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Understanding the regulation of cereal grain filling: The way forward[FA]

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ABSTRACT

During grain filling, starch and other nutrients accumulate in the endosperm; this directly determines grain yield and grain quality in crops such as rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*). Grain filling is a complex trait affected by both intrinsic and environmental factors, making it difficult to explore the underlying genetics, molecular regulation, and the application of these genes for breeding. With the development of powerful genetic and molecular techniques, much has been learned about the genes and molecular networks related to grain filling over the past decades. In this review, we highlight the key factors affecting grain filling, including both biological and abiotic factors. We then summarize the key genes controlling grain filling and their roles in this event, including regulators of sugar translocation and starch biosynthesis, phytohormone-related regulators, and other factors. Finally, we discuss how the current knowledge of valuable grain filling genes could be integrated with strategies for breeding cereal varieties with improved grain yield and quality.

Keywords: cereals, grain filling, grain yield, phytohormone regulation, starch synthesis, sugar translocation

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INTRODUCTION

Rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.) are major cereal crops which greatly contribute to human nutrition worldwide. The Food and Agriculture Organization (FAO) estimated global cereal production to be 2,791 million tons in 2021 (OECD-FAO, 2021). Rice and wheat production have greatly improved over the past decades due to the widespread application of "Green Revolution" genes. However, a yield ceiling has emerged, especially for rice. In China, the "super hybrid rice" and "super rice" programs were initiated to increase rice yields via the combined use of ideotype and heterosis breeding strategies, leading to the development of numerous rice varieties with large panicles (Cheng et al., 2007; Peng et al., 2008). However, some of these rice varieties do not produce high yields due to incomplete grain filling (Yang and Zhang, 2010; Sekhar et al., 2015). Therefore, grain filling capacity is a key determinant of crop yield.

Cereal seeds primarily comprise endosperm, which contains large numbers of starch granules. In grain filling, the plant translocates assimilates (carbohydrates produced by photosynthesis) to the endosperm, where a series of enzymes converts sucrose to starch (Krishnan and Dayanandan, 2003). Carbohydrates are translocated from source organs (leaves) to sink organs (seeds) where they are stored in the form of starch (Oparka and Gates, 1981; Patrick, 1997). Therefore, grain filling is closely related to

sugar translocation, metabolism, and starch biosynthesis. Disrupting genes related to these pathways leads to severe defects in grain filling, such as *Grain Incomplete Filling 1* (*GIF1*) in rice, *Miniature1* (*Mn1*) in maize, *ZmSweet4c* and *OsSweet4* (Wang et al., 2008a; Kang et al., 2009; Sosso et al., 2015).

Phytohormone levels in seeds also play vital roles in controlling grain filling (Yang et al., 2001; Xu et al., 2007; Zhou et al., 2013; Doll et al., 2017). Auxin regulates seed development; the indole-3-acetic acid (IAA) content rapidly increases in rice grains after pollination (Yang et al., 2001; Zhang et al., 2009; Uchiumi and Okamoto, 2010). Cytokinin and brassinosteroid (BR) levels regulate the division (and thus number) of endosperm cells in cereals (Yang et al., 2002; Wu et al., 2008). Abscisic acid (ABA) and gibberellin are also involved in grain filling in rice (Schmidt et al., 2014; Qin et al., 2021).

Transcription factor (TF) genes are specifically expressed in seeds, and endosperm development and grain filling require key TFs, such as OPAQUE2 (O2), the No Apical Meristem (NAC) domain TFs OsNAC127 and OsNAC129, Nuclear Factor YB1 (NF-YB1) and NF-YC1, or the B3 domain TF ABA-INSENSITIVE 19 (ZmABI19), to name a few (Li et al., 2015; Bai et al., 2016; Ren et al., 2021; Yang et al., 2021). These TFs likely function as key regulators of grain filling by connecting sugar translocation and metabolism, starch biosynthesis, phytohormonal regulation, and other biological and physiological events.

Poor grain filling reduces grain yield and quality. Understanding the factors that restrict grain filling and dissecting the underlying genes will lay the foundation for improving grain yield and quality by altering cultivation practices or by breeding. In this review, we describe grain filling and the factors that directly and indirectly affect this process. We discuss representative genes involved in different aspects of grain filling and present a molecular network linking key aspects of grain filling. Finally, we propose strategies for improving grain filling in cereals during cultivation and breeding.

WHAT IS GRAIN FILLING?

In cereal seeds, the endosperm accumulates starch and other nutrients. Most nutrients in seeds are stored in the endosperm (Olsen et al., 1999; Consonni et al., 2005). Endosperm development in cereals is generally described as grain filling, representing the final stage of cereal development during which fertilized ovaries develop into caryopses. Grain filling in cereals such as rice (Figure 1A) is characterized by dynamic changes in grain weight along with seed development. The quality of grain filling directly establishes the final yield and quality of grains. Grain filling is also associated with seed size and seed setting rate; some mutants with defects in grain filling exhibit smaller grains and a lower seed setting rate than the wild type (Nallamilli et al., 2013; Gui et al., 2014).

During grain filling, the starchy endosperm in the central area of the caryopsis primarily accumulates starch, along with a small portion of storage proteins (Wu et al., 2016a). The carbon for starch biosynthesis in cereal grains originates from photosynthesis in leaves, which produce assimilates that can be transferred directly to grains or reallocated to reserve pools in vegetative tissues (Schnyder, 1993). Once these assimilates (mainly sucrose) are transported to the caryopsis via specific transporters, sucrose can be converted into monosaccharides by hydrolysis and used for starch biosynthesis (Wang et al., 2008a). These processes reflect the relationships and interactions of well-defined source-flow-sink activities, which largely determine grain filling. In detail, the source represents tissues such as mature leaves and sheaths, which produce assimilates. The sink represents tissues such as grains/kernels, which accept and utilize assimilates. Flow occurs through phloem tissues, which translocate assimilates from source to sink tissue (Li et al., 2018b). Grain filling and production are enhanced in plants via coordinated source-sink-flow activities. For example, high rates of leaf photosynthesis and nutrient remobilization are indispensable for increased panicle and seed size (Yu et al., 2015; Chang et al., 2017). Moreover, the sucrose from leaves must be efficiently unloaded into the endosperm, which is also important for grain filling and crop yields (Ruan, 2014).

The filling caryopsis contains different types of tissues derived from maternal or filial components (Figure 1B). The endosperm is a triploid filial tissue consisting of an inner starchy endosperm and an outer aleurone layer. The embryo occupies a small portion of the caryopsis and comprises diploid filial tissue. Around the endosperm and embryo are the diploid maternal tissues, including the pericarp, seed coat, vascular bundle, and nucellus tissues (Wu et al., 2016a, 2016b). The vascular bundle and nucellus tissues (nucellar epidermis and nucellar projection) are fused with each other and play vital roles in the loading, transfer, and exchange of nutrients between maternal and filial tissues (Krishnan and Dayanandan, 2003; Liu et al., 2022).

During grain filling, cell layers of different maternal tissues exhibit dynamic changes in differentiation and degeneration. Some outer tissues of the caryopsis degenerate prior to starch accumulation, suggesting they have little effect on grain filling. The vascular bundles, nucellar projection, and nucellar epidermis remain alive for a longer time and degenerate at 21 d after fertilization. This duration matches the key stage of starch accumulation, highlighting the roles of these tissues in nutrient transport and grain filling (Wu et al., 2016b). However, the differentiation of the aleurone layer and starchy endosperm begins immediately after fertilization. These tissues accumulate starch throughout the grain filling stage. Only the aleurone layer and embryo retain live cell layers in mature grains (Krishnan and Dayanandan, 2003; Liu et al., 2022). Genes with specific expression patterns in the above-mentioned tissues might be directly involved in controlling grain filling in cereals, such as *GIF1* (expressed in

