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Effects of knockout of CO₂-responsive CCT protein, a regulator of starch synthesis on physiological characteristics of rice

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ABSTRACT

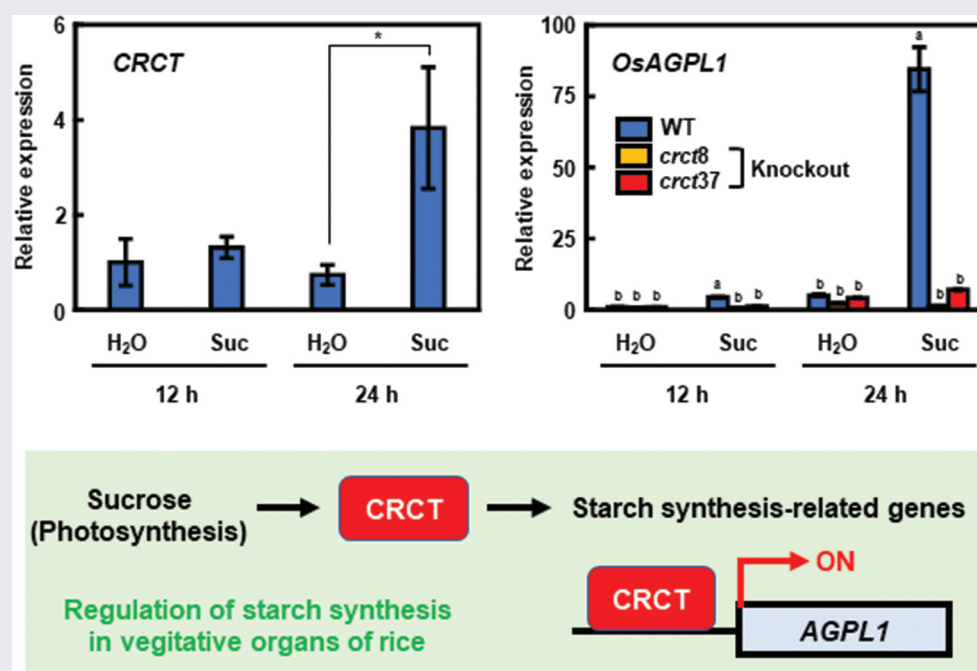
CO₂-Responsive CCT Protein (CRCT) is suggested to be a positive regulator of starch synthesis-related genes in the vegetative organs of rice. To investigate the physiological function of CRCT, we produced CRCT knockout lines by CRISPR/Cas9 system. CRCT knockout tended to slightly reduce photosynthetic performance and had some adverse effects on growth parameters such as tiller number and straw dry weight. The starch content in the leaf sheath of CRCT knockout lines was significantly lower than that of wild-type. qRT-PCR and RNA-seq analysis showed that sucrose treatment induced the expression of *CRCT*, which, in turn, induced many starch synthesis-related genes in wild-type, whereas this induction did not occur in CRCT knockout lines. In conclusion, our results suggest that sugar induction of starch synthesis-related genes is almost fully dependent on CRCT, making it a necessary factor for normal photosynthesis and growth of rice.

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CCT Protein; growth; photosynthesis; Rice (*Oryza sativa*); sugar response; starch



1. Introduction

Rice (*Oryza sativa* L.) is a staple crop addressing a large proportion of the global food demand. Improvement of rice yield and quality is crucial to meet the ever-

increasing global food demand accompanied by growing world's population. In plants including rice, starch is a major photosynthate and is highly accumulated as a storage polysaccharide in the reproductive organs

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such as seeds. Starch is also stored transiently in vegetative organs after photosynthesis (Stiff & Zeeman, 2012). In rice, starch is highly accumulated in the leaf sheath and culm (stem) during the vegetative stage and translocated to grain at the ripening stage (Perez et al., 1971). No less than 30% of grain carbon is derived from this carbon translocation (Cook & Yoshida, 1972), which can be particularly important for normal ripening under limited solar radiation conditions (Okamura et al., 2013). A greater capacity of starch accumulation in the stem can prevent grain chalkiness under high temperature (S. Morita & Nakano, 2011). Thus, starch accumulation in the stem is important to stabilize rice yield and quality.

Starch is a carbohydrate polymer composed of amylose and amylopectin. Amylose consists of a long α -1,4-linked linear glucose chain, which has a very small number of α -1,6-linked branch points. In contrast, amylopectin consists of relatively short α -1,4-linked glucose chains and many α -1,6-linked branch points. Starch biosynthesis is processed by various kinds of enzymes (Fujita et al., 2022). ADP-glucose pyrophosphorylase (AGPase) is a key starch synthesis-related enzyme that catalyzes the rate-limiting reaction to change glucose-1-phosphate into ADP-glucose. After the synthesis of ADP-glucose, a fine structure of amylopectin is constructed by coordinated actions of multiple isozymes of starch synthases (SS), starch branching enzymes (BE), and starch debranching enzymes (DBE). In detail, SSs elongate α -1,4-linked linear glucose chains, BEs generate α -1,6-linked branch points, and DBEs trim off inappropriate branches. In contrast to amylopectin, amylose is synthesized by only one kind of enzyme, granule-bound starch synthase (GBSS). Besides these enzymes, α -glucan phosphorylase (Pho) functions in the initiation step of α -1,4-linked glucan formation (Nakamura et al., 2012).

In addition to these enzymes involved in starch synthesis, its transcriptional regulators have also been identified. For example, the APETALA2/Ethylene-Responsive Element-Binding Protein family transcription factor, Rice Starch Regulator1 (RSR1) negatively regulates the expression of starch synthesis-related genes in seeds (Fu & Xue, 2010). Also, the rice DNA-binding protein OsBP-5 interacts with the ethylene-responsive element-binding protein OsEBP-89 down-regulating the expression of *GBSSI* (Zhu et al., 2003). By contrast, FLOURY ENDOSPERM2 (FLO2), which has a tetratricopeptide repeat (TPR) motif, can positively affect the expression of *GBSSI* and *BEI* (She et al., 2010). In addition, the basic leucine zipper (bZIP) transcription factor, OsbZIP58, directly binds to the promoters of some starch synthesis-related genes

(*OsAGPL3*, *GBSSI*, *OsSSIIa*, *BEI*, *BEIIb* and *OsISA2*) (J. C. Wang et al., 2013). More recently, the NAC transcription factors, OsNAC20 and OsNAC26, were reported to regulate the expression of *SSI* and *pullulanase* (*Pul*) and seed storage proteins (J. Wang et al., 2020). As an example of vegetative organs, it was reported that BioMass Yield1 and BioMass Yield3 (PvBMY1 and PvBMY3) positively regulate photosynthesis and starch synthesis, determining the biomass yield in switch grass (Ambavaram et al., 2018). However, compared to reproductive organs, information on the regulation of starch synthesis-related genes in vegetative organs is limited.

