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ORIGINAL ARTICLE

Mutation of *TL1*, encoding a novel C_2H_2 zinc finger protein, improves grains eating and cooking quality in rice

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Abstract

Key message **The cloning and characterization of a novel C_2H_2 zinc finger protein that affects rice eating and cooking quality by regulating amylose content and amylopectin chain-length distribution in rice.**

Abstract One of the major objectives in rice breeding aims to increase simultaneously yield and grain quality especially eating and cooking quality (ECQ). Controlling amylose content (AC) and amylopectin chain-length distribution (ACLD) in rice is a major strategy for improving rice ECQ. Previous studies show that some starch synthesis-related genes (SSRGs) are required for normal AC and ACLD, but its underlying regulating network is still unclear. Here, we report the cloning and characterization of a novel C_2H_2 zinc finger protein TL1 (Translucent endosperm 1) that positively regulates amylose synthesis in rice grains. Loss of *TL1* function reduced apparent amylose content (AAC), total starch, gel consistency, and gelatinisation temperature, whereas increased viscosity, total lipid, and ratio of amylopectin A chains with degree of polymerization (DP) 6–12 to B_1 chains with DP 13–24, resulting in an enhanced grain ECQ. The improved ECQ was accompanied by altered expression patterns of several tested SSRGs in *tl1* mutant grains. Furthermore, knockout of *TL1* in the high-yielding rice variety JiaHua NO.1 reduced AAC without obvious side effects on major agronomic traits. These findings expand our understanding of the regulating networks of grain starch metabolism and provide new insights into how rice ECQ quality can be improved via genetic approach.

Abbreviations

AAC	Apparent amylose content
AC	Amylose content
ACLD	Amylopectin chain-length distribution
DAF	Days after flowering
DB	Degree of branching
dCAPs	Derived cleaved amplified polymorphic sequence
DP	Degree of polymerization
ECQ	Eating and cooking quality
GBSS 1	Granule-bound starch synthase 1
GC	Gel consistency
GT	Gelatinization temperature
MAS	Marker-assisted selection
SEM	Scanning electron microscopy
SSRGs	Starch synthesis-related genes

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Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops, serving as a staple food for more than half of the world's population (https://www.ers.usda.gov/topics/crops/rice/rice-sector-at-a-glance/). Over the last couple of decades, rice yield has been greatly increased. However, with economic and social development, an increase in rice eating and cooking quality (ECQ) has become an important consideration of rice customers and breeders (Zeng et al. 2017).

Starch, accounting for approximately 90% of the rice grain endosperm, consists of linear amylose and branched amylopectin. The physicochemical properties of starch, amylose content (AC), gel consistency (GC), gelatinisation temperature (GT), and viscosity, determine rice ECQ; among them, AC is the primary determinant (Tian et al. 2009). Rice grains with a high AC are usually hard and have poor glossiness, but rice grains with an excessively low AC are usually too sticky after cooking (Liu et al. 2009). In addition, previous studies demonstrated that amylopectin A chains with degree of polymerization (DP) 6–12 and B1 chains with DP 13–24 also affect rice ECQ; it is proposed that an increase in the ratio of amylopectin A chains to amylopectin B1 chains reduces GT and hardness and improves the stickiness of cooked rice, resulting in better texture and cooking properties (Li et al. 2016, 2020). Therefore, maintaining a suitable AC and an appropriate high ratio of amylopectin A chains to B1 chains is crucial for improving rice ECQ.

Consumers in different areas usually have distinct preferences to the so-called superior quality rice, which differs mainly with respect to AC (Zeng et al. 2017). In eastern Asia, especially in Japan and the Yangtze River basin of China, most people prefer the so-called soft rice with a relative low AC ranging from 8 to 12% (Li et al. 2018). Therefore, low AC is a desirable trait and is selected in rice breeding in these regions.

In rice endosperm, amylose synthesis is largely regulated by the *Wx* locus, which encodes granule-bound starch synthase I (GBSSI) (Wang et al. 1995). A series of allelic variants of *Wx* (such as Wx^{lv}, Wx^{a}, Wx^{b}, Wx^{mq}, $Wx^{hp/op}$, Wx^{in}, Wx^{mv}, and *wx*) have been reported to contribute to variations in rice AC (Zhang et al. 2019, 2021). Among these variants, Wx^{a} and Wx^{b} are two major *Wx* alleles in Asian cultivated rice and have been recognized as being distributed in the *indica* and *japonica* subspecies, respectively (Wang et al. 1995). Wx^{mq} is weak mutation in the Wx^{b} allele and is derived from the *Japanese* soft rice cultivar ''Milky Queen'' with low-AC trait (Sato et al. 2002), which has been wildly used for soft rice breeding (Zhao et al. 2017a). The rice varieties with the $Wx^{hp/op}$ allele, derived from the opaque endosperm mutants, also show a low AC (Mikami et al. 1999; Liu et al. 2009); and, the $Wx^{hp/op}$ allele is evolutionarily closely related to but not derived from the Wx^{a} allele (Zhang et al. 2019). In addition to *Wx* alleles, *Du* genes, including *du1* (Zeng et al. 2007), *du3* (Isshiki et al. 2008), and *lowac1* (Igarashi et al. 2021), also control low-AC traits in rice by mediating *Wx* gene expression. *Du1*, encoding a pre-mRNA processing (Prp1) protein, regulates AC biosynthesis by affecting splicing of the Wx^{b} pre-mRNA, instead of Wx^{a} pre-mRNA (Zeng et al. 2007). *Du3*, encoding a 20-kDa subunit of the mRNA cap-binding protein, plays a role in Wx^{b} pre-mRNA splicing (Isshiki et al. 2008). Furthermore, *LowAC1*, encoding an RNA recognition motif (RRM) protein, is correlated with low-AC trait in rice (Igarashi et al. 2021). Moreover, *SSSII-2* regulates AC as well, rice grains of its RNAi lines exhibit a significantly reduced AC, similar to that of soft rice (Li et al. 2018).

Despite the abovementioned advances in the identification of multiple genes regulating low-AC traits in rice, the understanding of its genetic base is still very limited. Previous studies suggest that grain endosperm transparency is positively correlated with AC, therefore, the translucent endosperm can be considered as an indicator for low-AC traits in rice (Zhang et al. 2017; Huang et al. 2021a). To identify new low-AC traits genes, here, we screened the Yangtze River Delta rice landraces in China and identified a *japonica* soft rice cultivar, YingXiang38 (YX38), with a low apparent amylose content (AAC), though analyzing translucent endosperm phenotype. We revealed that the low AAC in YX38 is controlled by a novel gene *TL1* (*Translucent endosperm 1*) that encodes a C_2H_2 zinc finger protein and that *TL1* negatively regulates ECQ by regulating AC and fine amylopectin structure through regulating expressions of several *SSRGs*. Notably, *TL1* mutation in the high-yielding rice variety JiaHua NO.1 (JH1) reduced grain AAC without obvious side effect on main agronomic traits. These findings expand our understanding of the regulating networks of grain starch metabolism and provided new insights into how rice quality can be improved via genetic approach.

