Transcriptome and Metabolome Analyses Reveal the Pathway and Metabolites of Grain Quality Under *Phytochrome B* in Rice (*Oryza sativa* L.)

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**Abstract**

**Background:** Grain size and chalkiness is a critical agronomic trait affecting rice yield and quality. The application of transcriptomics to rice has widened the understanding of complex molecular responsive mechanisms, differential gene expression, and regulatory pathways under varying conditions. Similarly, metabolomics has also contributed drastically for rice trait improvements. As master regulators of plant growth and development, phy influence seed germination, vegetative growth, photoperiodic flowering, shade avoidance responses. *OsPHYB* can regulate a variety of plant growth and development processes, but little is known about the roles of rice gene *OsPHYB* in modulating grain development.

**Results:** In this study, rice *phytochrome B* (*OsPHYB*) was edited using CRISPR/Cas9 technology. We found that *OsPHYB* knockout increased rice grain size and chalkiness, and increased the contents of amylose, free fatty acids and soluble sugar, while the gel consistency and contents of proteins were reduced in mutant grains. Furthermore, *OsPHYB* is involved in the regulation of grain size and chalk formation by controlling cell division and complex starch grain morphology. Transcriptomic analysis revealed that loss of *OsPHYB* function affects multiple metabolic pathways, especially enhancement of glycolysis, fatty acid, oxidative phosphorylation, and antioxidant pathways, as well as differential expression of starch and phytohormone pathways. An analysis of grain metabolites showed an increase in the free fatty acids and lysophosphatidylcholine, whereas the amounts of sugars, alcohols, amino acids and derivatives, organic acids, phenolic acids, alkaloids, nucleotides and derivatives, and flavonoids decreased, which were significantly associated with grain size and chalk formation.

**Conclusions:** Our study reveals that, *OsPHYB* plays an important regulatory role in the growth and development of rice grains, especially grain size and chalkiness. Furthermore, *OsPHYB* regulates grain size and chalkiness formation by affecting gene metabolism interaction network. Thus, this study not only revealed that *OsPHYB* plays a vital role in regulating grain size and chalkiness of rice but reveal new functions and highlighted the importance and value of *OsPHYB* in rice grain development and provide a new strategy for yield and quality improvement in rice breeding.

**Keywords:** *OsPHYB*, Chalkiness, Grain size, Transcriptome, Metabolome, Correlation analysis

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**Background**

Rice (*Oryza sativa* L.) is consumed by more than half the world’s population, and the improvement in varieties and cultivation techniques has significantly improved
the yield and quality of rice (Tang et al. 2019). Moreover, rice is an important source of protein, energy, fibers, minerals, and bioactive compounds in many countries around the world (Ferreira et al. 2021). As living standards improve, consumer expectations of rice quality also increase. Grain chalkiness is a critical trait that determines the quality of rice (Hoshikawa 1989). Grain chalkiness in rice refers to the opaque part of endosperm, and its formation is controlled by multiple factors, including starch synthesis, the structure and arrangement of starch granules, phytohormones, and various external stresses during grain filling (Nevame et al. 2018; Yang et al. 2022). Mutations have been introduced to many of the genes involved in starch synthesis and carbon metabolism, such as glycolysis. Such genes include SSI, Wx, SSIIa|FLO5, BEIIb, OsAPL2, OsPK3 and OsPPDKB that result in a chalky phenotype (Hu et al. 2020; Xie et al. 2021; Zhang et al. 2022). Moreover, other factors related to the development of amyloplasts, such as FLO2, FLO7, SSG4 and ISA1, also play important roles in the formation of chalkiness (Matsushima et al. 2014; Xie et al. 2021). Protein and lipid metabolism also affect the formation of chalkiness (Lin et al. 2010; Wang et al. 2015a; Wu et al. 2019; Yu et al. 2021a).

In addition, multiple complex regulatory pathways are involved in grain size and the formation of chalkiness. Higher levels of auxin, cytokinins (CKs) and gibberellins (GAs) could result in more chalkiness, while brassinosteroids (BRs) could reduce chalkiness (Zhang et al. 2020). Changes in the contents of abscisic acid (ABA) and ABA/GA had significant effects on grain filling and chalkiness (Xiao et al. 2017). The formation of grain chalkiness can also be influenced by various external stresses during the grain-filling stage. Recent studies showed that reactive oxygen species (ROS) may play a critical role in the regulation of rice endosperm chalkiness (Lin et al. 2022; Liu et al. 2010; Xu et al. 2010). Grain size is directly related to changes in the composition, growth, and accumulation of the endosperm because it affects yield and quality, which are usually negatively correlated with each other (Wang et al. 2012). Genes such as GIF1, qGW8, GL7, OsMADS1, can regulate the quality of rice while regulating grain size (Liu et al. 2018; Wang et al. 2008, 2012, 2015b). Although some progress had been made in the research on chalk formation, particularly grain size, the research on rice grain size and chalk formation still merits further study, particularly the role of phytochromes.

The phytochrome family primarily senses red and far-red light to regulate a range of developmental processes throughout the life cycle of plants. The phytochrome gene family in rice is composed of three members that include PHYA, PHYB and PHYC (Sun et al. 2017). Current studies have shown that rice PHYB can adjust the de-yellowing, leaf angle, flowering stage, hypocotyl elongation, shade avoidance, and fertility of seedlings by receiving red light (Osugi et al. 2011; Takano et al. 2009, 2005). In addition, PHYA has unique and overlapping roles with PHYB in the regulation of photosynthesis in rice. The light-regulated overexpression of an Arabidopsis PHYA gene in rice has been reported to alter plant architecture and increase grain yield (Garg et al. 2006). By regulating the photosynthetic process, the phytochrome system also influences the source-sink relationship, grain quality, and yield. However, the regulation of PHYB on rice grain characters and quality has not been reported.

In this study, rice phytochrome B (OsPHYB) was edited using CRISPR/Cas9 technology. After screening and identification, homozygous OsPHYB loss-of-function mutants were obtained, and their grain shape and quality traits were determined to study the effect of OsPHYB on grain traits, and transcriptomic and metabolomic analyses were utilized to investigate the molecular mechanism underlying the variation in traits.

