Down-regulation of the Genome Uncoupled 4 Retards Starch Biosynthesis in Rice

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Research

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Abstract

Background

Starch is the major storage carbohydrate in rice, with essential physical functions for plant growth. The starch biosynthesis in rice employs the cooperation of nucleus and plastid, which requires regulation of the signals from nucleus to plastid. However, the plastid-to-nucleus retrograde signals for starch biosynthesis is partly mediated by tetrapyrrole intermediates, i.e., heme, but the underlying mechanism is largely unknown. In previous studies, we revealed that the Genome Uncoupled 4 (OsGUN4) mutation in rice have been revealed to greatly affect tetrapyrrole intermediates but retain a high photosynthetic capacity.

Results

Here, we further found that down-regulation of OsGUN4 promoted to accumulate sucrrose but reduce the total starch, attributing to abnormal performance of metabolisms and enzyme activities of starch biosynthesis in leaves of gun4epi. Besides, the exogenous sucrrose led to induced starch synthesis but reduced sucrrose contents in wild-type, while norflurazon(NF) treatments could eliminate or weaken these inductions. Nevertheless, no changes were detected between check and sucrrose treatments in the gun4epi, whereas NF treatment enhanced the trends of increased sucrrose but reduced starch, suggesting the roles of OsGUN4 on balance of photosynthesis and starch biosynthesis. Dynamic activity changes of starch biosynthetic enzymes were in accordance with the contents of carbon metabolites. Moreover, RNA sequencing revealed that a great deal DEGs were associated with starch metabolic pathways, with 62 genes being up-regulated and 25 down-regulated in gun4epi. Many genes involved in starch biosynthesis performed down-regulated expression, including the transcription factor of bZIP58 and its target genes of OsBEIIb and OsSSI, which are vital for the formation of amylopectin and starch granules, while displayed up-regulated expression of OsSIIa and OsGBSSI that promotes the formation of amyllose.

Conclusion

In conclusion, these findings confirm that OsGUN4 play regulatory roles on biosynthetic genes and enzyme activity in starch biosynthesis.

Background

Plants assimilate atmospheric CO₂ during photosynthesis using light energy to produce sugars and chemical energy (ATP) for plant growth [1]. In leaves, sugars are partly retained in chloroplasts during the day to synthesize transitory starch for short term storage, and then exported to non-photosynthetic organs during the subsequent night for long-term storage [2]. Starch is the major storage carbohydrate in higher plants, with essential physical functions and economical importance. As a major factor for plant growth, starch biosynthesis buffers metabolism and growth against the daily light/darkness alternation to avoid a shortfall of carbon at the end of the dark period [3–4].

Transient starch is photosynthetized synthesized during the day but degraded at night to provide carbon and energy under inactive photosynthesis [5]. Leaf starch mainly accumulates in the photosynthetic palisade and mesophyll (M) cells [6], and major mesophyll cells in mature leaves are source for sucrrose transport into sink tissues [7–8].

Transient starch in leaves is usually found in the plastid of photosynthetic organs [5]. While enzymatic functionality of the respective plastids depends largely on its own specialized proteome, and corresponding shifts of these proteome determine the transitions of different plastid types along with changes from environmental conditions and tissues [9–10]. The vast majority of plastid proteome is encoded by nucleus, but the expression of plastid genes is essential for metabolic processes such as photosynthesis and lipid biosynthesis [11–12]. Thus, the establishment of plastid multi-subunit protein complexes need a tight cooperation between nucleus and plastid genes [13]. Besides, high morphological and functional diversity of plastids in different tissues of multicellular plants are tightly connected to the function of the corresponding tissue [10], which can explain the manifestations of the same cell organelle in an individual plant.

