Rice TSV2 Encoding Threonyl-tRNA Synthetase is Needed for Early Chloroplast Development and Seedling Growth under Cold Stress

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Original article

Keywords: Albino phenotype, Chloroplast development, Cold stress, Rice, Threonyl-tRNA synthetase(ThrRS)

Posted Date: August 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-64012/v1

Version of Record: A version of this preprint was published at G3 Genes|Genomes|Genetics on July 30th, 2021. See the published version at https://doi.org/10.1093/g3journal/jkab196.

Abstract

Background

The chloroplast is a vital photosynthetic organelle for plant growth and development. However, the genetic factors involved in chloroplast development and its relationship with environment factors are largely unknown. Threonyl-tRNA synthetase (ThrRS), one of aminoacyl-tRNA synthetases (AARSs), plays a crucial role in protein synthesis. To date, there are few studies for AARS function on chloroplast development and plant growth, much less ThrRS in rice.

Result

In this paper, we characterized a thermo-sensitive virescent mutant *tsv2*, which showed albino phenotype and could not survive after the 4-leaf stage when grown at 20 °C, but recovered the normal phenotype when the temperature rose. Map-based cloning and complementation tests showed that *TSV2* encoded a chloroplast-located ThrRS protein in rice and the Lys-to-Arg mutation in the anticodon-binding domain affected chloroplast development under cold stress. Furthermore, the loss-of-function of the core domain in TSV2 led to seedling death regardless of temperatures. In addition, *TSV2* had a tissue-specific expression, and its disruption resulted in an evidently down-regulation of certain genes associated with chlorophyll biosynthesis, photosynthesis and chloroplast development at cold stress.

Conclusion

The *TSV2* encodes a rice threonyl-tRNA synthetase, located in chloroplasts, which is essential for cold-responsive regulation for chloroplast development and plant growth and closely related to the assembly of chloroplast ribosomes and functions at the first step of chloroplast differentiation.

Background

Aminoacyl-tRNA synthetase (AARS) is an important component of protein synthesis in ribosomes and required for translation in three different compartments of the plant cell: chloroplasts, mitochondria and the cytosol (Berg et al. 2005, Sang et al. 2005). AARSs generally consists of catalytic core domain, binding zinc ion domain, insertion domain, anticodon-binding domain, editing domain, etc (Greenberg et al. 2008) and involves in the process of amino acid transfer to its homologous tRNA(O'Donoghue et al. 2003). AARS and tRNA play a key role in the first step of protein synthesis. According to homology and folding pattern, AARSs can be divided into Class I and Class II, each AARS can be further divided into three subclasses: A, B and C (Martinis et al. 1999).

Previous studies have shown that AARS not only plays a key role in protein synthesis, but also participates in and regulates various biological processes, including RNA transcription and splicing,

protein translation, signal transduction, cell apoptosis, etc (Hausmann et al. 2008). In *Arabidopsis* thaliana, several mutants involving in AARS genes have been identified. In *edd1* mutant, inactivation of *GLYRS* encoding glycyl-tRNA synthetase causes embryonic development to stagnate, ultimately leading to the death (Uwer et al. 1998). Mutations in the *PRORS1* encoding prolyl-tRNA synthetase in *Arabidopsis* thaliana lead to abnormal transcription levels of the photosynthesis and plastid-synthesis genes, also leading to seedling death (Pesaresi 2006). The mutations of *NbERS* and *NBRS*, encoding glutamyl-tRNA synthetase and serine-tRNA synthetase, respectively, led to abnormal chloroplast and reduction of chlorophyll content, and finally the leaf presents etiolated phenotype (Kim 2005). As for ThrAS in *Arabidopsis* thaliana, only the phenotype (albinism and embryo death) observation of the mutant and specific expression localization have been carried out, and the molecular mechanism is still unclear (Berg et al. 2005; Duchene et al. 2005).

Rice is the most important food crop in Asia and has been set up as a model species for genome research. Low temperature is a serious stress in rice production, which hinders a broad spectrum of cellular components (e.g. chloroplast), metabolisms (e.g. photosynthesis), plant growth and yield. In spite of the facts that some indispensable genes for chloroplast development/seedling growth in rice at low temperatures have been identified, such as *TCD9* (Jiang et al. 2014), *V1* (Kusumi et al. 2010b), *V2* (Sugimoto et al. 2007), *V3*(Yoo et al. 2009), *OsV4* (Gong et al. 2014), *TCD5* (Wang et al. 2016a), *TCD10* (Wu et al. 2016), *TCD11* (Wang et al. 2017), *TSV3* (Lin et al. 2018), *TCM12* (Lin et al. 2019), *TCD33*(Wang et al. 2020), *TCD3*(Lin et al. 2020) and so on, the molecular mechanism of cold resistance and its influencing factors are not completely clear (Wang et al. 2020). In addition, few studies involving mutants caused by AARS mutations have been reported in rice, except for *OsValRS2* encoding Val-tRNA synthetase, its mutation led to damage of chloroplast development and chloroplast ribosome biogenesis (Wang et al. 2016b) and *OsERS1* encoding Glutamyl-tRNA synthetase, its mutation led to male-sterility (Yang et al. 2018). To our knowledge, any thermo-sensitive lethal mutants involving in threonyl-tRNA synthetase (ThrRS) gene have not been reported in rice yet. Here, we reported a *tsv2*, a thermo-sensitive virescent mutant of ThrRS gene that exhibited albino-lethal seedling under cold stress, but did normal growth under usual conditions. The chloroplast-localized TSV2 appears to play a vital role in early chloroplast development and seedling growth under cold stress in rice.

Results

Phenotypic Characterization of the tsv2 Mutant

The growth status of *tsv2* and WT seedlings were observed under four temperatures (20 °C, 24 °C, 28 °C and 32 °C) (Fig. 1). Needless to say, all WT seedlings always displayed green normal phenotype, regardless of temperatures and leaf-stage. However, the *tsv2* mutant was albino from the beginning and died after 4-leaf stage at 20℃ (Fig. 1A). At 24℃, the *tsv2* mutant turned to yellow and was not lethal (Fig. 1B). At 28℃, leaves of *tsv2* was close to green (Fig. 1C). Interestingly, the *tsv2* mutants at 32℃ exhibited the same green as WT plants (Fig. 1D). The results indicated that *tsv2* is a low temperature sensitive lethal mutant.

Consistent with the phenotype, the contents of photosynthetic pigments in *tsv2* mutants were the lowest at 20℃, and gradually recovered to the WT level (Fig. 2B-D) with the increase of temperatures. It is shown that chlorophyll accumulation in *tsv2* mutant was blocked under low temperature. By transmission electron microscope (TEM), WT mesophyll cells contained a lot of uniform chloroplasts, regardless of temperatures (Fig. 3A-D). However, at 20℃, the majority of cells in *tsv2* mutants contained only few chloroplasts, and the grana in the inner capsule decreased obviously and the structure was abnormal (Fig. 3E,F). Interestingly, at 32℃, the *tsv2* chloroplast structures tended to be normal and had no obvious difference from WT plants (Fig. 3G, H). Therefore, it can be deduced that aberrant chloroplast of *tsv2* resulted in reduced accumulation of chlorophyll and mutant phenotype under cold stress.

Map-based Cloning of TSV2

To understand the molecular mechanism underlying the *tsv2* mutant phenotype, map-based cloning was performed to identify the *TSV2* locus. In view of an approximately 3 (green): 1 (albino) ratio ($\chi^2 = 0.488$ < $\chi^2_{0.05} = 3.84$) in F_2 segregating population (Additional file 1: Table S1), consisting of 231 green plants and 70 albino plants at 20 °C, it was shown that the mutant phenotype was controlled by a recessive nuclear gene (*tsv2*). First, ninety-two F_2 mutant individuals were used for initial mapping, the target gene *TSV2* was located between ID12613 and MM3298 molecular markers on chromosome 2 (Fig. 4A). Subsequently, the mapping F_2 population was expanded to 1308 individuals, the *TSV2* gene was narrowed to 131 kb between ID12947 and ID13097,including eight candidate genes(Fig. 4B). We sequenced and analyzed all candidate genes and found only a A-to-G mutation in the 8th exon of *LOC_Os02g33500*, resulting in Lys(K)-to-Arg(R) mutation of the 614th site in TSV2 (Fig. 4C).

