

The salt tolerance of maize is linked to the uptake and transport of nutrients (such as K^+ and NO_3^-) and other indices. Cao et al. (2019) demonstrated that *qkc3/ZmHKT2*, which encodes a K^+-preferring transporter, functioned as a negative regulator of shoot K^+ content and salt tolerance. A domestication-associated non-synonymous SNP was shown to reduce the K^+ transport efficiency of ZmHKT2, thereby enhancing salt tolerance in modern maize cultivars (Cao et al., 2019). Using survival rate and biomass as phenotypic indices, SAG4 (a protein transport protein), SAG6 (the double-strand break repair protein, MRE11), ZmCLCg (a CLC-family chloride channel), and ZmPMP3 (a PM protein) have also been shown to be involved in the response of maize to salt stress, but the mechanisms and causal variants remain unclear (Luo et al., 2021; Luo et al., 2019b).

Nutrient use efficiency

Nutrient use efficiency (NUE) also represents a complex yet important trait regarding maize genetic improvement. Maize is one of the most widely grown cereals, which requires a substantial amount of fertilizers to obtain high yields. It is estimated that only 30% of the applied nitrogen (N) and phosphorus (P) fertilizers are used by maize in China, and excessive use of N and P fertilizers causes severe environmental pollution. About 20% of China's potassium (K) fertilizer (2.7 Mt) was consumed by maize, which was higher than any other crop.

The application of K fertilizer for maize was about 71 kg ha^{-1} in China, which was much higher than that of the world's average (39 kg ha^{-1}). Therefore, elucidation of the molecular mechanisms of maize nutrient acquisition, assimilation, and remobilization is a prerequisite for the genetic improvement of maize NUE.

N uptake, transport, and metabolism

The initial stage of nitrogen use involves the uptake and transport of nitrogen, including modifying root architecture and adjusting nitrate uptake and transport processes (Figure 4D). The uptake of nitrate by maize roots is mainly mediated by two key transporter families: NPF (NRT1) and NRT2. These two families function as high- and/or low-affinity nitrate transport systems, allowing plants to adapt to varying environmental N availability (Garnett et al., 2013). The maize genes *ZmNPF6.6* and *ZmNPF6.4* are capable of transporting NO_3^- and chloride in *Xenopus oocytes*, but the in-planta function still remains to be elucidated (Wen et al., 2017). ZmNRT1.1B mediates high-affinity nitrate (NO_3^-) uptake in roots and facilitates root-to-shoot NO_3^- translocation. Overexpression of *ZmNRT1.1B* enhances grain yield in modern maize hybrids, even under reduced N fertilizer application (Cao et al., 2024). For ammonium uptake, two rhizodermis-localized proteins ZmAMT1;1a and ZmAMT1;3, are most probably the major high-affinity transporters in maize roots (Gu et al., 2013). Expressions of ZmAMT1s are induced by ammonium availability, and overexpression of ZmAMT1;1a gene in transgenic maize can improve ammonium uptake capacity under low ammonium conditions (Zhao et al., 2018). Besides direct nitrogen uptake by roots, maize plants can establish symbiotic associations with mycorrhizal fungi that capture and deliver nitrogen to roots. A mycorrhiza-specific ammonium transporter ZmAMT3;1, transporting ammonium across the peri-arbuscular membrane, mediates mycorrhiza-dependent nitrogen uptake in maize roots (Hui et al., 2022).

In the maize genome, there are four genes encoding nitrate reductase (NR) and two genes encoding nitrite reductase (NiR). The promoter regions of ZmNR and ZmNiR contain nitrate (NO_3^-) response elements (NREs), which allow their transcription to be activated by nitrate (Liu and Chu, 2023). Nevertheless, the functional roles of these genes in maize remain poorly characterized. Nitrogen remobilization primarily relies on asparagine (Asn)/aspartate (Asp) and glutamine (Gln)/glutamate (Glu). The aforementioned *THP9* gene, encoding asparagine synthetase 4 and contributing to the divergence in protein content between modern maize and its wild relative teosinte, demonstrated a significant role in nitrogen use efficiency, suggesting its potential to enhance crop productivity in nitrogen-limited environments (Huang et al., 2022b)

P uptake and transport

Maize has evolved a series of strategies to adapt to fluctuations in environmental Pi supply, such as increased root biomass or root-to-shoot ratio, secretion of APases and organic acids, and variations of metabolite profiling (Li et al., 2022b; Luo et al., 2019a; Sun et al., 2016). Pi acquisition from soil solutions is an energy-mediated process, mainly via Pi/H^+ symporters such as PHT1 family members. There are thirteen putative *PHT1* genes in the maize genome, and most *ZmPHT1* genes are up-regulated during Pi starvation (Liu et al., 2016a). Among them, ZmPHT1;9 is a mycorrhizal-induced phosphate transporter (Liu et al., 2018a), and ZmPT7 (also named ZmPHT1;1) participates in Pi uptake in roots and Pi redistribution from source to sink leaves, and its Pi transport activity is activated by kinase ZmCK2s (Wang et al., 2020d).

K uptake and transport

Unlike N and P, K is not metabolized and always exists as ion forms (K^+) in plants. Therefore, K^+ uptake and transport in maize depend on the different kinds of K^+ channels and K^+ transporters that show diverse expression profiles (Zhang et al., 2023d). In maize roots, the K^+ transporters ZmHAK5 and ZmHAK1 were reported to play important roles in K^+ acquisition and allocation in maize under low K^+ (LK) stress (Han et al., 2021; Qin et al., 2019). The two K^+ channels in maize leaves, KZM2 and KZM3, are localized at the PM of the guard cells and control stomatal movement. They exhibit distinct channel kinetics. KZM3 functions as an inward K^+ channel, but KZM2 inhibits K^+ currents by forming a heteromeric channel with KZM3 (Gao et al., 2017; Gao et al., 2019b). In maize seeds, the transporter ZmNPF7.9/ZmSUGCAR1 is specifically expressed in the basal endosperm transfer layer, where it contributes to K^+ loading from maternal tissues into the maize endosperm. In addition to K^+, ZmSUGCAR1 transports sucrose and glucose into the endosperm (Yang et al., 2022a). Therefore, ZmSUGCAR1 plays a crucial role in the coordination of potassium and sugar transport during maize grain filling.

N signaling network

The perception of N signals rapidly modulates the activity of multiple cellular components, especially transcription factors, triggering swift transcriptional reprogramming and forming an intricate N regulatory network. As a member of the AGL17-like MADS-box transcription factor family that contains orthologs of *Arabidopsis* ANR1, ZmTMM1 plays important roles in the nitrate-induced lateral root development (Liu et al., 2020h). ZmCHB101, a core component of the SWI/SNF type ATP-dependent chromatin remodeling complex, can impact the binding of ZmNLP3.1 to NREs, negatively regulating the expression of nitrate transport genes ZmNRT2.1 and ZmNRT2.2 (Meng et al., 2020). The transcription factor ZmEREB97, which is primarily expressed in the primary root and lateral root primordia, plays a key role in NO_3^- acquisition by directly binding to and regulating the expression of six *ZmNRT* genes (Wu et al., 2024). Additionally, the maize gene *ZmNRG2.7* is strongly upregulated by NO_3^- in leaves. Overexpression of *ZmNRG2.7* in the *atnrg2* mutant rescues defects in NO_3^- signaling and metabolism, leading to increased biomass and improved nitrate use efficiency (Li et al., 2024b).

Several NLP members in maize, such as ZmNLP3.1/5/6/8, are found to regulate primary NO_3^- signaling and assimilation. ZmNLP5 plays a central role in the nitrogen signaling and metabolic network, exhibiting a rapid induction in response to NO_3^- treatment (Ge et al., 2020). Transcriptome analysis indicates that ZmNLP3.1 and ZmNRT1.1B function within the same signaling cascade, cooperatively regulating NO_3^--responsive genes (Cao et al., 2024). ZmNLP3.2 promotes root biomass accumulation under low N conditions by attenuating ZmARF19-mediated activation of ZmIAA14 (Wang et al., 2024c). Underlying a QTL associated with leaf senescence and nitrogen remobilization, a transcription factor ZmNAC7 functions as a negative regulator of nitrogen allocation from vegetative source to reproductive sink tissues (Zhang et al., 2019a). In maize seeds, the prolamin-box-binding factor 1 (ZmPBF1) binds to multiple genes involved in nitrogen (N) and carbon storage product biosynthesis, regulating nitrogen and carbon metabolism during endosperm development in a nitrogen-dependent manner (Ning et al., 2023).

P signaling network

Transcriptome reprogramming is an important way for maize to respond to Pi starvation (Lin et al., 2013; Sun et al., 2016). To date, a series of Pi-starvation response genes and regulators have been characterized in maize. Maize microRNA399 (ZmmiR399) is strongly induced during Pi starvation, and the *MIR399b*-overexpressing lines accumulate excessive P contents in shoots and display Pi-toxicity phenotypes (Du et al., 2018). Three low-Pi induced *ZmPHT1* genes, *ZmPHT1;1*, *ZmPHT1;3*, and *ZmPHT1;13*, are targets of ZmmiR399, and a Pi-deficiency-induced long-noncoding RNA *PILNCR2* modulates ZmmiR399-guided cleavage of *ZmPHT1s* (Wang et al., 2023k). ZmmiR399 also post-transcriptionally represses *ZmPHO2*, which encodes a ubiquitin-conjugating E2 enzyme (UBC), and the Pi-deficiency-induced long-noncoding RNA *PILNCR1* inhibits ZmmiR399-guided cleavage of *ZmPHO2* (Du et al., 2018). Transcription factor INDETERMINATE1 (ID1) directly inhibits the transcript of ZmmiR399, alleviating the repression of *ZmPHO2* by ZmmiR399 (Du et al., 2018; Wang et al., 2023h). Two NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR1/HYPERSENSITIVE TO LOW Pi-ELICITED PRIMARY ROOT SHORTENING1 proteins (NIGT1/HRS1s), *ZmNIGT1.1* and *ZmNIGT1.2*, are transcriptionally up-regulated under Pi-deficient conditions, and they modulate Pi and nitrate acquisition via regulating the transcripts of *ZmPHT1s* and *NPFs* to maintain the P and N balance in maize (Wang et al., 2020d). Transcription factors ZmPHR1 and ZmPHR2 are two homologs of AtPHR1 and OsPHR2 (Zhong et al., 2020b). ZmPHR1 modulates the transcripts of numerous Pi-starvation response genes (Tian et al., 2024b), such as Pi transporter genes *ZmPT4* and *ZmPT7*, amino acid transporter genes *ZmAAP2* and *ZmLHT1*, and carotenoid cleavage dioxygenase gene *ZmCCD10a* (Tian et al., 2024b; Wang et al., 2021d; Zhong et al., 2020b), suggesting that ZmPHR1 plays an important role in maize adaption to Pi deficiency

K signaling network

CIPK activity is controlled by CBL proteins, Ca^{2+}, potential protein kinases, and phosphatases. In maize, the specificity of CBLs and CIPKs is a key factor in the regulation of K signaling pathways. For example, K^+ transporter ZmHAK5 can improve K^+ uptake capacity in maize, whose activity is regulated by ZmCBL-ZmCIPK complexes (Qin et al., 2019). The ZmCBL1-ZmCIPK23 complex phosphorylates and activates ZMK1. The constitutive expression of ZMK1/ZmCIPK23 in shoots disrupts K^+ homeostasis in leaves, which leads to an oversensitive phenotype (Han et al., 2021). ZmCPK35 and ZmCPK37, two calcium-dependent protein kinases, modulate the channel activity of KZM3 (Li et al., 2022h).

Perspective of abiotic stress tolerance and NUE

Accumulative research demonstrates that abiotic stressors and the availability of nutrient exert fundamental impacts on maize growth and yields, and there is a great demand on genetic enhancement of the yield stability and NUE in maize. In future research, efforts should be invested in identifying key genes/alleles with significant breeding value, and comprehensive multi-omics data and artificial intelligence should be extensively employed to decipher mechanism and propose the strategy to break the trade-off between the stress resistance and grain yield. In addition, emerging evidences show that exogenous application of plant growth regulators (PGRs), including metabolites, could increase maize drought tolerance (Ma et al., 2022a; Zhang et al., 2021b), and inoculation of rhizobiome enhances maize abiotic stress tolerance (Zhang et al., 2022d). Besides cloning key genes for maize stress-tolerance breeding, investigating how PGRs and rhizobiome regulate maize stress responses and producing commercial bio-agents or biopesticides to help crops cope with environmental adversity would be a promising direction for future study.

Although substantial progress has been made in understanding the mechanism underlying NUE and its regulatory networks in maize, NUE improvement promoted by this knowledge is still limited. There are four key questions that require special attention in the future. First, clues for N, P, and K sensing are still missing. How maize senses these nutrient signals is the most urgent question to be addressed; however, this is challenging due to limited research approaches currently available. Second, fine-mapping QTLs associated with NUE in maize has remained challenging, largely due to the complexities involved in phenotyping. It is thus necessary to develop new measuring techniques and methods to capture precise phenotyping of NUE-related traits. Third, to improve NUE through genetic engineering, research should focus on discovering genes that suppress NUE, allowing precise genome editing instead of overexpression strategies. Finally, many NUE-related alleles were largely lost in modern maize cultivars due to the overloading of fertilizer. Retracing more beneficial alleles from wild relatives or maize landraces would be an inspiring strategy for future maize breeding toward meeting both yield and feed demand in a more sustainable manner.

Insights into maize resistance to diseases and pests

The major diseases/pests and their damages

Diseases and pests are important limiting factors of maize production in China, which cause about 10 million tons of corn loss every year. In the meanwhile, they lead to a decline in quality and food security (Wang et al., 2010). In recent years, the occurrence and severity of diseases and pests have been increasing, with a global level of yield loss estimated of 22.5% for maize (Deutsch et al., 2018; Savary et al., 2019). In China, there are more than 20 kinds of diseases and pests with high frequency and severe damage. The major maize diseases include northern corn leaf blight (NCLB), southern corn leaf blight (SCLB), southern corn rust (SCR), grey leaf spot (GLS), Curvularia leaf spot (CLS), white spot, stalk rot, ear rot, head smut, common smut, banded leaf and sheath blight, and maize rough dwarf (Figure 5). These diseases occur all year round or intermittently in different ecological zones and often lead to substantial economic losses at the household and national levels. The outbreaks of NCLB and GLS often cause susceptible maize varieties to experience yield declines of 10%–50% and 10%–60%, respectively, in the northeast and southwest regions of

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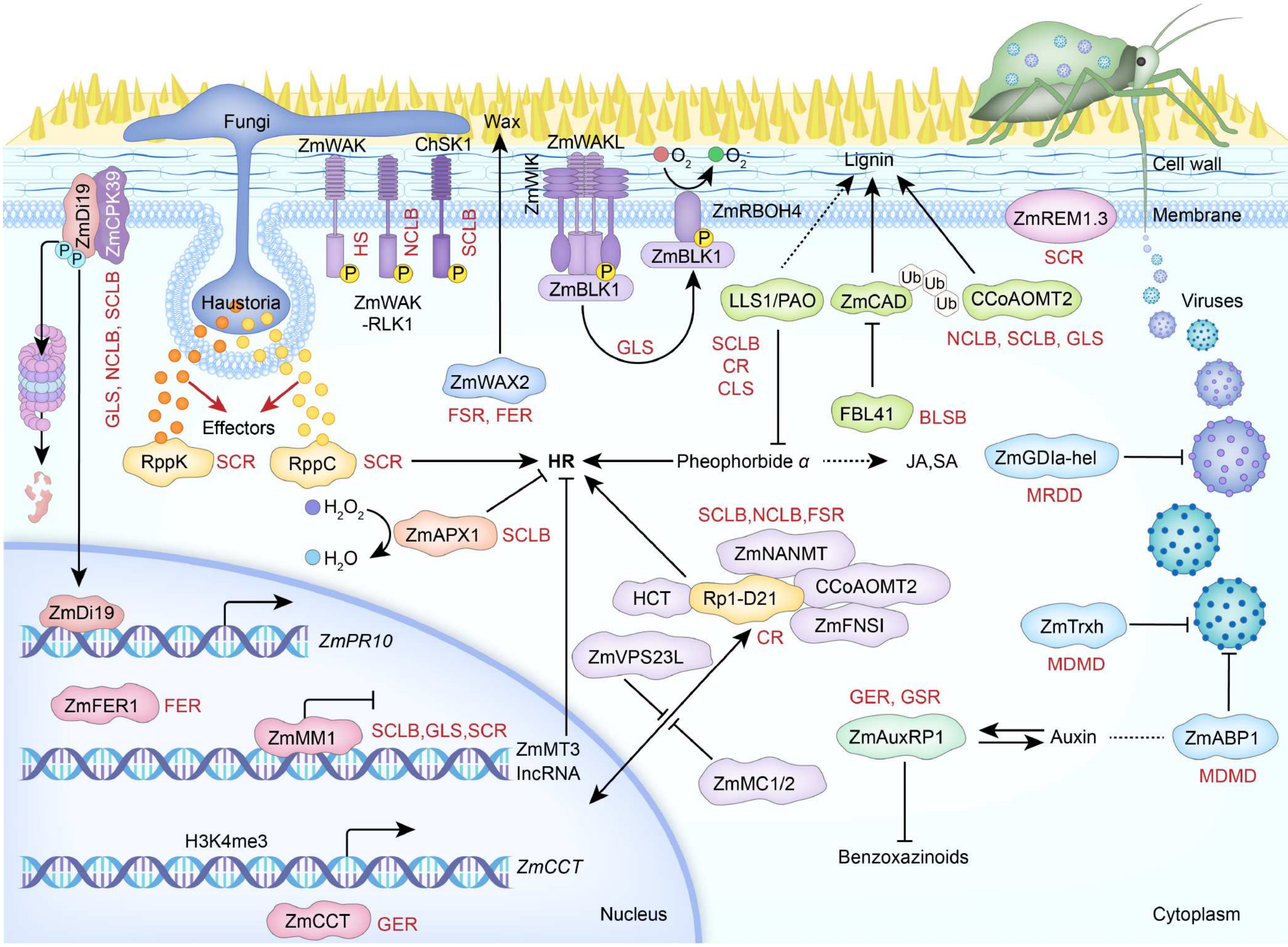

