The Component of the TAC Complex, TCD7, Controls Rice Chloroplast Development at the Early Seedling Stage under Cold Stress

Dongzhi Lin
Shanghai Normal University
Licheng Kang
Shanghai Normal University
Wenhao Zhou
Shanghai Normal University
Yulu Wang
Shanghai Normal University
Yu Chen
Shanghai Normal University
Jianlong Xu
Chinese Academy of Agricultural Sciences
Yanjun Dong (dong@shnu.edu.cn)
Shanghai Normal University

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Abstract

Transcriptionally active chromosome (TAC) is a component of protein-DNA complexes with RNA polymerase activity found in chloroplasts. Although TAC in Arabidopsis thaliana has been extensively investigated, how the rice (Oryza sativa L.) TAC complex functions remains largely unknown. We report the characterization of the mutant thermosensitive chlorophyll-deficient7 (tcd7) and the cloning of TCD7. tcd7 mutant seedlings displayed an albino phenotype specifically at low temperatures and before the four-leaf stage. We identified TCD7 by map-based cloning followed by transgenic rescue and genome editing tests, showing that TCD7 encodes the putative TAC component FRUCTOKINASE-LIKE 2 (OsFLN2). TCD7 transcripts were highly abundant in green tissues, and the protein localized to chloroplasts. In agreement with the albino phenotype, transcript levels of genes controlling chloroplast development and the establishment of photosynthetic capacity were severely reduced in tcd7 seedlings at low temperatures, but were expressed as in the wild type at high temperatures, implying that TCD7 regulates the PEP pathway and chloroplast development. Moreover, TCD7 interacted with the thioredoxin OsTRXz to form an OsTRXz-TCD7 regulatory module, which might regulate plastid transcription under cold stress. Our results demonstrate that the nucleus-encoded TAC protein TCD7 protects chloroplast development from cold stress via a TRXz-FLN regulatory module.

Introduction

The chloroplast is a semi-autonomous organelle whose genome encodes 100-250 proteins associated with photosynthesis and other physiological and biochemical functions (Wicke et al. 2011). The expression of chloroplast genes relies on two distinct RNA polymerases: a nucleus-encoded phage-type RNA polymerase (NEP) and a plastid-encoded prokaryotic-type RNA polymerase (PEP) (Toyoshima et al. 2005). NEP is mainly responsible for modulating the transcription of plastid housekeeping genes during early chloroplast development, including those encoding PEP core subunits, as well as genes involved in chloroplast functions. PEP activity is itself required for fully active chloroplasts, as it controls the expression of photosynthesis genes (Hajdukiewicz et al. 1997). PEP core proteins can be detected in two chloroplast protein fractions: the soluble RNA polymerase (sRNAP), and the insoluble fraction known as transcriptionally active chromosome (TAC) (Krause et al. 2000).

TAC proteins play essential roles in chloroplast development (Pfalz et al. 2006; Steiner et al., 2011; Pfalz and Pfannschmidt 2013), as revealed by studies in Arabidopsis (Arabidopsis thaliana). For instance, pTAC3 is a chloroplast-localized transcription factor that mediates DNA/RNA binding within the PEP complex (Yagi et al. 2012), while pTAC4 contributes to the formation of the thylakoid membrane (Zhang et al. 2012; Feng et al. 2014). Other sets of TAC proteins, such as pTAC10, pTAC12, pTAC6 and pTAC7, participate in PEP-mediated plastid gene expression and promote chloroplast development (Peter and Åsa, 2015; Chang et al. 2017). pTAC8 (also named PSI-P) is the P subunit of photosystem I (PSI) and influences the formation of thylakoid grana (Armbruster et al. 2013; Hansson et al. 2003). Likewise, pTAC5 plays an important role during chloroplast development under heat stress (Zhong et al. 2013). Importantly, the components pTAC12 and pTAC14 coordinate light signaling with chloroplast

development, as ptac12 and ptac14 mutants are albino (Yu et al. 2013). The TAC fraction is characterized by protein-protein interactions between the subunit pTAC10 and other TACs, such as FE SUPEROXIDE DISMUTASE 2 (FSD2) and FSD3, THIOREDOXIN Z (TRXz), pTAC7 and pTAC14, to regulate chloroplast development (Chang et al. 2017). Arabidopsis FRUCTOKINASE-LIKE PROTEIN2 (FLN2) interacts with FLN1, another member of the fructokinase-like (pfkB) protein family, and forms heterodimers that interacts with TRXz to regulate PEP activity. Notably, FLN2 also interacts with pTAC5, and the fln2-2 mutant is albino, although the mutant returns to a more wild-type appearance and accumulates chlorophyll upon exogenous application of 2% sucrose (Huang et al. 2013). Although much of the work on TAC components has been carried out in Arabidopsis, whether and how rice (Oryza sativa) TAC genes influence chloroplast development, especially under cold stress, remains largely unclear. Low temperatures can severely and negatively affect rice production by targeting a range of cellular components (e.g. chloroplasts) and metabolic pathways (e.g. photosynthesis) and thus reducing yield. Many genes required for chloroplast development in rice are reported to be induced during cold stress, such as THERMO-SENSITIVE CHLOROPHYLL DEFICIENT3 (TCD3; Lin et al. 2020), TCD5 (Wang et al. 2016b), TCD10 (Wu et al. 2016), TCD11 (Wang et al. 2017), TCD33 (Wang et al. 2020), VIRESCENT1 (V1; Kusumi et al. 1997 and 2010b), V2 (Sugimoto et al. 2004 and 2007), V3 (Yoo et al. 2009), OsV4 (Gong et al. 2014), THERMO-SENSITIVE VIRESCENT3 (TSV3; Lin et al. 2018a), THERMOSENSITIVE CHLOROPHYLL-DEFICIENT MUTANT1 (TCM1; Lin et al. 2018b) and TCM12 (Lin et al. 2019). However, the mechanism(s) by which these genes modulate cold resistance and chloroplast development have not been explored in detail (Wang et al., 2020).

To date, only a few TAC genes have been reported in rice: OspTAC2, TCM1 and WHITE LEAF AND PANICLE2 (WLP2). OspTAC2 encodes a protein homologous to Arabidopsis TAC2. As in Arabidopsis, the rice ptac2 mutant is albino and seedling lethal (Wang et al. 2016a), Similarly, TCM1 is the rice homolog to Arabidopsis TAC12, and its mutation leads to chloroplast damage in seedlings exposed to cold stress (Lin et al. 2018), WLP2 (also named OsFLN1) is homologous to Arabidopsis FLN1, and its mutation results in seedlings with white leaves and panicles specifically under heat stress (Lv et al. 2017). To our knowledge, thermo-sensitive chlorophyll-deficient mutants, occurred only in specific early-leaves, have not been reported in rice yet. Herein, we describe thermo-sensitive chlorophyll-deficient 7 (tcd7), a rice albino mutant with a conditional phenotype that affects leaves before the four-leaf stage and only under cold stress. We cloned TCD7, which encodes a pfkB family protein that is a constituent of the rice TAC complex. In addition, we show that the transcript levels of genes associated with chlorophyll biosynthesis, photosynthesis and chloroplast development are severely impaired in tcd7 mutants before the four-leaf stage during exposure to cold stress. Our work indicates that rice TCD7 plays an important role in chloroplast development and maintenance of PEP activity under specific conditions.

Results

Phenotypic Characterization of the tcd7 Mutant

Seedlings for the wild-type japonica rice variety Jiahua 1 and the tcd7 mutant were grown at four distinct temperatures (20ºC, 24ºC, 28ºC and 32ºC). Seedlings from WT displayed normal green leaves regardless of the growth temperature and leaf-stages, as expected. In sharp contrast, the tcd7 mutant produced albino leaves when grown at 20ºC, up to the fourth leaf. Surprisingly, all subsequent leaves starting with the fifth leaf took on a normal appearance, similar to WT seedlings (Fig. 1A). In addition, the albino phenotype seen in the tcd7 mutant was conditional, as we observed white leaves only when seedlings were grown at 20ºC, but not at 24ºC, 28ºC or 32ºC (Fig. 1B-D). We failed to rescue the albino phenotype when tcd7 seedlings were transferred from 20ºC to 32°C, as third and fourth white leaves did not turn green (data not shown). These results indicated that the mutant phenotype is temperature sensitive during the early seedling stage.

In agreement with our visual phenotypic assessment, seedling contents of the pigments chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (Car) before the four-leaf stage were much lower in white tcd7 leaves than in WT seedlings when grown at 20°C (Fig. 2A), but were similar to WT (Fig. 2B) at the higher growth temperature of 32°C. This result suggested that chlorophyll accumulation in younger tcd7 seedling may be blocked under cold stress. To determine whether the observed reduction of photosynthetic pigments in tcd7 seedlings at 20ºC might be due to altered chloroplast ultrastructure, we observed chloroplasts in seedlings of both genotypes grown at 20ºC or 32ºC by transmission electron microscopy (TEM). WT mesophyll cells contained numerous uniform chloroplasts, regardless of the growth temperature (Fig. 2C, 2D). By contrast, leaves from tcd7 seedlings grown at 20°C were characterized by far fewer grana and abnormal grana structure (Fig. 2E), whereas chloroplasts in leaves from tcd7 mutant seedlings grown at 32ºC had normal morphology, consistent with their lack of a distinct phenotype at this temperature (Fig. 2F). We therefore presume that the abnormal chloroplast structures seen in seedlings grown at 20°C may lead to reduced chlorophyll content in the mutant.

Map-based Cloning of TCD7

To identify TCD7, we generated a mapping population by crossing tcd7 plants with the polymorphic variety Peiai64S (indica). Leaves of F1 hybrid seedlings were a healthy green, indicating that tcd7 is a recessive mutation. In the subsequent F2 generation, the mutant phenotype segregated as a monogenic recessive Mendelian trait, with a wild-type to mutant phenotype ratio of 3:1 (Additional file 1: Table S1, $\chi 2 = 0.21 < \chi 20.05 = 3.84$). We then selected 94 F2 seedlings with the mutant phenotype for initial mapping, which placed TCD7 between markers MM3645 and MM3833 on chromosome 3 (Fig. 3A, B). We then increased the size of the mapping population to 624 F2 seedlings with the mutant phenotype for fine mapping, allowing us to narrow the mapping interval containing TCD7 to a 123-kb region between markers ID14867 and RM15419. This interval was covered by four bacterial artificial chromosomes (BACs AC097276, AC092778, AC097276 and AC109601) and contained six predicted genes (Fig. 3C). We amplified all candidate genes by PCR from tcd7 genomic DNA and sequenced all PCR amplicons to detect a polymorphism relative to the wild-type sequence: only LOC_Os03g40550, which encodes the TAC protein most similar to Arabidopsis FLN2 (thus also named OsFLN2 in this study), carried a 9-bp (GTTG

CTCTT) deletion in exon 3, 775 bp downstream from the translation start codon (Fig. 3D, 3E). Because of its length, this deletion does not disrupt the open reading frame of OsFLN2/TCD7, but results in a predicted protein lacking three amino acids (Val, Ala, Leu) compared to WT.