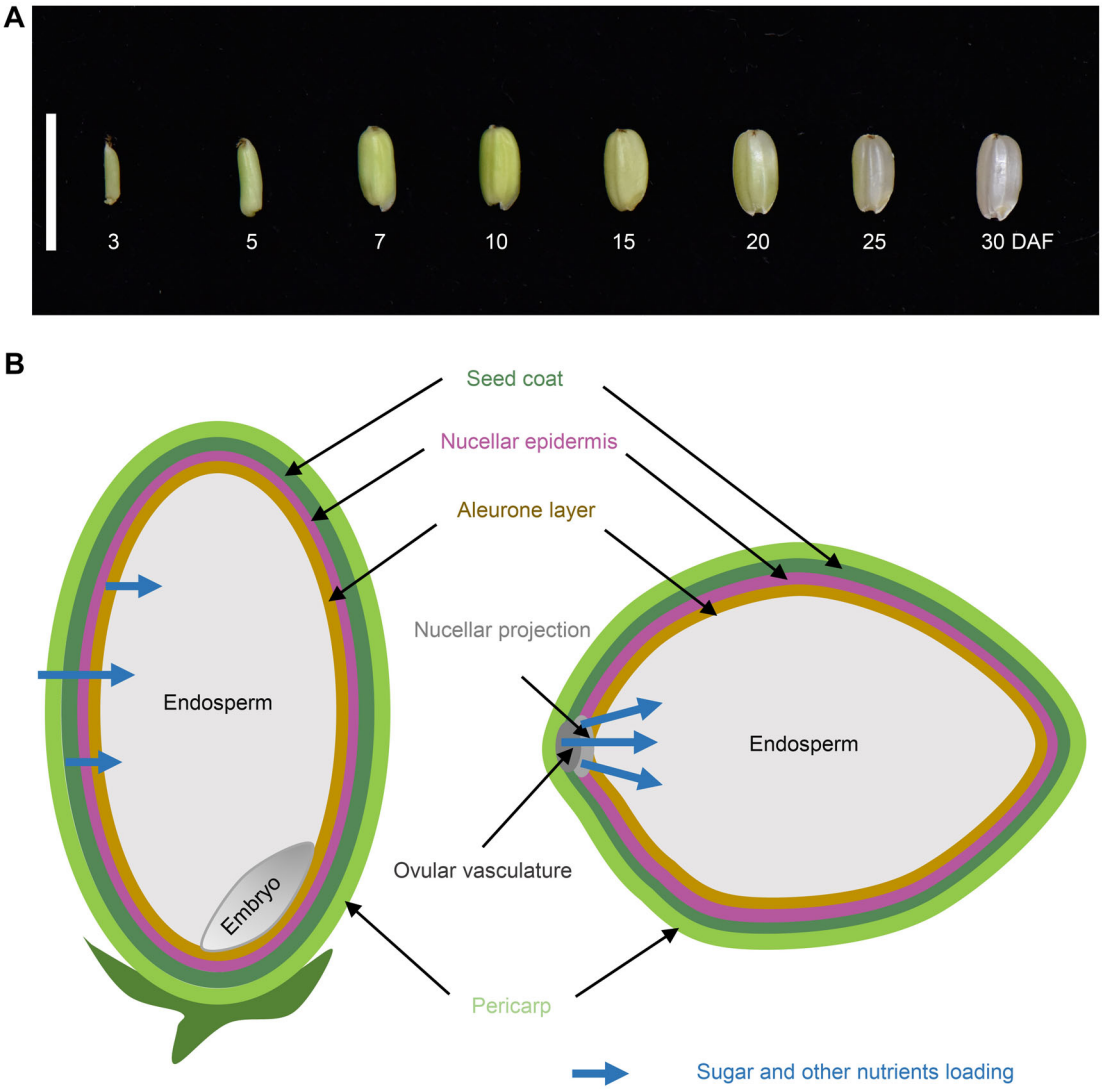

Figure 1. Dynamic of filling rice grains and the anatomical structure
(A) Morphology of developing seeds at successive endosperm development stages. DAF means days after fertilization. Scale bar, 1 cm. **(B)** The diagram of developing seeds with different maternal tissue and filial tissues at vertical and horizontal cutting surfaces. Tissues of pericarp, seed coat, nucellar epidermis, ovular vasculature, nucellar projection, aleurone layer, endosperm and embryo are indicated.

dorsal vascular bundles) (Wang et al., 2008a) and *OsPHO1;2* (specially expressed in ovular vasular bundles and the nucellar epidermis) (Ma et al., 2021).

FACTORS AFFECTING GRAIN FILLING IN CEREALS

To improve the yield potential of cereals, it is important to understand the factors that affect grain filling. Numerous studies in recent years have shown that cereal grain filling can be affected by many factors. Some factors are involved in the biological pathways that directly determine grain filling, including photosynthetic capacity (source), assimilate transport (flow), starch biosynthesis, and cell proliferation (sink). Other factors including phytohormone levels, nutrient levels, abiotic stress, and panicle and grain morphology affect grain filling indirectly via the above-mentioned four biological processes (Figure 2).

FOUR BIOLOGICAL PATHWAYS THAT DIRECTLY AFFECT GRAIN FILLING

Photosynthetic capacity

Photosynthesis contributes 60%–100% of the carbon source during grain filling in cereals (Zhai et al., 2002). Assimilates utilized for grain filling are provided by photosynthesis taking place in flag leaves and ears/panicles, along with some

Figure 2. Key factors involved in cereals grain filling control
The inner panels show the biological processes directly involving grain filling control, including photosynthesis capacity, assimilates transportation, starch biosynthesis and cell proliferation. The outer panels show the indirect factors impacting grain filling by the biological processes shown in inner panels, including phytohormone levels, nutrient levels, abiotic stress, panicle, and grain morphology.

assimilates stored in the culm prior to anthesis (Tambussi et al., 2007). Several studies have confirmed the large contribution of photosynthesis in ears/panicles to grain filling (Maydup et al., 2012; Sanchez-Bragado et al., 2016), and grain yield has been positively correlated with photosynthetic capacity in ears (Merah and Monneveux, 2015; Merah et al., 2017). Chlorophyll content in grains functions as an indicator of the photosynthetic capacity of developing grains, and both chlorophyll contents and net photosynthetic rate in grains are positively correlated with grain filling rate in rice (Chen et al., 2020). Another study suggested that photosynthesis in ears is the major contributor to grain filling in wheat and barley (*Hordeum vulgare*) (Sanchez-Bragado et al., 2020). However, the relationship between leaf photosynthesis and grain filling requires additional investigation. Murchie et al. (2002) looked for associations between grain-filling rate and photosynthesis in the flag leaves of several rice cultivars, but no obvious associations were identified (Murchie et al., 2002). Nevertheless, another study detected complete synchronization between grain filling and highly efficient photosynthetic function in leaves after heading (Zhai et al., 2002). Assimilates produced in leaves might not be fully transported to grains for filling in some varieties, making their relationship unclear.

Assimilate translocation

The transport of assimilates produced by leaf photosynthesis relies on the long-distance phloem pathway. During this step, effective coordination among photosynthesis, assimilate (mainly sucrose) transport efficiency, and sink activity can greatly increase grain filling. The efficiency of sucrose translocation is determined by three factors: the phloem loading, long-distance transport, and unloading of sucrose. The transport of sucrose from source to sink tissue is affected by properties of the vascular bundles, including their size, number, and flow capacity (Lemoine et al., 2013). The loading of sucrose into phloem and its unloading in seeds are coordinated by different transporters, such as sucrose transporters (SUTs) or sugar transporters (Sugars will eventually be exported transporters (SWEETs)), which strongly affect seed development and grain filling (Braun et al., 2014). Improving the efficient loading of sucrose from leaf tissues

into the phloem is thought to be crucial for enhancing the translocation stream, thereby affecting grain filling (Smith et al., 2018).

Starch biosynthesis

Starch production is divided into two major steps: the unloading of the substrate generated by sucrose catabolism into seeds, and its use for starch biosynthesis (Emes et al., 2003). Many enzymes are involved in early starch metabolism, including invertase (INVase), sucrose synthase (SUS), glucokinase (GKase), fructokinase (FKase), phosphoglucoisomerase (PGIase), phosphor-glucomutase (PGMase), uridine diphosphoglucose pyrophosphorylase (UGPase), and adenosine diphosphoglucose pyrophosphorylase (AGPase). Sucrose from source organs can be converted into uridine 5′-diphospho (UDP)-glucose and fructose by SUS or into glucose and fructose by INVase. UDP-glucose and fructose are the reaction substrates of the key steps in the starch biosynthesis cascade and serve as potential indicators of the sink strength of grains and grain-filling capacity (Sung et al., 1989; Counce and Gravois, 2006).

The substrates generated in the above early steps are converted into either amylose by granule-bound starch synthase (GBSS) or amylopectin by soluble starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) (Nakamura, 2017; Liu et al., 2022). The activities of SUS, UGPase, and phosphorylase 1 (Pho1) are key indicators reflecting the differences in sink strength and grain filling capacity between *indica* and *japonica* rice (Wakabayashi et al., 2021). Moreover, starch biosynthesis genes, such as *OsAGPL3, GBSSI, OsSSIIa, SBEI, ISOAMYLASE 2* (*ISA2*), and *OsBEIIb*, are under the control of different TFs that affect grain filling (Wang et al., 2013; Schmidt et al., 2014; Wang et al., 2020a; Feng et al., 2022). Although the above-mentioned metabolic factors are key indicators of grain filling, few studies have conducted a combined analysis of all enzymes and intermediate metabolites and their effect on grain filling.

Cell proliferation

Some cellular events such as cell division and endoreduplication affect grain filling as well. Many mutants with specific defects in cell fate in starchy endosperm have been isolated, most of which show severely reduced filling of starchy endosperm (Olsen, 2004). The volume of mature endosperm is determined by cell proliferation during the early grain-filling stage. The mitotic division of endosperm cells lasts for a certain period to support endosperm development. The cell division rate and its duration are closely associated with endosperm development and the capacity for grain filling (Shaw et al., 2022). Rice cultivars with dense panicles contain a large proportion of poorly filled grains that show greatly reduced cell division rates and ploidy but a longer division duration (Sahu et al., 2021). Therefore, changes in endosperm cells affect the grain-filling ability of cereals.

FOUR OTHER FACTORS INDIRECTLY INFLUENCE GRAIN FILLING

Phytohormones levels

Phytohormones are central regulators of seed development (Kende and Zeevaart, 1997). Most well-known phytohormones, including auxin, cytokinin, ABA, gibberellin, ethylene, and BRs, play essential roles in regulating seed-related traits (Anfang and Shani, 2021). ABA promotes grain filling by enhancing the mobilization of carbon assimilates into grains (Yang et al., 2001). Poor grain filling results from low IAA and ABA levels in the developing endosperm; the external application of ABA during the early grain filling stage increased endogenous ABA levels, thereby enhancing the grain filling rate (Zhang et al., 2009). Cytokinin plays important roles in promoting cell division during grain filling. High cytokinin levels are usually maintained in the endosperm to sustain cell division (Yang et al., 2002; Zhang et al., 2009). Indeed, increased cytokinin levels enhance grain filling in rice cultivars with large panicles containing numerous spikelets (Panda et al., 2018). BRs also positively regulate grain weight by stimulating the flow of assimilates (Wu et al., 2008) and promoting mitotic cell division in the lemma/palea (Xu et al., 2015). Moreover, BRs promote spikelet differentiation, thus increasing the total number of panicle spikelets (Zhang et al., 2019b). In contrast to the other phytohormones, the gaseous phytohormone ethylene negatively regulates grain filling (Sekhar et al., 2015; Sahu et al., 2021); the cell division rate in endosperm was reduced via the exogenous application of ethylene (Panda et al., 2009).

Polyamines (PAs) are hormone-like substances that function as endogenous plant growth regulators in many physiological processes. High PA levels were detected during seed development, and PA levels were significantly lower in aborting maize kernels than in normal kernels (Liang and Lur, 2002). Several studies have shown that the maximum cell number and cell division rate, grain weight, and grain-filling rate are all correlated with PA contents. The application of PAs to panicles enhanced endosperm cell division and grain filling (Yang et al., 2008; Feng et al., 2011; Wang et al., 2012). Another study found that PAs together with cytokinin coordinately regulate grain filling in wheat, pointing to the possible interactions of these plant growth regulators (Liu et al., 2013).

