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Molecular Networks of Seed Size Control in Plants

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seed size, grain size, cell proliferation, cell expansion, maternal tissues, endosperm development

Abstract

The size of seeds affects not only evolutionary fitness but also grain yield of crops. Understanding the mechanisms controlling seed size has become an important research field in plant science. Seed size is determined by the integrated signals of maternal and zygotic tissues, which control the coordinated growth of the embryo, endosperm, and seed coat. Recent advances have identified several signaling pathways that control seed size through maternal tissues, including or involving the ubiquitin-proteasome pathway, G-protein signaling, mitogen-activated protein kinase (MAPK) signaling, phytohormone perception and homeostasis, and some transcriptional regulators. Meanwhile, growth of the zygotic tissues is regulated in part by the HAIKU (IKU) pathway and phytohormones. This review provides a general overview of current findings in seed size control and discusses the emerging molecular mechanisms and regulatory networks found to be involved.

Contents

1. INTRODUCTION

In angiosperms, a mature seed consists of the embryo, the endosperm, and the seed coat. The embryo and the endosperm are originated from the fertilized egg cell and central cell, respectively, while the seed coat develops from the sporophytic integuments. Thus, seed growth is coordinately controlled by both maternal tissues and zygotic tissues. How plants determine their seed size is an important question in developmental biology. In addition, seed size influences plant fitness and adaption and determines seed yield in crops. Therefore, understanding the mechanisms of seed size control has become an important research field in plant science.

In *Arabidopsis thaliana* (*Arabidopsis*) and many other dicot plants, the outer and inner integument primordia initiate from the flanks of the chalaza and undergo cell division and cell elongation to envelop the nucellus, where the female gametophyte is generated (**Figure 1**). After fertilization, the integuments grow coordinately with the embryo and endosperm and form the seed coat. Because the integuments set the volume of the cavity within which the embryo and the endosperm develop, growth of the integuments affects the final size of the seeds. In rice (*Oryza sativa*) and many other monocot plants, the grain grows inside the spikelet hull (the husk) that encases the developing grain and limits grain growth (**Figure 1**). Therefore, grain size is influenced by growth of the spikelet hull in these monocot plants. Generally, growth of integuments or husks is controlled maternally and influences zygotic seed growth. In this case, seed size is determined by the maternal genotype but not the genotype of the zygote.

During double fertilization, the egg cell fuses with one sperm cell and gives rise to the diploid embryo, and the central cell is fertilized by another sperm cell and develops to form the triploid endosperm. In *Arabidopsis*, growth of the endosperm precedes embryo growth. Endosperm nuclei continue to divide without cytokinesis, causing a rapid enlargement of seed volume and resulting in a multinucleated cell termed the syncytium. Subsequently, cellularization occurs to divide the syncytium to individual cells. The seed almost reaches its final size when the embryo is in the globular stage and occupies only a small part of the seed (57, 105). At later stages, the endosperm is consumed while the embryo grows, and the embryo replaces most of the mature seed (**Figure 1**). By contrast, in monocot plants such as rice and wheat (*Triticum aestivum*), the endosperm occupies most of the volume of a mature grain. Therefore, the endosperm has a crucial role in determining seed size in both dicot plants and monocot plants. Several excellent reviews have provided very

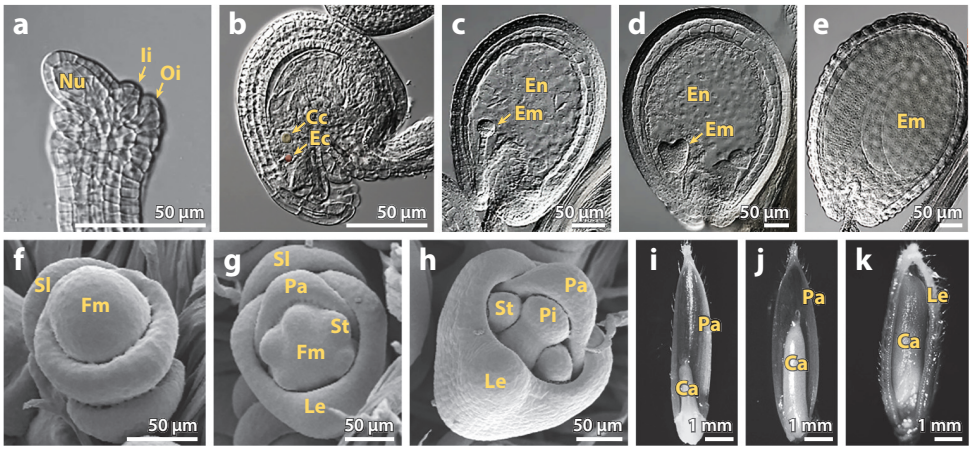

Figure 1

Seed development in *Arabidopsis* and rice. (*Top row*) Ovule and seed development in *Arabidopsis*. (*a*) A developing ovule. (*b*) A mature ovule. The nuclei of the egg cell (*red*) and the central cell (*yellow*) are indicated. Also shown are developing seeds in the (*c*) preglobular stage, (*d*) heart stage, and (*e*) mature green stage. (*Bottom row*) Grain development in rice. (*f–h*) Developing spikelets. (*i–k*) Developing grains after fertilization. Abbreviations: Ca, caryopsis; Cc, central cell; Ec, egg cell; Em, embryo; En, endosperm; Fm, floral meristem; Ii, inner integuments; Le, lemma; Nu, nucleus; Oi, outer integuments; Pa, palea; Pi, pistil; Sl, sterile lemma; St, stamen.

useful information about seed size control in plants (59, 86, 105, 134, 157). In this review, we focus on recent research progress on seed size control and discuss the possible molecular mechanisms and regulatory networks of seed growth.

2. CONTROL OF SEED SIZE BY MATERNAL TISSUES

Several signaling pathways have been shown to control seed size by regulating the growth of maternal tissues. These include or involve the ubiquitin-proteasome pathway, G-protein signaling, mitogen-activated protein kinase (MAPK) signaling, phytohormone perception and homeostasis, and some transcriptional regulators (**Figure 2**; **Table 1**). In this section, we discuss recent progress in understanding the roles of these signaling pathways in seed size control.

G-protein: a guanine nucleotide-binding protein that acts as a molecular switch inside cells

Mitogen-activated protein kinase (MAPK): a serine/threonine-specific protein kinase involved in various forms of cell signaling

Ubiquitin-interacting motif (UIM): a sequence motif of about 20 amino acid residues with ubiquitin-binding activity

2.1. Control of Seed Size by the Ubiquitin-Proteasome Pathway

Protein ubiquitination controls many aspects of cellular processes by influencing protein stability, activity, and localization. Ubiquitination requires the action of a series of special enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). The ubiquitin or ubiquitin chain can be removed by deubiquitinating enzymes. Recent studies have revealed that the ubiquitin-proteasome pathway has an important role in seed size regulation.

2.1.1. Control of seed size by the DA1 pathway. In *Arabidopsis*, the DA1 pathway has a key role in the maternal control of seed size by regulating cell proliferation in the integuments. The *da1-1* mutant produces large seeds and organs, indicating that DA1 negatively regulates seed and organ growth (66). DA1 is a ubiquitin receptor containing two ubiquitin-interacting motif (UIM) domains, a LIM domain, and a C-terminal peptidase domain (14, 66). DA1 acts redundantly with

