Mutation of OsGUN4 Uncoupled the Sugar-dependent Signals to Regulate Starch Biosynthesis in Rice

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Abstract

Background

Starch biosynthesis requires plastid-to-nucleus signals to ordinate the flow of carbon, which is partly mediated by tetrapyrrole intermediates. We previously revealed that mutation of the Genomes Uncoupled 4 (OsGUN4) would affect tetrapyrrole intermediates, but the underlying mechanisms for regulatory roles of OsGUN4 on starch biosynthesis remains largely unknown.

Results

In this study, we revealed that the OsGUN4 mutation not only retarded the carbon flow from sucrose to starch but also disabled the sensitive response to exogenous feeding sucrose. Moreover, extra addition of norflurazon (NF) would aggravate insensitivity to the sucrose-dependent induction in gun4epi, especially exhibiting collapse declines of the starch biosynthetic enzymes’ activities. However, genes encoding starch synthetic enzymes performed discordance with the activities of starch biosynthesis associated enzymes upon on the scarce expression of OsGUN4 in gun4epi. These results indicated that OsGUN4 played regulatory roles on biosynthetic genes and enzyme activity in starch biosynthesis. Furthermore, we also concluded that \(^1\text{O}_2\) derived from GUN4/protoporphyrin IX (proto) might be responsible for the sugar-dependent signals to regulate starch biosynthesis, due to positive correlations between the singlet oxygen (\(^1\text{O}_2\)) and many starch biosynthetic genes that were subjected to the control of three reported transcriptional factors (TFs) during starch biosynthesis. Eventually, the OsGUN4 mutation would also relieve the repression of glucose on Snf1-related protein kinases (SnRK1) but seem to negatively mediate the functioning of ADP-Glc pyrophosphorylase (AGPase).

Conclusion

In summary, we demonstrated that OsGUN4 serve as a broker to activate \(^1\text{O}_2\)-mediated signals in the sugar signaling cascade, possibly functioning upstream through TFs, and that OsGUN4 played roles in the SnRK1A-mediated signals, partly through the accumulations of sugars, e.g. glucose or sucrose.

Background

Plants assimilate atmospheric CO\(_2\) during photosynthesis using light energy to produce sugars and chemical energy (ATP) for plant growth (Graf and Smith 2011). 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 (Sulpice et al. 2009). 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 (Smith and Stitt 2007; Sitt and Zeeman 2012). Besides, transient starch is photosynthetic synthesized during the day to provide carbon and energy under inactive photosynthesis (Bahaji et al. 2014).

Leaf starch mainly accumulates in the photosynthetic palisade and mesophyll (M) cells (Tsai et al. 2009; Van Bel 2003; Geiger 2011), and major mesophyll cells in mature leaves are source for sucrose transport into sink tissues (Fig. 1). Moreover, plastids of the photosynthetic organs are responsible for the temporarily synthesize of starch in leaves (Bahaji et al. 2014). 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 (Enami et al. 2011; Lopez-Juez and Pyke 2005). 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 (Jarvis and Lopez-Juez 2013; Lyska et al. 2013). Thus, the establishment of plastid multi-subunit protein complexes need a tight cooperation between nucleus and plastid genes (Pogson et al. 2015). Besides, high morphological and functional diversity of plastids in different tissues of multicellular plants are tightly connected to the function of the corresponding tissue (Lopez-Juez and Pyke 2005), 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 (Chan et al. 2016). The GUN (genome uncoupled) proteins were identified for plastid-to-nucleus signaling studies (Susek et al. 1993). Thereinto, GUN4 have been found to be involved in the retrograde signaling pathway in Arabidopsis (Larkin et al. 2003) and rice (Li et al. 2017). Besides, the mutation of OsGUN4 in rice have also been revealed to deregulate transcription of photosynthesis-associated nuclear genes (PhANGs) depending on disruption of singlet oxygen ($^{1}$O$_{2}$)-induced signaling pathway (Li et al. 2017). This model suggested that accumulation of heme in active chloroplast can activate a mechanism to induce the expression of PhANGs (Larkin 2016). Interestingly, the plastid-to-nucleus retrograde signals is also revealed to regulate expression of nuclear starch biosynthetic genes, which is partly mediated by tetrpyrrole intermediates, i.e., heme (Enami et al. 2011). Besides, the mutation of OsGUN4 in rice have also been revealed to greatly affect tetrpyrrole intermediates, including heme, protoporphyrin IX (proto) and Mg-Proto (Li et al. 2017). 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 greatly deregulated biosynthesis of tetrpyrrole intermediates and functioning in $^{1}$O$_{2}$-induced signaling pathway in rice (Li et al. 2014; Li et al. 2017). Here, we further employed the rice epi-genetic mutant gun4$^{epi}$ to examine carbon metabolites, starch biosynthetic enzymes, genes involved in starch biosynthesis and eventually plan to disclose the roles of OsGUN4 on starch biosynthesis during vegetative stages. In conclusion, these findings would confirm that OsGUN4 plays regulatory roles in starch biosynthesis.
Methods