**Results**

**OsPHYB Mutation Increased the Grain Size and Formation of Chalkiness in Rice**

To investigate the effects of OsPHYB on grain size, yield, and quality of rice, we used CRISPR/cas9 to edit the first exon of OsPHYB to obtain OsPHYB mutants (Figs. 1A and Additional file 1: S1A). Three independent lines of homozygous knockout plants (T3 generation), osphyb-2 (single base “T” insertion), osphyb-4 (double base “GA” deletion) and osphyb-9 (single base “A” deletion) were adopted in the study (Fig. 1B). All three types of base mutations resulted in the termination of translation of their amino acids after the putative PAM domain, resulting in the loss of function of OsPHYB (Additional file 1: Fig. S1B, C).

Two-year field data analysis showed that the deletion of OsPHYB significantly improved the grain size and 1,000-grain weight of rice, particularly the grain length and grain width. Compared with NIP, the average grain length, grain width, 1,000-grain weight and 1,000-brown rice weight increased by 8.54%, 3.47%, 9.74% and 6.43%, respectively (Fig. 1C–I). In addition, the OsPHYB mutants also increased the grain chalkiness of transgenic plants, with an average increase of 305.65% and 371.61% in chalky grain rate and chalkiness, respectively, compared with those of NIP (Fig. 1D, J–K). The head rice rate of osphyb was reduced by an average of 15.24% compared with that of NIP (Fig. 1L). In addition, compared with NIP, the osphyb mutant also significantly decreased plant height, tiller number, seed setting rate, panicle length, number of primary
branches and secondary branches (Additional file 1: Fig. S2A–F, H–K). It was also found that the flowering phase of the osphyb mutant was about 3 weeks earlier relative to NIP (Additional file 1: Fig. S2A, G). The contents of nutrients in the NIP and OsPHYB deletion mutant rice were also determined. The knockout of OsPHYB significantly increased the contents of amylose, free fatty acids (FFA), and soluble sugar \( (P<0.01) \), whereas the content of protein and gel consistency significantly decreased \( (P<0.01) \). The contents of amylopectin and soluble starch increased slightly, but the results were not significant (Table 1).

OsPHYB Regulates the Number of Grain Cells and Complex Starch Granule Morphology in Rice

To elucidate how OsPHYB regulates grain length, we investigated cell size and cell number in the outer epidermis of spikelets using SEM (Fig. 2). It was observed that in the osphyb mutant, more cells were observed along the longitudinal axis of the grain lemma, a 28.76% increase relative to the number of NIP cells, while the
We mainly interpret carbon metabolism-related pathways that primarily included glycolysis, the tricarboxylic acid (TCA) cycle, amino acid metabolism and starch synthesis along with fatty acid synthesis, oxidative phosphorylation, antioxidant pathways and cyclins, plant hormone signal and transduction, which involved in regulating rice grain quality and size mediated by OsPHYB (Fig. 3). The expression of transcriptome genes was further confirmed by qRT-PCR. The samples we used for qRT-PCR were the same transgenic lines as the transcriptome, which were rice grain (12 days after flowering) samples of the same period in the second year. qRT-PCR quantitative validation results are shown in Additional file 1: Fig. S5.

**OsPHYB Mutation Results in Differential Expression of Multiple Carbon Metabolism Pathway Genes**

In this study, the pathway involved in glycolysis was found to be activated by up-regulating the levels of expression of genes encoding the enzymes involved in glycolysis, including hexokinase, phosphoglucomutase, phosphofructokinase, aldolase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, enolase, and pyruvate kinase, and decreased the levels of expression of gluconeogenesis rate-limiting enzymes, such as phosphoenolpyruvate carboxykinase, and one pyruvate phosphate dikinase gene (PPDK) in osphyb compared with those in NIP (Fig. 3 and Additional file 2: Table S2). The TCA cycle is the final metabolic pathway of the three major nutrients (carbohydrates, lipids, and amino acids) and also the hub of the connections between carbohydrate, lipid, and amino acid metabolism. The succinyl-CoA ligase (CoAoSCSb) and two malate dehydrogenases (Os05g0574400, FLO16) in the TCA cycle were up-regulated, whereas succinyl dehydrogenase (OsSDH2-2) was down-regulated (Fig. 3 and Additional file 2: Table S2). And, compared with NIP, the OsPHYB mutants exhibited changes in the expression of 33 starch metabolic genes. For example, genes involved in the metabolism of sucrose, cellulose, and hemicellulose were significantly up-regulated, whereas the genes for sucrose (SUS4) and starch (OsSSIIIa/Flo5) synthesis were significantly down-regulated. In addition, FLO2, FLO7 and FLO11 were also significantly reduced in osphyb (Fig. 3).

### Table 1 Analysis of seed quality traits parameters of osphyb and NIP

| Lines   | Amylose contents (mg/g) | Amylopectin contents (mg/g) | Starch contents (mg/g) | Soluble sugar contents (mg/g) | Protein contents (mg/g) | Free fatty acid contents (mg/g) | Gel consistency (mm) |
|---------|-------------------------|----------------------------|------------------------|-------------------------------|------------------------|-------------------------------|-----------------------|
| NIP     | 16.13 ± 0.28            | 256.32 ± 35.09             | 469.63 ± 22.86         | 5.92 ± 0.04                   | 302.39 ± 2.49          | 10.00 ± 0.46**                | 86.25 ± 0.72          |
| osphyb-2| 21.39 ± 0.09**          | 260.44 ± 31.64             | 511.29 ± 33.04         | 6.87 ± 0.02**                 | 204.75 ± 9.66**        | 13.20 ± 0.46**                | 76.81 ± 0.99**        |
| osphyb-4| 20.03 ± 0.72**          | 271.15 ± 17.18             | 524.54 ± 26.17         | 6.44 ± 0.10**                 | 266.54 ± 5.22**        | 13.33 ± 0.48**                | 71.54 ± 0.95**        |
| osphyb-9| 21.26 ± 0.40**          | 270.88 ± 27.48             | 499.49 ± 10.10         | 6.22 ± 0.03**                 | 207.30 ± 3.66**        | 12.80 ± 0.23**                | 69.17 ± 1.38**        |