Recently, we identified a novel transcriptional regulator of starch synthesis-related genes, designated CO₂-responsive CCT protein (CRCT), which is significantly upregulated under elevated CO₂ conditions where the starch level increases substantially in vegetative organs of plants (R. Morita et al., 2015). CRCT controls the expression of starch synthesis-related genes, such as *AGPase* and *BEI*. Accordingly, *CRCT* overexpression lines accumulated more starch in the leaf sheaths than wild-type, while *CRCT* knockdown lines accumulated less starch. Recently, chromatin immunoprecipitation analysis showed that CRCT can directly bind to the promoter regions of the starch synthesis-related genes (Fukayama et al., 2021). In addition, increased starch accumulation in the vegetative organs of *CRCT* overexpression lines resulted in higher photosynthesis capacity when grown under elevated CO₂ conditions (R. Morita et al., 2016). Moreover, the expression level of *CRCT* affects the level of short chains with a degree of polymerization (DP) from 5 to 14 (R. Morita et al., 2019). These results suggest that CRCT can control not only quantity of starch but also its structure in the vegetative organs.

In this study, we produced CRCT knockout lines by the CRISPR/Cas9 system to better evaluate the physiological and molecular function of CRCT. It was found that CRCT knockout strikingly decreased the starch content of the leaf sheath and negatively affected the growth. In addition, expression analysis was performed to clarify the relationship between sucrose-inducible expression of CRCT and starch synthesis-related genes. Our results revealed that CRCT plays a central role in sucrose induction of starch synthesis-related genes in rice.

2. Materials and methods

2.1. Plant material and growth condition

Wild-type rice (*Oryza sativa* L. cv. Nipponbare) and its CRCT knockout lines *crct8* and *crct37* (Fukayama et al., 2021) were grown in paddy soil under natural

light and temperature conditions in a temperature-controlled greenhouse (30°C day/25°C night). On 19 July 2019 (two weeks after sowing), rice seedlings at the 4.5 leaf stage were transplanted into 1 L pots supplemented with 2.0 g chemical fertilizer (N:P:K = 8:8:8), 0.5 g slow-release fertilizer, 0.4 g micronutrient and 1.5 g silicate fertilizer. After transplanting, plant heights, leaf ages, and tiller numbers were measured every week (from July 26th). On 100 days after sowing, panicle and straw were harvested and dried at 50°C and 80°C for 3 days, respectively. Then, its panicle and straw dry weights were measured.

For determination of leaf constituents and carbohydrates, the eighth leaf blades and leaf sheaths at 11.0 leaf stage at 66 days after sowing were sampled at 2 hours before the end of day (daytime 5:00–21:00 controlled by artificial light at evening). For qRT-PCR and RNA-Seq analyses, seventh leaf blades of wild-type and CRCT knockout lines at 7.2 leaf stage were detached before dawn, immersed in water or 50 mM sucrose solution and sampled at 12 h and 24 h. These samples were frozen in liquid nitrogen and stored at –80°C until use.

2.2. SDS-PAGE and immunoblotting

The soluble protein was extracted from leaf sheath as described previously (Fukayama et al., 2021). The extracted proteins were separated by SDS-PAGE (12%) and subjected to immunoblotting using antiserum raised against CRCT (R. Morita et al., 2019). Immunoreacted bands were visualized using ImmunoStar LD (Fujifilm Wako, Osaka, Japan) and exposed to X-ray films as described previously (Fukayama et al., 2021).

2.3. Measurement of photosynthetic parameters

The CO₂ gas exchange rate was measured with an open gas exchange system (LI-6400, LI-COR, Nebraska). The measurements were carried out at a photosynthetically active photon flux density (PPFD) of 1,500 μmol m^{–2} s^{–1}, a leaf temperature of 25°C, O₂ concentration of 21% and a leaf-to-air vapor pressure difference (VPDL) of 0.85–1.2 kPa. The CO₂ response curve of CO₂ assimilation was obtained from a single leaf by changing the ambient CO₂ partial pressure from 45 to 1,000 μL L^{–1}. The maximum carboxylation rate (V_{cmax}) was calculated using the model of von Caemmerer and Farquhar (1981) using the initial slope of the CO₂ response curve. The maximum electron transfer rate (J_{max}) was calculated as described by Ishikawa et al. (2011).

2.4. Determination of Rubisco, soluble protein, and chlorophyll contents

The leaf blade was ground using a mortar and pestle in the extraction buffer (50 mM Hepes-KOH, 5 mM MgCl₂, 5 mM DTT, 1 mM EDTA, 4 mM amino-n-caproic acid, 0.8 mM benzamidine-HCl, 0.1% (w/v) PVPP, 0.05% (v/v) Triton X-100, 5% (w/v) glycerol, pH 7.5). A portion of this homogenate was collected and acetone was added to it with a final concentration of 80% (v/v) for measurement of chlorophyll content. The rest of the homogenate was centrifuged at 14,500 g for 5 min, and the supernatant was used for measurement of soluble protein and Rubisco contents.

Chlorophyll content was measured spectrophotometrically as described by Porra et al. (1989). Soluble protein content was measured by the method of Bradford (1976) with bovine serum albumin as the standard. Rubisco content was measured by assuming the stoichiometric binding of [¹⁴C] carboxyarabinitol bisphosphate as described by Ishikawa et al. (2011).

2.5. Determination of starch and soluble sugar contents

The leaf blades and leaf sheaths were ground in liquid nitrogen using a mortar and pestle. The extraction of carbohydrate was basically carried out as described in R. Morita et al. (2015). The soluble sugar content was measured by the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1951). The starch content was measured by the Iodine method using potato starch as a standard (Suzuki et al., 2013).