Panicle and grain morphology

Panicle size in rice shows great diversity among varieties, reflecting different panicle branching types, densities, and overall grain number. In general, dry matter accumulation occurs more rapidly and earlier in spikelets on primary rachis branches than those on secondary branches (Liang et al., 2001), and assimilate partitioning is poor in grains located on the basal region of the panicle (Panda et al., 2015). Therefore, not all spikelets develop into well-filled grains. Upper early

spikelets with a high grain-filling rate are referred to as superior spikelets, and lower later spikelets with poor grain filling are referred to as inferior spikelets. The contrast between these spikelet types is greatest in super hybrid rice cultivars with large panicles (Yang and Zhang, 2010; Zhu et al., 2011). A recent study showed that the inter-grain space in panicles may lead to different rates of grain filling; small inter-grain spaces (<0.55 cm) have a negative effect on grain filling (Sahu et al., 2021). The trade-off between spikelet number and grain filling has been linked to ethylene, as a high spikelet number leads to high ethylene production, which is detrimental to grain filling (Panigrahi et al., 2019). Analysis of near isogenic lines (NILs) of *Grain Number per Panicle1* (*GNP1*) revealed the negative effect of grain number per panicle on grain filling. High *GNP1* transcript levels greatly increased grain number but led to poor grain filling, which can be explained by the reduced activities of key enzymes involved in carbon metabolism and reduced carbohydrate accumulation in culms and leaf sheaths (Wu et al., 2016c).

Seed size also affects grain filling. Many seed size-related quantitative trait loci (QTLs) have large effects on grain filling in rice. Plants with larger seeds showed accelerated grain filling under most conditions (Song et al., 2007; Li et al., 2011; Zhang et al., 2012b); one possible explanation is that the larger spikelet hulls in these plants provided more space for endosperm growth (Song et al., 2007). In wheat, both carpel size at anthesis and final grain dimension are positively correlated with the grain filling rate; a larger carpel usually leads to earlier grain filling and a longer grain filling period (Xie et al., 2015).

Abiotic stress

Environmental factors strongly influence grain filling. One of the most important such factors is water/drought stress. Water stress greatly decreases kernel weight by reducing endosperm cell number and starch granule accumulation, but it enhances the transport of dry matter to kernels, leading to early senescence and a short grain-filling period (Nicolas et al., 1985; Plaut et al., 2004). Interestingly, Zhang et al. found that an irrigation regime involving alternate soil wetting and moderate drying (WMD) improved grain filling of inferior spikelets by increasing cytokinin contents in shoots (Zhang et al., 2010a). The authors subsequently discovered that WMD enhanced the activities of key enzymes involved in sucrose-to-starch conversion, including SUS, AGPase, SS, and SBE, thereby increasing grain filling rate and grain weight (Zhang et al., 2012a). PAs are closely associated with improved grain filling in wheat under drought. PAs counteract the inhibitory effects of drought on grain filling by enhancing ABA accumulation in grains (Liu et al., 2013, 2016).

High temperature events have occurred more often and more intensely in recent years due to global warming, which greatly affects the growth and development of major cereal crops. Short episodes of high temperature stress during grain filling significantly reduce grain weight and increase yield loss in wheat, mainly due to increased thylakoid membrane damage (Djanaguiraman et al., 2020). High nighttime temperatures are more harmful to grain filling in rice than high daytime temperatures (Morita et al., 2002). High nighttime temperatures significantly reduce endosperm cell number and cell area, ultimately leading to decreased grain filling (Morita et al., 2005). Shi et al. performed a proteomic study of rice to study the effect of elevated nighttime temperatures on grain filling. Under these conditions, nitrogen and nonstructural carbohydrate (NSC) transport were reduced in susceptible rice cultivars, resulting in reduced maximum and average grain-filling rates. The increased levels of heat shock proteins and calcium signaling proteins in resistant cultivars might explain their resistance to high nighttime temperatures (Shi et al., 2013). In addition to high temperatures, low temperatures at the filling stage also affect grain filling. Under low temperatures, grain filling is inhibited, mainly due to a reduced starch biosynthesis rate (Xu et al., 2021a).

Nutrient levels

Nitrogen (N) and phosphorus (P) fertilizers are essential agronomic resources that affect crop growth, yield, and quality as well as grain filling. The application of N improves sucrose production by leaf photosynthesis, which might affect the grain filling rate (Wei et al., 2018). In rice, low N levels promote grain filling in superior grains rather than inferior grains. The activities of starch biosynthesis-related enzymes, the accumulation of NSCs in stems, and the redistribution of NSCs from stems to grains were all enhanced by low N treatment (Li et al., 2018a). Another study found that N fertilization during the middle and later stages of grain filling improved the grain-filling rates of both superior and inferior grains, as higher N levels increased the carbohydrate contents of leaves and enhanced the unloading of sucrose to grains. This enhanced carbohydrate partitioning increased the uniformity of inferior grain filling and therefore reduced the overall rate of chalkiness (Guo et al., 2022). P fertilizer also affects grain filling, as the exogenous application of P during grain filling greatly increased grain weight compared to plants with a minimum P supply (Jeong et al., 2017).

GENES AND MOLECULAR MECHANISMS UNDERLYING GRAIN FILLING IN CEREALS

Both internal and external factors affect the performance of cereal grain filling, making cloning of the underlying genes quite challenging. Nevertheless, with the increasing development of molecular and genetic methodologies, the list of cloned genes is getting longer each year, with more than 70 genes that participate in cereal grain filling (Table 1). To obtain an overall view of the roles of these genes in grain filling, we constructed a diagram of the key genes involved in sugar transport, starch biosynthesis, and phytohormonal regulation and their molecular connections. We also highlight some

Table 1. Identified grain filling regulators in cereals

Gene name	Function description	Mutant phenotype	References
Sugar translocation and unloading related regulators			
Sh1	Sucrose synthase	Shrunken endosperm	Chourey et al., 1998
GIF1/CIN2	Cell wall invertase	Incomplete grain filling	Wang et al., 2008a
Mn1	Cell wall invertase	Incomplete grain filling	Kang et al., 2009
OsINV3	Vacuolar invertase	Reduced grain size and weight	Morey et al., 2018
OsSWEET4	Sugar transporter	Empty pericarp phenotype	Sosso et al., 2015
OsSWEET11	Sugar transporter	Defective grain filling	Ma et al., 2017
OsSWEET14	Sugar transporter	No grain filling phenotype	Fei et al., 2021
OsSWEET15	Sugar transporter	No grain filling phenotype	Yang et al., 2018
SWEET4c	Sugar transporter	Empty pericarp phenotype	Sosso et al., 2015
ZmSWEET11	Sugar transporter	—	Shen et al., 2022
OsSUT1	Sucrose transporter	Impaired grain filling	Scofield et al., 2002
ZmSUT1	Sucrose transporter	—	Shen et al., 2022
OsMST4	Monosaccharide transporter	—	Wang et al., 2007
OsMST6	Monosaccharide transporter	—	Wang et al., 2008b
ZmSUGCAR1	Sugar transporter	Shrunken kernels	Yang et al., 2022a
GFD1	MATE (Multidrug and toxic compound extrusion) transporter	Long grain filling duration	Sun et al., 2022
Phytohormones related regulators			
OsRR4/OsRR6	Type-A response regulator	—	Panda et al., 2018
ZmYuc1	YUCCA (YUC) flavin-containing monooxygenase	Defective endosperm	Bernardi et al., 2012
OsYUC11	YUCCA (YUC) flavin-containing monooxygenase	Defected grain filling	Xu et al., 2021b; Zhang et al., 2021b
TGW6	Indole-3-acetic acid (IAA)-glucose hydrolase	Enhanced grain filling	Ishimaru et al., 2013
qGL11/OsGH3.13	IAA-amido synthetase	Increased grain weight	Wang et al., 2021a
BG1	Cytoplasmic membrane-associated protein	Decreased grain size	Liu et al., 2015b
ZmEHD1	C-terminal Eps15 homology domain (EHD) proteins	Shrunken kernel	Wang et al., 2020b
OsABA8ox2	Abscisic acid (ABA) 8′-hydroxylase	Improved grain filling	Teng et al., 2022
DG1	ABA efflux transporter	Defected grain filling	Qin et al., 2021
OsNAP	NAC (No Apical Meristem) transcriptional activator	Extended grain filling with increased grain yield	Liang et al., 2014
CYP	Sterol C-22 hydroxylases	—	Wu et al., 2008
OsI-BAK1	BRI1 associated kinase I (BAK1) homolog	Unfilled grains with defect grain filling	Khew et al., 2015
Starch synthesis related regulators			
OsAGPL2	Adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGP) large subunit	Shrunken endosperm	Tang et al., 2016; Wei et al., 2017
OsAGPS2b	AGP small subunit	Shrunken endosperm	Lee et al., 2007
Sh2	AGP large subunit	Shrunken kernels	Greene and Hannah, 1998
Bt2	AGP small subunit	Shrunken kernels	Greene and Hannah, 1998
OsGBSSI/Waxy	Granule-bound starch synthase I	Starchy endosperm with reduced starch content	Sano 1984; Sato et al., 2002; Zhang et al., 2019a
OsSSIIa	Starch synthase II	Increased chalkiness	Zhang et al., 2011
OsSSIIIa	Starch synthase III	White-core floury endosperm	Ryoo et al., 2007; Zhang et al., 2011
OsSBEI	Starch branching enzyme I	No obvious differences	Zhu et al., 2012; Sun et al., 2017
OsSBEIIb	Starch branching enzyme II	Complete floury endosperm	Tanaka et al., 2004
OsISA1	Isoamylase	Shrunken endosperm	Shufen et al., 2019
OsPho1	Phosphorylase	Shrunken endosperm	Satoh et al., 2008
RSR1	APETALA2 (AP2)/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN (EREBP) transcription factor (TF)	Increased amylose content	Fu and Xue, 2010