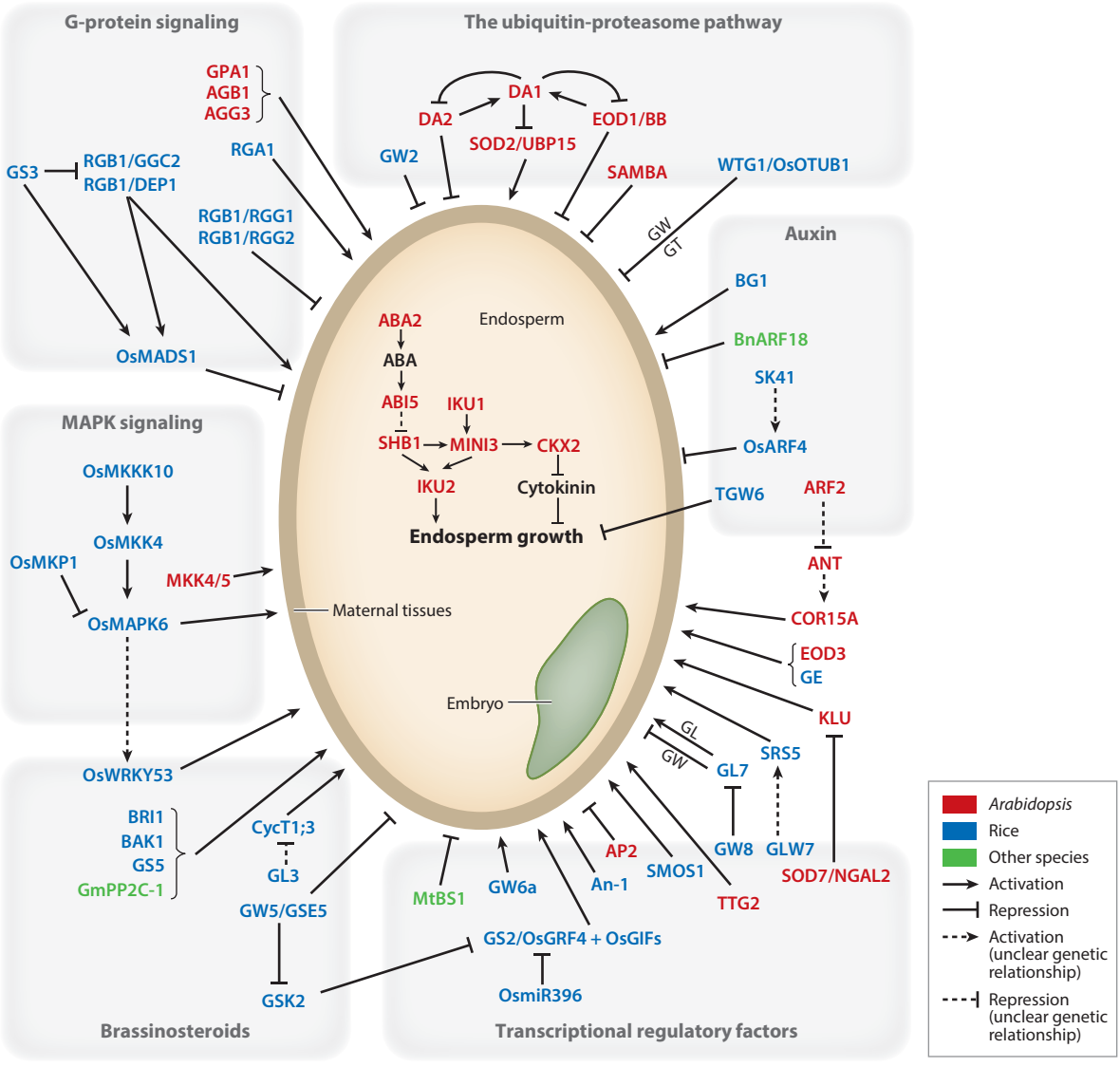

Figure 2

The major signaling pathways of seed size control. Seed size is coordinately regulated by the integrated signals of maternal and zygotic tissues. Regulators and signaling pathways that act through maternal and zygotic tissues are shown around and inside the seed. Dashed lines represent unclear genetic relationships. The seed size regulators in *Arabidopsis*, rice, and other species are shown in red, blue, and green, respectively. Abbreviations: ABA, abscisic acid; GL, grain length; GT, grain thickness; GW, grain width; MAPK, mitogen-activated protein kinase.

its closest homolog, DA1-related (DAR1), to control seed size. The *da1-1* allele encodes a mutant DA1 protein ($DA1^{R358K}$) that has a negative effect toward DA1 and DAR1. Simultaneous disruption of DA1 and DAR1 also results in increased seed and organ size.

Two RING-type E3 ligases, DA2 and ENHANCER OF DA1 (EOD1)/BIG BROTHER (BB), physically interact with DA1 to control seed and organ size (14, 130). Both *da2-1* mutants and *eod1/bb* mutants have large seeds and organs, suggesting that DA2 and EOD1/BB are negative

Table 1 Main regulators of seed size control

Species	Protein name	Protein category	Accession number	References
The ubiquitin-proteasome pathway				
Arabidopsis	DA1	Ubiquitin receptor	At1g19270	14, 66
Maize	ZmDA1	Ubiquitin receptor	GRMZM2G017845	133
Arabidopsis	DAR1	Ubiquitin receptor	At4g36860	66
Maize	ZmDAR1	Ubiquitin receptor	GRMZM2G099328	133
Arabidopsis	DA2	E3 ubiquitin ligase	At1g78420	14, 130
Arabidopsis	EOD1/BB	E3 ubiquitin ligase	At3g63530	13, 14, 66
Rice	GW2	E3 ubiquitin ligase	Os02g0244100	98
Maize	ZmGW2	E3 ubiquitin ligase	EU968771, FJ573211, EU962093	61
Wheat	TaGW2	E3 ubiquitin ligase	JN896622, JN896623	100
Arabidopsis	SOD2/UBP15	Ubiquitin-specific protease 15	At1g17110	15
Arabidopsis	SAMBA	APC16-like protein; negative regulator of the APC/C complex	At1g32310	19
Arabidopsis	RPT2A	26S proteasome regulatory particle AAA-ATPase	At4g29040	55
Rice	WTG1/OsOTUB1	Deubiquitinating enzyme homologous to human OTUB1	Os08g0537800	40, 119
G-protein signaling				
Arabidopsis	GPA1	Gα subunit	At2g26300	8
Rice	RGA1/D1	Gα subunit	Os05g0333200	3, 25
Arabidopsis	AGB1	Gβ subunit	At4g34460	8
Rice	RGB1	Gβ subunit	Os03g0669100	112
Arabidopsis	AGG3	Atypical Gγ subunit	At5g20635	8, 63
Rice	RGG1	Typical Gγ subunit	Os03g0635100	72
Rice	RGG2	Gγ subunit	Os02g0137900	72, 79
Rice	GS3	Atypical Gγ subunit	Os03g0407400	7, 20, 72, 77, 104
Rice	DEP1	Atypical Gγ subunit	Os09g0441900	41, 72, 104, 116, 156
Rice	GGC2	Atypical Gγ subunit	XP_015649592.1	104
The mitogen-activated protein kinase signaling pathway				
Rice	SMG2/OsMKKK10	Mitogen-activated protein kinase kinase kinase	Os04g0559800	32, 138
Rice	SMG1/OsMKK4	Mitogen-activated protein kinase kinase	Os02g0787300	17, 32, 138
Arabidopsis	MKK4	Mitogen-activated protein kinase kinase	At1g51660	148
Arabidopsis	MKK5	Mitogen-activated protein kinase kinase	At3g21220	148
Rice	OsMAPK6	Mitogen-activated protein kinase	Os06g0154500	32, 73, 138
Rice	LARGE8/OsMKP1	Mitogen-activated protein kinase phosphatase	Os05g0115800	32, 139
Phytohormone signaling and homeostasis				
Rice	D61/OsBRI1	LRR-RLK; brassinosteroid receptor	Os01g0718300	81
Rice	D11	CYP724B1; involved in brassinosteroid biosynthesis	Os04g0469800	108, 128, 154
Rice	SLG	BAHD acyltransferase-like protein	Os08g0562500	23
Rice	XIAO	LRR-RLK	Os04g0576900	47

(*Continued*)

Table 1 (*Continued*)

Species	Protein name	Protein category	Accession number	References
Rice	GS5	Putative serine carboxypeptidase	Os05g0158500	65, 135
Maize	ZmGS5	Putative serine carboxypeptidase	GRMZM2G123815	69
Rice	qGL3/GL3.1/ OsPPKL1	Protein phosphatase kelch family serine/ threonine phosphatase	Os03g0646900	89, 149
Rice	GSK2	SHAGGY-like kinase; regulator of brassinosteroid signaling kinase	Os05g0207500	111
Rice	GSE5/GW5/qSW5	Calmodulin-binding protein	Os05g0187500	18, 68, 95, 114, 125
Soybean	PP2C-1	Phosphatase 2C-1	Glyma17g33690	74
Arabidopsis	ARF2/MNT	Auxin response factor	At5g62000	85, 92
Rice	GL3.3/TGW3/ qTGW3/OsSK41	SHAGGY-like kinase	Os03g0841800	39, 129, 143
Rice	OsARF4	Auxin response factor	Os01g0927600	39
Rapeseed	ARF18	Auxin response factor	BnaA09g55580D	70
Jatropha	JcARF19	Auxin response factor	KX988008	106, 141
Rice	BG1	Unknown protein	Os03g0175800	71
Medicago	DASH	Endosperm-specific DOF transcription factor	Medtr2g014060	83
Transcriptional regulators				
Rice	GLW7	OsSPL13	Os07g0505200	96
Rice	GW8	OsSPL16	Os08g0531600	118, 120
Rice	GS2/GL2/GLW2/ PT2	OsGRF4	Os02g0701300	9, 16, 37, 62, 103
Rice	OsGIF1	Rice GRF-interacting protein 1	Os03g0733600	9, 16, 33, 62
Arabidopsis	SOD7/NGAL2	B3 domain transcriptional repressor	At3g11580	150
Arabidopsis	DPA4/NGAL3	B3 domain transcriptional repressor; NGAL3	At5g06250	150
Rice	OsMADS1	MADS-domain transcription factor	Os03g0215400	72, 145
Rice	OsWRKY53	WRKY family transcription factor	Os05g0343400	110
Rice	GS9	Transcriptional activator	Os09g0448500	152
Arabidopsis	ANT	AP2-like family transcription factor	At4g37750	78
Medicago	BS1	TIFY family transcription regulators	KM668032	28
Arabidopsis	AP2	AP2/EREBP family transcription factor	At4g36920	51, 84
Rice	SMOS1	Unusual AP2-type transcription factor	Os05g0389000	4
Arabidopsis	TTG2	WRKY family transcription factor	At2g37260	26, 52
Foxtail millet	LP1	WRKY family transcription factor	Seita.2G369500	131
Rice	An-1	Basic helix-loop-helix transcription factor	Os04g0350700	75
Rice	GW6a	Histone H4 acetyltransferase	Os06g0650300	99
Regulators influencing endosperm growth				
Arabidopsis	IKU1	VQ motif protein	At2g35230	27, 76, 115
Arabidopsis	IKU2	LRR receptor kinase	At3g19700	27, 76
Arabidopsis	MINI3	WRKY family transcription factor	At1g55600	76
Arabidopsis	SHB1	SYG1 family protein; transcription coactivator	At4g25350	53, 155
Arabidopsis	ABA2	Short-chain dehydrogenase/reductase	At1g52340	11

(*Continued*)

Table 1 (*Continued*)

