CTP synthase is essential for early endosperm development by regulating nuclei spacing

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Summary
Cereal grain endosperms are an important source of human nutrition. Nuclear division in early endosperm development plays a major role in determining seed size; however, this development is not well understood. We identified the rice mutant endospermless 2 (enl2), which shows defects in the early stages of endosperm development. These phenotypes arise from mutations in OsCTPS1 that encodes a cytidine triphosphate synthase (CTPS). Both wild-type and mutant endosperms were normal at 8 h after pollination (HAP). In contrast, at 24 HAP, enl2 endosperm revealed that wild-type nuclei were evenly distributed by microtubules while the enl2-2 nuclei were tightly packed due to their reduction in microtubule association. In addition, OsCTPS1 interacts with tubulins; thus, these observations suggest that OsCTPS1 may be involved in microtubule formation. OsCTPS1 transiently formed macromolecular structures in the endosperm during early developmental stages, further supporting the idea that OsCTPS1 may function as a structural component during endosperm development. Finally, overexpression of OsCTPS1 increased seed weight by promoting endosperm nuclear division, suggesting that this trait could be used to increase grain yield.

Introduction
The endosperm is a major component of cereal grains that stores starch, proteins and several other types of nutrients required for seed germination and early growth (Lopes and Larkins, 1993). One of the discriminating features of endosperm development is that fertilized central cells undergo rapid nuclear division without cell wall formation during the early developmental stages (syncytium stages), resulting in the formation of a multinucleate cell (Olsen, 2004). The numbers of nuclei in endosperm syncytium are greater in cereals than in the model plant Arabidopsis thaliana, in which the syncytial endosperm carries approximately 200 nuclei (Bennett et al., 1975). In contrast, during the first 1–2 days after pollination (DAP), rice endosperms produce 4,000–8,000 nuclei without accompanying cell division, which then migrate towards the periphery of the chalazal region of the embryo sac. Next, at 3–4 DAP, the syncytial endosperm is cellularized and cell walls are formed (Sabelli and Larkins, 2009). Endosperm cells then undergo rapid mitotic division from 4 to 10 DAP, endoreduplication from 8 to 10 DAP and programmed cell death from 14 to 20 DAP (Olsen, 2004).

Although several genes associated with cereal endosperm storage have been investigated, only a few genes affecting early endosperm development have been identified in rice (Kurata et al., 2005; Zhou et al., 2013). Rice plants with mutations in the gene LEAF AND FLOWER-related (OsLFR), which encodes an interaction partner of SWITCH/SUCROSE NON-FERMENTABLE (SWISNF) in the ATP-dependent chromatin-remodelling complex, display abnormal endosperm development (Qi et al., 2020). While the number of syncytial nuclei in osfr-1 and wild-type (WT) endosperm is similar at 24 hours after pollination (HAP), at 32 HAP and beyond, the number of free nuclei at the periphery of osfr-1 endosperm is much lower than in WT endosperm, suggesting that this gene functions in syncytial nuclei formation.

Regulatory genes affecting endosperm cellularization have also been identified. Suppression of CycB1;1 expression creates an aberrant endosperm phenotype beginning from 2 DAP (Guo et al., 2010). Although the CycB1;1-knock-down plants show normal distribution of endosperm nuclei to the sac periphery, cell walls fail to properly form, suggesting that CycB1;1 is involved in cellularization. In addition, mutants defective in MAD578 or MAD579 exhibit precocious endosperm cellularization and the mads78 mads79 double mutant is sterile (Paul et al., 2020). Overexpression of MAD578 and MAD579 results in delayed endosperm cellularization, indicating that these transcription factors inhibit endosperm cellularization. Both MAD578 and

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MADS79 interact with MADS89, which enhances their nuclear localization, suggesting MADS89 is also involved in endosperm development (Paul et al., 2020).

Genes involved in the later stages of endosperm development have also been identified. The endosperm of the rice mutant endospermless (enl1) is normal at 3 DAP but degenerates by later stages, resulting in the production of a large embryo (Hong et al., 1996). This mutant also develops enlarged nuclei due to a malfunction in mitotic chromosomal segregation in the syncytial endosperm. These phenotypes were later found to arise from a defect in an SNF2 family chromatin-remodelling helicase that is orthologous to human Plk1-interacting checkpoint helicase (Hara et al., 2015). The transgenic plants with reduced expression of NF-Y carry a reduced number of endosperm cells, suggesting that this gene functions during mitosis as part of endosperm development (Sun et al., 2014). Finally, endosperm mutants with defects in OsCCS52A display abnormalities during endoreduplication, reducing the amount of endosperm produced (Su’udi et al., 2012).

Enlarged embryos have been observed for several endosperm mutants and appear to be associated with a lack of endosperm (Guo et al., 2010; Hara et al., 2015; Nagasawa et al., 2013; Su’udi et al., 2012; Sun et al., 2014; Yang et al., 2013). More specifically, endosperm-specific suppression of cell cycle inhibition creates endosperm defects and increased embryo size (Guo et al., 2010). In addition, interactions between the embryo and endosperm at interface tissues appear to play critical roles in controlling relative embryo size (Nagasawa et al., 2013).

Certain molecules form non-membrane-bound macromolecular structures that play important cellular roles, including histone locus bodies (Liu et al., 2006), cytoplasmic processing bodies (Sheth and Parker, 2006), uridine-rich small nuclear ribonucleoprotein bodies (Liu and Gall, 2007) and purinosomes (An et al., 2008). In addition, cytidine triphosphate synthase (CTPS) also appears to form macromolecular structures based on work in the bacterial species Caulobacter crescentus and Escherichia coli (Ingression-Mahar et al., 2010), Drosophila (Liu, 2010), budding yeast (Saccharomyces cerevisiae) (Noree et al., 2010) and human tissue (Carcamo et al., 2011; Chen et al., 2011). In addition, inosine monophosphate dehydrogenase, which catalyses the rate-limiting reaction of de novo GTP biosynthesis, also forms macromolecular structures (Carcamo et al., 2011).