Molecular Complementation of the tsv2 Mutants

To confirm that the mutation of *LOC_Os02g33500* was responsible for the *tsv2* phenotype, an expression plasmid containing the entire *TSV2* (*LOC_Os02g33500*) driven by its endogenous promoter was constructed and transformed into the *tsv2* mutants. To quickly obtain results, we intentionally induced the differentiation of rice callus at 20 °C companying the uninfected calli as a control. Resultantly, fifteen T_o transgenic seedlings harboring pCAMBIA1301:TSV2 appeared green as WT plants, whereas control seedlings remained albino phenotype (Fig. 4D), showing that *LOC_Os02g33500* can rescue the mutant phenotype. Additionally, at 20 °C, we found segregation of albino phenotype in the transgenic T_1 population (Fig. 4D). Taken together, these results confirm that *LOC_Os02g33500* is *TSV2*.

Characterization of TSV2 Protein

Bioinformatic assay revealed that *TSV2* encodes ThrAS protein, consisting of 675 amino acids, with a molecular mass of approximately 76.9 kDa, which belongs to Class A in Class II of AARS family. The ThrAS contains at least two domains, including core threonyl-tRNA synthetase domain and anticodon-binding domain (Fig. 4C; Additional file 2: Figure S 1).

Orthologs of rice TSV2 were identified in *Zea mays, Sorghum bicolor, Setaria italic, Aegilops tauschii*, *Brachypodium distachyon*, *Arabidopsis thaliana*, *Cicer arietinum*, *Nicotiana sylvestris*, *Vitis vinifera*, *Ziziphus jujuba* (Fig. 5A), showing that TSV2 was very similar to many species and highly conserved in different higher plants. Phylogenetic analysis showed that the evolutionary relationships of TSV2 homologs were consistent with the taxonomy (Fig. 5B). It was noted that the mutated amino acid (Lys, K) in TSV2 was highly conserved among higher plants (Fig. 5B) and that its Lys-to-Arg mutation led to the change of a β sheets structure (Additional file 3: Figure S2), suggesting the vital role of this site for the functional integrity of the TSV2 protein.

Expression Pattern and Subcellular Localization of TSV2

To clarify the expression pattern of TSV2 in rice, a semi-quantitative RT-PCR was carried out on various tissues (Fig. 6). Consistent with the rice gene expression profiling data in the RiceXPro database (Additional file 4: Figure S3), *TSV2* was highly expressed in the 1st and 2nd leaves, and low in flag-leaf, root stem, and panicle (Fig. 6A), showing the tissue specificity and a vital role in early leaf chloroplast development. In addition, the TSV2 protein was predicted to be localized to the chloroplast using TargetP program (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000). To verify the actual subcellular localization, the pMON530:CaMV35S:TSV2-GFP plasmid was introduced into tobacco cells in the transient expression assay, with the pMON530:CaMV35S-GFP vector as control. Observationally, the GFP fluorescence was co-localized with chlorophyll auto-fluorescence (Fig. 6B,C). This affirmed that TSV2 was localized in the chloroplast.

Versatility for TSV2 Function

In view of the occurrence of Lys-to-Arg substitution mutation site in terminal anticodon binding domain in *tsv2* mutants, which still remains the integrity of other domains, such as threonine tRNA synthetase (ThrRS) domain (Fig. 4C; Additional file 2: Figure S1), thus, perhaps there exists other functions except for cold-inducible *TSV2* for chloroplast development. We performed targeted mutation of *TSV2* gene in WT plants by CRISPR/Cas9 system. Surprisingly, all homozygous T_o edited transgenic seedlings, grown at 20℃, with two different edited-sites (5 and 7 base deletion, respectively) on the 1st exon of *TSV2* gene (Additional file 5: Figure S4), displayed albino phenotype and eventually died (Fig. 7A). In addition, all T_1 edited homozygous seedlings from T_o edited heterzygous transgenic seedlings, grown at 20℃ and 32℃, displayed all albino phenotype, and finally died (Fig. 7B). Owing to the exon 1 sequence determined the ThrRS core domain (Fig. 4C; Additional file 2: Figure S1), thus, the loss of function in the core domain of *TSV2* led to albino death, regardless of temperatures. Therefore, expression of the core domain is co-regulated by other domains and temperatures, showing versatility for TSV2 function for early chloroplast development and seedling growth.

The Disruption of TSV2 Alters the Transcript Levels of Associated Genes

To elucidate the effect of the *tsv3* mutation on the expression of genes related to chloroplast development and to explore the regulating pathway, we performed RT-qPCR analysis of 20 genes involved in chlorophyll biosynthesis, photosynthesis, chloroplast development, and temperature sensitivity in rice (Additional file 6: Table S2). It was observed that levels of all tested genes for Chl biosynthesis, i.e., *chlorophyllide a oxygenase1* (*CAO1*), *glutamyl tRNA reductase* (*HEMA*), *NADPH-dependent protochlorophyllide oxidoreductase* (*PORA*) and *Chl synthetase* (*YGL1*) and for photosynthesis (*Cab1R, RbcL, PsaA, PsbA, Lhcpll*) were significantly down-regulated under low temperatures (20℃) (Fig. 8A, B), consistent with the observed albino phenotype of *tsv2* mutants (Fig. 1); Also, at 20 °C, *16SrRNA* (small subunit) and *23SrRNA* (large subunit) involved in ribosome assembly, and *Rps20*, encoding the small ribosomal subunit S20, in chloroplast, was seriously blocked (Fig. 8C). Furthermore, at 20 °C, except for *TCD9* encoding chloroplast chaperone protein OsCpn60α subunit (Jiang et al. 2014), three temperature-sensitive genes (*TCD5, TCD10* and *TCD11*) for chloroplast development (Wang et al. 2016a, 2017; Wu et al. 2016) showed a significant downward trend, in particular, *TCD10*, encoding PPR protein, and *TCD11*, encoding plastid ribosomal protein S6, were obviously down-regulated (Fig. 8D). By contrast, all transcripts of the reduced genes at 20 °C in the *tsv2* seedlings recovered to WT levels (within twofold range) or exceeded the WT level when grown at 32 °C (Fig. 9A-D), consistent with the recovery of leaf-color (Fig. 1D) and chloroplast development (Fig. 3G,H) in *tsv2* at 32 °C. Thus, our data indicated that the *tsv2* mutation leads to dramatic downregulation of many genes for chlorophyll biosynthesis, photosynthesis, and chloroplast development, under cold stress. In addition, the *TSV2* influences chloroplast ribosome assembly thereby affecting the process of chloroplast development and accumulation of photosynthetic pigment.

Discussion

In the current research, we identified and characterized rice *tsv2* mutants with imperfect chloroplasts and albino lethal phenotype at low temperatures, resulting from the abnormal expression of genes associated with chlorophyll biosynthesis, photosynthesis, and chloroplast development. The *tsv2* mutant phenotype was caused by the Lys-to-Arg mutation in anticodon binding domain in *TSV2* gene, encoding threonyl-tRNA synthetase protein (ThrRS). However, the loss of function in core domain for TSV2 led to seedling death regardless temperatures. Our results provide evidence that *TSV2* plays a key role in chloroplast development and plant growth in rice.

Incomplete of Anticodon-binding Domain in TSV2 Leads to the Albino Phenotype Under Cold Stress

To date, a lot of temperature-sensitive seedling-colour mutants of rice similar to *tsv2* phenotype have been reported. They displayed white or yellow leaves at low temperatures while became normal green at high temperatures. Mutant *v1* and *v2* showed yellow leaf at 20℃, and recovered to wild-type plants at 30℃ (Kusumi et al. 1997; Iba et al. 2010). The coding protein of *V1* gene participates in the regulation of plastid RNA metabolism and protein translation (Kusumi et al. 1997). Guanylate kinase encoded by *V2* gene plays a special important role in the early development of chloroplasts (Sugimoto et al. 2007). Also, at 22℃ *osv5a* leaves yellowed and whitened, but recovered to green phenotype over 28℃. *OsV5A* gene