Plant Materials

The following materials were used in this study: wild-type (Longtepu B, LTB) and its gamma ray-induced xantha mutant line Huangyu B (HYB), which was underlied by the epigenetic mutation of OsGUN4, gun4epi (Zhou et al. 2006; Li et al. 2014).

After germination, seedlings were grown on soil at 30 °C for 34 days under 16 h/8 h light/dark and at low light intensity (LL) of 100 μmol photons m⁻² s⁻¹ and were then transferred to 1× MS liquid medium (Murashige and Skoog 1962) containing 200 μM exogenous sucrose (exSuc) or 10 μM norflurazon (NF) plus with 200 μM exogenous Suc (exSuc+NF) for another 24 hours. Above samples at 35 days after germination (DAG) were collected at midday for further assays of carbon metabolites, enzyme activities, proto, singlet oxygen and quantitative real-time PCR (RT-qPCR).

Quantification of Proto

Briefly, 0.3 g fine powder from grinded leaves were immediately mixed with pre-cold alkaline acetone containing 0.1 N NH₄OH (9:1; v/v), and centrifugated at 16,000 × g for 5 min. Subsequently, the supernatants were collected for extraction of Proto (Papenbrock and Grimm 2001). The concentrations of proto was determined using 0.3 g fresh leaf tissues with the commercial enzyme linked immunosorbent assay kits (ELISA) method following the manufacturer's instructions.

Determination of Singlet Oxygen Contents

The concentrations of ¹⁰₂ was determined using the SOSG (singlet oxygen sensor green) method (Hideg et al. 2002) with 300 mg leaf samples. The fluorescence spectra were detected at excitation of 485 nm and emission of 520 nm using a fluorescence spectrophotometer (359S, Lengguang Tech., China).

Analysis of Metabolites

The concentrations of amylose, starch, and protein were determined with the methods described previously (Han et al. 2012). Sucrose, fructose, and glucose were analyzed following the methods described previously (Tang et al. 2016).

Determination of Enzyme Activity

Activities of enzymes, including sucrose synthase (SS), sucrose phosphate synthase (SPS), ADP-Glc pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SSS) and starch branching enzyme (SBE), were determined by using the commercial enzyme-linked immunosorbent assay (ELISA) kits (Mlbio Tech., China). Briefly, after homogenized in ice-cold buffer [50 mM HEPES-NaOH, pH 7.4, 2 mM MgCl₂, 50 mM mercaptoethanol, 12.5% (v/v) glycerol] with 100 mg leaf
tissues, the homogenate was then centrifuged at 20000 × g for 10 min at 4 °C, and the supernatants were sequentially used for enzyme activity assays according to the manufacturer’s instructions.