In this study, we identified rice endosperm mutants containing alterations in rice CTP synthase 1 (OsCTPS1). These mutants stopped the endosperm nuclei production at 24 HAP, with mutant nuclei remaining clustered and lacking sufficient radial microtubule systems (RMSs) between nuclei. In addition, mutant nuclei failed to migrate to the endosperm periphery, with these defects suggesting that OsCTPS1 is involved in microtubule formation. This hypothesis is further supported by evidence that OsCTPS1 interacts with tubulins. In addition, OsCTPS1 transiently forms macromolecular structures in syncytium endosperm.

Results
Identification of a mutant defective in endosperm development

We identified an individual from a japonica rice cultivar with defect in seed development. In this line, about 1/4 of seeds were abnormal and some germinated precociously (Figure 1a). Progeny exhibited entirely defective seeds, which indicated that the seed defects were due to a recessive mutation that was stably inherited. The ventral side of each seed was severely shrunken compared to the dorsal side (Figure 1b,c), and longitudinal sections of the developing seeds at 10 DAP showed that mutant seeds contained altered endosperm. In addition, the embryos of the mutant seeds were much bigger than those of WT seeds (Figure 1d). We thus named the mutant endospermless2-1 (enl2-1). Most mutant seeds germinated; however, their initial growth was slower than that of segregated WT seedlings. In addition, root development was significantly reduced in enl2-1 mutants, as mutant primary roots were 41% shorter than those of WT controls at 10 days after germination (DAG) (Figure 1e,f). To examine whether the enl2-1 mutants had decreased meristem activity, 7 DAG seedling roots were incubated with ethynyl deoxyuridine (EdU), which is incorporated into DNA during active DNA synthesis in S-phase cells. EdU visualization showed that enl2-1 had a smaller root apical meristem than WT plants due to a decreased number of meristem cells (Figure 1g,h). However, the growth of enl2-1 ultimately recovered and enl2-1 plants flowered at the same time as their WT siblings.

Map-based cloning of enl2-1

In order to identify the gene responsible for enl2-1 mutant phenotypes, we crossed the enl2-1 mutant line with the indica type rice cultivar Milyang 23. Bulked segregant analysis was conducted with 77 simple sequence repeat (SSR) markers distributed over 12 chromosomes using segregating F2 seeds (Ji et al., 2006), with R1M30 on chromosome 1 being the closest marker to the phenotype-linked region (Figure 2a). To validate the identity of this phenotype-linked marker, 704 individual F2 plants derived from normal and mutant seeds were genotyped with six InDel markers surrounding R1M30. Of the 251 resulting mutants, 228 (90.84%) possessed a homozygous japonica marker at R1M30 while 12 out of 453 normal seed-derived plants (2.65%) had the japonica marker at R1M30, indicating that the enl2-1 phenotypes are tightly linked to R1M30. Initial mapping indicated that the enl2-1 mutations were present in a 1.45-Mb region between RM9 and R1M30 (Figure 2a). Genotyping of 1086 seeds (568 normal and 518 mutant) by the border marker to the phenotype-linked region (Figure 2a). To validate the identity of this phenotype-linked marker, 704 individual F2 plants possessed a homozygous japonica marker at R1M30 while 12 out of 453 normal seed-derived plants (2.65%) had the japonica marker at R1M30, indicating that the enl2-1 phenotypes are tightly linked to R1M30. Initial mapping indicated that the enl2-1 mutations were present in a 1.45-Mb region between RM9 and R1M30 (Figure 2a). Genotyping of 1086 seeds (568 normal and 518 mutant) by the border markers RM9 and R1M30 identified 43 recombinants. Mapping of these recombinants narrowed the position of the mutant locus to a ~ 43-kb region between the S9697 and CBI12 markers that contains five annotated genes: Os01g42970 (zinc finger domain protein), Os01g42980 (ribosomal protein L22), Os01g42990 (pentatricopeptide repeat domain protein), Os01g43010 (transposon protein) and Os01g43020 (CTP synthase) (Figure 2a).

Among these annotated genes, the coding regions of all genes except Os01g43010 were sequenced from parental japonica and enl2-1 mutant plants. Sequence analysis revealed a single nucleotide substitution from A to T in the third exon of Os01g43020, causing a change of Asn (N) to Ile (I) at 86 th amino acid in a conserved CTP synthase domain (Figure 2b,c). No other alterations of the sequence were found in the four genes, indicating that the altered phenotypes of enl2-1 were caused by this point mutation in Os01g43020. Since this locus encodes a protein that is highly homologous to CTP synthase and contains both conserved CTP synthase and glutamine amide transfer domains (Choi and Carman, 2007) (Figure 2c), we re-named Os01g43020 as Orzya sativa CTP synthase 1 (OsCTPS1). CTP synthase catalyses the ATP-dependent transfer of the amide nitrogen of glutamine to the C-4 position of UTP to generate CTP, which is the rate-limiting final step in de novo pyrimidine-nucleotide biosynthesis (Figure 2d) (Choi and Carman, 2007).
Active CTP synthase is a tetrameric enzyme composed of a dimer of dimers, and its formation is favored by the presence of the substrate nucleotides ATP and UTP (Figure 2e) (Iben et al., 2011). To determine whether enl2-1 exhibits altered homodimerization, the homodimerization of OsCTPS1 and OsCTPS1-N86I (the OsCTPS1 form found in enl2-1) was examined using a co-immunoprecipitation (Co-IP) assay (Figure 2f). OsCTPS1-N86I appeared to dimerize at similar levels as OsCTPS1 (Figure 2f). Analysis of enzyme activity showed that OsCTPS1-N86I had significantly reduced CTP synthase activity compared to OsCTPS1 (Figure 2g), revealing that the amino acid substitution N86I in the enl2-1 mutant largely affects the enzyme activity of OsCTPS1, leading to a lack of endosperm.