Complementation and Genome Editing of TCD7

To assess whether the mutation we identified in LOC_Os03g40550 might be responsible for the tcd7 mutant phenotype under cold stress, we used two complementary approaches. First, we tested for rescue of the albino phenotype upon introduction of a functional copy of LOC_Os03g40550 in the tcd7 mutant, driven by the cauliflower mosaic virus (CaMV) 35S promoter. All T0 transgenic plants produced only green leaves when grown at 20ºC (Fig. 4B), whereas independent T0 transgenic plants transformed with the empty vector pCAMBIA1301 retained the mutant phenotype (Fig. 4A). During the subsequent T1 generation, T1 plants with green leaves also carried a copy of the transgene when grown at 20ºC, whereas none of the T1 plants with albino leaves did (Fig. 4C), indicating that the overexpression of LOC_Os03g40550 in the tcd7 mutant background rescued the albino phenotype characteristic of the mutant. Independently, we generated two homozygous transgenic T0 plants by CRISPR/Cas9 genome editing. One edited plant harbored the same 9-bp (CATCAGAAG) deletion detected in TCD7; we designated this line as T0-a. The other edited plant, which we designated T0-b, carried multiple deletions and mutations in TCD7 (Additional file 2: Fig. S1). Importantly, both T0 plants exhibited the same albino phenotype as the tcd7 mutant when grown at 20ºC (Fig. 4D). T0 plants heterozygous at these two edited sites were fully green, indicating that both mutations are recessive, like the original tcd7 mutation. Moreover, the T1 progeny of these heterozygous edited T0 transgenic plants segregated for the albino phenotype when grown at 20°C (Fig. 4E, 4F). Taken together, these results confirm that LOC_Os03g40550 is TCD7.

Analysis of TCD7 Expression and Subcellular Localization of TCD7

We next determined the expression pattern of TCD7 in various tissues in wild-type seedlings by semi-quantitative RT-PCR. TCD7 was highly expressed in leaves (second, third and flag leaves), but much less expressed in roots, stems or panicles (Fig. 5A). These results were consistent with the rice gene expression atlas available in the RiceXPro database (Additional file 2: Fig. S2), and were broadly in agreement with a tissue-specific expression pattern and a vital role in leaf chloroplast development for TCD7.

As TCD7, one of the subunits of the TAC complex, we investigated its subcellular localization. The TargetP 1.1 Server predicted that TCD7 localizes to the chloroplast (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000). To test this hypothesis, we fused the first 149 amino acids of TCD7, including any potential signal targeting sequence, to the N terminus of

green fluorescent protein (GFP). The resulting clone was driven by the 35S promoter and transiently expressed in tobacco protoplasts. Confocal microscopy revealed co-localization of GFP fluorescence and chlorophyll auto-fluorescence (Fig.5B), demonstrating that TCD7 is a chloroplast-localized protein.

Characterization of the TCD7 Protein

The TCD7 locus consists of five exons and six introns (Fig. 3D) and encodes a protein of 589 amino acids with a predicted molecular mass of about 65 kDa. A search of the Pfam database revealed that TCD7 belongs to the transcriptionally active chromosome (TAC) complex and contains a pfkb domain (Additional file 2: Fig. S3). In addition, the tcd7 mutation results in the deletion of three conserved amino acids (Val, Ala, Leu) within the pfkb domain, which is predicted to disrupt the α-helical structure of TCD7, possibly leading to a compromised overall structure (Additional file 2: Fig. S3B). TCD7 is highly conserved across land plants, and was closest to its putative ortholog from the monocot purple false brome (Brachypodium distachyon) (Fig. 6B). Notably, TCD7/OsFLN2 shared only 55% identity with Arabidopsis FLN2 (Huang et al. 2013) (Additional file 2: Fig. S4), which differed at the three amino acids deleted in tcd7 (Val, Ala, Ile instead of Val, Ala, Leu). The conservative change of a leucine to isoleucine might result in different functions in rice and Arabidopsis.

Expression of Chloroplast- and photosynthesis-related Genes in tcd7

We observed ultrastructural defects in tcd7 chloroplasts when seedlings were grown at 20ºC (Fig. 2C-2F), which might explain the albino phenotype. However, this possibility does not preclude an effect of the tcd7 mutation on the expression of genes involved in photosynthesis or chlorophyll biogenesis or chloroplast development. Therefore, we surveyed relative expression levels for 26 genes associated with chlorophyll biosynthesis (PORA, HEMA1, CAO1, YGL1), photosynthesis (Cab1R, RbcS, rbcL, psaA, psbA, LhcpII) and chloroplast development (RpoTp, rpoA, rpoB, rpoC1, rpoC2, FtsZ, aptA, 23S rRNA, 16S rRNA, rps7, rps20, V1, V2, OsV4, TSV3, petA) in WT and tcd7 seedlings at the third-leaf stage grown at 20ºC or 32ºC. In the tcd7 mutant grown at 20°C, we measured drastically reduced transcript levels for chlorophyll biosynthesis genes encoding glutamyl tRNA reductase (HEMA), chlorophyllide A oxygenase (CAO1), protochlorophyllide oxidoreductase (PORA) and the CHLG subunit of chlorophyll synthase (named Yellow-Green Leaf1 [YGL1] in rice) (Fig. 7A), in agreement with the reduced chlorophyll contents seen earlier (Fig. 2A and the albino phenotype (Fig. 1A).

Although Cab1R (encoding the light harvesting Chla/b-binding protein) and RbcS (encoding the small subunit of Rubisco) showed 50-70% higher expression levels in the tcd7 mutant relative to WT, other photosynthesis-related genes(rbcL, psaA, psbA and LhcpII) were expressed at levels 30% of WT or below in the tcd7 mutant, suggesting that tcd7 should also hinder photosynthesis (Fig. 7B). Of 16 genes involved in chloroplast development, eight (rpoA, ropB, rps7, rps20, V1, V2, OsV4 and petA) were

expressed at low levels at low temperatures (Fig. 7C), which may lead to malformed chloroplasts (Fig. 2E). The PEP subunits rpoA and ropB (Kusumi et al. 2011) were especially downregulated in tcd7, with expression levels only reaching about 20% of WT levels. Notably, the downregulation of gene expression noted above in tcd7 seedlings grown at 20ºC was largely abrogated when the seedlings were grown at 32ºC (Fig. 8), with gene expression levels now more comparable between the WT and the mutant.

To explore the molecular mechanism behind the stage specificity of the albino phenotype exhibited by the tcd7 mutant, we next determined the relative transcript levels of PORA, HEMA1, CAO1, rbcL, psaA, psbA and rpoA in WT and mutant seedlings grown at 32ºC until the fourth- or fifth-leaf stage. Although all genes were significantly downregulated in the fourth leaves (Fig. 9A, 9B), their relative expression returned to nearly normal levels in the fifth leaves (Fig. 9C, 9D). We then turned to the PSI protein PsaA and the PSII protein D1, encoded by psaA and psbA, respectively. Indeed, we had established that expression of psaA and psbA was low in the third (Fig. 7B) and fourth leaves (Fig. 9B) of the tcd7 mutant at 20ºC, but was largely normal in seedlings grown at 32ºC (Fig. 8B) and in the fifth mutant leaf from seedlings grown at 20ºC (Fig. 9D). The accumulation patterns of PsaA and D1 were largely congruent with the levels of their corresponding transcripts, with very little protein detected in the fourth leaves of seedlings grown at 20ºC (Fig. 9E), but comparable accumulation in the fifth leaves of WT and tcd7 seedlings grown at 32ºC. In conclusion, these results suggest that the altered expression of chlorophyll biosynthesis and chloroplast biogenesis genes may contribute to the albino phenotype seen in the mutant until the four-leaf stage under cold stress.

TCD7 Interacts with OsTRXz in Yeast

Arabidopsis FLN2, which shares 55% identity with TCD7, interacts with TRXz and FLN1 (Arsova et al. 2010, Huang et al. 2013 and 2015). The putative rice ortholog to Arabidopsis TRXz is OsTRXz, encoded by LOC_Os08g29110, while AtFLN1 is most similar to OsFLN1, also named WPL2, which is encoded by LOC_Os01g63220 (Lv et al. 2017). We therefore tested whether TCD7 might interact with OsTRXz and OsFLN1 in a yeast-two hybrid assay. We removed the chloroplast targeting sequences from all proteins during cloning. Unexpectedly, TCD7, interacted only with OsTRXz, and not with OsFLN1, suggesting that TCD7/OsFLN2 and Arabidopsis FLN2 may function via distinct mechanisms (Fig. 10).

A possible biochemical basis for the tcd7 mutant phenotype may stem from a disrupted interaction between TCD7 and its partner OsTRXz. However, the loss of the three amino acids in FLN2tcd7 did not affect the TCD7-OsTRXz interaction (Fig. 10). Structurally, the mutated TCD7, lacking three amino acids in tcd7 mutants, could still interact with OsCITRXz (Fig. 10A), indicating that the tcd7 mutation may affect the function much more than the structure of rice FLN2.

tcd7 Mutant Phenotype is not due to Insufficient Energy Supply

Arabidopsis fln2 mutants display an albino and seedling-lethal phenotype at any growth temperature; however, chlorophyll accumulation can be partially rescued by exogenous supplementation with 2% sucrose, as evidenced by delayed greening (Huang et al. 2013 and 2015). We therefore wondered whether insufficient sucrose or energy supply might underlie the albino phenotype of tcd7 mutant seedlings exposed to cold stress before the fifth-leaf stage. However, no amount of exogenous sucrose from 2% to 8% succeeded in turning tcd7 albino seedlings green at the third- or fourth-leaf stage when grown at 22ºC (Fig. 11). On the contrary, higher sucrose concentrations appeared to inhibit seedling growth. We conclude that the albino phenotype is not the result of insufficient energy (sucrose) supply in tcd7 mutant seedlings.