2.6. Qrt-PCR and RNA-seq analyses

The total RNA was isolated from rice tissues using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first strand cDNA was synthesized from the total RNA with an oligo (dT)₁₈ and a random hexamer as primers using the PrimeScript II first Strand cDNA Synthesis Kit (Takara, Kyoto, Japan). The qRT-PCR was carried out using gene-specific primers shown in Supplemental Table S1, TBgreen Premix Ex Tag GC (Takara), and a thermal cycler (MyGoPro, IT-IS Life Science, Dublin, Ireland). The expression of *Actin* was used as an internal control.

RNA-seq analysis was conducted using a mixture of equal amount of total RNA from leaf blades of two plants for WT and one plant each of *crct8* and *crct37* for CRCT knockout line showing average values of *CRCT* expression in 24 h sucrose treatment by qRT-PCR. RNA-seq was performed on the Illumina NovaSeq 6000 platform at

Macrogen Japan Corporation (Tokyo, Japan). Raw data were trimmed using the Trimmomatic program (Bolger et al., 2014), mapped to the Rice Annotation Project Database reference genome IRGSP-1.0 using the HISAT2 program, and assembled using the StringTie program (Pertea et al., 2016). Expression profiles were represented as read count and normalized by TPM (transcript per kilobase million) and TMM (trimmed mean of M-value). Differentially expressed genes were defined as fold change ≥ 2 and $p < 0.05$ analyzed by exact test using edgeR (Robinson et al., 2010). GO enrichment analysis was carried out using the g:Profiler program (Raudvere et al., 2019). Raw RNA-seq data in this study were deposited to SRA (ID: SUB13203159).

3. Results

3.1. Growth and photosynthetic parameters

Several CRCT knockout lines were generated by CRISPR/Cas9 method (Fukayama et al., 2021) (Figure 1). All CRCT knockout lines contain biallelic frameshift mutations in the coding region of CRCT (Figure 1(b)). Thus, no CRCT band was detected by immunoblot analysis in these knockout lines (Figure 1(c)).

To study the effect of CRCT knockout on rice growth, the plant height, leaf number and tiller number were compared between CRCT knockout lines and wild-type. The plant height and leaf number of CRCT knockout lines were comparable with wild-type, whereas the tiller

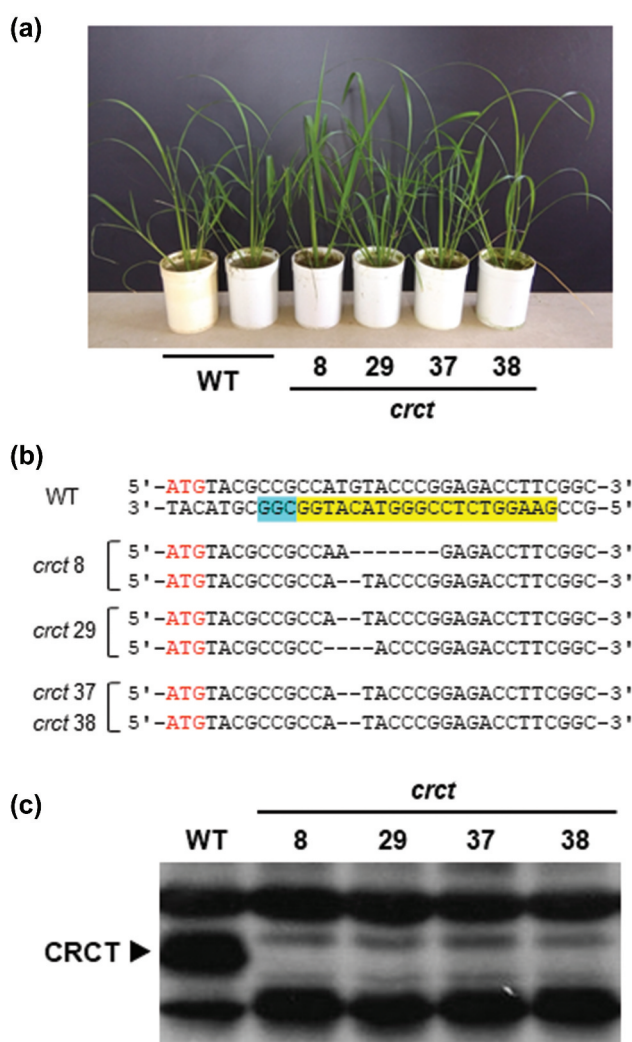


Figure 1. CRCT knockout lines produced by CRISPR/Cas9 system. (a) the pictures of wild-type and CRCT knockout lines at 60 days after sowing. (b) the nucleotide sequences of CRISPR/Cas9 target region in CRCT knockout lines. The target sequence (shown in yellow background) was designed on antisense strand just below the translation initiation codon (ATG, shown in red) in the coding sequence of CRCT. PAM sequence is shown by blue background. (c) Immunoblot analysis of CRCT in CRCT knockout lines. CRCT protein was detected using anti-CRCT antibodies. The arrowhead indicates the position of CRCT. Others are non-specific bands. WT, wild-type; crct, CRCT knockout line.

number of CRCT knockout lines was significantly lower than that of wild-type (Figure 2(a)). After these lines were harvested, panicle and straw dry weights were determined (Figure 2(b)). The panicle dry weight of CRCT knockout lines was comparable with that of wild-type. On the other hand, the straw dry weight of CRCT knockout lines was significantly lower than that of wild-type. The reduction of the tiller number of CRCT knockout line would cause the reduction of straw weight. However, there was no significant difference in the panicle/straw dry weight between genotypes (Supplemental Figure s2).

Using these plants, the photosynthetic parameters were also analyzed (Figure 3). The CO_2 assimilation rate of CRCT knockout lines tended to be slightly lower than that of wild-type (Figure 3A). Although there was no significant difference, the maximum carboxylation rate (V_{cmax}) and maximum electron transport rate (J_{max}) of CRCT knockout lines were also slightly lower than those of wild-type.