Expression patterns of OsCTPS1

Six putative CTP synthase genes were found in the rice genome, which we named OsCTPS1 (Os01g43020), OsCTPS2 (Os01g46570), OsCTPS3 (Os05g49460), OsCTPS4 (Os05g49520), OsCTPS5 (Os05g49770) and OsCTPS6 (Os12g36950). Five OsCTPS1-5 are clustered to monocot CTPS genes, while one OsCTPS6 is closely related to dicot CTPS genes (Figure S1 and S2). Expression data found in public domains indicated that all six genes are ubiquitously expressed (Figure S3). We measured the expression levels of all six CTPS genes in developing seeds at 1 DAP, when enl2-1 defects begin to appear, and saw that OsCTPS1 was the most highly expressed CTPS family member (Figure 3a). The OsCTPS1 expression pattern was then determined to evaluate whether the mutant phenotypes were caused by tissue specific expression. Quantitative RT-PCR analyses showed that OsCTPS1 was expressed ubiquitously in all of the samples and in developmental stages ranging from seedlings to mature plants (Figure 3b). However, OsCTPS1 expression levels in both shoot apical meristems (SAMs) and panicles were approximately twofold higher than in leaves and internodes, although it is uncertain whether this difference is large enough to explain enl2-1 mutant phenotypes.

To examine cellular level OsCTPS1 expression, we generated transgenic rice plants expressing the GUS reporter gene under the control of the OsCTPS1 promoter. The GUS expression was universally detected in all the organs examined (Figure 3c–h), with preferential expression in the stele and quiescent centre (QC) regions of roots (Figure 3d). Reporter expression levels were also higher in the SAM (Figure 3e) as well as the anthers and veins of developing spikes (Figure 3g,h). These results suggest that OsCTPS1 expression is higher in dividing cells. In developing seeds, the GUS reporter was expressed throughout the embryo sac, with stronger staining observed in the endosperm and
embryo (Figure 3i–l). Cross sections of developing seeds at 2 DAP showed that the reporter was uniformly expressed in the endosperm and nucellus, as well as pericarp cells (Figure 3l). These observations indicate that the mutant phenotypes were not likely caused by tissue specificity of OsCTPS1 expression.

Generation and characterization of additional OsCTPS1 alleles

To confirm that the phenotypes observed in the enl2-1 mutant were indeed due to a defect in OsCTPS1 activity, we generated additional alleles of the gene using CRISPR/Cas9. The sgRNA target site was selected in the second exon to generate premature stop codons, thereby resulting in null alleles. Sequencing of several plants independently transformed with the CRISPR/Cas9 vector showed that two different deletions occurred at or near the target site in four plants (lines #1, #2, #3 and #4). We also selected one plant (Line #5) that showed no alterations in the sequence as a WT control (Figure S4a).

All five transgenic plants grew normally throughout their entire lifetimes. However, all of the seeds from lines #1–5 containing OsCTPS1 deletions were abnormal, whereas the WT control plant (Line #5) produced normal seeds (Figure S4b).
appeared to be biallelic at the primary transgenic stage due to two different deletions in each plant, and all of the seeds from each plant were abnormal (Figure S4 and Figure S5). We selected lines #1 (enl2-2) and #4 (enl2-3) for further analyses. While seeds from the enl2-2 mutant germinated normally, the seedlings of gene-edited mutants grew slower than the WT control plants (Line #5) (Figure S6). However, growth was recovered at later developmental stages and headed at a similar time to WT (Figure S5a).

WT and enl2-2 seeds were harvested at different developmental stages and compared. No significant difference was observed at 2 DAP; however, the developing mutant seeds were narrower in width compared to WT seeds starting from 4 DAP (Figure S5b). This reduced width was more significant at the upper part of the seed. The mutant phenotype was more severe when seeds were dried, with mutant seeds appearing severely shrunk while WT seeds appeared oval shaped (Figure S5b).

FIGURE 3 Expression patterns of OsCTPS genes. (a) Quantitative RT-PCR analyses of expression levels of six OsCTPS genes in developing seeds at 1 DAP. Error bars indicate standard deviation $n = 4$. (b) Quantitative RT-PCR analyses of expression levels of OsCTPS1 in various organs. Error bars represent standard deviation $n = 4$. (c-l) Representative GUS expression patterns in a seedling leaf (c), seedling root (d), shoot apical meristem (e), internode (f), spikelet in 3-cm panicle (g), mature spikelet (h), embryo sac from a developing seed at 1 DAP (i-k) and in a cross-sectional image of a developing seed at 2 DAP (l). Scale bars, 2 mm (c), (e), (f), (h); 1 mm (g); 100 µm (d, i, l); 50 µm (j, k). st, stele; QC, quiescent centre; SAM, shoot apical meristem; an, anther; vb, vascular bundle; es, embryo sac; en, endosperm; em, embryo; n, nucellus; p, pericarp. Red arrows indicate GUS signal.
Longitudinal sections of the developing seeds indicated that the mutant endosperm did not develop. No significant difference between mutant and WT seeds was observed at 1 DAP (Figure S7a, f); however, differences became obvious at 4 DAP (Figure S7b,g). Whereas WT seeds were filled with a large number of endosperm cells, mutant seeds did not exhibit endosperm development and the central part of each seed remained empty. At later developmental stages, mutant embryos divided but failed to differentiate normally (Figure S7 and S8). Overall, these observations suggest that mutant seeds were defective starting in the early stages of development.

**Mutant seeds were defective in syncytial endosperm**

Early endosperm development consists of the syncytial stage from 1 to 2 DAP and the cellularization stage at 3 DAP in rice (Lopes and Larkins, 1993; Hara et al., 2015; Qi et al., 2020). To examine potential defects in detail, WT and mutant seeds were harvested in the early stages of development (Figure 4). In WT seeds, the endosperm nuclei started to divide at 1 DAP (Figure 4a,g) and at 2 DAP were rapidly dividing and separated by the formation of interzonal phragmoplasts between sister nuclei. This directed the distribution of endosperm nuclei to the peripheries of the endosperm cells (Figure 4b,h). At 3 DAP, the syncytial endosperm was cellularized (Figure 4c,i), as previously reported (Sabelli and Larkins, 2009). In enl2-2 seeds, a clump of several endosperm nuclei had formed at 1 DAP (Figure 4d,j) and the number of nuclei had slightly increased by 2 DAP. However, these nuclei were not spaced apart and did not migrate to the periphery (Figure 4e,k). At 3 DAP, most mutant endosperm nuclei had degenerated (Figure 4f,l). In contrast, embryo development did not significantly differ between the mutant and WT during the early developmental stages. Longitudinal sections of the embryos from seeds at 1 to 3 DAP showed that the embryos in the mutant ovaries increased in size in a similar manner as WT embryos and formed globular embryos at 3 DAP (Figure 4m-r).