encodes chaperone protein interacting with PORA and PORB and stabilizing PORB protein (Liu et al. 2016; Zhou et al. 2013). Besides, the mutant *dua1* showed a pale phenotype at 19 °C, PPR protein *DUA1* is essential for a chloroplast ribosome development under cold stress (Cui et al. 2018). Moreover, our research group also reported some thermo-sensitive leaf color mutants of rice. For example, *tcd9* mutant showed the albino phenotype before the 3-leaf stage over 24℃, *TCD9* encodes chloroplast chaperone protein OsCpn60α subunit (Jiang et al. 2014). Also, *tcd3*, *tcd5, tcd9, tcd10, tcd11, tcd33, tsv3* and *osv4* mutants (Gong et al. 2014; Jiang et al. 2014; Lin et al. 2018, 2020; Wang et al. 2016a,b, 2007, 2020; Wu et al. 2016) exhibited the albino or white phenotype and malformed chloroplast at 20 °C, but did the normal phenotype at 32 °C. More interestingly, *TCD5, TCD10* and *OsV4 all* encodes respective novel PPR protein(Wang et al. 2016b; Wu et al. 2016; Gong et al. 2014). However, different from the above mutants, the edited-mutant seedlings for *TSV2* gene in this study had not temperature-sensitivity for leaf-color and died at high temperatures (Fig. 7B). Thus, it was interesting to explore the reason why the *TSV2*-edited mutant phenotype was different from the *tsv2* mutant. Sequencing analysis showed that the edited-deletion sites of transgenic plants was located in the 1st exon, which led to the premature termination and made the core domain of ThrRS protein disappear (Additional file 2: Figure S1; Additional file 5: Figure S4). However, the mutation site of *tsv2* was located on the terminal exon 8, which only destroyed an original β sheet of the anticodon-binding domain in TSV2 (Additional file 3: Figure S2). Among AARSs, anticodon-binding domain is found in histidyl, glycyl, threonyl and prolyl tRNA synthetases (Wolf et al. 1999) and involved in anticodon stem-loop binding and recognition (Aberg et al. 1997). In this study, the destruction of the core domain will lead to the full loss of the function of TSV2, and its mutant phenotype has nothing to do with temperatures. Interestingly, the Lys-to-Arg mutation in the anticodon-binding domain only resulted in whitening under only cold stress, and the increase of temperature can make up for the defect of its domain function. Therefore, the mutations of different domains in TSV2 will result in different phenotype. Taken together, incomplete of anticodon-binding domain is responsible for the *tsv2* albino phenotype under cold stress.

TSV2 **Functions at the First Step of Chloroplast Development**

It is well known that chloroplast development is divided into three steps (Kusumi et al. 2010a, b; Kusumi and Iba, 2014). The first step involves the activation of plastid replication and plastid DNA synthesis. The second step involves the establishment of chloroplast genetic system. The last step involves the high expression of plastid and nuclear targets encoding the photosynthetic apparatus, resulting in the synthesis and assembly of the photosynthetic apparatus. At present, through utilization of thermo-sensitive leaf-color rice mutants, it was shown that *V3* (Yoo et al. 2009), *TCD9* (Jiang et al. 2014), *TCD10* (Wu et al. 2016), *TCD11* (Wang et al. 2017), *TSV3* (Lin et al. 2018a), *TCD33*(Wang et al., 2020) and *TCD3* (Lin et al. 2020) function in the first step; *V1* (Kusumi et al. 1997), *V2* (Sugimoto et al. 2007), *TCD5* (Wang et al. 2016a) and *OsV4* (Gong et al. 2014) function in the second step; and *TCM12* (Lin et al. 2019) in the third step. Theses results showed that cold stress affects all three steps of chloroplast development, and its regulatory pathways are complex and diverse. In this study, to explore which step of chloroplast development was regulated by *TSV2*, the transcript levels of certain known genes for the first step (*TCD9, TCD10, TCD11*), the second step (*TCD5*), and the third step (*Cab1R, RbcL, PsaA, PsbA, LhcpII*) of

chloroplast development in *tsv2* albino leaves under stress were observed. Obviously, the transcript levels of the first step *TCD10* and *TCD11* genes were largely down-regulated(Fig. 8D), indicating that *TSV2* functions in the first step of chloroplast development. However, as to why *TCD9*, involved in the same first step (Jiang et al. 2014), still was highly expressed, we speculate that the reason may be that *TSV2* is between the downstream of *TCD9* and the upstream of *TCD10* and *TCD11*, and was not regulated by *TCD9*. In addition, those inhibitions (*TCD10, TCD11*), caused by *TSV2* dysfunction, in the first step, definitely would lead to the inhibition of certain associated-genes in its second (*TCD5*) and third (*Cab1R, RbcL, PsaA, PsbA, LhcpII*) stage (Fig. 8A,B), resulting in the reduction of chlorophyll accumulation and photosynthesis, thereby causing the seedling death.

Possible Role of TSV2 in Chloroplast Ribosome Assembly and Protein Synthesis

Chloroplast development is a complex biological process, in which many key proteins are translated and formed in chloroplasts. The plastid ribosomal proteins are crucial to ribosome biosynthesis, plastid protein biosynthesis, chloroplast development and ribosome assembly (Zhao et al. 2016). In the past, several genes for chloroplast ribosome assembly were reported in rice, such as *WLP1* (encoding ribosome protein L13, RPL13) (Song et al. 2014), *ASL1* (encoding ribosomal protein S20, RPS20) (Gong et al. 2013), *ASL2* (encoding ribosomal protein L21, RPL21) (Lin et al. 2015), *TCD11* (encoding plastid ribosomal protein S6, RPS6) (Wang et al. 2017). As known, the mutation of *TCD11/Rps6* (Wang et al., 2017) and *WLP1/Rpl13* (Song et al. 2014) both produced the thermo-sensitive leaf-color as the *tsv2* mutants, but, the mutation of *ASL1/Rps20* (Gong et al. 2013) and *ASL2/Rpl21* (Lin et al. 2015) both led to seedling death, regardless of temperatures, showing that chloroplast ribosome subunits have different functions or responses to temperatures. In this study, the expression of *Rps20/ASL1*, *Rpl21/ASL2* and *Rps6/TCD11* in *tsv2* mutants at low temperatures decreased significantly, showing that *TSV2* affected chloroplast ribosome synthesis under cold stress. Notably, *16SrRNA* and *23SrRNA* were severely hampered(Fig. 8C), definitely resulting in impaired translation and protein synthesis in chloroplasts. With the increase of temperature, all expressions of three ribosome subunit genes (*Rps20/ASL1*, *Rpl21/ASL2*, *Rps6/TCD11*) basically returned to normal levels and the expression of chloroplast ribosome *16SrRNA* and *23S rRNA* was up-regulated strongly. As known, chloroplast translation occurs in 30S and 50S ribosomes, both which are important components in ribosomes and their changes will directly affect ribosome assembly. Therefore, in view of the reduced transcripts of *16SrRNA, 23SrRNA, Rps20*, *Rpl21, TCD11/Rpl6* in albino *tsv2* seedlings (Fig. 8C,D), we can infer that *TSV2* is involved in the chloroplast ribosomes assembly and protein synthesis.

Conclusions

The *TSV2* encodes a rice threonyl-tRNA synthetase, which is located in chloroplasts and is closely related to the assembly of chloroplast ribosomes and functions at the first step of chloroplast differentiation. Mutation in the anticodon-binding domain of TSV2 causes chloroplast ribosomes to fail to assemble normally, resulting in impaired chloroplast development and lethal phenotype of rice plants, under cold stress. But, the loss-of-function in the core domain led to seedling death regardless of temperatures.

Further work on TSV2 will involve an understanding of how it participates in chloroplast development and plant growth.

Materials And Methods

Plant Materials and Growth Conditions

The *thermo-sensitive virescent mutant tsv2* was discovered in rice mutant pool from Jiahua 1 (wild type, WT, *japonica* variety) treated with Cobalt-60 gamma rays. By the way, the mutant phenotype can be distinguished from normal green at Hainan island, China (winter season, subtropical climate) and Shanghai, China (spring season, temperate climate) under local conditions during the early seeding stage. The *tsv2* mutant was crossed with Peiai64S (*indica* variety) and the obtained F_2 seeds were used for genetic analysis and gene cloning. WT and *tsv2* plants were cultured in incubators under controlled 12 h of light and 12 h of dark at a constant temperature of 20 °C, 24 °C, 28 °C and 32 °C, respectively, for phenotypic characterization, pigment analysis, and DNA and RNA extraction.

Measurement of Photosynthetic Pigments and Transmission Electron Microscopy

For photosynthetic pigment analysis, 200 mg fresh leaves were taken from the 3-leaf-stage seedlings cultured at 20℃, 24℃, 28℃ and 32℃, respectively and incubated with 5 mL of extraction buffer (ethanol: acetone: water = 5:4:1) at 4 °C in the dark for 18 h. Using spectrophotometer (Beckman Coulter, Danvers, MA, USA), chlorophyll a, b and Car contents were measured according to the modified methods of Arnon (1949) and Wellburn (1994). This experiment were performed out with three biological replicates.

To observe ultrastructure of chloroplast, tissues were sampled from the 3rd leaves of the 3-leaf-stage WT and *tsv2* seedlings grown at 20 °C and 32 °C, respectively, were treated with the mixed solution of 3% glutaraldehyde solution and 2.5% paraformaldehyde. The observation of chloroplast was performed based on previous methods (Jiang et al. 2014). Samples were viewed with a Hitachi765 (Hitachi, Tokyo) transmission electron microscope.