*p<0.05, **p<0.01

size of the cells did not change significantly (Fig. 2A–E). Grains produced from the osphyb transgenic plants had a highly chalky appearance compared with those from NIP (Fig. 1D and 2F). Notably, a cross-sectional analysis showed that the belly region of osphyb mutant grain appeared floury-white, while the inner endosperm was translucent as in the NIP endosperm (Fig. 2F). Moreover, the abdominal endosperm cells of osphyb mutants were filled with loosely arranged complex starch granules with larger spaces, which were distinct from the densely arranged endosperm cells of NIP and exhibited large spherical shapes (Fig. 2G). Endosperm cells on the other side of osphyb were consistent with irregular polyhedral starch granules compared with those of NIP (Fig. 2I). At the excessive position of the farinaceous to hyaline regions, a very small number of small, rounded starch complex granules were observed in the osphyb (Fig. 2H).

**Overview of Transcriptional Profiles of All Rice Samples**

Grain size and quality are complex quantitative traits that are regulated by multiple genes. To study the gene regulatory network of OsPHYB on grain size and quality in more detail, a transcriptomic analysis of NIP and osphyb mutants was performed. A fragments per kilobase of transcript per million mapped fragments (FPKM) box plot, FPKM density distribution, biological repeats (Pearson’s correlation coefficient), and differentially expressed gene (DEG) different heat map results are shown in Additional file 1: Fig. S3. The volcano plot and differential heatmap of the DEGs showed that 51.86% of the 2137 differential genes were up-regulated, whereas 48.14% were down-regulated (Additional file 1: Fig. S3, D, E). The carbohydrate transport and metabolism, fatty acid synthesis and metabolism, oxidative phosphorylation, multiple amino acid synthesis, cell cycle, ribosome, RNA polymerase, protein translation and modification, and various organic acid metabolic pathways, among others, were annotated or enriched between osphyb and NIP material based on KOG annotation and KEGG pathway enrichment (Additional file 1: Fig. S4). We mainly interpret carbon metabolism-related pathways that primarily included glycolysis, the tricarboxylic acid (TCA) cycle, amino acid metabolism and starch synthesis along with fatty acid synthesis, oxidative phosphorylation, antioxidant pathways and cyclins, plant hormone signal and transduction, which involved in regulating rice grain quality and size mediated by OsPHYB (Fig. 3).

NIP 16.13 ± 0.28
osphyb-2 21.39 ± 0.09**
osphyb-4 20.03 ± 0.72**
osphyb-9 21.26 ± 0.40**

NIP 256.32 ± 35.09
osphyb-2 260.44 ± 31.64
osphyb-4 271.15 ± 17.18
osphyb-9 270.88 ± 27.48

NIP 469.63 ± 22.86
osphyb-2 511.29 ± 33.04
osphyb-4 524.54 ± 26.17
osphyb-9 499.49 ± 10.10

NIP 5.92 ± 0.04
osphyb-2 6.87 ± 0.02**
osphyb-4 6.44 ± 0.10**
osphyb-9 6.22 ± 0.03**

NIP 302.39 ± 2.49
osphyb-2 204.75 ± 9.66**
osphyb-4 266.54 ± 5.22**
osphyb-9 207.30 ± 3.66**

NIP 10.00 ± 0.46**
osphyb-2 13.20 ± 0.46**
osphyb-4 13.33 ± 0.48**
osphyb-9 12.80 ± 0.23**

NIP 86.25 ± 0.72
osphyb-2 76.81 ± 0.99**
osphyb-4 71.54 ± 0.95**
osphyb-9 69.17 ± 1.38**
The α/β-amylase genes (OsAmy3E and Os09g0569200) were up-regulated in osphyb, while the α/β-amylase repressor genes (RAG1, RAG2, and RA5B) were down-regulated (Fig. 3 and Additional file 2: Table S2). Moreover, ISA1 was also found to be significantly up-regulated in osphyb, whereas SSG4 was repressed (Fig. 3 and Additional file 2: Table S2). The results indicated that glycolytic activation, changes in the TCA cycle, and starch morphology and composition affected grain size and chalk formation.

Further analysis indicated that the genes for amino acid synthesis, such as OsSAMS3 and OsDHQS, and...
Fig. 3 Schematic model of gene regulatory networks for the grain size and chalkiness formation of osphyb. Pathway: Glycolysis, TCA cycle, starch synthesis, amino acid metabolism, fatty acid synthesis, plant hormones, oxidative phosphorylation, antioxidation, translation regulation. Genes encoding enzyme(s) are indicated in orange boxes, metabolites and other materials in white boxes. The expression patterns of the genes encoding corresponding enzyme(s) are indicated above or below the arrows. Each row (6 boxes) represents a DEG. Six boxes in each row represent 3 biological repeats of one gene in NIP (first three boxes) and osphyb (last three boxes), respectively. The enriched DEGs boxes for 40S, 50S, 60S, Ribosome biogenesis and RNA polymerase are rotated 90° clockwise. Log₂ (fold changes) are represented by a colour scale from green (down-regulated) to red (up-regulated).
those for amino acid metabolism, such as OsMTK2, OsGDH2, and OsAdSS, were significantly up-regulated in osphyb. Three amino acid transporter (OsATL6, OsCAT6, OsCAT11) and four storage protein genes (Os02g0224300, Os02g0456100, OsEn5S1, Os06g0507150) were down-regulated (Fig. 4 and Additional file 2: Table S2). Storage lipids are vital components to maintain the structure of seed storage substances and valuable for rice quality and food texture. The deletion of OsPHYB resulted in the significant up-regulation of all 6 differential fatty acid synthesis genes, as well as 11 lipid transporter and nine glyceride hydrolase genes compared with NIP (Fig. 3 and Additional file 2: Table S2). The results indicated that the OsPHYB mutation leads to changes in the level of expression of amino acids, storage proteins and fatty acids in grains, which could be one of the reasons for the increase in the number of grains and their chalkiness.