Figure 5. Current progress on maize disease resistance genes in China. This figure illustrates maize disease resistance genes that have been cloned and confirmed, and the underlying mechanisms of disease resistance have been uncovered by scientists in China. BLSB, Banded leaf and sheath blight; CLS, *Curvularia* leaf spot CLS; CR, common rust CR; FER, *Fusarium* ear rot; FSR, *Fusarium* stalk rot; GER, *Gibberella* ear rot; GLS, grey leaf spot; GSR, *Gibberella* stalk rot; HS, head Smut; MDMD, maize dwarf mosaic disease; MRDD, maize rough dwarf disease; NCLB, northern corn leaf blight; SCLB, southern corn leaf blight; SCR, southern corn rust. These proteins are subdivided into several categories: (i) Membrane-associated proteins, including ZmCPK39, ZmWAK, ZmWAK-RLK1, ChSK1, ZmREM1.3, ZmWAKL, and ZmWAKL's interacting protein ZmWIK and ZmBLK1, which confer resistance to HS, NCLB, SCLB, SCR, and GLS, respectively. (ii) NLR-type R proteins RppK and RppC, which confer resistance to SCR by recognizing the effectors AvrRppK and AvrRppC secreted by the fungal pathogens. (iii) The NLR-type R protein Rp1-D21, which confers a hypersensitive response (HR) and enhanced resistance to CR. Related proteins repressing Rp1-D21-mediated HR include ZmVPS23L, ZmMC1/2, HCT, CCoAOMT2, ZmFNSI, and ZmNANMT. ZmNANMT also confers resistance to SCLB, NCLB, and FSR. (iv) Proteins involved in secondary metabolism, including the lignin biosynthetic enzyme ZmCAD and its regulator FBL41 that confers resistance to BLSB, CCoAOMT2 that confers resistance to NCLB, SCLB and GLS, the wax biosynthetic protein ZmWAX2 that confers resistance to FSR and FER, and the chlorophyll degradation enzyme LLS1/PAO that regulates resistance to SCLB, CR, and CLS. (v) The nucleus-localized proteins ZmFER and ZmCCT that confer resistance to FER and GER, respectively, and ZmMM1 that confers resistance to SCLB, GLS, and SCR by regulating the transcription of *ZmMT3*, which transcribes a long non-coding RNA (lncRNA). (vi) ZmAuxRP1 confers resistance to GER and GSR by controlling benzoxazinoid levels. (vii) ZmGD1a-hel, ZmTrxh, and ZmABP1 confer resistance to viruses, including MRDD and MDMD, which are transmitted by insects. Proteins of each category are indicated by different colors.

China. In the Huang-Huai-Hai region, the epidemics of SCLB and SCR have caused corn losses of 10%–30% and 10%–60%, respectively (Lu et al., 2020). Additionally, a new maize leaf disease called white spot, caused by *Epicoccum latusicollum*is, has become increasingly severe in the southwest of China in recent years (Zhang, 2022). *Pythium* stalk rot (PSR) and *Gibberella* stalk rot (GSR), mainly caused by *Pythium inflatum* and *Fusarium graminearum*, respectively, are soil-borne diseases that severely affect maize production. The incidence of stalk rot for susceptible varieties can be as high as 50%–100%, leading to yield losses of 30%–50% in epidemic years (Duan et al., 2019; Khokhar et al., 2014). Ear rot, caused by a variety of fungi, is one of the most destructive and widespread diseases of maize, often leading to a considerable reduction in yield and quality (Duan et al., 2022a; Lanubile et al., 2017; Zhou et al., 2018). Besides, the pathogenic fungi can produce dangerous mycotoxins such as fumonisins, trichothecenes, and aflatoxin that are toxic to humans and animals (Duan et al., 2016; Xia et al., 2022).

The main pests in maize are the Asian corn borer (ACB) (*Ostrinia furnacalis*), fall armyworm (*Spodoptera frugiperda*), oriental armyworm (*Mythimna separata*), yellow peach moth (*Conogethes punctiferalis*), green corn aphid (*Rhopalosiphum maidis*), and others. The ACB is one of the most destructive corn pests widely distributed in China, and it has seriously reduced corn production and quality, posing a threat to food security. The ACB feeding and tunneling inside the stem and ear have caused a 10%–30% loss of annual maize production. Furthermore, it increases the risk of ear rot incidence and severity. The green corn aphid has been rampant in recent years, and the number of aphids per plant reaches 10,000–25,000 in severe fields, resulting in a yield loss as high as 30%. The fall armyworm (FAW) is an important migratory agricultural pest worldwide, which has invaded extensive areas since 2016 and now poses a severe threat to food security. This pest has caused an annual yield reduction of 8.3–20.6 million tons of corn in 12 African countries, with the economic losses ranging from $2.48 billion to $6.19 billion (Day et al., 2017; Eschen et al., 2021). FAW arrived in Yunnan, China, in December 2018 and spread to 26 provinces within a year, exhibiting a strong preference for infesting corn and hence severely threatening corn production in China (Sun et al., 2021b).

Qualitative disease resistance

Plant disease resistance is typically recognized as either qualitative resistance or quantitative resistance. Qualitative resistance is usually controlled by one single resistance (*R*) gene or a few major *R* genes with a large effect, providing complete or near-complete resistance. Therefore, qualitative resistance traits tend to segregate in a Mendelian manner (Corwin and Kliebenstein, 2017; Nelson et al., 2018). Most *R*-genes underlying qualitative resistance encode nucleotide-binding, leucine-rich repeat (NB-LRR or NLR) immune receptor proteins. Canonical NLRs comprise an N-terminal coiled-coil (CC) domain or a Toll/interleukin 1 receptor (TIR) domain, a middle NB-ARC (APAF1, certain *R* gene products, and CED-4) domain, and a C-terminal LRR domain (Jones and Dangl, 2006; Sun et al., 2020c). NLRs can directly or indirectly recognize pathogen-secreted effectors to confer effector-triggered immunity (ETI), and the recognition often triggers the hypersensitive response (HR), a rapid localized cell death at the pathogen infection sites (Balint-Kurti, 2019; Sun et al., 2020c).

Maize contains fewer than 150 NLR proteins, which is lower than other cereal crops (Sarris et al., 2016). Recently, NLRs in 26 founder lines of the NAM population were explored, and it was found that NLRs exhibited very high sequence diversity and presence/absence variation among different NAM founder lines, with a large NLR cluster on chromosome 10 representing a diversity hotspot (Thatcher et al., 2023a).

One classic example of NLRs systematically investigated in maize is the *Rp1* locus on chromosome 10. This locus consists of a cluster of tandemly repeated NLR-encoding genes that confer resistance to common rust caused by specific races of *Puccini sorghi* (Hu et al., 1996; Hulbert, 1997). The *HRp1-D* haplotype of this locus contains *Rp1-D*, a well-known common rust resistance gene, and eight paralogs from *Rp1-dp1 to Rp1-dp8* (Sun et al., 2001). Intragenic recombination between *Rp1-D* and *Rp1-dp2* generated a chimeric variant *Rp1-D21*, which combines the N-terminus of *Rp1-dp2* with the C-terminal LRR region of *Rp1-D* (Smith et al., 2010; Sun et al., 2001). Maize lines carrying *Rp1-D21* exhibit a spontaneous lesion mimic phenotype and confer a nonspecific response to several rust species (Hu et al., 1996; Smith et al., 2010). The activities of NLRs are usually determined by the intra-molecular and inter-molecular interactions. The structural basis of how Rp1-D21 confers autoactivity has been elucidated (Wang et al., 2015b). The CC domain of Rp1-D21 (CC_{D21}) confers autoactivity, and the NB-ARC domain interacts with CC_{D21} to suppress its activity. The recombinant LRR domain of Rp1-D21 competitively interacts with the NB-ARC domain, relieving CC_{D21} to confer HR in Rp1-D21 (Wang et al., 2015b). The severity of Rp1-D21-mediated HR depends on temperature and genetic background (Chintamanani et al., 2010). *Rp1-D21* has been used as an effective tool to identify candidate genes modulating Rp1-D21-mediated HR and maize disease resistance. GWAS identified genes encoding homologs of hydroxycinnamoyltransferase (HCT) and caffeoyl CoA *O*-methyltransferase (CCoAOMT), two enzymes in the lignin biosynthesis pathway, as candidate genes (Olukolu et al., 2014). HCT1806, HCT4918, and CCoAOMT2 were further functionally verified to interact with CC_{D21} in suppressing Rp1-D21-mediated HR (Wang and Balint-Kurti, 2016; Wang et al., 2015a). Interestingly, ZmFNSI-1 and ZmFNSI-2, two homologs of flavone synthase I (FNSI) in the flavone biosynthesis pathway, also interact with HCT and suppress Rp1-D21-mediated HR (Zhu et al., 2020c). Additionally, the maize nicotinate *N*-methyltransferase (ZmNANMT), a homolog of *Arabidopsis* NANMT responsible for the conversion of nicotinate to trigonelline, interacts with CC_{D21} and suppresses Rp1-D21-mediated HR, forming a complex with HCT1806 and CCoAOMT2 (Liu et al., 2021d). Interestingly, putative enzymes from different pathways (lignin biosynthesis, flavone biosynthesis, and nicotinate pathways) form complexes in modulating NLR activity. All these proteins act as negative regulators in Rp1-D21-mediated HR, suggesting that they are susceptibility genes in maize.

The proper subcellular localization is critical for NLR activity. Rp1-D21 and CC_{D21} are predominantly localized in the cytoplasm and the nucleus, and both localizations are required for their autoactivity (Wang and Balint-Kurti, 2015). ZmVPS23L and its homolog ZmVPS23, components of the endosomal sorting complex required for transport (ESCRT) machinery, are predominantly localized in the endosomes (Sun et al., 2023b). They suppress Rp1-D21-mediated HR likely through a physical interaction with CC_{D21} and relocate CC_{D21} from the nucleo-cytoplasm to the endosomes (Sun et al., 2023b). Metacaspases (MCs) belong to a cysteine protease family, structurally related to metazoan caspases (Uren et al., 2000). ZmMC1 and ZmMC2, but not ZmMC9, display a punctate distribution and physically associate with CC_{D21} to suppress Rp1-D21-mediated HR (Luan et al., 2021). In summary, the newly identified NLR regulators ZmVPS23L/ZmVPS23 and ZmMC1/ZmMC2 negatively modulate Rp1-D21-mediated HR, likely by changing the subcellular localization via physical interaction.

SCR is caused by *Puccinia polysora* Underw., and at least nine resistance quantitative trait loci (*RppC*, *RppCML470*, *RppD*, *RppM*, *RppP25*, *RppQ*, *RppS*, *RppS313*, and *RppK*) with major effect on SCR have been reported in maize (Sun et al., 2021a). Recently, three of them (*RppC*, *RppK*, and *RppM*) have been cloned using a map-based cloning strategy, and all of them encode typical CC-NLR proteins (Chen et al., 2022b; Deng et al., 2022; Wang et al., 2022d). Screening of the pathogen effectors results in the identification of AvrRppC and AvrRppK, which interact with RppC and RppK, respectively. Both AvrRppC and AvrRppK contain predicted secretion signal peptides but lack any other known structural domains (Chen et al., 2022b; Deng et al., 2022). Variation in AvrRppC determines RppC resistance against SCR. RppC confers high resistance to the prevalent isolate PP.CN1.0, but shows moderate resistance to PP.CN2.0 and susceptibility to PP.CN3.0 (Deng et al., 2022). AvrRppK can suppress chitin-triggered immunity to increase susceptibility to SCR, but elicit effector-triggered immunity (ETI) when interacting with RppK. Interestingly, different from AvrRppC, AvrRppK is conserved and has no sequence variation in all examined isolates, which leads to high resistance against multiple *P. polysora* isolates (Chen et al., 2022b).

Another example is *Rxo1*, which also encodes an NLR protein in maize. *Rxo1* confers nonhost resistance to *Xanthomonas oryzae pv. oryzicola*, which causes bacterial streak disease in rice (Zhao et al., 2005). It also controls resistance to *Burkholderia andropogonis*, which causes bacterial stripe in sorghum and maize (Zhao et al., 2005; Zhao et al., 2004b). Some isolates of both bacteria induce HR in the maize lines containing *Rxo1*, and genetic control of the HR to both bacteria segregated as a single dominant gene (Zhao et al., 2004b). Rxo1 interacts with the effector avrRxo1 secreted from *X. oryzae pv. oryzicola* to induce a typical HR in maize leaves (Zhao et al., 2004a; Zhao et al., 2004b).

Most NLR genes provide race-specific resistance to pathogens. However, in maize, the CC-NLR proteins mentioned earlier mostly contribute to quantitative resistance rather than qualitative resistance. This is likely because disease phenotypes are often evaluated under field conditions, where a mixture of different pathogen races exists. Due to the complexity of pathogens and environmental effects, finding a typical qualitative resistance gene for most maize diseases is challenging.

Quantitative disease resistance

Quantitative disease resistance (QDR) often exhibits partial but more durable and non-race-specific resistance, and has been extensively utilized in maize breeding to control diseases (Gou et al., 2023; Nelson et al., 2018). A large number of studies have been performed to characterize disease resistance QTL against different pathogens in maize (Gupta et al., 2023; Zhu et al., 2021). Due to the complex inheritance of QDR, only a few QDR genes have been functionally characterized in maize, most of which confer quantitative resistance to fungal pathogens and viruses. *ZmWAK* encodes a cell-wall-associated kinase consisting of a cytoplasmic serine/threonine kinase domain, a calcium-binding epidermal growth factor (EGF_CA) domain, and an extracellular galacturonan-binding (GUB) domain. It confers quantitative resistance to *Sporisorium reilianum*, the causal agent of head smut in maize (Zuo et al., 2015). *ZmWAK* is expressed highly in the mesocotyl in the seedling stage to prevent the spreading of *S. reilianum* through the plant (Zuo et al., 2015). *ZmWAK* has been introgressed into different backgrounds to improve head smut resistance for both inbred lines and hybrids (Zhao et al., 2012). Interestingly, *ZmWAKL*, encoding a cell-wall-associated receptor kinase-like protein, was recently identified as the causative gene at the major QTL against gray leaf spot in maize (Zhong et al., 2024). ZmWAKL interacts with a leucine-rich repeat immune-related kinase ZmWIK and phosphorylates the receptor-like cytoplasmic kinase (RLCK) ZmBLK1, triggering a ROS burst by phosphorylating ZmRBOH4 (Zhong et al., 2024). The maize ZmWAKL-ZmWIK-ZmBLK1-ZmRBOH4 receptor/signaling/executor module involved in GLS resistance is one of the few elucidated modules of the QDR molecular mechanism in maize. Another GLS QDR gene was also characterized as an RD-WAK gene, *ZmWAK02* (Dai et al., 2024). A wall-associated receptor-like kinase gene *ZmWAK-RLK1* is underlying a major northern leaf blight resistance locus *Htn1* (Hurni et al., 2015). *Htn1* could reduce fungal penetration into host tissues and reduce benzoxazinoid secondary metabolites (Yang et al., 2019b). *Ht2* and *Ht3* have been found to be identical and allelic to *Htn1* (Yang et al., 2021a). Recently, Ht1 has been identified as a CC-NLR, distinguishing it from other NLB resistance genes (Thatcher et al., 2023b).

