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Phytohormone dynamics in developing endosperm influence rice grain shape and quality

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Abstract Hormones are important signaling molecules regulating developmental processes and responses to environmental stimuli in higher plants. Rice endosperm, the portion of the seed surrounding the embryo, is the main determinant of rice grain shape and yield; however, the dynamics and exact functions of phytohormones in developing endosperm remain elusive. Through a systemic study including transcriptome analysis, hormone measurement, and transgene-based endosperm-specific expression of phytohormone biosynthetic enzymes, we demonstrated that dynamic phytohormone levels play crucial roles in the developing rice endosperm, particularly in regard to grain shape and quality. We detected diverse, differential, and dramatically changing expression patterns of genes related to hormone biosynthesis and signaling during endosperm development, especially at early developmental stages. Liquid chromatography measurements confirmed the dynamic accumulation of hormones in developing endosperm. Further transgenic analysis performed on plants expressing hormone biosynthesis genes driven by an endosperm-specific promoter revealed differential effects of the hormones, especially auxin and brassinosteroids, in regulating grain shape and quality. Our studies help elucidate the distinct roles of hormones in developing endosperm and provide novel and useful tools for influencing crop seed shape and yield.

INTRODUCTION

Endosperm is an important and common component of angiosperm seeds, which provides embryos with nutrients, influences their development, and is crucial for seed germination. The endosperm originates from the union of the diploid central cell with a sperm cell, separated from the sperm cell that fertilizes the haploid egg cell. The endosperms of monocots, including maize, rice, barley and wheat, provides the most important food sources for humans, feed, and industrial raw materials. Although the fate of the endosperm in most monocots differs from that in eudicots, the early developmental processes of endosperm, through the phases of syncytial growth and cellularization, are similar and relatively evolutionarily conserved. The syncytial phase, also called the free nuclear stage, is characterized by successive divisions of nuclei without cytokinesis. The endosperm then becomes cellularized through the formation of cell walls. After cellularization, endosperm in eudicots is gradually degraded through programmed cell death, and their component nutrients are transported into and accumulate in cotyledons, whereas the endosperm of monocot seeds proliferates and differentiates to form starchy endosperm. The period of endosperm cellularization is crucial owing to its close association with nuclear proliferation, which influences seed size and grain weight (Olsen 2004).

Plant hormones are essential signaling molecules that regulate various aspects of plant growth and development. Several recent studies suggested that phytohormones, particularly brassinosteroids (BRs) and auxin, are crucial for endosperm development, and many genes regulating grain size or/and endosperm development are correlated with levels and signaling of BRs and auxin (Tanabe et al. 2005; Morinaka et al. 2006; Schruff et al. 2006; Tong et al. 2012; Yin and Xue 2012; Ishimaru et al. 2013; Zuo and Li 2014; Li and Li 2015; Klosinska et al. 2016; Hu et al. 2018). In both *Arabidopsis* (*A. thaliana*) and rice (*Oryza sativa*), BR-insensitive or BR-deficient mutants produce small seeds, while plants with high BR levels due to overexpression of BR synthetic genes have large seeds (Zuo and Li 2014; Li and Li 2015).

The auxin biosynthesis genes *YUCCA10* (*YUC10*) and *TRYPTOPHAN AMINOTRANSFERASE RELATED 1* (*TAR1*) are targets of the FIS-PRC2 complex,

and both are paternally expressed post-fertilization to initiate endosperm development in *Arabidopsis* (Klosinska et al. 2016). In rice, *THOUSAND-GRAIN WEIGHT 6* (*TGW6*, which encodes an IAA-glucose hydrolase protein) is crucial in regulating grain weight. *TGW6* controls IAA supply, and ultimately limits the cell number in endosperm (Ishimaru et al. 2013). BIG GRAIN1 (BG1), a regulator of auxin response and transport, increases the size of rice grains (Liu et al. 2015). In addition, both BRs and auxin play important roles in early endosperm after fertilization (Figueiredo et al. 2015). A study of the maize (*Zea mays*) *invertase-deficient miniature1* (*mn1*) mutant indicates that the cytokinins (CKs) regulate cell number in seeds and influence seed size (Rijavec et al. 2009). Notably, abscisic acid (ABA) is critical for seed maturation and dormancy, yet it also is involved in regulating cellularization. In barley (*Hordeum vulgare*), decreased ABA content causes abnormal growth of endosperm during the cellularization and differentiation stages (Sreenivasulu et al. 2010). In *Arabidopsis*, endosperm cellularization is delayed in the ABA-deficient mutant *aba2-1* (Cheng et al. 2014).

Although studies reveal complex regulation and crucial roles of phytohormones in rice endosperm development, there remains a deficiency of systemic studies, and much remains unknown regarding the effects of hormones at early stages of endosperm development, and particularly the distinct roles of various hormones. Here, by systemically analyzing the expression patterns of genes related to hormone biosynthesis and signaling, and measuring hormone contents in seeds, we elucidated the dynamics of phytohormones in endosperm at different developmental stages. Furthermore, a transgenic approach in which we analyzed the grain size and quality of rice plants expressing the auxin, gibberellin (GA), BR and CK biosynthetic genes driven by an endosperm-specific promoter revealed diverse effects of phytohormones on grain size and quality, showing that phytohormones have distinct and crucial roles in endosperm/seed development. These results should provide helpful tools for improving the grain yields of crops.

RESULTS

Dynamic hormone biosynthesis and signaling during seed development

Using the transcriptome data of rice early endosperm, in particular the endosperm at 2 days after fertilization (DAF) (Xue et al. 2012; Xing et al. 2015), we characterized the differentially expressed genes (DEGs) involved in hormone biosynthesis and signaling (Figure S1) during endosperm development. Given that final levels of each hormone are determined by several key enzymes, we analyzed the rate-limiting enzymes involved in the biosynthesis of the various phytohormones.

The *YUCCA* (*YUC*) gene family, encoding the key enzyme in auxin synthesis, consists of 14 members, of which 4 (*OsYUC1*, *OsYUC9*, *OsYUC11* and *OsYUC12*) show significant differential expression and seemed likely to be involved in regulating endosperm development. We observed that the expression profiles of the *OsYUC* genes displayed one of two tendencies: a gradual increase over the course of endosperm development, or expression specifically in endosperm at 2 DAF. *OsYUC1*, *OsYUC9* and *OsYUC11* manifested the former pattern (gradually increasing in endosperm after 6 DAF), with their levels perhaps correlating with the accumulation of storage substances, while *OsYUC12* showed the latter pattern (Figure 1A).