OsPHYB Regulates the Expression of Genes Involved in Hormone Signaling

The deletion of OsPHYB altered the expression of 64 hormone signaling genes, including 10 genes related to auxin, four related to CK, 15 related to GA, 12 related to ABA, seven related to BRs, 16 related to ethylene (ETH) compared with the levels of expression in NIP (Fig. 3 and Additional file 2: Table S2). Among them, IAA (OsIAA7, OsIAA11, and OsCLIP), GA (OsGAE1, OsGASR1, OsGRAS9, OsGRAS32, and OsGIF2), BR (OsBZR1, OsBLE1, and OsGSK3) and CK (OsAHP1) signaling genes in osphyb were all up-regulated, while the IAA efflux protein OsPIN9 and the GA signaling inhibitor SLRL1 were down-regulated, indicating that the mutation in OsPHYB promoted the signaling and accumulation of IAA, GA, BR and CK. Further analysis found that the ABA synthesis gene OsNCED1, ABA receptor genes OsPYL4 and OsPYL5, 11 ethylene-responsive factors (ERFs) and OsEIL3 ETH signaling genes in osphyb were
down-regulated, indicating that the OsPHYB mutation inhibited the synthesis of ABA and the signal transduction and accumulation of ETH. The results indicated that changes in the expression of phytohormones in grains caused by OsPHYB mutations are important regulators of osphyb grain gain and chalky formation.

**OsPHYB regulates the Differential Expression of Oxidative Phosphorylation and Antioxidant Pathway Genes**

A previous study found that FLO16 was significantly up-regulated in the TCA cycle after the deletion of OsPHYB, whereas OsSDH2-2 was down-regulated (Fig. 3 and Additional file 2: Table S2). Further analysis revealed that deletion of OsPHYB up-regulated the expression of both cyclin (OsCycB2, OsCycB2;2, OsCycD3;2) and cyclin-dependent kinase (OsCDKB1;1, OsCDKB2;1) genes but significantly inhibited the expression of the cyclin-dependent kinase inhibitor gene OsKRP6. In addition, 17 F-box genes were differentially expressed, and 14 of them were down-regulated (Fig. 3 and Additional file 2: Table S2). The results indicated that the enhanced expression of transcription-translation and cell cycle genes could be related to grain size and chalk formation.

**Metabolic Pathways Contributed to Grain Size and Quality in osphyb**

There are 59 metabolic pathways related to grain size and quality traits, and varying rice traits are regulated by different metabolic pathways (Fig. 4). Among them, ascorbic acid metabolism, fatty acid metabolism, amino acid metabolism, and phytohormone signal transduction among others were consistent with the results in Sect. 3.3. This further confirmed the importance of these metabolic pathways in the regulation of grain size and quality by OsPHYB. The correlation analysis between metabolic pathways and grain traits showed that 25 metabolic pathways affected rice yield, and 14 were positively correlated, whereas 11 were negatively correlated (Fig. 4). The chalkiness trait was affected by 10 metabolic pathways; one was positively correlated, and nine were negatively correlated (Fig. 4). Ten metabolic pathways significantly affected the processing quality (head rice rate); six were positively correlated, and one was negatively correlated (Fig. 4). Taste and nutritional quality were affected by 24 metabolic effects with 14 positively correlated and 10 negatively correlated (Fig. 4). The correlation between amylose and other nutritional qualities and metabolic pathways was the opposite; it was affected by a total of five metabolic pathways with three positively correlated and two negatively correlated (Fig. 4). The results indicated that amino acid metabolism, organic acid metabolism, fatty acid metabolism, alkaloid metabolism, purine metabolism, and phenylpropane and flavonoid metabolism played an important role in the regulation of grain size and quality by OsPHYB mutation.

**A Correlation Analysis of the Effect of Different Metabolites from osphyb on Grain Size and Quality**

Compared with NIP, the deletion of OsPHYB altered 201 significantly different metabolites that were detected in 10 categories, including amino acids and derivatives, flavonoids, phenolic acids, lipids, nucleotides, and derivatives, organic acids, alkaloids, and tannins. A total of 99 metabolites were significantly up-regulated,
whereas 102 metabolites were down-regulated (Fig. 5A, Additional file 1: Fig. S6, Additional file 3: Table S3). Among them, the contents of FFA, lysophosphatidylcholine (LPC), vitamins, and most organic acids significantly increased. Saccharides and alcohols, flavonoids and nucleotides, and derivatives significantly decreased (Fig. 5A and Additional file 3: Table S3). Changes in these metabolic pathways were caused by changes in transcription, such as fatty acid synthesis, amino acid synthesis and metabolism, and the TCA cycle (Fig. 3, Additional file 2: Table S2). In addition, nucleotides, flavonoids, and organic acids among others were also regulated by their abundance of expression at the transcriptional level (Additional file 1: Fig. S7).

The correlation analysis of 201 differential metabolites and four grain traits in rice found that 110 metabolites were significantly associated with four grain traits, including the 1,000-grain weight, grain length, grain chalkiness rate, and head rice rate ($P < 0.05$) (Fig. 5B and Additional file 3: Table S3). An additional analysis with a Venn diagram indicated that 76 of the 110 metabolites significantly correlated with the yield traits of 1,000-grain weight and grain length ($P < 0.05$), whereas only 29 in 76 significantly correlated with the 1,000-grain weight and grain length ($P < 0.05$) (Fig. 5C, E). A total of 65 of the 110 metabolites significantly correlated with the rates of chalky grains and whole milled rice ($P < 0.05$), whereas 12 of the 65 only significantly correlated with the rates of chalky grains and whole milled rice ($P < 0.05$) (Fig. 5D, F). A total of 29 in the metabolic profile of 110 were significantly associated with all four grain traits ($P < 0.05$) (Fig. 5G).

Among them, the increase in all FFA and almost all LPC metabolites in the significantly related lipids significantly positively correlated with the 1,000-grain weight and grain length of osphyb, whereas the head rice rate negatively correlated. The increase in FFA positively correlated with the rate of grain chalkiness (Fig. 5B). The decrease of all the saccharides and alcohols that significantly correlated in others was almost negatively correlated with the 1,000-grain weight, grain length, and grain chalkiness rate of osphyb, whereas the head rice rate generally significantly positively correlated. All the amino acids and derivatives, organic acids, phenolic acids, alkaloids, nuclei and derivatives, and flavonoids that decreased were almost significantly negatively correlated with the 1,000-grain weight, grain length, and grain chalkiness rate but positively correlated with the head rice rate (Fig. 5B).