Isolated maize QDR genes have been shown to be involved in diverse processes with a range of mechanisms (Gou et al., 2023). An F-box protein gene, *ZmFBL41*, involved in the E3 ligase proteasome system, is a susceptibility gene targeted by *Rhizoctonia solani* in maize. Mutation of *ZmFBL41* inhibits degradation of ZmCAD, leading to the accumulation of lignin and improved resistance to banded leaf and sheath blight (Li et al., 2019h). *ZmTrxh* and *ZmABP1*, underlying *SCMV1* and *SCMV2*, which encode an atypical h-type thioredoxin protein and an auxin-binding protein, respectively, confer QDR to sugarcane mosaic virus (SCMV) in maize (Leng et al., 2017; Liu et al., 2017b). Both ZmTrxh and ZmABP1 function as molecular chaperones. An ascorbate peroxidase ZmAPX1 has been shown to confer QDR to southern leaf blight by decreasing H_2O_2 accumulation and activating the JA-mediated defense signaling pathway (Zhang et al., 2022a). Some NLR genes were identified as QDR genes in maize, such as *Rcg1*, which confers resistance to anthracnose stalk rot (Broglie et al., 2006). *ZmFER1* is a homolog of the wheat *TaHRC* gene, encoding a histidine-rich calcium-binding protein, which suppresses the calcium-mediated immune response and triggers wheat *Fusarium* head blight susceptibility (Chen et al., 2022c; Li et al., 2019c; Liu et al., 2022b). Mutation of *ZmFER1* could improve resistance against *Fusarium* ear rot in maize (Liu et al., 2022b). The *ZmWAX2* gene confers quantitative resistance to *F. verticillioides*, which causes seed rot and stalk rot in maize. A lack of *ZmWAX2* compromises maize resistance to *F. verticillioides* by reducing cuticular wax deposition, while the *ZmWAX2* overexpression lines show significantly increased immunity to *F. verticillioides* (Ma et al., 2023b).

Transposon insertions in functional genes have been found to be associated with QDR to different diseases. A *CACTA*-like transposon insertion in the promoter of a CCT domain-containing gene *ZmCCT* causes quantitative disease susceptibility to *Gibberella* stalk rot through selective depletion of H3K4me3 to suppress the pathogen-induced *ZmCCT* expression (Wang et al., 2017a). The same variation is also responsible for photoperiod response in maize, which accelerates maize adaptation to temperature regions (Yang et al., 2013). A helitron transposon insertion in *ZmGDIα* causes quantitative recessive resistance to maize rough dwarf disease by perturbing vesicle trafficking to restrict virus spreading (Liu et al., 2020f). A harbinger-like transposable element insertion in the first exon of *ChSK1*, a susceptibility gene encoding a leucine-rich repeat receptor kinase, confers quantitative recessive resistance to southern corn leaf blight (Chen et al., 2023a).

In some cases, QDR genes confer resistance to multiple diseases. A plastid stroma-localized auxin-regulated protein ZmAuxRP1 confers QDR to *Gibberella* stalk rot and *Fusarium* ear rot (Ye et al., 2019). Silencing of *ZmAuxRP1* reduces the biosynthesis of indole-3-acetic acid (IAA) and promotes benzoxazinoid formation to increase resistance (Ye et al., 2019). *ZmABP1* confers resistance to sugarcane mosaic virus, maize dwarf mosaic virus, and wheat streak mosaic virus (Leng et al., 2017). Though significant progress has been made over the past decades, our understanding of the mechanisms underlying QDR is still lacking in maize.

Broad-spectrum and multiple disease resistance

With the escalating threats posed by various maize diseases, there is a growing need for broad-spectrum resistance (BSR) and multiple disease resistance (MDR) in the maize breeding program. BSR provides resistance against multiple races of a specific pathogen, while MDR confers resistance against various diseases caused by different pathogens. Therefore, identifying loci or genes for BSR or MDR holds great value for maize resistance improvement. To date, two BSR genes (*Rp1-D21*, and *RppK*) and six MDR genes (*ZmCCoAOMT2*, *ZmMM1*, *LLS1*, *ZmNANMT*, *ZmPropep1*, and *ZmCPK39*) have been cloned in maize (Chen et al., 2022b; Huffaker et al., 2011; Li et al., 2022d; Li et al., 2023g; Smith et al., 2010; Wang et al., 2021a; Yang et al., 2017b; Zhu et al., 2024). These gene discoveries hold great promise to enhance maize resistance for sustainable and resilient agricultural practices. *Rp1-D21* and *RppK* encode CC-NBS-LRR proteins, but they show different functions and mechanisms. The autoimmunity activated by Rp1-D21 contributes to nonspecific resistance against the common rust fungus *Puccinia sorghi* (Smith et al., 2010). In contrast, RppK confers maize resistance against multiple races of *P. polysora* (the causal agent of southern corn rust) through recognizing the core effector AvrRppK, which is widely distributed and 100% conserved in all tested *P. polysora* isolates (Chen et al., 2022b). Unlike *Rp1-D21* that suppresses plant growth, *RppK* presents great values for breeding as it does not affect major agricultural traits in the absence of SCR, and it significantly increases yield without affecting other major agricultural traits in the presence of SCR (Chen et al., 2022b).

The six MDR genes encode different proteins and exhibit different resistance mechanisms. *ZmCCoAOMT2*, encoding a caffeoyl-CoA *O*-methyltransferase, confers quantitative resistance against southern leaf blight (SLB), northern leaf blight (NLB), and gray leaf spot (GLS); and its resistance is mainly associated with the accumulation of lignin (a metabolite from the phenylpropanoid) and oxylipins (a metabolite from the lipoxygenase pathway) (Yang et al., 2017b). ZmMM1, a MYB-transcription repressor, positively regulates resistance against NLB, GLS, and SCR by enhancing ROS accumulation. This is achieved by directly suppressing the transcription level of *ZmMT3* by ZmMM1. Moreover, the variation in the 3′ UTR region of *ZmMM1* in the $ZmMM1^{C117}$ haplotype activates stronger resistance responses by enhancing its translation level than those in the $ZmMM1^{Mo17}$ haplotype (Wang et al., 2021a). *LETHAL LEAF SPOT1* (*LLS1*) encodes the pheophorbide *a* oxidase (PAO) that converts pheophorbide *a* to red chlorophyll catabolites, and its loss-of-function mutants *lls1* and *les30* display lesion mimic phenotypes and are resistant to multiple necrotrophic and biotrophic pathogens, including *Cochliobolus heterostrophus*, *Puccinia sorghi*, *Colletotrichum graminicola*, and *Curvularia lunata* (Wakker) Boed (Li et al., 2022d; Simmons et al., 1998; Yang et al., 2004). Further, the MDR conferred by *lls1* and *les30* mutants is associated with the over-accumulation of phytohormones, jasmonic acid (JA), and salicylic acid, as well as the phytoalexins phenylpropanoids, lignin, and flavonoids (Li et al., 2022d). *ZmNANMT*, encoding a nicotinate N-methyltransferase, was identified as a suppressor of *Rp1-D21*-mediated HR. Its knockout mutants showed significantly enhanced resistance against SLB, NLB, and stalk rot compared with wild type plants with no fitness cost (Li et al., 2023g). The *ZmPROPEP1* gene encodes the precursor of peptide ZmPep1. Pretreatment with ZmPep1 prior to infection promotes the biosynthesis of hormones JA and ethylene, as well as transcription levels of defense genes. Consequently, it significantly enhances maize resistance against southern leaf blight and anthracnose stalk rot (Huffaker et al., 2011). Notably, a recent study reported that six non-conventional peptides (NCPs) in maize showed broad-spectrum antifungal activity, indicating that peptides might play important roles in MDR (Tian et al., 2021b). *ZmCPK39*, encoding a calcium-dependent protein kinase, negatively regulates maize resistance against GLS, NLB, and SLB. Phosphorylation of the transcription activator ZmDi19 by ZmCPK39 promotes its degradation and results in the reduced expression level of its target gene *ZmPR10*. The resistance allele $ZmCPK39^{Y32}$ exhibits a lower transcription level than the susceptible allele $ZmCPK39^{Q11}$ and enhances resistance responses by increasing the accumulation of ZmDi19 and ZmPR10 after pathogen infection (Zhu et al., 2024).

In summary, ROS accumulation, defense gene expression, and biosynthesis of secondary metabolites are generally activated in BSR and MDR genes. This suggests that employing the same or similar strategies by gene-editing or other means could improve maize BSR and MDR.

Herbivore insect resistance

In China, Asian corn borer (*Ostrinia furnacalis*) and corn leaf aphid (*Rhopalosiphum maidis*) are the two major pests of maize, causing significant yield and quality losses (Figure 6). Current pest control relies heavily on pesticides, which raise concern about human food safety and insect resistance (Guo et al., 2019). Increasing maize resistance to these pests is a sustainable and effective strategy. Thus, in the effort to breed insect-resistant maize varieties, it is crucial to identify insect resistance genes and dissect their molecular mechanisms.

In recent decades, a number of studies have been conducted to isolate QTLs involved in insect resistance in maize. For example, in a QTL analysis of ACB resistance using the H21×Mo17 F_2 and F_3 populations, nine QTLs were found to be associated with the degree of infestation and the number of stalk holes (Yu et al., 2003). In the analysis of a Zi330×K36 population, 14 QTLs related to the resistance to ACB were detected (Yu et al., 2007). Additionally, several QTL loci for aphid resistance were also identified in other studies (Betsiashvili et al., 2015; Meihls et al., 2013; Song et al., 2017; Tzin et al., 2015; Wang et al., 2023f).

Plants and insects engage in a co-evolutionary "arms race", during which plants have evolved various defensive strategies to cope with the constant threat from various pests. In maize, herbivore-induced plant volatiles (HIPVs) can mediate tritrophic defense and prime neighboring maize against *Ostrinia furnacalis* (Foba et al., 2023). MAPKs are among the earliest-activated, fast-responding components of plants to herbivores. Knocking out *ZmMPK6* reduces ethylene levels, while *ZmMPK6* silencing increases DIMBOA and DIMBOA-Glc levels. Further analysis indicates that ZmMPK6 likely mediates the transcriptional regulation of *BX1* by affecting MYB transcription factors from the ethylene pathway, which, in turn, causes the activation of benzoxazine synthesis (Zhang et al., 2021a).

The JA pathway plays a vital role in defending against herbivorous insects, with MYC2 acting as a key regulatory transcription factor. In maize, the *myc2ab* double mutant shows increased insect susceptibility compared with the wild type. ZmMYC2s regulate the levels of benzoxazines and volatile

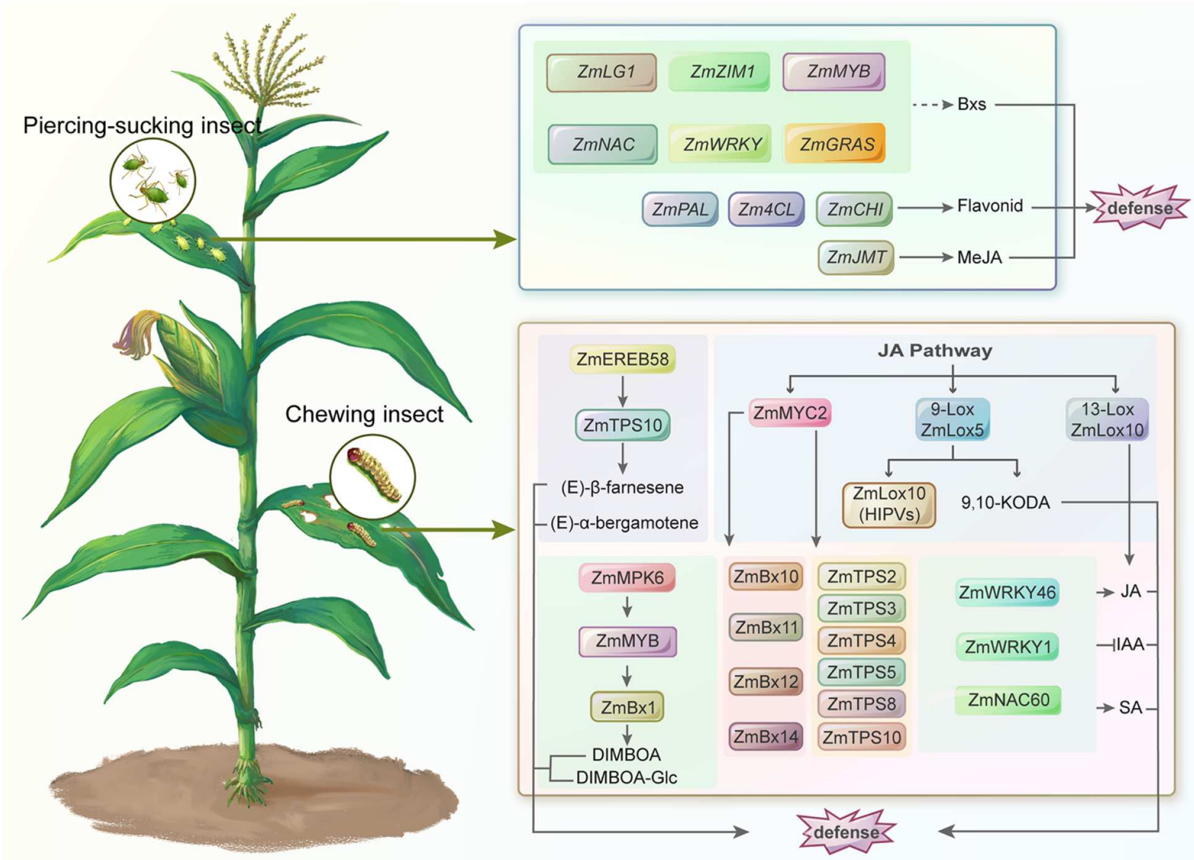

Figure 6. Summary of research progress on insect resistance in maize. Top: piercing-sucking pests, e.g., aphids, affect the genes such as *ZmLG1*, *ZmZIM1*, *ZmMYB*, and *ZmNAC*, thereby activating the benzoxazine synthetic pathway. These pests also regulate flavonoid content through *ZmPAL*, *Zm4CL*, and *ZmCHI* genes, and affect MeJA content by targeting *ZmJMT*. Bottom: chewing pests, e.g., corn borers, infest maize and activate genes involved in various pathways, including benzoxazinoid, JA (jasmonic acid), SA (salicylic acid), and terpene pathways. The expression of *ZmLOX5* can be induced, leading to the accumulation of 9,10-KODA. The diverse compounds mentioned above may contribute to pest defense in maize.

terpenoids by directly binding to promoters of *IGPS1/3*, *BX10/11/12/14*, and *TPS10/2/3/4/5/8* (Ma et al., 2023a). MeJA treatment induces the expression of pathogenicity-associated protein 1 (PR1) and thioredoxin M-type chloroplast precursor (TRXM), both of which can inhibit the development of ACB larvae and pupae (Zhang et al., 2015b). ZmLOX10, a member of the 13-lipoxygenases (LOXs), initiates JA synthesis. Feeding on *Zmlox10* mutants benefits the growth of *Spodoptera exigua*, while the *Zmlox10* mutant is less appealing to the parasitoid wasp larvae (*Cotesia marginiventris*) (Christensen et al., 2013). The *Zmlox5* mutant greatly reduces the accumulation of various oxidized lipids and defense metabolites, including benzoxazines, abscisic acid (ABA), and JA-isoleucine (JA-Ile). As a result, the insects fed on *Zmlox5* plants grow better. Consistently, the artificial diets supplemented with 9,10-KODA, a compound produced by ZmLOX5, inhibit the growth of fall armyworm larvae. Moreover, ZmLOX5 can protect insects by regulating ZmLOX10-mediated volatile signal transduction in green leaves (Yuan et al., 2023).

Transcription factors also play an important role in pest defense alongside secondary toxic metabolites and hormonal signaling pathways. EREB58 (GRMZM2G381441), an AP2/ERF-type transcription factor, has been shown to bind to the promoter of *TPS10* and mediate jasmonic acid-induced synthesis of sesquiterpenoids, which confer resistance to herbivorous insects (Li et al., 2015d; Qi et al., 2016a). By combining ACB larval bioassays of 10 inbred maize lines with transcriptome and metabolome analyses, three transcription factors, NAC60, WRKY1, and WRKY46 were identified to be associated with benzoxazinoid biosynthesis. These transcription factors can regulate the defense-growth trade-off by increasing benzoxazinoids, JA and SA levels while decreasing the IAA level in maize (Guo et al., 2023a). Moreover, correlation analysis on transcriptome levels identified four transcription factors (MYB61, GRAS37, NAC35, and WRKY75) that might account for the high Bx levels in the aphid-resistant line Mo17 (Song et al., 2017). Integrating QTL mapping and transcriptome data revealed that *ZmJMT*, associated with the jasmonic acid pathway, might be the candidate aphid resistance gene. A transcriptomic and metabolomic association analysis identified several key regulatory genes, such as *ZmPAL*, *Zm4CL*, and *ZmCHI*, related to flavonoid biosynthesis, which may respond to aphid infestation in maize (Wang et al., 2023f). Interestingly, loss-of-function mutations in *LIGULELESS1* (*LG1*), a well-known regulator of leaf angle, were found to activate JA signaling pathways, thereby enhancing aphid resistance in maize (Huang et al., 2024a).