Discussion

In this study, we described the albino mutant tcd7, which is characterized by an absence of chlorophyll accumulation and disrupted chloroplast development up to the fourth leaf when grown at the lower temperature of 20ºC. We cloned TCD7 by map-based cloning and determined that it encodes a chloroplast-localized component of the TAC complex with a pfkB domain. The deletion of three amino acids within the TCD7 pfkB domain resulted in a thermo-sensitive virescent phenotype, and drastically reduced the expression levels of genes associated with chlorophyll biosynthesis, photosynthesis and chloroplast development before the fourth-leaf stage under cold stress (Fig. 7). This study demonstrates that rice TCD7 plays important roles during early chloroplast development under cold stress.

TCD7 Acts during the Second Step of Chloroplast Development

Chloroplast development in rice involves the coordination of nuclear and chloroplast genes and can be classified into three main steps (Kusumi et al. 2011; Kusumi and Iba 2014). The first step consists of the activation of plastid replication and plastid DNA synthesis and involves OsPOLP1 (encoding a plastid-localized DNA polymerase) and FtsZ (encoding a component of the plastid division machinery) (Vitha et al. 2001; Takeuchi et al. 2007). The second step determines chloroplast buildup and the establishment of the chloroplast genetic system, which relies on OsRpoTp (encoding NEP), and rpoA and rpoB (encoding PEP core α and β subunits, respectively) (Hiratsuka et al. 1989; Kusumi et al. 2011). The third step sets up the long-term the function of photosynthetic organs and involves PEP-transcribed plastid genes (e.g. psaA, psbA and rbcL) (Kusumi et al. 2011). In previous studies, mutants similar to tcd7 have been isolated, including v1 to v4, several tcd and tcm mutants and tsv3 (Iba et al. 1991; Kusumi et al. 1997; Sugimoto et al. 2007; Yoo et al. 2009; Jiang et al. 2014; Gong et al. 2014; Wang et al. 2016b; Wu et al. 2016; Wang et al. 2017; Lin et al. 2018a, b; Lin et al., 2019, Lin et al., 2020;Wang et al. 2020). The corresponding genes have been assigned to one of the three steps of chloroplast development, based on the mutant phenotype and the expression pattern of the gene: V3, TCD3, TCD9, TCD10, TCD11, TCD33 and TSV3 function in the first step; V1, V2, V4, TCD5 and TCM1 act during the second step; and TCM12 functions in the third step. Cold stress thus likely affects all three steps of chloroplast development, via

multiple regulatory pathways. During exposure to cold temperatures, the tcd7 mutation severely repressed the expression of genes involved in the second (rpoA, rpoB, OsV4, V1, V2) and third (rbcL, psaA, psbA) steps of chloroplast development (Fig. 7), but not the first step (e.g. OsRpotp, FtsZ, TSV3). Thus, TCD7 may function during the second step, and later in the third phase as a logical progression of altered chloroplast development. In addition, the expression of all genes that were repressed under cold stress were restored to nearly WT levels when the plants were grown at higher temperatures, providing a molecular basis for the thermosensitivity of the mutant (Fig. 1). It is possible that another protein replaces TCD7 to carry out its function past the fourth leaf, which would explain the normal chloroplast development beyond this stage. The TCD7 paralog PFKB1 (Zhu et al., 2020) might fulfill such a role.

TCD7 is a Component of the TAC Complex Regulating PEP Activity and Photosynthesis under Cold Stress

Transcriptionally active chromosomes (TACs) are involved in replication, transcription, translation, detoxification, protein modification and plastid metabolism (Pfalz et al. 2006). Arabidopsis mutations in the TAC subunit FLN2 (encoded by At1g69200) lead to an albino, pale-green or seedling-lethal phenotype resulting from defective chloroplast development in a temperature-independent manner (Arsova et al. 2010; Huang et al. 2013). Other Arabidopsis TAC components (e.g. pTAC2, pTAC5, pTAC6, pTAC7, pTAC10, pTAC12, pTAC14, FLN1 and FLN2) are also required for proper function of the PEP transcriptional machinery and participate in post-transcriptional processes in the chloroplast; their loss of function is also associated with a temperature-independent albino phenotype (Pfalz et al. 2006; Arsova et al. 2010; Huang et al. 2013). In rice, the TAC proteins OsFLN1/WPL2 (Lv et al. 2017) and TCM1 (the rice homolog to Arabidopsis pTAC12)(Lin et al. 2018b) are reported to regulate PEP activity in a temperature-dependent manner, although in opposite directions. Indeed, the wpl2 mutant had an albino phenotype under heat stress (Lv et al. 2017), whereas the tcm1 mutant (like the tcd7 mutant) was characterized by an albino phenotype under cold stress(Lin et al. 2018b). In view of the reduced expression levels of rpoA, rpoB and PEP-dependent plastid genes (rbcL, psaA and psbA) (Fig. 7C, Fig. 10B), and the reduced PsaA (PSI) and D1 (PSII) protein abundance (Fig. 9E), we conclude that TCD7 regulates PEP activity and photosynthesis under cold stress.

The rice TRX-FLN Regulatory Module Mediates PEP Activity and Chloroplast Development under Cold Stress

As stated above, our data clearly indicated that TCD7/OsFLN2 is involved in the second step of chloroplast development by modulating PEP activity under cold stress. A hypothetical model for TCD7 function is shown in Fig. 12. PEP is the dominant RNA polymerase in chloroplasts, with a complex and still elusive regulatory mechanism (Kremnev and Strand 2014; Pfalzet al. 2006). In Arabidopsis, TRXz and its two interactors FLN1 and FLN2 form a complex that regulates PEP activity during PEP-dependent transcription (Arsova et al. 2010). The Arabidopsis fln1 and fln2 mutants have an albino phenotype at all

growth temperatures, with FLN1 possibly playing a more important role than FLN2 (Arsova et al. 2010; Gilkerson et al. 2012). In addition, chlorophyll accumulation can be partially restored in fln2 mutant seedlings upon provision of exogenous sucrose (Huang et al. 2013 and 2015). Importantly, the expression of neither Arabidopsis FLN1 nor FLN2 was regulated by temperature. By contrast, the rice tcd7 (this study) and wlp2 mutants (Lv et al. 2017) had an albino phenotype only at specific temperatures, raising the possibility that TCD7 and WLP2 expression might respond to temperature. Notably, the expression of PFKB1, a TCD7 paralog, regulates the expression of PEP-encoding genes irrespective of the temperature and may therefore compensate for the loss of TCD7 when its expression subsides (Zhu et al. 2020).

Multiple lines of evidence suggest that TCD7 and Arabidopsis FLN2 may function via at least partially distinct mechanisms. First, WLP2/OsFLN1 was shown to interact with OsTRXz to regulate PEP activity under cold stress(Lv et al. 2017); Arabidopsis FLN1 can also interact with FLN2 (Huang et al. 2013). In sharp contrast, we found here that TCD7/OsFLN2 did not physically interact with WLP2 (OsFLN1), which is consistent with previous results (Lv et al. 2017). Furthermore, WLP2/OsFLN1 can stabilize OsTRXz to mediate regulation of chloroplast development in the face of heat stress (Lv et al. 2017), whereas TCD7/OsFLN2 appears to stabilize OsTRXz to regulate chloroplast development during exposure to cold (Fig. 10A). Second, adding sucrose to the growth medium did not rescue the albino phenotype of the tcd7 mutant (Fig. 11), unlike for the Arabidopsis fln2 mutant. Third, TCD7/OsFLN2 has only 55% identity with Arabidopsis FLN2 (Additional file 2: Fig.S 4), which might be expected to correspond to distinct functions. It is worth noting here that the transcript levels of OsFLN1 and OsFLN2 have been incorrectly assumed to maintain a relative dynamic balance to regulate PEP activity and that OsFLN2 may partially compensate for loss of OsFLN1 function (Lv et al. 2017) due to no mutants of OsFLN2 as tcd7 mutant.

Protecting PEP activity and chloroplast development from cold or heat stress is essential and is mediated via the coordination of the OsTRXz-OsFLN complex. Undoubtedly, additional pathways may also regulate or protect chloroplast development in rice. For instance, the other TAC subunit, TCM1, regulates PEP activity under cold stress, although whether TCM1 can interact with OsTRX remains to be tested. Likewise, the Obg-like GTPase protein TSV3 regulates the biogenesis of the chloroplast ribosome 50S subunit under cold stress (Lin et al. 2018a). Importantly, the putative chloroplast-localized oxidoreductase TSV is also reported to interact with OsTRXz and form a OsTRXz-TSV module for the protection of chloroplast development from cold stress (Sun et al. 2017). One attractive possibility therefore calls upon OsTRXz as a central signaling hub that is absolutely critical for chloroplast development, especially under heat or cold stress, as a function of its interacting partners. Gaining a better understanding of TCD7 function and how TACs interact with OsTRXz to coordinate chloroplast development according to cell type, developmental stage or environmental conditions merits further investigation.

Conclusion

The TCD7 encodes chloroplast-targeted TAC protein which is important for chloroplast development and regulating PEP activity and photosynthesis, and its disruption would lead to a defective chloroplast at

early rice seedling stage under cold stress. Our results demonstrate that the TCD7 protein protects chloroplast development from cold stress via a TRXz-FLN regulatory module.

Materials And Methods

Plant materials and Growth Conditions

We identified the thermo-sensitive chlorophyll-deficient mutant tcd7 in our mutant pool in the japonica rice variety Jiahua 1 exposed to 60Co γ-radiation. An F2 mapping population was generated by crossing the tcd7 mutant and the indica variety Peiai64S. The mutant phenotype was easily distinguished from the wild type at the early seeding stage at Hainan island, China (winter season, subtropical climate) and Shanghai, China (early spring season, temperate climate) under local growth conditions. All rice plants were cultivated in paddy fields. Jiahua 1 (wild type, WT) and tcd7 mutant seedlings were also grown in growth incubators (12h light/12 h dark; light intensity 180 μmol m-2 s-1) at a constant temperature of either 20ºC, 24ºC, 28ºC or 32ºC for phenotypic characterization, pigment analysis, and DNA and RNA extraction.

Analysis of Photosynthetic Pigments and Transmission Electron Microscopy

Contents of leaf photosynthetic pigments were determined according to the methods of Arnon (1949) and Wellburn (1994), with slight modifications. Briefly, fresh leaves of tcd7 and WT seedlings were collected, ground to a powder in liquid nitrogen and incubated in chlorophyll extraction solution (ethanol:acetone:water 5:4:1 [v/v/v]). The absorbance of the extracts was measured at 470 nm, 645 nm and 663 nm with a METASH-UV5100 spectrophotometer. Results are reported as the average of three parallel experiments.