Materials and methods

Plant materials, growth and harvest

YingXiang38 (YX38) is one of the rice landraces in Shanghai, China, a *japonica* soft rice cultivar with preferred ECQ. It was developed by Bright Seed Industry Co Ltd in Shanghai, China, in 2017 (http://ricedata.cn) from a cross between the *japonica* cultivars RuanYu NO.1 and WuYu NO.19. JiaHua NO.1 (JH1) is a high-yielding *japonica* rice cultivar that is widely planted in Zhejiang Province and neighboring regions, China (http://ricedata.cn). Two mapping populations were constructed by crossing YX38 as the male parent with 9311 or JH1 as female parents. *tl1* mutants were generated in both Nipponbare (Nip) and JH1 using CRISPR/Cas9. The abovementioned rice materials were cultivated in the paddy field of Shanghai Normal University in Shanghai (May to October) or in paddy field of Shanghai Academy of Agricultural Sciences in Lingshui Li Autonomous County of Hainan (December to April). For sampling, developing grains were harvested at 5, 10, 15, 20, and 25 days after flowering (DAF), respectively, each sample was a collection of three independent main tillers, each from a different plant. Mature grains were harvested at about 45 DAF.

The population development and inheritance mode of *TL1*

180 F_2 progenies of 9311 × YX38 and 126 F_2 progenies of JH1 × YX38 were used to determine the inheritance mode of *TL1*. Genetic analysis revealed segregation of endosperm characters in F_2 grains. Chi-square ($\chi 2$) tests were used to evaluate the goodness of fit of the observed and expected segregation ratios in genetic analysis (Table 1).

Grain phenotype observation and endosperm iodine staining

The hulls of rice seeds were removed to observe the external appearance of the grains in 9311, JH1, YX38, YX38 complement lines, Nip, *tl1* mutant and F_2 progenies of JH1 × YX38. Grains were cut through the center to expose the endosperm and 0.2% iodine reagent (0.2 g I and 2 g KI, which were dissolved into sterile water, and the final volume is 100 mL) was dropped on the endosperm cut surface, and photographs were taken after 3–5 min staining.

Measurement of physicochemical properties

Rice grain physicochemical properties including AAC, GC, GT, DB, viscosity, total starch, total lipid, taste value, and amylopectin CLD were measured using mature seeds. For AAC, the milled rice grains were crushed to a fine powder, defatted and dispersed in sodium hydroxide solution. After the addition of 0.2 mL iodine reagent, absorbance was measured at 620 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, America). For total starch content, 100 mg of milled rice powder was used using a microplate reader (Multiskan GO, Thermo Fisher Scientific, America) as described previously (Holm et al. 1986). For amylopectin CLD, 5 mg rice powder was dissolved in 5 mL water in a boiling water bath for 60 min. Sodium azide solution (10 uL, 2% w/v, Sangon Biotech), acetate buffer (50 uL, 0.6 M, pH 4.4), and isoamylase (10 uL, 1400 U) (catalog number: 08124, sigma) were added to the starch dispersion, and the mixture was incubated in a water bath at 37℃ for 24 h. The hydroxyl groups of the debranched glucans were reduced by treatment with 0.5% (w/v) of sodium borohydride under alkaline conditions for 20 h. The preparation about 600 uL was dried in vacuo at room temperature and allowed to dissolve in 20 uL NaOH solution (1 mol/L) for 60 min. Then, the solution was diluted with 580 uL distilled water. The sample extracts were analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-100 anion-exchange column (4.0 × 250 mm) using a pulsed amperometric detector (PAD, Dionex ICS 5000 system). Data were acquired on the ICS5000 (Thermo Fisher Scientific, USA) and processed using chromeleon 7.2 CDS (Thermo Fisher Scientific, USA) as described previously (Zhou et al. 2015). For starch viscosity properties, 3 g of milled rice powder was transferred into a container with 25 mL of distilled water, mixed and measured with a Rapid Visco Analyzer (RVA Super 4; Newport Scientific, Sydney, NSW, Australia), and the concrete procedures were described in Supplementary Table S4. For GC, 100 mg milled rice flours were used and measured following the method of Tan et al. (1999). For the taste value analysis, 30 g polished rice was soaked with 40.5 g distilled water for 30 min in a stainless steel tank and then cooked for 30 min. The cooked rice was analyzed with an RCTA-11A Taste Analyzer (Satake, Japan) according to the manufacturer's protocol. For GT, 5 mg of rice powder was placed in an aluminum sample cup, mixed with 30 µL distilled water and sealed. The samples were analyzed by a DSC (DSC, Q2000, TA Instruments, USA) following the manufacturer's protocols. For the DB analysis, 10 mg of rice powder was dissolved in 1 mL dimethyl sulfoxide-D6 at 80 ℃ for 12 h, the samples were analyzed with an NMR spectrometer (Bruker BioSpin GmbH) according to the manufacturer's protocol. The total lipid in the grains was measured as described previously (Kang et al. 2005).

Table 1 Segregation of endosperm characters in F_2 populations derived from the cross of YX38 with two different parents

Cross	Brown rice phenotype of F_2 seeds		Exp. ratio	$\chi 2$	*P* Valve
	Transparent	Translucent			
9311/YX38	142	38	3:1	1.25	> 0.05
JH1/YX38	93	33	3:1	0.03	> 0.05

Scanning electron microscopy of starch granules

For scanning electron microscopy (SEM) observations of the shape and size of the starch granules, milled grains were cut into halves, mounted on an aluminum stub using double-sided conductive carbon tape, coated with gold after air-drying and examined using a scanning electron microscope (S-3000n, Hitachi, Japan) at 15 kV. Micrographs of each starch sample were taken at a magnification of 25×, 500×, 1500× and 3000×, respectively.

Molecular cloning of *TL1*

Linkage analysis was carried out in an F_2 population consisting of 22 individuals with translucent endosperm using simple sequence repeat (SSR) markers. Subsequently, fine mapping was carried out using the other 3028 F_2 or F_3 individuals with normal or translucent endosperm. Two flanking markers (RM3617 at 28.61 Mb and RM4124

at 31.08 Mb on chromosome 6) were used to identify recombinant plants. These recombinant plants were genotyped with ten insertion-deletion (InDel) markers located between the flanking SSR markers. *TL1* was eventually defined between DW1 and ID17407 within a 140-kb region (Fig. 1). (The primer sequences for the SSR and InDel markers are listed in Supplementary Table S1.)

dCAPS analysis

A functional marker primer was designed to amplify the *TL1* sequence of rice varieties YX38, and JH1 simultaneously (Table S3), according to the sequence alignment of the *TL1*/*tl1* alleles (Fig. S3A). PCR was performed with *TaKaRa Ex Taq* DNA Polymerase following the manufacturer's protocols (Takara). PCR amplification products were purified using PureLink PCR Micro Kit according to the