Development from undifferentiated proplastid to functional plastid is coordinately achieved between plastid and nucleus, requiring cooperation between nucleus-to-plastid antegrade signaling and plastid-to-nucleus retrograde signaling [14]. The GUN (genome uncoupled) proteins were identified for plastid-to-nucleus signaling studies [15]. Thereinto, GUN4 have been found to be involved in the retrograde signaling pathway in Arabidopsis [16] and rice [17]. Besides, the mutation of OsGUN4 in rice have also been revealed to deregulate transcription of PhANGs depending on disruption of 1O₂-induced signaling pathway [17]. This model suggested that accumulation of heme in active chloroplast can activate a mechanism to induce the expression of PhANGs [18]. Interestingly, the plastid-to-nucleus retrograde signals is also revealed to regulate expression of nuclear starch biosynthetic genes, which is partly mediated by tetrapyrrole intermediates, i.e., heme [9]. Besides, the mutation of OsGUN4 in rice have also been revealed to greatly affect tetrapyrrole intermediates, including heme, Mg-Proto and Proto [17]. Above on, retrograde signaling may play important roles in starch biosynthesis of leaves, but the underlying mechanism remains largely unknown.

In previous studies, we revealed that the OsGUN4 mutation in rice have been revealed to greatly affect tetrapyrrole intermediates and function in 1O₂-induced signaling pathway [17, 19]. Here, we further employed the rice epi-genetic mutant gun4epi to examine carbon metabolites, starch biosynthetic enzymes, genes involved in starch biosynthesis in order to investigate the role of OsGUN4 on starch biosynthesis during vegetative stages. In conclusion, these findings confirm that OsGUN4 play regulatory roles in starch biosynthesis.

Results

Mutation of OsGUN4 produced aberrant starch metabolism
Previous studies revealed the positive effects of OsGUN4 mutation on photosynthetic capacity during vegetative stages [20–21], but no details were focused on the relationship between photosynthetic products-sucrose and starch biosynthesis. To determine the effects of OsGUN4 mutation on starch biosynthesis during vegetative stage, the carbon metabolites and relative starch biosynthetic enzymes were investigated in 35 days after germination (DAG) seedlings (Fig. 1–2 and Additional file 5–6: Table S1-S2).

Compared to the wild-type, both of the sucrose and amylose contents were increased in gun4epi (Fig. 1a, e), while the fructose, glucose, total starch and protein contents were decreased (Fig. 1b, c, d, f), suggesting that the OsGUN4 mutation promoted to accumulate the sucrose but decrease starch synthesis.

To reveal why the OsGUN4 mutation led to abnormal starch metabolism existed, the 35 DAG seedlings were treated with exogenous sucrose (exSuc). In the wild-type, contents of the sucrose and amylose were reduced, whereas the fructose, glucose, total starch and protein contents were increased after treatment (Fig. 1). However, in gun4epi, no difference was detected before and after treatment, but both of sucrose and amylose concentration were higher than that in the wild-type, whereas the fructose, glucose, total starch and protein contents were lower, indicating the retardative transformation of sucrose to starch in gun4epi leaves (Fig. 1a-f).

We next treated the 35 DAG seedlings were with exSuc added norflurazon (NF; the agent for blocking photosynthesis, causing the gun phenotype). After exposed to exSuc added with NF, contents of carbon metabolites were little induced compared with the control, but greatly inhibited in relative to the single sucrose treatment in wild-type, indicating that NF blocked the sucrose-induced signaling (Fig. 1). Nevertheless, no difference was still detected between the control and the sucrose treatment in gun4epi, but there were significant differences for the treatment of sucrose added with NF compared to the other two treatment (Fig. 1a-f). All these results suggested that OsGUN4 mutation influenced the starch biosynthesis in leaves.

Mutation of OsGUN4 deregulated activities of enzymes responsible for starch biosynthesis

Dynamic activity changes of enzymes involved in starch biosynthesis were in accordance with the contents of carbon metabolites (Fig. 2 and Additional file 6: Table S2). In consistent with the results as shown in Fig. 1, activities of ADP-Glc pyrophosphorylase (AGPase), soluble starch synthase (SSS) and starch branching enzyme (SBE) showed significant increases, but activities of sucrose synthase (SS), sucrose phosphate synthase (SPS) and granule-bound starch synthase (GBSS) were increased in gun4epi (Fig. 2a-f).