Chloroplast structures were observed from the third leaf of tcd7 and WT seedlings grown at 20ºC (restrictive temperature for the mutant phenotype) and 32ºC (permissive temperature) using transmission electron microscopy (TEM) as previously described (Jiang et al. 2014) with minor modifications. Samples were visualized with a Hitachi765 (Hitachi, Tokyo) transmission electron microscope.

Mapping and Cloning of TCD7

Genomic DNA from fresh rice leaves was extracted by the CTAB method, as described previously (Murray and Thompson 1980). To fine-map the TCD7 locus, 624 individuals with the mutant phenotype were selected from the F2 mapping population and genotyped with simple sequence repeat (SSR) or InDel markers (Additional file1: Table S2). Candidate genes within the final mapping interval were then PCR-amplified and sequenced (SinoGenoMax, Shanghai, China). Last, the functions and open reading frames for candidate genes were obtained from the Rice Genome Annotation Project

(http://rice.plantbiology.msu.edu/). Conserved domains were predicted using the online tool SMART (http://smart.embl-heidelberg.de/).

Genetic Complementation of the tcd7 Mutant

Total RNA was extracted from WT seedlings and transcribed into first-strand cDNAs for use as PCR template. Then, a 1.8-kb cDNA corresponding to LOC_Os03g40550 was PCR-amplified with the primers 5′-GGGGTACCATGCACCGAATGGCTTCTCTT-3′ (underlined sequences indicate a KpnI site) and 5′-GCGTCGACTCACTCCACATATAAAAGCTCACTC-3′ (underlined sequences indicate a SalI site). The resulting PCR amplicon was then digested with KpnI and SalI before being subcloned into the expression vector pCAMBIA1301-CaMV35S to generate the binary vector pCAMBIA1301-CaMV35S-TCD7(cDNA). The pCAMBIA1301-CaMV35S-TCD7(cDNA) construct was then introduced into Agrobacterium (Agrobacterium tumefaciens) strain EHA105 ahead of Agrobacterium-mediated transformation of the tcd7 mutant, as described previously (Hiei et al. 1994), with the temperature used for in vitro plant culture set to 20ºC. The genotype of transgenic seedlings was determined by PCR amplification of the hygromycin phosphotransferase (HPT) gene with the primers HPTF (5′-GGAGCATATACGCCCGGAGT-3′) and HPTR (5′-GTTTATCGGCACTTTGCATCG-3′) and primers GUSF (5′-GGGATCCATCGCAGCGTAATG-3′) and GUSR (5′-GCCGACAGCAGCAGTTTCATC-3′) as selection markers. The resulting T0 transgenic plants were grown in a paddy field after screening for hygromycin resistance and confirmation by DNA sequencing. After T1 seeds were collected from T0 plants, all T1 transgenic seedlings were grown at 20ºC and used to score the segregation ratio of the mutant phenotype.

Genome editing of TCD7

As an independent validation of LOC_Os03g40550 as the causal gene for the tcd7 mutant, the genomic locus was edited via clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease (Cas9) genome editing in WT plants. The targeting guide RNAs (gRNAs) for LOC_Os03g40550 were designed using a CRISPR Primer Designer software (http://www.crispr.dbcls.jp/) (Ma et al., 2015) and are listed in Additional file 1:Table S3. The recognition sequences were cloned between the OsU6 promoter and the gRNA scaffold (from the pYLgRNA-OsU6 vector) in the Cas9 expression backbone vector pYL-CRISPR/Cas9-MH at the BsaI sites, as previously described (Ma et al. 2015). The resulting construct (CRISPR/Cas9 expression) and the empty vector were introduced into Agrobacterium strain EHA105 and used to infect calli from WT plants via Agrobacterium-mediated transformation (Hiei et al. 1994), with the temperature used for in vitro culture set to 20°C. After seeds were collected from the resulting T0 plants, T1 seedlings were grown and used to determine the segregation of genome-edited seedlings and their associated phenotypes at 20ºC.

RT-PCR and Real-time PCR (qPCR) Analysis

Total RNA was extracted from the roots, stems and leaves of WT at the seedling stage, as well as from flag leaves and young panicles at the heading stage, using an RNA Prep Pure Plant kit (Tiangen Co., Beijing, China). The purified total RNA was then used for first-strand cDNA synthesis with the ReverTra Ace kit (ToYoBo, Osaka, Japan) following the manufacturer's instructions. RT-PCR analysis was carried out to assess TCD7 transcript levels. For RT-qPCR of TCD7 and 26 genes associated with chlorophyll biosynthesis (PORA, HEMA1, CAO1, YGL1), photosynthesis (Cab1R, RbcS, rbcL, psaA, psbA, LhcpII) and chloroplast development (RpoTp, rpoA, rpoB, rpoC1, rpoC2, FtsZ, aptA, 23SrRNA, 16SrRNA, rps7, rps20, V1, V2, OsV4, TSV3, petA), total RNA was extracted from the third, fourth and fifth leaves of WT and tcd7 seedlings. qPCR analyses were performed using the SYBR Premix Ex TaqTM kit (TaKaRa) on an ABI7500 Realtime PCR System (Applied Biosystems; http://www.appliedbiosystems.com). Relative transcript levels were calculated as described by Livak and Schmittgen (2001). The primers for qPCR were designed according to either Wu et al (2007) and NCBI-published sequences and are listed in Additional file1:Table S4. The rice ACTIN gene was used as a reference.

Subcellular localization

A cDNA fragment encoding the N-terminal region of TCD7 (amino acids 1-149) was amplified using the primer pair 5′-CCATCGATATGCACCGAATGGCTTCTCTT-3′ (the ClaI site is underlined) and 5′-GGGGTACCTCTGCCCCTCTTCTTTGGTTC-3′ (the KpnI site is underlined). The PCR amplicon was then subcloned into the binary vector pMON530-GFP to generate pMON530-TCD7-GFP. Constructs (pMON530-GFP and pMON530-TCD7-GFP) were transiently transfected into tobacco protoplasts and incubated in the dark at 28ºC for 16 h and then their fluorescence was observed with a Leica SP8 confocal laser scanning microscope. Protoplast isolation followed a previously published method (Zhang et al. 2011).

Yeast Two-hybrid Assay and Immunoblot Analysis

Yeast two-hybrid techniques were performed as described previously (Gao et al. 2011). The coding regions for LOC_Os03g40550 (TCD7) in WT and tcd7 were PCR-amplified and cloned into the bait vector pGBKT7. The coding regions for OsTRXz (LOC_Os08g29110) and OsFLN1/WLP2 (LOC_Os01g63220) were cloned into the prey vector pGADT7. Yeast two-hybrid assays were conducted following the manufacturer's instructions (Clontech). The primers used for vector construction are listed in Additional file 1:Table S5

For immunoblot analyses, chloroplast proteins (D1 and PsaA) were extracted from the fourth and fifth leaves of WT and tcd7 seedlings grown at 20ºC or 32ºC. Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, immunoblotted with corresponding antibodies (anti-D1 or anti-PsaA), and detected using High-sig ECL Western Blotting Substrate (Tanon, China).

Sugar Assays for Seeding Growth

Mature dried seeds of WT and tcd7 were surface-sterilized with 75% ethanol and NaClO disinfectant (3:1 [v/v] ddH2O:NaClO). Seeds were allowed to germinate in transparent bottles filled with sterile Murashige and Skoog (MS) medium supplemented with 2%, 3%, 5% or 8% sucrose. Bottles containing seedlings were then placed in a light incubator set to 20ºC under a 12 h light/12 h dark photoperiod and a light intensity of 180 μmol photons m-2 s-1. Seedling growth was determined until the five-leaf stage.

Abbreviations

Chla: Chlorophyll a ; Chl b: Chlorophyll b; FLN1:FRUCTOKINASE-LIKE PROTEIN1; FLN2: FRUCTOKINASE-LIKE PROTEIN2; NEP: Nucleus-encoded phage-type RNA polymerase; PEP: Plastid-encoded prokaryotic-type RNA polymerase; TAC: Transcriptionally active chromosome

Declarations

Funding

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Authors’ Contributions

YJD and JLX provided the research materials and designed the research. DZL, LCK, WHZ, YLW, YC and YJD performed the experiments. YJD and YC analyzed data. YJD, DZL and JLX wrote the manuscript. All authors reviewed the manuscript.

Availability of Data and Materials

All relevant data are provided in tables within the paper in the additional files.

Ethics approval and consent to participate

This study complied with the ethical standards of China, where this research was conducted.

Consent for Publication

All authors have consented to the publication of this manuscript.

Competing Interests

The authors declare that they have no competing interests.

Author details

1College of Life Sciences, Shanghai Normal University, Shanghai 200234, China

2The Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, 12 South Zhong-Guan Cun Street, Beijing 100081, China