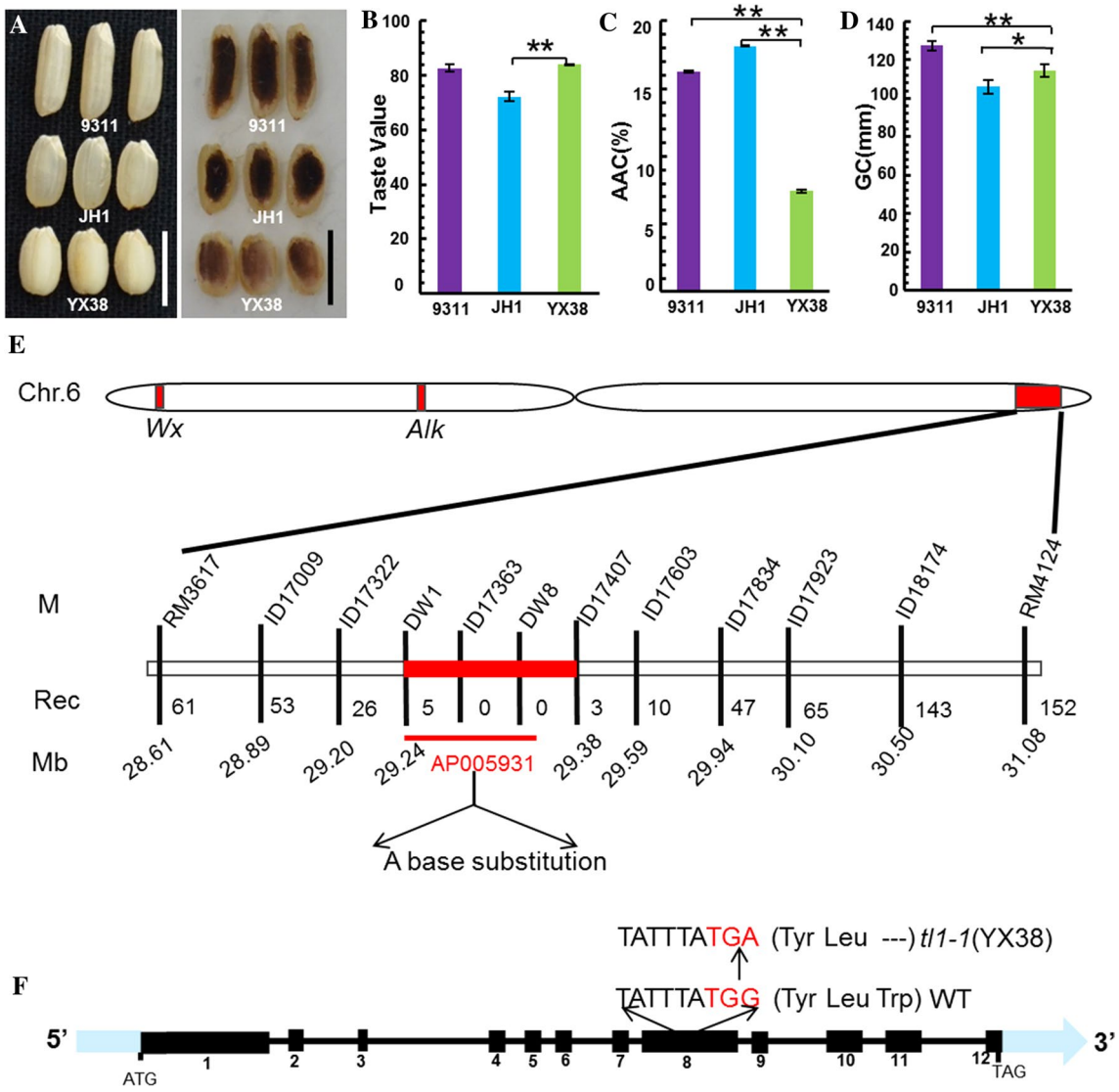

Fig. 1 Characterization of YX38 with preferred ECQ and positional cloning of the causal gene. **A** Comparisons of the appearance of 9311, JH1 (JiaHua NO.1) and YX38 (YingXiang38) of milled grains (left) and starch iodine stained grains (right). **B**–**D** Comparison of the physicochemical properties in milled grains of YX38 with 9311 and JH1. **B** Taste value; **C** AAC; **D** GC. **E**–**F** Map-based cloning of causal gene. **E** Location of the TL1 locus for low amylose in a 140-kb genomic region on chromosome 6 determined using 3050 F_2 or F_3 plants; (F) A schematic representation of the exon and intron organization of *TL1* (LOC_Os06g48530). ATG indicates the putative starting nucleotide of translation, and TGA is the stop codon. The black boxes indicate exons, and intervening lines indicate introns. BAC, Bacterial artificial chromosome. M, Molecular marker; Rec, number of recombinations; *Wx* encodes granule-bound starch synthase 1; *Alk*, encodes soluble starch synthase II-3. Bars, 7 mm. **Significant difference ($P < 0.01$); *Significant difference ($P < 0.05$). Data are means ± standard deviations from at least three independent measurements

manufacturer's protocols (Thermo Scientific) and then were digested with the *Mae*II enzyme (Thermo Scientific). The digestion mixture (20 uL) containing 2 uL of 10 × buffer, 5 uL of PCR product, 1 uL of *Mae*II enzyme, and 12 uL of ddH_2O_2 was incubated at 65 ℃ for 4 h, and the *MaeII* digested products were separated by electrophoresis for 30 min on 3% agarose gel.

Complementation vector construction and plant transformation

For generation of the *tl1* functional complementation vector, 2,679 bp of the *TL1* upstream region (termed pTL1), and full-length *TL1* CDS (termed TL1), were synthesized by whole gene synthesis method in Shanghai Generay Biotech Co., Ltd, and the base sequence of pTL1 and *TL1* CDS was confirmed by sanger sequencing and then inserted into pCAMBIA1301-eYFP to construct the plasmid pCAMBIA1301-pTL1:TL1-eYFP. Calli induced from young panicles of the homozygous YX38 plants were used for transformation with *Agrobacterium tumefaciens* (EHA105) that carries pCAMBIA1301-pTL1: TL1-eYFP plasmid. The grains from T_1 lines were used to perform complementation analysis.

qRT-PCR assay

Total RNA was isolated from developing rice grains harvested at 5, 10, 15, 20, and 25 DAF of Nip and homozygous *tl1-2* plants using Omni Plant RNA kit as described by the manufacturer. qRT-PCR procedures and data analysis were performed as previous described (Zhao et al. 2017b). Rice housekeeping gene *Actin* (LOC_Os10g36650) was used as the reference gene. Primers for qRT-PCR are listed in Supplemental Table S2.

Results

Phenotypic evaluation

In a journey to look for low AC soft rice cultivars, we screened the Yangtze River Delta rice landraces in China and identified a *japonica* soft rice cultivar, YX38, which is characterized by preferred ECQ (Fig. 1B; Tables S5, S6) with translucent endosperm phenotype (Fig. 1A). Milled YX38 grains contained a significantly lower AAC than the *japonica* cultivars JH1 and the *indica* cultivars 9311 with the Wx^b allele (responsible for intermediate AAC) (Fig. 1C). When stained with 0.2% iodine reagent, endosperms in cross sections of JH1 and 9311 seeds turned dark brown, while those in the soft rice YX38 looked slight red-brown (Fig. 1A, Right), confirming that YX38 grains have less amylose.

Map-based cloning of the gene underlying lower ACC in YX38

The low transparency was used as the target trait for genetic analysis. Two different rice cultivars, 9311 (*indica*) or JH1 (*japonica*), were crossed with YX38. All F_1 grains produced were transparent and segregation ratios of transparency in F_2 grains were 3:1 (transparent: translucent endosperm) as expected (Table 1). These data indicated that the translucence trait in YX38 is controlled by a single recessive gene. The transparent or translucent endosperm in F_2 progenies of JH1 × YX38 (Fig. S1A) was confirmed by 0.2% iodine staining, in which endosperms with transparent traits turned dark brown, while endosperms with translucent traits looked slight red-brown (Fig. S1B). This result also suggests that grain endosperm transparency is positively correlated with AC, which was in agreement with previous studies (Zhang et al. 2017; Huang et al. 2021a). A map-based cloning approach was used to identify causal gene based on 3050 individuals of an F_2 or F_3 mapping population. The mutated gene was finally mapped on the chromosome 6 between two markers, DW1 and ID17407, defining a 140 kb region (Fig. 1E). Sequence analysis revealed a base substitution from G to A at the position 4561 in the exon 8 of an annotated gene LOC_Os06g48530 (Fig. 1, S2–S3) that encodes a C_2H_2 zinc finger protein with DNA and protein binding function (http://rice.uga.edu/), named as *Translucent endosperm 1* (*TL1*), which introducing a premature stop codon and a truncated protein of 407 amino acid residues (Fig. S3). Genomic sequence analysis showed that *TL1* in Nip is 7044 kb in length, comprising 12 exons with a 2,172 bp coding region encoding a predicted protein of 723 amino acid residues (Fig. 1F, S3).