Mapping and Cloning of TSV2 Gene

Total genomic DNA from rice fresh leaves were extracted by CTAB method described in previous studies (Murray and Thompson 1980). DNA specific fragments were amplified by EDC-810 PCR instrument (Eastwin, Shanghai, China). PCR products were transferred to 2–4% agarose gel containing ethidium bromide for electrophoresis. The bands were observed and recorded under UVP imager. The F_2 population of 1308 individuals with the mutant phenotype was used for fine mapping of the *TSV2* locus. First of all, ninety-two SSR primers based on the Gramene database (http://www.gramene.org) were used to investigate the chromosome of the target gene, and then developed SSR and InDel markers (Additional file 7: Table S3)

were used for fine-mapping of *TSV2*. Next, DNA fragments of the candidate genes were PCR amplified and sequenced (SinoGenoMax, Shanghai, China). Lastly, the function and open reading frames of the candidate genes were obtained from TIGR (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) and conserved domain structures were predicted using SMART (http://smart.embl-heidelberg.de/).

Complementation Experiment

First of all, the WT genomic DNA fragment covering the entire *TSV2*(*LOC_Os02g33500*) coding region (3.9 kb), plus a 1.4 kb upstream region and a 0.6 kb downstream sequence was amplified using the specific primers, pF: 5’-TACGAATTCGAGCTCGGTACCTCCACCAAAGTTTACGAAGC-3’ (*Kpn*I) and pR: 5’-GCTGTGAAGAACCTCCCTATGTCGACCTGCAGGCATGCAAG-3’ (*Sal*I). The underlined sequences stand for cleavage sites of the restriction enzymes. Then, the amplified fragment was cloned onto vector pCAMBIA1301 (CAMBIA, http://www.cambia.org.au), the pCAMBIA1301-TSV2 plasmids were transferred into *Agrobacterium* EHA105 and introduced into the *tsv2* mutant by Agrobacterium tumefaciens-mediated transformation (Hiei et al. 1994), except that the temperature used for in *vitro* plant differentiation was set at 20 °C. The genotype of transgenic plants was determined using PCR amplification of the *hygromycin phosphotransferase* gene (*hpt*) with primers *HPTF* (5'-GGAGCATATACGCCCGGAGT-3') and *HPTR* (5'-GTTTATCGGCACTTTGCATCG-3') and GUS gene with primers GUSF (5'-GGGATCCATCGCAGCGTAATG-3') and GUSR (5'-GCCGACAGCAGCAGTTTCATC-3') as selection. In addition, all T_1 seedlings were cultured at 20 °C and were used to examine the segregation of the mutant phenotype.

Targeted Mutation of TSV2 Gene

To determine if the novel allelic mutants in *TSV2* display the similar or more severe phenotype compared with the *tsv2* mutant, CRISPR/Cas9 technique was used for the targeted mutation. First of all, adaptor primers (pF1: 5’-GCCGGGCTCAGCTCCGTCTCGTT-3’ and pR1: 5’-AAACAACGAGACGGAGCTGAGCC-3’; pF2: 5’-GTTGGCGGGATCCGACGGCAAGG-3’ and pR2: 5’-AAACCCTTGCCGTCGGATCCCGC-3’) were designed by CRISPR Primer Designer (Naito et al. 2015). The sequence was inserted into the region between the OsU6 promoter and the gRNA scaffolds, from pYLgRNA-OsU6vector, of Cas9 expression backbone vector (pYLCRISPR/Cas9-MH) at the *Bsa*I sites according to the previous methods (Ma et al. 2015). The vector was transformed into the WT callus through *Agrobacterium tumefaciens*-mediated infection (Hiei et al. 1994), except that the temperature used for in *vitro* plant differentiation was set at 28 °C. Transgenic T_0, T_1 plants were selected and sequenced, all T_1 seedlings were cultured at both 20℃ and 32℃, respectively, to observe the phenotype and the segregation.

Subcellular Localization of TSV2

To determine the subcellular localization of TSV2, the cDNA fragment of *TSV2* gene was amplified by PCR using the corresponding primer pairs (pF: 5’-GAAGATCTATGGCGGCCGCCGTCTCCGC-3’ and pR: 5’-GGGGTACCCCGGTGTGGCGGATGCGGAGCA-3’). The PCR products were cloned into the pMON530-GFP vector, which was transformed into tobacco (*Nicotiana* tabacum) mesophyll cells through Agrobacterium-

mediated infection. Meanwhile, empty pMON530-GFP vector was used as control. The analysis was carried out based on previously described method (Jiang et al. 2014).

Sequence and Phylogenetic Analyses

The protein domain prediction website InterPro was used to analyze the sequence and domain of TSV2, and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/) was used to predict the three-dimensional structure of protein. NCBI (http://www.ncbi.nlm.nih.gov) was used to query the homologous proteins of *TSV2* in other species, and MEGA6 and DNAMAN were used for phylogenetic tree analysis and homologous sequence alignment.

Transcriptional Expression Analysis

Total RNA was isolated from fresh tissues (root, stem, leaf, panicle) of WT and *tsv2* plants using by an RNA Prep Pure Plant kit (TianGen, Beijing, China). The transcription of chlorophyll synthesis, chloroplast development, photosynthesis-associated genes (*Cab1R, CAO, HEMA, YGL1, LHCP*II, *PORA, PsaA, PsbA, RpoB, RpoC, Rps20, Rps21,16SrRNA, 23SrRNA*) and temperature-sensitive genes for chloroplast development (*TCD5*, *TCD9*, *TCD10*, *TCD11*) (Additional file 6: Table S2), in rice was assessed using quantitative real-time PCR (RT-qPCR). The specific primers for qPCR were listed in Table S2. A SYBR Premix Ex Taq™ RT-PCR Kit (Takara, Japan) was performed according to manufacturer's instructions. ABI-7500on Real-Time PCR System (Applied Biosystems; http://www.appliedbiosystems.com) was used to perform the analysis. The relative quantification of gene expression data was analyzed as previously described (Livak and Schmittgen 2001). *Actin* was used as an internal control. This experiment were carried out with four biological replicates.

Abbreviations

AARSs: aminoacyl-tRNA synthetases (AARSs)

Chl: Chlorophyll

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9

ThrRS: Threonyl-tRNA synthetase

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of supporting data

All data generated or analyzed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by Minister of Science and Technology of China (MOST) (2016YFD0100902), the Shanghai Municipal Science and Technology Commission (19391900200), Innovation Program of Shanghai Municipal Education Commission (2017-01-07-00-02-E00039), the Natural Science Foundation of China (No.30971552) and Taizhou Municipal Science and Technology Bureau of Zhejiang Province, China (No.1901ny02).

Authors' contributions

DL and YD provided the mutant seeds and generated F_1 and F_2 seeds for genotyping and phenotyping. DL, WZ, JS, YW, XP and YD performed the experiments of phenotype assays and molecular analysis. DL, XP and YD designed and discussed the research. DL, YD and XP wrote the manuscript. All authors approved the manuscript.

Acknowledgments

We thank Prof. Yaoguang Liu (Southern China Agriculture University, China) for kindly providing CRISPR/Cas9 vector and system. We are grateful to Prof. Zhongnan Yang (Shanghai Normal University, China) for kindly providing the pMON530-GFP vector and Dr. Zhiming Zheng (Canadian Department of Agriculture) for her critical reading of and suggestions for our manuscript.