3Shanghai Key Laboratory of Plant Molecular Sciences, Shanghai 200234, China

4Institute of Genetics, Shanghai Normal University, Shanghai 200234, China

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Figures

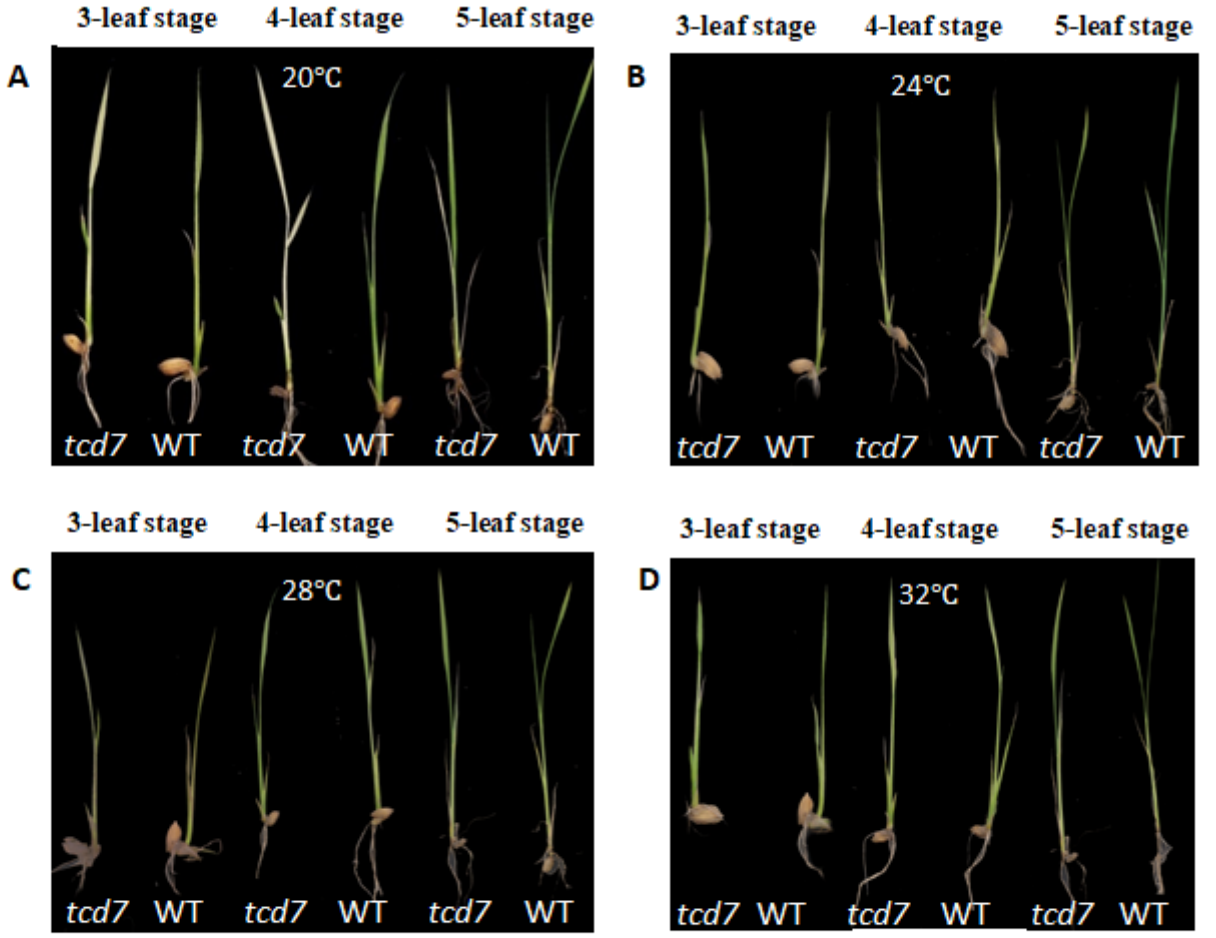

Fig. 1 Phenotypic characterization of the *tcd7* mutants. Seedlings of *tcd7* mutant (left) and Jinhua 1 (WT, right) at the three-, four- and five-leaf stages grown at 20°C (A), 24°C (B), 28°C (C) and 32°C (D), respectively.

Figure 1

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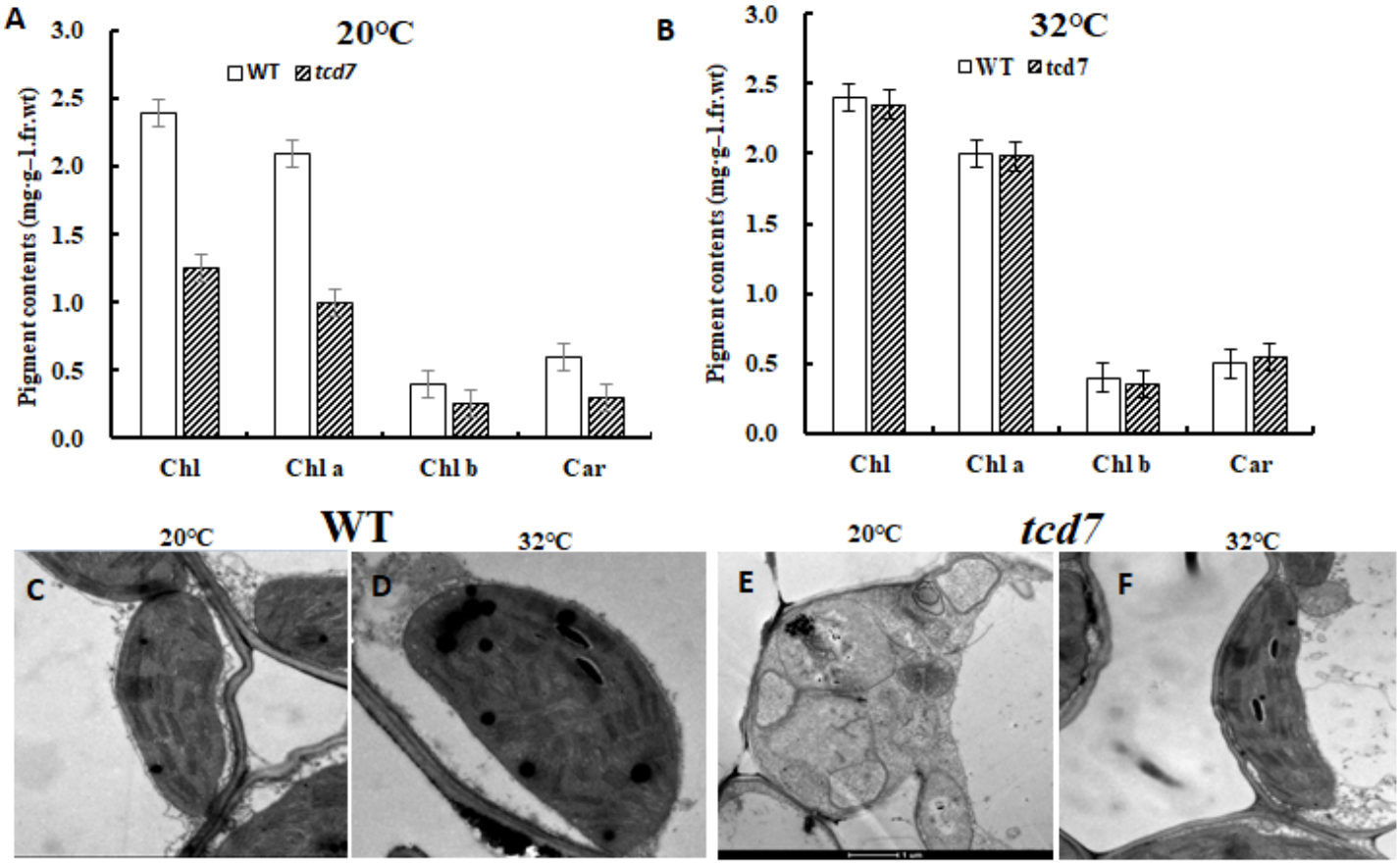

Fig. 2. Photosynthetic pigment contents and chloroplast structure of the third leaf in wild-type (WT) and *tcd7* mutant. *A,B:* Photosynthetic pigment contents of WT and mutants grown at 20°C (A) and 32°C; (B). Chloroplast ultrastructure of third-leaf cells from seedlings grown at 20°C (C and E) and at 32°C (D and F). Data are means ± SD ($n = 3$).

Figure 2

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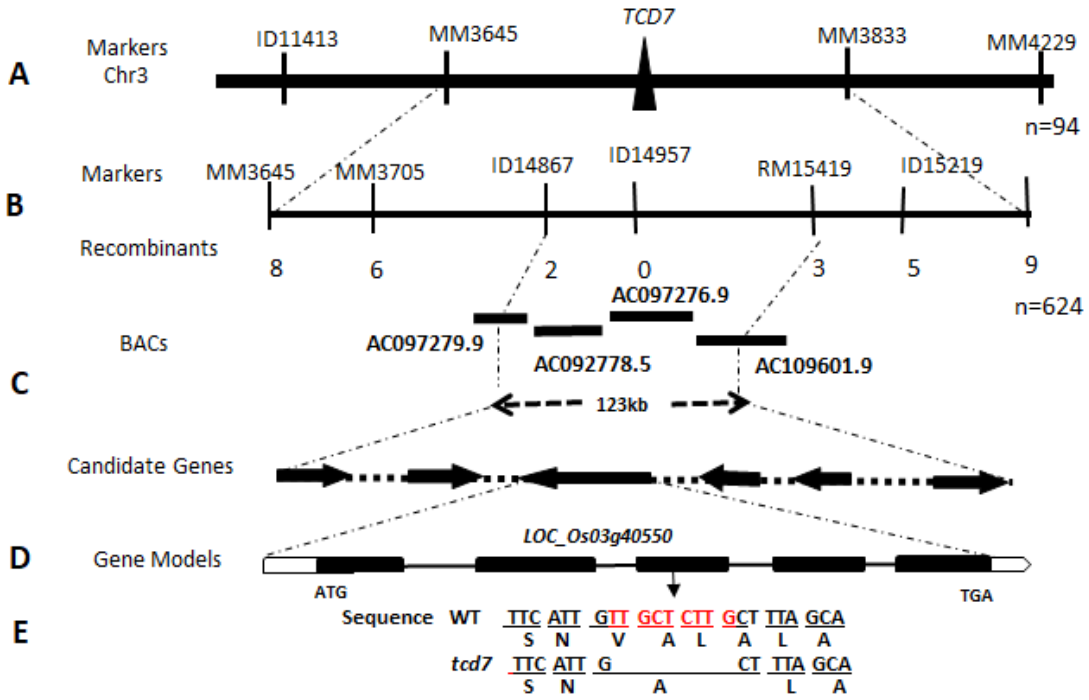

Fig. 3. Map-based cloning of the *TCD7* gene. (A) The location of *TCD7* was mapped to chromosome 3. (B) The location of *TCD7* was narrowed to a region between MM3645 and MM3833. (C) Predicted locations of four BACs in the 123-kb genome region. (D) Sequence analysis showed that *LOC_Os03g40550* is the *TCD7* gene. Black boxes represent exons; the lines between them represent introns.