Auxin is produced mainly via the indole-3-pyruvic acid pathway (Ljung 2013), which is catalyzed by YUCs as well as by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) and the related aminotransferases TAR1 and TAR2 (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011) (Figure S1A). Comparison of gene expression revealed differential auxin biosynthesis in endosperm and embryo. The step catalyzed by YUCs occurred in both embryo and endosperm, whereas that catalyzed by TAA1 occurred mainly in endosperm (Figure 1A). Notably, the expression of auxin-response genes, including *Aux/IAA*, *GH*3 and *SAUR* (Jain et al. 2006a, 2006b, 2006c), followed patterns similar to those of either *OsYUC12* or *OsYUC1* (Figure S2), indicating that auxin, too, utilizes two distinct synthesis and response modules functioning, respectively, in early central cell proliferation and in late accumulation of storage substances.

Isopentenyl transferase (IPT) family members are the rate-limiting enzymes of CK synthesis. Analysis of the expression patterns of the *OsIPT* genes suggest that CKs have more crucial roles in endosperm than in embryo, as *OsIPT4*, *OsIPT5*, and *OsIPT7* were specifically expressed in endosperm, especially before 9 DAF (Figure 1B). This also suggests that CKs are produced at the early stage of endosperm development to initiate and maintain cell division or syncytium formation. Genes involving in CK signaling showed similar patterns in embryo and endosperm (Figure S3A, D) and, notably, had maximal expression in endosperm at 2 DAF (Figure S3A).

Expression of BR-synthesis genes showed patterns similar to that of the *OsIPT* genes, i.e. maximal expression in ovary and endosperm at 2 or 3 DAF (and relatively low expression by 6 DAF, Figure 1C). Further analysis revealed that BR signaling genes also exhibited similar patterns (Figure S3B). Notably, all BR-synthesis and -signaling genes had higher expression levels in embryo than in endosperm (Figures 1C, S3B) and showed fairly uniform expression profiles.

Analysis of GA-synthesis genes showed that *GA20ox* and *GA3ox* genes were expressed uniquely in embryo (Figure 1D), which suggests that GA is synthesized in embryo and then transported into endosperm, a result that is in line with those of a previous study (Xue et al. 2012). Two GA-deactivating genes, *elongated uppermost internode* (*EUI*) and *EUI Like3* (*EUIL3*), were expressed from ovary to 9 DAF endosperm, implying that GA levels were low in early endosperm development and that *EUI* might be more crucial to GA levels than *GA2ox*, another GA-deactivating gene, in endosperm (Figure 1D).

Expression of ABA-signaling and -synthesis genes was not detected until 3 DAF (Figure S4A, C), which is consistent with previous studies showing that ABA promotes cellularization and that ABA-response-related genes are up-regulated during the cellularization of rice endosperm (Cheng et al. 2014; Xing et al. 2015). Expression of ethylene signaling genes gradually increased in the course of endosperm development (Figure S4B, D), but few ethylene-synthesis genes showed decreased expression in endosperm (Figure S4D). Finally, analysis of the expression

of genes involved in jasmonic acid (JA) biosynthesis indicated that few of them displayed noteworthy changes during endosperm development (Figure S4E).

Dynamic hormone contents in developing endosperm

To pinpoint the actual hormone contents *in vivo* and to assess whether these expression profiles can predict the hormones' dynamic variations, we measured some detectable hormones in rice endosperms, including indoleacetic acid (IAA), zeatin (Z), zeatin riboside (ZR), ABA and jasmonic acid (JA). The expression profiles of auxin-related genes suggested that auxin increases early in endosperm development, reaching a maximum at 6 DAF. Indeed, high-performance liquid chromatography (HPLC) measurement confirmed a rapid rise of IAA concentration in endosperm at 6 DAF (Figure 2A). Both Z and ZR are CK hormones, and measurement of Z and ZR showed that CKs were detected in endosperm only at 3 and 6 DAF, which is consistent with the expression patterns of the *OsIPT* genes (Figures 1B, 2B, 2C). ABA levels gradually increased, peaked at 6 DAF and then decreased thereafter (Figure 2D), again similar to the expression patterns of ABA-synthesis genes.

In contrast to the strong correlations between the actual levels of auxin, CKs, and ABA and the microarray data, the JA content showed little correlation. Although JA biosynthetic genes showed relatively little variation in expression over the course of endosperm development, the JA content rapidly declined at 6 DAF, implying that JA probably has no function in endosperm at later developmental stages (Figure 2E). Considering that JA is synthesized in ovary and pollen, and JA-related genes showed highest expression in ovary (Figure S4E) and in endosperm at 3 DAF, we suspect that JA may remain in the endosperm during the early stages and thus retain a relatively high concentration at 3 DAF.

Altered hormone levels in endosperm influence grain shape and quality

By analyzing the transcriptomic data of genes specifically expressed in the aleurone and starchy endosperm, we identified the gene *OsTFSE1*, encoding a transcription factor (TF) that is highly expressed in starchy endosperm. *OsTFSE1* was highly

expressed in caryopses from 4 to 21 DAF (C4 to C21), and abundant *OsTFSE1* expression was detected in starchy endosperm collected in caryopses at 11 DAF (but not in mixed aleurone-testa sample). No expression of *OsTFSE1* was detected in vegetative tissues (Figure 3A). Further promoter-reporter gene fusion studies performed by analyzing the GUS expression in different tissues confirmed that *OsTFSE1* was highly expressed in starchy endosperm at different stages, but not in embryo, leaf blade, leaf sheath, node, internode, floret, stamen or ovary (Figure S5), indicating that *OsTFSE1* expression is specific to starchy endosperm. We then used the promoter of *OsTFSE1* to drive genes catalyzing the rate-limiting step of various hormones biosynthetic pathways, including bacterial *IaaM* and rice *IPT3*, *Dwarf4* and *GA20ox*2 (all previously reported as being crucial for hormone biosynthesis; Comai and Kosuge 1982; Ashikari et al. 2002; Monna et al. 2002; Spielmeyer et al. 2002; Sakamoto et al. 2006a, 2006b), to promote hormone synthesis based on the dynamics of various hormones and to investigate the distinct functions of auxin, CKs, BR, and GA in endosperm. qRT-PCR analysis confirmed the increased transcription of these genes in seeds (Figure S6), and further HPLC analysis confirmed increases in auxin and cytokinin levels in endosperm upon expression of *IaaM* and *IPT3* driven by the *OsTFSE1* promoter (Figure 3B).