Quantitative Real-time PCR Analysis

Quantitative real-time PCRs were performed with the methods as previously described (Li et al. 2017). Relative gene expression was calculated in relation to the rice Ubiquitin gene using the 2ΔΔCt method (Livak and Schmittgen 2001). Genes were subjected to RT-PCR analysis by using gene-specific primers (Additional file 1: Table S1).

RNA Sequencing

The cDNA libraries were constructed with RNA extracted from seedlings of 35 DAG and sequenced on an Illumina Hiseq 2000 platform (Beijing Novogene Bioinformatics Technology Co., Ltd. Beijing, China). For mapping, after removing adapter sequences from the raw reads, the Tophat v2.0.9 program (Trapnell et al. 2012) with E-value ≤ 10^{-5} as cut-off point was employed to align the cleaned data to the reference’s genome sequences (www.gramene.org). Simultaneously, 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 ≥1 as the threshold to detect differentially expressed genes (DEGs). Besides, the GOseq R package with P ≤ 0.05 and WEGO software were used to conduct gene ontology (GO) enrichment, while KEGG pathways were employed for DEG analysis with a FDR ≤ 0.05 as significant levels.

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/).

Statistical Analysis

Values were expressed as means ± standard deviations (n=6) and analyzed using two-way ANOVA test followed by the Tukey’s Multiple Comparison Test with P < 0.05.

Results

Mutation of OsGUN4 Performed Aberrant Starch Metabolism

Previous studies revealed the positive effects of OsGUN4 mutation on photosynthetic capacity during vegetative stages (Zhou et al. 2006; Wu et al. 2007), but no detail was focused on the relationship between photosynthetic products, e.g. 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 seedlings (Fig. 2, Fig. 3, Additional file 1: Table S2 and Table S3). Compared to the wild-type, both of the sucrose and amylose contents were increased in
**Mutation of OsGUN4 Deregulated Activities of Starch biosynthetic Enzymes**

Dynamic activity changes of enzymes involved in starch biosynthesis were in accordance with the contents of carbon metabolites (Fig. 3, Additional file 1: Table S3). In consistent with the results as shown in Fig. 2, activities of AGPase, SSS and SBE showed significant increases, but activities of SS, SPS and GBSS were increased in gun4epi (Fig. 3).

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. 3). However, after exposed to exSuc+NF, activities of the related enzymes (Fig. 3), 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 detected between the control and the exSuc treatment in gun4epi, however, the sucrose added with NF treatment greatly affected the enzyme activities in relative to other treatments (Fig. 3). 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 DEGs were identified between gun4epi and WT by RNA-seq, with 203 genes being up-regulated and 265 down-regulated in gun4epi (Fig. 4a, Additional file 1: Table S4).

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. 4b, Additional file 1: Fig. S1), including genes for fructose-1,6-bisphosphate aldolase (FBA, OsFEA), fructose 1,6-bisphosphate (FBP), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), SPS, SPP, SS and GBSS (OsGBSSI), as well as genes for fructose and glucose synthesis, such as fructokinase (Frk) and hexokinase (HxK), SSS and SBE. Besides, genes for isoamylases (ISA), pollulanase (PUL) and \( \alpha \)-amylase (Amy3B, 3C, 3D), and representative genes for storage proteins, including protein disulfate isomerase (PDI), prolamin (CysR10), PPDKB, glutelins (GluA1, GluA2, GluA3), alanine aminotransferase (AlaAT1, AlaAT4), 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. 4b, Additional file 1: Fig. S1c-f). 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. 5–6, Additional file 1: Table S5). Compared to the wild-type, gene expression for sucrose synthesis, including genes for FBA (OsFEA), FBP (OsCFR, OsFBP2, OsFBP3), PGI (OsPGI), PGM (OspPGM), SPS (OsSPS2, OsSPS11), SPP (OsSPP1, OsSPP2), SuS (OsSUS1, 2, 5, 6, 7) and GBSS (OsGBSSI) were significantly increased (Additional file 1: Fig. S2-3), while genes for fructose and glucose synthesis, such as Frk (OsFrK1) and HxK (OsHxK1, OsHxK4 and OsHxK7), were greatly reduced in gun4epi (Fig. 5). Moreover, gene expression for AGPase (OsAGPS1), SS (OsSSIVa, OsSSV) and BE (OsBElla, OsBEllb) were also decreased in gun4epi compared to that in wild-type (Fig. 6 and Additional file 1: Fig. S2-3). Nonetheless, OsSUS4, OsAGPL1, OsAGPL3, OsAGPL4 and OsSSIIc showed increased expression in gun4epi (Fig. 5–6). All these results were consistent with the results as shown in Fig. 4b, 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. 5–6), whereas gene expression for APGase, SSS and SBE were little increased in the wild-type (Fig. 6). However, sucrose treatment induced no significant difference with the control in gun4epi (Fig. 5–6). 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. 5–6). All these results were consistent with the results as shown in Fig. 2 and Fig. 3, suggesting the regulatory role of OsGUN4 on expression of starch biosynthetic genes.
Effects of OsGUN4 Mutation on Some Reported Sugar-dependent signals