The content of leaf constituents related to photosynthesis in the knockout mutants was almost the same as that in wild-type (Figure 4). The contents of

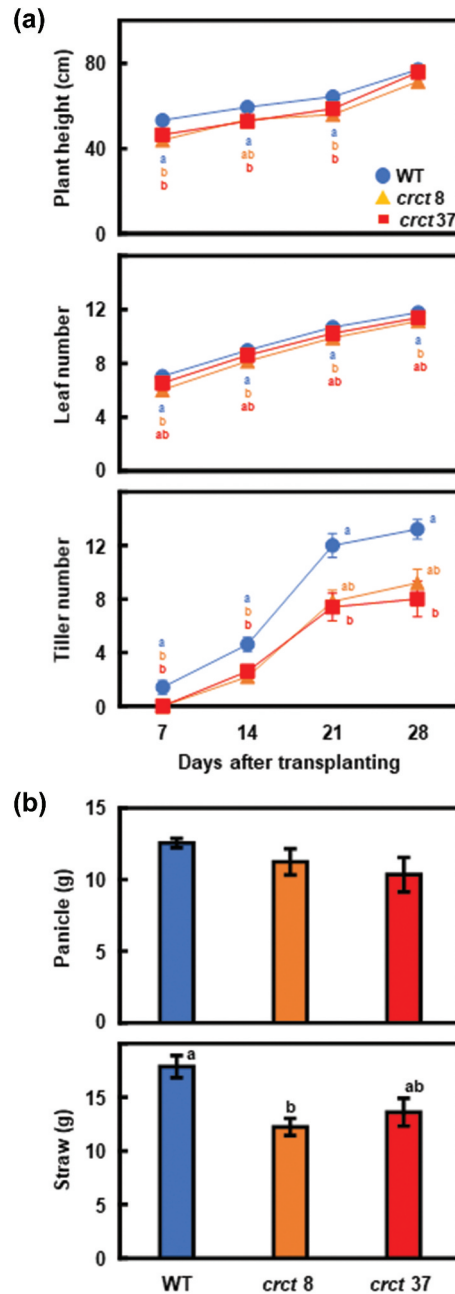


Figure 2. Growth parameters of CRCT knockout lines. (a) the plant length, leaf number and tiller number. (b) the panicle and straw dry weights. The dry weights were measured on the harvest stage, 100 days after sowing. The data represents the means \pm SD of five independent plants. Different letters above columns indicate significant differences between genotypes by Tukey's test ($P < 0.05$).

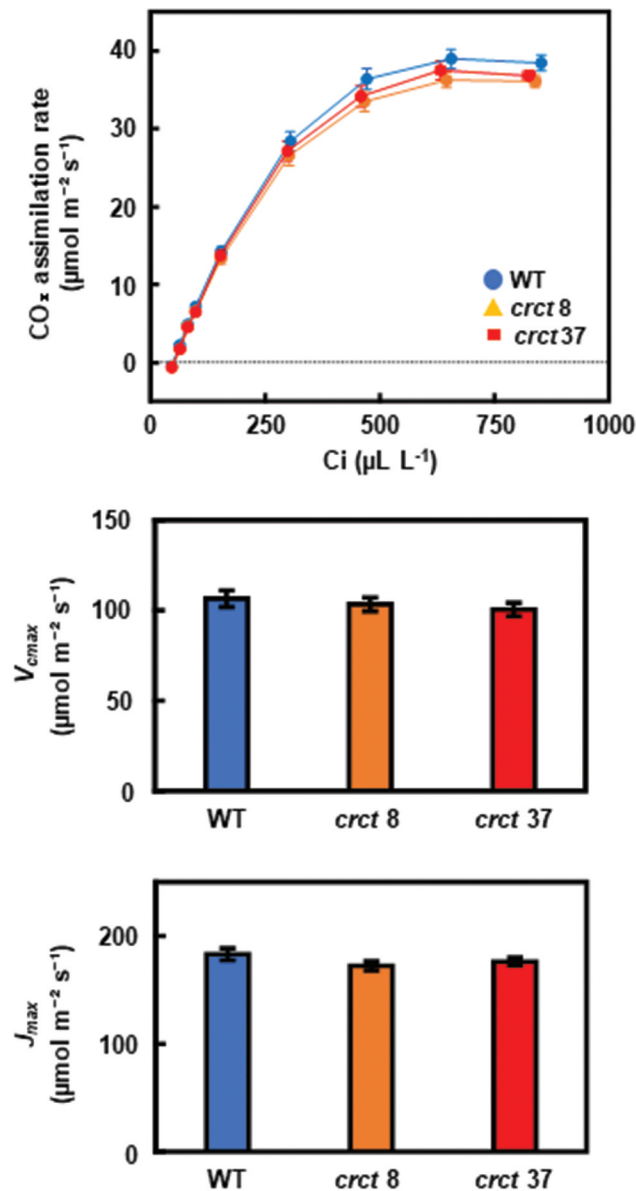


Figure 3. Photosynthetic parameters of CRCT knockout lines. The eighth leaf blades at 8.2 leaf stage were used for the measurement of CO₂ assimilation rate. The maximum carboxylation rate (V_{max}) and maximum electron transfer rate (J_{max}) were calculated using the CO₂ assimilation rates and kinetic parameters shown in Ishikawa et al. (2011). The data represents the means \pm SD of five independent plants. There were no significant differences between genotypes by Tukey's test ($P < 0.05$).

Rubisco and chlorophyll in CRCT knockout lines were comparable with those of wild-type. CRCT knockout lines have slightly higher soluble protein content than wild-type. One of the CRCT knockout lines, *crct8* showed significantly higher soluble protein content than wild-type. Hence, Rubisco/soluble protein in the CRCT knockout lines tended to be lower than that in wild-type.

3.2. Starch and soluble sugar contents

The soluble sugar and starch contents were analyzed in the leaf sheath and leaf blade (Figure 5). The starch content in the leaf sheath of CRCT knockout lines was

markedly lower than that of wild-type (Figure 5(a), Supplemental Figure s1), which was consistent with CRCT knockdown lines (R. Morita et al., 2015). Although there was no significant difference, there was also a tendency for the leaf blade to have a lower starch content in the CRCT knockout lines (Figure 5(b)). In addition, the soluble sugar content of CRCT knockout lines tended to be lower in the leaf sheath and higher in the leaf blade than those of wild-type. These results further highlighted that CRCT regulates starch content in the vegetative organs such as leaf sheath of rice, as proposed previously (R. Morita et al., 2015).