Figure 3

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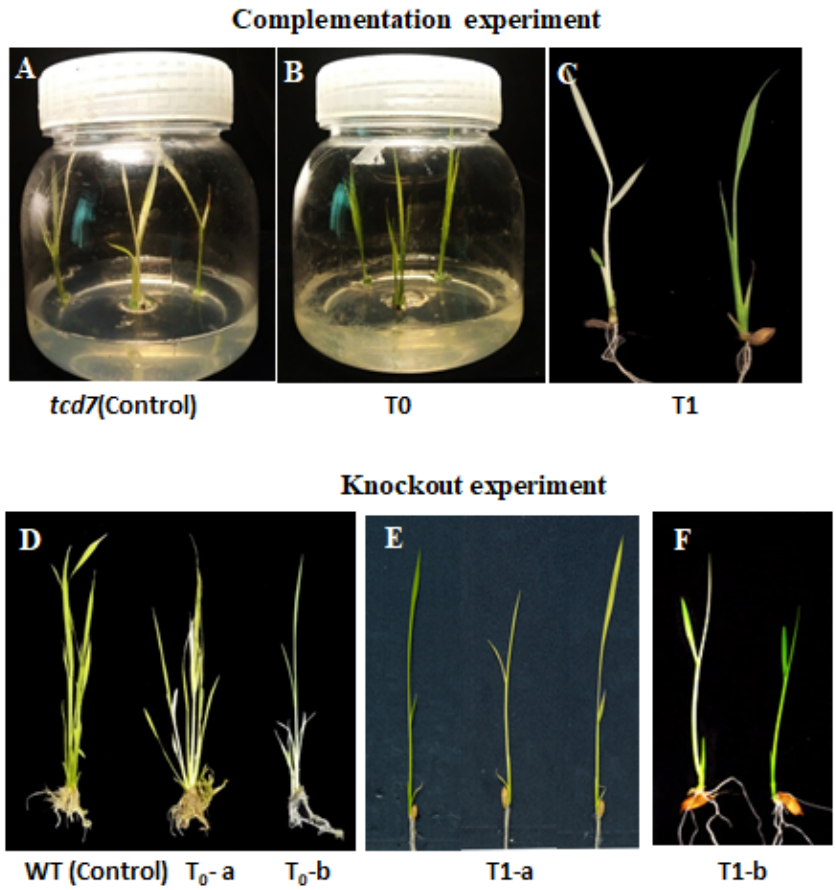

Fig. 4 Complementary (A, B,C) and knockdown (D, E) validation of *TCD7* genes. All seedlings were grown at 20°C. A, *tcd7* seedlings transformed with the empty vector pCAMBIA1301 as control; B, The obtained T_0 transgenic plants transformed with pCAMBIA1301-TCD7 in the *tcd7* mutant; C, The segregation of T_1 plants obtained from T_0 plants transformed with pCAMBIA1301-TCD7; D, WT seedlings served as control; the T_0-a and T_0-b transgenic lines were two type of homozygous transgenic T_0 plants carrying different edited mutation in the *TCD7* gene by CRISPR/Cas9 genome editing; E, F, the segregation of T_1 seedlings obtained from transgenic T_0-edited plants.

Figure 4

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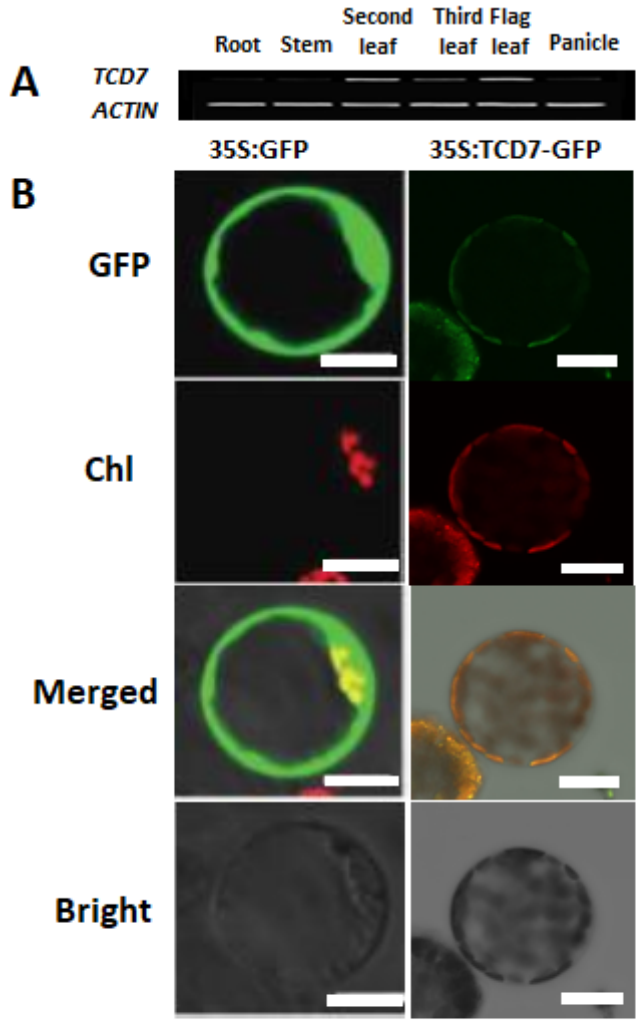

Fig. 5. Expression pattern and subcellular localization of the *TCD7* gene. (A) The expression of *TCD7* in various tissues. (B) GFP signal in tobacco chloroplasts transiently transformed with the empty GFP vector and TCD7-GFP fusion protein. Bars = 5 μm.

Figure 5

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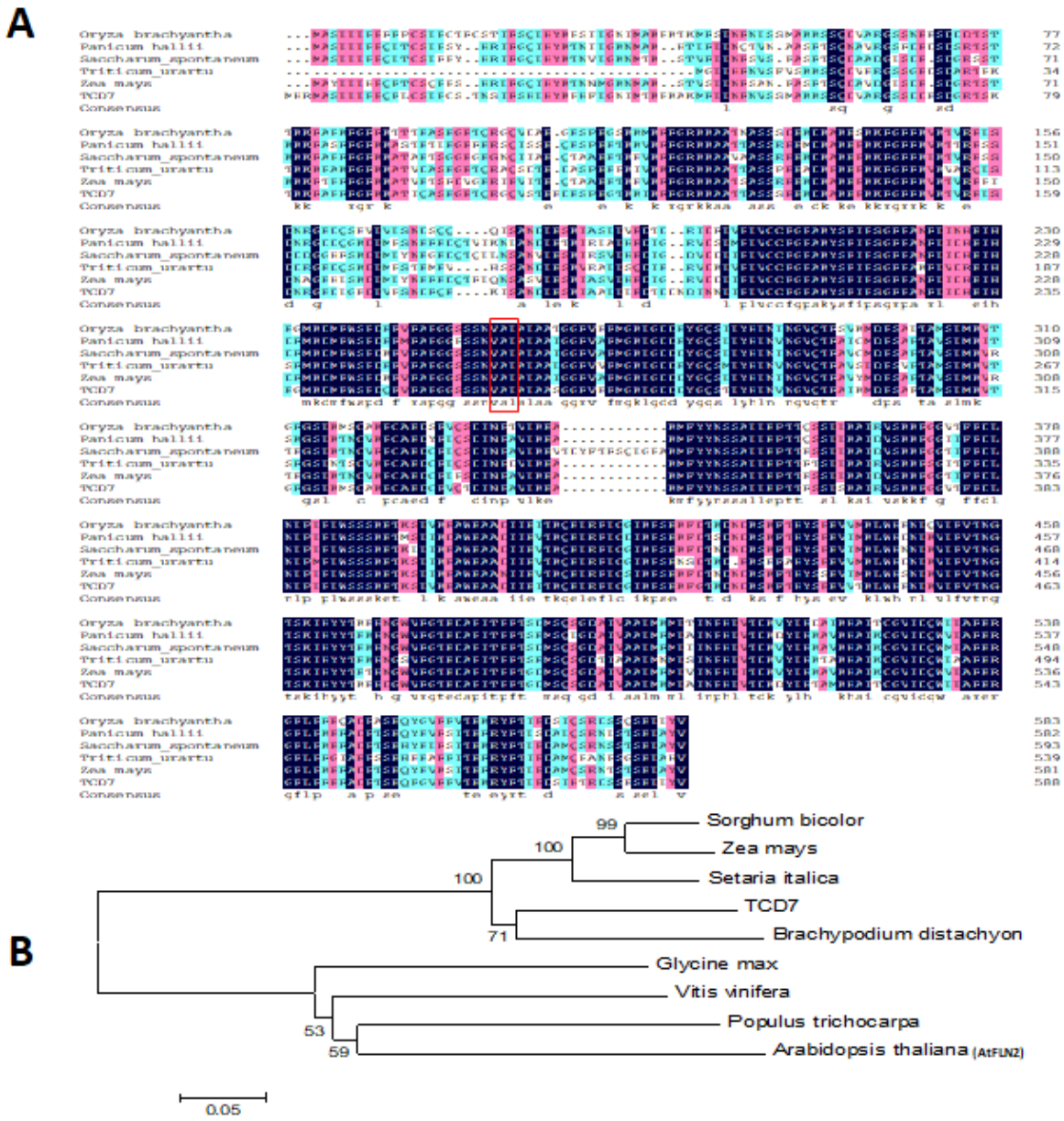

Fig. 6. Phylogenic analysis of TCD7 protein. (A) Amino acid sequence alignment of TCD7 with the six homologous proteins from other plants; amino acids fully or partially conserved are shaded black and gray, respectively. (B) Phylogenic tree of TCD7 and homologous proteins. Scale represents percentage substitution per site. Statistical support for the nodes is indicated.

Figure 6

Please See image above for figure legend.