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 OsGUN4-mediated signals from plastid to nucleus for gene expression, the reported singlet oxygen- (Li et al. 2017) and sucrose-mediated (Lu et al. 2007) signals in rice were used for further investigation (Fig. 7, Additional file 1: Table S6). In WT, there were significant accumulation of $^{1}$O$_{2}$ after exSuc treatments in relation to LL, but no obvious difference was detected between exSuc and exSuc$^{+}$NF treatments (Fig. 7a). However, in gun4$^{epi}$, the accumulation of $^{1}$O$_{2}$ showed no changes after exSuc treatments, but additional NF made lower $^{1}$O$_{2}$ contents than that in WT, indicating that OsGUN4 might function in response to sucrose via $^{1}$O$_{2}$-mediated signals (Fig. 7a). Moreover, accumulations of proto in WT showed positive relation to the $^{1}$O$_{2}$ contents, whereas more proto accumulated in gun4$^{epi}$, especially after exposed to exSuc$^{+}$NF (Fig. 7b). These results suggested GUN4 and proto was essential for the generation of $^{1}$O$_{2}$-mediated signals.

Furthermore, to clarify the possible connection of $^{1}$O$_{2}$-mediated signals to TFs, three reported TFs, including starch biosynthetic bZIP58, and sucrose-dependent NAC36 and MYB14, were further investigated in seedlings. Expression of OsbZIP58 was significantly decreased in gun4$^{epi}$, but, after sucrose treatment, there was no obvious expression difference of bZIP58 in gun4$^{epi}$ (Fig. 7c). However, in wild-type, expression of bZIP58 was significantly induced by sucrose, while showed no obvious changes with CK after addition of NF (Fig. 7c). Moreover, exSuc induced enhanced the expression of NAC36 and MYB14, but additional NF greatly relieved these changes in WT (Fig. 7d and Additional file 1: Fig. S4). Nonetheless, in the absence of OsGUN4, expression of NAC36 and MYB14 performed no response to exSuc, and the phenomenon would be aggravated with supplement of NF in gun4$^{epi}$ (Fig. 7d and Additional file 1: Fig. S4). Thus, it can be seen that expression of bZIP58, NAC36 and MYB14 showed consistent trends with contents of $^{1}$O$_{2}$ and proto, suggesting the possible connection of $^{1}$O$_{2}$ and these three examined TFs.

However, genes encoding Snf1-related protein kinases (SnRK1s) and its down-stream TF of MYBS1, were significantly down-regulated after exSuc treatments in WT, but could be restored to higher expression with the additional NF (Fig. 7e and f). The similar phenomenon was also showed in gun4$^{epi}$, while the expression of SnRK1A and MYBS1 in gun4$^{epi}$ were greatly induced compared to that of WT under the exSuc$^{+}$NF treatments (Fig. 7e and f). These results indicated that no direct evidence suggested the connection of $^{1}$O$_{2}$-mediated signals to SnRK1A-mediated signals, but that GUN4 performed roles in the SnRK1A-mediated signals, possibly via the accumulations of sugars, e.g. glucose or fructose.