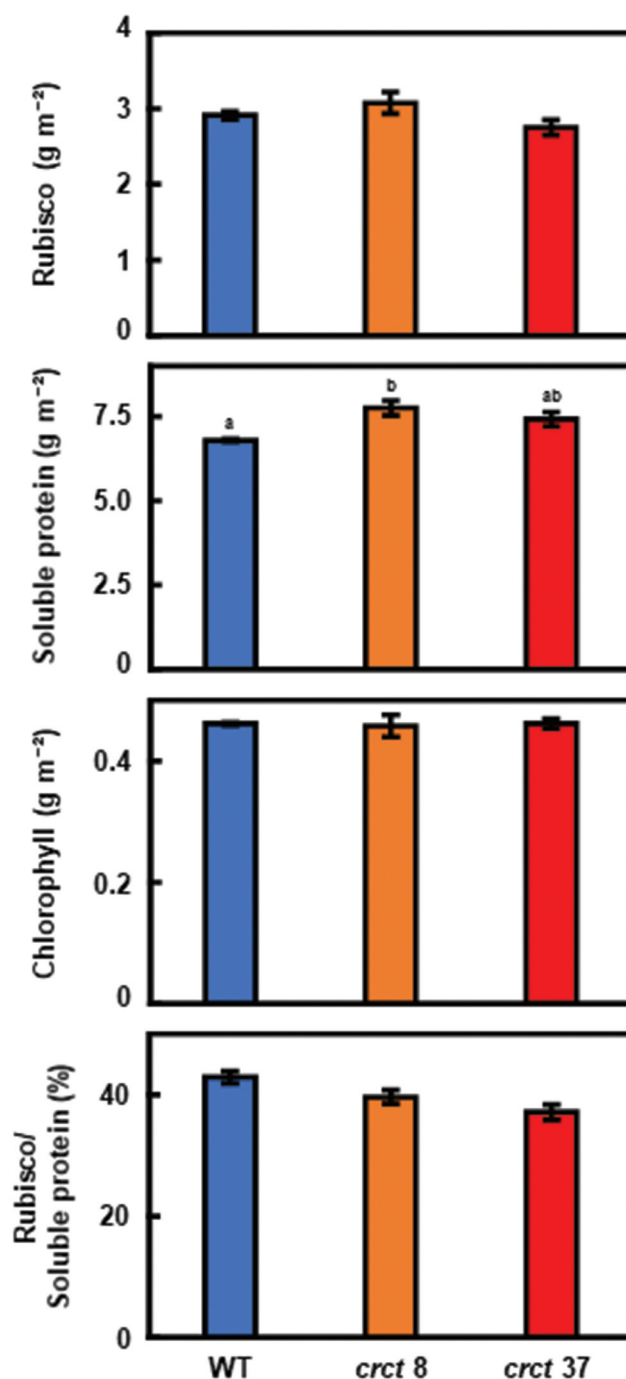


Figure 4. Leaf constituents of CRCT knockout lines. Rubisco, soluble protein, and chlorophyll contents of the eighth leaf blade at 11.0 leaf stage were shown. The data represents the means \pm SD of five independent plants. Different letters above columns indicate significant differences between genotypes by Tukey's test ($P < 0.05$).

3.3. Sucrose induction of the expression of CRCT and starch synthesis-related genes

Previously, we showed that the expressions of *CRCT* and hence starch synthesis-related genes were induced by sugars, especially sucrose (R. Morita et al., 2015). To clarify the contribution of CRCT to sugar induction of starch synthesis-related genes, sucrose-dependent

induction of starch synthesis-related genes was analyzed using CRCT knockout lines (Figure 6). As expected, the expression of *CRCT* was notably increased by sucrose treatment in wild-type, and this increase was more pronounced at 24 h than at 12 h after sucrose treatment. Concomitantly, in wild-type, the expression of *OsAGPL1* was markedly increased by sucrose treatment, approximately 16-fold over the control (water treatment) at 24

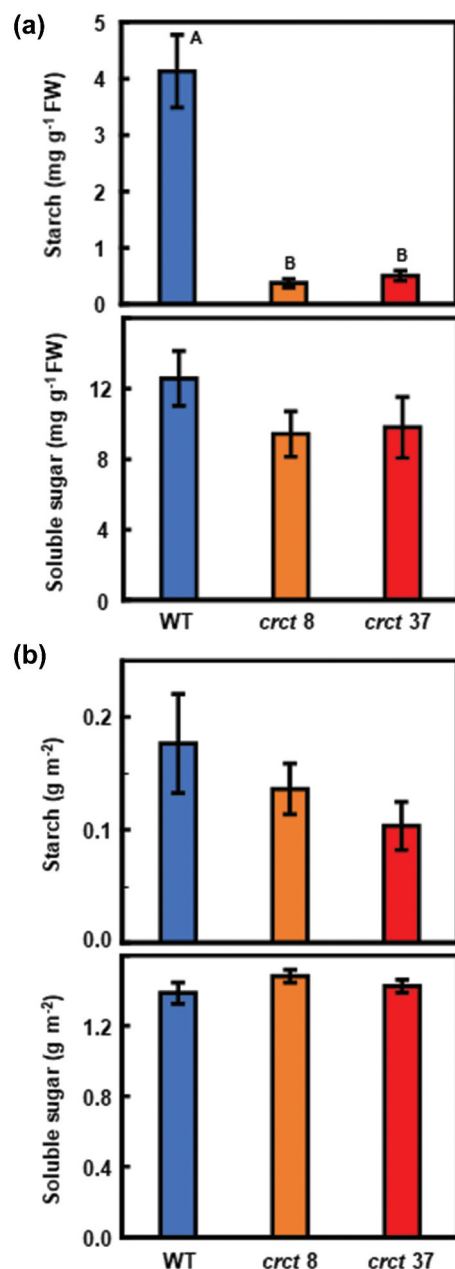


Figure 5. Soluble sugar and starch contents of CRCT knockout lines. Soluble sugar and starch contents in the leaf sheath (A) and leaf blade (B). Eighth leaf sheath and leaf blade at 11.0 leaf stage were sampled at the end of day and used for the measurement of sugar and starch contents. The data represents the means \pm SD of five independent plants. Different capital letters above columns indicate significant differences between genotypes by Tukey's test ($P < 0.01$).

h. On the other hand, in CRCT knockout lines, the expression of *OsAGPL1* was not increased by sucrose treatment. Although there was a difference in degree, a similar tendency was observed in other starch synthesis-related genes except for *OsBE1* and *OsGPT2*. Among these genes, the tendency was most remarkable in *OsAGPL1*, followed by *OsAGPS1* and *OsPho1*. These results indicated that CRCT responds to sugar and promotes the expression of starch synthesis-related genes, whereas responsiveness to CRCT varies by genes.