Continued

Table 1. Continued

Gene name	Function description	Mutant phenotype	References
OsbZIP58	Basic leucine zipper factor TF	Opaque grains	Wang et al., 2013
SERF1	SALT-RESPONSIVE ETHYLENE RESPONSE FACTOR 1	Enhanced grain filling	Schmidt et al., 2014
OsNCA20/26	NAC TFs	Decreased starch	Wang et al., 2020a
OsNF-YB1	Nuclear factor-Y TF subunit B	Small grains with chalky endosperm	Bai et al., 2016; Xu et al., 2016
OsMADS6	MADS-box TF	Lacked and aborted starch filling	Zhang et al., 2010b
OsMADS29	MADS-box TF	Shrunken endosperm	Yin and Xue 2012
OsMADS14	MADS-box TF	Shrunken and chalky grains	Feng et al., 2022
Other regulators			
ONAC127/129	NAC domain TF	Incomplete grain filling and shrunken grains	Ren et al., 2021
OsNAC23	NAC domain TF	Small grain size	Li et al., 2022
Opaque2 (*O2*)	bZIP-type TF	Opaque endosperm	Cord Neto et al., 1995; Li et al., 2015
ZmABI19	B3 domain-containing TF	Small and shrunken kernels	Yang et al., 2021
ZmbZIP29	Basic Leucine Zipper 29	Small kernels with delayed endosperm development	Yang et al., 2022b
ZmGRAS11	GRAS domain-containing protein	Reduced kernel size and cell expansion	Ji et al., 2022
miR1432	microRNA	Enhanced grain filling	Zhao et al., 2019
KRP1	Cyclin-dependent kinase (CDK) inhibitor	—	Barroco et al., 2006
DEK15	Cohesion-loading complex subunit	Reduced endosperm	He et al., 2019
OsPK2	Pyruvate kinase	Defective grain filling	Cai et al., 2018
OsPK3	Pyruvate kinase	Compromised grain filling	Hu et al., 2020
FLO19	Pyruvate dehydrogenase complex E1 component subunit	Slower grain filling rate and reduced grain weight	Lei et al., 2022
OsGS1;1	Glutamine synthetase	Retardation of grain filling	Tabuchi et al., 2005
Gln1-3/4	Glutamine synthetase	Reduced kernel weight	Martin et al., 2006
PHO1;2	PHO1 (phosphorylase 1)-type Pi transporter	Shrunken endosperm	Ma et al., 2021
OsFIE2	WD40-containing component of polycomb repressive complex 2	Smaller seeds	Nallamilli et al., 2013
OsCPK31	Ca^{2+} sensor protein kinases	Partial unfilled grains	Manimaran et al., 2015
GSD1	A putative remorin protein	Reduced grain setting	Gui et al., 2014
OsQUA2	A putative pectin methyltransferase	Reduced grain yield	Xu et al., 2017
GFR1	Membrane-localized protein	Decreased grain filling rate	Liu et al., 2019
EDR1	Uridine 5′-diphospho (UDP)-glucosyltransferase	Incomplete filled grains	Wu et al., 2022
GW2	RING-type protein	Increased grain size	Song et al., 2007
qGL3/OsPPKL1	Putative protein phosphatase	Long grains	Zhang et al., 2012b
GS5	Putative serine carboxypeptidase	Small grain size and decreased grain filling	Li et al., 2011; Xu et al., 2015
OsAsp1	Aspartic protease	Eliminated grain filling difference between SS and IS	Chang et al., 2020
GF14f	14-3-3 protein	Increased grain weight	Zhang et al., 2019c

other important regulators linking different pathways that regulate grain filling (Figure 3).

Genes for sugar transport and unloading participate in grain filling

Grain filling largely depends on the efficient transport and unloading of sucrose from source tissues to sink tissues. Several genes involved in sucrose transport and unloading have been shown to regulate grain filling. *Shrunken1* (*Sh1*), encoding SUS, is a key regulator of grain filling in maize; the *sh1* mutant shows typical shrunken endosperm (Chourey et al., 1998). *GIF1* (also named *CELL WALL INVERTASE 2* (*OsCIN2*)) and *Mn1*, encoding cell wall invertase, are key grain filling-related genes in rice and maize, respectively; the loss of *GIF1* and *Mn1* function led to an approximately 24% and 70% reduction in seed weight, respectively (Wang et al., 2008a;

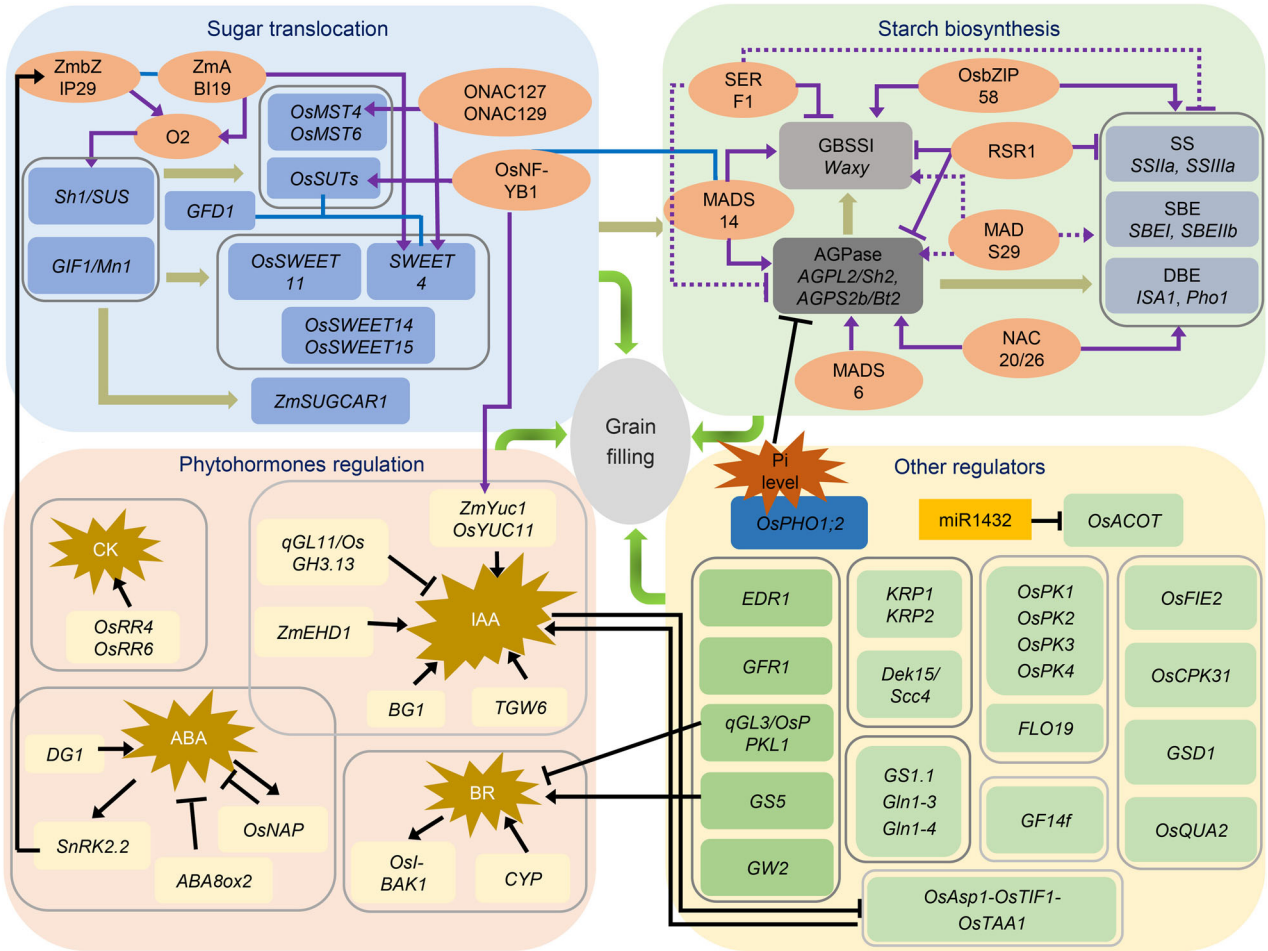

Figure 3. Regulation networks of reported grain filling genes in cereals

The key grain filling genes are categorized into four major groups, including sugar translocation, phytohormones regulation, starch biosynthesis, and other regulators. In addition, some group regulators may interact with each other, and the regulation networks of different genes are indicated.

Li et al., 2013). In developing rice seeds, *GIF1* is primarily expressed in the ovular vascular trace, where sucrose is unloaded for starch biosynthesis in the endosperm. The tissue-specific expression of *GIF1* is important for normal grain filling, as its ectopic overexpression driven by the cauliflower mosaic virus 35S promoter led to poorly filled and severely shrunken seeds, in contrast to the enhanced grain weight in *GIF1*-overexpression lines driven by the native promoter (Wang et al., 2008a). The constitutive overexpression of either *GIF1* or *Mn1* significantly increased grain weight and yield in maize, pointing to the functional conservation and ubiquitous expression of maize invertase genes (Wang et al., 2008a; Li et al., 2013). The vacuolar invertase gene *OsINV3* plays key roles in cell expansion and in driving the transport of assimilates for grain filling, and its mutation leads to greatly reduced grain size and weight (Morey et al., 2018).

Sugar transporters of the SWEET family play important roles in grain filling. Maize ZmSWEET4c, the first SWEET identified in cereals, mediates the transport of transepithelial hexose across the basal endosperm transfer layer (BETL). *ZmSWEET4c* shows signatures of selection during domestication (Sosso et al., 2015). Mutants of *ZmSWEET4c* and its rice ortholog *OsSWEET4* show defects in grain filling. Interestingly, *SWEET4* encodes a hexose transporter that likely functions downstream of *GIF1/Mn1* (Sosso et al., 2015).