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 (Liebers et al. 2017). Despite displaying diverse and tissue-dependent functions, each differentiated form of plastid shares a set of genomes (Lopez-Juez and Pyke 2005). Chloroplasts are the location for photosynthesis and biosynthesis of transient starch (Bahaji et al. 2014). Also, the aberrant chloroplasts usually would cause abnormal photosynthesis and starch metabolism (Bahaji et al. 2014; Liebers et al. 2017). The OsGUN4 mutation performed aberrant chloroplast morphology as reported previously (Zhou et al. 2006), and also indeed reduced the accumulation of starch at here (Fig. 2). 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. 2), which is partly related to the positive effects of OsGUN4 mutation on photosynthetic capacity during vegetative stages (Zhou et al. 2006; Wu et al. 2007). On the other hand, this is due to the deregulated enzyme activities involved in starch biosynthesis (Fig. 3). For example, in gun4epi, the enhanced activities of SS and SPS was responsible for the accumulation of sucrose (Fig. 3), whereas the decreased AGPase, SSS and SBE activities made neglect effects on starch synthesis (Fig. 3). 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 (Ragel et al. 2013; Guo et al. 2017). 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 (Ragel et al. 2013; Guo et al. 2017). This is consistent with the results as shown in Fig. 4 and Fig. 6, which could also explain the enhanced AGPase 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. 2). This was due to the dynamic activity changes of enzymes involved in starch biosynthesis, which were in accordance with the contents of metabolites (Fig. 3).

Furthermore, NF treatment is usually used for explore the uncoupled phenomenon of PhANGs transcriptional levels from chlorophyll accumulation in Arabidopsis (Susek et al. 1993) and C. reinhardtii (Formighieri et al. 2012). Our previous studies also revealed that the mutation of OsGUN4 deregulate transcription of PhANGs depending on disruption of 1O2-induced signaling pathway in rice (Li et al. 2017). 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. 2-3, Additional file 1: Fig. S3-5). All these results suggested OsGUN4 functions in response to sugar signals during starch biosynthesis.

Roles of OsGUN4 in the Regulation of Starch Biosynthesis
Functioning of the plastids requires cooperation of plastid genes and nuclear genes, which could reach the balance of photosynthesis and starch biosynthesis (Chan et al. 2016). Although GUN4 have been revealed in the plastid-to-nucleus signaling pathway (Li et al. 2017), 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, Additional file 1: Fig. S5). 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 (Enami et al. 2011). 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 (Enami et al. 2011). In our previous studies, the OsGUN4 mutation greatly affect tetrapyrrole intermediates, including heme, Mg-Proto and Proto in rice (Li et al. 2017). The blocking of photosynthesis and starch biosynthesis in gun4epi also illustrated this from Suc added with NF treatment (Fig. 1-3, Additional file 1: Fig. S3-5), suggesting the suppressive 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 (Xiao et al. 2017), MYB14 (Zhang et al. 2014). OsGUN4 is localized in plastid in our previous studies, so it is impossible for OsGUN4 to regulate gene expression as TFs in nucleus. Instead, OsGUN4 might function in regulation of genes participating in starch biosynthesis via transcription factors, e.g. bZIP58 (Fig. 7). 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 amylopectin and starch granules, while displayed up-regulated expression of OsSSIiia and OsGBSSI that promotes the formation of amylose (Fig. 3, Fig. 6 and Additional file 1: Fig. S3). Thus, the retardative transformation from sucrose to starch mostly depends on the deregulated expression of genes for starch biosynthetic enzymes.