To study defects in the syncytial nucleus structure of the early endosperm, we performed propidium iodide (PI) staining with whole-mounted developing ovaries and analysed the endosperm nuclei by confocal laser-scanning microscopy. Embryo development appeared similar for WT and enl2-2 at 3, 8 and 24 HAP (Figure 5 a1, a3, b1, b3, c1, c3, d1, d3, e1, e3, f1 and f3). At 3 HAP, the endosperm nuclei began dividing in both WT and enl2-2 (Figure 5 a2, a4, b2 and b4). At 8 HAP, the number of WT and enl2-2 endosperm nuclei was 8 to 16 (Figure 5 c2, c4, d2 and d4) as previously reported (Hara et al., 2015). At 24 HAP, the number of WT endosperm nuclei had significantly increased, with some nuclei migrating to the endosperm periphery (Figure 5 e2, e4). In contrast, enl2-2 endosperm still contained approximately 10–16 nuclei, forming a clumped structure that failed to migrate.

![FIGURE 4 Morphological comparisons of developing WT and enl2-2 endosperm and embryos at 1–3 DAP.](image-url)
OsCTPS1 has an important role in early endosperm development

**FIGURE 5** Early endosperm and embryo development in WT and enl2-2 seeds. (a and b) PI staining of whole-mount developing WT (a) and enl2-2 (b) ovaries at 3 HAP. Developing WT (a1 and a3) and enl2-2 (b1 and b3) embryos. Developing WT (a2 and a4) and enl2-2 (b2 and b4) endosperm. Scale bars, 50 μm (a1, a2, b1 and b2); 20 μm (a3, a4, b3 and b4). (c and d) PI staining of whole-mount developing WT (c) and enl2-2 (d) ovaries at 8 HAP. Developing WT (c1 and c3) and enl2-2 (d1 and d3) embryos. Developing WT (c2 and c4) and enl2-2 (d2 and d4) endosperm. Scale bars, 50 μm (c1, c2, d1 and d2); 20 μm (c3, c4, d3 and d4). (e and f) PI staining of whole-mount developing WT (e) and enl2-2 (f) ovaries at 24 HAP. Developing embryo of WT (e1 and e3) and enl2-2 (f1 and f3). Developing WT (e2 and e4) and enl2-2 (f2 and f4) endosperm. Scale bars, 100 μm (e1, e2, f1 and f2); 20 μm (e3, e4, f3 and f4). Arrows indicate critical differences at 24 HAP.
(Figure 5 f2,14). These results indicate that enl2-2 endosperm nuclei divided three to four times before stopping.

Quantitative RT-PCR analysis of the genes involved in cell cycle and cell division (CycB1;1, Cyclin A1;1, CDKA1, CDKA2, Cyclin B2;2, OsKRP1, OsKRP3, OsKRP4, OsKRP5 and OsKRP6) in the developing seeds at 3 HAP and 24 HAP showed that the expression levels of the genes were not significantly changed in enl2-2 mutant (Figure S9). However, the expression of MADS79 and MADS89, which are markers of cellularization, was decreased at 24 HAP (Figure S9), supporting that mutation of OsCTPS1 caused defects in nuclear spacing rather than mitosis.

The OsCTPS1 protein associates with microtubules

Since the main differences between enl2-2 and WT endosperm appeared to arise from a lack of nuclei spacing and migration in enl2-2 endosperm, OsCTPS1 may be involved in the formation of the RMS required for nuclei distribution. To evaluate this hypothesis, we visualized microtubules using anti-tubulin antibodies and stained nuclei with DAPI at 1 DAP. WT endosperm nuclei were evenly spaced by RMSs that radiate the nuclei (Figure 6a). In contrast, enl2-2 endosperm nuclei were compactly packed, likely due to a reduced amount of associated RMS (Figure 6b). These observations suggest that OsCTPS1 may involve in microtubule organization during endosperm development. To determine whether OsCTPS1 associates with microtubules, we generated transgenic plants expressing the OsCTPS1-sGFP fusion protein.

OsCTPS1 transiently forms macromolecular structures in syncytial endosperm

Macromolecular CTPS protein structures have previously been reported in both microorganisms and animals. To evaluate whether OsCTPS1 forms such structures, we constructed the fusion protein OsCTPS1-sGFP. When this fusion protein was expressed in S. cerevisiae and the signal visualized using a fluorescent microscope, one to several rod-shaped macrostructures were present in each cell (Figure 7a). The length of each rod-shaped structure was about 10–50% of the cell diameter, which is similar to native macromolecular CTPS structures previously described in budding yeast. Control cells expressing

Immunoprecipitation experiments involving leaf extracts mixed with either α-tubulin or β-tubulin antibodies showed that OsCTPS1 binds to both α-tubulin and β-tubulin (Figure 6c). To confirm these interactions, full-length OsCTPS1 cDNA was fused to a Myc tag while α-tubulin and β-tubulin were fused to an HA tag and then co-expressed in the protoplasts prepared from rice callus (Oc) suspension cells. After immunoprecipitation by anti-HA antibodies, the interacting proteins were detected using anti-Myc antibodies. Results of this experiment confirmed that OsCTPS1 interacts with α-tubulin and β-tubulin (Figure 6d), which is consistent with a previous observation that CTPS binds to tubulin in human embryonic kidney cells and that CTPS colocalizes with microtubules (Higgins et al., 2008).