After exposed to exSuc, significant increased activities of AGPase, SSS and SBE, but decreased activities of SS, SPS and GBSS were showed in wild-type, whereas no difference was detected in gun4epi, suggesting the retardative accumulation of starch from sucrose in gun4epi (Fig. 2a-f).

After exposed to exSuc added with NF, activities of the related enzymes (Fig. 2a-f), was little induced compared with the control, but greatly inhibited in relative to the single sucrose treatment in wild-type, indicating that NF blocked the sucrose-induced signaling. Still, no difference was still detected between the control and the exSuc treatment in gun4epi, but the sucrose added with NF treatment greatly affected the enzyme activities in relative to other treatments (Fig. 2a-f). All these results suggested that OsGUN4 mutation influenced activities of the starch biosynthetic enzymes in leaves.

Differently expressed genes related to starch biosynthesis revealed by RNA-seq

To analyze the detailed regulation of OsGUN4 on starch biosynthesis in vegetative leaves, RNA-seq was performed in the wild-type and gun4epi. According to the mapping results using the metabolism overview pathways in MapMan, a total of 468 differentially expressed genes (DEGs) were identified between HYB and LTB by RNA-seq, with 203 genes being up-regulated and 265 down-regulated in gun4epi (Fig. 3a).

To investigate the expression profiles in lodicules of the wild-type and gun4epi, DEGs with adjusted \( P < 0.001 \) were selected for further analysis, and many of the DEGs were associated with sucrose and starch metabolic pathways, with 62 genes being up-regulated and 25 down-regulated in gun4epi (Fig. 3b and Additional file 1: Fig. S1), including genes for fructose-1,6-bisphosphate aldolase (FBA, OsFBA), fructose 1,6-bisphosphate (FBP), phosphoglucomutase (PGM), sucrose-phosphate-synthase (SPS), sucrose phosphate phosphatase (SPP), sucrose synthase (SSP) and GBSS (OsGSS), as well as genes for fructose and glucose synthesis, such as fructokinase (Frk) and hexokinase (HxK), soluble starch synthase (SS) and branching enzyme (BE). Besides, genes for isoamylases (ISA), pullulanase (PUL) and c-amylose (Amy3B, 3C, 3D), and representative genes for storage proteins, including protein disulfate isomerase (PDI), prolamin (CysR10), PPDKB, glutelins (GluA1, GluA2, GluA3), alanine aminotransferase (AaAT1, AaAT4), major allergenic protein (RA16, RA17, RA5B, RAG2 and RG21), globulins (globulin1, globulin2, 11 s-globulin, 10kD-, 13kD-, 17kD-, 19kD-globulin) also deregulated expression in gun4epi in relative to WT (Fig. 3b and Additional file 1: Fig. S1). These results suggested that OsGUN4 mutation affected expression of many genes participating in starch and protein biosynthesis in leaves.

Mutation of OsGUN4 deregulated gene expression responsible for starch metabolism in leaves

To ensure the sequencing results, expression for genes of log2 fold changes more than 0.5 folds were further detected by RT-qPCR (Fig. 4–5 and Additional file 7: Table S3). Compared to the wild-type, gene expression for sucrose synthesis, including genes for FBA (OsFBA), FBP (OsCFB, OsFBP2, OsFBP3), PGI (OsPGI), PGM (OsPGM), SPS (OsSPS2, OsSPS1), SPP (OsSPP1, OsSPP2), SuS (OsSUS1, 2, 5, 6, 7) and GBSS (OsGSS) were significantly increased (Additional file 2: Fig. S2 and Additional file 3: Fig. S3), while genes for fructose and glucose synthesis, such as Frk (OsFrK7) and HxK (OsHxK1, OsHxK4 and OsHxK7), were
greatly reduced in gun4epi (Fig. 4). Moreover, gene expression for AGPase (OsAGPS1), SS (OssSIVa, OssSIV) and BE (OssBEIIa, OssBEIIb) were also decreased in gun4epi compared to that in wild-type (Fig. 5 and Additional file 2–3: Fig. S2 and Fig. S3). Nonetheless, OsSUS4, OsAGPL1, OsAGPL3, OsAGPL4 and OssSSLc showed increased expression in gun4epi (Fig. 4–5). All these results were consistent with the results as shown in Fig. 3b, indicating that the mutation of OsGUN4 affected expression of starch biosynthetic genes.