Except for the corn borer and aphid, the fall armyworm *Spodoptera frugiperda*, which invaded China in 2019, is another important and destructive insect pest. However, the identification of functional resistance genes or genetic loci in maize is less progressed (Sun et al., 2021b; Wang et al., 2023c). With the application of GC-EAD and GC-MS/MS techniques in maize and rice, researchers identified ten and eleven compounds, respectively. One of them is a green leaf volatile, (Z)-3-hexenyl-acetate, which was shown to mediate differential oviposition by *Spodoptera frugiperda* (Wang et al., 2023d).

Genetically modified insect-resistant maize

Chinese researchers have made significant advancements in developing genetically modified maize with independent intellectual property rights. These varieties are designed to resist pests and herbicides, and are now in the industrialization pilot stage. In 2019, Beijing Da Bei Nong Biotechnology Co., Ltd. (DBNBC) obtained a national production safety certificate for their event DBN9936, using the cry1Ab insect resistance gene and the CP4 epsps herbicide resistance gene. Concurrently, Zhejiang University and Hangzhou Ruifeng Biotechnology Co., Ltd. (RF) also obtained a national production safety certificate for the event Ruifeng 125, incorporating the cry1Ab/cry2Aj insect resistance gene and the G10 EPSPs herbicide resistance gene. In 2020, other events like DBN9858 (CP4 epsps and pat herbicide resistant genes), DBN9501 (vip3Aa19 insect resistant genes and pat herbicide resistant genes), and ND207 (mcry1Ab and mcry2Ab insect resistant genes) were approved with national production safety certificates. In 2021, RF acquired a national production safety certificate for the event ZheDa Ruifeng 8, which incorporated cry1Ab and cry2Ab insect resistance genes. In 2022, the event nCX-1, incorporating CdP450 and cp4 epsps herbicide resistance genes, was also granted a national production safety certificate. In 2023, the event of BFL4-2, containing cry1Ab, cry1F insect resistance genes, and cp4 epsps herbicide resistance genes, received a national production safety certificate from the Institute of Biotechnology, Chinese Academy of Agricultural Sciences. In the same year, the event CC-2, generated by China Agricultural University using the maroACC herbicide resistance gene, was approved with a national production safety certificate (http://www.moa.gov.cn/ztzl/zjyqwgz/spxx/). These insect-resistant events demonstrate high efficacy against target pests like corn borer, armyworm, and cotton bollworm, while the herbicide-resistant events can tolerate target herbicides such as glyphosate and glufosinate at 4 times the regular dose (Li et al., 2022c). Multiple varieties derived from DBN9936, Ruifeng 125, and ND207 have been pilot-planted in various provinces, including Inner Mongolia, Jilin, Hebei, Yunnan, and Sichuan, with a planting area of 4 million acres.

Perspective of disease and insect resistance

To date, great progress has been achieved in understanding the genetic basis of disease resistance in maize, with numerous genes associated with maize disease resistance being cloned, including *ZmWAK* (Zuo et al., 2015), *ZmCCT* (Wang et al., 2017a), *ZmCCoAOMT2* (Yang et al., 2017b), *ZmREM1.3* (Wang et al., 2019), *ZmFBL41* (Li et al., 2019h), *ZmAuxRP1* (Ye et al., 2019), *ZmMM1* (Luan et al., 2021), *RppC* (Deng et al., 2022), *RppK* (Deng et al., 2022), *ZmFER1* (Liu et al., 2022b), *ZmAPX1* (Zhang et al., 2022a), *ZmWAX2* (Ma et al., 2023b), *ZmNANMT* (Li et al., 2023g), *ZmVPS23L* (Sun et al., 2023b), *ChSK1* (Chen et al., 2023a), *ZmWAK02* (Dai et al., 2024), and *ZmWAKL* (Zhong et al., 2024) for fungal diseases, *ZmTrxh* (Liu et al., 2017b), *ZmABP1* (Leng et al., 2017), and *ZmGDIα-hel* (Liu et al., 2020f) for viral diseases. Among them, *ZmWAK*, *ZmGDIα-hel* (Xu et al., 2020b), *RppK* (Deng et al., 2022), *ZmCCT* (Tong et al., 2022), *RppC*, and other genes/QTL have been widely used in maize breeding. Additionally, resistance to *Lepidoptera* pests has been greatly improved by using exogenous *Bt* genes from bacteria, with a total of nine transgenic events having accomplished the biosafety assessments and obtained the safety certificates for production and application.

The maize responses to disease or pest are complex and multilayered processes. Future research may focus on the following issues: (i) how to clone genes conferring multiple resistance and elucidate their functions? (ii) How to balance maize disease or pest resistance with yield? (iii) How to use new biotechnologies, such as high-throughput genomic approaches, to detect and utilize the key susceptible genes? (iv) How to engineer NLR genes with expanding recognition species? (v) How to elucidate the interactions between the environmental microbiome and resistance? (vi) How to identify novel exogenous genes conferring resistance to *Coleopteran* pests? (vii) How to reconstruct resistance pathways for disease and insect through Synthetic biology? Due to the co-evolution of pathogens and insects, novel resistant genes are needed for maize improvement. Molecular technologies have great values in breeding resistant cultivars, such as gene pyramiding, genomic selection, transgenic techniques, RNA interference, and genome editing. Combining multiple resistant genes into a few elite inbred lines using the above technologies holds great potential for breeding varieties with strong and durable resistance to multiple pathogens and pests.

Male sterility and its applications in maize

Male sterility, including genic male sterility (GMS), thermosensitive genic male sterility (TGMS), and cytoplasmic male sterility (CMS), is a very useful trait for heterosis utilization and hybrid seed production in maize. GMS is caused by defects of nuclear genes and related to anther and pollen development, which can be divided into 14 stages (Wan et al., 2019). CMS is regulated by both cytoplasmic and nuclear genes, and it has been used in commercial hybrid maize production (Yang et al., 2022b). However, CMS suffers from poor genetic diversity, increased disease susceptibility, and unreliable restoration. GMS can overcome the drawbacks, but it cannot be produced on a large scale through self-pollination. With the advances of GMS gene isolation and biotechnology, several efficient biotechnology-based male sterility (BMS) systems have been achieved and have potential applications in maize hybrid breeding and seed production (Wan et al., 2019). In addition, the identification of TGMS genes provides opportunities for the two-line system in maize hybrid seed production. In this section, we systematically summarize the latest advances of male sterility (GMS, CMS, and TGMS) in maize and outline their application strategies in maize hybrid seed production.

Genic male sterility

To date, more than 50 GMS genes have been identified in maize (Table S3). Most of them are recessive GMS genes, except *ms44*

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(Fox et al., 2017), *Mei025* (Huang et al., 2022a), and *Ms42* (Li et al., 2022i). Based on their encoding proteins and involved biological processes, these GMS genes could be classified into four classes: transcription factors (19 genes), lipid metabolism (20 genes), sugar metabolism (three genes) and other processes (12 genes). The involved biological processes cover four key anther developmental events (Figure 7), including cell fate specification (stages S1–S2), somatic cell differentiation (stages S3–S5), tapetum development and pollen mother cell (PMC) meiosis (stages S6–S8b), and pollen maturation and anther dehiscence (stages S9–S14).

Cell fate specification

Anther wall formation is initiated from L1-d (layer1-derived) and L2-d (layer2-derived) cells of the stamen primordium. L1-d cells differentiate into the epidermis, while L2-d cells divide into primary parietal cells and archesporial (AR) cells; the former further divides into the endothecium and secondary parietal cells, then the secondary parietal cells divide again to form the middle layer and tapetum (Marchant and Walbot, 2022).

The plant germline, serving as progenitors of gametes, is generally considered to derive from AR cells. *MALE STERILE CONVERTED ANTHER1* (*ZmMSCA1*) encodes a glutaredoxin and is required for maize AR cell specification. It promotes the AR specification from the inner L2-d cells under hypoxia conditions (Kelliher and Walbot, 2012). When ZmMSCA1 is reduced under hypoxia conditions, it subsequently reduces TGA TFs (such as ZmTGA9-1/-2/-3), facilitating their nucleus localization, activating the expression of genes locking in AR cell specification, and initiating somatic cell differentiation (Marchant and Walbot, 2022).

Somatic cell differentiation

After the specification of AR cells, MULTIPLE ARCHESPORIAL CELLS1 (ZmMAC1) is secreted to brake AR cell proliferation and cause neighboring cells to become supportive tissues (Wang et al., 2012). ZmMAC1 is recognized by and interacts with the protein kinase MULTIPLE SPOROCYTE1 (ZmMSP1) in the outer L2 layer cells to promote the specification of the L2 layer cells into primary parietal cells (van der Linde et al., 2018). During the differentiation of primary parietal cells into the endothecium and secondary parietal cells, the HD-ZIP IV transcription factor ZmOCL4 (OUTER CELL LAYER4) suppresses periclinal division within the endothecium. Anthers of the *ocl4* mutant develop an ectopic cell layer exhibiting endothecium characteristics adjacent to the middle layer and endothecium, resulting in partial male sterility (Vernoud et al., 2009). *ZmMs23* and *ZmMs32* encode basic helix-loop-helix (bHLH) TFs and regulate tapetal cell division and differentiation. *ZmMs23* transcripts localize initially to the precursor secondary parietal cells, then predominantly to daughter tapetal cells (Nan et al., 2017). Loss of *ZmMs32* results in the failure of tapetum precursor cell differentiation and an extra tapetum layer formation (Moon et al., 2013), and *ZmMAC1* expression is down-regulated in the *ms32* mutant compared with that in the wild type, indicating that *ZmMs32* is required for *ZmMAC1* expression. ZmTGA10, a bZIP TF, also affects somatic cell differentiation, as the loss function of *ZmTGA10* leads to non-dehiscent anthers and thus complete male sterility (Jiang et al., 2021a). *ZmMs33*, encoding a glycerol-3-phosphate acyltransferase, catalyzes the first step of the glycerolipid synthetic pathway, which is critical for maintaining the typical structure and function of endothecium chloroplasts (Xie et al., 2018; Zhang et al., 2018d; Zhu et al., 2020b). *ZmMs28* encodes an ARGONAUTE family protein ZmAGO5c, and its transcripts primarily accumulate in pre-meiosis anthers. *ZmMs28* may regulate tapetal cell development through small RNA-mediated epigenetic regulatory pathways (Li et al., 2021d). However, the regulatory relationship of these genes remains unclear.

Tapetum development

Tapetum, the neighboring cells of the developing microspores, is crucial for pollen development as it supplies most components and nutrients required for pollen wall formation. Any defects in the tapetum often lead to pollen aberrations and ultimately result in male sterility (Wan et al., 2020; Wan et al., 2019). In maize, many identified GMS genes are involved in tapetal development regulation (Figure 7).

Firstly, eight TFs essential for tapetum development are reported. Besides *ZmMs9* encoding a MYB TF (Albertsen et al., 2016), seven other genes encoding TFs, including three MYB TFs ZmMYB84 and ZmMYB33-1/-2, two bHLH TFs ZmbHLH51 and ZmbHLH122, one PHD-finger TF ZmPHD11, and one LBD TF ZmMS1/ZmLBD30, are proven to be required for anther development and male fertility (Hou et al., 2023; Jiang et al., 2021a). Notably, a transcriptional cascade of ZmMS23-ZmMS32-ZmbHLH122-ZmbHLH51-regulated pathway has been reported to be essential for the tapetal cell differentiation during early anther development (Nan et al., 2022). Refer to the conserved regulatory pathways AtDYT1-AtTDF1-AtAMS-AtMs188-AtMs1 in *Arabidopsis* and OsUDT1-OsTDF1-OsTDR-OsMYB80-OsPTC1 in rice (Cai et al., 2015; Lou et al., 2018), a similar regulatory pathway (ZmMS32-ZmMS9-ZmbHLH51-ZmMYB84-ZmMS7) required for controlling tapetal development in maize has been validated, and the newly identified *ZmMs1/ZmLBD30* regulated by ZmMYB84 and ZmMS7 serves as a feedback repressor to shut down the upstream regulatory cascade, ensuring timely tapetal PCD and pollen exine formation (Hou et al., 2023; Wan et al., 2019).

Secondly, 11 lipid metabolism-related genes that are highly expressed from stages S6 to S8b have been identified as GMS genes and proven to be involved in tapetal development and anther cuticle and pollen exine development in maize (Figure 7; Table S3). *ZmMs30* and *ZmIPE2* encoding GDSL lipases are required for pollen exine (especially foot layer) development (An et al., 2019; Huo et al., 2020). *ZmDFR1/2* encoding tetraketide α-pyrone reductases and *ZmACOS5-1/-2* encoding acyl-CoA synthetases play redundant roles in controlling maize pollen fertility, respectively (Liu et al., 2022d). *ZmMs2/ZmABCG26* encodes an ATP-binding cassette G (ABCG) transporter and is required for the lipid transport to the anther and microspore surfaces (Jiang et al., 2021b; Xu et al., 2021c). *ZmMs13/ZmABCG2a* exhibits stage-specific expression in anthers, with temporal expression peaking at stages S5, S8b, and S10. These distinct peaks are orchestrated by the stage-specific regulators ZmbHLH122, ZmMYB84, and ZmMYB33-1/-2, respectively. This precisely timed, triphasic transcriptional control of *ZmMs13* is essential for the sequential processes of callose dissolution, tapetal PCD, pollen exine development, and anther cuticle formation (Fang et al., 2023). *ZmMs44*, *ZmLTPg11*, and *ZmLTPx2* encoding lipid transport proteins (LTPs) are responsible for lipid transport from the tapetum to the locule, but *ZmLTPg11*

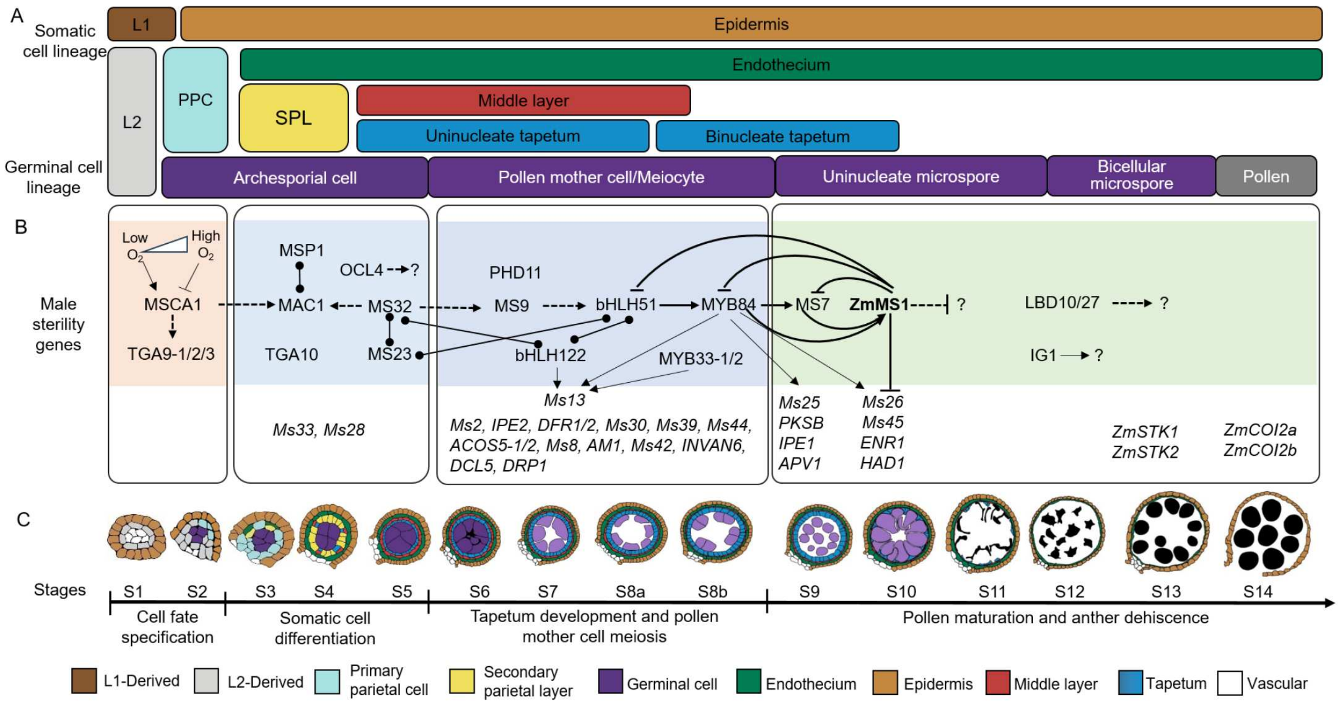

Figure 7. Maize genic male sterility genes and their roles in controlling anther and pollen development. A, The diagram apex depicts anther cell lineage specification, originating from layer 1 cells (L1) and L2 cells. While the L1 exclusively gives rise to the epidermis, all internal anther lobe cell types descend from the L2 via sequential periclinal divisions. This layered ontogeny initiates when primary parietal cells (PPCs) undergo periclinal division, producing the endothecium and secondary parietal layer (SPL). Subsequently, the SPL divides periclinally to yield the middle layer and tapetum. B, Within the phase boxes are lists of the cloned genic male sterility genes, corresponding to the developmental stages in the lower part of the figure and the upper part of the figure based on their expression patterns. C, At the bottom of the diagram, the 14 sequential stages (S1–S14) of anther development are displayed in cartoons. Below the stages are the terms used to describe the four phases, which are highlighted in different color shades in (B).

and *ZmLTPx2* have little effect on pollen fertility (Fox et al., 2017; Li et al., 2021a).