**FIGURE 6** The association of OsCTPS1 with microtubules. (a and b) Immunolocalization of β-tubulin in microtubules at 24 HAP. Microtubules were immunolabelled with anti-β-tubulin and the nuclei stained with DAPI (blue) in WT (a) and enl2-2 (b) endosperm. Scale bar, 20 µm. RMS, radial microtubule system. (c) Immunoprecipitation of α- and β-tubulin using OsCTPS1-GFP. Leaf blades of transgenic plants expressing OsCTPS1-GFP and WT plants were collected for immunoprecipitation assays. After immunoprecipitation with α- or β-tubulin antibodies, samples were detected using GFP antibodies following SDS-PAGE. 1, WT; 2, OsCTPS1-GFP transgenic plants. (d) Co-immunoprecipitation of OsCTPS1 with α- and β-tubulin antibodies. OsCTPS1 was Myc-tagged and α- and β-tubulin were HA-tagged. OsCTPS1-HA and α- or β-tubulin-HA were co-expressed in Oc cell protoplasts. Total protein extracts were immunoprecipitated with anti-HA antibodies, and interaction signals were detected using anti-Myc antibodies following SDS-PAGE.
OsCTPS1 has an important role in early endosperm development

**FIGURE 7** Filamentous structures of OsCTPS1 in yeast and rice. (a and b) GFP fluorescence signal in *S. cerevisiae* expressing OsCTPS1-sGFP (a) and sGFP (b). DIC image (a1), GFP signal (a2) and merged images (a3) of *S. cerevisiae* expressing OsCTPS1-sGFP. Scale bar, 5 µm. (c and d) Subcellular localization of OsCTPS1-GFP in untreated (c) and 4 µg/ml DON-treated (d) rice protoplasts. DIG images of rice protoplasts (c1 and d1), GFP signal (c2 and d2) and merged images of OsCTPS1-GFP and NLS-mRFP (c3 and d3). NLS-mRFP was used as a nuclear marker (c3 and d3). Scale bar, 10 µm. (e and f) Co-localization of OsCTPS1 and β-tubulin. OsCTPS1:GFP and β-tubulin:RFP were co-expressed in untreated (e) or 4 µg/ml DON-treated (f) Ocs cells. Rice protoplasts DIG (e1 and f1), GFP signal (e2 and f2), RFP signal (e3 and f3) and merged images of OsCTPS1-GFP and β-tubulin:mRFP (e4 and f4). Scale bar, 10 µm. (g-j) Filamentous structures of OsCTPS1 in developing endosperm. Fluorescence of developing endosperm at 8 HAP (g), 24 HAP (h), 72 HAP (i) and 96 HAP (j) from transgenic plants expressing OsCTPS1-GFP. Scale bar, 10 µm.
sGFP alone showed evenly distributed GFP signal in each cell (Figure 7b).

To investigate whether OsCTPS1 forms macrostructures in rice, the OsCTPS1-sGFP fusion protein was co-expressed with NLS-RFP, a nuclear marker, in protoplasts prepared from Ocs rice suspension cells. Visualization of OsCTPS1-sGFP signal revealed that OsCTPS1 is evenly distributed between the cytoplasm and the nucleus (Figure 7c). However, when Ocs culture protoplasts were treated with the glutamine analog 6-diazo-5-oxo-L-norleucine (DON), the GFP signal became condensed and formed macromolecular structures (Figure 7d), which is similar to results involving DON-induced formation of CTPS filamentous structures in Drosophila and human cells (Chen et al., 2011). We also observed co-localization of OsCTPS1-sGFP and β-tubulin-RFP signals in normal and DON treatment conditions (Figure 7e,f). These results support the association of OsCTPS1 and tubulins to form microtubules.

To study whether these macromolecular structures are related to the phenotypes observed in ocspts1 mutants, we generated transgenic rice plants that expressing OsCTPS1-sGFP protein under the control of the constitutive maize promoter Ubi1 Since the mutant phenotype was most prominent in the endosperm during early developmental stages, we analyzed developing endosperms at 8, 24, 72 and 96 HAP. A large number of GFP signal puncta were observed in endosperm cytoplasm at 8 HAP (Figure 7g). In addition, long macromolecular structures were detected in the cytosol at 24 HAP (Figure 7h) while short, rod-like structures were visible at 72 HAP (Figure 7i). Moreover, the GFP signal had almost completely diminished at 96 HAP (Figure 7j). These result indicated that OsCTPS1 transiently forms macromolecular structures during the early stage of endosperm development when enl2 displayed the endosperm defect phenotypes.

Overexpression of OsCTPS1 improves grain yield

In order to see whether increased expression of OsCTPS1 promotes endosperm development, OsCTPS1-overexpressing plants were produced in which OsCTPS1 expression was controlled by the maize ubiquitin promoter. Out of six independently transformed plants, we selected two lines (#2 and #6) that expressed OsCTPS1 at high levels for further examination (Figure 8a). The typical grain size, length, width, thickness and 1000-grain weight of both overexpression lines was greater than that of WT (Figure 8b-f). Most agronomic traits of OsCTPS1-overexpressing plants were similar to WT, except for plant height, which was slightly decreased in transgenic plants (Figure S10). When the endosperms were observed at 60 HAP, more endosperm cells had formed in the grains of the transgenic plants than in WT grains (Figure 8g-i). These results suggest that the increased expression of OsCTPS1 enhances nuclear division in the endosperm, resulting in increased grain size.

Discussion

Rice is a major food crop in many regions of the world (Lo et al., 2020); thus, as the world population continues to grow, increasing rice grain yield is important. Grain yield is determined by several factors including grain size, panicle number per plant, grain number per panicle and fertility (Xing and Zhang, 2010; Lo et al., 2020; Yang et al., 2020). In addition, several studies have indicated that spikelet hull size depends on both cell proliferation and expansion (Lyu et al., 2020; Yang et al., 2020). Although endosperm development plays a major role in controlling the quality and quantity of rice grains, the mechanisms underlying the early stages of cereal endosperm development remain largely unknown (Qi et al., 2020).