OsSWEET11 is another regulator of grain filling in which the knockout mutant shows severe defects in grain filling. *OsSWEET11* is primarily expressed in the ovular vascular trace and nucellar epidermis, and regulates the release of sucrose from maternal tissues to filial tissues in the developing caryopsis (Ma et al., 2017). The *Ossweet11 Osweet15* double mutant shows more severe grain filling defects than the *Ossweet11* single mutant, and the double mutant accumulates more starch in the pericarp. The similar tissue-specific expression patterns of *OsSWEET11* and *OsSWEET15* suggest their function in both sugar efflux from the nucellar projection and sugar influx into the nucellar epidermis/aleurone interface (Yang et al., 2018). Unlike SWEET4, OsSWEET11, and OsSWEET15 primarily function during the later filling stages (Sosso et al., 2015; Yang et al., 2018). A recent study revealed

that OsSWEET14 and OsSWEET11 cooperatively contribute to grain filling in rice. The expression profile of *OsSWEET14* is similar to that of *OsSWEET11*, and the phenotype of the *Ossweet14 Ossweet11* double knockout mutant was much more severe than that of the *Ossweet11* single mutant (Fei et al., 2021). Although OsSWEET11, OsSWEET14, and OsSWEET15 might function redundantly in regulating grain filling, all three transporters are likely required to cooperatively regulate grain filling during seed development.

Once sugars arrive at the aleurone layer, they are transported into the endosperm by aleurone-specific SUTs. The rice genome contains five *SUT* genes: *OsSUT1-5* (Aoki et al., 2003). Antisense suppression of *OsSUT1* resulted in strongly reduced sucrose uptake in seeds, as well as decreases in both grain filling rate and grain weight (Scofield et al., 2002). In addition to SUTs, monosaccharides including glucose and fructose could be immediately transported into the endosperm by monosaccharide transporters (MSTs). Among rice *MST* genes, *OsMST4*, and *OsMST6* are expressed in the dorsal vascular bundle, nucellar epidermis, and aleurone layer and play important roles in the early and middle grain-filling stages (Wang et al., 2007, 2008b).

In addition to classic sugar transporters, a recent study demonstrated that an NRT1 (NITRATE TRANSPORTER)/PTR (PEPTIDE TRANSPORTER)-type transporter named SUGCAR (SUCROSE AND GLUCOSE CARRIER) functions in both sucrose and glucose transport in maize (Yang et al., 2022a). *ZmSUGCAR1* is specifically expressed at the BETL. Mutations of *ZmSUGCAR1* decrease the accumulation of sucrose and glucose in the kernel and lead to a shrunken kernel phenotype. The sugar transport activities of SUGCAR homologs were also demonstrated in wheat and sorghum (*Sorghum bicolor*), suggesting a conserved mechanism in cereals (Yang et al., 2022a). MATEs (Multidrug and toxic compound extrusion transporters) are cation antiporters that constitute one of the largest transporter families in most organisms. The rice MATE transporter GRAIN FILLING DURATION MUTANT 1 (GFD1) determines the grain filling by interacting with two sugar transporters, OsSWEET4 and OsSUT2, with OsSWEET4 mediating its effect on the grain filling duration, with OsSUT2 regulating grain size and grain number (Sun et al., 2022). These findings shed light on the sugar transporter interactome that controls grain filling.

Phytohormone-related regulators of grain filling

Cytokinin increases endosperm cell number during grain filling (Werner et al., 2001). *OsRR4* and *OsRR6* encode type-A response regulators involved in cytokinin signaling. Overexpressing either genes improved cytokinin levels in the developing caryopsis, likely leading to enhanced grain filling in rice cultivars with large panicles (Panda et al., 2018), although this hypothesis requires experimental validation.

Auxin is closely related to endosperm development and grain filling (Zhao, 2018). The developing kernels of mutants with abnormal grain filling (such as *mn1*) show reduced auxin levels (Le et al., 2010). YUCCA (YUC) flavin monooxygenases catalyze a rate-limiting step in auxin biosynthesis. In maize, *ZmYuc1* plays an essential role in endosperm development by affecting IAA biosynthesis; in agreement, the *Zmyuc1* mutant has defective endosperm (Bernardi et al., 2012). The mutation of *OsYUC11* leads to defects in grain filling and reduced auxin biosynthesis, confirming the notion that auxin biosynthesis is essential for grain filling (Xu et al., 2021b; Zhang et al., 2021b). In addition, OsYUC11 might promote grain filling by mitigating the effects of dry soil, as *OsYUC11* is upregulated under drought stress (Teng et al., 2022).

The rice QTL *THOUSAND-GRAIN WEIGHT 6* (*TGW6*) is caused by a polymorphism in a gene that encodes an IAA-glucose hydrolase and catalyzes the conversion of IAA-glucose to free IAA in the developing caryopsis. The functional *TGW6* allele accelerates endosperm cellularization combined with increased auxin accumulation, while the loss of function allele of *TGW6* enhances rice grain filling and grain weight (Ishimaru et al., 2013). *OsGH3.13*, encoding an IAA-amido synthetase, is the underlying gene of the QTL *GRAIN LENGTH 11* (*qGL11*). NIL with lower expression of *OsGH3.13* showed increased grain weight and higher IAA content. Knocking out of *OsGH3.13* led to enhanced grain weight, confirming its role in seed development (Wang et al., 2021a).

Auxin transport is also involved in seed development and grain filling, as blocking auxin transport led to abnormalities in different seed tissues (Forestan et al., 2010). *Big Grain1* (*BG1*) encodes a membrane-localized protein that regulates auxin transport in rice. Overexpression of *BG1* increased basipetal auxin transport and altered auxin distribution, thereby increasing grain size, whereas knockdown of *BG1* resulted in decreased auxin transport and smaller grains (Liu et al., 2015b). *ZmEHD1* encodes a C-terminal Eps15 homology domain (EHD) protein that regulates auxin homeostasis, as the *ehd1* mutant showed shrunken kernels and reduced IAA levels in kernels (Wang et al., 2020b).

Numerous studies have shown that higher ABA levels in grains result in increased grain filling and grain weight in various crops (Seiler et al., 2011; Zhang et al., 2018b). *OsABA8ox2* encodes ABA 8′-hydroxylase, which catalyzes the committed step of ABA catabolism. In the *aba8ox2* mutant, the grain filling of inferior spikelets is greatly enhanced, while overexpressing *OsABA8ox2* significantly reduced grain filling (Teng et al., 2022). *DEFECTIVE GRAIN-FILLING 1* (*DG1*) in rice mediates the long-distance transport of ABA from leaves to the caryopsis. Mutants in *DG1* fail to accumulate leaf-derived ABA, which activates starch biosynthesis genes, leading to the formation of incompletely filled, floury seeds (Qin et al., 2021). Similar grain-filling defects were observed in mutants of the maize *DG1* ortholog, pointing to a conserved role for *DG1* in cereals (Qin et al., 2021). *PREMATURELY SENILE 1* (*PS1*, also named *OsNAP* for *NAC activated by APETALA3/PISTILLATA*) encodes a plant-specific NAC transcriptional activator that links ABA with leaf senescence and grain filling. *OsNAP* expression is specifically induced by ABA, while ABA levels were significantly reduced in *OsNAP* overexpression lines, pointing

to a feedback loop between *OsNAP* and ABA. Notably, the downregulation of *OsNAP* contributes to yield improvement due to delayed leaf senescence and an extended grain-filling period (Liang et al., 2014).

BRs also positively regulate grain filling (Xu et al., 2015). *CYTOCHROME P450* (*CYP*) genes encode sterol C-22 hydroxylases, which function in BR biosynthesis. Overexpressing *CYP* genes significantly enhanced grain filling in rice, particularly in basal inferior grains (Wu et al., 2008). BRI1-ASSOCIATED KINASE I (BAK1) interacts with the BR receptor BR-INSENSITIVE 1 (BRI1) to function in BR perception and BR signal transduction. The rice *BAK1* homolog *Osl-BAK1* is highly expressed after heading, and silencing of *Osl-BAK1* led to the production of green and unfilled grains (Khew et al., 2015).

Although these studies established the relationships between phytohormones and grain filling, the molecular network underlying phytohormone-mediated regulation of grain filling requires further study.

Starch biosynthesis regulators control grain filling

Starch in cereals is composed of a mixture of amylose and amylopectin. Starch biosynthesis in cereals is catalyzed by a complex system composed of five classes of enzymes: AGPase, GBSS, SS, SBE, and DBE (Jeon et al., 2010; Huang et al., 2021). AGPase catalyzes the rate-limiting step of starch biosynthesis in cereal plants, which produces adenosine diphosphoglucose (ADP-glucose) and pyrophosphate (PPi) from glucose-1-phosphate (G1P) and adenosine 5'-triphosphate (ATP) (Ballicora et al., 2004). Several studies have revealed that *AGPase* genes play key roles in grain filling, including *AGPL2* and *AGPS2b*, which are specifically expressed in endosperm. Loss of function of either *AGPL2* or *AGPS2b* in rice and their maize orthologs *Sh2* or *Brittle-2* (*Bt2*) seriously disrupts starch biosynthesis and leads to shrunken grains (Greene and Hannah, 1998; Lee et al., 2007; Tang et al., 2016; Wei et al., 2017).

In cereals, amylose is synthesized by GBSS, which has two isoforms: GBSSI and GBSSII (Jeon et al., 2010). GBSSII is mainly present in leaf tissues and maternal tissues of developing seeds and is responsible for transitory starch accumulation in these tissues. GBSSI is only present in filling endosperm and is responsible for amylose synthesis in seeds (Vrinten and Nakamura, 2000; Dian et al., 2003; Hirose and Terao, 2004). GBSSI is encoded by *Waxy* (*Wx*), with different natural alleles (Zhang et al., 2019a, 2021a). Null mutants of *Wx* do not have significantly altered total starch contents, but they have significantly reduced amylose contents in starchy endosperm (Sano, 1984; Fujita et al., 2001; Sato et al., 2002).