Finally, a callose synthase, ZmMs39/ZmCalS12, plays a role in sugar metabolism during early anther development. The tapetum cells of the *ms39* mutant are abnormal from the dyad (S8a) stage (Zhu et al., 2018). *ZmMs8*, encoding a putative β-1,3-galactosyltransferase, modulates cell division, expansion, and differentiation during early anther development (Wang et al., 2013).

Pollen mother cell meiosis

Within the anther lobes, the innermost AR cells mature into PMCs, also referred to as microspore mother cells (Walbot and Egger, 2016). PMCs undergo meiosis to generate haploid gametes via two rounds of chromosome segregation that follow one round of chromosome replication (Hamant et al., 2006). In maize, several elements regulating meiosis progression, sister chromatid cohesion, homologous pairing and synapsis, meiotic recombination, and chromosome distribution were characterized. *ZmAM1* is likely required for the initiation of meiosis (Pawlowski et al., 2009), while the meiotic exit in maize is determined by a tetratricopeptide repeat domain protein ZmTDM1 (Zhang et al., 2023e). After meiosis is initiated, the meiotic chromatids are stepwise organized into higher-order chromosome structures. *ABSENCE OF FIRST DIVISION* (*AFD1*), encoding a meiosis-specific cohesin subunit, is essential for establishing prophase chromosome morphology and sister-chromatid cohesion (Golubovskaya et al., 2006). Another cohesin subunit, Structural Maintenance of Chromosome 3 (SMC3), is essential for sister chromatid cohesion in both mitosis and meiosis. Especially, ZmSMC3 participates in centromere pairing during meiosis (Zhang et al., 2020c). In early prophase I, sister chromatids form loops and are anchored on the proteinaceous axial element (AE). The AE serves as a framework for synaptonemal complex (SC) assembling, and plays a central role in meiotic recombination (Mercier et al., 2015; Zickler and Kleckner, 1999). Maize *DESYNAPTIC2* (*DSY2*), encoding a coiled-coil protein, is an AE protein that is required for DNA double-strand breaks (DSBs) formation and is directly involved in synaptonemal complex (SC) assembly in maize (Lee et al., 2015). During meiosis, one of the most important events is meiotic recombination. It is initiated by the programmed formation of DNA double-strand breaks (DSBs), which is catalyzed by an evolutionarily conserved SPO11 as the catalytic core and accessory proteins (Hamant et al., 2006; Robert et al., 2016). In maize, DSB formation involves ZmSPO11-1 (Ku et al., 2020), ZmSPO11-2 (Li et al., 2022f), ZmMTOPVIB (Jing et al., 2020), PHS1 (Ronceret et al., 2009), and PRD1 (Wang et al., 2022i). SPO11 family proteins (ZmSPO11-1, ZmSPO11-2, and ZmMTOPVIB), PHS1, PRD1, PRD2, and PRD3a were assembled into a DSB-forming complex, which is linked with the chromosome axes via the interaction of PRD3a and axial element of the synaptonemal complex ASY1 (Wang et al., 2023j). DSB ends need to be processed and loaded with RAD51 and DMC1, which facilitates homologous pairing and single-end invasion of a non-sister chromatid (Hamant et al., 2006; Mercier et al., 2015). In maize, RAD51a, RAD51b, and RAD51c are essential for DSB repair in meiocytes (Jing et al., 2019; Li et al., 2007). Conservatively, RAD51 and DMC1 are recruited onto DSB sites in maize meiocytes, which depends on the normal function of

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ZmCOM1, BREAST CANCER SUSCEPTIBILITY PROTEIN 2 (BRCA2), and AAA-ATPase FIDGETIN-LIKE-1 (FIGL1) (Li et al., 2007; Wang et al., 2018e; Zhang et al., 2023f). Besides, ZmRAD17, a replication factor C (RFC)-like DNA damage sensor protein, is required for accurate DSB repair in PMCs (Zhang et al., 2021g). Eventually, DSB repair gives rise to either crossovers (COs) or non-crossovers (NCOs) (Mercier et al., 2015). An F-box protein ACOZ1 functions in CO formation by controlling chromosome compaction during meiosis in maize (Jing et al., 2022).

Pollen maturation and anther dehiscence

Many GMS genes functioning during late anther developmental stages (S9 to S14) have been identified in maize, including several TFs, lipid metabolism-related and other types of GMS genes (Figure 7). For instance, *ZmMs7* encodes a PHD finger TF and is required for tapetal cell death and late pollen development (Zhang et al., 2018b). ZmMS7 associates with maize nuclear factor Y (NF-Y) subunits, assembling into ZmMS7-NF-YA6-YB2-YC9/12/15 heterotrimeric complexes. These complexes directly bind the CCAAT box within promoter regions to activate downstream target genes (An et al., 2020a). Concurrently, the tapetum-specific lateral organ boundary domain (LBD) transcription factor ZmLBD30, encoded by *ZmMs1*, is initially triggered by a ZmbHLH51-ZmMYB84-ZmMS7 transcriptional activation cascade. Subsequently, ZmMS1/ZmLBD30 acts as a transcriptional repressor, attenuating this cascade through negative feedback to precisely coordinate timely tapetal PCD and pollen exine formation (Hou et al., 2023). *ZmIG1* also encodes an LBD TF, and mutation of this gene results in haploid embryo production and male sterility (Evans, 2007). *ZmLBD10* and *ZmLBD27* double-gene mutant displays partial male sterility, of which 32.18% pollen grains are aborted, indicating their redundant roles in the late anther development (Jiang et al., 2021a).

At least eight GMS genes are involved in lipid biosynthesis and modification in anther tapetal cells. *ZmMs25*/*ZmMs6021* is a plastid-localized fatty acyl reductase that reduces fatty acyl-coenzyme A (CoA) or -acyl carrier protein (ACP) substrates to generate primary fatty alcohols, and it is regulated by ZmMYB84 directly (Tian et al., 2017; Zhang et al., 2021f). *ZmAPV1*/*ZmMs10* encodes a cytochrome P450 fatty acid hydroxylase and catalyzes C12 fatty acids to hydroxylated C12 fatty acids (Somaratne et al., 2017). *ZmMs26* encodes a long-chain fatty acid ω-hydroxylase that catalyzes C16/C18 fatty acids to C16/C18-hydroxylated fatty acids (Djukanovic et al., 2013). *ZmIPE1*/*ZmMs20* encodes a glucose-methanol-choline oxidoreductase and is involved in C16/C18-hydroxylated fatty acid oxidation (Chen et al., 2017b; Wang et al., 2019d). *ZmPKSB* encodes a polyketide synthase that catalyzes C16/C18 fatty acyl-CoA to triketide and tetraketide α-pyrone. *ZmPKSB* is directly activated by ZmMYB84, and this regulatory module controls a trade-off between anther cuticle and pollen exine formation (Liu et al., 2022c). *ZmMs45* encodes an atypical strictosidine synthase participating in the pollen exine thickening and anther cuticle development (Cigan et al., 2001). Recently, a plastid-localized enoyl-acyl carrier protein (ACP) reductase/β-hydroxyacyl-ACP dehydratase (ZmENR1/ZmHAD1) complex has been reported to enhance the efficiency of *de novo* fatty acid biosynthesis during maize pollen and anther development. Furthermore, the ZmENR1/ZmHAD1 complex is governed by a ZmMS1-mediated feedback repression loop, safeguarding the proper formation of the anther cuticle and pollen exine (Zhang et al., 2024a).

Finally, five GMS genes are involved in other processes during late anther developmental stages. *ZmSTK1* and *ZmSTK2* encode receptor-like cytoplasmic kinases and indirectly participate in the glycolytic pathway to regulate maize pollen development. Mutation in *ZmSTK1* or *ZmSTK2* severely disrupts pollen transmission, ultimately compromising male fertility (Fan et al., 2018). *ZmCOI2a* and *ZmCOI2b* encode F-box proteins, function as key JA receptors, and redundantly regulate gametophytic male fertility and anther dehiscence in maize. The pollen of *coi2a coi2b* double mutant shows severe defects in both pollen germination and pollen tube elongation (Qi et al., 2022). *Desiccation-Related Protein 1* (*DRP1*) is specifically expressed during the tetrad to the uninucleate microspore stage in both the tapetum and microspores. Mutation of *DRP1* results in abnormal Ubisch bodies, defective tectum of the pollen exine, and complete male sterility (Han et al., 2022; Hu et al., 2022). Nevertheless, the molecular functional mechanism of these GMS genes needs to be further explored.

Cytoplasmic male sterility

Three major CMS types are distinguished in maize and designated as CMS-T, CMS-S, and CMS-C, respectively, according to their mitochondrial DNA and fertility restoration patterns (Figure 8A). CMS-T and CMS-C are sporophyte sterility, while CMS-S is gametophyte sterility. Except for the molecular bases of CMS-T, those of CMS-S and CMS-C have been elucidated only in recent years.

The mitochondrial gene *T-urf13*, a key determinant of CMS-T male sterility, encodes a 13-kD polypeptide (URF13) integral to the inner mitochondrial membrane (Levings, 1990). Full fertility restoration of CMS-T requires two dominant nuclear genes, *restorer of fertility1* (*Rf1*) and *Rf2*. The *Rf2* gene encodes a mitochondrial aldehyde dehydrogenase, which is critical for anther development in maize (Cui et al., 1996). Additionally, *Rf8* and *Rf** have similar but distinct effects on the expression of the *T-urf13* gene to restore pollen fertility of the CMS-T maize (Dill et al., 1997).

The *orf355* gene is the causal gene of CMS-S maize and is positively regulated by nuclear transcription factor ZmDREB1.7 (Xiao et al., 2020). The mitochondrial *orf355* gene drives ROS overproduction and microspore abortion (Xiao et al., 2020). The main restorer gene *Rf3* of CMS-S, encoding a pentatricopeptide repeat (PPR) protein, decreases the orf355 transcript abundance to restore fertility (Qin et al., 2021). Compared with Rf3, Rf9 is a less effective fertility restorer and acts indirectly by reducing linear mitochondrial subgenomes containing *orf355* (Gabay-Laughnan et al., 2009).

The *atp6c* gene is the causal gene of CMS-C maize. Mitochondrial ATP6C disrupts F_1Fo-ATP synthase assembly, reducing ATP synthesis and triggering tapetal PCD and pollen abortion (Yang et al., 2022b). Fertility of CMS-C is restored redundantly by Rf4 (a bHLH transcription factor) and Rf5 (Jaqueth et al., 2019), and Rf-I antagonizes Rf5 activity (Hu et al., 2006), although the detailed mechanism remains unclear.

Thermosensitive genic male sterility (TGMS)

Although the phenomenon of TGMS in maize has been reported for many years, its molecular mechanism has been less studied

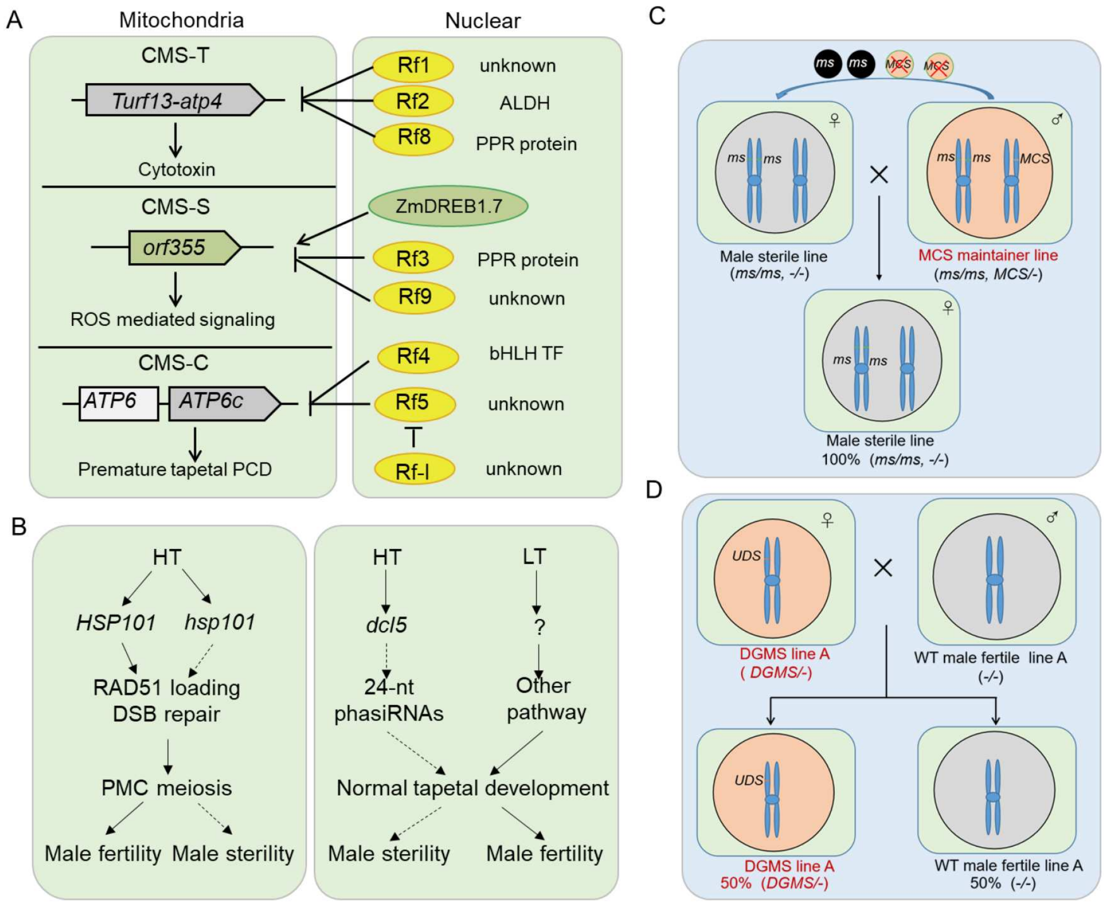

Figure 8. The working models of CMS, TGMS, and two representative biotechnology-based male sterility systems in maize. A, Three CMS/restoration systems in maize. The mitochondrial genes *Turf13*, *orf355*, and *ATP6C* specifically disrupt anther and pollen developmental processes in their respective CMS-T, CMS-S, and CMS-C lines. Fertility restoration occurs in hybrids through cognate nuclear *Rf* genes, which suppress the expression of these mitochondrial factors to restore male fertility. B, Working models of two representative TGMS (*HSP101* and *DCL5*) genes. HT and LT represent high and low temperatures, respectively. C and D, Two representative BMS systems (MCS and DGMS) in maize.

(Figure 8B). Recently, maize cytosolic invertase ZmINVAN6 has been reported to play a crucial role in ensuring meiotic fidelity under heat stress; its absence causes chromosome bridges and micronuclei (Huang et al., 2022a). *ZmMs42*, encoding a member of the heat shock protein family HSP101, is critical for thermotolerance by ensuring RAD51-mediated DSB repair during meiosis (Liu et al., 2022d). *Dicer-like5* (*ZmDCL5*) generates 24-nt phasiRNAs essential for pollen viability, and *dcl5* mutants show temperature-sensitive male sterility (Teng et al., 2020). *ZmTMS5*, the maize ortholog of rice *TMS5*, confers thermosensitive male fertility: loss-of-function mutants display male sterility under high temperatures but remain fertile under low-temperature conditions (Li et al., 2017d). Furthermore, liquid-liquid phase separation (LLPS) may play a potential role in high-temperature-induced male sterility based on *Arabidopsis* studies (Buttress et al., 2022; Zhu et al., 2022), which represents a promising future direction to explore the mechanism of TGMS in maize and other crops.

Application strategies of male sterility in maize

Using male-sterile line is an efficient strategy instead of the manual detasseling of the female parent in maize hybrid production, along with reducing the seed production cost and greatly improving the hybrid seed purity. CMS revolutionized hybrid seed production by eliminating manual detasseling. However, CMS-T fell out of favor after the 1970s southern corn leaf blight epidemic, which targeted URF13-containing lines (Levings, 1990). Recent focus has shifted to CMS-S and CMS-C due to improved understanding of restoration gene, enabling safer three-line breeding systems.

In addition, the GMS lines can be a better alternative for hybrid maize seed production in the future. Several next-generation GMS systems have been developed by utilizing GMS genes in combination with new technologies in maize, such as the Multi-Control Sterility (MCS), Dominant Genic Male Sterility (DGMS), and other BMS systems (Figure 8C and D) (An et al., 2020a; Zhang et al., 2018b).