We identified rice mutants defective in the early stages of endosperm development. Null OsCTPS1 mutants showed halted nuclear division at 24 HAP during syncitial endosperm formation. Endosperm nuclei in ocspts1 plants did not migrate to the periphery of the cell and degenerated, resulting in the loss of endosperm. This endosperm defect in enl2 occurred earlier than defects associated with other rice endosperm mutants. Among the several genes that have been identified as playing important roles during endosperm development, OsLFR was the gene with the earliest known function. Mutants defective in OsLFR function start to display endosperm defects at 32 HAP, when they possess fewer free nuclei at the periphery of the endosperm than WT plants (Qi et al., 2020). Since our enl2 mutant showed endosperm defects at 24 HAP, OsCTPS1 seems to function at least 8 h before OsLFR. The nuclei from oslfr mutants were able to migrate to the periphery of endosperm cells, whereas enl2 endosperm nuclei did not migrate, also supporting the earlier function of OsCTPS1.

The enl2 mutants showed endosperm defects earlier than the mutants with mutations in genes associated with cellularization, including knock-down mutants CycB1;1 that exhibit issues with cell wall formation (Guo et al., 2010) and mutants lacking MAD57B or MAD579 that display precocious endosperm cellularization (Paul et al., 2020). The enl2 mutants also showed altered phenotypes before the mutants with altered mitotic chromosomal segregation or endoreduplication arising from defects in en1, NF-Y and OsCCS52A (Hara et al., 2015; Su’udi et al., 2012; Sun et al., 2014).

Our enl2 mutants also displayed enlarged embryos, which is consistent with previous observations associating enlarged embryos with other endosperm mutants (Guo et al., 2010; Hara et al., 2015; Nagasawa et al., 2013; Su’udi et al., 2012; Sun et al., 2014; Yang et al., 2013). These increases in embryo size appear to be linked with the absence of the endosperm. Endosperm-specific suppression of cell cycle inhibition has been shown to cause endosperm defects and increases in embryo size (Guo et al., 2010), and interactions between the embryo and endosperm at interface tissues appear to play critical roles in controlling embryo size (Nagasawa et al., 2013). Although the embryos were large in our enl2 mutant seeds, they developed normally, including during the early developmental stages, indicating that the endosperm is not needed for early embryo development. Whereas the overall embryo structure was normal in enl2-1, enl2-2 embryos were occasionally defective and seeds sometimes failed to germinate, indicating that this null mutation caused more severe phenotypes than the less severe mutation in enl2-1.

The rice genome contains six CTPS genes, of which two (OsCTPS3 and OsCTPS4) were expressed at very low levels in all organs tested. The remaining four CTPS genes were ubiquitously expressed in various tissues. This redundancy may contribute to the lack of apparent phenotypic changes at most developmental stages in the enl2 mutants. However, enl2 mutants showed defects in nuclear division in the endosperm, suggesting that more than one CTPS gene is needed for rapid nuclear division when a large number of nucleotides are needed for DNA synthesis.

Unlike the distant spacing of nuclei in WT endosperm, the nuclei in enl2 endosperm were closely packed and appeared to clump, which appeared to arise due to a lack of sister nuclei.
OsCTPS1 has an important role in early endosperm development

**FIGURE 8** Characterization of OsCTPS1-overexpressing plants. (a) OsCTPS1 expression levels in OsCTPS1-overexpressing transgenic plants. RNA samples were collected from seedling leaf blades. Numbers indicate individual transgenic plants. (b) Phenotype of OsCTPS1-overexpressing and WT plant grains. Scale bar, 5 mm. (c) 1000-grain weight of OsCTPS1-overexpressing plants compared with WT. n = 4. Statistical significance is indicated by *** (P < 0.001). (d) Grain length in OsCTPS1-overexpressing plants compared to WT. n = 10. Statistical significance is indicated by *** (P < 0.001). (e) Grain width in OsCTPS1-overexpressing plants compared to WT. n = 10. Statistical significance is indicated by *** (P < 0.001). (f) Grain thickness in OsCTPS1-overexpressing plants compared to WT. n = 10. Statistical significance is indicated by *** (P < 0.001) and ** (P < 0.05). (g-i) Z-stack images obtained by confocal laser-scanning microscopy of WT (g) and OsCTPS1-overexpressing plants (h and i). Scale bar, 100 μm. (j-l) Enlarged images of red boxes in Figure 8-g-i. Scale bars, 50 μm.
separation by microtubules in the endosperm. In addition, enl2 endosperm nuclei failed migration to the cell periphery. Since this nuclei movement to the sac periphery is likely mediated by microtubules, the major defect in enl2 mutant endosperm may be due to an insufficient amount of functional microtubules. We showed that OsCTPS1 interacts with tubulin proteins, supporting the possibility that OsCTPS1 is involved in microtubule function. It was previously reported that plant microtubules associate with several proteins during endosperm development (Huang et al., 2019; Pignocchi et al., 2009). For example, endosperm defective1 (EDE1) is a microtubule-associated protein that is essential for microtubule function during mitosis and cytokinesis in Arabidopsis endosperm and embryo (Pignocchi et al., 2009). In addition, the kinesin-14 subfamily protein VKS1 colocalizes with microtubules to enable free nuclei migration in the developing endosperm in maize (Huang et al., 2019).

Cells of transgenic plants expressing OsCTPS1-GFP fusion proteins exhibit rod-shaped macromolecular structures. Although transient expression of Arabidopsis CTPS in Nicotiana benthamiana protoplasts produced aggregates, these clusters did not form uniformly organized structures (Daumann et al., 2018). Furthermore, Arabidopsis CTPS did not form macromolecular structures when expressed in S. cerevisiae. In contrast, we showed that rice OsCTPS1 formed macromolecular structures in S. cerevisiae and in stably transformed plants, indicating that CTPS transiently forms macromolecular structures.

Although we used the constitutively expressed Ubi promoter, macromolecular structures were only detected in endosperm cells undergoing rapid nuclear division, indicating that such division may be a requirement for OsCTPS1 macromolecular structure formation. CTPS macromolecular structures specifically formed at a stage when large amounts of CTP were needed, suggesting that these structures increase enzyme activities. Our observations are consistent with previous reports indicating that CTPS macromolecular structures in animals are formed at specific stages of development (Liu, 2016). In Drosophila, these structures are observed in early-stage neuroblasts of the larval central nervous system (Aughey et al., 2014). In mice, such structures preferentially arise in fast-growing cells and in the axons of rat neurons (Noree et al., 2010). The requirement of a large number of macromolecular CTPS structures during rapid nuclear division thus appears to be evolutionarily conserved in both animals and plants.