Species	Protein name	Protein category	Accession number	References
Arabidopsis	ABI5	bZIP transcription factor	At2g36270	11
Arabidopsis	BRI1	LRR-RLK; brassinosteroid receptor	At4g39400	46
Arabidopsis	BZR1	Transcription factor	At1g75080	46
Arabidopsis	DET2	Steroid-5-α-reductase; involved in brassinosteroid biosynthesis	At2g38050	46
Arabidopsis	DWF4	CYP90B1; involved in brassinosteroid biosynthesis	At3g50660	46
Arabidopsis	AHKs	Histidine kinases; cytokinin receptors	At5g35750 At1g27320 At2g01830	90
Arabidopsis	AHPs	Histidine phosphotransfer proteins; regulators of cytokinin signaling	At3g21510 At3g29350 At5g39340 At3g16360 At1g03430	43
Arabidopsis	CKX2	Cytokinin oxidase/dehydrogenases	At2g19500	58, 126
Rice	TGW6	IAA-glucose hydrolase	Os06g0623700	45
Other regulators				
Arabidopsis	COR15a	Cold-regulated protein localized in chloroplast	At2g42540	78
Rice	SRS5	α-Tubulin	Os11g0247300	94
Arabidopsis	KLU/CYP78A5	Cytochrome P450	At1g13710	1
Arabidopsis	EOD3/CYP78A6	Cytochrome P450	At2g46660	22
Arabidopsis	CYP78A9	Cytochrome P450	At3g61880	22
Rice	GE/CYP78A13	Cytochrome P450	Os07g0603700	82, 136, 140
Rice	CYP704A3	Cytochrome P450	Os04g0573900	109
Soybean	CYP78A72	Cytochrome P450	Glyma19G240800	151
Soybean	CYP78A10	Cytochrome P450	Glyma05G019200	121
Rice	GAD1	EPIDERMAL PATTERNING FACTOR-LIKE family secretary signal peptide	Os08g0485500	49
Rice	GL7/GW7/SLG7	TON1 RECRUITING MOTIF-containing protein	Os07g0603300	118, 122, 153

regulators of seed and organ size (13, 66, 130). The interaction of these two E3 ligases with DA1 is not likely to mediate DA1 degradation because the *da2-1* or *eod1/bb* mutations synergistically enhance the seed and organ size phenotypes of *da1-1* (66, 130). Interestingly, both DA2 and EOD1/BB can monoubiquitinate DA1 in multiple sites, which activates the peptidase activity of DA1. The active DA1 peptidase can then cleave diverse growth regulators to regulate seed and organ growth (14). In addition, DA1 can cleave and destabilize EOD1/BB and DA2, which is likely a feedback regulation of the E3 ubiquitin ligases. The activation of DA1 characterizes a coupled ubiquitin-binding and ubiquitylation model within which UIM domains promote monoubiquitination of the proteins that contain them (34). This coupled ubiquitylation of DA1 may cause a conformation change, leading to either activation of its peptidase domain or substrate recruitment. Determination of the three-dimensional structure of DA1 by computational prediction or X-ray crystallography analysis will help elucidate the mechanism of DA1 activation in the future.

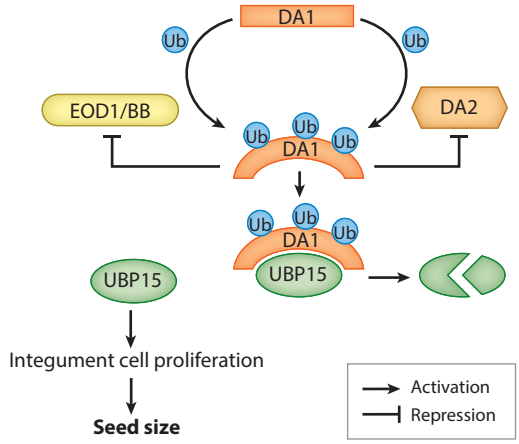

Figure 3

Control of seed size by the DA1 pathway. DA1 is a ubiquitin-activated protease that negatively regulates seed size by restricting integument cell proliferation. The E3 ubiquitin ligase DA2 or EOD1/BB can monoubiquitinate DA1 in multiple sites to activate its peptidase activity. The active DA1 can cleave DA2 or EOD1/BB, which leads to destabilization of the E3 ligases. The active DA1 cleaves its downstream target UBP15 to control seed growth. UBP15 promotes seed growth by enhancing cell proliferation in the integuments. Abbreviations: BB, BIG BROTHER; EOD1, ENHANCER OF DA1; Ub, ubiquitin; UBP15, UBIQUITIN-SPECIFIC PROTEASE15.

One of the targets of DA1 is UBIQUITIN-SPECIFIC PROTEASE15 (UBP15)/SUPPRESSOR2 OF DA1 (SOD2), which acts downstream of DA1 to promote seed growth by regulating cell proliferation in the integuments (15). The *ubp15/sod2* mutant was isolated as a suppressor of *da1-1* with small seeds and organs. UBP15 acts as a deubiquitinating enzyme. DA1 directly interacts with UBP15 and cleaves UBP15 to modulate its protein stability. The *ubp15* mutant is epistatic to *da1-1* with respect to seed and organ size, and plants overexpressing *UBP15* mimic the seed and organ size phenotypes of *da1-1*, suggesting that DA1 and UBP15 act antagonistically in a common genetic pathway to regulate seed and organ growth. UBP15 may promote seed and organ growth by targeting unknown cell-proliferation regulators for deubiquitination. Further identification of UBP15-interacting partners will help to uncover these downstream targets. In addition, it will be interesting to investigate whether UBP15 can deubiquitinate the monoubiquitinated DA1 to modulate its peptidase activity through feedback. Genetic analyses suggest that UBP15 acts independently with DA2 and EOD1/BB with respect to seed and organ size, indicating that UBP15, DA2, and EOD1 may have different targets that control seed and organ growth (**Figure 3**).

The function of the DA1 pathway in seed size control is likely conserved among different species. Firstly, overexpression of a negative interfering mutant of *Arabidopsis DA1* (*AtDA1*^{R358K}) can increase seed size and seed yield in both *Arabidopsis* and *Brassica napus* (66, 117). Secondly, overexpression of mutated *ZmDA1* or mutated *ZmDAR1* in maize (*Zea mays*) increases kernel yield (133). Moreover, the homolog of *DA2* in rice, *GRAIN WIDTH AND WEIGHT 2* (*GW2*), was identified as a major quantitative trait locus (QTL) for grain width and weight (98). GW2 negatively regulates grain width and weight by restricting cell division in the spikelet hull. A 1-base-pair (bp) deletion in the coding region of *GW2* causes the loss of function of *GW2*, resulting in increased grain width and weight. This mutant allele can increase grain yield without affecting grain quality, and it therefore has great potential for rice yield improvement. In addition, GW2 homologs in maize and wheat are involved in grain size control (61, 100). Because manipulating

Quantitative trait locus (QTL): a DNA locus that correlates with variation in a phenotype

the DA1 pathway can increase seed yield, it will be important to identify other components of the DA1 pathway in different species and evaluate their effects on crop yield.

SQUAMOSA promoter binding protein-like (SPL): a family of transcription factors defined by a plant-specific DNA-binding domain binding to a *SQUAMOSA* promoter

2.1.2. The anaphase-promoting complex regulator SAMBA limits seed growth. The anaphase-promoting complex (APC) is a multi-subunit E3 ubiquitin ligase that regulates mitotic progression by marking cell cycle proteins for degradation (97). A plant-specific negative regulator of the APC, SAMBA, restricts seed and organ growth by controlling cell proliferation (19). The *samba* mutant displays large seeds and organs, and accumulates CYCA2;3, indicating that SAMBA regulates seed and organ size by modulating the stability of A-type cyclins. Interestingly, *samba* synergistically enhances the seed and organ size phenotypes of *da1-1 eod1-2*, suggesting that SAMBA might function redundantly of or in parallel with DA1 and EOD1/BB to control seed size (113). It is also possible that SAMBA functions with DA1 and EOD1/BB in a complex to control seed growth. In the future, it will be worthwhile to test whether SAMBA could physically interact with DA1 and EOD1/BB.

2.1.3. The 26S proteasome affects seed size. The 26S proteasome consists of the 20S core protease and the 19S regulatory particle (RP). One of the subunits of the RP, RPT2, is involved in seed size control in *Arabidopsis* (55). The *Arabidopsis* RPT2 is encoded by two homologous genes, *AtRPT2a* and *AtRPT2b*. A loss of function of *AtRPT2a* affects 26S proteasome activity, resulting in large seeds and organs. Petals and cotyledons of the *rpt2a* mutant contain larger and fewer cells than those of the wild type, indicating that *AtRPT2a* regulates organ size by restricting cell expansion. Since many studies suggest the important role of the ubiquitin-proteasome pathway in seed size control, it would be interesting to analyze whether other subunits of the 26S proteasome influence seed size.

2.1.4. The rice deubiquitinase OsOTUB1/WIDE AND THICK GRAIN 1 regulates grain size. The rice *wide and thick grain 1-1* (*wtg1-1*) mutant shows wide, thick, and short grains with increased grain weight and increased grain number per panicle. *WTG1* encodes a deubiquitinating enzyme homologous to human OTUB1 that belongs to the ovarian tumor domain protease (OTU) family (40, 119). Overexpression of *WTG1/OsOTUB1* leads to thin and long grains with decreased grain weight. WTG1/OsOTUB1 controls grain size and shape mainly by affecting cell expansion in the spikelet hull, suggesting that it functions in a distinct pathway with GW2, which influences grain width by regulating cell division. WTG1/OsOTUB1 also regulates plant architecture by promoting the degradation of rice SQUAMOSA promoter binding protein-like 14 (OsSPL14)/IDEAL PLANT ARCHITECTURE 1 (IPA1)/WEALTHY FARMER'S PANICLE (WFP) (48, 80, 119). WTG1/OsOTUB1 limits K63-linked ubiquitination and in turn promotes K48-linked ubiquitination of OsSPL14 to modulate its stability. It is possible that WTG1/OsOTUB1 also targets downstream cell-expansion regulators to regulate grain size. Identifying novel targets of WTG1/OsOTUB1 and testing whether WTG1/OsOTUB1 affects the stability of known grain size regulators would help to uncover its downstream components of grain size control.