Furthermore, the exogenous sucrose induced the gene expression for AGPase, GBSS, SSS, SBE, whereas reduced the expression of genes for SS and SPS in the wild-type (Fig. 4–6). After exposed to sucrose added with norflurazon, the gene expression for SS, SPS and GBSS still remained higher expression than that of sucrose treatment (Fig. 4–5), whereas gene expression for AGPase, SSS and SBE were little increased in the wild-type (Fig. 5). However, sucrose treatment induced no significant difference with the control in gun4epi (Fig. 4–5). But the sucrose supplement with norflurazon treatment intensified the trend of gene expression changes, and showed more enhanced dynamics in gun4epi than that in the wild-type (Fig. 4–5). All these results were consistent with the results as shown in Fig. 1 and Fig. 2, suggesting the regulatory role of OsGUN4 on expression of starch biosynthetic genes.

Abnormal effecting of OsGUN4 mutation on the transcriptional expression of OsbZIP58

OsGUN4 is localized in plastid in our previous studies, so it is impossible for OsGUN4 to regulate gene expression as transcriptional factors (TFs) in nucleus. Thus, to clarify the signaling of OsGUN4 from plastid to nucleus, the reported TF of bZIP58 was used for further investigation (Fig. 6 and Additional file 7: Table S3).

In 35 DAG seedlings, expression of OsbZIP58 was significantly decreased in gun4epi (Fig. 6). Also, after sucrose treatment, there was no obvious expression difference of OsbZIP58 in gun4epi (Fig. 6). But in wild-type, expression of OsbZIP58 was significantly induced by sucrose, while showed no obvious changes with CK (Fig. 6). These results suggested that OsGUN4 mutation down-regulated the transcriptional expression of OsbZIP58.

Discussion

OsGUN4 is involved in regulation of starch biosynthesis

In plant cells, plastids display a high morphological and functional variations, and include four major forms of etioplast, chloroplast, chromoplast and amyloplast [22]. Despite displaying diverse and tissue-dependent functions, each differentiated form of plastid share a set of genome [23]. Chloroplasts are the location for photosynthesis and biosynthesis of transient starch [5]. Also, the aberrant chloroplasts usually would cause abnormal photosynthesis and starch metabolism [5, 22]. The OsGUN4 mutation performed aberrant chloroplast morphology as reported previously [20], and also indeed reduced the accumulation of starch at here (Fig. 1d). Nonetheless, the mutation of OsGUN4 did not cause the decrease of sucrose derived from photosynthesis in gun4epi, and inversely, the OsGUN4 mutation led to the accumulation of sucrose (Fig. 1a), which is partly related to the positive effects of OsGUN4 mutation on photosynthetic capacity during vegetative stages [20–21]. On the other hand, this is due to the deregulated enzyme activities involved in starch biosynthesis (Fig. 2). For example, in gun4epi, the enhanced activities of SS and SPS was responsible for the accumulation of sucrose (Fig. 2a-b), whereas the decreased AGPase, SSS and SBE activities made neglect effects on starch synthesis (Fig. 2c-f). All these results suggested that OsGUN4 mutation blocked the accumulation of starch from sucrose in leaves.

Generally speaking, over-accumulations of starch biosynthetic intermediates, i.e. ADPglucose and sucrose would result in photo-oxidative stresses [24–25]. For example, the mutation of TaSSIVb-D in wheat induced the reduction of starch granule number and photosynthetic efficiency, this may be attributed to high contents of the substrate ADPglucose [24–25]. This is consistent with the results as shown in Fig. 4 and Fig. 6, which could also explain the enhanced AGP activity and increased expression of OssSIV in gun4epi. Besides, the addition of exogenous sucrose also indicated that GUN4 played a role in the normal synthesis of starch. Exogenous sucrose could greatly promote the transient starch and protein biosynthesis in wild-type, but could not induce the accumulation of sucrose and amylose in gun4epi (Fig. 1). This was due to the dynamic activity changes of enzymes involved in starch biosynthesis, which were in accordance with the contents of metabolites (Fig. 2).