**Network Diagram and Correlation Analysis of Primary Metabolic Pathways**

A Pearson’s correlation coefficient analysis was conducted based on the metabolome and transcriptome profiles and resulted in a Pearson correlation coefficient (PCC) $> 0.9$, PCC-$P < 0.01$). The screening results showed that 42 metabolites significantly correlated with 101 genes (Fig. 6, Additional file 4: Table S4). Eight pathway network sets related to carbon metabolism, lipid metabolism, amino acids and derivatives, nucleotides, organic acids, phenylpropane and flavonoids, oxidative phosphorylation and hormone signaling were obtained (Fig. 6).

As more genes or metabolites connected, their range of influence became larger. In the carbon metabolism pathway, only mws0281 (citrate) positively correlated with these pathway genes (Fig. 6A). In the lipid metabolism pathway, all 15 metabolites positively correlated with genes (Fig. 6B). In the amino acids and derivatives pathway, three out of 14 metabolites (mws0281, pmp000967, pmp001213) positively correlated, and the rest, such as mws0001, mws0230, mws0254, mws0671, pme0026, pme0193, negatively correlated (Fig. 6C). In the nucleotide, organic acid, phenylpropane, and flavonoid pathways, six, three, and four metabolites were associated with these pathway genes, respectively (Fig. 6D–F). In the oxidative phosphorylation pathway, mws0376 (fumaric acid) negatively correlated with these pathway genes, whereas mws0192 (succinic acid) positively correlated (Fig. 6G). In the hormone signaling pathway, mws1050 (O-acetylsalicylic acid) negatively correlated with these pathway genes, while Hmln001566 (salicylic acid) positively correlated (Fig. 6H).

**Discussion**

Increasing grain yield and improving grain quality are two important goals of rice breeding. Particle size is not only a yield trait but also an important appearance quality of rice, and chalkiness is one of the most important

(See figure on next page.)

**Fig. 5** Correlation analysis of four grain traits and 201 different metabolites of osphyb. A Analysis of the different metabolite types and increase and decrease levels between osphyb and NIP. B Analysis of 110 differential metabolites significantly related to osphyb 4 grain traits. Correlation coefficient are represented by a colour scale from green (negative correlation) to red (positive correlation). *$P < 0.05$, **$P < 0.01$ C Analysis of metabolites significantly correlated with 1000 grain weight, grain length of osphyb among 201 differential metabolites. D Analysis of metabolites significantly correlated with grain chalkiness rate, head rice yield of osphyb among 201 differential metabolites. E Among the 201 differential metabolites, only the metabolites significantly correlated with the 1000 grain weight and grain length of osphyb were analyzed. F Among the 201 differential metabolites, only the metabolites significantly correlated with the grain chalkiness rate, head rice yield of osphyb were analyzed. G Metabolite analysis of 201 differential metabolites significantly correlated with osphyb 4 grain traits.
indicators of grain quality (Jiang et al. 2022; Yang et al. 2022). In this study, *OsPHYB* mutations increased the kernel size and chalkiness, while altering the composition of kernel nutrients. Furthermore, these alterations were shown to be associated with the physiological, transcriptomic, and metabolomic changes caused by *OsPHYB*.
mutations. Therefore, our findings may contribute to future molecular breeding of rice that focuses on improving grain yield and quality.

**OsPHYB Plays a Key Role in the Regulation of Grain Quality**

Chalkiness is closely related to kernel size, often negatively correlating (Miyazaki et al. 2018). This is consistent with our findings that the deletion of OsPHYB resulted in increased grain size and chalkiness (Fig. 1). Kernel size is determined by the endosperm cell number and area, while chalkiness is closely related to abnormal endosperm cell development (Morita et al. 2005), uneven accumulation of starch, and shape (Ji et al. 2019; Lei et al. 2022). Changes in the contents of amylose,
fatty acids, and soluble sugar can alter the quality of rice grains (Dong et al. 2014; Nakata et al. 2017; Wang et al. 2015a). These results were consistent with the results that OsPHYB mutation resulted in significant changes in the number of grain cells, the morphology of complex starch granules, and the content of nutrients (Fig. 2; Table 1). Taken together, the knockout of OsPHYB increased the contents of FFA, soluble sugars, amylose metabolites, and promoted the assimilation of material, leading to increased grain size and a higher 1,000-grain weight, but the rapid enlargement of grains could result in loosely packed starch granules and thus, a highly chalky appearance in osphyb.

**OsPHYB Could Affect Grain Quality Through Carbon Metabolism Genes**

Previous research reported that carbon metabolism plays vital roles in starch synthesis and the formation of chalkiness (Liu et al. 2010). As an end product of glycolysis and a key substrate of mitochondrial respiration, pyruvate plays a pivotal role in plant carbon metabolism (O’Leary 2021). The OsPPDKB/FLO4 mutation increases the contents of amylose and lipids in the endosperm and causes a powdery endosperm, while increasing the supply of pyruvate for lipid synthesis (Lappe et al. 2018; Wang et al. 2020). The overexpression of FLO16 significantly increased the grain size and weight (Teng et al. 2019), whereas mutations in FLO2, OsSSIIIa/Flo5, and FLO7 have been reported to cause changes in starch-related traits in grains, resulting in sily endosperm (Ryoo et al. 2007; She et al. 2010; Zhang et al. 2016). The changes in levels of expression of ISA1, SSG4, and RAG2 and the sucrose, cellulose, hemicellulose, and amylase genes were all key triggers of grain size and chalkiness in grain development (Hakata et al. 2012; Matsushima et al. 2014; Peng et al. 2014b; Zhou et al. 2017). The changes of expression of these genes were consistent with the results of this study (Table 1, Figs. 2G–I and 3).