Maize MCS System

Several MCS systems have been established by transforming a single MCS construct into *ms7*, *ms30, or ms33* recessive male-sterile mutants in maize (An et al., 2019; Zhang et al., 2018b; Zhu et al., 2019). For example, an MCS construct comes from the integration of several functional modules, including fertility restoration (*ZmMs33*), pollen disruption (*ZmAA/Dam*), fluorescence markers (*DsRed2*), and herbicide resistance (*Bar*), and it has been transformed into the corresponding recessive GMS

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mutant (e.g., *ms33*). Transgenic pollen fails to germinate, allowing mechanical seed sorting for 100% sterile female lines. The MCS construct integrates dual male-fertility disruptors, severely compromising transgene transmission via pollen. Concurrently, its *Bar* gene cassette ensures efficient propagation of genetically pure seed stocks within transgenic maintainer lines.

For instance, among the pollen grains produced by the MCS maintainer line under *ms33* genetic background, the non-transgenic and MCS transgenic grains are separated as 1:1. Thus, self-pollination of the MCS maintainer line produces seeds with the genotype of both the MCS maintainer line (*ms33*/*ms33* +MCS-T-DNA) and the male-sterile line (*ms33*/*ms33*). When the male sterile line (*ms33*/*ms33*) is crossed with the MCS maintainer line, pure male-sterile seeds with the *ms33*/*ms33* genotype can be produced and used as male-sterile female lines for maize hybrid seed production (Figure 8C).

Maize DGMS system

The DGMS system is a simpler but efficient and broadly applicable BMS system, and it has been developed from the early expression of *ZmMs7* under the p5126 promoter and accompanied by a red fluorescence seed-sorting marker (i.e., *p5126-ZmMs7M* construct) (An et al., 2020a). Transgenic maize carrying the *p5126-ZmMs7M* construct exhibits complete, stable DGMS with abolished pollen production yet unimpaired vegetative growth and female fertility. When pollinated by its fertile maintainer sibling, this DGMS line yields a near 1:1 ratio of transgenic fluorescent DGMS seeds to non-transgenic wild-type male-fertile seeds (Figure 8D). Due to hemizygous integration of the DGMS construct, F_1 hybrid seeds from crosses with male-fertile lines segregate 1:1 into male-sterile and male-fertile progeny. The DGMS system can enhance the grain yield of male-sterile F_1 hybrid plants under stress conditions by reallocating resources from tassels to ears (Wan et al., 2021).

Other BMS systems

Besides the MCS and DGMS systems, other similar BMS systems have also been developed in recent years. For example, the Manipulated GMS Maintainer (MGM) system employs CRISPR/Cas9 technology to produce male-sterile lines and transgenic maintainer lines via a single maize transformation (Qi et al., 2020b). In addition, this strategy can be extended to any hybrid crop other than maize.

Conclusion and perspective on male sterility

In summary, decoding male sterility genes has illuminated maize reproductive biology and spurred innovative breeding technologies. DGMS systems, combined with genomic selection, promise to elevate hybrid yields sustainably. Future work should explore LLPS in TGMS and optimize CRISPR-based platforms for global maize genetic improvement.

Hybrid breeding and mechanisms of heterosis in maize

Hybrid maize exhibits pronounced heterosis (hybrid vigor), which is successfully harnessed via hybrid breeding. Following the rediscovery of heterosis by Shull and East in the first few decades of the 20th century (East and Jones, 1919; Shull, 1908), maize breeding has switched from landraces and open-pollinated varieties (OPVs) (prior to 1930s) to "double-cross hybrids" (1930s–1950s) and then eventually to the "single-cross hybrids" stage (since 1960s). Nowadays, single-cross hybrids predominate worldwide production due to superior performance in yield and stress tolerance (Duvick, 2005; Wang et al., 2011).

The genetic and molecular basis of heterosis

Since the 1960s, empirical breeding has classified maize inbred lines into distinct pools of germplasm, called "heterotic groups" (Lu et al., 2009; Mikel and Dudley, 2006), and it is gradually becoming clear that hybrids produced by crossing specific pairs of heterotic groups display stronger heterosis than intra-group hybrids or hybrids produced by crossing other combinations of heterotic groups. Such female-male pairs with pronounced heterosis are called "heterotic patterns" (Melchinger and Gumber, 1998; Zhang et al., 2018f). Later, various molecular markers (RFLPs, SSRs, and SNPs) were developed and used to guide the assignment of inbred lines into various heterotic groups to achieve the maximum utilization of heterosis (Lu et al., 2009; Melchinger and Gumber, 1998; Tian et al., 2021a; Wu et al., 2015). For temperate hybrid breeding, the various germplasm is generally classified into the Stiff Stalk (SS), Non-Stiff Stalk (NSS, also called Lancaster), Iodent, PA (also named Domestic Reid), PB (also called Tem-tropic I group), Tangsipingtou (TSPT), X-group, and LvDa Red Cob (LRC) groups (Li et al., 2022a; Romay et al., 2013; Tian et al., 2021a; Wang et al., 2023a; Wang et al., 2020a; Wu et al., 2015; Zhang et al., 2018f). In general, the SS, PA, and X-group are used as the female parents, and the NSS, PB, and SPT are utilized as the male parents. In addition, SS×NSS and PA×SPT have become the predominant heterotic patterns widely used in the United States and China, respectively (Li et al., 2022a; Zhang et al., 2018f).

Recent molecular studies revealed that the female and male heterotic groups have differentiated genetically over time (Reif et al., 2003; Unterseer et al., 2016); nevertheless, a systematic assessment of the genetic basis of heterotic group formation and improvement during modern maize breeding has been lacking until recently. Li et al. (2022) recently conducted re-sequencing and phenotypic analyses of 21 agronomic traits for a panel of 1,604 modern elite maize lines. They made several interesting observations. (i) Both the female heterotic groups (FHGs, like SS, and PA) and male heterotic groups (MHGs, like NSS, Iodent, PB, and SPT) experienced convergent selection for earlier flowering time and a set of yield and plant architecture related traits, but underwent divergent selection for three traits potentially related to seed dehydration rate. Accordingly, they revealed complementary and divergent selection in the genomes of the MHGs and FHGs. (ii) GWAS analysis identified 4,329 genes associated with various agronomic traits and demonstrated a positive correlation between trait improvement and accumulation of favorable alleles during maize breeding. (iii) They found increased genetic differentiation between the US_SS and US_NSS groups, and between the PA and SPT heterotic groups, during modern maize breeding. Moreover, they demonstrated a promoting role of accumulated heterozygous superior alleles of the differentiated genes for maize heterosis (Li et al., 2022a).

Much effort has been devoted to dissecting the genetic and molecular basis of heterosis. Maize is particularly suitable for this purpose due to its out-crossing habit and agricultural importance. Dominance (complementation), single-locus overdominance, and epistasis are the major hypotheses postulated to explain heterosis, and each of them has gained empirical support from recent genetic and molecular studies (Birchler and Veitia, 2010; Chen et al., 2015; Flint-Garcia et al., 2009b; Guo et al., 2013; Liu et al., 2020b; Liu et al., 2020e; Ma et al., 2016; Tian et al., 2021c; Wan et al., 2022; Wang et al., 2016c; Wei et al., 2016; Wu et al., 2021a; Xing et al., 2016; Zhang et al., 2023b; Zhang et al., 2016b; Zhou et al., 2022a). Nevertheless, these studies are largely based on limited population size, limited traits studied, or limited information on the genetic variations of the samples under study. Thus, the exact mechanism of maize heterosis still remains enigmatic.

A major breakthrough in this field was recently made by a collaborative effort among a group of Chinese scientists led by Haiyang Wang, who constructed a pangenome for maize and exploited pair-wise genetic variations (SNPs, InDels, SVs, and PAVs) via *de novo* genome assembly and analyses of 12 key maize founder inbred lines (FILs). In combination with eQTL mapping and association studies, they validated a number of SVs as potential causal variants for the differentiation of several important agronomic traits (flowering time, kernel row number, kernel number per row, tassel branch number, ear height, and disease resistance) in various heterotic groups. Further, using a set of diallel-cross F_1 hybrids, they demonstrated a high positive correlation of heterosis with SV number, PAV number, and the length of non-syntenic regions between the parental lines, while SNPs and InDel number were only weakly associated with heterosis. In addition, they identified two ethylene-related genes, *ZAR1* and *ZmACO2*, which promote heterosis in an overdominant fashion (Wang et al., 2023a). These results provide concrete and compelling evidence supporting the notion that dominance plays a major role, but overdominance also contributes to maize heterosis.

Although the exact molecular mechanisms of maize heterosis await to be further elucidated, efforts have been made to develop various genome-wide prediction models of hybrid performance by integrating phenomics, genomics, transcriptomics, and metabolomics (Jiang et al., 2020; Westhues et al., 2017). For example, a recent study developed prediction models for seven biomass- and bioenergy-related maize traits using 56,110 genome-wide SNPs and 130 metabolites, with accuracies ranging from 0.72 to 0.81 for SNPs and from 0.60 to 0.80 for metabolites (Riedelsheimer et al., 2012). More recently, a group of scientists led by Jianbing Yan developed an algorithm called TOP (for target-oriented prioritization) that integrates multi-omics data with machine learning to predict and select the best breeding candidates with high accuracy (up to 91%) (Yang et al., 2022d). These efforts should help to improve the efficiency of identifying inbred lines with strong combining ability to create superior hybrids. Nevertheless, it is expected that a better comprehension of the molecular mechanisms that underlie heterosis will definitely help to develop more accurate genomic prediction models for hybrid maize breeding in the future.

Progress in unilateral cross-incompatibility of maize

Maize unilateral cross-incompatibility (UCI) is a pre-zygotic reproductive barrier known to restrict pollen flow among maize populations (Zhang et al., 2018g). Three major UCI systems, *Gametophyte factor 1* (*Ga1*), *Ga2*, and *Teosinte crossing barrier 1* (*Tcb1*), are well documented (Chen et al., 2022e; Lu et al., 2019; Wang et al., 2022h; Zhang et al., 2023h; Zhang et al., 2023i; Zhang et al., 2018g). *Tcb1* is predominantly present in teosinte, while *Ga1* and *Ga2* are in both maize and teosinte (Chen et al., 2022e). All three UCI loci are governed by a pair of male and female determinants that form three different haplotypes: -*S* haplotype (*Ga1-S*, *Ga2-S*, or *Tcb1-S*) possessing both male determinant and female determinant, -*M* haplotype (*Ga1-M*, *Ga2-M* or *Tcb1-M*) harboring only male determinant, and wild type (*ga1*, *ga2* or *tcb1*) having neither. In the process of fertilization, wild-type pollen is blocked by the *S* haplotype because of the female barrier, but wild-type silk accepts pollen of the *S* and *M* haplotypes. The *M* haplotype serves as a cross-neutral mediator, showing bidirectional cross-compatibility with both the *S* haplotype and the wild-type (Figure 9A) (Chen et al., 2022e; Zhang et al., 2018g). Genetically, the male determinants of three loci are dominant and gametophytic in nature, while the female determinants are recessive and sporophytic in nature (Figure 9B) (Chen et al., 2022e; Lu et al., 2019; Zhang et al., 2023h; Zhang et al., 2023i; Zhang et al., 2018g).

Exploration of UCI genes is preliminarily required to uncover the mystery of UCI, and major progress has been achieved in recent years. Transcriptomic analysis of the silks from the near-isogenic lines of *Ga1-S* and *ga1* revealed that the silk-expressed *ZmPME3* gene is a candidate of female determinant in the *Ga1* locus (Moran Lauter et al., 2017). This gene (referred to as *ZmGa1F* thereafter) was subsequently confirmed as the *Ga1* female determinant by direct genetic evidence (Wang et al., 2022h; Zhang et al., 2023h). A male determinant gene of the *Ga1* locus, *ZmGa1P*, was cloned and shown to endow wild-type pollen with the ability to break through the *Ga1-S* female barrier (Zhang et al., 2018g). The *Tcb1-f* gene was identified as a *Tcb1* female determinant, and it exhibits 98.84% sequence identity with *ZmPME3* (Lu et al., 2019). Interestingly, the *Tcb1-m* gene, exhibiting 96.59% sequence identity compared with *ZmGa1P*, was identified as the *Tcb1* male determinant (Zhang et al., 2023h). *Tcb1* and *Ga1* loci are in genetic linkage in teosinte, and diversification of them appears to have occurred after maize domestication (Zhang et al., 2023h). A pair of genes, *ZmGa2P* and *ZmGa2f*, was identified as the male and female determinants of the *Ga2* locus, respectively (Chen et al., 2022e). Furthermore, the *Ga1* locus was proven to contain one silk-expressed *ZmGa1F* and eight pollen-expressed *ZmGa1P*-like genes (including *ZmGa1P*) (Zhang et al., 2023i). Two *Ga1-S* lines (SK and HP301) and five *Ga1-M* lines (CML52, CML333, NC350, NC358, and Tzi8) from the maize pan-genome (Hufford et al., 2021; Yang et al., 2019a) provide additional evidence for the presence of the above nine genes (Wang et al., 2022h; Zhang et al., 2023i). An organizational model in which "multiple pollen-expressed male determinants and a silk-expressed female determinant are tightly linked to control the *Ga1* locus" was proposed. The *ZmGa2P*-like and *Tcb1-m*-like gene cluster was also identified in the *Ga2* and *Tcb1* locus using the maize pan-genome (Chen et al., 2022e; Hufford et al., 2021). This indicates that all three UCI loci possess the same organizational model (Figure 9C). The existence of multiple male determinants offers evolutionary benefits because the loss of function of male determinants would be lethal. Though all the male and female determinants of the three loci have been identified, the maize UCI is more complex than expected. Some maize lines contain the intact *ZmGa1F* or *Tcb1-f*, while they are either not expressed or highly expressed

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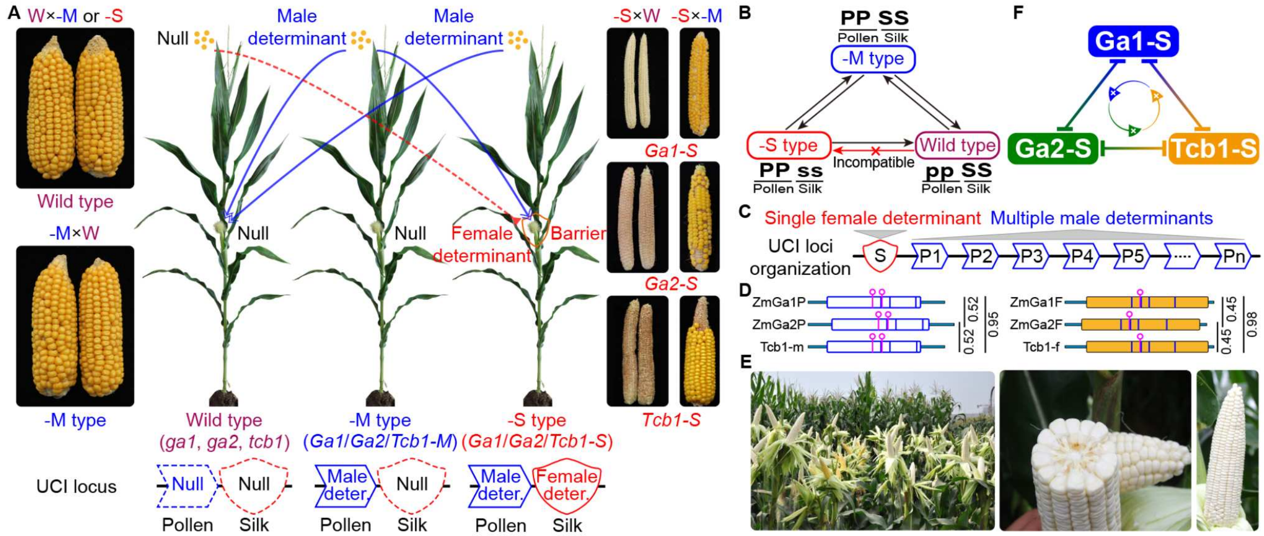

Figure 9. Advances in maize UCI and their practical applications in commercial breeding programs. A, The genotypes of maize inbred lines and their cross-compatible/-incompatible relationships. Three maize UCI loci, *Ga1*, *Ga2*, and *Tcb1*, were identified in maize, and each of them possesses three haplotypes, *-S* type (*Ga1-S*, *Ga2-S* or *Tcb1-S*), *-M* type (*Ga1-M*, *Ga2-M* or *Tcb1-M*), and wild type (*ga1*, *ga2* or *tcb1*), according to the presence/absence of pollen-expressed male determinant and silk-expressed female determinant. During fertilization, wild-type pollen is rejected by *-S* due to the female barrier; however, the pollen of wild type is accepted by *-M*. B, The genetic relationships of three genotypes. The male determinants of three loci are dominant and gametophytic in nature (indicated by PP), while the female determinants are recessive and sporophytic in nature (indicated by ss). C, The organizational model of maize UCI loci. Each locus is modulated by a single female determinant and multiple male determinants, which are tightly linked. S indicates female determinant and *P1-Pn* indicate male determinants. D, Protein sequence identification and catalytic site analysis of maize UCI determinants. *ZmGa1P*, *ZmGa2P*, and *Tcb1-m* are the male determinants, while *ZmGa1F*, *ZmGa2F*, and *Tcb1-f* are the female determinants of the three UCI loci, respectively. All maize UCI determinants encode PME protein. Five conserved amino acids in the catalytic sites are indicated by vertical lines. Mutations of these conserved residues are marked by pink color. E, Application of the maize UCI in commercial breeding programs. The white kernel line harboring the UCI locus rejected wild-type pollen (yellow kernel) from adjacent plants without physical isolation. F, The combination of the three genetically distinct and reciprocally incompatible loci (*Ga1-S*, *Ga2-S*, and *Tcb1-S*) offers a more robust reproductive barrier than any single UCI system alone.

but lack the cross-incompatibility barrier (Lu et al., 2019; Zhang et al., 2023i). Maize UCI is potentially regulated at the transcriptional, translational, and post-translational levels (Zhang et al., 2023i). Possible loci/modifiers or genes, such as another silk-expressed gene *ZmPRP3* encoding a pathogenesis-related protein that promotes pollen tube growth (Wang et al., 2022h), may be involved in maize UCI.