Moreover, NF treatment is usually used for explore the uncoupled phenomenon of photosynthesis-associated nuclear genes (PhANGs) transcriptional levels from chlorophyll accumulation in Arabidopsis [15] and C. reinhardtii [26]. Our previous studies revealed that the mutation of OsGUN4 deregulate transcription of PhANGs depending on disruption of 02-induced signaling pathway in rice [17]. Here, after NF treatment, the induction by exogenous sucrose were nearly eliminated or weakened in the wild-type, whereas the OsGUN4 mutation aggravated no response to sucrose signals in gun4epi (Fig. 1–2, and Additional file 4: Fig. S4). All these results suggested OsGUN4 functions in response to sugar signals during starch biosynthesis.

Roles of OsGUN4 in regulation of starch biosynthesis

The normal functioning of plastids require cooperation of plastid genes and nuclear genes, which could reach the balance of photosynthesis and starch biosynthesis [14]. Although GUN4 have been revealed in the plastid-to-nucleus signaling pathway [17], it has not yet been reported its similar functions on starch biosynthesis. As is shown in preceding part of the text, OsGUN4 indeed function in the starch biosynthesis, and the OsGUN4 mutation also greatly deregulated many genes for key enzymes in starch biosynthesis (Fig. 4–5, and Additional file 4: Fig. S4). Thus, we can conclude that OsGUN4 may regulate the genes encoding key enzymes in starch biosynthesis. There are mainly two ways to employ the regulation, by tetrapyrrole intermediates and by sucrose signals.

Inhibitors of plastid gene expression could repress amyloplast differentiation and starch biosynthesis in tobacco (Nicotiana tabacum) Bright Yellow-2 (BY2) cultured cells [9]. This indicated a plastid-to-nucleus retrograde signals from plastid gene expression to the regulation for expression of nuclear starch
biosynthesis genes, partly mediated by tetrapyrrole intermediates, i.e., heme [9]. In our previous studies, the OsGUN4 mutation greatly affect tetrapyrrole intermediates, including heme, Mg-Proto and Proto in rice [17]. The blocking of photosynthesis and starch biosynthesis in gun4epi also illustrated this from Suc added with NF treatment (Fig. 1–3 and Additional file 3–4: Fig. S3, Fig. S4), suggesting the retardative signals from plastid to nucleus to promote starch biosynthesis.

Regulation of sucrose signals on starch biosynthesis can realized via transcription factors in cereal crops, e.g. NAC36 [27], MYB14 [28]. OsGUN4 is localized in plastid in our previous studies, so it is impossible for OsGUN4 to regulate gene expression as transcriptional factors (TFs) in nucleus. Instead, OsGUN4 might function in regulation of genes participating in starch biosynthesis via transcription factors, e.g. bZIP58 (Fig. 6). RNA-seq and RT-qPCR assays revealed that many genes for key enzymes in starch biosynthesis were significantly down-regulated in gun4epi, including including the transcription factor of bZIP58 and target genes of OsBEIIb and OsSSI, which are vital for the formation of amylepectin and starch granules, while displayed up-regulated expression of OsSSIIIa and OsGBSSI that promotes the formation of amylase (Fig. 3 and Additional file 3: Fig. S3). Thus, the retardative transformation from sucrose to starch mostly depends on the deregulated expression of genes for starch biosynthetic enzymes.