Multiple genes involved in protein metabolism have been reported to mediate chalky formation (Xie et al. 2021), and multiple amino acid transporters can improve the quality of rice by regulating the contents of amino acids and storage proteins (Guo et al. 2020; Ji et al. 2020; Lin et al. 2017a; Lu et al. 2018). Thus, changes in the abundance of the expression of amino acid metabolism and the repression of expression of storage protein genes led to the increased chalkiness of osphyb. Fatty acids have also been reported to be key factors that affect grain size, yield and quality (Guo et al. 2013; Sandoval 2012; Wang et al. 2015a; Zhao et al. 2019). Therefore, the differential expression of these genes resulted in changes in protein and FFA content in osphyb grains (Table 1, Fig. 3). Lipids are closely related to starch synthesis, and changes in the biosynthesis of lipids can significantly affect the changes in starch content (Cai et al. 2018; Xi et al. 2020). In conclusion, the mutation of OsPHYB mediated the levels of expression of the genes in carbon metabolic pathways, the coordinated regulation of carbon allocation by these dynamic regulatory processes resulted in changes in grain size and the formation of chalkiness in osphyb.

**OsPHYB Could Affect Grain Quality Through Phytohormone Signaling Genes**

Plant hormones are important regulators of grain size and chalkiness formation in rice (Sou et al. 2006; Xie et al. 2021). This study indicated that OsPHYB mutations activated IAA, GA, CK, and BR signaling and accumulation, while ABA and ETH synthesis and/or signaling were inhibited (Fig. 3). This is consistent with previous studies that reported that IAA, GA, CK, BR and ABA regulate grain shape and chalkiness (Jiang et al. 2022; Xiao et al. 2017; Zhang et al. 2020).

*BG1* is involved in the regulation of auxin transport and response, and the overexpression of *BG1* improves grain size and weight (Liu et al. 2015). *OsIAA7, OsIAA11*, and *OsCLIP* in *osphyb* were up-regulated, whereas *OsPIN9* was downregulated (Fig. 3). The overexpression of *OsGASR9* and *OsBZR1* increased grain size and weight (Li et al. 2019; Zhu et al. 2015), whereas the inhibition of ETH could enhance the level of expression of starch synthase and cell cycle genes and lead to increased grain size (Panda et al. 2016). In addition, high levels of ethylene and 1-aminocyclopropane-1-carboxylate (ACC) in grain inhibited cell division of the endosperm, resulting in decreased grain weight and increased chalkiness (Zhang et al. 2009). The overexpression of *OsPYL/RCAR5* severely reduced grain yield, whereas *OsPYL/RCAR5* was significantly inhibited in *osphyb* (Kim et al. 2014). The results of this study are consistent with those of previous studies (Fig. 3). *OsAO3* is involved in the biosynthesis of ABA and negatively regulates the yield of rice grains (Shi et al. 2021). The ABA synthesis gene OsNCED1 in *osphyb* was significantly down-regulated, and the ABA receptor gene was also significantly down-regulated (Fig. 3). In conclusion, phytohormones play an important role in grain size and chalkiness during endosperm development in rice. The differential expression of genes in phytohormonal signaling also results in changes in the expression of responsive genes, which could be an additional reason for grain size and chalkiness formation.

**OsPHYB Could Affect Grain Quality by Promoting ATP Production, Regulating pH Homeostasis and Enhancing Oxidative Tolerance**

Recent studies have identified a close correlation between redox homeostasis and the development and chalkiness
of rice grains (Liu et al. 2010; Suriyasak et al. 2017; Xu et al. 2010), suggesting that ROS may play a critical role in the regulation of rice endosperm chalkiness (Liu et al. 2010; Xu et al. 2010). Existing studies have shown that the overexpression of Chalk5 can perturb the pH homeostasis of the endosperm membrane transport system, thereby affecting proteosome biogenesis and increasing the chalkiness of endosperms (Li et al. 2014). The PM H\(^{+}\).ATPases are also involved in cellular growth, sugar transport, mineral nutrient translocation, and grain filling among others and are involved in cell growth by reducing the apoplast pH and activating the expansins responsible for cell expansion (Falhof et al. 2016; Gaxiola et al. 2007). This was consistent with changes in the expression of Chalk5 and PM H\(^{+}\)-ATPase (OsA7, Os02g0313900) in osphyb (Fig. 3). This study also indicated that the mutation of OsPHYB can also activate the expression of oxidative phosphorylation complexes I, III, and IV to promote the production of ATP (Fig. 3).

Ascorbic acid deficiency leads to the accumulation of hydrogen peroxide (H\(_2\)O\(_2\)), which affects antioxidant capacity and photosynthetic function, alters enzyme activities and gene transcript abundances related to starch synthesis, and ultimately leads to increased grain chalkiness (Yu et al. 2017), while its increased content enhanced the tolerance of oxidatively stressed grains and reduced chalkiness (Lin et al. 2022). ROS can lead to rice grain chalkiness by pathways that overlap with seed germination (Suriyasak et al. 2017). In this study, the levels of expression of antioxidant enzyme-related genes, such as OsAPXs, OsDHAR1, OsGPX3, OsGSTs, OsMDAR3, OsSOD1, and OsPRXs, in osphyb increased significantly, while the expression of oxygen burst genes (Osrbho7 and Osrbho9) was down-regulated (Fig. 3, Additional file 2: Table S2). Thus, the increase in osphyb grain and chalkiness could be regulated by activating oxidative phosphorylation, enhancing oxidative tolerance, and disrupting pH homeostasis.