OsGUN4 Might Serve as a Broker of the Sugar-dependent Signals to Regulate Starch Biosynthesis

Sugars not only play key roles in metabolism and structural constituents of plant cells but also serve as essential components of signaling pathway in sugar responses (Rolland et al. 2006; Lu et al. 2007). Here, as shown in above, the OsGUN4 mutation led to accumulation of sucrose (Fig. 2), attributing to the seriously shrinking activities of starch biosynthetic enzymes, especially for AGPase (Fig. 3), which is the key regulatory enzyme of starch biosynthesis (Tiessen et al. 2003). The reduced activity of AGPase would retard the carbon flow from sucrose to starch in the scarce presence of OsGUN4 (Fig. 8). This could be concluded from results of exSuc^+NF in Fig. 3. Although extra NF caused the decreased AGPase activity in both of WT and gun4epi, while it made the much more seriously downregulated activities of AGPase in gun4epi (Fig. 3). However, genes encoding the large subunit of AGPase, e.g. AGPL1, AGPL3 and AGPL4,
performed enhanced expression in \textit{gun4epi} compared to wild-type (Fig. 6), displaying the uncoupled phenomenon that the inconsistent performance of chloroplast and nucleus. Correspondingly, many genes for SS, SPS, SSS and SBE also showed similar phenomenon (Fig. 5-6 and Additional file 1: Fig. S3), indicating OsGUN4 might be an important intermediate in the signaling cascade to regulate starch biosynthesis.

Many transcription factors have been reported to mediate sucrose signals during starch biosynthesis in cereal crops, e.g. bZIP58 (Wang et al. 2013; Kim et al. 2017), NAC36 (Xiao et al. 2017), MYB14 (Zhang et al. 2014). Interestingly, all of them were suggested to be as the regulator of genes encoding AGPase in rice (Wang et al. 2013) or in maize (Xiao et al. 2017; Zhang et al. 2014). Thus, genes encoding bZIP58, as well as genes for the orthologs of NAC36 and MYB14 in rice, were also investigated at here (Fig. 7 and Additional file 1: Fig. S4). All of bZIP58, NAC36 and MYB14, not only showed insensitive response to the sucrose but also would be aggravated immunity to sucrose with additional NF in \textit{gun4epi} (Fig. 7d and Additional file 1: Fig. S4). Correspondingly, downstream genes of the examined TFs, including \textit{AGPL2, SSI, AGPS1} and \textit{OsBEIIb}, also displayed similar expression trends (Fig. 6). Therefore, we can conclude that OsGUN4 plays regulatory roles in starch biosynthesis, possibly via TFs, but the details of OsGUN4 mediated mechanism underlying sugar-regulated transcription remain mostly unclear (Fig. 8).

The tetrapyrrole intermediates, e.g. heme, proto, have been reported to be functions as constitutives for signals from chloroplast to nucleus (Larkin, 2016; Tabrizi et al. 2016). We also previously reported one light-dependent singlet oxygen-mediated signal for regulating PhANGs in rice, attributing to the collision of GUN4 and proto (Li et al. 2017). Here, we also demonstrated that \textsuperscript{1}O\textsubscript{2} derived from GUN4 and proto might be also responsible for the sugar-dependent signals to regulate starch biosynthesis. On the one hand, accumulations of proto in WT showed positive relation to the \textsuperscript{1}O\textsubscript{2} contents, whereas more proto accumulated in \textit{gun4epi}, especially after exposed to exSuc\textsuperscript{+NF} (Fig. 7b), suggesting GUN4 and proto was essential for the generation of \textsuperscript{1}O\textsubscript{2}-mediated signals. On the other hand, expression of \textit{bZIP58, NAC36} and \textit{MYB14} showed positive and consistent trends with contents of \textsuperscript{1}O\textsubscript{2} and proto (Fig. 7), indicating the possible connection of \textsuperscript{1}O\textsubscript{2} and these three examined TFs.