Conclusion

In conclusion, we here found that down-regulation of OsGUN4 promoted to accumulate sucrose but reduce the total starch (Fig. 1), attributing to abnormal performance of metabolisms and enzyme activities of starch biosynthesis (Fig. 2) in leaves of gun4epi. Moreover, the exogenous sucrose induced starch synthesis but inhibited sucrose contents in wild-type, while norflurazon eliminated the changes (Fig. 1–2). However, no changes were detected between check and sucrose treatments in the gun4epi, but NF treatments enhanced the trends of increased sucrose but reduced starch (Fig. 1–2). Dynamic activity changes of enzymes involved in starch biosynthesis were in accordance with the contents of carbon metabolites, suggesting the roles of OsGUN4 on balance of photosynthesis and starch biosynthesis (Fig. 1–2). Moreover, RNA sequencing revealed that many of the DEGs were associated with starch metabolic pathways, with 62 genes being up-regulated and 25 down-regulated in gun4epi (Fig. 3). A great deal of genes involved in starch biosynthesis performed down-regulated expression (Fig. 4–6), including the transcription factor of bZIP58 and its target genes of OsBEIIb and OsSSI, which are vital for the formation of amylepectin and starch granules, while displayed up-regulated expression of OsSSIIIa and OsGBSSI that promotes the formation of amylase. All these findings suggest that OsGUN4 plays vital roles in the biosynthesis of transient starch in leaves.

Materials And Methods

Plant materials

The following materials were used in the present study: wild-type (Longtepu B, LTB) and its gamma ray-induced xantha mutant line Huangyu B (HYB) [20]. The epigenetic mutation of OsGUN4, gun4epi, underlies the xantha phenotype of HYB [19].

After germination, seedlings were raised by growing on soil at 30°C for 35 days under 16 h/8 h light/dark and at low light intensity (LL) of 100 μmol photons m−2 s−1. Above seedlings grown under the LL condition for 28 days were then transferred to 1 × MS liquid medium [29] containing 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc + NF) for another 24 hours. The samples at midday were collected for assays for carbon metabolites, enzyme assays and quantitative real-time PCR (RT-qPCR).

Analysis of metabolites

Determination of amylase, starch, and protein were performed as described previously [30]. Sucrose, fructose, and glucose were analyzed using the methods described previously [31].

Enzyme activity assays

0.1 g samples were homogenized in ice-cold buffer [50 mM HEPES-NaOH, pH 7.4, 2 mM MgCl2, 50 mM mercaptoethanol, 12.5% (v/v) glycerol], and the homogenate was then centrifuged at 20000 × g for 10 min at 4 °C. The supernatants were used for enzyme activity assays according to the manufacturer’s instructions with the plant enzyme-linked immunosorbent assay (ELISA) kits (Mlbio, Shanghai) as follows: sucrose synthase (SS, NO. mi077397), sucrose phosphate synthase (SPS, NO. mi062647), ADP-Glc pyrophosphorylase (AGPase, NO. mi076671), granule-bound starch synthase (GBSS, NO. mi076667), soluble starch synthase (SSS, NO. mi076670), starch branching enzyme (SBE, NO. mi076664).

Quantitative real-time PCR analysis

Total RNA was extracted from tissues using the Qiagen Spin Plant RNA Mini Kit (Qiagen, Hilden, Germany). The first-strand cDNA was made by reversely transcribing 1 μg total RNA using oligo-dT18 Primer and GoScript™ Reverse Transcription System Kit (Promega, WI, U.S.A.) according to the manufacturer’s instructions. Quantitative RT-PCR was performed using a SYBR Green GoTaq® qPCR Master Mix containing ROX as internal control (Promega, WI, U.S.A.) and an ABI 7300 sequence detection system (Applied Biosystems). All RT-PCRs used the following cycling conditions: 10 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. Relative gene expression was calculated in relation to the rice Ubiquitin gene using the 2−ΔΔCt method [32].

RNA sequencing analysis

RNA extracted from seedlings of 35 DAG were used for the construction of cDNA libraries, which were subsequently sequenced on an Illumina Hiseq 2000 platform (Beijing Novogene Bioinformatics Technology Co., Ltd. Beijing, China). For mapping, the raw reads were cleaned by removing adapter sequences and then aligned to the references genome sequences (www.gramene.org) by using the Tophat v2.0.9 program with E-value < 10−5 as cut-off point [33]. For detection of differentially expressed genes (DEGs), the DESeq package (ver 2.1.0) was used with a false discovery rate (FDR) ≤ 0.005 and the absolute value
of the log2 (fold change) with RPKM $\geq 1$ as the threshold to determine significant differences of gene expression. For DEG analysis, gene ontology (GO) enrichment were conducted by the GOseq R package with $P \leq 0.05$ and functionally classified by WEGO software, while KEGG pathways were analyzed with a FDR $\leq 0.05$ as significant levels of differential expression.