**OsPHYB Could Affect Grain Quality by Activating Transcription-Translation and Cell Cycle Genes**

Ribosomes are the cellular machinery that performs protein synthesis by translating the information contained in mRNA molecules (Hammerling et al. 2020). RNA polymerase activity plays an important role in rice development and yield-related traits (Jha et al. 2021; Zhao et al. 2020). GL6 interacts with RPCS3 and TFC1, participates in the RNA polymerase III transcription machinery, and regulates the expression of genes involved in rice grain development (Wang et al. 2019). Ribosomal gene mutations primarily manifest as a variety of developmental defects, such as OsRPL3B and OsRPS3A (Byrne 2009; Uzair et al. 2021; Zheng et al. 2016). In this study, the mutation of OsPHYB activated the expression of a large number of RNA polymerase and ribosomal genes (Fig. 3). Translational regulation is ubiquitous in cellular processes, and the cell cycle is a highly orchestrated process that is extensively regulated to ensure accurate DNA replication and proper chromosome segregation (Stumpf et al. 2013). OsmiR396a/OsGRFs have been reported to act between SLR1 and the cell cycle-related genes OsCycB2 and OsCycB2.2 to mediate the proliferation of rice cells (Lu et al. 2020). The expression of OsCycB1;1 is critical for endosperm formation via the regulation of mitotic division, and the endosperm plays an important role in the maintenance of embryo development in rice, whereas OsCYCD3;1 promotes branch formation (Guo et al. 2010; Ohyama et al. 2022). The levels of expression of OsKRP1 and OsKRP2 play an important role in rice endosperm development, grain filling, and cell proliferation (Ajadi et al. 2019). Therefore, the grain enlargement and chalky formation of osphyb could be owing to the regulatory role of RNA polymerase, ribosome and cell cycle pathways in growth and development, which are achieved by regulating translation and cell cycle.

**OsPHYB Mutation Alters Grain Metabolic Pathways and the Content of Metabolites, Such as FFA and LPC Among Others**

It has been reported that the formation of chalky endosperm is related to a reduction in the metabolites related to carbon and nitrogen metabolism that are involved in starch storage and protein synthesis, whereas the alkaloids, phenolic acids, amino acids, flavonoids, and starch metabolism are all closely related to the biosynthesis of proteins and the accumulation of starch in chalky endosperm (Chen et al. 2020; Kim et al. 2013; Lin et al. 2017b). Starch lipids are specific to cereal endosperm starch and are primarily composed of FFA and LPC; these fatty acids strongly influence the assembly and properties of cereal starch (Gayral et al. 2015). OsACOT is a key enzyme in fatty acid desaturation and elongation, particularly the transformation of palmitic acid (16:0) to linoleic acid (18:2), and miR1432-OsACOT regulates the increase in particle size and grain filling by regulating the biosynthesis of long-chain fatty acids (Zhao et al. 2019). Therefore, the increases in FFA and LPC caused by the mutation in OsPHYB could affect the development and structure of amyloplasts and starch granules in grains, resulting in larger kernels and increased chalkiness in osphyb.

The increase in content of soluble sugar in high chalky grains comes at the expense of starch degradation (Nakata et al. 2017). The decrease of saccharides and alcohols in osphyb could be caused by the changes in related metabolic enzymes, such as amylase, at the
transcriptional level. Aspartate aminotransferase and amino acid permease can regulate the contents of rice grain proteins, nutritional quality, and yield (Lu et al. 2012; Peng et al. 2014a). Adjusting the concentration of amino acids can promote bud growth and increase the number of tillers, which could improve grain yield (Lu et al. 2018). Almost all the decreased metabolism of amino acids and derivatives in osphyb were significantly associated with increased chalkiness, which could be caused by the effect of changes in the composition of amino acids on grain development. Maltodisaccharides, linear glucans, monosaccharides, and organic acids are involved in starch biosynthesis in rice endosperm (Nakamura et al. 2020). Fumarase catalyzes the reversible hydration reaction between fumarate and L-malate in the TCA cycle. Therefore, the organic acids that are significantly related to grain and chalkiness in osphyb were significantly reduced, whereas these changes in organic acids could affect the synthesis of starch during grain development, whereas the reduction in fumaric acid affected the normal metabolism of the TCA cycle. The phenolic ring can stabilize and delocalize unpaired electrons, conferring an antioxidant property to phenolic acids. Phenolic acids are considered to be natural antioxidants that can scavenge the free radicals that could increase oxidative stress and potentially damage the large biological molecules, such as lipids, proteins, and nucleic acids (Adom and Liu 2002; Ratseewo et al. 2019). Therefore, the content of phenolic acids positively correlated with the antioxidant capacity of rice (Yu et al. 2021b). Flavonoids constitute a major group of plant phenolic compounds. Rice flavonoids primarily have antioxidant properties, although some of them have not been evaluated for their antioxidant activities (Cho et al. 2013; Goufo and Trindade 2014). OsCOP1 regulates flavonoid biosynthesis and embryogenesis in rice seeds, thereby regulating the development of pericarp and embryos (Kim et al. 2021). The reduction in organic acids, phenolic acids, and flavonoids significantly related to grain traits in osphyb could be related to the enhanced ROS scavenging activated by the ROS antioxidant capacity of osphyb. Purine bases and nucleosides are produced by the turnover of nucleotides and nucleic acids, as well as from some cellular metabolic pathways (Ashihara et al. 2018). The decrease in adenine, guanine, and other nucleotides and derivatives significantly related to their grain traits in osphyb could be the result of OsPHYB mutations on its genetic level of metabolism and a series of related cellular metabolic pathways. A network analysis of the metabolites and genes of eight major metabolic pathways reaffirmed these results, particularly the lipid metabolism pathways. In conclusion, the increase of grain size and chalkiness caused by the deletion of OsPHYB was regulated by various metabolic pathways and metabolites, which ultimately led to changes in the composition of endosperm and the development and accumulation of starch.

**Conclusions**

A mutation in OsPHYB increased grain size and chalky formation in rice, which are complex traits controlled by multiple genes and metabolic pathways. The transcriptomic and metabolomic analyses showed that the induction in grain enlargement and the formation of chalkiness that resulted from the deletion of OsPHYB were complex and owing to a multi-factor coordinated network regulation (Fig. 7). In particular, a variety of key carbon metabolic genes, starch synthesis, and starch grain development genes, plant hormone genes, cell cycle genes, transcriptional regulation of oxidative phosphorylation and fatty acid synthesis pathways, and the increase in free fatty acid metabolites are critical for the grain size and chalk formation regulated by OsPHYB. Among them, the repressed expression of OsPPDKA, OSSSIIA/Flo5, Flo2, Flo7, SSG4, RAG2 genes and the up-regulated expression of Chalk5 gene are extremely important for OsPHYB-involved rice quality regulation. The up-regulation of OsBZR1, OsGASR9, OsCycB2, OsCycB2;2, OsCycD3;2,
OsCDKB1;1, OsCDKB2;1 genes which induced by OsPHYB mutation positively contributed to grain size increase of osphyb. Taken together, these results revealed new functions of OsPHYB in rice grain development and provide a new strategy for yield and quality improvement in rice breeding.