Phenotypic observations showed that, compared to those of ZH11, there was no difference in the height, tiller number, or heading day of the transgenic plants, which is consistent with endosperm-specific changes in phytohormones; however, the length of brown grains of all transgenic plants was increased, while the width was changed to a variable degree (Figure 3C). Higher auxin levels led to longer seeds due to the enhancement of seed length but not width, and higher CK levels increased the length and suppressed the width of seeds, resulting in slender grains (Figure 3D). Altered grain shape due to increased auxin and CK promotes higher grain weight. Like auxin, GA affected only the length of brown grains and promoted higher seed weight (Figure 2D). Among the four types of hormones, BR exerts the most profound effects, and thus both the length and width of seeds were significantly increased upon enhanced expression of *Dwarf4* (Figure 3D). Although BR- and CK-related genes show similar expression profiles, observations of grain shape indicated that they might have different functions in regulating grain shape.

Noticeably, increased GA at early stage due to *GA20ox*2 overexpression promoted grain length, which is similar to the effect of auxin. A possible explanation for this similarity is that auxin and GA might have analogous functions in endosperm at later developmental stages.

In addition to grain shape, hormone alterations also result in changes in grain quality. Measurement of standard rice grain quality indexes showed that increased auxin, CKs, and GA resulted in a greater degree of chalkiness, a commercially undesirable trait (Figure 4A), as evidenced by a higher percentage of grains with chalkiness (PGWC) and a higher degree of endosperm chalkiness (DEC) (Figure 4B). Notably, seeds with higher BR showed less chalkiness (Figure 4A), including lower PGWC and DEC (Figure 4B). In addition, the apparent amylose content (AAC) of seeds was reduced by increased amounts of any of the four hormones (Figure 4B).

DISCUSSION

Our expression profile analysis, measurement by liquid chromatography, and phenotypic observations revealed crucial and distinct roles of dynamic phytohormone levels, particularly those of auxin, CKs, BRs and GA, in rice endosperm in determining grain shape and quality. We conclude that auxin and GA promote grain length, while CKs lead to slender grains by enhancing the length but suppressing the width of grains. Among the hormones we examined, BRs had the most profound effects, boosting both grain length and width (Figure 5A). Notably, altered grain shape due to increased levels of auxin, CKs, and GA in endosperm also led to greater chalkiness, while BRs reduce chalkiness in addition to the increasing grain size. Consistent with this, a recent study in *Arabidopsis* showed that BR mediates plant reproductive development and, in particular, optimizes the seed yield by modulating BR signaling (Zu et al. 2019).

The differences in phytohormone levels and distinct patterns of phytohormone-related gene expression revealed complex and distinct roles of the various phytohormones during endosperm development (Figure 5B). At an early stage (2 DAF), auxin and CKs influence endosperm syncytium formation, during

which the fertilized central cell undergoes repeated mitoses without cell wall formation. In addition, *Arabidopsis YUC10*, the ortholog of *OsYUC12*, is paternally expressed in fertilized endosperm at the initiation stage and links central cell proliferation and auxin synthesis (Klosinska et al. 2016). Recent studies indicate that *OsYUC12* and *ZmYUC1* (the ortholog gene of *OsYUC12* in maize) are also imprinted in rice endosperm (Luo et al. 2011; Du et al. 2014) or maize (Waters et al. 2013; Xin et al. 2013), indicating that *YUC* members are commonly imprinted in the endosperm of monocots and dicots and suggesting a conserved role of auxin biosynthesis in early central cell proliferation.

Besides influencing seed development, ABA also begins to regulate cellularization at 3 DAF. BRs may regulate cell division at both the free nuclear and cellularization stages, and facilitate cell enlargement at 3 DAF and 6 DAF. Although a relatively higher JA level was detected at 3 DAF, expression profile analysis suggested that JA biosynthetic genes were not highly expressed, and thus, the high JA content was maintained from the ovary before fertilization and in mature pollens. In endosperm after 6 DAF, auxin levels rapidly increased, which is consistent with the start of nutrient accumulation at that stage. In conclusion, CKs and auxin play important roles at the free nuclear stage and ABA functions in cellularization. BRs are involved in both cell division and cell enlargement during early endosperm development.

Our investigations of the dynamics of hormones in developing seeds confirmed the distinct effects of hormones on grain yield and appearance quality traits. This information could provide novel tools to improve seed shape, yield or quality by magnifying hormone synthesis, particularly auxin, or GA synthesis, at distinct developmental stages. Chalkiness is correlated with grain shape (negative correlation in most cases); however, the alteration of final grain quality is mainly caused by the influence of glume development. By using the endosperm-specific promoter we developed, we excluded the effects of altered glume on chalkiness and thus uncovered the crucial role of endosperm development on this aspect of rice quality. Notably, BRs not only boost grain length and width but also improve grain appearance (i.e., reduce chalkiness), indicating that BRs may offset the divergence

between these two traits through other mechanisms. It is possible that the effects of auxin, CK, and GA on grain size exceed their effects on grain quality. In other words, the conversion and accumulation of nutrients might lag behind the enlargement of storage space, possibly leading to the loose arrangement of starch granules that gives grains a chalky quality. BRs promote the transport of nutrients such as sugar and are involved in source-sink translocation. BRs may thus improve seed size and grain quality by enhancing grain filling, suggesting that the ability to accurately regulate BR levels in specific regions or developmental stages could carry potential for agronomic improvement of crops.

Endosperm development is a complex process that includes various stages. Considering the diverse functions of phytohormones, modulating phytohormone levels through the use of an endosperm-specific promoter provides informative insights into the effects of phytohormones on endosperm. However, a single promoter is insufficient to allow detailed and exact elucidation of the physiological functions of these hormones. For example, in transgenic lines overexpressing *GA20ox2*, the low GA level in WT endosperm at 4 DAF is changed, which is diverted into cellularization and remained until seed maturation, while according to microarray analysis, the normal status of low GA level is maintained since the beginning to 9 DAF. The lack of the delicate promoters expressed at specific developmental stages during endosperm development, especially at early central cell proliferation stage, make it difficult to detect the exact physiological effects of GA on early seed development and to differentiate at which stage GA mainly functions in regulating the grain shape and quality. Finding unique promoters acting even at early stages of endosperm development, particularly stage-specific promoter, remains a challenge, which restricts the relevant studies. Transcriptomic studies and bioinformatics analysis may help to identify more appropriate promoters and facilitate further investigations.