The SNP G/A at the 4561 site of the *TL1*/*tl1* alleles resulted in the *Mae* II enzyme recognition sequence 'ACGT' (Fig. S3A). Therefore, a dCAPS functional marker using the *Mae* II restriction enzyme (dCAPS/*Mae*II) was designed to identify the *TL1* and *tl1* alleles. The pair of primers, designed to flank the 4561 site of the *TL1*/*tl1* (Fig. S3; Table S3), was used to amplify the target region using the genomic DNA of YX38, JH1, and F_1 plants (derived by crossing YX38 with JH1), which revealed one band (a 313-bp fragment) in the JH1 rice variety, two bands (82 and 231-bp fragments) in the YX38 rice variety, and three bands (82, 231, and 313-bp fragments) in the F_1 (Fig. S4A). We also genotyped F_2 populations from the cross of YX38 with JH1 to show cosegregation of the *TL1* genotypes and the matching phenotypes using dCAPS/*Mae* II (Fig. S4A) and found that the dCAPS/*Mae* II is also polymorphic between

YX38 and the other fourteen high-yielding rice cultivars (Fig. S4B).

TL1 affects amylose content and fine structure of amylopectin in rice

To confirm that LOC_Os06g48530 is the causal gene of reduced AAC in YX38, a binary plasmid pCAMBIA1301-pTL1:TL1-eYFP was introduced into the YX38 homozygous plants (pTL1 and TL1CDS from Nip). The data showed that low transparency and low amylose content phenotype of YX38 was complemented in transgenic plants (Fig. 2B, C). Furthermore, to better understand the role of *TL1*, we generated two knockout mutant *tl1-2* and *tl1-3* in the Nip variety using CRISPR/Cas9 technique (Fig. S5). Grains of both *tl1-2* and *tl1-3* mutants displayed translucent endosperm compared with those of Nip with decreased AAC from 17.50% to 5.83% and 5.73%, respectively (Fig. 2A, left and C).

In addition to decreased AAC, *tl1-2* and *tl1-3* grains displayed also decreased total starch and GC as compared

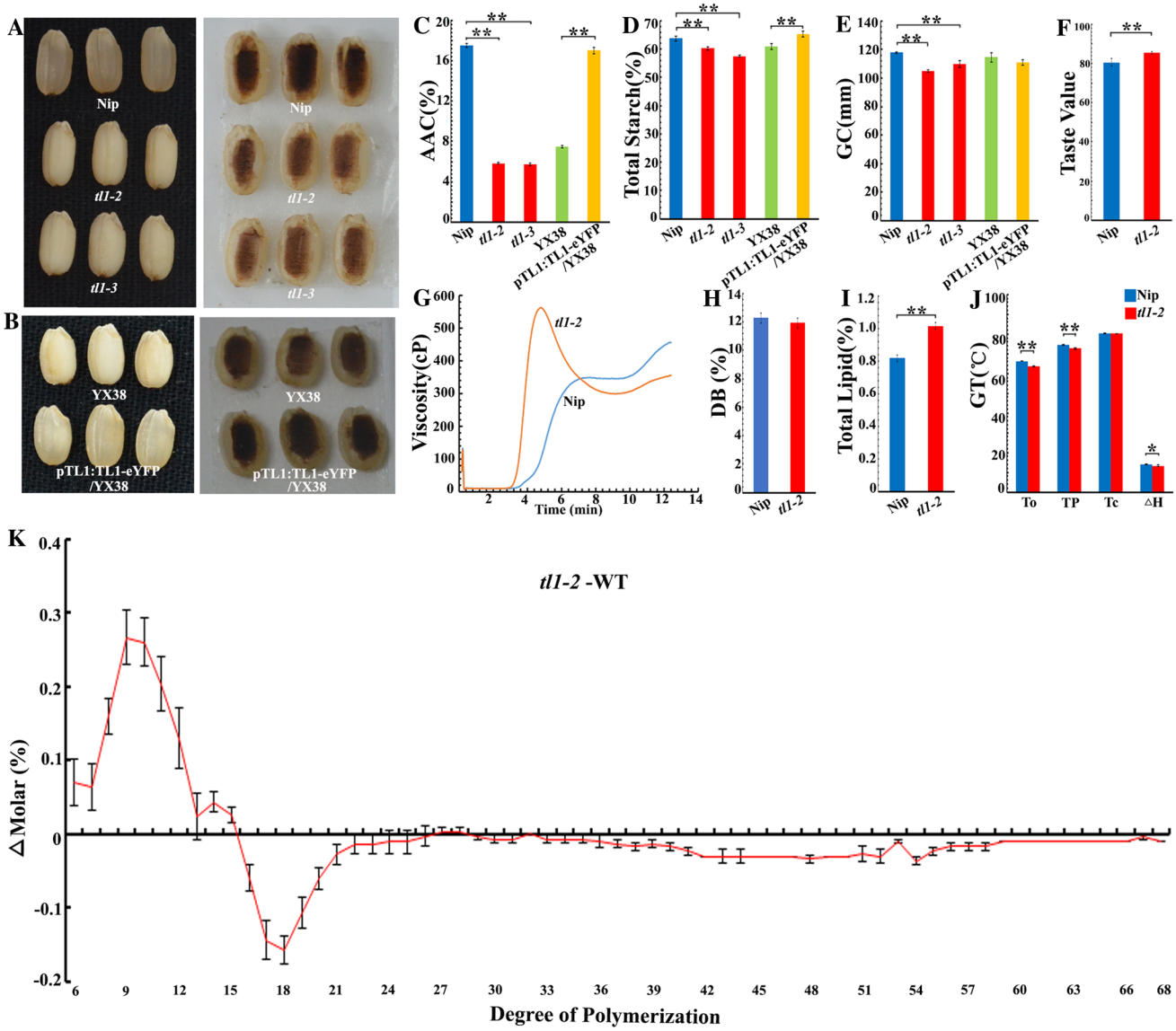

Fig. 2 Physicochemical properties of starch from grains of plants with different *TL1* genotypes. **A**, **B** Comparisons of the appearance of milled grains of Nip, *tl1-2, tl1-3*, YX38, and pTL1-ACF-eYFP/YX38 (left) and starch iodine stained grains (right). **C**–**K** Physicochemical properties of milled rice in Nip, *tl1-2, tl1-3*, YX38, and pTL1-ACF-eYFP/YX38 **C** AAC; **D** total starch; **E** GC; **F** taste value; **G** viscosity; **H** DB; **I** total lipid; **J** GT; **K** differences in the amylopectin chain-length distributions between the WT and *tl1-2*, % in **K** indicates percentage of differences of relative peak area in the amylopectin molecule with different chain length between the WT and the *tl1-2*. **Significant difference ($P < 0.01$); *Significant difference ($P < 0.05$). Data are means ± standard deviations from at least three independent measurements

with wild type (Fig. 2D–E). Compared to YX38, the complementation lines expressed *TL1* from Nip elevated AAC and total starch, but not GC (Fig. 2C–E), indicating that GC is likely affected by diverse genetic factors. Meanwhile, *tl1-2* showed also increased taste value, viscosity, and total lipid compared with wild type (Fig. 2F–G and I; Table S7). However, the DB levels of milled rice starch are similar between *tl1-2* and wild type (Fig. 2H). Furthermore, the onset (To), and peak (Tp) temperatures, and enthalpy (ΔH) of gelatinization of milled rice starch in the *tl1-2* mutant were all significantly lower than those in the wild type when GTs of milled rice from wild type and *tl1-2* were analyzed by differential scanning calorimetry (Fig. 2J).