The function of CTPS filaments remains under debate (Liu, 2016). Cells may form these filamentous macromolecular structures to store inactive enzymes so they can be released when required. This idea is supported by the observation that CTPS polymerization inhibits CTPS activity in bacteria. In addition, destabilization of the active tetrameric arrangement to form dimers increases filament formation in S. cerevisiae, suggesting that the filaments are comprised of inactive CTPS dimers (Noree et al., 2014). In contrast, CTPS polymerization increases catalytic activity in human cells (Lynch et al., 2017). These varied regulatory mechanisms may arise from structural differences between microbes and human cells observed using cryo-electron microscopy (Lynch et al., 2017). In this study, we provided a possibility that CTPS filaments may involve in nuclei spacing by interacting with microtubules.

**Experimental procedures**

**Plant materials and growth conditions**

The endospermless mutant enl2-1 (Line no. PFG_1C-15623) was identified from a mutant population of *Oryza sativa* var. *japonica* cultivar Hwayoung. Deletion mutants were generated in the *japonica* rice cultivar *Oryza sativa* var. *japonica* cultivar Nipponbare. Seeds were either germinated on 1/2 Murashige and Skoog (MS) medium containing 3% sucrose or directly in soil as previously reported (Yi and An, 2013). Plants were grown in a controlled growth room (14 h light, 28°C/10 h dark, 22°C, humidity approximately 60%) or a paddy in Yongin, Korea, as previously described (Cho et al., 2016).

**5-Ethynyl-2′-deoxyuridine staining**

S-phase cells were visualized by 5-ethyl-2′-deoxyuridine (EdU) staining as previously reported (Kotogány et al., 2010; Yoon et al., 2020). Plants grown on MS medium were transferred to new MS medium containing 10 µM EdU and incubated for 4 h. Samples were then fixed for 1 h in 4% paraformaldehyde in PBS (pH 7.2) and permeabilized in a PBS solution containing 0.5% Triton X-100 for 30 min. Next, samples were incubated for 30 min in an EdU detection cocktail (C10338, Click-it EdU Alexa Fluor 555; Invitrogen). Images were obtained using the RFP channel of a LSM 800 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Map-based cloning**

Homozygous enl2-1 mutant plants (male parent) were crossed with the *indica* type cultivar Milyang 23 (female parent). F1 hybridity was confirmed using the polymorphic SSR markers R1M7 and R7M7 (Shen et al., 2004). F2 seeds harvested from 25 F1 plants were de-husked for the selection of mutant and normal seeds. For bulked segregant analysis, 20 mutants and 20 normal seeds were grown on MS agar media. Genomic DNA was extracted from each resulting plant using the CTAB method and the same amount of genomic DNA from each of four plants was pooled into one tube, resulting in five tubes for mutant seeds and another five tubes for normal seeds. The pooled DNA was genotyped using SSR markers distributed across 12 chromosomes. For fine mapping, normal and mutant seeds were germinated on MS media and genomic DNA isolated from each seedling using a simple, non-toxic DNA preparation protocol (Kim et al., 2016). Individual plants were genotyped by RM9 and R1M30 markers and only recombinants between these two markers further analysed. New markers, including InDel type polymorphic markers and SNP markers, were developed using reference genomic sequences of *japonica* (Nipponbare IRGSP 1.0) and *indica* rice variants (93-11 variety). All of the primers used for fine mapping are listed in Table S1.

**Co-immunoprecipitation (Co-IP) analysis**

Co-IP assays were performed as previously described (Cho et al., 2016; Yoon et al., 2017). Briefly, fusion proteins were co-expressed in rice Oc cell protoplasts then total proteins extracted in IP buffer [75 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 20 µM MG132, protease inhibitor cocktail (Roche)]. Expressed proteins were immunoprecipitated using anti-HA mouse monoclonal antibodies (12CA5; Roche) or anti-Myc mouse monoclonal antibodies (#2276; Cell Signaling) conjugated with A and G agarose beads (Millipore, Billerica, MA, http://www.EMDmillipore.com). For protein detection, horseradish peroxidase (HRP)-conjugated anti-HA monoclonal antibody (#2999; Cell Signaling) or anti-Myc monoclonal antibody (#2040; Cell Signaling) were used.
CTP synthase activity assay

Full-length cDNA encoding OsCTPS1 was amplified using the primer pairs listed in Table S2. The cDNA was cloned into the BamHI and XhoI sites of the 6X His-fusion protein expression vector pET28a(+). The bacterial strain E. coli BL21 (DE3) was used as a host for recombinant protein expression. The N-terminal 6X His-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen, USA; https://www.qiagen.com) according to the manufacturer’s instructions. The CTP synthase activity was determined by comparing changes in extinction coefficients of UTP and CTP (182 M/cm and 1520 M/cm, respectively) at 291 nm. This assay for measuring CTP synthase activity examines the relative conversion of UTP to CTP by the same concentration of purified OsCTPS1-WT or OsCTPS-N81I. The standard reaction mixture was 0.1 ml total and consisted of 50 mM Tris-Cl (pH 8.0), 10 mM MgCl2 and 100 mM β-mercaptoethanol with final substrate concentrations of 2 mM L-glutamine, 2 mM ATP, 2 mM UTP, 0.1 mM GTP and an appropriate dilution of purified protein, as previously described (Chang et al., 2007). During the reaction mixture incubation at 37°C, the UV absorption was measured every 5 or 10 min for 1 h total. At least three independent replicates were conducted for each assay each involving three biological replicates.