Materials and Methods
Plant Materials and Growth Conditions
The CRISPR/Cas9 expression vector pBUN411 used to create the osphyb mutation was provided by Prof. Qijun Chen, China Agricultural University (Beijing, China) (Xing et al. 2014). A 19 bp sequence (5'-TCCTCCACC GCATCGATGT-3') was selected as the target site before the protospacer adjacent motif (PAM) sequence (CGG) near the N-terminal of the first exon of OsPHYB. Nipponbare (NIP) was the variety of japonica rice that was transformed.

All the rice plants used in this study were grown naturally in 2019–2020 during the growing season and maintained at the Henan Agricultural University, Zhengzhou (E 112°42′–114°14′, N 34°16′–34°58′), Henan Province, China. The planting density was 13 × 30 cm, with one plant per hill.

Analysis of Rice Grain Phenotypes
The harvested grains were air-dried at room temperature and stored for 3 months. Thirty plump grains were randomly selected from transgenic and wild-type plants, and their grain length, width, and thickness were measured using a Vernier caliper. The weight of 1000 grains was determined using a granulometer (Canoscan 5600F; Canon, USA) and electronic balance. The rate of chalkiness was measured using a rice appearance quality detector (JMWT12; Dongfu JiuHeng, Beijing, China). At least five biological replicates were analyzed.

Scanning Electron Microscopy Analysis (SEM)
They were observed with a SEM (SU8100; Hitachi, Tokyo, Japan) after gold was sprayed on the natural fracture cross-section of rice grains and polished rice. They were then photographed with an accelerating voltage of 3.0 kV. All the procedures were conducted according the manufacturer’s instructions.

Gene Expression Analysis
Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and then reverse transcription was performed using reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR (real-time quantitative reverse transcription PCR) was performed using GoTaq, a CFX 96 real-time system qRT-PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions for the quantitative PCR master mix (Promega). The gene-specific primers are listed in Additional file 5: Table S1. The following conditions were used for the PCR: initial denaturation at 95 °C for 3 min, then 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. The relative level of expression was calculated using the 2−ΔΔCT method (Livak and Schmittgen 2001). The primers for transcriptome qRT-PCR verification are shown in Additional file 5: Table S1.

Transcriptomic Analysis
RNA-seq was performed using RNA that was extracted from the endosperm samples of wild-type and OsPHYB knockout plants 12 days after flowering (DAF) using the TRIzol reagent. The purity and quality of the total RNA were checked according to the manufacturer’s instructions. Qualified and high-quality total RNA samples were reverse transcribed into cDNA used to construct the cDNA library. An Illumina HiSeq high-throughput sequencing platform (Illumina, San Diego, CA, USA) was used to sequence the cDNA library based on sequencing by synthesis (SBS) technology. Three biological replicate samples were prepared for NIP and the mutant plants. After sequencing, the data were analyzed using the rice genome sequence (https://rapdb.dna.affrc.go.jp/download/irgsp1.html) as a reference. Differential expression analysis, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses and quantitative qRT-PCR analysis were conducted as previously described (Zhu et al. 2018).

Metabolite Profiling and Data Analysis
The metabolites extracted from the rice 12 DAF grains sampled were measured using UPLC-MS/MS technology combined with data-dependent acquisition (Chen et al. 2013). XCMS software was used for peak extraction and metabolite identification, and multidimensional statistical analysis of the mass spectrometry data was conducted using SIMCA software (Fraga et al. 2010). An orthogonal partial least squares discriminant analysis (OPLS-DA) was used to filter out noises (Thevenot et al. 2015). The UPLC conditions are shown in the Additional file 1.

Widely Targeted Metabolomics Assay
A correlation analysis was performed between the differential metabolite data and rice grain traits, and the Pearson correlation coefficient was calculated using R (Foundation for Statistical Computing, Vienna, Austria). The correlation heatmap was visualized using the pheatmap package in R. A Venn diagram was plotted using the VennDiagram package of R to visualize the significantly correlated genes.
Abbreviations
ABA: Abscisic acid; ACC: 1-Aminocyclopropane-1-carboxylate; BRs: Brassinosteroids; CKs: Cytokinins; DAF: Days after flowering; DEG: Differentially expressed gene; ERF: Ethylene-responsive; ETH: Ethylene; FFA: Free fatty acids; FPKM: Fragments per kilobase of transcript per million mapped fragments; GAs: Gibberellins; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LPC: Lysophosphatidylcholine; NIP: Nipponbare; Oryza sativa phytochrome B; OPLS-DA: Orthogonal partial least squares discriminant analysis; PCC: Pearson correlation coefficient; PM: Plasma membrane; PPDK: Pyruvate phosphate dikinase gene; qRT-PCR: Real-time quantitative reverse transcription PCR; ROS: Reactive oxygen species; SBS: Sequencing by synthesis; SEM: Scanning electron microscope; TCA: Tricarboxylic acid.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12284-022-00600-5.

Additional file 1. Supplementary Materials and Figures.
Additional file 2. Table S2. Gene names and numbers for differential pathways.
Additional file 3. Table S3. The name and number of metabolites.
Additional file 4. Table S4. Network statistics of differential metabolites and related genes.
Additional file 5. Table S5. List of primers required for this study.

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Author Contributions
FL wrote the manuscript text, conceptualization, review & editing. YL prepared Fig. 4 and 5. XZ, LL, YY and FK material planting, data curation, formal analysis. XJ material creation. YZ, JL, TP and HS review and editing. QZ and YD supervision, fund acquisition, resources, review & editing. All authors read and approved the final manuscript.

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Availability of Data and Materials
The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations
Ethics Approval and Consent to Participate
Not applicable.

Consent for Publication
All authors are consent for publication.

Competing Interests
The authors declare that they have no competing interests.

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