The modulation of phytohormone levels in specific developmental stages or tissues has shown potential as a means of crop improvement (Zhang et al. 2011). Thus, our elucidation of the dynamic phytohormone levels and functions in rice

endosperm expands the knowledge of phytohormone functions in specific tissues and should aid future crop breeding.

MATERIALS AND METHODS

Plant materials and growth conditions

Japonica rice (*Oryza sativa* cv. Zhonghua11, ZH11) was used for all transformations. Transgenic rice plants were grown in a phytotron with a 12-h light (28^{o}C) / 12-h dark (22^{o}C) cycle. The plants used for measurement of the grain-related traits were grown in the field under natural conditions in either Shanghai or Lingshui (in Hainan province, with a different photoperiod from Shanghai). Homozygotes of transgenic plants were used for phenotypic observation and quality traits analysis. Plants from the different locations (Shanghai or Lingshui, at least two years' plantings) were analyzed.

Microarray data normalization

Rice gene expression data were collected from public databases, including GEO database accessions GSE11966 (data from seedling, leaf, root, embryo and endosperm at 6 DAF of ZH11), GSE27856 (data from ovary, embryo and endosperm of other developmental stages of ZH11) and GSE57615 (data from endosperm at 2 DAF), and analyzed using the R software package (http://www.rproject.org) and packages from Bioconductor (Gentleman et al. 2004; http://www.bioconductor.org). The raw data have been evaluated in previous study (Xue et al. 2012; Xing et al. 2015). The hybridization signals were normalized using GC-Robust Multichip Average (GC-RMA) from Bioconductor.

Data statistical analysis

Differentially expressed genes involved in hormones biosynthesis and signaling were identified. The ANOVA tool in TIGR MeV (version 4.0, http://www.tm4.org/mev.html) was used to identify genes differentially expressed during the course of endosperm development. The threshold was set at a *P* value <0.01. Significantly differentially expressed genes between endosperm and embryo

were identified by the linear statistical model in the Limma package (Diboun et al. 2006; Ritchie et al. 2015), which was set to fold change >2 and *P* value <0.01.

Quantification of various hormones

Ovary before fertilization, seed at 3 DAF and endosperm at 6, 9, and 16 DAF of ZH11 were collected for quantification assays of auxin (IAA), ABA, JA, and zeatin (cytokinin) contents by liquid chromatography-tandem mass spectrometry (Liu et al. 2010). Sample preparation and measurement of contents of various hormones were performed according to previous descriptions (Zhou et al. 2017).

Analysis of *TFSE1* promoter

Total RNAs were extracted with TRIzol reagent (Invitrogen, 15596-018), and mRNAs were isolated from at least 30 μg total RNA with a PolyATtract® mRNA isolation system (Promega, Z5310). The resultant mRNA was used to synthesize cDNA with a first-strand cDNA synthesis kit (Toyobo, FSK-100).

Real-time PCR (qRT-PCR) was performed using SYBR Green premix (Takara, DRR081) with the following program: 95^{o}C for 10 min, then 40 cycles total of 95^{o}C for 20 s, 56^{o}C for 20 s, and 72^{o}C for 20 s (to capture the fluorescence signal in this step). *OsActin1* was used as an internal standard to normalize the expression of the examined genes.

Materials for qRT-PCR analysis included young leaves and roots harvested from 28-day-old seedlings cultured in Hoagland nutrient solution, panicles with lengths of 18–21 cm before heading, caryopses collected at 4, 7, 11, 14, and 21 DAF, starchy endosperm collected from 11 DAF caryopsis, and aleurone and testa tissues peeled from 11 DAF caryopsis. Panicles and caryopses were collected from plants grown in the fields in Beijing.

For GUS activity assays, ZH11 were transformed with the construct *pTFSE1::GUS* (see below) and the transformants were grown in a phytotron and cultivated to produce T_1 transgenic lines. The leaf blade, leaf sheath, node, internode, floret, stamen, ovary and 4, 7, 11, 14, 21 and 28 DAF caryopses sectioned

longitudinally were collected from given T_1 transgenic lines grown in the fields in Beijing. The tissues were fixed in acetone for 1 h at -20^{o}C and washed once with GUS staining buffer. The reaction was initiated by addition of 1 mg/mL X-glucuronide sodium salt in GUS staining buffer at 37^{o}C and incubated overnight. The samples were observed and images were acquired using a Nikon anatomical lens (Nikon, SMZ800) with a digital camera (Nikon, DS-Ri1).

Constructs and rice transformation

To generate the pTFSE1::GUS construct, a 2,674-bp DNA fragment (from bp –28 to bp –2,701 upstream of the ATG of *OsTFSE1*) was amplified from ZH11 genomic DNA using KOD FX DNA polymerase (Toyobo, KFX-101) and inserted into the pPLV15 vector by a ligation-independent cloning method (De Rybel et al. 2011).

To generate constructs driven by the endosperm-specific promoter pTFSE1, the backbone vector pUN1301 was first digested with HindIII, blunted-ended with T4 DNA polymerase, and ligated with the *TFSE1* promoter DNA fragment. cDNAs encoding *IaaM*, *Dwarf4*, *IPT3* and *GA20ox* were then individually amplified and subcloned into the plasmid to generate *pTFSE1::IaaM*, *pTFSE1::Dwarf4*, *pTFSE1::IPT3* and *pTFSE1::GA20ox2* constructs. The resultant constructs were co-transformed into electrocompetent *Agrobacterium tumefaciens* EHA105, and positive clones were transformed into immature ZH11 embryos.

The transgenic status of plants was confirmed by quantitative RT-PCR (qRT-PCR) analysis of total RNAs extracted from the 7 DAF endosperm using TRIzol reagent (Life Technologies) and reverse transcribed into cDNA according to the manufacturer's instructions (Toyobo). qRT-PCR analysis was performed by using SYBR Green Realtime PCR Master Mix (Toyobo). The expression level of rice *UBQ5* was used as an internal control for normalization. Sequences of the primers used are listed in Table S1.

Measurement of grain quality

Analyses of grain weight and apparent amylose content (AAC) were performed as described previously (Fu and Xue 2010; Xu et al. 2016). At least 500 milled grains

were used for measurement of the percentage of grains with chalkiness (PGWC) and the degree of endosperm chalkiness (DEC).