The maize UCI in incompatible silk is caused by the cessation of pollen tube growth (Chen et al., 2022e; Lu et al., 2014; Zhang et al., 2018g). The male determinants and female determinants of the *Ga1*, *Ga2*, and *Tcb1* loci all encode type-II/group1 pectin methylesterase (PME) proteins (Figure 9D) (Chen et al., 2022e; Lu et al., 2019; Zhang et al., 2023h; Zhang et al., 2023i; Zhang et al., 2018g), which are known to modulate the pectin methyl esterification level of the pollen tube cell wall at the tip region and affect its structure and properties, thus regulate the growth of pollen tube (Duan et al., 2020). A balanced pectin methyl esterification level is required for proper pollen tube growth- too much or too little disrupts normal development (Zhang et al., 2023h). In the *Ga1* and *Ga2* mediated UCI systems, suppressed PMEs activity elevates pectin methyl esterification levels at the pollen tube apex, leading to growth arrest (Chen et al., 2022e; Zhang et al., 2018g). The crystal structure of plant PME revealed five evolutionarily conserved residues, Q113, Q135, D136, D157, and R225, where mutations caused a significant reduction in enzymatic activity (Jolie et al., 2010; Pelloux et al., 2007). Maize UCI-related PMEs harbor mutations in these conserved residues (Figure 9D), suggesting that they may lack functional PME activity. Together with the significantly altered methyl esterification of pollen tube, other active PME components in pollen and/or silk, such as ZmPME10-1 (a pro-region harboring type-I/group 2 PME that interacts with male determinants and female determinants of *Ga1* and *Ga2*), are likely involved in the control of maize UCI (Chen et al., 2022e; Zhang et al., 2018g). These results reveal the mechanism of PME-mediated maize UCI. Unlike the self-incompatibility (SI) system, where direct interactions of male and female determinants enable pistils to discriminate self/non-self pollen, maize UCI employs a distinct mechanism, as evidenced by the lack of physical interaction between the male determinants and female determinants. In addition to the similarities, uniqueness also exists among the *Ga1*, *Ga2*, and *Tcb1* loci. Three maize UCI loci are genetically distinct and have significantly different pollen tube morphologies, suggesting that the molecular mechanisms of the maize UCI vary (Chen et al., 2022e; Lu et al., 2014). Moreover, the molecular mechanism underlying the specificity of male-female determinant interactions in maize UCI remains an open question.

Maize UCI acts as a natural reproductive barrier, limiting gene flow among maize populations. As most dent and flint maize varieties carry the wild type haplotypes, making this system a potential tool for commercial breeding (Zhang et al., 2018g). Introduction of the maize UCI locus into dent and flint maize varieties offers an effective method to isolate undesired pollen in commercial maize production (Figure 9E) (Chen et al., 2022e; Zhang et al., 2018g). Nevertheless, the identification of *S* haplotypes and *M* haplotypes among elite maize varieties greatly compromises their utilization as reproductive barriers in maize breeding programs (Chen et al., 2022e; Zhang et al., 2018g). *Ga1*, *Ga2*, and *Tcb1* maize lines are cross-incompatible with each other (Figure 9F). Pyramiding of *Ga1*, *Ga2*, and *Tcb1* and development of lines homozygous at two or three loci presents an effective solution to the limitations observed when employing a single UCI locus as a reproductive barrier (Zhang et al., 2023h; Zhang et al., 2018g). Together, the mechanism of how male determinants and female determinants, including uncharacterized components, coordinately regulate maize UCI remains to be elucidated. Significant progress in understanding of UCI in maize will not only shed light on the identity and specificity of the three UCI loci, but also promote the application of these systems in crop improvement.

Theory and practice of molecular breeding by design

Crop molecular breeding by design is a strategic approach to plant breeding that integrates modern genetic technologies and molecular biology tools to create improved crop varieties in a precise manner (Liu et al., 2021b). Over the last ten years, progress in maize genome editing technologies has enabled efficient generation of desirable mutants for both functional studies and breeding practices. With advancements in computational biology and artificial intelligence, predictive modeling and machine learning are increasingly applied to inform breeding decisions, adding further sophistication to molecular breeding by design. In addition, double haploid breeding, enabling fast development of homozygous inbred lines, has made great progress in establishing a robust breeding process.

Maize genome editing

A programmable RNA-guided DNA endonuclease functioning in bacterial immune defense had been harnessed to develop diverse CRISPR/Cas genome editing tools (Jinek et al., 2012). These tools have significantly revolutionized maize basic research, enabling researchers to dissect the genetic architecture of polygenic traits, understand gene function, and uncover underlying molecular mechanisms. At the same time, these technologies have demonstrated immense potential in breeding applications (Figure 10) by facilitating precise genetic modifications to improve crop yield, stress resilience, and disease resistance in maize, thereby opening up new possibilities and directions for sustainable and resilient agricultural production (Chen et al., 2019a; Xie et al., 2020).

Development of tools for efficient genome editing in maize

Single-guide RNA expression in the nucleus is one of the most important components for efficient targeting through CRISPR/Cas effector (Figure 10A). To meet this end, all seven native Pol III-recognized promoters for RNA-guide-based genome editing systems had been characterized to construct highly efficient CRISPR/Cas systems in maize (Qi et al., 2018). The *dmc1* promoter was used to drive Cas9 expression, and an efficiency of 66% homozygous or bi-allelic mutants was obtained in maize (Feng et al., 2018a).

CRISPR/Cas9 systems can be applied in a high-throughput manner to generate thousands of targeted mutations simultaneously. This is particularly useful when combined with a barcode-based deep sequencing method that enables fast tracing of the target mutation from the huge mutant libraries (Liu et al., 2020c). CRISPR/Cas systems can also be engineered as programmable transcription factors to either activate or inhibit transcription, the idea of which has been demonstrated for parthenogenesis induction in maize (Qi et al., 2023).

SNPs contribute to substantial phenotypic diversity in maize. Several tools have been developed to generate targeted SNP mutations, such as base substitutions (prime editing), cytosine (CBE), and adenine (ABE) base editors (Figure 10B). A base editor combining CRISPR/Cas9 nickase, cytidine deaminase, and uracil DNA glycosylase inhibitor (UGI) enabled targeted conversion of cytosine to thymine at both *ZmALS1* and *ZmALS2* genes, resulting in high-level herbicide resistance (Li et al., 2020h). Prime editing is a precise genome editing technique that can introduce all possible base substitutions along with short insertion and deletion. An efficient ePE5max system had been developed to efficiently generate heritable mutations in maize (Qiao et al., 2023).

Except Cas9, some enzymes from Cas12 and Cas13 families, for instances, AsCas12a, LbCas12a, LwaCas13a, and RfxCas13d, have been developed for sensitive pathogen nucleic acid detection including rice black-streaked dwarf virus, *Fusarium graminearum*, and *Fusarium verticillioides* (Li et al., 2022e)

The rapid visualization of transgenic material in seed, called seed fluorescence reporters, was also well established to improve the selection efficiency of both edited events and transgene-free individuals (Xu et al., 2021b; Yan et al., 2021b)

Genotype-independent transformation and genome editing

Existing technologies have shown that genotype-independent genome editing is no longer an obstacle. First, the overexpression of the wheat gene *TaWOX5* from the *WUSCHEL* family (Wang et al., 2022a), along with other reported maize *WUSCHEL*, can dramatically increase transformation efficiency with less genotype dependency in wheat, rye, barley, and maize. Second, a recent study revealed that the signaling molecule REF1, which promotes plant regeneration, can boost the regeneration and transformation efficiency of the maize line B104 by 6-fold and 4-fold, respectively. Compared with other strategies that improve transformation by expressing developmental regulatory factors, the use of exogenous ZmREF1 peptide represents a simpler and more practical approach to enhancing transformation (Yang et al., 2024b). Additionally, a novel method that utilizes magnetic nanoparticles to deliver foreign genes into maize pollen through germinal pores has been reported. This approach effectively addresses the critical bottlenecks of tissue culture dependency and genotype limitations in maize genetic transformation (Wang et al., 2022j). These techniques have laid an important foundation for the precise genome editing of varieties used in maize production.

Precision genetic improvement by employing CRISPR/Cas systems

The CRISPR/Cas9 system has revolutionized the field of precision genetic improvement in crops, providing a robust platform to engineer diverse traits in maize. From plant architecture and yield-related traits to grain quality and disease resistance, this transformative technology is reshaping maize breeding and advancing sustainable agricultural practices.

CRISPR/Cas9 has demonstrated remarkable success in enhancing yield-related traits in maize. By generating weakened promoter alleles of CLE genes and null alleles for partially redundant compensating CLE genes, researchers achieved significant improvements in grain size and plant biomass (Liu et al., 2021c). Optimizing plant architecture is critical for

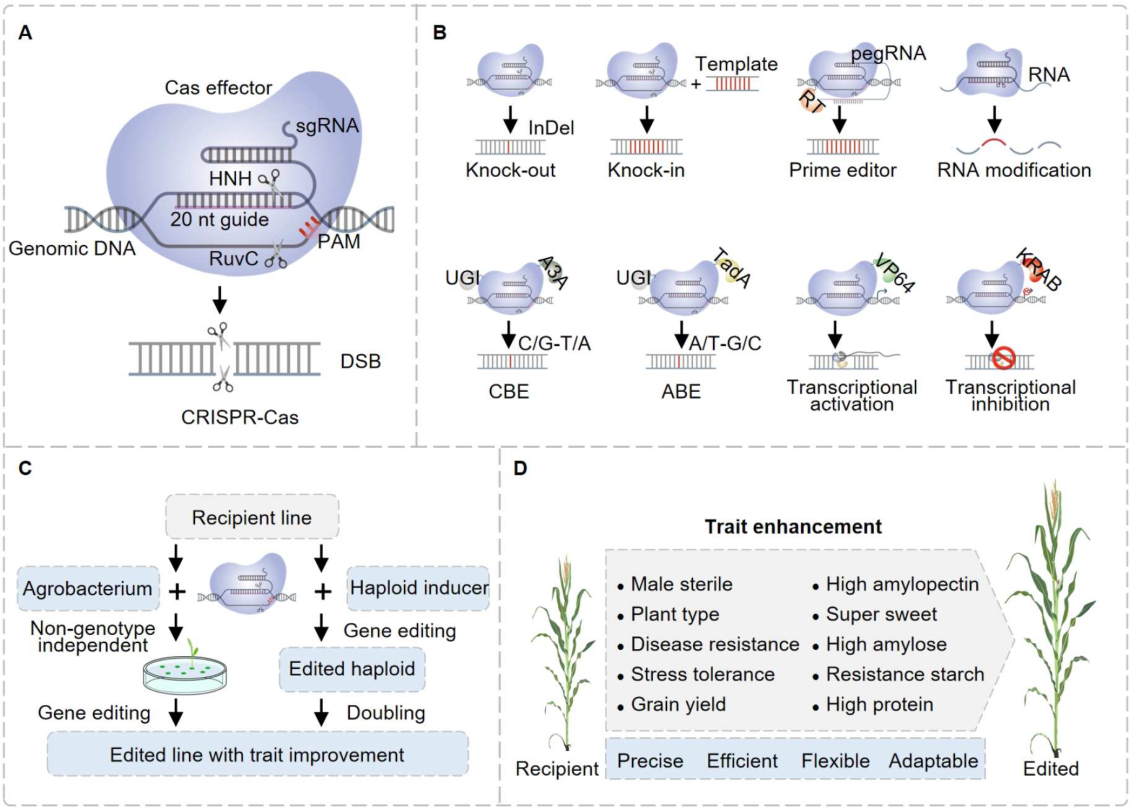

Figure 10. The development and application of CRISPR/Cas tools transform maize genetic improvement and breeding. A, The basic components of CRISPR/Cas effectors that could be extended into diverse and precise genome editing tools in (B). B, Development of diverse genome editing tools that could introduce precise and different types of targeted mutations in the maize genome. C, Two major CRISPR/Cas delivery strategies to introduce desirable mutations without genotype dependence. D, Overview of reported CRISPR/Cas-based genetic improvement and breeding applications in maize.

improving productivity in high-density planting systems. Using a highly efficient *in vivo* targeted mutator, researchers achieved precise mutations in the LIGULELESS1 (LG1) locus, generating compact plant architecture without the negative effects of linkage drag. The system achieved mutation frequencies of 51.5%–91.2% in T_0 stable transformants and 11.79%–28.71% in progeny crossed with recipient lines, underscoring its effectiveness in modifying plant architecture (Li et al., 2017a). Efforts have also been made to generate a series of maize lines with reduced plant height by precise editing of the *Brachytic2* (*Br2*) gene. Moreover, the editing of *Br2* in multiple genetic backgrounds by using the Haploid-Inducer Mediated Genome Editing (IMGE) system could generate plants with optimized plant height, which can potentially facilitate molecular breeding of maize cultivars suitable for high-density planting (Zhao et al., 2025).

The rising demand for specialty maize varieties, such as waxy maize and supersweet maize, has driven the application of CRISPR/Cas9 in quality improvement. For instance, precise edits to the *WX1* gene enabled the conversion of normal parental lines into waxy maize, streamlining the development of specialty germplasm (Qi et al., 2020a). Furthermore, multiplex genome editing targeting *Sh2* and *Wx* genes has facilitated the creation of supersweet, waxy, and dual supersweet-waxy maize varieties, paving the way for advancements in specialty corn breeding (Dong et al., 2019).

Biotic stress, including fungal infections and viral diseases, continues to pose significant challenges to maize production worldwide. CRISPR/Cas9 has emerged as a powerful tool to address these threats. For example, null mutants of *ZmGDIa*, created through CRISPR/Cas9 editing, conferred enhanced resistance to maize rough dwarf disease (MRDD), outperforming natural *ZmGDIa-hel* alleles. Importantly, these mutants displayed no agronomic disadvantages, highlighting the feasibility of targeting *RabGDIa* for resistance to rice black-streaked dwarf virus (RBSDV) in other monocot crops (Liu et al., 2022a). Similarly, CRISPR/Cas9 has been employed to develop maize varieties resistant to ear rot caused by *Fusarium verticillioides*, further demonstrating the potential of this technology in breeding disease-resistant maize (Liu et al., 2022b).

While off-target effects of gene editing are documented, their agricultural impact is often mitigated by scalable and low-cost genotyping to isolate precise edits in crops. Unlike medical applications, crop systems tolerate the off-target risks due to efficient selection of edited individuals from large populations. Advances in sgRNA design tools further reduce such risks, enabling practical implementation in maize breeding without prohibitive technical barriers.

Genomic selection and its application

Genomic selection (GS) is a breeding technique using genome-wide molecular markers to construct a prediction model from “genotype” to “phenotype” and estimate the breeding value of an individual to assist in selecting the best inbred lines and designing hybrid combinations. Compared with molecular marker-assisted selection breeding technology, GS does not need to identify sites significantly related to target traits. Even if the effect of a single site is small, all genetic effects leading to phenotypic variation can be captured by high-density genetic markers, and the breeding value can be evaluated when individual genotypes are obtained, which can greatly shorten the breeding cycle and improve breeding efficiency. Genome selection technology was first applied to breeding high-yield dairy cattle, and has been gradually extended to plant breeding, such as predicting maize heterosis, rice yield, and wheat stress resistance (van Dijk et al., 2021). The application of GS technology can maintain population genetic diversity and obtain high genetic gain in a short period of time, which has become the mainstream technical means of foreign maize commercial breeding systems (Crossa et al., 2017). CIMMYT has implemented GS technology in the global maize breeding program, and multinational companies such as Corteva and Bayer have applied GS technology in the breeding process to improve the efficiency of maize breeding.