To evaluate the possible effect of *TL1* on the fine structure of amylopectin, we measured the glucan CLD. Compared with wild type, the proportion of short or intermediate chains with DP between 6 and 15 was significantly elevated, whereas the proportion of intermediate or long chains with DP values in the range 16–68 was significantly decreased in the *tl1-2* mutant, with the exception of the intermediate chains with DP values 27, 28, 32 (Fig. 2K). Previous reports suggested that an increase in the ratio of amylopectin short chains to amylopectin intermediate chains reduces GT (Li et al. 2016, 2020). Meanwhile, levels of onset (To), peak (Tp) temperatures, and enthalpy (ΔH) of gelatinization of milled rice starch in the *tl1-2* were all significantly lower than those in the wild type (Fig. 2J). Given AAC also decreased in *tl1-2* (Fig. 2C), and, AC and GT were two main physicochemical characteristics of rice starch (Tian et al. 2009), so, above analysis indicated that *TL1* affects not only amylose content but also fine structure of amylopectin, two important polyglucans in rice starch granules.

Starch granule morphology in *tl1* mutant grains is altered

To determine the effect of *TL1* on grains starch granule morphology, scanning electron microscopy (SEM) was used to examine fractured surfaces of rice grains with different *TL1* genotypes. The wild type Nip showed uniform sized polygonal starch granules with sharp edges, smooth flat surfaces, and compound starch granules (Figs. 3, S6). In contrast, the granules in *tl1-2* and *tl1-3* were variable in size and shape with irregular surfaces. Granules in complementary lines expressing *TL1* in YX38 (pTL1:TL1-eYFP/YX38) appeared similar to those of Nip, whereas those of YX38 rice were similar to those of *tl1-2* and *tl1-3* (Figs. 3, S6). Meanwhile, small holes were also observed in the centers of the starch granules of YX38, *tl1-2, tl1-3,* and *tl1-4*, which was consistent with the reduced transparency observed in grains of those lines (Figs. 3, 7D–E).

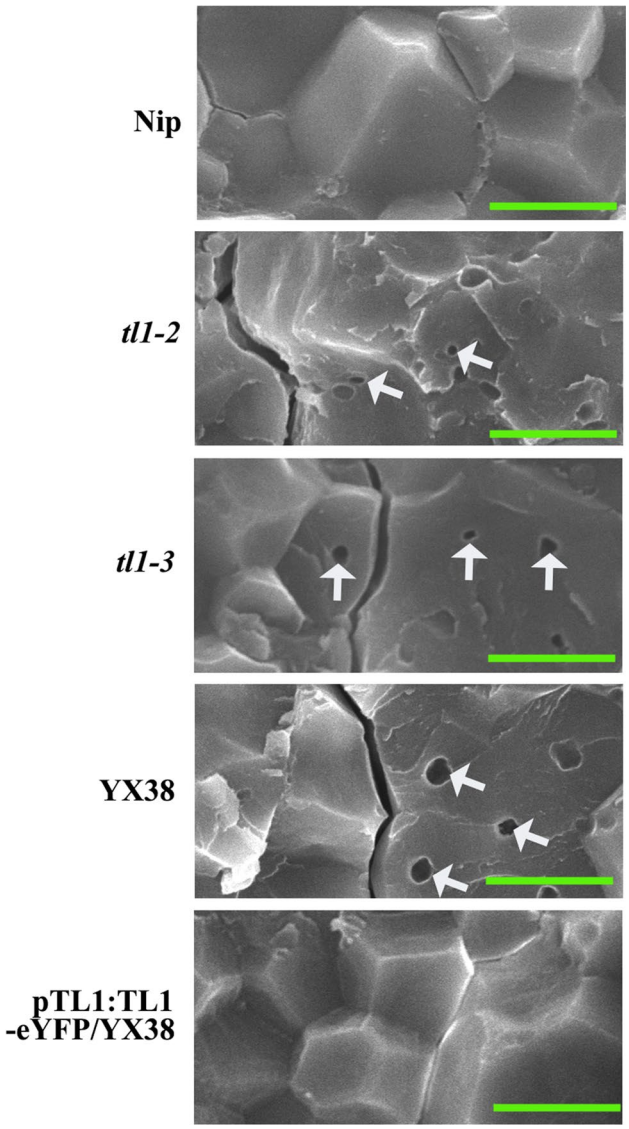

Fig. 3 Scanning electron micrographs analysis of endosperm from plants with different TL1 genotypes. The white arrows indicate small holes in the starch granules of *tl1-2*, *tl1-3*, and YX38. For each *TL1* genotype, at least five endosperms were observed by scanning electron micrographs, all showing the same morphological changes in the granules, and the pictures shown in here are representatives of each genotype. Bars, 0.5 um

TL1 is ubiquitously expressed in developing seeds

To further characterize the function of *TL1*, we examined the *TL1* expression. Quantitative reverse transcription (qRT-PCR) results revealed that *TL1* is ubiquitously expressed in both vegetative and reproductive organs including roots, stems, leaves, and developing seeds (Fig. 4). In developing seeds, the expression of *TL1* was detectable at 5 DAF, which increased along the developmental stages, peaked at 15 DAF, and then declined (Fig. 4).

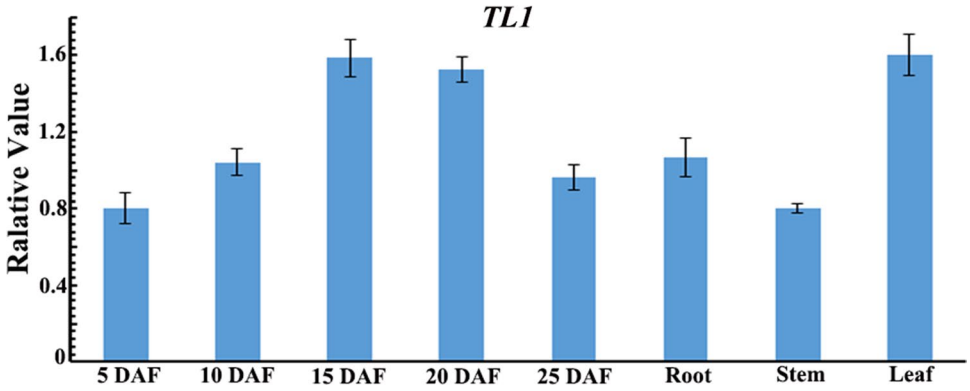

Fig. 4 Expression pattern of *TL1*. qRT-PCR analysis of *TL1*. RNA was extracted from developing seeds, roots stems and leaves, Error bars indicate SD, and each reaction had three biological repeats

TL1 encodes a C_2H_2 zinc finger protein with a single zinc finger motif

To further functional characterize *TL1*, we conducted phylogenetic analysis and found that TL1s can be divided into dicotyledonous group and monocotyledons group (Fig. 5A) and that TL1 is mostly close to its orthologs in *Panicum virgatum*, *Zea mays* and *Sorghum bicolor* (Fig. 5A). Notably, the TL1 open reading frame was found to encode a 723 amino acid protein predicted to contain a domain of unknown function 3546 (DUF3546), and an Arsenite-resistance protein-2a (ARS2) domain near its C terminus, the ARS2 domain included a single C_2H_2-type zinc finger (Fig. 5B).