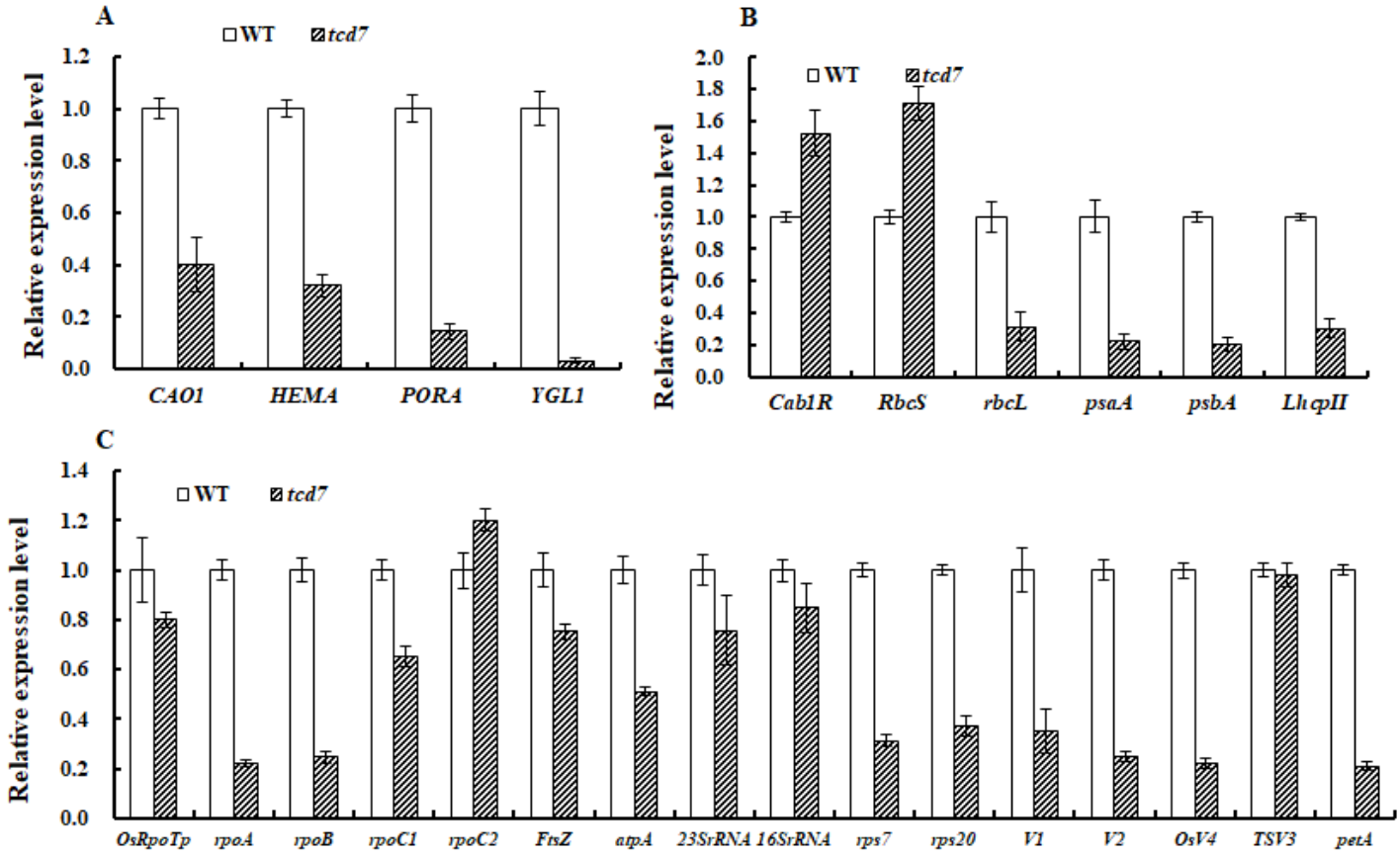

Fig. 7. qRT-PCR analysis of genes associated with Chl biosynthesis **(A)**, photosynthesis **(B)** and chloroplast development**(C)** in wild type (WT) and *tcd7* at the three-leaf stage at 20°C; the relative expression level of each gene in WT and *tcd7* was analyzed by qRT-PCR and normalized using *OsActin* as an internal control. Data are means ± SD *(n* = 3).

Figure 7

Please See image above for figure legend.

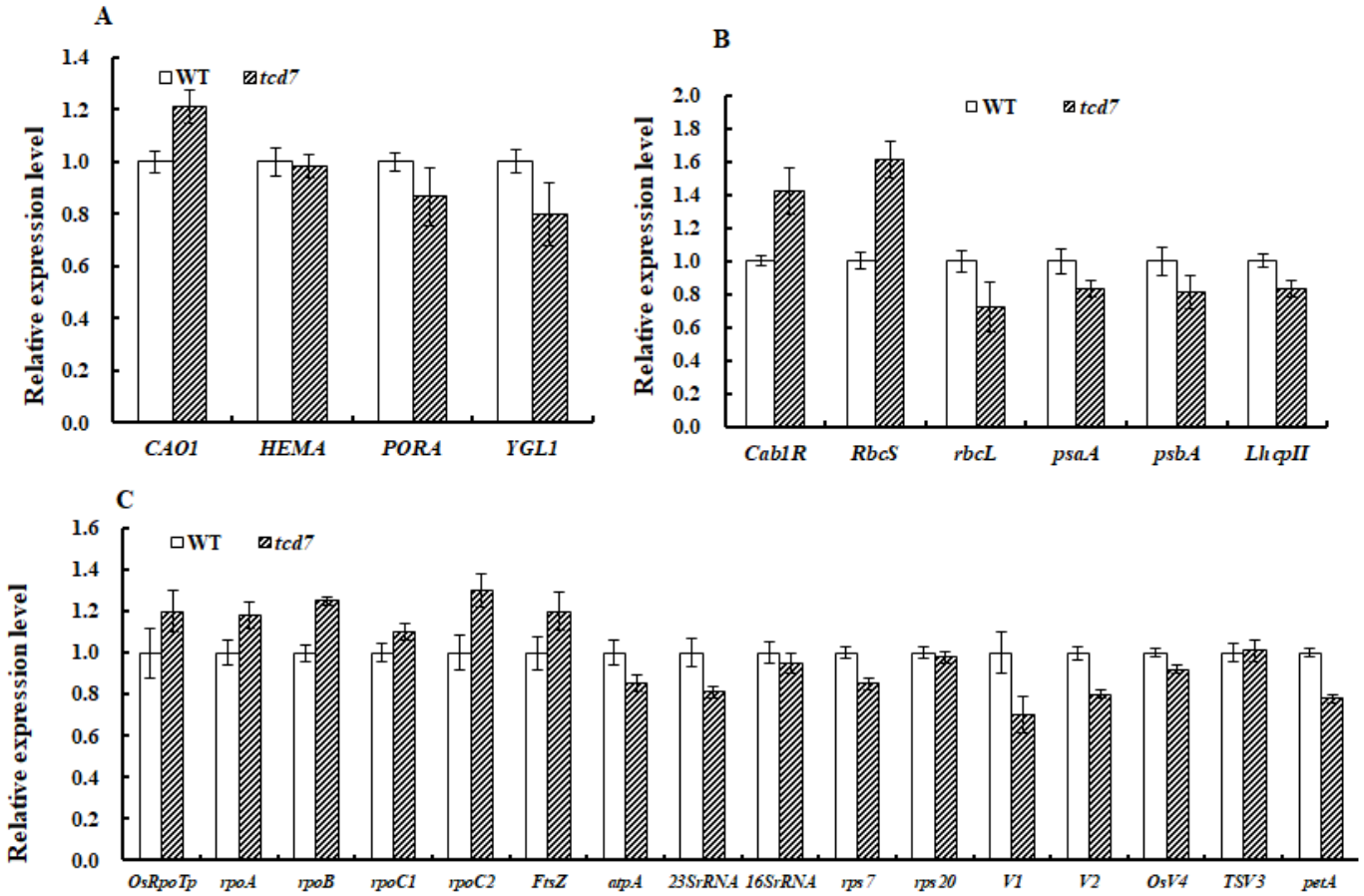

Fig. 8. qRT-PCR analysis of genes associated with Chl biosynthesis **(A)**, photosynthesis **(B)**, and chloroplast development in wild type (WT) and *tcd7* at the 3-leaf stage at 32°C; the relative expression level of each gene in WT and *tcd7* was analyzed by qRT-PCR and normalized using *OsActin* as an internal control. Data are means ± SD ($n = 3$).

Figure 8

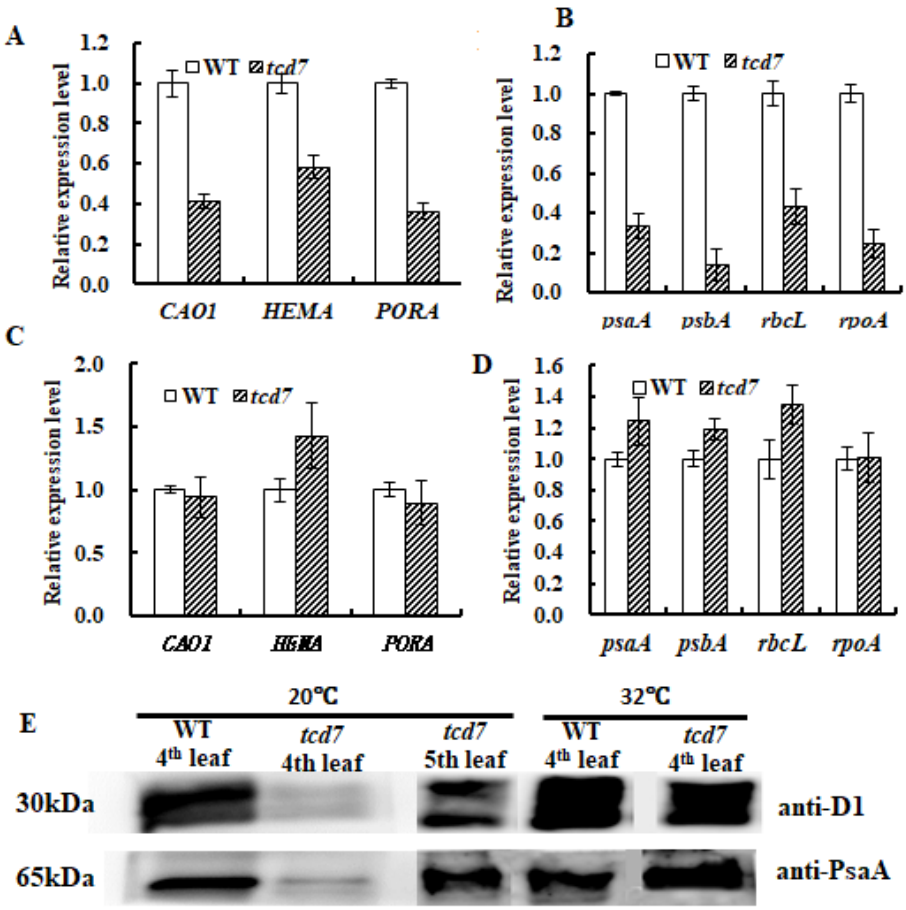

Fig. 9. Transcript expressions of related genes in WT and *tcd7* mutant grown under 20°C and Western-blot analysis of photosynthetic protein D1 and PsaA. (A) Expression of chlorophyll synthesis related-genes (*CAO1, HEMA, PORA*) in white fourth leaf (A) and green fifth leaf (A, C); (B,D) Expression of photosynthesis-related genes (*psaA*, *psbA*, *rbcL*) and a chloroplast development gene (*rpo A*) in the fourth (B) and fifth leaf; (E) Immunoblot analyses of D1 and PsaA in the fourth and fifth leaves of *tcd7* and WT seedling grown at 20°C and 32°C.

Figure 9

Please See image above for figure legend.