**Accession numbers**

Genes investigated on transcription is were identified through homolog search of the following databases: The Rice Annotation Project (RAP) Database (https://rapdb.dna.affrc.go.jp/) and GenBank/EMBL database (https://www.ncbi.nlm.nih.gov/). These genes were subjected to RT-PCR analysis by using gene-specific primers (Additional file 8: Table S4).

**Statistical analysis**

All experiments were performed with six independent biological repeats. Values were expressed as means ± standard deviations and analyzed using two-way ANOVA test followed by the Tukey's Multiple Comparison Test with $P < 0.05$.

**Declarations**

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Contributions

R.L. and H.Z. conceived the study. R.L., M.J., and H.Z. carried out the experimental analysis data analysis. R.L., H.Z. and M.J. finished the first draft, and R.L. finished the final draft. All authors reviewed and approved the final manuscripts.

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**Ethics declarations**

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have agreed to the publication of this manuscript.
Availability of data and material
The dataset supporting the conclusions of this article are included within the article and its additional files.

Competing interests
The authors declare no competing financial interests.

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Figures

**Figure 1**

Contents of metabolites related to starch biosynthesis. The contents of (a) sucrose, (b) glucose, (c) fructose, (d) total starch, (e) amylose and (f) protein were investigated in leaves of 35 days after germination (DAG) wild-type (black bar) and gun4epi (blank bar) seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF).

**Figure 2**

Activities of enzymes involved in starch biosynthesis. Activities of (a) sucrose synthase (SS), (b) sucrose phosphate synthase (SPS), (c) ADP-Glc pyrophosphorylase (AGPase), (d) granule-bound starch synthase (GBSS), (e) soluble starch synthase (SSS) and (f) starch branching enzyme (SBE) were analyzed in leaves of 35 DAG wild-type (black bar) and gun4epi (blank bar) seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF).
Figure 3

Different-expressed genes involved in starch biosynthesis by RNAseq in 35 DAG leaves. (a) Metabolism overview maps showing differences in transcript levels between gun4epi and wild-type. (b) Metabolism overview maps showing transcriptional differences for genes involved in starch biosynthesis between gun4epi and wild-type. log2 fold change ratios for average transcript abundance were calculated based on three replicates of WT (Longtepu B, LTB) and the epigenetic gun4 mutant gun4epi (Huangyu B, HYB). The resulting file was loaded into the MapMan Image Annotator module to generate the metabolism overview map. On the logarithmic color scale, red represents downregulated and blue represents upregulated transcripts.
Figure 4

Expression levels of the genes involved in sucrose biosynthesis. Expression of representative genes involved in sucrose, glucose and fructose biosynthesis in leaves of wild-type (black bar) and gun4epi (blank bar) at 35 DAG seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF). Abbreviations: SPS, Sucrose-phosphate-synthase; SUS, Sucrose synthase; FrK, Fructokinase; HxK, Hexokinase.
Figure 5

Expression levels of the genes involved in starch biosynthesis. Expression of representative genes involved in ADP-glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSS), soluble starch synthase (SS) and starch branching enzyme (BE) in leaves of wild-type (black bar) and gun4epi (blank bar) at 35 DAG seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF). Abbreviations: AGPL, ADP-glucose pyrophosphorylase large subunit; AGPS, ADP-glucose pyrophosphorylase small subunit; SSII, IV, V, Soluble starch synthase II, IV, V; BE II, III, Starch branching enzyme II, III.

Figure 6

Transcriptional levels of OsbZIP58 in leaves. Expression levels of gene encoding transcriptional factors of bZIP58 in wild-type (black bar) and gun4epi (blank bar) at 35 DAG seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF).

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