Amylopectin biosynthesis is catalyzed by SS, SBE, and DBE. SS functions in linear glucan chain elongation by catalyzing the transfer of the glucosyl unit of ADP-glucose to the nonreducing end of a glucan chain. Four isoforms of SS have been identified in cereal endosperm: SSI, SSII, SSIII, and SSIV (Jeon et al., 2010). Mutants defective in these genes (such as *SSIIa* and *SSIIIa*) show abnormalities in endosperm-stored starch, leading to grain chalkiness and poor grain filling (Ryoo et al., 2007; Zhang et al., 2011). Cereals contain three isoforms of SBE: SBEI, SBEIIa, and SBEIIb. *SBEIIb* is specifically expressed in endosperm; its null mutant produces thin, floury grains (Tanaka et al., 2004). Simultaneously inhibiting both *SBEI* and *SBEIIb* expression resulted in heavy, opaque grains and reduced grain weight (Zhu et al., 2012), suggesting that *SBEI* and *SBIIb* are essential for normal grain filling. DBE consists of ISA, pullulanase (PUL), and Pho. ISA has three isoforms: ISA1, ISA2 and ISA3. ISA1 is essential for endosperm development and grain filling, as the *isa1* mutant displays shrunken endosperm with abnormal starch granules (Shufen et al., 2019). The *pho1* mutants show shrunken endosperm and severely reduced starch content, indicating that Pho1 regulates grain filling (Satoh et al., 2008).

The expression of genes encoding enzymes for seed starch biosynthesis is regulated by different TFs and regulatory proteins. *Rice Starch Regulator1* (*RSR1*) encodes an APETALA 2 (AP2)/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN (EREBP) family TF, deficiency of which results in enhanced expression of starch biosynthesis genes in seeds. *rsr1* mutants produce larger seeds with a higher seed mass and amylose content than the wild type but with round, loosely packed starch granules, leading to a chalky phenotype (Fu and Xue, 2010). Basic leucine zipper 58 (OsbZIP58) directly binds to the promoters of six starch biosynthesis genes: *OsAGPL3, Wx, SSIIa, SBEI, SBEIIb*, and *ISA2*. The *Osbzip58* null mutant produces white belly grains (Wang et al., 2013). SALT-RESPONSIVE ETHYLENE RESPONSE FACTOR 1 (SERF1) directly regulates the expression of *GBSSI*. Loss of function of *SERF1* enhances starch biosynthesis and grain filling (Schmidt et al., 2014).

The NAC TFs OsNAC20 and OsNAC26 mainly localize to the aleurone layer and starchy endosperm. The *Osnac20 Osnac26* double mutant has significantly reduced starch content compared to the wild type. Both OsNAC20 and OsNAC26 directly activate the expression of *SSI, PUL, AGPL2*, and *AGPS2b*, thus playing essential and redundant roles in regulating starch biosynthesis (Wang et al., 2020a). NF-YB1 directly binds to a G-box in the *Wx* promoter and activates its transcription (Xu et al., 2016). NF-YB1 also binds to CCAAT boxes in the *SUT1, SUT3*, and *SUT4* promoters and activates their expression, thereby regulating grain filling (Bai et al., 2016; Xu et al., 2016). Additionally, NF-YB1 also regulates the expression of *OsYUC11* (Xu et al., 2021b), suggesting that NF-YB1 has wide regulatory targets.

MADS-box TFs also transcriptionally regulate starch biosynthesis genes. *MADS6* is highly expressed in rice spikelets and endosperm. MADS6 regulates the expression of *AGPase* genes to affect grain filling. The mutation of *MADS6* severely affects endosperm, as 32% of the mutant seeds lacked starch or were aborted (Zhang et al., 2010b). *MADS29* is preferentially expressed in the nucellus and the nucellar projection. Suppressing the expression of *MADS29* resulted in abnormal seed development with shrunken seeds, a

reduced grain-filling rate, and suppressed starch biosynthesis (Yin and Xue, 2012). A recent study determined that OsMADS14 regulates grain filling by interacting with NF-YB1 to promote the transcription of *OsAGPL2* and *Wx*. Mutations of *OsMADS14* result in a shrunken and chalky grain phenotype (Feng et al., 2022).

OTHER GENETIC REGULATORS OF GRAIN FILLING

Key TFs and microRNAs

In addition to genes involved in starch biosynthesis, TFs directly regulate the expression of other types of genes important for regulating grain filling. Two seed-specific NAC domain TFs, ONAC127 and ONAC129, are responsive to heat stress and function in rice grain filling by forming heterodimers. *ONAC127* and *ONAC129* are mainly expressed in the pericarp, and both single and double knockouts and overexpression plants showed incomplete grain filling and shrunken grains. ONAC127 and ONAC129 directly bind to the promoters of *OsMST6* and *OsSWEET4*, suggesting these TFs regulate grain filling by affecting sugar transport (Ren et al., 2021). Trehalose-6-phosphate (Tre6P) mediates the sensing of carbon status and adjusts the sucrose levels in both source and sink organs (Figueroa and Lunn, 2016). OsNAC23 was recently shown to regulate Tre6P signaling in rice. OsNAC23 increases Tre6P levels by directly targeting and repressing the Tre6P phosphatase gene *TPP1*. Both *OsNAC23* overexpression lines and *tpp1* mutants showed enhanced leaf sucrose efflux, which facilitated grain filling and improved grain yield (Li et al., 2022). Therefore, the OsNAC23-TPP1-Tre6P regulatory module might be an ideal target for rice breeding.

The endosperm-specific TF Opaque2 (O2) is a central regulator of endosperm filling in maize. O2 binds to the SUS-encoding genes *Sh1, Sus1*, and *Sus2* to enhance SUS-mediated endosperm filling (Deng et al., 2020). Moreover, O2 directly activates the expression of *ZmGRAS11*, encoding an endosperm-specific GRAS domain-containing protein, to facilitate grain filling by coordinating cell expansion (Ji et al., 2022). ZmABI19 is a B3 domain TF that activates the expression of both *O2* and *SWEET4c* by directly binding to their promoters. *Zmabi19* mutants display opaque mature kernels and reduced grain size, indicating that ZmABI19 regulates grain filling (Yang et al., 2021). The TF ZmbZIP29 interacts with ZmABI19 to regulate *O2* expression; *Zmbzip29* seeds develop more slowly and are smaller at maturity than the wild type. More severe seed phenotypes were observed in the *Zmbzip29 Zmabi19* double mutant compared to the single mutants, and their storage reserves were also greatly reduced, indicating that ZmABI19 and ZmbZIP29 serve as hubs to coordinate grain filling. Overexpressing *ZmABI19* or *ZmbZIP29* increased both storage-reserve accumulation and kernel weight, pointing to their potential for breeding applications (Yang et al., 2022b). The activities of ZmABI19 and ZmbZIP29 are enhanced by ABA, as ABA induces the accumulation of Sucrose non-fermenting-like kinase 2.2 (SnRK2.2), which interacts with both proteins and enhances their transactivation of *O2* (Yang et al., 2022b). These findings uncover a molecular connection between ABA signaling and the control of grain filling.

MicroRNAs (miRNAs), a large class of gene expression regulators, affect grain filling by regulating the transcript levels or translatability of their target genes at different stages of endosperm development. The dynamic expression patterns of miRNAs during rice grain filling have been fully explored. The accumulation of many miRNAs is negatively correlated with the grain filling rate (Xue et al., 2009; Peng et al., 2013). Some miRNAs differentially accumulate between superior and inferior rice grains and may be involved in pathways regulating phytohormone metabolism, carbohydrate metabolism, and cell division (Peng et al., 2011, 2014). miR1432 negatively regulates grain filling, as transgenic lines with suppressed miR1432 expression showed enhanced grain filling and grain yield. The acyl-CoA thioesterase gene *OsACOT*, a major cleavage target of miR1432, functions in the biosynthesis of medium-chain fatty acids. Rice plants overexpressing a miR1432-resistant form of *OsACOT* showed an up to 46.7% increase in grain weight due to an improved grain filling rate, indicating that the miR1432-*OsACOT* module has great potential as a target for yield improvement (Zhao et al., 2019).

Cell cycle-related genes

Genes involved in the cell cycle and cell proliferation also regulate grain filling. The cyclin-dependent kinase inhibitor Orysa;KRP1 (Kip-related protein 1) plays an important role in regulating grain filling. Overexpressing Orysa;*KRP1* dramatically reduced seed filling due to disturbed endosperm cell production (Barroco et al., 2006). By contrast, *krp2* and *krp1 krp2* mutants exhibited significantly reduced seed width, seed length, and grain weight compared to the wild type, suggesting that KRP1 and KRP2 might function in grain filling by inhibiting cell proliferation and enlargement (Ajadi et al., 2019). The maize gene *Defective kernel 15* (*Dek15*) encodes a homolog of SISTER CHROMATID COHESION PROTEIN 4 (SCC4). The mitotic cell cycle and endoreduplication are disrupted in the *dek15* mutant, resulting in reduced endosperm size and embryo lethality (He et al., 2019).

Pyruvate metabolism-related genes

Pyruvate kinase (PK) is a key enzyme that regulates the final step of the glycolysis pathway. Several PK genes were recently shown to function in rice grain filling. The *Ospk2* mutant displays reduced grain weight and starch content, pointing to a role for *OsPK2* in starch biosynthesis and grain filling, but the exact molecular mechanism is still unclear (Cai et al., 2018). The *Ospk3* mutant also shows defects in grain filling, with a large proportion of unfilled grains and a markedly reduced grain filling rate, grain thickness, and grain weight. More sucrose and transitory starch were retained in mutant leaves than the wild type, leading to reduced

accumulation of storage materials in grains. Therefore, OsPK3 regulates grain filling by coordinating sucrose translocation (Hu et al., 2020). OsPK3 interacts with the PK isozymes OsPK1 and OsPK4. All three enzymes are located in mitochondria. Disrupting *OsPK3, OsPK1*, or *OsPK4* led to different degrees of defective grain filling, suggesting that OsPK3-OsPK1/OsPK4 form heterodimers *in vivo* to coordinately regulate grain filling (Hu et al., 2020).

The pyruvate provided by the glycolytic pathway can be converted into acetyl-CoA and nicotinamide adenine dinucleotide hydrogen by the pyruvate dehydrogenase complex. *FLOURY ENDOSPERM 19* (*FLO19*) encodes a plastid-localized pyruvate dehydrogenase complex E1 component subunit in rice. The *flo19* mutant has a slower grain filling rate and lower 1 000-grain weight compared to the wild type. Developing endosperm of the *flo19* mutant contains altered membrane lipid contents, which might affect starch biosynthesis and starch grain development. Overexpressing *FLO19* increased grain size and grain yield in rice, suggesting that this gene could be valuable for rice breeding (Lei et al., 2022).