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Figures

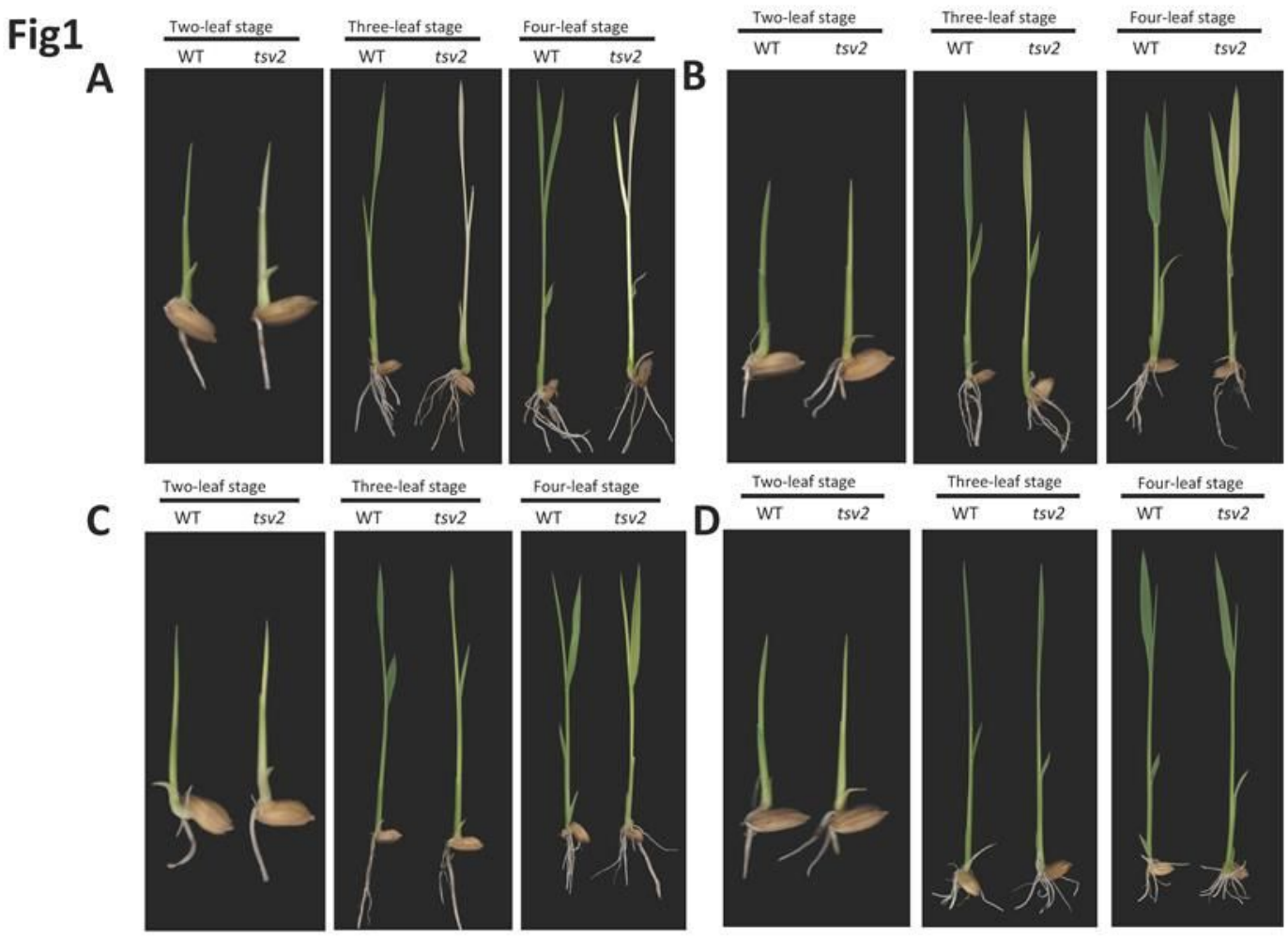

Figure 1

Phenotypic characterization of the tsv2 mutants. Seedlings of Jiahua1 (WT, left) and tsv2 mutant (right) at the 2-, 3- and 4-leaf stage grown at (A) 20°C, (B) 24°C, (C) 28°C and (D) 32°C, respectively.

Fig.2

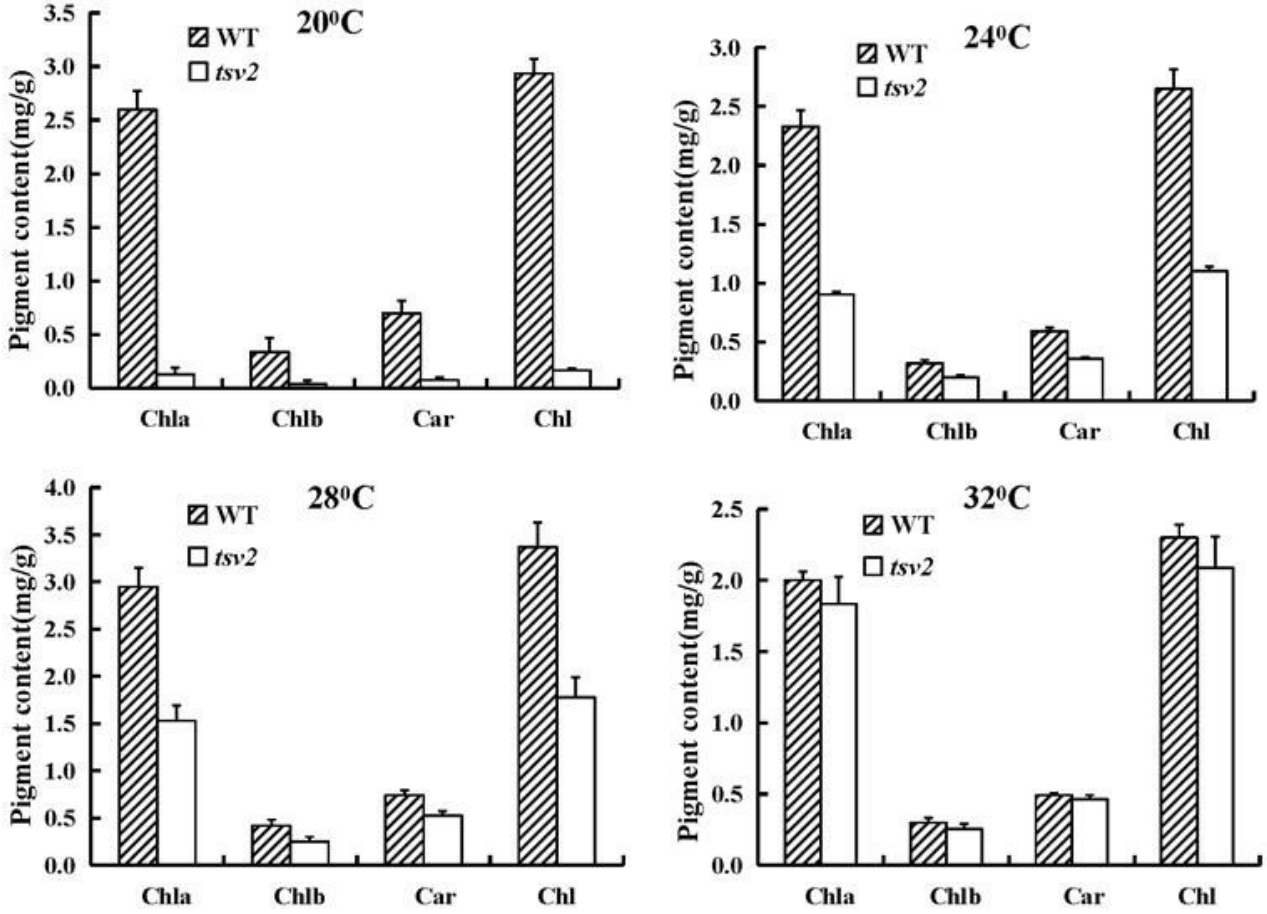

Figure 2

Chlorophyll contents of three-leaf stage of wild-type (WT) and tsv2 seedlings grown at (A) 20°C, (B) 24°C, (C) 28°C and (D) 32°C, respectively. Error bars represent standard deviation (n = 3).