Vector construction and plant transformation

To generate transgenic plants expressing OsCTPS1-sGFP, full-length OsCTPS1 CDNA was amplified by PCR using the primers listed in Table S2 and then digested with the restriction enzymes BsiWI and SpeI and inserted into the binary vector pGA3427 under the control of the maize ubiquitin 1 promoter (Kim et al., 2009). To generate pOsCTPS1-GUS transgenic plants, a 2.55-kb fragment of the OsCTPS1 promoter region was amplified using the primer pair listed in Table S2. Genomic DNA from ‘Nipponbare’ rice was used as a template. The amplified promoter fragment was inserted between the HpaI and XhoI restriction sites of the pGA3383 binary vector carrying the GUS coding sequence followed by the nopalline synthase terminator (Kim et al., 2009). osctps1 knockout plants were generated in the ‘Nipponbare’ background using CRISPR/Cas9 with the CRISPR/Cas9 vector previously described in Cho et al., 2018). The spacer sequence 5‘-GGGCACCTTTCCATCTACAGATGC-3‘ was cloned into the entry vector pOs-sgRNA to monitor sgRNA expression. The resulting recombinant entry vector, pOs-sgRNA, was then cloned into the destination vector pHap1 and XhoI restriction sites of the pGA3383 binary vector carrying the GUS coding sequence followed by the nopaline synthase terminator (Kim et al., 2009). osctps1 knockout plants were generated in the ‘Nipponbare’ background using CRISPR/Cas9 with the CRISPR/Cas9 vector previously described in (Cho et al., 2018). The spacer sequence 5‘-GGGCACCTTTCCATCTACAGATGC-3‘ was cloned into the entry vector pOs-sgRNA to monitor sgRNA expression. The resulting recombinant entry vector, pOs-sgRNA, was then cloned into the destination vector pHap1 and XhoI restriction sites of the pGA3383 binary vector carrying the GUS coding sequence followed by the nopaline synthase terminator (Kim et al., 2009). OsCTPS1 has an important role in early endosperm development and plant growth. Numerous studies have demonstrated that OsCTPS1 is involved in various processes such as seed development, carbohydrate metabolism, and stress response. OsCTPS1 expression is induced by stress conditions, including heat, drought, and salinity, indicating its role in stress tolerance. Furthermore, OsCTPS1 expression is regulated by various signaling pathways and hormones, such as auxin and brassinosteroids, which are important for plant development and growth. Therefore, understanding the role of OsCTPS1 in plant development and stress response is crucial for improving crop productivity and resilience.
Immunofluorescence localization

Experiments were performed based on a modified protocol (Pignocchi et al., 2009). To perform immunofluorescence localization, developing seed samples were fixed in 4% (w/v) paraformaldehyde in microtubule-stabilizing buffer/DMSO (MTSB; 50 mM PIPES, 5 mM EGTA and 5 mM MgCl2 in 5% DMSO, pH 6.7 to 7.0). Samples were then dehydrated by an ethanol series (50, 70, 90 and 100%) and then treated with a t-butyl alcohol series and infiltrated with paraﬃn. After embedding, samples were cut to a thickness of 10 μm with a microtome and sectioned samples rehydrated and then digested with 2% (w/v) disrélase (Sigma-Aldrich) for 30 min at room temperature. Next, samples were permeabilized with 1% (v/v) Triton X-100 in 10% DMSO-MTSB for 30 min at room temperature. Samples were blocked with 1% (w/v) BSA in MTSB for 1 h at room temperature and incubated with an anti-β-tubulin polyclonal antibody (#PA5-16863; Invitrogen) diluted 1:50 in 3% (w/v) BSA in MTSB at 4°C overnight. After washing with MTSB buffer, samples were incubated in Alexa Fluor 594 goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody (#A11037; Invitrogen) at a 1:300 dilutions in 3% (w/v) BSA in MTSB. DNA was stained with NucBlue® Fixed Cell ReadyProbes® Reagent (#R37606; Invitrogen). Samples were observed using a BX61 microscope (Olympus) with 360 to 370 nm (excitation) and 420 to 460 nm (emission) for DNA detection and 488 nm (excitation) and 610 nm (emission) for β-tubulin signal detection.

Yeast expression assay

To express OsCTPS1 in yeast, the corresponding full-length cDNA was amplified by PCR using the primers listed in Table S2. The cDNA fragment was then inserted into the pPS808 vector (Addgene, plasmid #8856) containing a GFP coding sequence. Haploid Saccharomyces cerevisiae YM4271 cells were transformed using a modified lithium acetate transformation protocol (Cho et al., 2016). Transformants were grown on minimal medium that lacking uracil for selection. Fluorescence was observed using a BX61 microscope (Olympus) with 360 to 370 nm (excitation) and 420 to 460 nm (emission) for GFP channels.

Statistical analyses

The P values were calculated by using one-way analysis of variance (ANOVA; Tukey HSD test) for the test groups with R program (Cohen and Cohen, 2008).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

J.Y., L.-H.C., S.-R.K and G.A. designed the project; J.Y., L.-H.C., S.-R.K, W.T., X.P., R.P., S.M., W.-J.H., H.J. performed experiments; K.-H.J., J.-S.J. and G.A. analysed and interpreted the data; J.Y., L.-H.C., S.-R.K and G.A. wrote the paper with significant input from all authors.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic tree of CTPS genes from 44 plant species.

Figure S2 Phylogenetic tree of CTPS genes from five plant species: Arabidopsis, rice, maize, Sorghum bicolor, and Glycine max.

Figure S3 Digital expression patterns of six CTPS genes in rice.

Figure S4 Generation of osctps1 null mutants using CRISPR/Cas9.

Figure S5 Phenotypes of WT and enl2-2 mutants.

Figure S6 Phenotypes of WT and enl2-2 mutant at seedling stages.

Figure S7 Seed phenotypes of WT and enl2-2 mutants.

Figure S8 Embryo development of WT and enl2-2.

Figure S9 Expression levels of genes involved in cell cycle and mitosis in WT and enl2-2 at 3 HAP and 24 HAP.

Figure S10 Characterization of OsCTPS1-overexpressing plants.

Table S1 Primers used for fine mapping and sequencing of Os01g43020.

Table S2 List of primers used in this study.