Additionally, SnRK1A functions upstream from the interaction between MYBS1 and αAmy3 in the sugar signaling cascade in rice (Lu et al. 2007). In this study, after exposed to exSuc, genes encoding SnRK1A and MYBS1 were significantly down-regulated both in WT and \textit{gun4epi} mutant, but could be restored to higher expression with the additional NF (Fig. 7e and f), with the decreased sucrose contents but accumulated glucose (Fig. 2). The repression of glucose on SnRK1A was consistent with the results as shown in Lu et al. 2007. However, the expression of SnRK1A and MYBS1 were greatly induced in \textit{gun4epi} compared to that of WT under the treatment of exSuc\textsuperscript{+NF} (Fig. 7e and f), depending on the greatly decreased glucose (Fig. 2). Nonetheless, feeding of sucrose have been reported to prevent AGPase redox inactivation in potato tubes (Tiessen et al. 2003), and we here also found that feeding of sucrose did cause the enhanced AGPase in WT, but it seemed to be diminished in \textit{gun4epi} mutant, especially after the
additional feeding of NF (Fig. 3). Thus, it can be concluded that GUN4 also performed roles in the SnRK1A-mediated signals, possibly via the accumulations of sugars, e.g. glucose or sucrose (Fig. 8).

**Conclusion**

In summary, we demonstrated that OsGUN4 serve as a broker to activate $^1O_2$-mediated signals in the sugar signaling cascade, functioning upstream from three examined TFs, e.g. bZIP58, NAC36, MYB14, and that OsGUN4 performed roles in the SnRK1A-mediated signals, possibly through the accumulations of sugars, e.g. glucose or sucrose. Consequently, OsGUN4 plays regulatory roles in starch biosynthesis via mediating biosynthetic gene expression and enzyme activity.

**Abbreviations**

- **GUN4**: Genome Uncoupled 4; **Chl**: chlorophyll; **ELISA**: enzyme linked immunosorbent assay; **HYB**: Huangyub B; **LL**: low light; **LTB**: Longtepu B; **MgCh**: Mg-chelatase; **NF**: norflurazon; **$^{1}O_2$**: singlet oxygen; **Proto**: protoporphyrin IX; **RT-qPCR**: quantitative real-time PCR; **TFs**: transcriptional factors; **WT**: wild-type; **AGPase**: ADP-Glc pyrophosphorylase; **AGPL**: ADP-glucose pyrophosphorylase large subunit; **AGPS**: ADP-glucose pyrophosphorylase small subunit; **AlaAT**: alanine aminotransferase; **Amy**: Alpha-amylase isozyme; **BE**: branching enzyme; **DAG**: days after germination; **DEG**: differentially expressed gene; **FBA**: fructose-1,6-bisphosphate aldolase; **FBP**: fructose 1,6-bisphosphate; **FDR**: false discovery rate; **FLO**: FLOURY ENDOSPERM; **Frk**: fructokinase; **GBSS**: granule-bound starch synthase; **HxK**: hexokinase; **ISA**: isoamylases; **PDI**: protein disulfate isomerase; **PGI**: phosphogluconeisomerase; **PGM**: phosphoglucomutase; **PhANGs**: photosynthesis-associated nuclear genes; **PUL**: pollulanase; **RSR**: RICE STRACH REGULATOR; **SBE**: starch branching enzyme; **SnRK1s**: Snf1-related protein kinases; **SS**: sucrose synthase; **SSS**: soluble starch synthase; **SPP**: sucrose phosphate phosphatase; **SPS**: sucrose phosphate synthase; **SuS**: sucrose synthase; **SUT**: Sucrose transporter; **UGP**: UDP-glucosepyrophosphorylase.

**Declarations**

**Ethics approval and consent to participate**

The authors declare that the experiments were performed in compliance with the current laws of China.

**Consent for publication**

All authors are consent for publication.

**Availability of data and material**

All data generated or analyzed during this study are included in this published article and its supplementary files.
**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors' contributions**

RL and HZ conceived the study. RL, MJ, and ML carried out the experimental analysis data analysis. RL and HZ finished the first draft, and RL finished the final version.