Traditional GS models

Best linear unbiased prediction (BLUP) is the most widely used GS method. The early BLUP model used the pedigree information of animals as input to construct the pedigree relationship matrix and train the model, which was not suitable for the population with incomplete pedigree information or no pedigree information in plant breeding. It was not until the early 2000s that genome-wide markers were formally used to estimate marker effects and train models. Two representative BLUP models are GBLUP (genomic BLUP) and rrBLUP (ridge regression BLUP). The former uses genomic information to construct an individual genetic relationship matrix instead of a pedigree relationship matrix, while the latter uses the ridge regression algorithm to estimate the marker effect. Wang et al. (2018c) extended the category of BLUP and proposed cBLUP (compressed BLUP) and sBLUP (super BLUP) based on compression markers and samples, respectively. In addition to the BLUP family, traditional GS methods also include (i) Bayesian models such as BayesA, BayesB, BayesC, and Bayes Cπ, (ii) penalized linear models such as LASSO (least absolute shrinkage and selection operator), Bayesian ridge regression, and Bayesian LASSO (iii) nonlinear models such as RKHS (reproducing kernels Hilbert spaces regression), random forest, SVM (support vector machine), and ANN (artificial neural network) (Tong and Nikoloski, 2021).

Advances of GS techniques

Although GS provides a new prospect for crop breeding, the accuracy of traditional GS models is still low for yield and related quantitative traits, which are heavily influenced by the environment. Researchers have tried new strategies to improve the predictive power of complex traits.

(1) Adopt ensemble learning and deep learning methods. The factors that affect the accuracy of GS are complex, including the heritability of traits, population size, density of molecular markers, selection of statistical models, population structure, and inter-population relationship. These factors have multi-dimensional complexity and interdependent interaction. Azodi et al. (2019) systematically evaluated 12 genome selection algorithms based on statistics and machine learning to predict 18 traits of 6 plant species, and they found that no algorithm had the best performance on all species and traits, and model tuning and ensemble helped to improve the prediction effect. Recently, Yan et al. (2021a) developed a one-stop crop smart breeding toolbox CropGBM, based on a new ensemble learning algorithm LightGBM, which not only improves the prediction accuracy, robustness, and operational efficiency of the model, but also performs well in real complex breeding populations with small training samples and serious population structure. In addition, it also has auxiliary functions such as automatic processing of genotype and phenotype data and automatic screening of features, which are suitable for large-scale breeding practice. Wang et al. (2023e) developed a GS model DNNGP based on the convolutional neural network framework, which carried out feature optimization through dynamic integration of different types of multi-omics data, and optimized the model through batch normalization and early stopping. Compared with the traditional GS method, the operation efficiency, prediction accuracy, and robustness were significantly improved. DNNGP accelerates the application of deep learning technology in GS and has been applied to identify rare and favorable alleles, which are valuable genetic targets for genomic design breeding to accelerate the development of novel maize varieties (Fan et al., 2025).

(2) Carry out multi-omics and multi-trait joint prediction. Compared with the direct prediction of phenotypes from genotypes, some studies have shown that the ability to predict complex traits such as yield can be effectively improved by integrating intermediate phenotypes such as transcriptome and metabolome, suggesting that intermediate omics data provide additional information in predicting complex traits. Hu et al. (2019) proposed MLLASSO, a phenotypic prediction method that integrates multi-omics data. Multi-layer LASSO model was used to predict gene expression through genotype, metabolite content through predicted gene expression, and phenotype through predicted metabolite content, completing the construction of a directed learning model supervised by intermediate omics data. After the establishment of the model, only the genotype data is needed to complete the phenotype prediction, which greatly improves the practicability of the model in crop breeding.

The combined prediction of multiple traits can improve the prediction ability of complex phenotypic traits by using genetic correlation information between traits, especially for low heritability traits. Recently, Yang et al. (2022d) developed a target-oriented prioritization (TOP) algorithm for multi-trait selection breeding through machine learning simulation, which can effectively carry out multi-trait selection and select breeding materials with advantages of specific target traits while maintaining other traits of existing commercial varieties. TOP can also integrate transcriptome and metabolome data to further improve the accuracy of multi-trait selection breeding.

(3) Construct GS models of genotype-environment interaction. In addition to genetic factors, complex quantitative traits of crops are affected by non-genetic factors such as the external environment. The contribution of environment to phenotypic variance is different for different traits. For example, environmental factors contribute to about 90% of the phenotypic variance of flowering time, while they contribute to less than 60% of the phenotypic variance of both plant height and grain yield (Fu and Wang, 2023). Therefore, adding environmental factors can generally improve the predictive power of complex traits. When using statistical methods such as GBLUP or rrBLUP, in addition to genotype, genotype-by-environment (G×E) effects can be directly modeled by including environmental factors as covariates. In contrast, machine learning is more flexible in

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integrating environmental factors. The gradient enhancement framework XGBoost and LightGBM were used to integrate genotype and environmental factors including weather, geographical location (latitude and longitude), and year to predict maize yield and plant height. Compared with the results of the linear random effects model considering the same environmental factors, the accuracy of the machine learning method was significantly improved (Arouisse et al., 2021). Xu et al. (2022b) proposed a new strategy of integrated genomic-enviromic prediction (iGEP), which accurately predicted the performance of specific genotypes in specific environments with the support of comprehensive information of genotype, phenotype, and environment type, and realized directional breeding adapted to climate change and specific environments in a true sense.

Doubled haploid breeding

Doubled haploid (DH) breeding technology can significantly accelerate the process of line development, thus possessing crucial value in breeding. DH technology has made rapid progress in both application and theoretical research in the past decade. A complete DH technology has been established and widely applied in enterprises. In terms of basic research, four genes related to maize haploid induction have been cloned. Moreover, based on the conservation of these genes in different species, haploid induction methods have been developed for other monocotyledonous crops like wheat and rice, as well as dicotyledonous plants like tomato, rapeseed, and tobacco. Furthermore, haploid breeding technology has demonstrated promising potential for integration with genome editing technology.

Milestones of DH breeding technologies

Three key steps were involved in DH breeding, i.e., haploid induction, identification of haploids, and chromosome doubling. Haploid induction depends on the haploid inducer line. The first haploid inducer line is named Stock6, which induces maize haploids at a frequency of 2%–3% in its self-pollinated progeny or as parents in hybrid crosses (Coe, 1959). Subsequently, new haploid inducer lines, such as ZMS, KEMS, UH400, and CAU5, were developed with enhanced haploid induction rate (HIR) (Li et al., 2009). There were more than 50 publicly reported haploid inducer lines globally until 2016, and all these inducers were derived from Stock6 (Hu et al., 2016), and the HIR of widely used haploid inducers was approximately 10%. These haploid inducers paved the way for industrial application of DH technology.

Most of the induced progenies are diploids, with only a few being haploids. It is necessary to distinguish them from haploid seeds before chromosome doubling. Sarkar and Coe (1966) introduced the *R1-nj* marker into the Stock6, which exhibits a dominant genetic trait that turns the aleurone and endosperm purple. This color marker system remains stable in temperate germplasms. However, in some tropical germplasms and a few temperate ones, the application of this identification system is limited due to the presence of inhibitory genes, such as C1-I, which prevent the expression of purple color in embryos and endosperms (Chaikam et al., 2015). To address the identification issue in large-scale haploid breeding processes, Wang et al. (2016a) proposed kernel oil content as a marker for haploid identification, and developed a fully automated haploid seed identification system, with a haploid identification accuracy over 90%. To further improve the efficiency of haploid identification, new marker systems, including fluorescent proteins, C1&R gene overexpression, and Betalain, were tested in maize (Chen et al., 2022a; Dong et al., 2018a; Wang et al., 2023b). These new makers make haploid identification convenient and easy.

Reduced fertility of haploids is precisely one of the main issues hindering the application of DH technology. Furthermore, compared with the female fertility of haploid plants, the male fertility is generally more severely affected (Röber et al., 2005). Currently, there are two main methods for haploid doubling: natural and chemical doubling. The efficiency of haploid doubling is closely related to the genetic background, and temperate materials typically exhibit higher haploid male spike fertility. In chemical doubling, colchicine is the main ingredient that restores fertility. The efficiency of chemical doubling is significantly higher than natural doubling. Chromosome doubling based on immature embryo tissue culture is a newly emerged efficient method; it not only enhances the efficiency of haploid doubling but also shortens the cycle of pure line development.

Genetic studies on haploid induction and chromosome doubling

The genetic basis of maize haploid induction has been a focal point of research for scientists. *ggi1*/*qhir1* is the first QTL that was detected by several early studies. Prigge et al. (2012) not only verified the effect of *qhri1*, but also detected 7 more QTLs contributing to HIR. Further, two QTLs, *qhir1* and *qhir8*, were mapped to the intervals of 243 and 789 kb, respectively, which laid an important foundation for the cloning of candidate genes (Dong et al., 2013; Liu et al., 2015a). In *qhir1*, a phospholipase (Patatin-like phospholipase) coding gene, *ZmPLA1*/*MATL*/*NLD*, which was specifically expressed in male flowers, contained an insertion of CGAG in the fourth exon, leading to frameshift mutations and premature translational termination. This results in the loss of gene function, leading to haploid induction (Gilles et al., 2017; Kelliher et al., 2017; Liu et al., 2017a). These works revealed the mystery behind haploid induction that had puzzled breeders for over sixty years (Jackson, 2017). A recent study showed that in *qhir8*, the 131st single base substitution (C-T) in *ZmDMP*, which encodes a protein with an unknown function of DUF679, leads to a significant increase in HIR. Knocking out the *ZmDMP* gene through gene editing can elevate HIR from 2% to 6%–7% (Zhong et al., 2020a). Based on the sequence of *ZmPLA1*/*MTL*/*NLD*, Li et al. (2021e) identified a phospholipase D (*ZmPLD3*) coding gene in pollen, and found that disrupting this gene further enhanced haploid induction efficiency. Jiang et al. (2022a) unraveled the molecular mechanism of haploid induction caused by the key gene *ZmPLA1* in maize. They identified an increase in ROS in the germ cells as a critical factor for maize haploid induction and discovered a novel gene, *ZmPOD65*, which is involved in haploid induction. Incorporating *ZmPLD3* and *ZmPOD65* mutant alleles into haploid inducer lines is expected to further improve the efficiency of maize haploid induction systems.

New technologies based on DH breeding

The genes that control haploid induction provided chances to build a DH breeding process in crop species other than maize. Recent studies showed that knocking out the *ZmPLA1*/*MATL*/*NLD* homologs in wheat, rice, and millet can induce haploids successfully at a frequency from 1% to 20% (Cheng et al., 2021; Liu et al., 2020a; Yao et al., 2018). The *ZmDMP* gene is conserved among dicot species. It was demonstrated that knocking out the *ZmDMP* homologs can induce haploids in many species including tomato, tobacco, rapeseed, watermelon, and Medicago (Wang et al., 2022b; Zhang et al., 2022b; Zhao et al., 2022b; Zhong et al., 2020a; Zhong et al., 2022). These studies opened the era of establishing a general DH breeding system for different crops.

Recent innovations in marker systems have significantly improved haploid identification. The RUBY reporter, a betalain-based visual marker, enables species-independent haploid screening in maize and tomato by producing vivid pigmentation in embryos/radicles within 10 days post-pollination, overcoming limitations of anthocyanin inhibitors (Wang et al., 2023b). In maize, the MAGIC1/2 system co-expresses transcription factors ZmC1 and ZmR2 to enhance anthocyanin accumulation, achieving 99.1% identification accuracy at early developmental stages (Chen et al., 2022a). Additionally, double-fluorescence markers (e.g., DsRED/eGFP) allow non-destructive, high-throughput screening via embryo-specific expression, which has been validated in diverse elite lines (Li et al., 2025). These systems collectively address challenges of efficiency, background dependency, and scalability in modern DH breeding.

Gene editing technology has emerged as a useful tool in the precise improvement of plant and animal germplasm. Nevertheless, only a limited genetic background can be used for transformation in maize, significantly limiting the application of gene editing. In 2019, Hi-Edit/IMGE, which combines gene editing with DH breeding, was developed. This technique involves introducing gene editing components into inducer lines. In the haploid plants obtained from the crosses with these lines, a small number of haploids with target genes edited can be selected and doubled to obtain an edited inbred line (Kelliher et al., 2019; Wang et al., 2019a). By integrating haploid breeding with gene editing, this strategy facilitates the rapid improvement of specific traits in breeding materials and presents significant potential for maize improvement. Despite its theoretical advantages, the practical adoption of HI-Edit/IMGE in the seed industry has been limited. The most significant bottleneck lies in the rapid development of haploid inducer lines that simultaneously possess haploid induction capability and gene-editing functionality. Conventional breeding strategies to stack these traits are both time-consuming and labor-intensive. Recent efforts have focused on screening haploid inducer lines with high genetic transformation efficiencies to construct HI-Edit/IMGE systems in maize (Delzer et al., 2024). Additionally, a genetic transformation system based on the haploid inducer line HI3 has been developed, achieving the goal of "one-step line creation". This method directly introduces gene-editing vectors into haploid inducer lines, enabling simultaneous haploid induction and gene editing within the same generation (Tian et al., 2024a). These advances underscore both the potential and the challenges of implementing HI-Edit/IMGE in maize breeding programs, offering a promising pathway for more efficient and precise crop improvement.

Prospect of molecular breeding by design technologies

Maize breeding technology has experienced the development process from GS 1.0 to GS 4.0. GS 1.0 to GS 3.0 aims to improve the evaluation efficiency of DH lines, while GS 4.0 aims to directly select promising hybrids (Fu et al., 2022). In the GS 4.0 era, model prediction accuracy may not be the only goal pursued in plant breeding practice. On the contrary, starting from breeding practice, the selection of breeding materials with comprehensive consideration of multiple traits, the ability to conduct a large number of sample tests with fewer training samples, and the robustness, scalability, and operation efficiency of breeding models are important factors to be considered (Yan and Wang, 2023). Jiang et al. (2020) proposed a "genome design breeding" scheme that combines DH line production, genome selection, genome optimization, and machine learning simulation, which provides a valuable paradigm for the engineering breeding process of maize in the GS 4.0 era. Furthermore, how to integrate multi-omics data (e.g., metabolomics and epigenomics) into genomic selection models for enhanced breeding accuracy is also an area that deserves more attention.

Perspectives

Over the past decade, significant progress has been made in understanding the composition and function of maize genomes, identifying the genes and molecular pathways underlying agronomic traits, and developing novel technologies for hybrid development. However, there are still lots of questions that need to be explored.

Although dozens of genome assemblies have been released, there is still a lack of understanding of the functional elements in the genome. Considering the larger genome size, the high proportions of repetitive sequences, and the frequent occurrence of long-distance interactions between *cis*-regulatory elements and their target genes, it remains a great challenge to catalog and characterize the functional elements in the genome. In addition to understanding and utilizing the existing variations in the maize genome, the exploitation of other diversity needs to be considered. For example, a lot of genetic variations have been lost during maize domestication, including variations that are important for agronomic traits. Also, novel variations created using cutting-edge genome editing tools contribute to maize diversity. How to efficiently integrate these variations with existing variations for maize improvement will be important in the future.

The methods for gene cloning from QTLs have been perfected over the past decades. However, it is still difficult to clone genes underlying QTLs with minor effects. The efficiency of QTL cloning needs to be improved. Instead of cloning QTLs one by one, intelligent methods that enable high-throughput cloning of multiple genes need to be developed. Besides, the traits for QTL mapping and cloning need to be carefully selected. Besides agronomic traits that are usually collected manually by people, high-throughput phenotyping techniques that enable fast and accurate collection of multiple data points should be developed. These techniques are the prerequisites for large-scale field tests across the maize growing seasons and under various environmental conditions. In the future, we aim to cultivate "ideal" maize varieties that meet the diverse needs of consumers. If maize is to be used as feed, we should fully take into account the requirements of the feed industry for maize. If it is used as an industrial raw material, we need to consider the demands of the industrial sector for maize. And if it is used for human consumption, we should consider the needs of different

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consumers for maize. It was shown that there is often a trade-off between traits, for example, between yield and quality. Identifying genes involved in balancing multiple traits and understanding the synergistic molecular mechanisms will provide insights not only into gene regulatory theories, but also into breeding varieties with desirable traits.

Integration of knowledge from functional genomics studies into maize breeding will be of great importance. In addition to marker-assisted selection of single favorable alleles, technologies that enable efficient pyramiding of multiple alleles from different genes involving various traits should be developed. Theories and breeding practices of genome selection are anticipated to be developed. The establishment of a genome-wide selection workflow involves several types of platforms, such as high-throughput genotyping platforms, haploid induction and doubling platforms, genomic selection platforms, and so on. The development of novel prediction models and algorithms, and the construction of user-friendly databases that integrate multi-dimensional datasets will aid the development of genome-wide breeding strategies. We understand that the development of these novel breeding strategies needs a systemic effort of the maize community. Moreover, cross-disciplinary efforts of people with different expertise in genetics, genomics, mathematics, molecular biology, and computer sciences are needed. In summary, with the ever-growing knowledge gained from functional genomics, the innovative technologies that continue to be developed, and a joint effort of the entire research community, a breakthrough in maize genetic improvement is anticipated in the future.

Compliance and ethics

The authors declare that they have no conflict of interest.

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Supporting information

The supporting information is available online at https://doi.org/10.1007/s11427-025-3022-6. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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