TL1 affects expressions of several starch biosynthetic genes in rice grains

SSRGs affect rice ECQ via their regulatory roles in physicochemical properties of starch. For example, *GBSSI* affects AC while *SSII-2* and *SSII-3* affect GT (Tian et al. 2009; Huang et al. 2009). Therefore, we performed qRT-PCR to determine if reduced AAC and increased ratio of amylopectin chains A to chains B1 in *tl1-2* mutant are related to expression changes of SSRGs (Fig. 6). We found that expression levels of 16 of 18 tested SSRGs are reduced in *tl1-2* at least one tested stages compared with wild type. For example, expression levels of *Wx* (*GBSSI*) and several amylopectin synthetic genes including *SSII-2* and *SSII-3* were significantly decreased in *tl1-2* at most tested stages, especially at 15 DAF (Fig. 6). However, expression levels of *ISAI* and *SSI* were increased and fluctuated, respectively, in *tl1-2,* showing different expression patterns from these 16 SSRGs. Notably, expression levels of *TL1* were also significantly decreased in *tl1-2* at all tested stages, indicating *TL1* dysfunction inhibits itself expression (Fig. 6).

TL1 mutation in the high-yielding rice variety JH1 reduces grains AAC without obvious side effect on main agricultural traits

To explore the breeding application value of *TL1* in rice, *TL1* null mutant (*tl1-4*) was generated in the high-yielding rice variety JH1 by CRISPR/Cas9 system (Fig. 7A, B). Phenotyping data indicated that *tl1-4* has similar starch physicochemical properties to those of *tl1-2* and *tl1-3*, including reduced AAC (Fig. 7C–G). It is worth to note that *tl1-4* has comparable morphological traits (Fig. 7H–J) with JH1 controls, though the grain length of *tl1-4* was significantly shorter than that of JH1 controls (Fig. 7N).

Discussion

C_2H_2 zinc finger proteins constitute one of the largest families and are highly involved in regulation of flowering induction, floral organ morphogenesis, pollen and pistil maturation, abiotic or biotic stress tolerance in plants (Han et al. 2018; Huang et al. 2009; Lyu and Cao 2018; Prigge and Wagner 2001; Xiao et al. 2009; Zhuang et al. 2020). Nevertheless, it is unknown whether C_2H_2 zinc finger proteins participate in starch biosynthesis of endosperm in cereal crops. Herein, we isolated and identified a novel C_2H_2 zinc finger protein gene, *TL1*, which is highly expressed in developing rice seeds and essential for starch granules formation (Fig. 2). Generally, C_2H_2-type zinc finger proteins contain one or multiple zinc finger motif that is thought to recognize and bind its target DNA sequence. Our data indicated that TL1 contains a single zinc finger motif (Fig. 5B).

First, TL1 affects ACC in rice grains. Compared with AAC (7.45%) in YX38, rice grains of the complementary

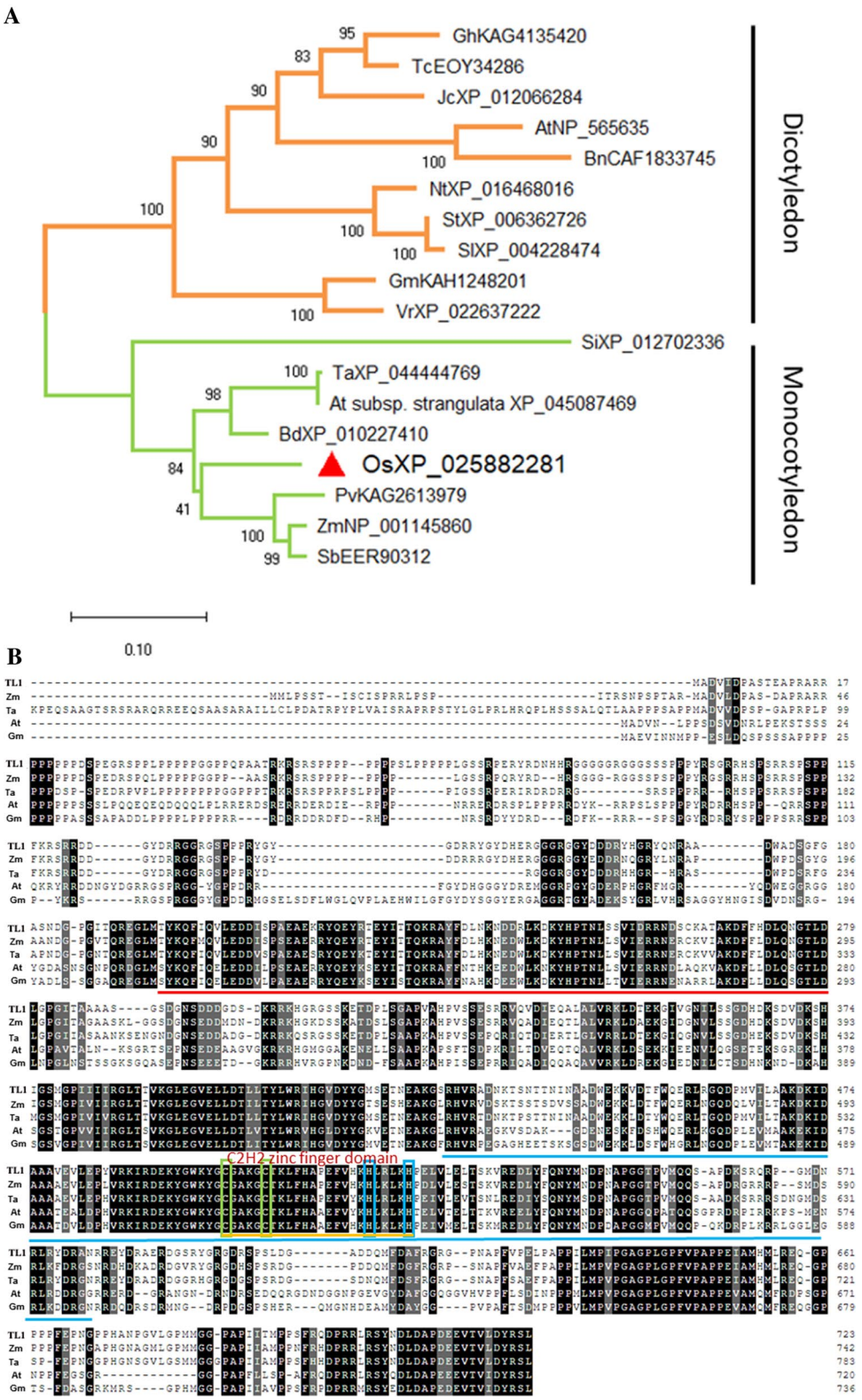

Fig. 5 TL1 encodes a zinc finger protein with a single C_2H_2 motif. **A** Phylogenetic tree for TL1-like proteins according to the maximum likelihood statistical method using the program MEGAX. Confidence in the nodes was tested by performing 1000 bootstrap replicates. Gh, *Gossypium hirsutum*; Tc, *Theobroma carao*; Jc, *Jatropha curcas*; At, *Arabidopsis thaliana*; At subsp strangulate, *Aegilops tauschi subsp strangulate*; Bd, *Brachypodium distachyon*; Bn, *Brassica napus*; Gm, *Glycine max*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pv, *Panicum virgatum*; Sb, *Sorghum bicolar*; Si, *Setaria italic*; Sl, *Solanum lycopersium*; St, *Solanum tuberosum*; Ta, *Triticum aestivum*; Vr, *Vigna radiate*; Zm, *Zea mays*. **B** Alignment of TL1-related sequences from plants. The C_2H_2 zinc finger domain is underlined in orange; the DUF3546 domain is underlined in red; the ARS2 domain is underlined in blue. Green and blue boxes, His amino acids sequence and Cys amino acids sequence, respectively