Interestingly, the induction of these three starch synthesis-related genes was greater than that of CRCT, the regulatory factor.

To further explore the influence of sucrose treatment on the gene expression, we performed RNA-seq analysis using total RNA of CRCT knockout lines and wild-type treated by sucrose for 24 h. There were 1,456 differentially expressed genes (fold change ≥ 2 , $P < 0.05$), of which 937 were upregulated and 519 were downregulated by CRCT knockout. Among differentially expressed

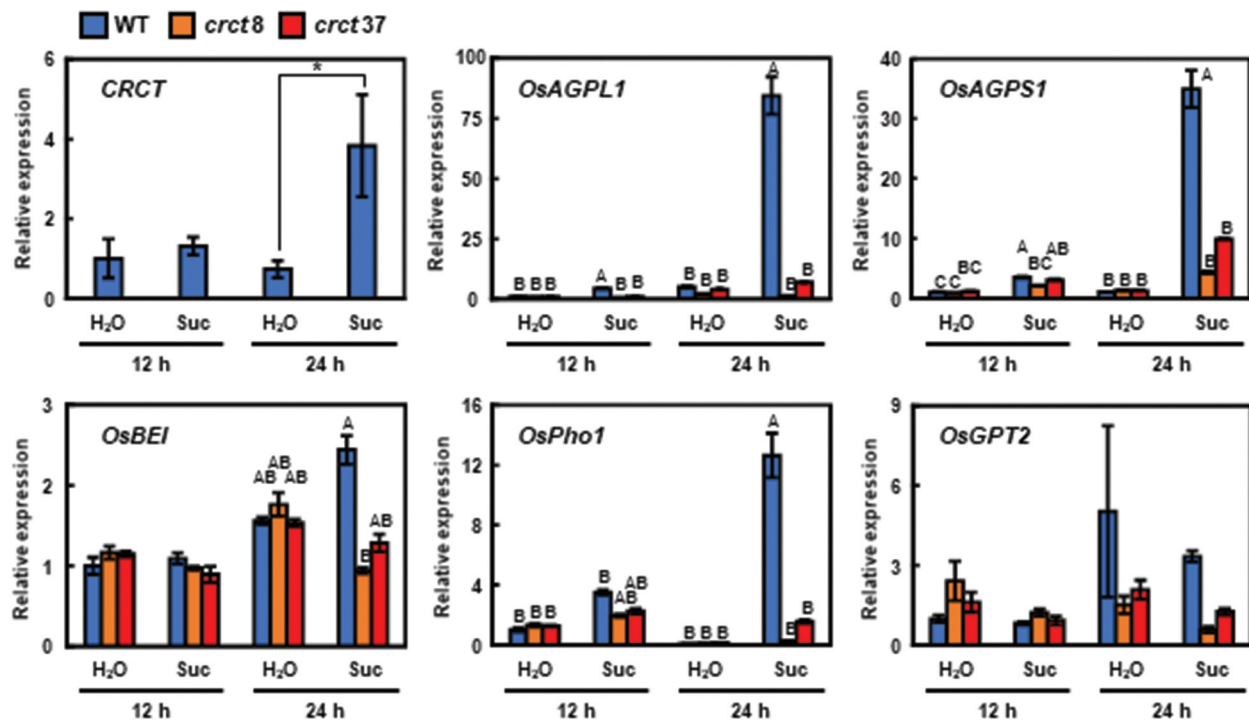


Figure 6. Effects of sucrose on the expression of starch synthesis-related genes. The leaf blades of wild-type and CRCT knockout lines were immersed in water (H₂O) or 50 mM sucrose solution (Suc). The relative expression of *CRCT* and starch synthesis-related genes (*OsAGPL1*, *OsAGPS1*, *OsBEI*, *OsPho1* and *OsGPT2*) were analyzed by Qrt-PCR. The expression of *Actin* was used as an internal control. The gene expressions are shown as relative expression to wild-type at 12 h on water. The data represents the means \pm SD of four independent samples. Asterisks indicate significant differences between treatments at the same incubation time by the Student's t-test (* $P < 0.05$). Different capital letters above columns indicate significant differences between genotypes at the same incubation time by Tukey's test ($P < 0.01$).

genes showing the TMM normalized count above 50 (assuming highly reliable), the top 15 of upregulated and downregulated genes are shown in Figure 7(a). Compared with wild-type, various categories of genes were upregulated in CRCT knockout lines such as *OsCRSH2* and *lipxygenase*. On the other hand, many starch synthesis-related genes such as *OsAGPL1* and *OsPho1* were downregulated in CRCT knockout lines. Among the top 15 downregulated genes, nine genes were related to starch synthesis.

Next, we performed GO enrichment analysis using all the differentially expressed genes (1,456 genes) and listed up significant GO terms (Figure 7(b), Supplemental Figures s3 and s4). Compared with wild-type, the processes related to secondary metabolism and photosynthesis were upregulated in CRCT knockout lines. In contrast, many downregulated processes were related to starch synthesis such as polysaccharide metabolic process and starch biosynthetic process. This result suggests that CRCT plays a central role in sucrose induction of starch synthesis-related genes and possibly other differentially expressed genes including up-regulated genes may not be a direct effect of CRCT.