Nitrogen and phosphate-related genes

Glutamine synthetase (GS) converts glutamate and ammonium ions to glutamine, the main form of transported N in plants. Rice contains three homologous but distinct genes encoding cytosolic GS: *OsGS1;1, OsGS1;2*, and *OsGS1;3*. Knockout mutants of *OsGS1;1* showed a severely retarded growth rate and grain filling, indicating that *OsGS1;1* is important for normal growth and grain filling. *OsGS1;1* is highly expressed in leaves, suggesting it indirectly affects grain filling by influencing plant type, photosynthesis, or nutrient transport (Tabuchi et al., 2005). Either single or double mutation of two GS isoforms in maize, *Gln1-3* and *Gln1-4*, strongly reduced kernel weight and yield, while overexpressing *Gln1-3* or *Gln1-4* significantly increased grain yield with enhanced GS activity (Martin et al., 2006). These observations suggest that GSs are closely related to grain filling in cereals, in part building a link between N levels and grain filling.

Phosphate (Pi) is another macroelement that is essential for plant growth and crop yields. A recent study showed that the PHO1-type Pi transporter PHO1;2 is a key regulator of grain filling in both rice and maize, clarifying the relationship between grain filling and Pi transport. *Ospho1;2* mutants show strongly reduced grain filling, with reduced starch contents and shrunken endosperm. The Pi efflux activity of OsPHO1;2 and its plasma membrane localization in seed tissues point to a specific role for OsPHO1;2 in Pi reallocation in filling grains. The mutation of *OsPHO1;2* leads to increased accumulation of Pi in the endosperm, thereby inhibiting both the expression of *AGPase* and the activity of the encoded protein and thus grain filling (Ma et al., 2021).

Polycomb complexes, protein kinases, remorin, and pectin methyltransferase

Polycomb complexes play key roles in regulating endosperm development in cereals via histone modifications. FERTILIZATION-INDEPENDENT ENDOSPERM 2 (OsFIE2) is responsible for the H3K27me3 modification of many endosperm-specific genes in rice. Reduced *OsFIE2* expression led to partially filled and smaller grains, highlighting its role in controlling grain filling. OsFIE2 controls the H3K27me3 modification at *OsMADS6*, which partially explains its effect on grain filling (Nallamilli et al., 2013). Ca^{2+} sensor protein kinases have been identified in most plant species including cereals. The rice cyclin-dependent protein kinase (CDPK) gene *OsCPK31* was functionally validated by overexpressing and silencing this gene in rice cultivar TP309. *OsCPK31*-overexpressing plants showed rapid grain filling and early maturation, while silencing of *OsCPK31* led to increased numbers of unfilled grains without any difference in maturity duration. However, the underlying mechanism of the role of OsCPK31 in regulating grain filling requires further investigation (Manimaran et al., 2015).

GRAIN SETTING DEFECT 1 (*GSD1*) encodes a putative remorin protein that affects grain setting and filling in rice. The dominant *gsd1-D* mutant shows reduced carbohydrate accumulation in leaves and a reduced grain setting rate. GSD1 specifically localizes to the plasma membrane and plasmodesmata of phloem companion cells, suggesting it regulates the translocation of photo-assimilates via the symplastic pathway to affect grain setting and grain filling in rice (Gui et al., 2014). Homogalacturonan (HG) is the main component of pectins. *QUASIMODO 2* (*OsQUA2*) encodes a putative pectin methyltransferase. *Osqua2* mutants exhibit a markedly reduced grain yield due to a lower degree of methylesterification and blocked sucrose translocation. OsQUA2 is indispensable for maintaining a high degree of HG methylesterification in the cell walls of sieve elements in culms, which might be required for efficient sucrose transport and grain filling (Xu et al., 2017). These findings confirm the notion that efficient sucrose transport and partitioning are critical for normal grain filling.

Important QTLs controlling grain filling

A large variation in grain filling can be found among natural crop varieties. QTLs are the major contributors to this variation. Several QTLs have been shown to affect grain filling in rice. *GRAIN-FILLING RATE1* (*GFR1*), the major QTL for grain filling rate, encodes a membrane-localized protein and is constitutively expressed. The $GFR1^{Ludao}$ allele improves the grain-filling rate mainly by increasing the initial Rubisco activity in the Calvin-Benson cycle. *GIF1* is upregulated in NIL-$GFR1^{Ludao}$, which promotes sucrose unloading during the grain filling stage (Liu et al., 2019). Nevertheless, the mechanism by which GFR1 controls grain filling requires further exploration. Another group found that *EDR1* (*ENDOSPERM DEVELOPMENT IN RICE*) underlies the QTL responsible for differential endosperm development between upland and paddy rice; *EDR1* encodes UDP-glucosyltransferase. The $EDR1^{YZN}$ allele from upland rice shows reduced UDP-glucosyltransferase activity compared to the $EDR1^{YD1}$ allele from paddy rice, resulting in abnormal

endosperm development, incomplete grain filling, and poor grain quality (Wu et al., 2022).

GW2, a rice QTL affecting both grain width and weight, encodes a RING-type protein with E3 ubiquitin ligase activity. The loss of GW2 function results in larger spikelet hulls with increased cell number, as well as an accelerated grain-filling rate (Song et al., 2007). *GRAIN SIZE 5* (*GS5*) encodes a putative serine carboxypeptidase that positively regulates grain size by enhancing grain width, grain filling, and grain weight (Li et al., 2011). *GRAIN LENGTH 3* (*qGL3*) encodes a putative protein phosphatase with a Kelch-like repeat domain (OsPPKL1). The *qgl3* allele exhibits a long grain phenotype, with a favorable effect on grain filling and grain weight (Zhang et al., 2012b). Both *GS5* and *qGL3* might determine grain size and filling via BR pathways. Enhanced *GS5* expression prevents the endocytosis of OsBAK1-7 by competitively inhibiting the interaction between OsBAK1-7 and MEMBRANE STEROID BINDING PROTEIN 1 (OsMSBP1), which may explain the positive association between grain size and *GS5* expression (Xu et al., 2015). qGL3 interacts with, dephosphorylates, and stabilizes OsGSK3 (Glycogen synthase kinase-3), thereby suppressing BR signaling. The *Osgsk3* mutant shows increased grain length and weight, confirming the role of OsGSK3 in regulating seed development (Gao et al., 2019).

Genes underlying differential grain filling between superior and inferior spikelets

Several studies have attempted to explain the mechanism responsible for superior and inferior spikelets during grain filling. Sahu et al. reported that cell cycle events and the expression of cell cycle regulators are factors determining differential grain filling in rice spikelets (Sahu et al., 2021). The differential filling rate between superior and inferior spikelets has also been associated with changes in the expression of *OsCIN4, INV1, OsINV3, SUS1*, and *AGPL2* (Ishimaru et al., 2005). Grain filling in inferior spikelets is restricted by that in superior spikelets, as the limited supply of assimilates and plant hormones results in poor grain filling of inferior spikelets. Indeed, removing superior spikelets improved grain filling in inferior spikelets by increasing sucrose and phytohormone levels (You et al., 2016).

Ethylene is also involved in the differential grain filling of superior and inferior spikelets. Ethylene biosynthesis genes are expressed at higher levels in inferior spikelets, leading to a significant increase in the ethylene evolution rate. The high concentrations of ethylene in inferior spikelets suppress the expression of starch biosynthesis genes and the activities of their encoded enzymes, and leading to poor grain filling (Zhu et al., 2011). Another study revealed that the initiation of grain filling occurred much later in inferior than superior spikelets due to poor sucrose unloading ability and the low efficiency of sucrose-to-starch metabolism (Jiang et al., 2021). Deng et al. showed that higher IAA contents are important for the initiation of grain filling in superior spikelets, suggesting that this auxin might function as a signal to control grain filling. The differential IAA levels between superior and inferior spikelets affect dorsal vascular cell development and sucrose unloading from the phloem, which is important for grain filling (Deng et al., 2021).

Although the poor grain filling of inferior spikelets has been associated with many physiological changes, the underlying molecular mechanisms remain obscure. A transcriptome-wide analysis determined that *ASPARTIC PROTEASE 1* (*OsASP1*) reaches an earlier and higher transcriptional peak in inferior than superior spikelets. The mutation of *OsASP1* abolished the difference in grain weight between superior and inferior spikelets, indicating that *OsASP1* is essential for balancing caryopsis development in superior and inferior spikelets. OsASP1 interacts with OsTIF1 (TAA1 transcriptional inhibition factor 1) to abolish its inhibition of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*OsTAA1*), which is responsible for IAA biosynthesis. The OsASP1-OsTIF1 complex maintains IAA-mediated apical dominance between superior and inferior spikelets. In turn, IAA reduces the interaction of OsASP1 with OsTIF1 to form a feedback loop, which further fine-tunes differences in IAA levels between superior and inferior spikelets (Chang et al., 2020).

Another study demonstrated that GF14f, a member of the 14-3-3 protein family, shows different temporal and spatial expression patterns between superior and inferior spikelets. Suppressing *GF14f* gene expression by RNA interference resulted in increased grain length and weight. Thus, the higher abundance of GF14f in inferior spikelets may be responsible for their poor grain filling. GF14f might affect grain filling by interacting with enzymes involved in sucrose breakdown, starch biosynthesis, the tricarboxylic acid cycle, and glycolysis (Zhang et al., 2019c). Finally, different levels of AGPase small subunit 2 (AGPS2) accumulate in superior versus inferior rice spikelets, as revealed by proteomic analysis. AGPS2 binds to SBE, PUL, and DBE, as well as GF14e, which might be involved in the poor grain filling of inferior spikelets (Zhang et al., 2019c; Zhao et al., 2021).