line expressing wild type *TL1* contained significantly higher AAC (17.01%), and those grains of loss-of-function of *TL1* in the backgrounds of Nip and JH1 contained significantly lower AAC (5.83, 5.73 and 7.94%, respectively) (Fig. 2C, 7F). As the key determinant of rice ECQ, amylose biosynthesis mechanism has been extensively explored in past decades, and some genes involved in amylose content, including enzymes and regulators, have been identified. Among them,

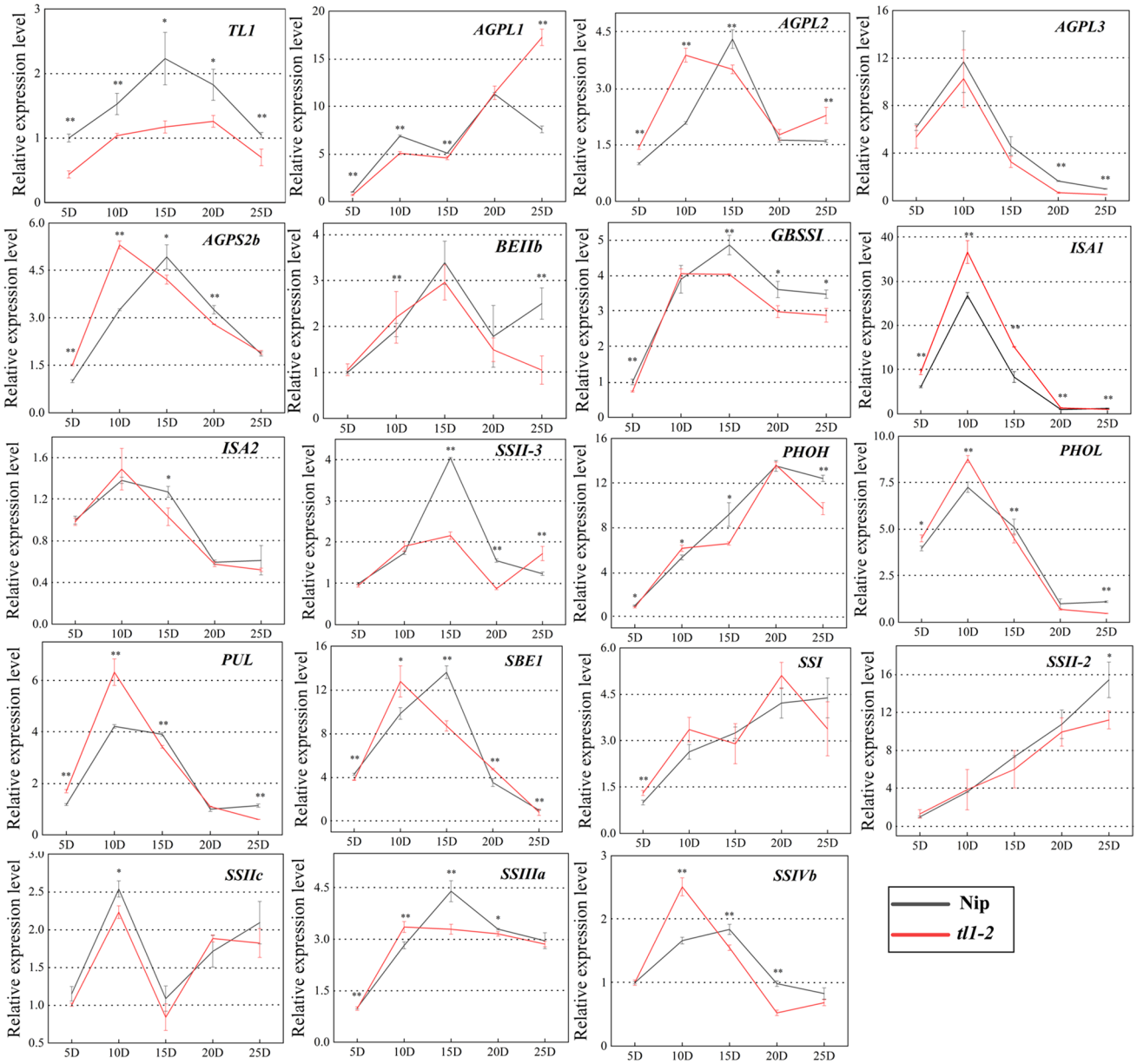

Fig. 6 Expression profiles of rice starch synthesis genes in developing grains of wild type and *tl1-2* mutant. Total RNA was extracted from seeds at 5, 10, 15, 20, and 25 DAF. **Significant difference ($P < 0.01$); *Significant difference ($P < 0.05$). Data are means ± standard deviations from at least three independent measurements

Wx regulates AAC and ECQ of rice grains (Tian et al. 2009), while *SSII-2* regulates AAC by affecting *Wx* expression levels (Huang et al. 2021b). The significantly reduced AAC together with obvious reductions in expression levels of *Wx* and *SSII-2* in *tl1* mutant (Fig. 6) indicated that *TL1* mediates rice amylose biosynthesis likely through regulating expression levels of SSRGs, such as *Wx* and *SSII-2*.

Second, *TL1* also affects amylopectin CLD in rice grains. Our data showed that mutation of *TL1* significantly increased the proportion of short or intermediate chains with DP 6–15 while significantly decreased the proportion of intermediate or long chains with DP values in the range 16–68, thus, significantly increased the ratio of short chains with small DP values to long chains with large DP values (Fig. 2K). This change in the amylopectin CLD property thus lowered GT in *tl1-2* mutant grains (Fig. 2J). The observed change of amylopectin CLD in *tl1-2* mutant grains was similar to those in grains of *ssii-3* and *sbe1* mutants (Satoh et al. 2003; Huang et al. 2021b) and was consistent with reduced expression levels *of SSII-3* and *SBE1* in *tl1-2* (Fig. 6). Expression levels of other SSRGs, including *SSI, PHOL*, *SSII-2*, and *BEIIb*, were also altered in *tl1-2* grains during seeds development