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AUTHOR CONTRIBUTIONS

H.X. conceived the project and designed experiments; X.Z., J.T. and A.B. performed experiments; H.X., L.X. and C.L. supervised the project; X.Z. and H.X. wrote the paper with input from all authors. All authors read and approved the contents of this manuscript.

REFERENCES

Ashikari M, Sasaki A, Ueguchi-Tanaka M, Itoh H, Nishimura A, Datta S, Ishiyama K, Saito T, Kobayashi M, Khush GS, Kitano H, Matsuoka M (2002) Loss-of-function of a rice gibberellin biosynthetic gene, GA20 oxidase (GA20ox-2), led to the rice 'green revolution'. **Breed Sci** 52: 143-150

Cheng ZJ, Zhao XY, Shao XX, Wang F, Zhou C, Liu YG, Zhang Y, Zhang XS (2014) Abscisic acid regulates early seed development in *Arabidopsis* by ABI5-mediated transcription of SHORT HYPOCOTYL UNDER BLUE1. **Plant Cell** 26: 1053-1068

Comai L, Kosuge T (1982) Cloning and characterization of IaaM, a virulence determinant of *Pseudomonas savastanoi*. **J Bacteriol** 149: 40-46

De Rybel B, van den Berg W, Lokerse A, Liao CY, van Mourik H, Möller B, Peris CL, Weijers D (2011) A versatile set of ligation-independent cloning vectors for functional studies in plants. **Plant Physiol** 156: 1292-1299

Diboun I, Wernisch L, Orengo CA, Koltzenburg M (2006) Microarray analysis after RNA amplification can detect pronounced differences in gene expression using limma. **BMC Genomics** 7: 252

Du MR, Luo M, Zhang RF, Finnegan EJ, Koltunow AMG (2014) Imprinting in rice: The role of DNA and histone methylation in modulating parent-of-origin specific expression and determining transcript start sites. **Plant J** 79: 232-242

Figueiredo DD, Batista RA, Roszak PJ, Kohler C (2015) Auxin production couples endosperm development to fertilization. **Nat Plants** 1: 15184

Fu FF, Xue HW (2010) Coexpression analysis identifies rice starch regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. **Plant Physiol** 154: 927-938

Hu Z, Lu SJ, Wang MJ, He H, Sun L, Wang H, Liu XH, Jiang L, Sun JL, Xin X, Kong W, Chu C, Xue HW, Yang J, Luo X, Liu JX (2018) A novel QTL qTGW3 encodes the GSK3/SHAGGY-like kinase OsGSK5/OsSK41 that interacts with OsARF4 to negatively regulate grain size and weight in rice. **Mol Plant** 11: 736-749

Jain M, Kaur N, Garg R, Thakur JK, Tyagi AK, Khurana JP (2006a) Structure and expression analysis of early auxin-responsive Aux/IAA gene family in rice (*Oryza sativa*). **Funct Integr Genomics** 6: 47-59

Jain M, Kaur N, Tyagi AK, Khurana JP (2006b) The auxin-responsive GH3 gene family in rice (*Oryza sativa*). **Funct Integr Genomics** 6: 36-46

Jain M, Tyagi AK, Khurana JP (2006c) Genome-wide analysis, evolutionary expansion, and expression of early auxin-responsive SAUR gene family in rice (*Oryza sativa*). **Genomics** 88: 360-371

Ishimaru K, Hirotsu N, Madoka Y, Murakami N, Hara N, Onodera H, Kashiwagi T, Ujiie K, Shimizu B, Onishi A, Miyagawa H, Katoh E (2013) Loss of function of the IAA-glucose hydrolase gene *TGW6* enhances rice grain weight and increases yield. **Nat Genet** 45: 707-711.

Klosinska M, Picard CL, Gehring M (2016) Conserved imprinting associated with unique epigenetic signatures in the *Arabidopsis* genus. **Nat Plants** 2: 16145

Li N, Li YH (2015) Maternal control of seed size in plants. **J Exp Bot** 66: 1087-1097

Liu L, Tong H, Xiao Y, Che R, Xu F, Hu B, Liang C, Chu J, Li J, Chu C (2015) Activation of Big Grain1 significantly improves grain size by regulating auxin transport in rice. **Proc Natl Acad Sci USA** 112: 11102-11107

Liu X, Yang YL, Lin WH, Tong JH, Huang ZG, Xiao LT (2010) Determination of both jasmonic acid and methyl jasmonate in plant samples by liquid chromatography tandem mass spectrometry. **Chinese Sci Bull** 55: 2231-2235

Ljung K (2013) Auxin metabolism and homeostasis during plant development. **Development** 140: 943-950

Luo M, Taylor JM, Spriggs A, Zhang HY, Wu XJ, Russell S, Singh M, Koltunow A (2011) A genome-wide survey of imprinted genes in rice seeds reveals imprinting primarily cccurs in the endosperm. **PLoS Genet** 7: e1002125

Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H, McSteen P, Zhao YD, Hayashi K, Kamiya Y, Kasahara H (2011) The main auxin biosynthesis pathway in *Arabidopsis*. **Proc Natl Acad Sci USA** 108: 18512-18517

Monna L, Kitazawa N, Yoshino R, Suzuki J, Masuda H, Maehara Y, Tanji M, Sato M, Nasu S, Minobe Y (2002) Positional cloning of rice semidwarfing gene, *sd-1*: Rice "Green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. **DNA Res** 9: 11-17

Morinaka Y, Sakamoto T, Inukai Y, Agetsuma M, Kitano H, Ashikari M, Matsuoka M (2006) Morphological alteration caused by brassinosteroid insensitivity increases the biomass and grain production of rice. **Plant Physiol** 141 :924-931.