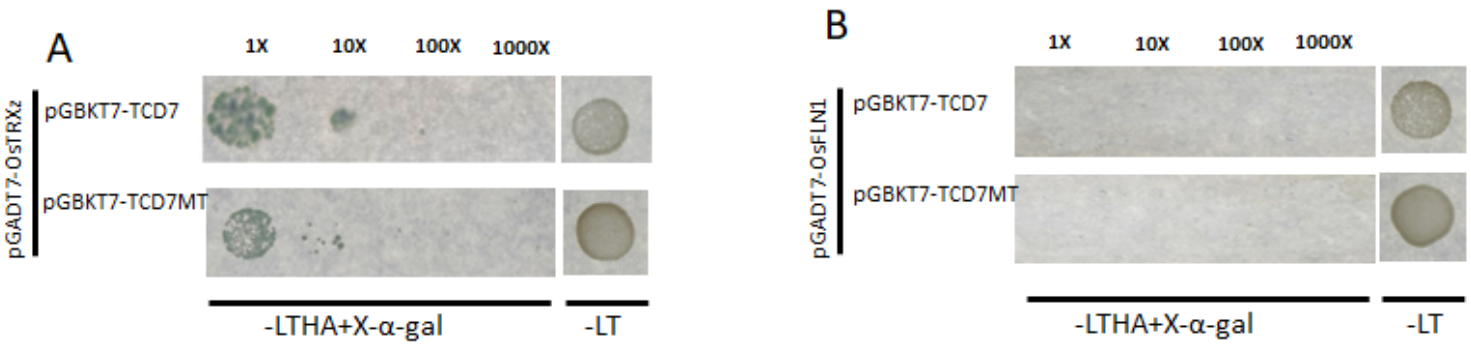

Fig. 10. Analysis of the results of yeast two-hybrid experiment and expression of TAC-related genes. (A-B): Interaction of TCD7, OsTRXz, and OsFLN1 in a yeast two-hybrid assay. Yeast were spotted onto control medium (SD/-Leu/-Trp [-LT]) and selective medium (SD/-Leu/-Trp/-His/-Ade + X-α-gal [-LTHA+X-α-gal]).

Figure 10

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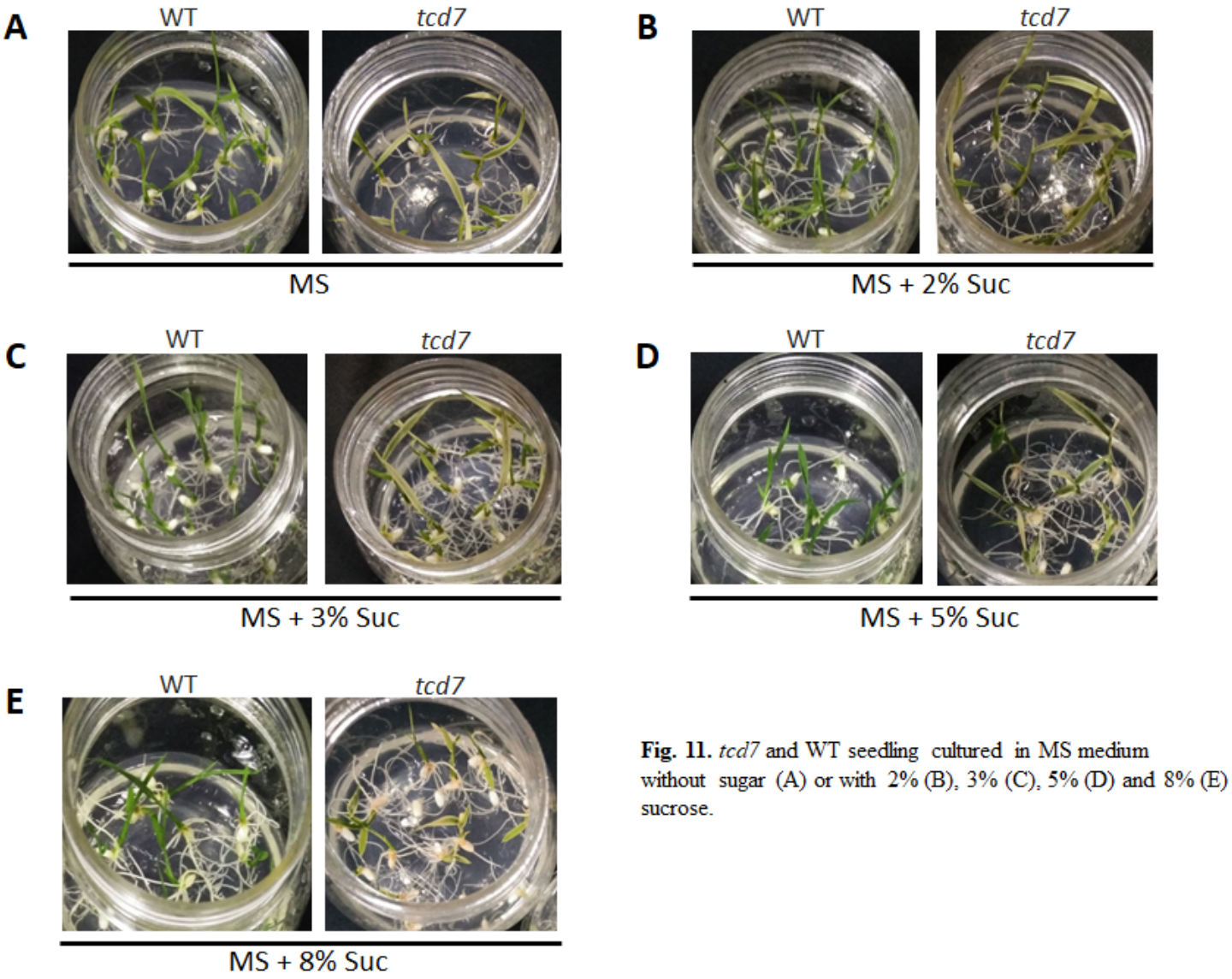

Fig. 11. *tcd7* and WT seedling cultured in MS medium without sugar (A) or with 2% (B), 3% (C), 5% (D) and 8% (E) sucrose.

Figure 11

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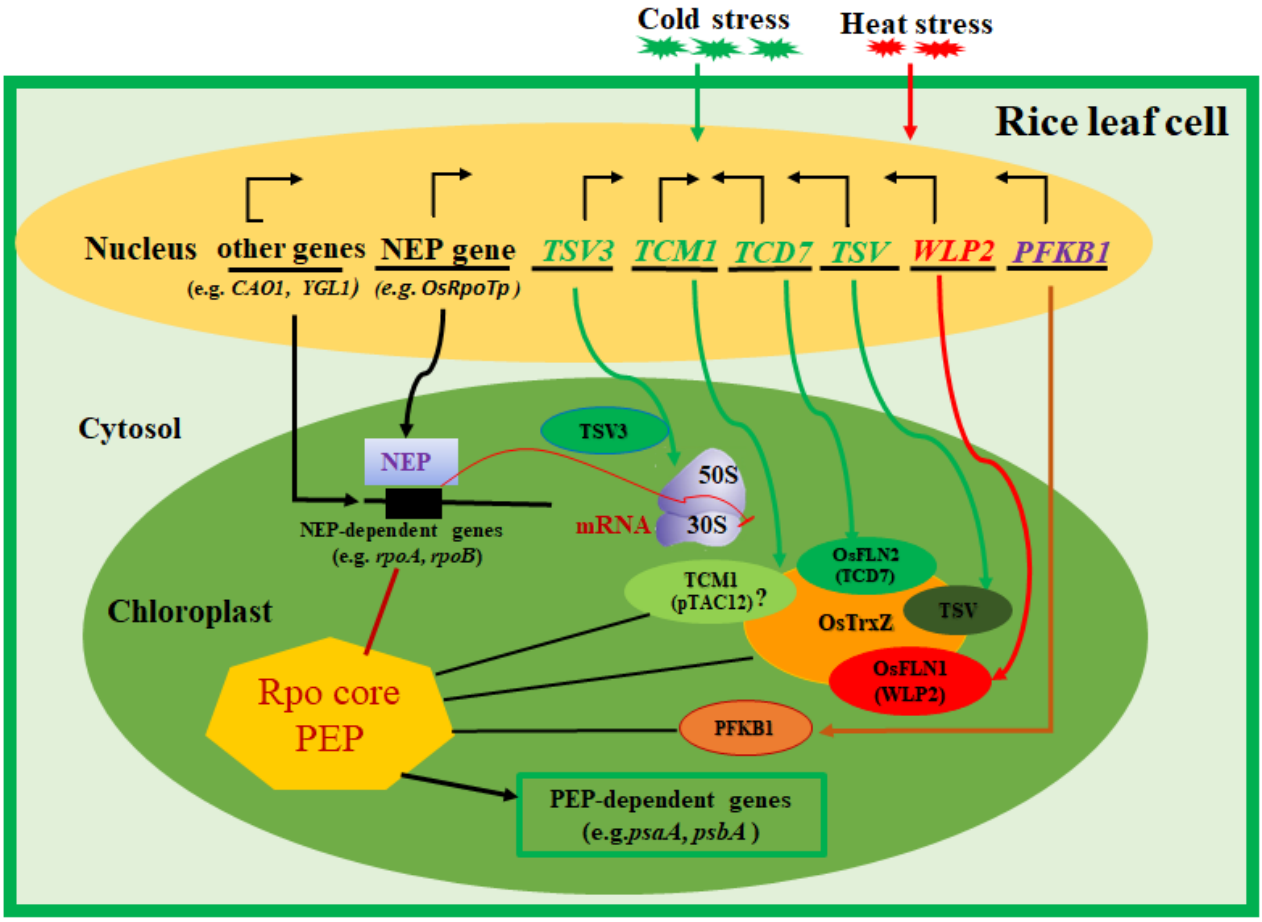

Fig. 12. Schematic illustration of *TCD7*, *TSV3* (Gong et al. 2018), *TCM1* (Lin et al. 2018), *TSV* (Sun et al. 2017), *PFKB1* (Zhu et al. 2020) and *WPL2* (*OsFLN1*) (Lv et al. 2017) functions on expression regulation of chloroplast-associated genes and chloroplast development in rice. Two types of RNA polymerases (NEP and PEP) have been identified in higher plant chloroplasts. TCD7 (OsFLN2) interacts with OsTRXz, not with WPL2(OsFLN1), to regulate the expression of PEP-encoded genes only under cold stress (Lv et al. 2017); TSV, a putative plastidic oxidoreductase, interacts with OsTRXz to regulate PEP activity under cold stress (Sun et al. 2017); *TCM1*, encoding one TAC of (pTAC12), regulates PEP activity under cold stress; *PFKB1*, encoding another pfkb protein, regulates the expression of PEP-encoded genes, regardless of temperature; *TSV3* regulates the chloroplast 50S ribosome subunit only under cold stress (Gong et al. 2018).

Figure 12

Please See image above for figure legend.

Supplementary Files

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