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**Figures**
Figure 1

Schematic diagram of starch biosynthesis in leaves. The blue typefaces represent the enzymes of starch biosynthesis pathway in rice. Enzymes involved in steps for cytosol and plastids are shown in yellow and purple, respectively. Abbreviations: F2BA, fructose-1,6-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; F2BP, fructose 1,6-bisphosphatase; F6P, fructose-6-phosphate; PGI, phosphoglucoseisomerase; G6P, glucose-6-phosphate; PGM, phosphoglucomutase; G1P, glucose-1-phosphate; UGP, UDP-glucose pyrophosphorylase; UDPG, UDPglucose; SPS, sucrose-phosphate-synthase; Suc-P, sucrose-phosphate; SPP, sucrose phosphate phosphatase; Suc: sucrose; Glu: glucose; Fru: Fructose; A/N-inv, alkaline/neutral invertase; SS, sucrose synthase; FrK, Fructokinase; pHK, plastidial hexokinase; BT1, Brittle 1; AGP, ADP-glucose pyrophosphorylase; ADPG, ADPglucose; Pho1: Plastidial
starch phosphorylase; DPE: Disproportionating enzyme; BE, Branching enzyme; LD, Linear dextrin; BG, Branched glucan; GBSSI, granule bound starch synthase I; BEI, Branching enzyme; SS: soluble starch synthase; ISA1, Isoamylase 1.
Contents of metabolites related to starch biosynthesis. The contents of sucrose, glucose, fructose, total starch, amylose and 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 3

Activities of enzymes involved in starch biosynthesis. Activities of sucrose synthase (SS), sucrose phosphate synthase, ADP-Glc pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SSS) and 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 4

Different-expressed genes involved in starch biosynthesis by RNAseq in 35 DAG leaves. Metabolism overview maps showing differences in transcript levels (a) for all genes and (b) for genes involved in starch biosynthesis between gun4epi and wild-type. Metabolism overview maps showing transcriptional
differences 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 5

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; SS, Sucrose synthase; FrK, Fructokinase; HxK, Hexokinase.
Figure 6

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, ADPG large subunit; AGPS, ADPG small subunit; SSII, IV, V, Soluble starch synthase II, IV, V; BE II, III, Starch branching enzyme II, III.
Figure 7

Effects of OsGUN4 mutation on reported sucrose-dependent signals. Concentration of (a) singlet oxygen (1O₂) and (b) proto, as well as expression levels of (c) bZIP58, (d) NAC36, (e) SnRK1A and (f) MYBS1 in wild-type and gun4epi at 35 DAG seedlings treated without (check, CK) or with 200 μM sucrose (Suc) or Suc plus with 10 μM norflurazon (Suc+NF).
Figure 8

Putative models for regulation of starch biosynthesis involved in OsGUN4. Feeding with sucrose would promote the carbon flow to starch biosynthesis, requiring the sugar-mediated signals from chloroplast to nucleus (Lu et al. 2007). Simultaneously, plants would slow down the chlorophyll biosynthesis to accumulate some intermediates, e.g. proto, heme, for the activation of plastid-to-nucleus signals (Li et al. 2017; Fig. 7). In particular, OsGUN4/proto serves as a broker to generate singlet oxygen-dependent signals (Fig. 7), which would possibly work upstream of transcriptional factors, e.g. bZIP58, NAC36, MYB14 (Fig. 7) in the sugar signaling cascade and hence mediate the enzyme activities (Fig. 3) via regulating gene in transcriptional levels (Fig. 5-6). Beyond this, OsGUN4 performed roles in the SnRK1A-mediated signals, possibly via the accumulations of sugars, e.g. glucose or fructose (Fig. 2). For example, feeding of sucrose suddenly led to the increases of glucose, which would repress SnRK1A and its downstream MYBS1 and AMY3. Nonetheless, feeding of sucrose did cause the enhanced AGPase with unknown mechanisms.

Supplementary Files

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