Olsen OA (2004) Nuclear endosperm development in cereals and *Arabidopsis thaliana*. **Plant Cell** 16: S214-S227

Rijavec T, Kovac M, Kladnik A, Chourey PS, Dermastia M (2009) A comparative study on the role of cytokinins in caryopsis development in the maize miniature1 deed mutant and its wild type. **J Integr Plant Biol** 51: 840-849

Ritchie ME, Phipson B, Wu D, Hu YF, Law CW, Shi W, Smyth GK (2015) *limma* powers differential expression analyses for RNA-sequencing and microarray studies. **Nucleic Acids Res** 43: e47

Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S, Tanaka H, Kitano H, Matsuoka M (2006a) Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. **Nat Biotechnol** 24: 105-109

Sakamoto T, Sakakibara H, Kojima M, Yamamoto Y, Nagasaki H, Inukai Y, Sato Y, Matsuoka M (2006b) Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. **Plant Physiol** 142: 54-62

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. **Development** 133: 251-261

Spielmeyer W, Ellis MH, Chandler PM (2002) Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. **Proc Natl Acad Sci USA** 99: 9043-9048

Sreenivasulu N, Borisjuk L, Junker BH, Mock HP, Rolletschek H, Seiffert U, Weschke W, Wobus U (2010) Barley grain development: Toward an integrative view. **Int Rev Cel Mol Bio** 281: 49-89

Stepanova AN, Yun J, Robles LM, Novak O, He WR, Guo HW, Ljung K, Alonso JM (2011) The *Arabidopsis* YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. **Plant Cell** 23: 3961-3973

Tanabe S, Ashikari M, Fujioka S, Takatsuto S, Yoshida S, Yano M, Yoshimura A, Kitano H, Matsuoka M, Fujisawa Y, Kato H, Iwasaki Y (2005) A novel cytochrome P450 is implicated in brassinosteroid biosynthesis via the characterization of a rice dwarf mutant, dwarf11, with reduced seed length. **Plant Cell** 17: 776-790

Tong H, Liu L, Jin Y, Du L, Yin Y, Qian Q, Zhu L, Chu C (2012) DWARF AND LOW-TILLERING acts as a direct downstream target of a GSK3/SHAGGY-like kinase to mediate brassinosteroid responses in rice. **Plant Cell** 24: 2562-2577

Waters AJ, Bilinski P, Eichten SR, Vaughn MW, Ross-Ibarra J, Gehring M, Springer NM (2013) Comprehensive analysis of imprinted genes in maize reveals allelic variation for imprinting and limited conservation with other species. **Proc Natl Acad Sci USA** 110: 19639-19644

Won C, Shen XL, Mashiguchi K, Zheng ZY, Dai XH, Cheng YF, Kasahara H, Kamiya Y, Chory J, Zhao YD (2011) Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in *Arabidopsis*. **Proc Natl Acad Sci USA** 108: 18518-18523

Xin MM, Yang RL, Li GS, Chen H, Laurie J, Ma C, Wang DF, Yao YY, Larkins BA, Sun QX, Yadegari R, Wang XF, Ni ZF (2013) Dynamic expression of imprinted genes associates with maternally controlled nutrienta allocation during maize endosperm development. **Plant Cell** 25: 3212-3227

Xing MQ, Zhang YJ, Zhou SR, Hu WY, Wu XT, Ye YJ, Wu XX, Xiao YP, Li X, Xue HW (2015) Global analysis reveals the crucial roles of DNA methylation during rice seed development. **Plant Physiol** 168: 1417-U1554

Xu JJ, Zhang XF, Xue HW (2016) Rice aleurone layer specific OsNF-YB1 regulates grain filling and endosperm development by interacting with an ERF transcription factor. **J Exp Bot** 67: 6399-6411

Xue LJ, Zhang JJ, Xue HW (2012) Genome-wide analysis of the complex transcriptional networks of rice developing seeds. **PLoS ONE** 7: e31081

Yin LL, Xue HW (2012) The MADS29 transcription factor regulates the degradation of the nucellus and the nucellar projection during rice seed development. **Plant Cell** 24: 1049-1065

Zhang M, Zheng XL, Song SQ, Zeng QW, Hou L, Li DM, Zhao J, Wei Y, Li XB, Luo M, Xiao YH, Luo XY, Zhang JF, Xiang CB, Pei Y (2011) Spatiotemporal manipulation of auxin biosynthesis in cotton ovule epidermal cells enhances fiber yield and quality. **Nat Biotechnol** 29: 453-458

Zhou LJ, Xiao LT, Xue HW (2017) Dynamic cytology and transcriptional regulation of rice lamina joint development. **Plant Physiol** 174: 1728-1746

Zu SH, Jiang YT, Hu LQ, Zhang YJ, Chang JH, Xue HW, Lin WH (2019) Effective modulating brassinosteroids signal to study their specific regulation of reproductive development and enhance yield. **Front Plant Sci** 10: 980

Zuo JR, Li JY (2014) Molecular genetic dissection of quantitative trait loci regulating rice grain size. **Annu Rev Genet** 48: 99-118

Figures

Figure 1. Differential expression profiles of phytohormone biosynthesis genes during rice seed development

Heat maps of the expression of hormone biosynthesis genes during rice seed development, including genes related to the synthesis of auxin **(A)**, cytokinins **(B),** brassinosteroids **(C)** and gibberellin **(D)**. Genes showing dynamic changes (ANOVA, $P < 0.01$) during endosperm development are illustrated in the upper panel, and genes show higher expression in endosperm than in embryo or vice versa (two-sided Student's *t*-test, $P < 0.01$) in the bottom panel. Gene expression was detected in ovary and embryo at 3, 6, 9 and 12 days after fertilization (DAF) and in endosperm at 2, 3, 6, 9, and 16 DAF.