4. Discussion

Previously, we reported that knockdown of *CRCT* by RNAi decreased starch content of the rice leaf sheath (R. Morita et al., 2015). However, how knockout of CRCT affects starch accumulation remained to be elucidated. In this study, we showed that CRCT knockout lines had significantly lower starch content of the leaf sheath than wild-type (Figure 5). This result was consistent with the previous study, which highlighted that CRCT regulates starch accumulation in the rice leaf sheath.

Among the growth parameters, the tiller number of CRCT knockout lines was notably lower than that of wild-type (Figure 2). In a previous study, it was shown that tiller numbers were slightly decreased in *CRCT* overexpression lines while slightly increased in *CRCT* knockdown lines (R. Morita et al., 2015). In the *CRCT* knockdown lines, the expression of *CRCT* was decreased, and starch accumulation in the leaf sheath was decreased. The cause of this increase in tiller numbers of *CRCT* knockdown lines might be explained by the increase in the allocation of carbohydrates to tiller formation, which should have been used for starch accumulation. It has been reported that there is a trade-

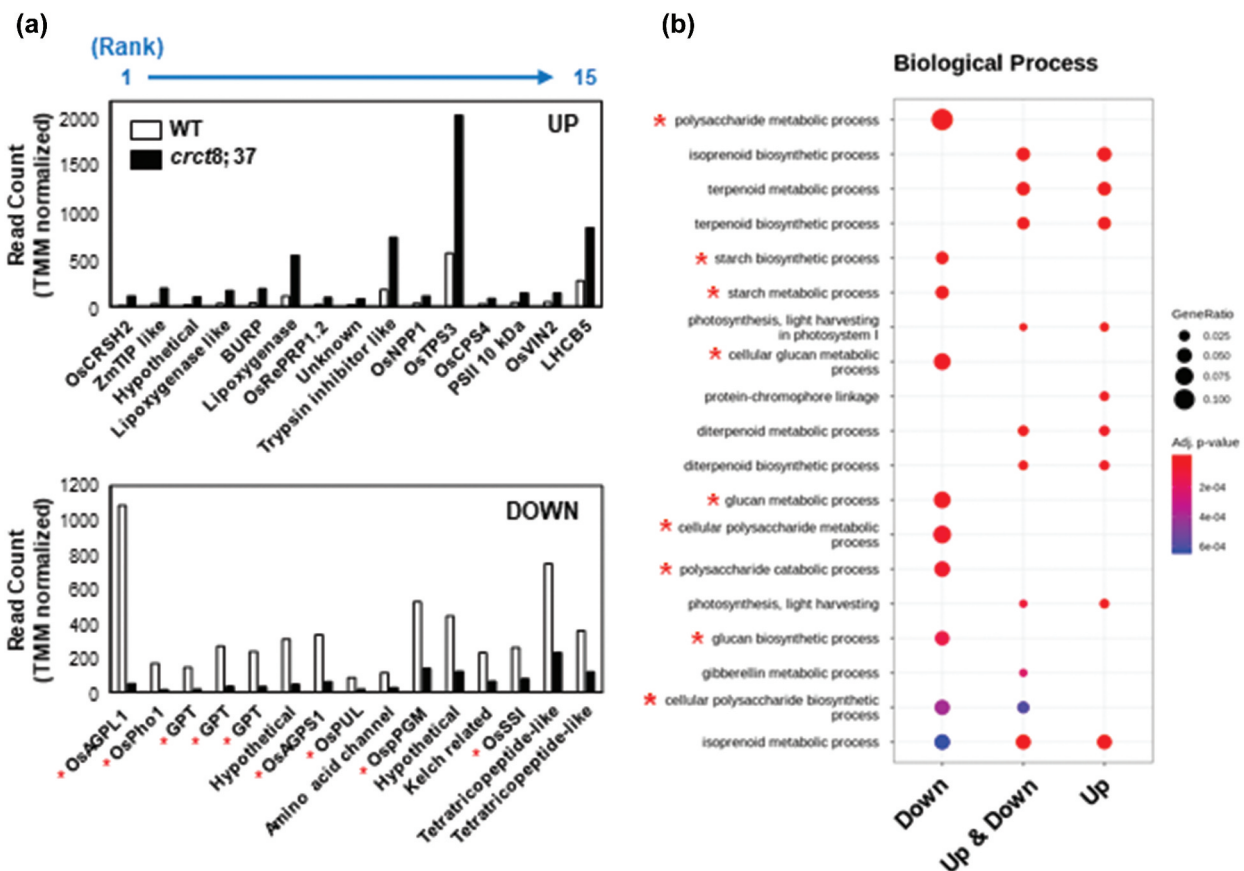


Figure 7. RNA-seq analysis. RNA-seq analysis was performed using total RNAs of wild-type and CRCT knockout lines (mixture of *crct 8* and *crct 37*) after sucrose treatment for 24 h. The top 15 differentially expressed genes between wild-type and CRCT knockout lines whose averages read counts normalized by TMM method above 50 are shown (a). Upper graph shows the upregulated genes and lower graph shows the downregulated genes in CRCT knockout lines. Red asterisk indicates starch synthesis-related genes. Based on the RNA-seq data, GO enrichment analysis was performed (b). Top 9–10 significant GO terms were listed up using differentially expressed genes from upregulated and downregulated gene data (fold change ≥ 2 , $P < 0.05$, term size : 10–500). Figure was created using g:Profiler program. Gene ratio and P-value are shown on the right. Red asterisk indicates starch synthesis-related processes.

off between plant growth and starch accumulation (Sulpice et al., 2009). For example, in the *agpl1* mutant, the starch content in the leaf sheath was decreased, while the tiller number was increased (Okamura et al., 2014). However, in this study, CRCT knockout lines had less tiller numbers than wild-type, which is inconsistent with previous reports. Considering these, the complete absence of CRCT may negatively influence tiller formation in some way independent of the trade-off. It has been reported that the tiller angle of *CRCT* overexpression lines was larger than that of wild-type (R. Morita et al., 2015). The expression of *CRCT* was quite high in the nonelongation internode and node where initial tiller formation occurs (R. Morita et al., 2015). Therefore, in any case, it is likely that CRCT is involved in the regulation of tiller formation, although the mechanism by which CRCT affects tiller formation remains to be elucidated.