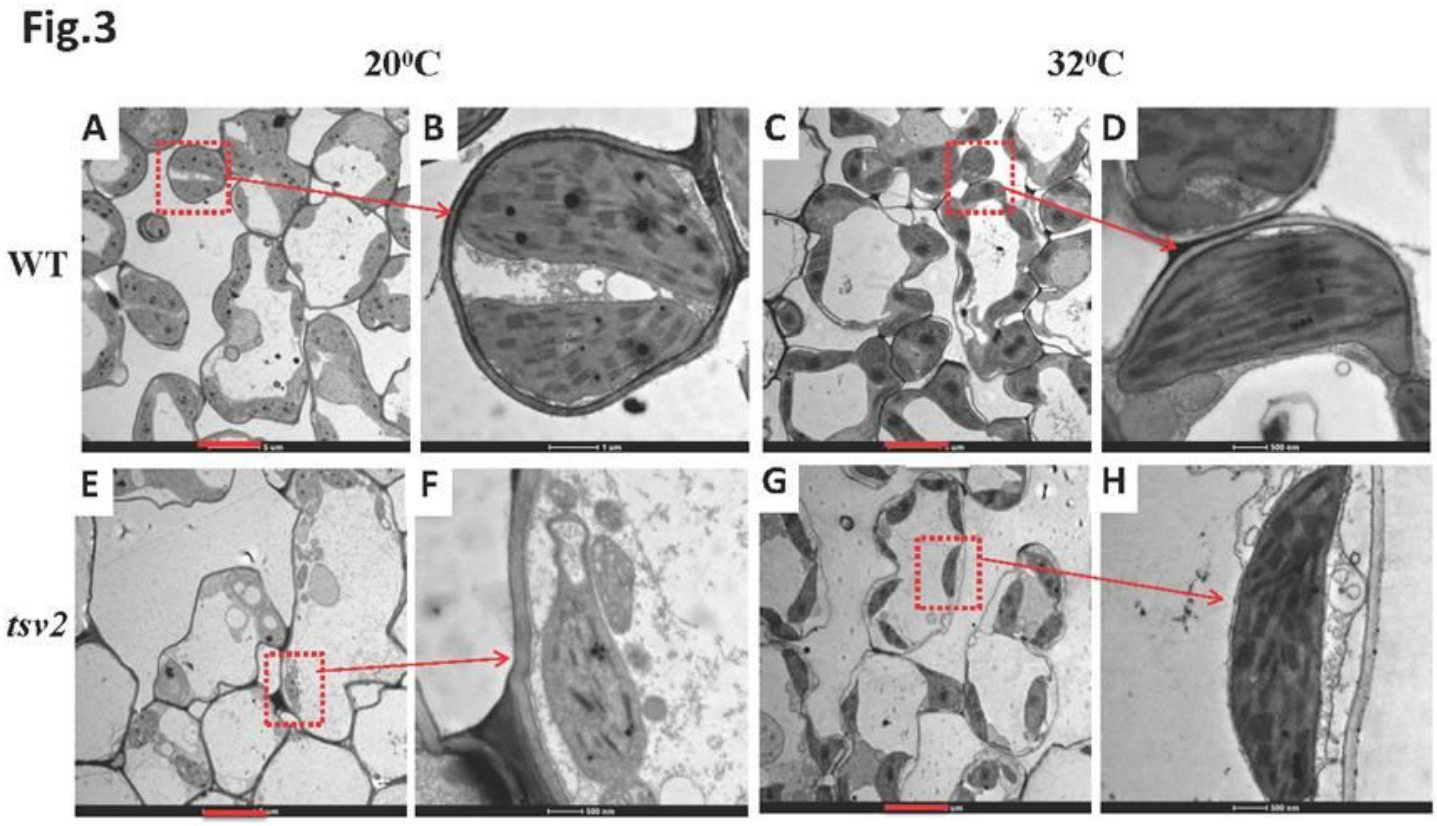

Figure 3

TEM images of three-leaf chloroplasts in the WT and tsv2 mutants grown at 20°C (A,B,E,F) and 32°C(C,D,G,H). The red scale bar represents 5μm.

Fig.4

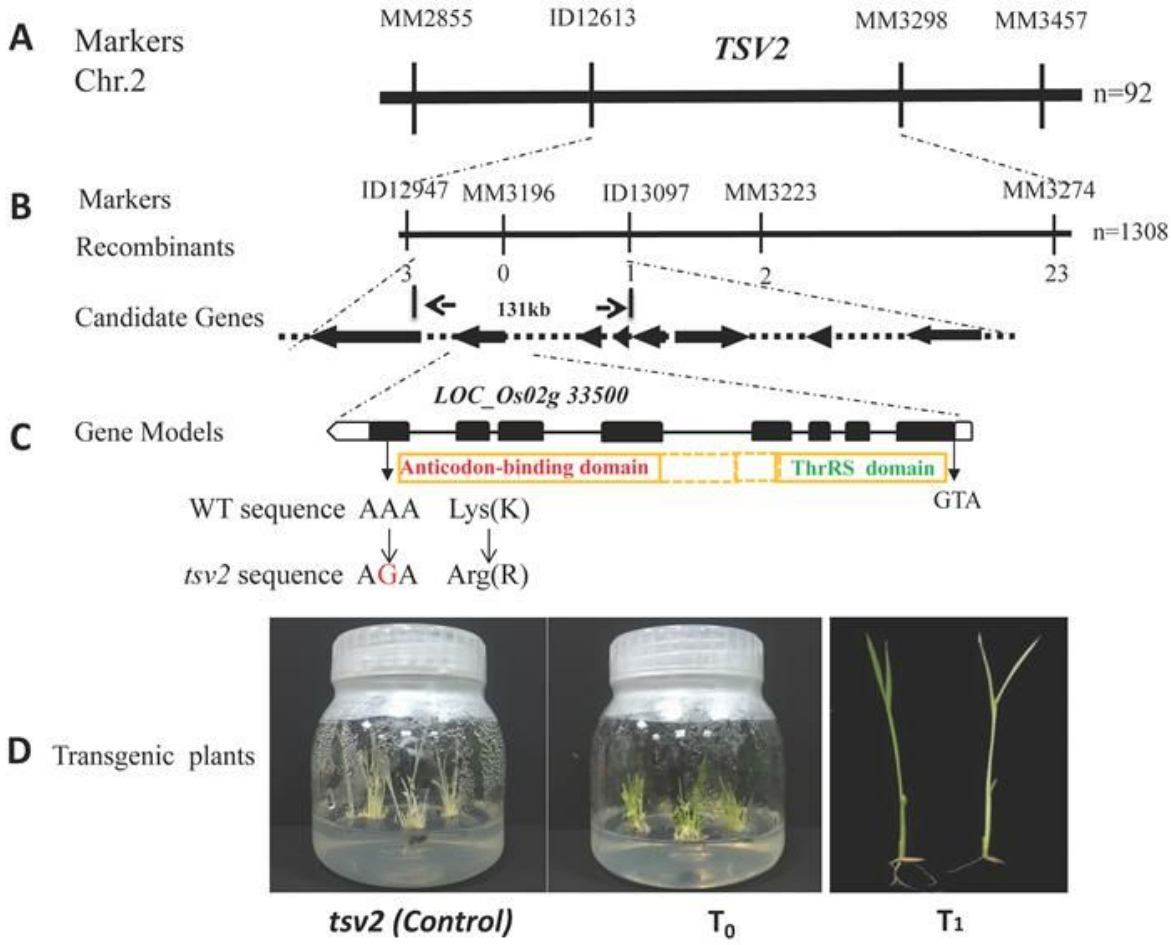

Figure 4

Map-based cloning of the TSV2 gene. (A) Location of the TSV2 was located on chromosome 2. (B) TSV2 gene was narrowed to 131kb region, containing eight candidate genes (C) Black boxes represent exons and the black lines between them represent introns. (D) T0 transgenic plants transformed with pCAMBIA1301(control) and pCAMBIA1301-TSV2 at 20°C. The T1 segregation from the T0 plants at 20°C. The genotypes of green phenotype are TSV2:TSV2/TSV2: tsv2 (left) and the genotype of the albino phenotype is tsv2:tsv2 (right).