FUTURE PERSPECTIVES

In the past decades, genes for different yield components have been identified, increasing our understanding of yield formation and providing the opportunity to further improve the yield potential of crops. Nevertheless, yield improvement is still a critical issue in agricultural production, as the production of major crops must double by 2050 in order to satisfy the needs of the increasing population (Ray et al., 2013). To break the yield ceiling, the New Plant Type strategy has been proposed to breed rice varieties with large panicles. Indeed, several super rice varieties with great yield potential have been bred using this strategy (Qian et al., 2016; Zeng et al., 2017). However, super rice varieties frequently do not meet their expected yield potentials due to poor grain-filling ability, as evidenced by a low grain-filling rate and many unfilled grains (Yang and Zhang, 2010). Therefore,

understanding the mechanisms of grain filling in cereals will greatly contribute to further increasing yields during crop cultivation and breeding.

Both cultivation and breeding strategies can be used to improve grain filling, which involves managing external environmental conditions and plant genetics, respectively (Figure 4). The proper control of mild soil drying in both rice and wheat during the later grain-filling period can enhance whole-plant senescence and the remobilization of carbon from vegetative tissues to grains, leading to better grain filling (Yang and Zhang, 2006). Therefore, it would be worthwhile practicing this method in the field using a variety of cultivars and planting locations. In addition, more field tests can be carried out to clarify the effects of N and P fertilizers on cereal grain filling, as most previous studies have not provided clear conclusions. High N fertilization causes the so-called "stay-green" phenomenon due to delayed grain filling, but mild soil drying can overcome this effect (Yang and Zhang, 2006), indicating that the combined application of different cultivation methods could improve grain filling. Although many studies have confirmed that applying different phytohormones promotes grain filling (Yang et al., 2001; Wu et al., 2008; Tang et al., 2009; Tamaki et al., 2015; Panda et al., 2018), suitable concentrations for use in the field require further study. Moreover, the widespread application of phytohormones in the field is difficult due to the high cost of this treatment, as most phytohormones are expensive. Nevertheless, it might be possible to identify less costly chemicals that promote grain filling in the future.

Compared to the cultivation strategy, the breeding strategy could be more efficient for long-term application. The grain filling ability of different cereals could be improved by introgressing beneficial genes. QTLs are ideal breeding targets, but little attention has been paid to identifying QTLs for grain filling traits, possibly due to the lack of efficient methods for trait evaluation. Rice QTLs strongly associated with increased filling percentage per panicle have been detected on chromosomes 8 and 12. These two QTLs overlap with QTLs for NSC content in culms and leaf sheaths during grain filling (Takai et al., 2005). Another study attempted to identify QTLs responsible for grain filling rate in 95 rice varieties by time-course association mapping (Liu et al., 2015a). However, no common QTLs for grain filling were identified in either study, pointing to the complexity of grain filling control among rice varieties. As mentioned above, *GFR1* and *EDR1* are the only two known QTLs directly involved in controlling

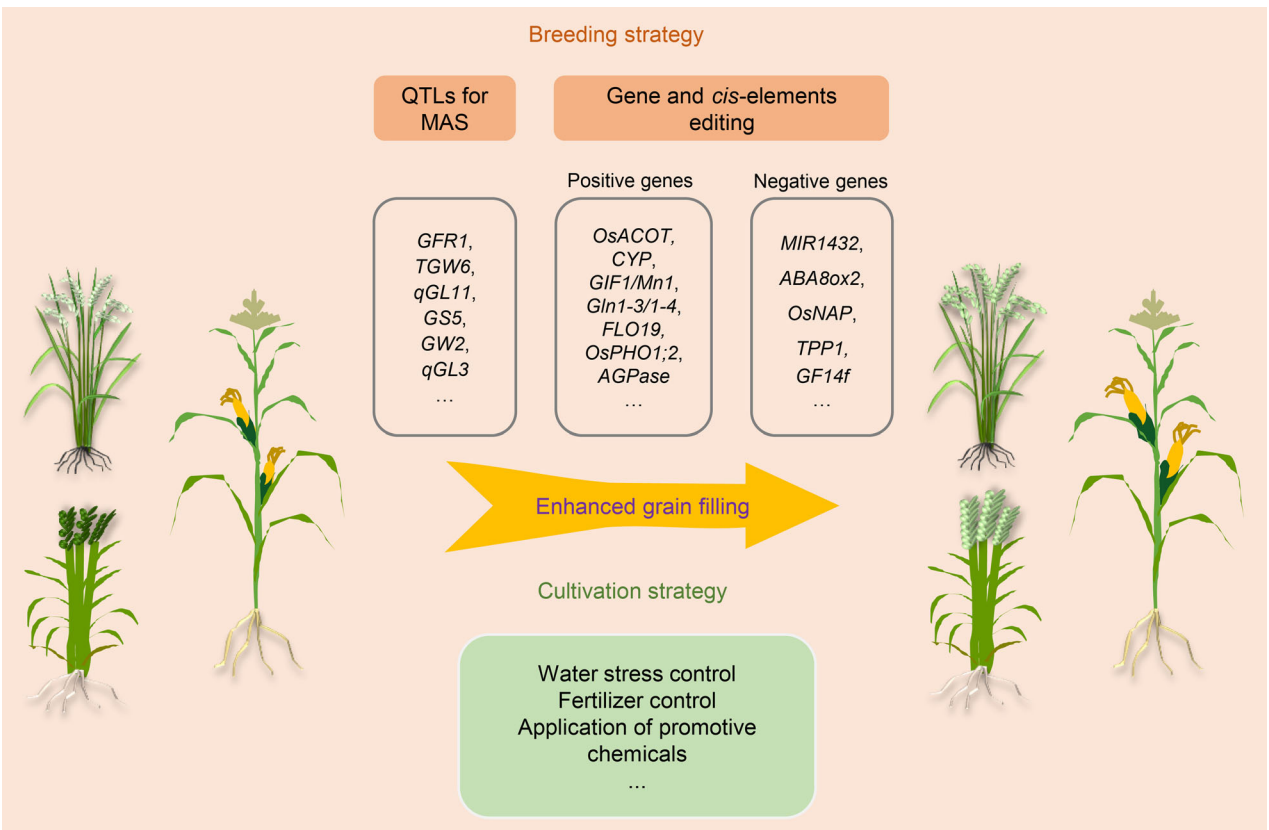

Figure 4. Strategies for improving cereals grain filling in breeding and cultivation.
We raise both breeding and cultivation strategies for improving grain filling in cereals. For cultivation, proper control of soil drying, adequate nitrogen and phosphate fertilizers, and application of different promotive chemicals could improve grain filling. For breeding, quantitative trait loci (QTLs) are the ideal targets for molecular assisted selection (MAS). Additionally, useful alleles either for the positive regulators or negative regulators of grain filling can be fast generated by gene editing techniques.

grain filling. The favorable allele of *GFR1* is an ideal target for improving grain filling in rice breeding. Some grain size QTLs also improve grain filling, including *TGW6, qGL11, GS5, GW2*, and *qGL3* (Song et al., 2007; Li et al., 2011; Zhang et al., 2012b; Ishimaru et al., 2013; Wang et al., 2021a), representing other possible selection targets for improving grain filling. Nevertheless, the efficiency of these QTLs for promoting grain filling requires further validation in different genetic backgrounds, as their effects might require the increased production of assimilates to support the filling of larger grains.

Among the genes identified from mutant studies or by reverse genetics, some can be used to increase grain filling via bioengineering. Enhanced grain filling and grain weight can be achieved by overexpressing positive regulators or knocking out negative regulators of grain filling. Here, we propose positive and negative regulators of grain filling that are suitable for bioengineering based on the phenotypic changes of different transgenic plants. The positive regulators include *OsACOT*, sterol C-22 hydroxylase (CYP), *GIF1/Mn1, Gln1-3/Gln1-4, OsPHO1;2, OsNAC23, FLO19, ZmABI19, ZmbZIP29* and *AGPase* genes, while the negative regulators include miR1432, *ABA8ox2, OsNAP, TPP1*, and *GF14f* (Figure 4). Many reports only describe the phenotypes of mutants with poor grain filling, such as mutants of genes involved in sugar transport (Scofield et al., 2002; Sosso et al., 2015; Ma et al., 2017; Yang et al., 2022a). It would be worth examining the contributions of these genes to grain filling via overexpression in the future. *ABA8ox2* and *GF14f* could be ideal targets to balance grain filling between superior and inferior spikelets, and their contributions could be validated by repressing their activities, especially in varieties with large panicles.

Either RNA interference or overexpression of target genes requires vector fragments to be integrated into the genome, raising safety issues associated with transgenic plants. Rapid breakthroughs in gene editing technology have made it possible to easily knock out negative regulators of grain filling without producing transgenic plants. Using this technique, novel alleles of different QTLs can be quickly generated in different elite cultivar backgrounds (Shen et al., 2018; Mao et al., 2021). In addition to coding regions, the promoter regions of genes can also be edited to generate useful alleles with altered expression patterns, as demonstrated by the efficient editing of the promoters of *SCM3, IPA1, Wx*, and *GWD1* in rice (Cui et al., 2020; Zeng et al., 2020; Wang et al., 2021b; Song et al., 2022) and *SlWUS* and *SlCLV3* in tomato (*Solanum lycopersicum*) (Rodriguez-Leal et al., 2017). Differential phenotypic variation can be identified among the edited plants, making it easy to identify optimal lines. It is possible to generate edited lines with elevated expression of these positive regulators of grain filling, thereby creating useful alleles. Upstream open reading frame (uORF) editing is another way to enhance gene function, as a functional uORF affects the translation of a protein from a downstream ORF. Successful uORF editing has been performed in *Arabidopsis thaliana* (*AtBRI1*), lettuce (*LsGGP1* and *LsGGP2*), and rice (*DTH2*). Phenotypic changes matching enhanced gene function were observed in the edited lines (Zhang et al., 2018a; Liu et al., 2021), confirming the efficiency of the uORF editing strategy. We suggest that the positive regulators of grain filling mentioned above would be ideal targets for uORF editing.

Finally, genome information for a large number of rice germplasms has been generated by high-throughput resequencing (Huang et al., 2012; Wang et al., 2018), and it provides the opportunity to identify superior alleles of positive regulators of grain filling, which produces similar effects of gene overexpression. Although current publications provide gene lists that can be searched to create useful alleles for breeding applications, it will be important to identify additional genes that regulate grain filling, as the molecular network of grain filling control is still unclear and requires further exploration.

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CONFLICTS OF INTEREST

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