There was no significant difference in the panicle dry weight between CRCT knockout lines and wild-type (Figure 2(b)). This result suggests that CRCT controls the starch accumulation mainly in the vegetative organs. In contrast, the straw dry weight of CRCT knockout lines was significantly lower than that of wild-type. This result may be due to the reduction of the tiller number in CRCT knockout lines.

The CO_2 assimilation rate and thus calculated parameters V_{cmax} and J_{max} of CRCT knockout lines were slightly lower than those of wild-type (Figure 3). These results indicate that knockout of CRCT could negatively affect photosynthetic capacity in rice. Previously, it was reported that the photosynthetic rate of *CRCT* knock-down lines was significantly lower than that of wild-type particularly when grown under elevated CO_2 conditions (R. Morita et al., 2016). In this report, a negative correlation was observed between the photosynthetic

rate and total carbohydrate content. Soluble sugar content tended to increase in the leaf blade of CRCT knockout lines (Figure 5(b)). This increase in sugar may have had a negative effect on photosynthesis as reported previously (Lobo et al., 2015).

The expression of *CRCT* was shown to be induced by sugar such as sucrose and glucose (R. Morita et al., 2015). It is also known that the expressions of some starch synthesis-related genes are upregulated by sugar (Akihiro et al., 2005; Dian et al., 2005). Therefore, it was expected that CRCT controls sugar responsivity of starch synthesis-related genes. In this study, the expression of starch synthesis-related genes was not upregulated by sucrose in CRCT knockout lines, while it was remarkably upregulated in wild-type. This result revealed that sugar inducibility of the expression of starch synthesis-related genes was almost fully dependent on CRCT, and strongly supports the previous hypothesis that CRCT is a positive regulator of starch accumulation in vegetative tissues, regulating coordinated expression of starch synthesis-related genes in response to the levels of photoassimilates (such as sucrose) (R. Morita et al., 2015). In the previous study, the expression of *CRCT* was not induced by non-metabolizable sugars such as 3-O-methylglucose and 2-deoxyglucose (R. Morita et al., 2015), suggesting that metabolism of sugar is necessary for the induction of CRCT. However, it is largely unknown how CRCT is induced by sugars. Sugar signaling pathways of plants have been found to include hexokinase-1 (HXK1), trehalose-6-phosphate (Tre6P), and target of rapamycin (TOR) for sensing high carbon availability and Snf1-related protein kinase-1 (SnRK1) and C/S1 bZIP transcription factors for responding to carbon starvation (Deprost et al., 2007; Dobrenel et al., 2016; Granot et al., 2014; Moore et al., 2003; Wingler, 2018). Recently, it was shown that AtCRCT (an ortholog of CRCT in *Arabidopsis thaliana*) was phosphorylated by SnRK1, and the phosphorylation was reduced by sucrose treatment in sucrose-starved *Arabidopsis* (Van Leene et al., 2022). Therefore, it is possible that SnRK1 is involved in the CRCT-related sugar response. In the previous study, it was shown that CRCT interacts with 14-3-3 proteins and hypothesized that the interaction of 14-3-3 protein may inactivate CRCT (Fukayama et al., 2021). The putative 14-3-3 binding motif, 'GLRRAYSEGD', is located upstream of the CCT domain of CRCT and matches the SnRK1 phosphorylation site of AtCRCT. Thus, it is assumed that CRCT activity is post-translationally regulated by the phosphorylation and the interaction with 14-3-3 proteins. This may be a reason why inductions of the expression of the starch synthesis-related

genes by sucrose were more remarkable than that of CRCT (Figure 6).

Since the sugar signaling pathway is very complicated, there can be another candidate to regulate sugar response of CRCT. Besides CRCT, some transcription factors related to sugar response have been reported. For example, SUSIBA2 reported to be a sugar-inducible WRKY transcription factor regulating starch synthesis in barley (Sun et al., 2003). AtWRKY20, the transcription factor regulating *APL3* was upregulated by sugar in *Arabidopsis thaliana* (Nagata et al., 2012). OsNAC23, which is the sugar-responsive transcription factor, Tre6P and SnRK1 form a feed-forward regulatory loop that regulates sugar homeostasis and grain yield in rice (Li et al., 2022). In addition, MYB75/PAP1, the transcription factor regulating anthocyanin biosynthesis-related genes was upregulated by sucrose in *Arabidopsis thaliana* (Teng et al., 2005). Furthermore, bZIP3, which is the transcription factor regulating leaf shape, was under the control of SnRK1 and downregulated by sugar in *Arabidopsis thaliana* (Sanagi et al., 2018). These transcription factors might be involved in the regulation of starch synthesis-related genes through regulation of sugar induction of CRCT. It is an important subject that needs to be elucidated.

RNA-seq analysis indicated that various categories of genes were upregulated in CRCT knockout lines (Figure 7). This upregulation may be a secondary effect to compensate for the reduction of starch synthesis in CRCT knockout lines. Among differentially expressed genes, genes related to photosystem, a plastoglobule, and apoplast tended to be upregulated in CRCT knockout lines. These can be responses to promote photosynthesis and photosynthate transport compensating for the reduction of starch accumulation in CRCT knockout lines. However, photosynthetic parameters of CRCT knockout lines were not notably different from wild-type (Figure 3). Thus, it is likely that CRCT is not a direct determinant of photosynthetic capacity, but a factor that indirectly influences photosynthesis by regulating starch synthesis.

In conclusion, we propose that CRCT is a major transcription factor regulating starch synthesis depending on the sugar level in the vegetative organs of rice. Through this function, CRCT indirectly affects photosynthesis and growth. These findings may help to understand the full picture of the regulatory mechanism of starch synthesis and finally to enhance plant productivity in the future.

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Disclosure statement

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Author contribution

Fumihiro Miyagawa, Ryutaro Morita and Hiroshi Fukayama designed the research. Fumihiro Miyagawa, Naoki Shibatani and Hiroshi Fukayama performed the experiment with the help from Daisuke Sasayama, Tomoko Hatanaka, Tetsushi Azuma. Fumihiro Miyagawa, Naoki Shibatani and Hiroshi Fukayama analyzed the data. Hiroshi Fukayama supervised the research. Fumihiro Miyagawa and Hiroshi Fukayama wrote the manuscript.

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