2.2. Control of Seed Size by G-Protein Signaling

The heterotrimeric G-protein complex consists of Gα, Gβ, and Gγ subunits that participate in multiple processes of plant growth and development. Loss of function and suppression of either Gα or Gβ result in short seeds in both *Arabidopsis* and rice, suggesting that Gα and Gβ positively

regulate seed length (3, 8, 25, 112). The *Arabidopsis* genome encodes three Gγ subunits (AGGs). Although the function of AGG1 and AGG2 in seed size control has not been characterized in detail, the *agg3* mutant shows short seeds and organs (8, 63). AGG3 is a noncanonical Gγ subunit that contains an N-terminal γ-like domain and a C-terminal cysteine (cys)-rich region (8). Overexpression of *AGG3* increases seed and organ size in *Arabidopsis*, indicating that *AGG3* promotes seed growth (63).

In rice, a major QTL for grain size, *GS3*, encodes a noncanonical Gγ homologous to AGG3 (7, 20). GS3 also contains an N-terminal γ-like domain and a C-terminal cys-rich domain. Different *GS3* alleles have been characterized in different rice varieties. Compared with the wild-type allele, the null allele carries a single nucleotide substitution (C-A), resulting in a premature stop codon and leading to a truncated protein with part of the N-terminal γ-like domain. This null allele causes long grains and is widely used by rice breeders (20, 107). Another allele encodes a truncated protein containing the N-terminal γ-like domain and lacking the C-terminal cys-rich region and is considered to be a gain-of-function allele (77). This gain-of-function allele causes very short grains.

A major QTL for rice panicle architecture, *DENSE AND ERECT PANICLE 1* (*DEP1*)/*PANICLE ERECTNESS* (*qPE9*), encodes another noncanonical Gγ, which contains N-terminal and C-terminal domains that are similar to those of GS3 (41, 116, 156). The *dep1/qpe9-1* allele encodes a truncated protein containing the complete N-terminal γ-like domain and lacking most of the C-terminal cys-rich region, resulting in erect panicles and slightly decreased grain size and weight (41, 142, 156). This *dep1* allele is considered to be a gain-of-function allele (72, 101). A recent study shows that overexpression of *DEP1* results in large grains, whereas downregulation or knockout of *DEP1* causes small grains and erect panicles (104). This result supports that the *dep1/qpe9-1* allele is a dominant negative or loss-of-function allele. The *dep1/qpe9-1* allele is widely utilized by breeders to improve panicle shape and grain yield in *O. sativa* ssp. *japonica* varieties (41). However, the effect of *dep1/qpe9-1* on grain yield is controversial. One study shows that *dep1/qpe9-1* enhances grain yield (41), while other studies reveal that *dep1/qpe9-1* has negative effects on grain yield (142, 156). Besides its functions in panicle architecture and seed size, *dep1/qpe9-1* also confers enhanced nitrogen uptake and assimilation (101). Most likely, artificial selection of the *dep1/qpe9-1* allele is due to its function in nitrogen-use efficiency and/or panicle architecture, rather than its effect on grain size and yield.

A recent study shows that GS3 acts antagonistically with DEP1 and another noncanonical Gγ, GGC2, to regulate grain size by competitively binding Gβ (RGB1) (104). DEP1 and GGC2 interact with RGB1 to positively regulate grain length, but GS3 competes with DEP1 and GGC2 to bind RGB1, thereby repressing the function of DEP1 and GGC2 (**Figure 4**). Genetic analyses suggest that the function of these Gγs in grain length control depends on Gα (RGA1) and RGB1. Interestingly, the degradation of GS3 is affected by its C-terminal tail. The protein encoded by the gain-of-function allele of *GS3* lacks the C-terminal tail and is therefore more stable, enhancing repression of DEP1 and GGC2 and resulting in extremely short grains. It was proposed that the N-terminal γ-like domains of different Gγs have similar functions in grain length regulation, which would suggest that the tail length of noncanonical Gγs might determine their functional differences. Investigating the function of chimeric proteins consisting of the N-terminal γ-like domains of GS3 and the C-terminal tails of DEP1 and GGC2 may help to further verify this interesting speculation. Notably, *Arabidopsis* plants likely do not distinguish differences in the C-terminal tail lengths of noncanonical Gγ proteins because rice *DEP1*, *GGC2*, and *GS3* genes can recover the short-organ phenotypes of the *Arabidopsis agg3* mutant. In addition, this study suggests that RGB1 is a limit component in promoting grain size (104). Therefore, overexpression of RGB1 should attenuate the competition between GS3 and DEP1/GGC2, leading to long grains.

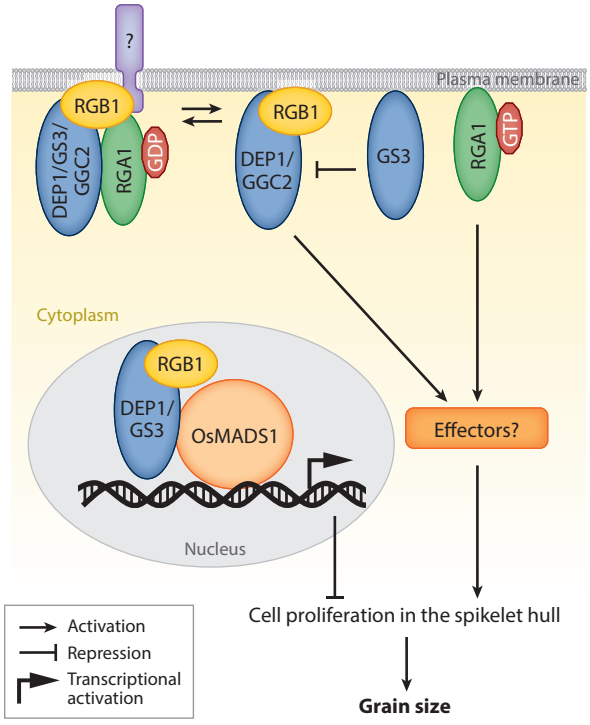

Figure 4

Control of grain size by G-protein signaling in rice. RGA1 protein is proposed to be self-activated. The dissociation of RGA1 with the RGB1–DEP1/GS3/GGC2 dimer is regulated by growth signals mediated by unknown G-protein-coupled receptors. Dissociated RGA1 and the RGB1–DEP1/GGC2 dimer may activate unknown downstream effectors to regulate grain size by promoting cell proliferation in the spikelet hull. GS3 competes with DEP1 or GGC2 to bind RGB1, leading to repression of their functions. In addition, the transcription factor OsMADS1 is proposed to be a downstream effector of G-protein signaling that negatively regulates grain size. DEP1 and GS3 interact with OsMADS1 in the nucleus to enhance its transcriptional activity, which in turn promotes the activation of target genes that limit grain growth. Recent studies have found inconsistently that DEP1 represses grain growth (72) and promotes grain growth (104). Abbreviations: DEP1, DENSE AND ERECT PANICLE 1; GGC2, noncanonical Gγ subunit 2; GS3, GRAIN SIZE 3; RGA1, Gα; RGB1, Gβ.

Unexpectedly, however, overexpression of RGB1 results in short grains in rice (72), although suppression of RGB1 also leads to short grains (104). In addition, overexpression of the typical Gγ subunits RGG1 or RGG2 has been shown to repress grain growth in rice, and loss of function of RGG2 increases grain size and yield (72, 79). It seems very interesting that RGB1/DEP1 and RGB1/GGC2 dimers promote grain growth (104), while RGB1/RGG1 and RGB1/RGG2 dimers restrict grain growth (72). Further studies need to clarify the functional divergence of different Gβ/γ combinations for grain size control.

Another study shows that DEP1 and GS3 physically interact with the transcription factor MADS1 in the nucleus to promote the transcription of downstream genes, thereby repressing grain growth (**Figure 4**) (72). OsMADS1 corresponds to a QTL for grain length in tropical *japonica* rice (72, 145). A mutation in the last intron of *OsMADS1* causes splicing defects and leads to long grains. This mutant allele of *OsMADS1* has undergone artificial selection during rice domestication (145). GS3 and DEP1 appear to act similarly in binding and enhancing the transcriptional

activity of OsMADS1 to restrict grain growth (72). Surprisingly, a loss of function of *OsMADS1* enhances the long-grain phenotype of the null allele of *GS3* (72). In contrast, other findings suggest that GS3 functions antagonistically with DEP1 to regulate grain growth (104), suggesting that GS3 and DEP1 might oppositely regulate the transcriptional activity of OsMADS1. Further studies need to clarify these inconsistent results and elucidate the function of G-proteins in grain size control.