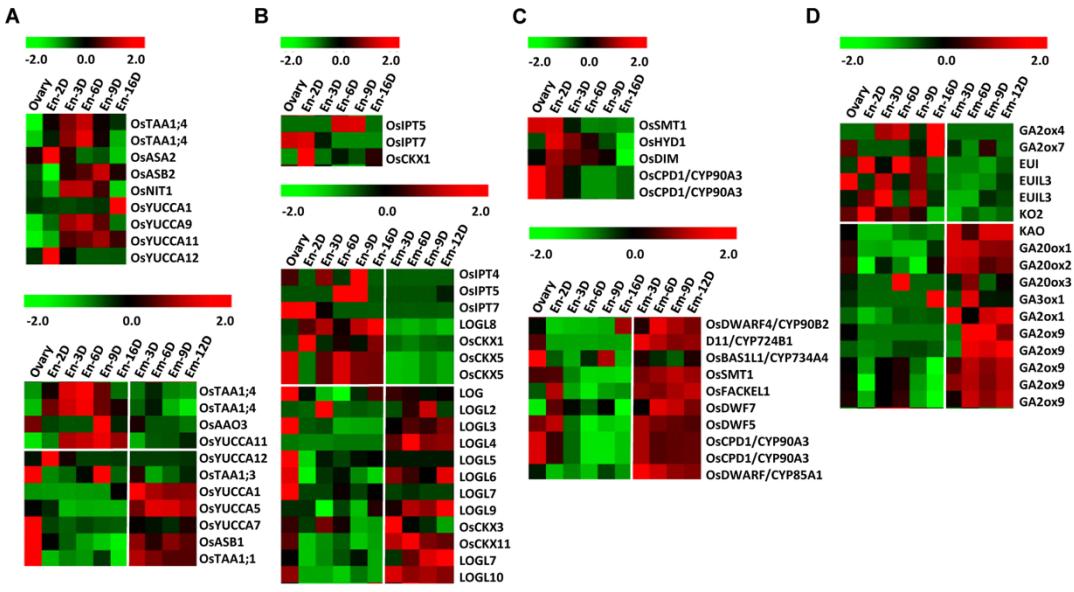

Figure 2. Differential levels of phytohormones during rice seed development

(A–E) Levels of indoleacetic acid (IAA, **A**), zeatin (ZT, **B**), zeatin riboside (ZR, **C**), abscisic acid (ABA, **D**) and jasmonic acid (JA, **E**) in ovary, caryopses at 3 DAF and endosperms at 6, 9, and 16 DAF.

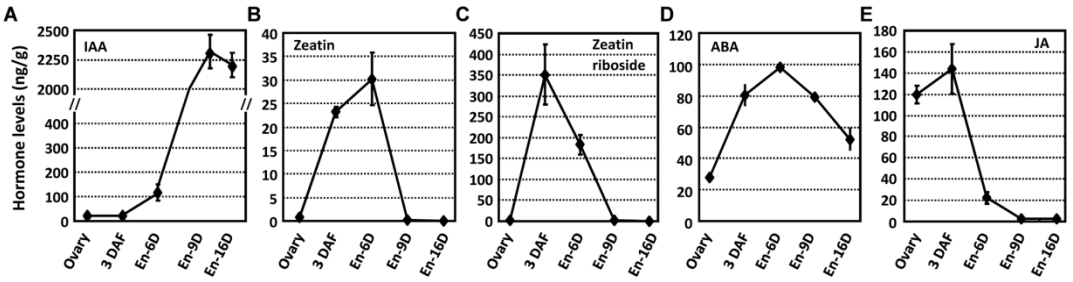

Figure 3. Increased specific hormone in endosperm results in altered grain size and yield

(A) Quantitative RT-PCR (qPCR) analysis showing that the *OsTFSE1* promoter is specifically expressed in developing endosperms. Vegetative tissues of leaf (L) and root (R) of 28-day-old seedlings, panicle (P, 18–21 cm in length), caryopses at 4, 7, 11, 14 and 21 days after pollination (DAF) and starchy endosperm (SE) and mixed aleurone-testa (A+T) at 11 DAF were collected for analysis. The expression levels of *OsTFSE1* were normalized to those of *OsActin1*. The experiments were repeated three times, and data are shown as means ± *SD*. **(B)** Expression of bacterial *IaaM* and rice *ITP3* driven by *OsTFSE1* promoter results in increased amounts of auxin (left) and cytokinin (right) in rice endosperms. Endosperms at 8 DAF were collected to measures the hormone levels. Data were statistically analyzed by one-sided Student's *t*-test and are presented as means ± *SD* ($n = 3$, **, $P < 0.01$). **(C)** Altered brown grains of transgenic plants expressing bacterial *IaaM* or rice *IPT3*, *Dwarf4, or GA20ox*. Bars = 1 cm. **(D)** Measurement and calculation of brown grain length, width, and 100-grain weight of transgenic plants expressing *IaaM*, *IPT3*, *Dwarf4, or GA20ox*. Experiments were repeated three times. Data were statistically analyzed by two-sided Student's *t*-test and are presented as means ± *SD* for grain length and width ($n > 100$, *, $P < 0.05$; **, $P < 0.01$) or as means ± *SE* for 100-grain weight ($n = 10$, **$P < 0.01$).