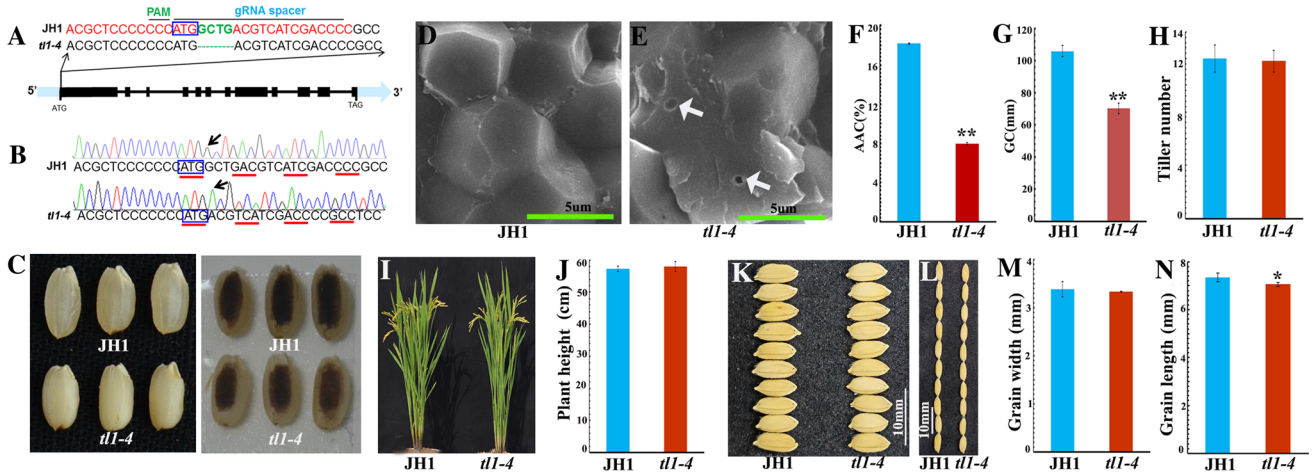

Fig. 7 The physicochemical properties of grain starch and main agronomic characteristic in *tl1-4*. **A** Schematic diagram of targeted sites in *TL1* and mutation site of the *tl1-4*. **B** Representative sanger sequencing chromatograms of JH1 and *tl1-4*. **C** Comparisons of the appearance of milled grains (left) and starch iodine stained of endosperm (right) of *tl1-4* and JH1. (D-E) Scanning electron micrographs of endosperms of JH1 (**D**), and *tl1-4* (**E**). **F** AAC. **G** GC. **H** Tiller number. **I** Morphological traits. **J** Plant height. **K**–**N** Grain width and grain length. The arrows of fig B indicate the begin sites of mutation in *tl1-4*. The ATG in blue boxes are initiation codon of *TL1* gene. The white arrows in fig **E** indicate small holes in the starch granules of *tl1-4*. For *TL1-4* and JH1, at least five endosperms were observed by scanning electron micrographs, the pictures shown in here are representatives of *TL1-4* and JH1. **Significant difference ($P < 0.01$); *Significant difference ($P < 0.05$). Data are means ± standard deviations from at least three independent measurements

(Fig. 6). Compared with *ssii-3* mutant, the absence of *SSII-2* results in a minor change in amylopectin CLD (Huang et al. 2021b). The mutation in *BEIIb* specifically alters the structure of amylopectin in the endosperm by sharply reducing short chains with DP 8–12 (Nishi et al. 2001), which was opposite to that of *tl1-2*. Increased amylopectin short chain A and decreased amylopectin medium chain were reported in *pho1* mutants (Satoh et al. 2008). Thus, the aberrant features of amylopectin in *tl1* mutant were likely the manifestation of its effects on multiple SSRG genes, including *SSII-3, SBE1, SSI, PHOL*, *SSIIb*, and *BEIIb*.

It is well known that amylose and lipid biosynthesis are interdependent in cereal plants (Baysal et al. 2020). Given both starch and lipids compete for the same precursors during grain filling, so it is possible that any deficiency in starch biosynthesis pathway will provide lipid pathway with additional substrates (Kang et al. 2005). Indeed, starch metabolism deficiency increases lipid accumulation in *Arabidopsis thaliana* and potato (Yu et al. 2018; Xu et al. 2019). In rice grains, the reduction in starch levels is accompanied with an increase in total lipids in *fse1* mutant and *SBEIIb* knockout mutant line E15 (Baysal et al. 2020). The increased total lipids together with decreased AAC in *tl1* mutant grains revealed in this study supported as well such an interdependence (Fig. 2I).

Similar to previously reported transcription factors, such as bZIP58 and NY-YB1 (Wang et al. 2013; Bello et al. 2019) that positively regulate amylose content via affecting expression levels of SSRGs, TL1 regulates amylose biosynthesis in rice through modulating expressions of some SSRGs as well (Fig. 6). Although both *bzip58* and *tl1* showed a higher proportion of amylopectin short chains with DP 6–11, and a lower proportion of amylopectin intermediate chains with DP 16–21 compared with wild type, different from *bzip58* showing reduced chains with DP 13–15 and slightly increased chains with DP 24–45, *tl1* had an opposite effect on chains with DP 13–15 and 24–45. This divergent effect may be derived from their unique target genes, for example, expression levels of *SSII-3* and *SSIIIa* were reduced in *tl1*(Fig. 6), but increased in *bzip58* (Wang et al. 2013). In addition, *TL1* mutation did not give rise to obvious side effects on major agronomic traits, including plant morphological traits (Fig. 7H–J), which exhibited great potentials to be used for rice breeding through inducing *tl1* in the high-yielding rice variety using CRISPR/Cas9 system or backcross breeding by MAS. However, dysfunction of *bZIP58* and *NY-YB1* led to serious side effects, such as a white belly that greatly limits their application in rice breeding programs (Wang et al. 2013; Bello et al. 2019).

In traditional breeding, soft rice was bred by introducing Wx^{mq} or $Wx^{hp/op}$ allele into high-yielding rice cultivars through MAS (Li et al. 2018; Liu et al. 2009; Zhao et al. 2017a), which is time-consuming and tedious. In addition, genetically modification of known AC regulation genes also helps to create new rice cultivars with desirable AC, for example, a novel soft rice with low AC was also generated by knocking down the expression of *SSSII-2* (Li et al. 2018). However, commercialization of genetically

modified crops is limited by long and costly regulatory evaluation processes as well as by public concerns. Recently, creating new low AC rice has been extensively carried out to improve grain quality by precise editing of *Wx* promoter using CRISPR/Cas9 system, or by nucleotide substitutions in *Wx* code region using adenine and cytosine base editors (Huang et al. 2020, 2021a; Xu et al. 2021; Zeng et al. 2020). However, above processes were relative tedious. Thus, cloning and identification of novel target genes suited for improving grain ECQ trait is stirring, which can be gained by directly editing its code region by CRISPR/Cas9 system, and is relatively simple compared with adenine and cytosine base editors. Our study confirmed the efficiency of this approach, in which when the *TL1* was mutated in the high-yielding rice variety JH1, grain AAC content was significantly reduced while major agricultural traits were not obviously affected, including plant morphological traits (Fig. 7). In addition, the discovered dCAPs/*Mae*II can be applied in the selection of backcross and self-bred progenies with a low AAC from YX38 and other high-yielding rice cultivars through MAS.

In summary, we identified a C_2H_2 zinc finger protein TL1 that is responsible for the low-AAC trait in superior quality *japonica* soft rice YX38. Our study also revealed the possible underlying mechanism of *TL1*-mediated improvements in rice ECQ and highlighted the key role of the coordinated regulation of amylose content and amylopectin CLD via regulation of the expressions of SSRGs. Furthermore, we also provided new strategies, germplasm resources, and molecular markers for breeding novel soft rice lines with both high yield and superior ECQ quality rice.

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Author contribution statement GC and JY designed the experiments. SF, SP and TW performed the experiments. GC and CJ analyzed the data. GC wrote the manuscript. GC and JX modified the manuscript.

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Data availability All data supporting the findings of this study are available within the paper and within its supplementary data published online.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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