As plant genomes encode few G-protein-coupled receptors, currently it is unclear how the G-protein complex mediates possible upstream signaling to regulate grain size. Several studies have demonstrated the interactions between G-protein components and receptor-like kinases (RLKs) (87) and suggest that G-protein may respond to signaling from RLKs to regulate grain growth. For instance, the brassinosteroid (BR) receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED KINASE 1 (BAK1) have been recently shown to phosphorylate Gβ and Gγ to regulate sugar response and plant growth in *Arabidopsis* (88). Considering that BR signaling has a critical role in seed size control (discussed in Section 2.4.1), it would be interesting to investigate whether G-proteins act downstream of OsBRI1 and OsBAK1 to regulate grain size in rice. In addition, the downstream effectors of the G-protein complex in grain size control are still largely unknown, with the exception of OsMADS1. Future studies are expected to identify the upstream signals, receptors, and downstream effectors of the G-protein complex in grain size control.

2.3. Control of Seed Size by Mitogen-Activated Protein Kinase Signaling

The MAPK cascades are composed of three tiers of protein kinases: a MAPK kinase kinase (MKKK), a MAPK kinase (MKK), and a MAPK (137). *Arabidopsis* MKK4/5 functions upstream of MAPK6 to control embryogenesis (148). The *mkk4 mkk5* double mutant exhibits a short-seed phenotype, whereas the seed size of *mpk6* is comparable to that of wild-type plants, probably due to functional redundancy between MPK6 and MPK3 (148). In rice, OsMKKK10, OsMKK4, and OsMAPK6 act as a cascade to regulate grain size (**Figure 5**) (138). The loss-of-function mutants *small grain2-1* (*smg2-1*)*/osmkkk10*, *smg1-1/osmkk4*, and *dwarf and small grain1* (*dsg1*)*/osmapk6* form small grains due to reduced cell proliferation in the spikelet hull, whereas activation of OsMKKK10 or OsMKK4 increases grain size as a result of increased cell proliferation (17, 73, 138). OsMKKK10 can sequentially phosphorylate and activate OsMKK4 and OsMAPK6, and the activity of OsMAPK6 is positively associated with grain size (138). Further genetic analyses suggest that OsMKKK10, OsMKK4, and OsMAPK6 act in a common genetic pathway to control grain size in rice.

Dual-specific MAPK phosphatases (MKPs) function as negative regulators of MAPK signaling (5). MKPs can inactivate MAPKs by specifically dephosphorylating the phosphorylated activation loop of MAPKs. Rice OsMKP1 acts antagonistically with OsMKK4 to regulate grain size by regulating OsMAPK6 activity (32, 139). Loss of function of *OsMKP1* increases grain size due to increased cell proliferation in the spikelet hull, whereas overexpression of *OsMKP1* leads to small grains (32, 139). Thus, precise control of OsMAPK6 activity by reversible phosphorylation is critical in rice grain size control.

OsWRKY53 has been found to be a direct downstream target of the OsMKK4-OsMAPK6 cascade in wounding and defense responses (12, 38, 144). The *oswrky53* mutant forms small grains, while overexpression of *OsWRKY53* leads to large grains (110). Therefore, OsWRKY53 might act as a target of the OsMKKK10-OsMKK4-OsMAPK6 module to control grain growth. However, it should be noted that OsWRKY53 regulates grain growth by promoting cell expansion in spikelet hulls, whereas the OsMKKK10-OsMKK4-OsMAPK6 cascade controls grain size by enhancing

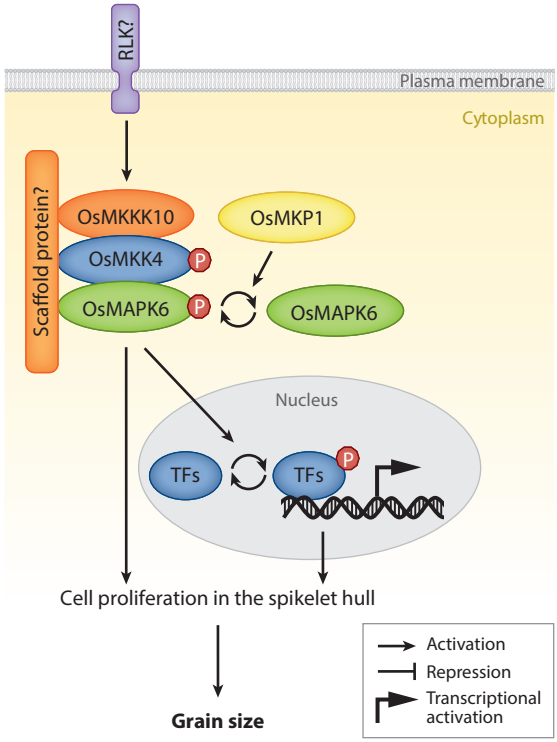

Figure 5

Control of grain size by mitogen-activated protein kinase (MAPK) signaling. Growth signals activate the OsMKKK10-OsMKK4-OsMAPK6 cascade through unknown receptor-like kinases (RLKs). The sequential phosphorylation activates OsMAPK6 to promote cell proliferation in spikelet hulls, thereby increasing grain size. OsMKP1 can dephosphorylate and deactivate OsMAPK6 to inhibit its function in grain size control. OsMAPK6 may phosphorylate transcription factors (TFs), which regulate grain size by influencing cell proliferation in the spikelet hull.

cell proliferation. Further studies are needed to address whether OsWRKY53 acts in the same genetic pathway as the OsMKKK10-OsMKK4-OsMAPK6 module to control grain size.

Some evidence suggests an interaction between MAPK signaling and G-protein signaling in *Arabidopsis*. For instance, a recent study showed that *Arabidopsis* RACK1 proteins function as scaffolds to connect G-protein and MAPK signaling in immune signaling (10). Furthermore, Gβ directly interacts with MPK3/6, MKK4/5, and YODA (an MKKK) to regulate zygote development, indicating that Gβ may also function as a scaffold (146). Considering that both G-protein signaling and MAPK signaling are involved in seed size control, it would be worthwhile to investigate whether they work in the same pathway to regulate seed growth. In addition, MAPK cascades generally act as signaling modules downstream of RLKs to regulate plant growth and development. It would be a worthwhile challenge to identify the RLKs and their ligands upstream of MAPK signaling in seed size control.

2.4. Control of Seed Size by Phytohormones

Phytohormones have various functions in plant growth and development, stress responses, and metabolism. BR and auxin have been shown to regulate seed growth through maternal tissues. In

addition, these two phytohormones can also affect endosperm development to influence seed size (discussed in Section 3).

2.4.1. The role of brassinosteroids in maternal control of seed size. In both *Arabidopsis* and rice, the BR-deficient mutants and BR-insensitive mutants form short seeds, suggesting that BR promotes seed growth (21, 36, 46, 81, 108, 147). Consistently, the *slender grain (slg-D)* mutant shows increased BR content and long grains (23), whereas a loss of function of *XIAO* results in typical BR-deficient phenotypes and reduced grain length (47). In rice, the small-grain phenotype of the BR-deficient mutants mainly results from decreased cell size in the lemma/glume, suggesting that BR regulates grain size by promoting cell expansion in the spikelet hulls (21, 128, 154). However, genetic analyses in *Arabidopsis* suggest that BR may control seed size by influencing endosperm development (discussed in Section 3.1.2) but affect seed shape through maternal tissues (46).

GS5 controls grain size in rice likely by modulating OsBAK1-7 endocytosis. GS5 is a putative serine carboxypeptidase encoded by a major QTL for grain size, *GS5* (65). GS5 regulates grain width by promoting cell proliferation and cell expansion in the palea/lemma. GS5 can bind to OsBAK1-7 and affect its endocytosis (135). Natural variation in the *GS5* promoter affects *GS5* expression and contributes to grain size diversity in rice. An increased amount of GS5 protein prevents the endocytosis of OsBAK1-7, resulting in enhanced BR signaling and increased grain size. However, the genetic interaction between GS5 and OsBAK1-7 is still unclear. In maize, the GS5 ortholog (ZmGS5) is also involved in kernel development, suggesting that the function of GS5 is conserved in seed growth (69).

The rice OsPPKL proteins share some similarity with *Arabidopsis* BSU1, which acts in BR signal transduction to promote cell elongation and cell division (149). Although the roles of OsPPKL proteins in BR responses are unclear, they are involved in grain size control (89, 149). OsPPKL1 is encoded by a major QTL for grain length, *qGL3/qGL3.1*. OsPPKL1 regulates grain size by restricting cell proliferation in the spikelet hull. The *qgl3* allele harbors a single nucleotide mutation that leads to an aspartate-to-glutamate change in a conserved domain of OsPPKL1, resulting in increased grain length and grain yield without affecting grain quality. Notably, *qgl3* is a rare allele that has not been artificially selected during rice domestication, and it therefore could be used in rice breeding. Furthermore, OsPPKL1 can interact with and dephosphorylate Cyclin-T1;3 (89). Downregulation of Cyclin-T1;3 reduces grain length in rice. Thus, OsPPKL1 may regulate grain size by modulating Cyclin-T1;3 activity. Intriguingly, OsPPKL1 and OsPPKL3 negatively regulate grain length, while their homolog OsPPKL2 promotes grain growth (149).

The rice GSK3/SHAGGY-like kinase GSK2 is one of the orthologs of *Arabidopsis* BIN2. Activation of GSK2 results in typical BR-deficient phenotypes and short grains in rice (111). GSK2 has been involved in different signaling pathways to control grain size. Firstly, GSK2 directly interacts with the transcriptional activator GRAIN SIZE 2 (GS2)/GROWTH-REGULATING FACTOR 4 (OsGRF4) and inhibits its activity, suggesting that GSK2 may regulate grain size through the OsGRF4–GRF-INTERACTING FACTORs (OsGIFs) regulatory module (discussed in Section 2.5.2). Secondly, GSK2 modulates the transcriptional activity of GRAIN SHAPE GENE ON CHROMOSOME 9 (GS9) (152). GS9 is a transcriptional activator. It forms a transcriptional complex with OsOFP8 and OsOFP14 to control grain shape by regulating cell division. OsOFP8 represses the transcriptional activity of GS9, whereas this repression is attenuated by GSK2. In addition, GSK2 can phosphorylate the GRAS family protein DWARF AND LOW-TILLERING (DLT/OsGRAS-32/D62/GS6) to mediate BR signaling (64, 102, 111).