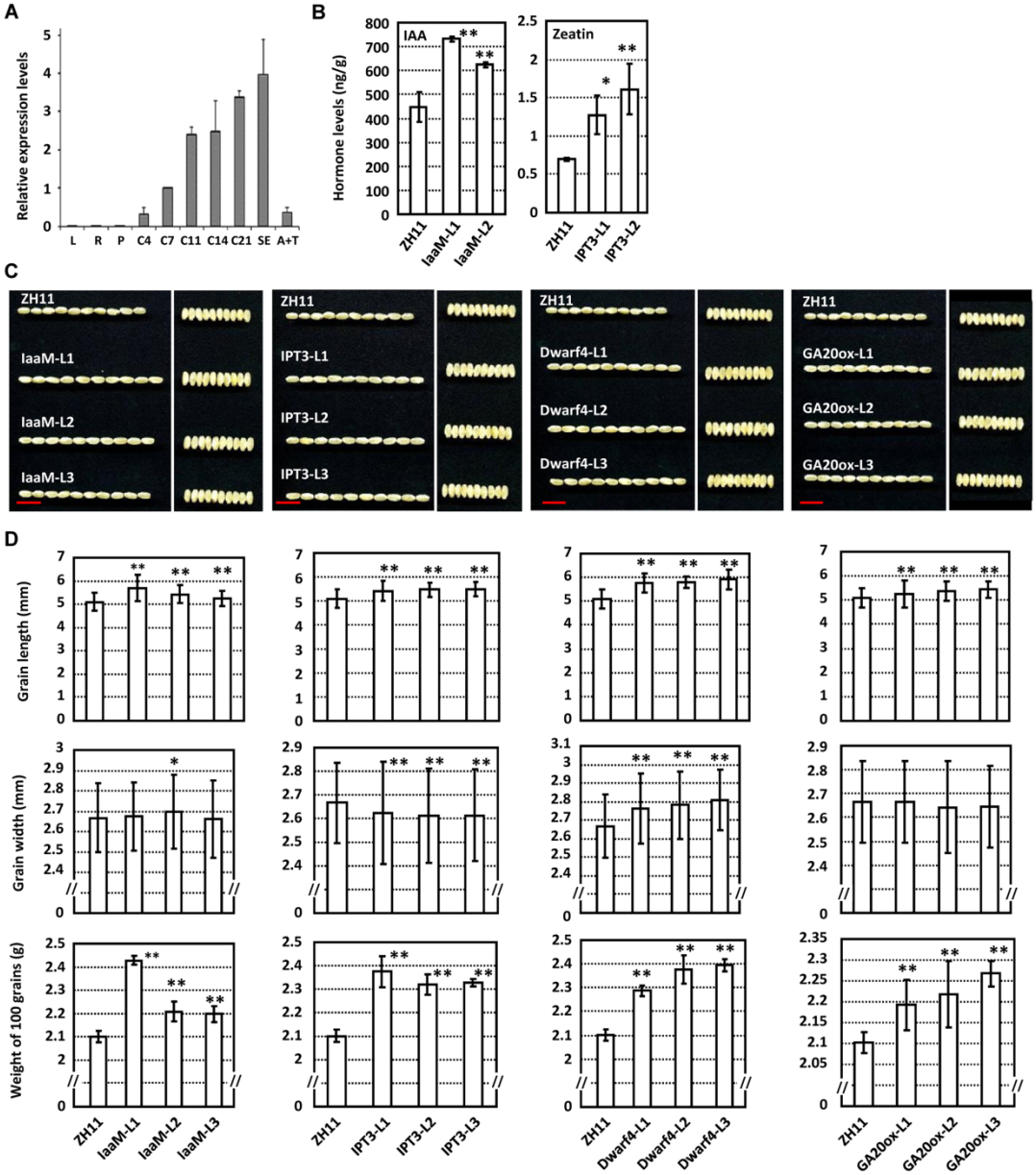

Figure 4. **Altered hormone levels in endosperm result in changes in grains quality**

(A) Phenotypic observations showing the increased or reduced grain chalkiness of transgenic plants expressing bacterial *IaaM* or rice *IPT3*, *Dwarf4* or *GA20ox*. Bar = 1 cm. **(B)** Analysis of the percentage of grains with chalkiness (PGWC), degree of endosperm chalkiness (DEC) and apparent amylose content (AAC) of transgenic plants expressing *IaaM*, *IPT3*, *Dwarf4*, *or GA20ox*. Experiments were repeated three times, and data are shown as means ± *SD* (*n* = 3). Statistical analysis by two-sided Student's *t*-test reveals significant differences (**P* < 0.05, ***P* < 0.01).

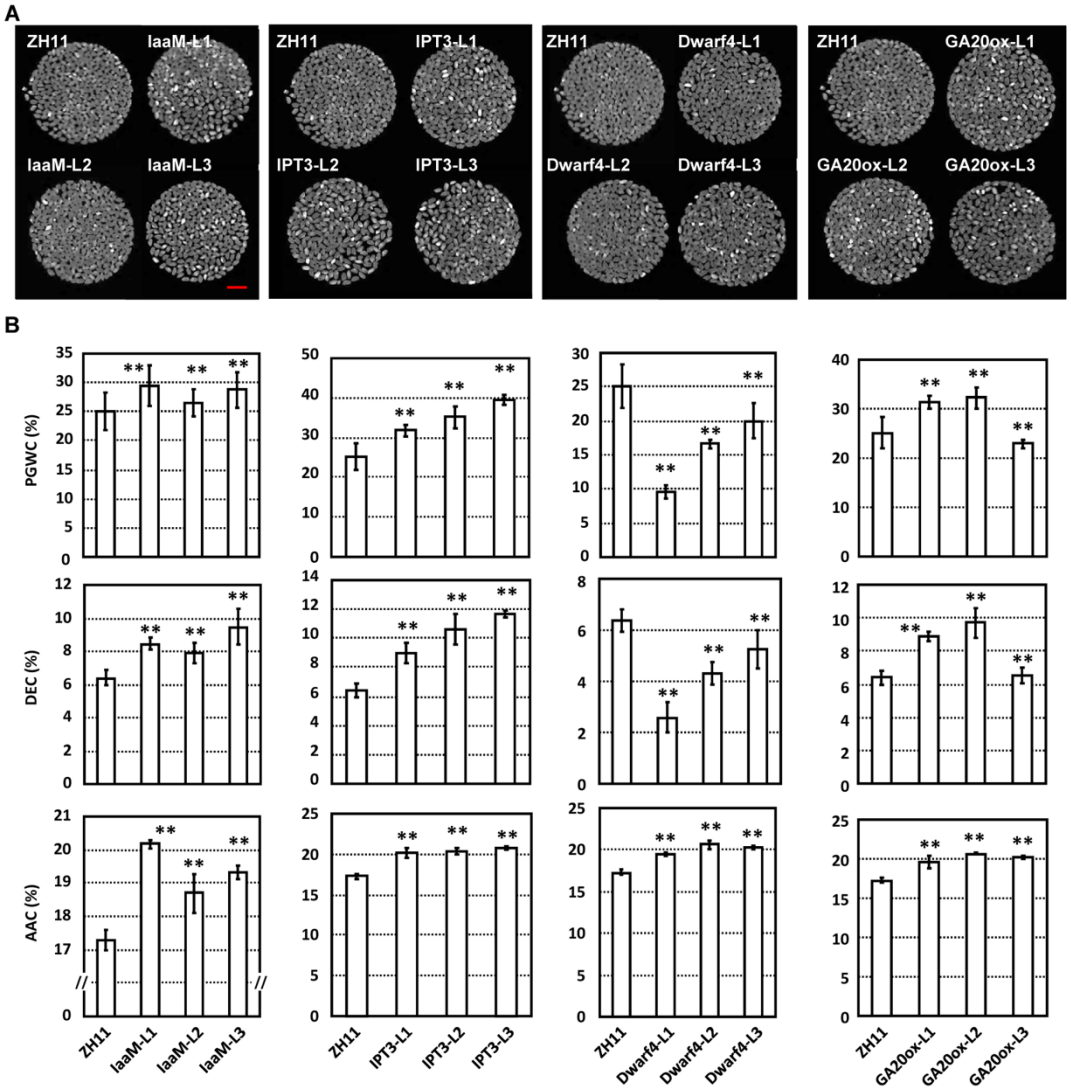

Figure 5. A hypothetical model showing the effects of dynamic hormones on rice endosperm development

(A) Plant hormones have diverse effects on endosperm development and hence brown grain size. CK may be specifically synthesized in endosperm, while GA is synthesized mainly in embryo. CK and BRs show opposite effects on grain width determination, and CK, BRs, auxin, and GA all promote grain length. **(B)** Dynamics and correlation of plant hormones with developmental/biological processes in developing endosperms. Solid lines, supported by measurements and microarray data; dotted line, supported by microarray data only.