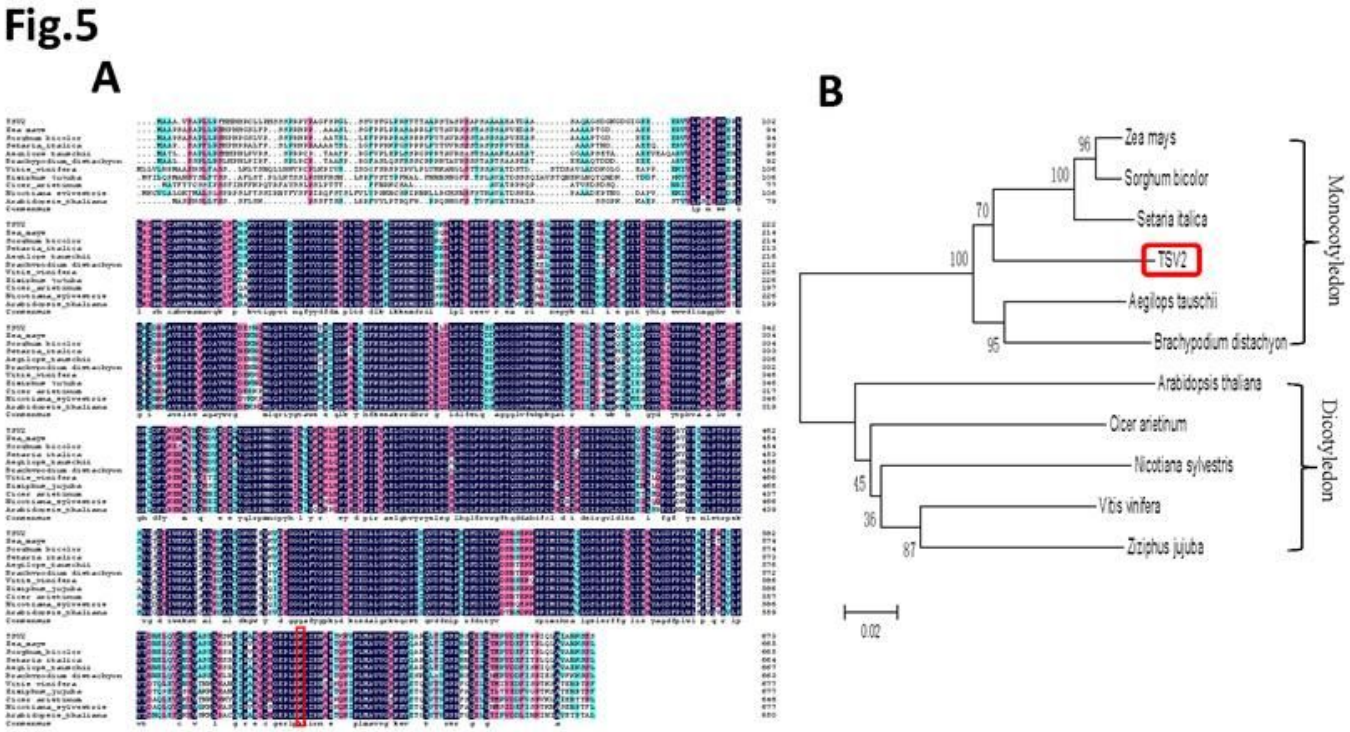

Figure 5

Phylogenic analysis of TSV2 protein. (A) Amino acid sequence alignment of TSV2 with the eleven homologous proteins from amino acids fully or partially conserved are shaded black and gray, respectively. (B) Phylogenic tree of TSV2 and homologous proteins. Scale represents percentage substitution per site. Statistical support for the nodes is indicated.

Fig.6

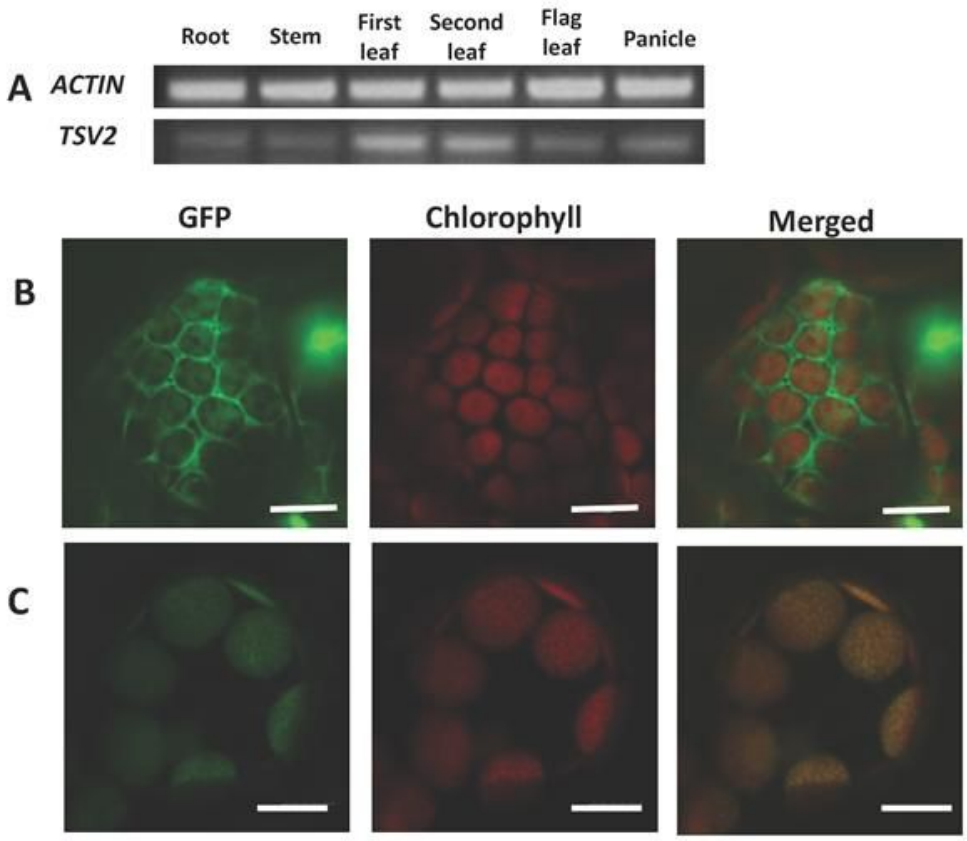

Figure 6

Expression pattern and subcellular localization of TSV2. (A)Analysis of expression of TSV2 in different tissues by RT-PCR. OsActin was used as a control (cycle number for OsActin was 28, cycle number for TSV3 was 35). (B) Empty GFP vector without a specific targeting sequence. (C) TSV2 -GFP fusion. The scale bar represents 20 μm.

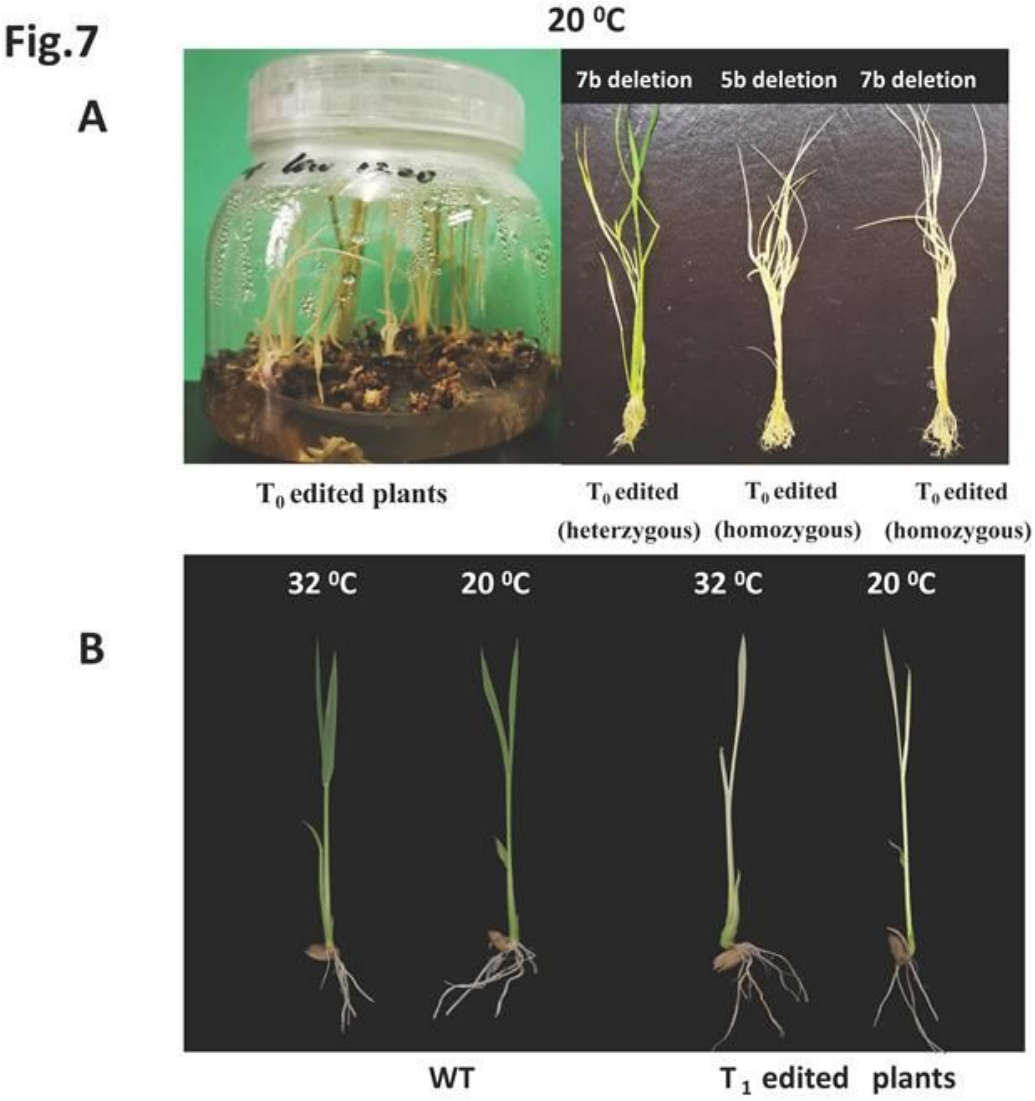

Figure 7

Phenotype of the edited transgenic plants by CRISPR/Cas9 technology. (A) To edited transgenic plants at 20°C. (B) The homozygous T1 plants from the heterzygous T0 plants grown at 20°C and 32°C.