The kinase activity of GSK2 is repressed by GW5/qSW5. *GW5/qSW5* is a major QTL for grain width that was located in an 11.2-kilobase-pair region (95, 114, 125). The gene encoding a

ubiquitin-binding protein was previously proposed to be *GW5*. Recent studies demonstrated that another open reading frame in this region (LOC_Os05g09520) encoding a calmodulin-binding protein is the bona fide *GW5* gene (68). This gene was also identified as a major QTL for grain size, *GSE5*, by a genome-wide association study (18). Natural variation in the promoter region of *GSE5/GW5* affects its expression and contributes to grain size diversity. Compared to most narrow-grain *O. sativa* ssp. *indica* varieties, most wide-grain *indica* varieties have a 950-bp deletion (DEL1) in the *GSE5/GW5* promoter, while most *japonica* varieties carry a 1,212-bp deletion (DEL2). These deletions cause decreased expression of *GSE5/GW5*, leading to wide and heavy grains due to enhanced cell proliferation in the spikelet hull. By contrast, overexpression of *GSE5/GW5* leads to narrow and long grains. The DEL1 and DEL2 alleles have been widely used in *indica* and *japonica* rice breeding, respectively. In addition, GSE5/GW5 interacts with GSK2 and represses its kinase activity, suggesting that GSE5/GW5 may control grain width by decreasing GSK2 activity. However, loss of function of *GSE5/GW5* causes wide and heavy grains, while downregulation of *GSK2* results in long and heavy grains but does not affect grain width (152). Further genetic and cellular analyses could help to address how GSE5/GW5 negatively regulates GSK2 activity to influence grain width and weight. Notably, although both GS9 and GSE5/GW5 interact with GSK2, genetic analyses suggest that GS9 and GSE5/GW5 act independently to control grain size (152).

Soybean (*Glycine max*) PP2C-1 likely regulates seed size by modulating GmBZR1 stability. In *Arabidopsis*, BZR1 is phosphorylated by BIN2 and dephosphorylated by PP2A and BSU1. A recent study shows that the accumulation of dephosphorylated soybean GmBZR1 is affected by PP2C-1. PP2C is encoded by a QTL for seed weight in soybean (74). The *PP2C-1* allele from wild soybean ZYD7 promotes soybean seed growth by enhancing integument cell expansion. PP2C-1 associates with GmBZR1 and facilitates the accumulation of dephosphorylated GmBZR1. By contrast, the *PP2C-2* allele produces a nonfunctional PP2C protein due to a few amino acid changes in the N terminus, resulting in small seeds. About forty percent of cultivated soybeans do not have the *PP2C-1* allele, indicating that this allele could be introduced to these cultivars for yield improvement (74).

Although BRs and many BR signaling components have been implicated in seed size control, it is still uncertain how BRs promote seed growth. Firstly, little is known about the genetic relationships between identified BR-related seed size regulators, leaving our understanding of the mechanisms of BRs in seed size control rather fragmented. Further studies are needed to integrate the network by which BRs regulate seed size. Secondly, some BR-signaling components affect seed size by controlling cell expansion in the maternal tissues, whereas others influence cell division. This suggests that BRs may regulate seed growth through distinct downstream pathways. It is necessary to elucidate how BRs coordinate cell proliferation and cell expansion to regulate seed growth. In addition, evidence from rice suggests that BRs regulate grain size by influencing the size of the spikelet hulls (21, 128, 154). However, it was shown that BRs act zygotically to control seed size in *Arabidopsis* (46). Further studies are expected to elucidate the different roles of BRs in seed size control in *Arabidopsis* and rice.

2.4.2. The role of auxin in maternal control of seed size. Auxin signaling is mediated by the Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors and the AUXIN RESPONSE FACTOR (ARF) transcription factors (124). The *Arabidopsis* genome encodes twenty-three ARF proteins, among which is ARF2/MEGAINTEGUMENTA (MNT), which is involved in seed size control (85, 92). ARF2/MNT acts maternally to regulate seed growth by limiting cell proliferation in the integuments (92). A loss of function of ARF2/MNT results in pleiotropic phenotypes, including increased seed and organ size, abnormal flower morphology, and reduced fertility. Interestingly, expression of *ARF2* driven by the *APETALA1* (*AP1*) promoter

in the *arf2* mutant improved fertility and retained the seed size phenotype, resulting in a significantly increased harvest index compared with *arf2* (42). The *arf2* mutation prolongs the expression of *AINTEGUMENTA* (*ANT*) and *CYCD3;1* in leaves and stems, suggesting that ARF2 may control seed growth by regulating the expression of *ANT* and *CYCD3;1* (92). A recent study showed that ARF2 binds to the *ANT* promoter, and ANT directly regulates the expression of *COLD-REGULATED15A* (*COR15a*) (78). The *cor15a* knockout mutant has smaller seeds than the wild type, suggesting that *COR15A* positively regulates seed size (78). The genetic relationships between *AP2*, *ANT*, and *COR15A* warrant further investigation.

A recent study showed that SHAGGY-like kinase 41 (OsSK41)/OsGSK5 interacts with OsARF4 to regulate grain size and weight (39). OsSK41/OsGSK5 is encoded by a major QTL for grain weight, *qTGW3/GL3.3* (39, 129, 143). It negatively regulates grain size by affecting cell expansion in the spikelet hulls. OsSK41 can phosphorylate OsARF4, which limits grain size. Loss of function of *OsSK41* or *OsARF4* results in large and heavy grains. OsSK41 and OsARF4 regulate the expression of some auxin-response genes, indicating that they likely control grain size through auxin signaling. Importantly, the loss-of-function allele of *qTGW3/GL3.3* has not been extensively selected in rice breeding, suggesting that it could be applied for rice improvement.

Evidence from other species also suggests the roles of ARF proteins in seed size control. In *B. napus*, natural variation in the *ARF18* gene affects seed weight and silique length (70). ARF18 acts as a repressor of auxin-responsive genes by forming homodimers and functions maternally to control seed size by restricting cell expansion in the silique wall. A mutant allele of *ARF18* causes a deletion in the ARF18 protein that prevents its homodimerization, leading to enhanced expression of downstream genes and increased seed weight. In addition, *JcARF19* was identified as a major QTL for seed length in the woody plant *Jatropha curcas* (141). Overexpression of *JcARF19* increases seed size and seed yield in *Arabidopsis* and *J. curcas* (106).

Auxin transport is also involved in seed size control. Rice BIG GRAIN1 (BG1) is a novel membrane-localized protein. *BG1* is preferentially expressed in the vascular tissue of culms and young panicles. The dominant mutant *bg1-D* shows increased auxin basipetal transport, altered auxin distribution, and extra-large grains, indicating that BG1 may be involved in auxin transport and grain size control. The increased grain size of *bg1-D* is due to enhanced cell proliferation and cell expansion in the spikelet hulls (71). Additionally, it was shown that fertilization of the central cell leads to auxin production, and auxin is exported to the maternal tissues to drive seed coat development (24). Defective auxin export from the zygotic tissue can also affect the growth of maternal tissue and seed size, as observed in the *dash* (*DOF acting in seed embryogenesis and hormone accumulation*) mutant of *Medicago truncatula*. *DASH* encodes an endosperm-specific DOF transcription factor that is expressed in the chalazal endosperm during the transition phase between embryogenesis and seed filling. Strong loss-of-function *dash* alleles cause severe defects in endosperm development, leading to impaired embryogenesis and resulting in aborted pods or mature seeds with reduced size. Interestingly, treatment of wild-type pods with the auxin efflux transport inhibitor TIBA mimicked the *dash* phenotype, while exogenous IAA can restore the pod and seed set phenotype of *dash*, indicating that auxin export to the maternal tissue is required for normal seed growth (83).

Even though auxin signaling and transport have been extensively studied in *Arabidopsis*, our current understanding of the role of auxin in seed size control is limited. This might be partly due to the functional redundancy of AUX/IAA and ARF proteins in auxin responses. In addition, some auxin-transport mutants are infertile or embryo-lethal and are therefore unavailable for seed size characterization. Thus, multiple mutant lines or overexpression lines of auxin-signaling mutants and weak alleles of auxin-transport mutants could be used in future work to analyze whether the key auxin-signaling or auxin-transport components affect seed growth.

2.5. Control of Seed Size by Transcriptional Regulators

Transcriptional regulation is crucial for multiple plant growth and developmental processes. Several transcriptional regulators have been identified as important factors of seed size control in plants, including transcription factors, transcriptional coactivators, and regulators involved in chromatin modification.

2.5.1. SQUAMOSA promoter binding protein-like family transcription factors. *GRAIN LENGTH AND WEIGHT ON CHROMOSOME 7* (*GLW7*), a major QTL for rice grain size, encodes OsSPL13 (96). OsSPL13 positively regulates grain size by promoting cell expansion in the spikelet hull. In tropical *japonica* rice, a tandem-repeat sequence in the 5′ UTR of *OsSPL13* leads to elevated expression of *OsSPL13*, resulting in large grains. This allele was thought to be introgressed from *indica* varieties under artificial selection. OsSPL13 can bind the promoter of *SMALL AND ROUND SEED 5* (*SRS5*) and promote its transcription. *SRS5* encodes an α-tubulin protein (93). A semidominant mutation of *SRS5* leads to short grains, whereas overexpression of *SRS5* increases grain length (94). Thus, OsSPL13 may influence grain growth by promoting the expression of *SRS5*.

OsSPL16 also acts as a positive regulator of grain size by enhancing cell proliferation and grain filling (120). *OsSPL16* corresponds to the QTL for grain width, *GW8* (120). In the Basmati rice variety, a 10-bp deletion in the promoter region of *OsSPL16* causes downregulation of *OsSPL16*, resulting in slender grains and better quality of appearance. OsSPL16 can directly bind to the *GRAIN WIDTH7* (*GW7*) promoter and repress its expression (118). The rice QTL for grain size, *GRAIN WIDTH7* (*GW7*)/*GRAIN LENGTH ON CHROMOSOME 7* (*GL7*)/*SLENDER GRAIN ON CHROMOSOME 7* (*SLG7*), encodes a protein that is homologous to *Arabidopsis* LONGIFOLIA1 (LNG1) and LNG2 (118, 122, 153), which regulate leaf morphology by promoting cell expansion in the leaf-length direction (56). One study showed that high expression of *GL7/GW7/SLG7*, due to a mutation in its promoter region, leads to narrow and long grains (118). In contrast, another study demonstrated that elevated expression of *GL7/GW7/SLG7* results from the increased gene copy number (122). This beneficial allele of *GL7* has been selected in rice breeding (122). Given that OsSPL16 directly regulates *GL7/GW7/SLG7* expression, it is possible that the two act in the same genetic pathway to regulate grain size. Notably, one study showed that *GL7/GW7/SLG7* regulates grain size and shape by increasing cell proliferation in the grain-length direction and decreasing cell proliferation in the grain-width direction (118), while another two studies revealed that *GL7/GW7/SLG7* controls grain size and shape by promoting cell expansion in the grain-length direction and repressing cell expansion in the grain-width direction but does not influence cell proliferation (122, 153). Further cellular analyses of the gain-of-function and loss-of-function mutants of *OsSPL16* and *GL7/GW7/SLG7* in the same genetic background could help resolve these apparent conflicting results. Considering that SPLs have a common binding site, it would be interesting to investigate whether both OsSPL13 and OsSPL16 can bind the promoter regions of *GL7/GW7/SLG7* and *SRS5* to regulate their expression.

2.5.2. The OsmiR396-OsGRF4-OsGIFs regulatory module. A major QTL for rice grain size, *GS2/GRAIN-LENGTH-ASSOCIATED 2* (*GL2*)/*GLW2*/*PANICLE TRAITS 2* (*PT2*), encodes the transcription factor OsGRF4, which regulates grain size by mainly promoting cell expansion and slightly promoting cell proliferation (9, 16, 37, 62, 103). *OsGRF4* is regulated by microRNA396 (OsmiR396). The $GS2^{AA}$ allele with mutations in the targeting site of OsmiR396 in *OsGRF4* disrupts the repression effects of OsmiR396 toward *OsGRF4*, leading to increased expression of *OsGRF4*, and resulting in large grains and high grain yield. OsGRF4 physically interacts with the transcription coactivators OsGIF1/2/3 (16, 62). Plants overexpressing *OsGIF1* form

large grains (9, 16, 33, 62). Thus, the OsmiR396-OsGRF4-OsGIFs module has an important role in grain size control. In addition, GSK2 physically interacts with OsGRF4 and inhibits its transcription activity (9). GSK2 has a critical role in BR signaling and negatively regulates grain size (111). Therefore, OsGRF4 may interact with BR signaling to regulate grain size. In *Arabidopsis*, AtGIFs physically interact with AtGRFs to control organ size by regulating cell proliferation, and the expression of AtGRFs is also regulated by miR396 (67, 91), suggesting that the function of the miR396-GRFs-GIFs module is conserved in the regulation of organ growth. It would be interesting to analyze whether the miR396-GRFs-GIFs module also controls seed size in *Arabidopsis* and other plant species.

WRKY: a family of transcription factors containing a DNA-binding domain that recognizes the W-box *cis*-regulatory element

2.5.3. The NGATHA-LIKE-KLUH pathway. Two *Arabidopsis* B3 domain transcriptional repressors, NGATHA-LIKE 2 (NGAL2)/SUPPRESSOR OF DA1 (SOD7) and DEVELOPMENT-RELATED PcG TARGET IN THE APEX4 (DPA4)/NGAL3 act maternally to regulate seed size by restricting cell proliferation in the integuments (150). The *sod7-2 dpa4-3* mutant produces large seeds, whereas the dominant *sod7-1D* mutation reduces seed size due to overexpression of *NGAL2*. NGAL2 binds to the promoter of *KLUH* (*KLU*) and represses its expression (150). *KLU* encodes the cytochrome P450 CYP78A5. The *klu* mutant forms small seeds, and *klu-4* is epistatic to *sod7-2 dpa4-3* with respect to seed size, indicating that NGALs act in a common pathway with KLU to regulate seed size (1, 150). Besides KLU, several CYP family members have also been involved in seed size control. Most of these CYPs act as positive regulators of seed size, including *Arabidopsis* CYP78A6/EOD3 and CYP78A9 (22), soybean CYP78A72 and CYP78A10 (121, 151), and rice CYP78A13/GE (82, 136, 140). By contrast, rice CYP704A3 limits grain size (109).

2.5.4. The KIX-PEAPOD regulatory module. *BIG SEEDS1* (*BS1*) encodes a plant-specific transcription regulator that has a critical role in seed size determination in legumes. In *M. truncatula*, the *bs1-1* mutant exhibits large and heavy seeds. Similarly, suppression of *BS1* orthologs in soybean (*G. max*) increases seed size (28). BS1 is homologous to *Arabidopsis* PEAPOD1 (PPD1) and PPD2, which regulate organ size by promoting meristemoid cell proliferation (127). Recent studies show that PPD proteins form a repressor complex with KIX proteins and TOPLESS (TPL) to regulate organ growth, and that PPD and KIX are targeted by the F-box protein STERILE APETALA (SAP) for degradation (30, 60, 123). It would be interesting to investigate whether SAP, KIXs, and PPDs regulate seed size in *Arabidopsis* and crops.

2.5.5. APETALA2-type transcription factors. The *Arabidopsis* AP2 transcription factor is well-known for its role in determining flower organ identity (50, 51, 84). A loss of function or suppression of *AP2* leads to large seeds with enlarged integument cells, indicating that AP2 also has a role in seed size control (51, 84). The unusual AP2-type transcription factor SMALL ORGAN SIZE1 (SMOS1) acts as an auxin-dependent regulator for grain and organ size in rice (4). The *smos1* mutant has reduced organ and grain size due to decreased cell size and abnormal microtubule orientation. SMOS1 directly regulates the expression of *PHOSPHATE-INDUCED PROTEIN 1* (*OsPHI-1*) that is involved in cell expansion. Recently it has been shown that SMOS1 forms a complex with DLT, which regulates BR responses and grain size in rice (35, 111), suggesting that SMOS1 may integrate auxin and BR signaling to control rice grain size.

2.5.6. WRKY family transcription factors. *Arabidopsis TRANSPARENT TESTA GLABRA2* (*TTG2*) encodes a WRKY family transcription factor (52). The *ttg2* mutants form small and round seeds due to reduced cell length in the integuments. Interestingly, the *ttg2* mutants also show a pale seed coat color compared with the wild type due to defective tannin and mucilage production.

Reciprocal crosses showed that *TTG2* acts maternally to regulate seed growth (26). The expression level of *WRKY15a* is also correlated with seed size variation in wild and cultivated soybeans, suggesting that WRKY15 also has a role in seed size control (31). In addition, foxtail millet *LOOSE PANICLE1* (*LP1*) encodes a WRKY transcription factor. Loss of function of LP1 leads to loose panicles and enlarged seeds, indicating that LP1 negatively regulates grain size in foxtail millet (131).

2.5.7. Basic helix-loop-helix family transcription factors. Rice *Awn-1* (*An-1*) encodes a basic helix-loop-helix transcription factor and regulates awn development as well as grain length (75). Upregulation of *An-1* expression causes long awns and long grains, while RNA interference of *An-1* results in short awns, short grains, and increased grain number. An-1 promotes grain growth by increasing cell division in the spikelet hull. The *An-1* locus has been proposed to be major target for artificial selection during rice breeding programs. This study discovered an important link between awn length and grain length, although the way in which An-1 regulates grain growth remains unclear. Consistent with this idea, GRAIN NUMBER, GRAIN LENGTH, AND AWN DEVELOPMENT1 (GAD1) regulates both awn length and grain length in rice (49). *GAD1* encodes a member of the EPIDERMAL PATTERNING FACTOR-LIKE family. Loss of function of *GAD1* causes decreases in awn length and grain length, likely as the result of decreased cell division.

2.5.8. Chromatin modification. A QTL for rice grain size *GRAIN WEIGHT ON CHROMOSOME 6a* (*GW6a*) encodes histone H4 acetyltransferase OsglHAT1 (99). Natural variation in the promoter region of *OsglHAT1* contributes to the variation in grain size and weight. Elevated expression of *OsglHAT1* increases grain size by promoting cell proliferation in the spikelet hull. OsglHAT1 influences global acetylation levels of histone H4, suggesting that seed size is also regulated by chromatin modification. Importantly, the rare allele that elevates *OsglHAT1* expression not only enhances grain weight but also increases grain yield and plant biomass. This allele has not yet been artificially selected and thus could be exploited for rice yield improvement.