Fig.8

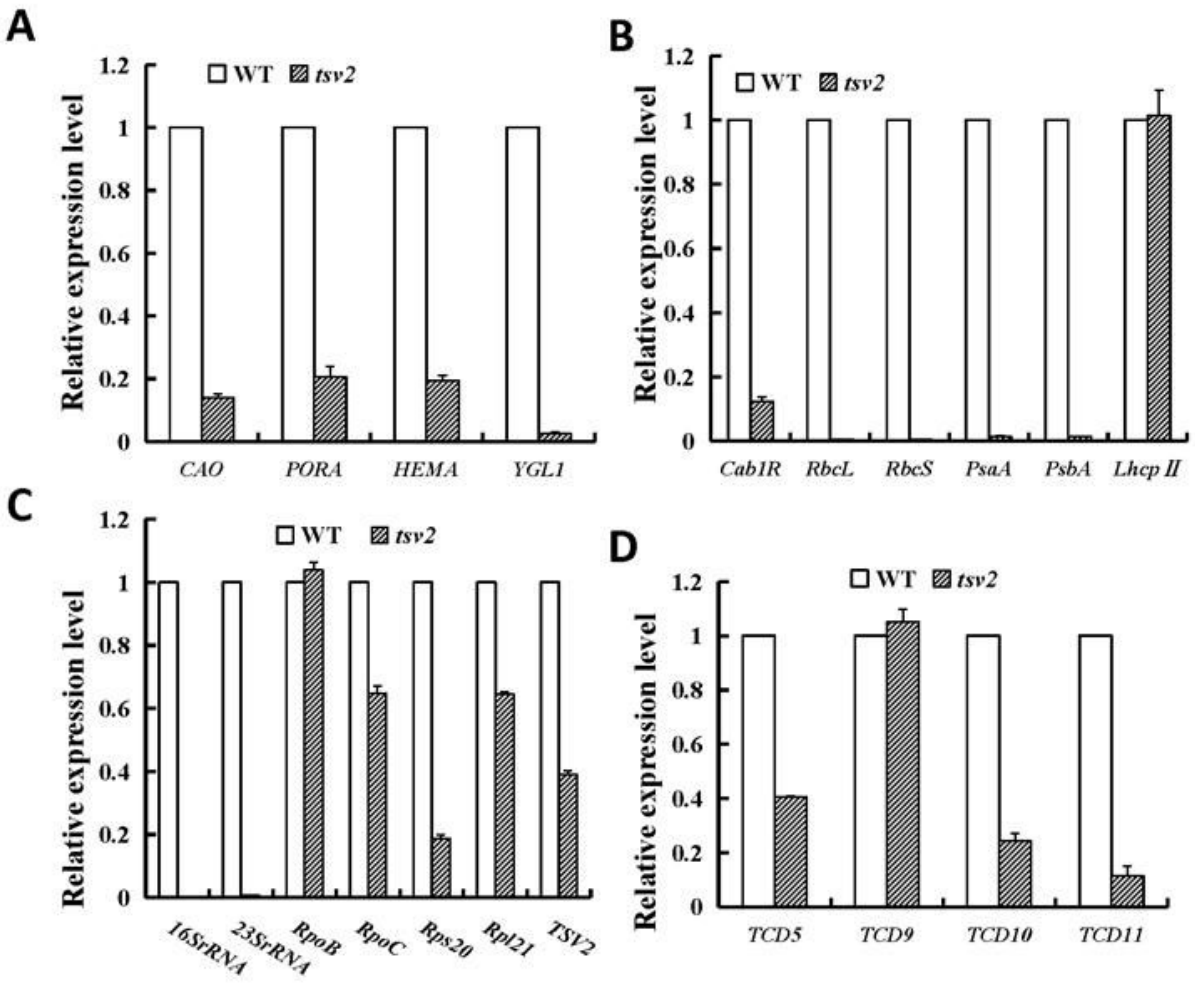

Figure 8

Phenotype of the edited transgenic plants by CRISPR/Cas9 technology. (A) To edited transgenic plants at 20°C. (B) The homozygous T1 plants from the heterzygous T0 plants grown at 20°C and 32°C.

Fig.9

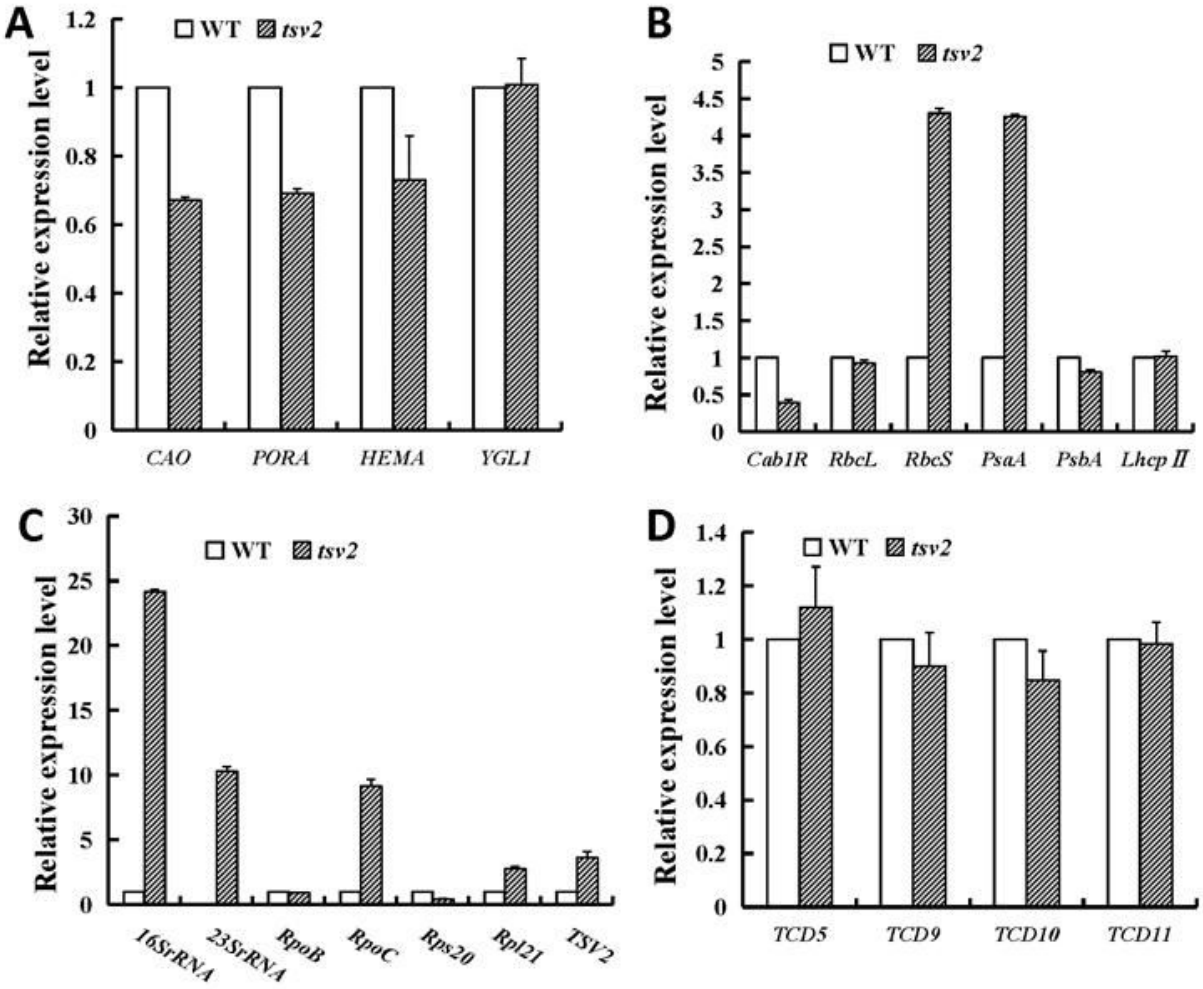

Figure 9

Quantitative expression analysis of those genes related to Chl biosynthesis, photosynthesis and chloroplast development in mutant at 32°C. A, B, C, D Expression levels of genes related to Chl biosynthesis, photosynthesis, chloroplast developmentand and temperature sensitivity in WT and the tsv2 mutant in the 3rd leaves, respectively. The relative expression level of each gene in WT and mutant was analyzed by qPCR and normalized using the OsActin as an internal control. Data are means±SD (n = 3).

Supplementary Files

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- supplementarymaterials.pdf