3. CONTROL OF SEED SIZE BY ZYGOTIC TISSUES

Seed size is also influenced by the growth of zygotic tissues, such as the endosperm and the embryo. It has been reported that growth of the endosperm is an important factor in setting the final seed size; however, the role of the embryo in determining seed size has not been studied in detail. It has been shown that the HAIKU (IKU) pathway and some phytohormones regulate seed size by affecting endosperm development. Endosperm growth is also affected by genomic imprinting, as reviewed previously (29). In this section, we mainly discuss the role of the IKU pathway and phytohormones in endosperm development and seed size control.

3.1. The HAIKU Pathway Controls Seed Size by Regulating Endosperm Development

Several studies indicate that endosperm development is influenced by the IKU pathway. The IKU pathway is regulated by abscisic acid (ABA) and BR, and cytokinin acts downstream of the IKU pathway to control seed growth.

3.1.1. HAIKU1, HAIKU2, MINISEED3, and SHORT HYPOCOTYL UNDER BLUE1 act in the same genetic pathway to control seed size by influencing endosperm development. The *Arabidopsis haiku1* (*iku1*), *iku2*, and *miniseed3* (*mini3*) mutants produce small seeds (27, 76). Pollination of these mutants with wild-type pollens results in normal-sized seeds,

suggesting that *IKU1*, *IKU2*, and *MINI3* function zygotically to regulate seed growth. Consistently, the mutants show precocious cellularization during endosperm development, leading to a premature arrest of seed growth. Genetic analyses have demonstrated that *IKU1*, *IKU2*, and *MINI3* act in the same genetic pathway to regulate seed growth (27, 76, 115). *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*) acts upstream of *MINI3* and *IKU2* to control endosperm development and seed size (155). The dominant *shb1-D* mutant forms large seeds due to delayed endosperm cellularization, and the seed size phenotype is suppressed by *iku2-4* or *mini3-2*. *MINI3* encodes a WRKY family transcription factor. MINI3 binds to the W-box in the promoter region of *IKU2* or *MINI3* and recruits SHB1 to promote the expression of *IKU2* and *MINI3* (53). *SHB1* encodes a protein containing an N-terminal SPX domain and a C-terminal EXS domain, which is also involved in cryptochrome signaling (54). *IKU1* encodes a VQ motif protein. IKU1 can also interact with MINI3 in a yeast-two-hybrid assay (115), and the expression of *MINI3* is promoted by IKU1 (76). However, it is unclear whether MINI3 can recruit IKU1 to activate the expression of *MINI3* and *IKU2*. Alternatively, IKU1 may form a complex with MINI3 and SHB1 to activate transcription. *IKU2* encodes a leucine-rich repeat (LRR) kinase that acts downstream of MINI3 to control seed size. LRR kinases participate in diverse signaling pathways to regulate cell processes. However, the downstream signaling of IKU2 in seed size control is still unclear. Moreover, overexpression of *Arabidopsis SHB1* in canola (*B. napus*) upregulates the expression of canola *MINI3* and *IKU2*, resulting in increased seed size and seed yield without significant changes to nutritional content. Thus, the function of the IKU pathway in seed size control appears to be conserved between *Arabidopsis* and canola and could be applied in breeding for seed yield improvement (132).

3.1.2. Abscisic acid and brassinosteroid control seed size by regulating the HAIKU pathway. The ABA-deficient mutant *aba2* shows increased seed size and elevated expression of *SHB1*, *MINI3*, and *IKU2*. Genetic analyses showed that *shb1* is epistatic to *aba2*, suggesting that ABA regulates seed size through the IKU pathway. ABA directly regulates *SHB1* expression through the ABA response transcription factor ABA-INSENSITIVE5 (ABI5). ABI5 binds to the ABA-response element (ABRE) in the promoter region of *SHB1* to repress its transcription. The *abi5* mutant exhibits large seeds and increased *SHB1* transcription levels (11).

In *Arabidopsis*, BRs have been reported to regulate seed size through zygotic tissues (46). BR-deficient mutants and BR-insensitive mutants have small and round seeds. Pollination of these mutants with wild-type pollens results in seeds with normal size but round shape, suggesting that BR regulates seed size through zygotic control. The BR-response transcription factor BZR1 can bind to the promoters of *SHB1*, *MINI3*, and *IKU2* to regulate their expression, suggesting that BR may regulate the IKU pathway to control seed size.

3.1.3. Cytokinin acts downstream of the HAIKU pathway to control seed size. The core cytokinin signaling components in *Arabidopsis* include the sensor histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs), and response regulators (44). In *Arabidopsis*, a loss of function of AHKs or AHPs reduces seed set and increases seed size (43, 90). Cytokinin homeostasis can also affect seed number and seed size in *Arabidopsis* and crop plants (2, 6). Therefore, cytokinin signaling may contribute to balancing seed size and seed number.

Cytokinin oxidase/dehydrogenases (CKXs) can modulate cytokinin homeostasis by degrading active cytokinin. Overexpression of *AtCKX* genes in *Arabidopsis* enhances cytokinin breakdown, resulting in large seeds and decreased seed number (126). Interestingly, *CKX2* is a direct transcription target of MINI3. MINI3 binds to the promoter of *CKX2* to regulate its expression. Moreover, the seed size phenotype of *iku2-2* can be partially rescued by the *ahk2 ahk3* mutant or overexpression of *CKX2*. Therefore, the IKU pathway acts, at least partially, through cytokinin

signaling to regulate seed size (58). In addition, the IKU pathway may also act through other signaling pathways, for instance the unknown downstream signaling of IKU2, to control endosperm development and seed size. Identification of these downstream signaling factors may be a worthwhile future challenge.

3.2. The Role of Auxin in Endosperm Development and Seed Size Control

Auxin signaling and transport act maternally to influence seed size, as discussed in Section 2.4.2. Some evidence suggests that auxin homeostasis can influence seed size by regulating endosperm development. In rice, a major QTL for thousand-grain weight, *TGW6*, encodes an IAA-glucose hydrolase that produces free IAA (45). TGW6 controls the timing of the transition from the syncytial phase to the cellular phase during early endosperm development by regulating IAA supply. A loss-of-function allele of *TGW6* results in increased grain length and grain weight due to the delayed onset of endosperm cellularization, indicating that auxin is required for normal endosperm development and grain size. TGW6 influences grain weight with no effect on husk size, confirming that it controls grain size by regulating endosperm development. Notably, this loss-of-function allele is likely not a selection target during rice domestication, and it could therefore be useful in rice breeding.

4. CONCLUSIONS AND PERSPECTIVES

Seed size is regulated by a complex network that integrates multiple developmental and environmental signals. Although recent studies have identified some key seed size regulators and several molecular pathways, our knowledge of the complete regulatory network remains limited and fragmented. One future challenge is to explore the upstream and downstream components of known seed size regulators and the possible connections between regulatory pathways that have been identified. This research process can be accelerated by the combination of traditional research methods with modern high-throughput approaches, such as phenomics, metabolomics, proteomics, and genome-wide association studies. Furthermore, it is noteworthy that the same gene or allele can have inconsistent effects on cell proliferation and expansion and grain yield in rice. One possible reason is that rice researchers often use near-isogenic lines, which may possess multiple other mutations, to perform genetic and physiological analyses. Genome-editing technology provides a convenient method of generating mutants and specific mutant alleles in the same genetic background, and this could help to clarify previously inconsistent results and facilitate the elaboration of seed size control networks in rice and other crops.

Seed size varies dramatically across different plant species, and this large variation has been described as an important adaptive character. In the future, a key challenge will be to establish an evolutionary understanding of seed size variation by identifying key genes determining seed size differences between species. This could be achieved by a comprehensive analysis of large-scale genetic and genomic data sets. Seed size is often negatively correlated with seed number and seed quality. Different plant species have evolved to balance fecundity and quality in distinct ways depending on the environment, and this has resulted in extensive natural variation of these traits between species and even within individual species. Obviously, these traits have undergone artificial selection during crop domestication to optimize crop yield and quality. With our greatly increased knowledge of the molecular mechanisms that determine these traits, the combination of beneficial alleles controlling these characters will help breeders develop desired crop varieties with higher yield and better quality. Interestingly, several regulators of seed size have similar functions between different plant species, implying that the mechanisms of seed size control are largely

conserved. Strategically, the main challenge we now face is to apply the findings from model plants to seed size improvement in crops.

SUMMARY POINTS

1. Seed size is determined by the coordinated growth of maternal and zygotic tissues.
2. The DA1 pathway controls seed size in *Arabidopsis* by regulating cell proliferation in the integuments.
3. G-protein subunits have important roles in seed size control in both *Arabidopsis* and rice.
4. The OsMKKK10-OsMKK4-OsMAPK6 cascade controls grain size in rice by promoting cell proliferation in the spikelet hull. The cascade is regulated by OsMKP1 through reversible phosphorylation of OsMAPK6.
5. Brassinosteroids (BRs) and auxin are involved in maternal control of seed/grain size.
6. Seed size is also regulated by some transcriptional regulators, including the SQUAMOSA promoter binding protein-like transcription factors, the OsmiR396-OsGRF4-OsGIFs regulatory module, and the NGATHA-LIKE-KLUH pathway.
7. The HAIKU (IKU) pathway controls seed size by influencing endosperm development. Abscisic acid and BRs regulate the IKU pathway to influence seed size. Cytokinin acts downstream of the IKU pathway to control seed size.

DISCLOSURE STATEMENT

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