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ORIGINAL PAPER

The involvement of long non-coding RNAs in the formation of high temperature-induced grain chalkiness in rice

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Abstract

A period of exposure to high ambient temperatures can damage the process of grain filling in rice, potentially inducing the endosperm to become chalky. Given the established involvement of long non-coding RNAs (lncRNAs) in regulating plant development and its stress response, the purpose here was to reveal the extent to which lncRNA activity contributes to the endosperm chalkiness syndrome. Among 578 lncRNAs identified in spikelets harvested ten days after fertilization from plants exposed to high temperature stress, 14 were found to be significantly up-regulated expressed than in control plants, while 45 were significantly down-regulated expressed. Of these 59 differentially expressed lncRNAs, 32 were predicted as interacting with five mRNAs involved in starch metabolism and catabolism, indicating an involvement of these lncRNAs in starch formation in the endosperm, and hence in causing the chalkiness syndrome.

Keywords Long non-coding RNA (lncRNA) · High temperature · Chalkiness · Starch metabolism · Rice

Introduction

Starch comprises > 80% of the dry matter present in the rice endosperm, so exerts a strong influence over the appearance of the mature grain. Grains exhibiting a chalky endosperm, a feature of both brown rice and milled rice, suffer from an overly loose packing of starch and protein (Sreenivasulu et al. 2015). The syndrome affects not only the appearance of the grain, but also its processing, cooking and nutritional values (Fitzgerald et al. 2009). Its genetic determination is complex, and is clearly strongly influenced by the growing environment, in particular by the ambient temperature to which the plant is exposed during the grain filling stage (Siebenmorgen et al. 2013).

Upon fertilization, the rice grain grows rapidly, reaching its maximum weight around 21 days after fertilization (DAF) (Wu et al. 2016). Major component of endosperm is starch, so the products of genes encoding the enzymes required for starch synthesis are critical to the expression of the chalkiness syndrome. Six classes of enzyme have been associated with starch synthesis, namely the ADP glucose pyrophosphorylases (AGPases), the starch synthases (SSs) and granule-bound starch synthases (GBSSs), the starch branching enzymes (BEs), the starch debranching enzymes (DBEs), various phosphorylases (PHOs) and the disproportionating enzymes (DPEs) (Ohdan et al. 2005); a number of the genes encoding these enzymes are at least partially repressed by high ambient temperature (Yamakawa et al. 2007). According to Nakata et al. (2017), excessive α-amylase activity occurring during the grain maturation phase underlies the chalkiness syndrome, while Yamakawa et al. (2007) have proposed that the high temperature-induced suppression of *GBSSI* and *BEIIb*, together with the promotion of α-amylase activity and the activation of heat shock proteins are responsible.

Many long (> 200 nt) non-coding RNAs (lncRNAs) are thought to have a regulatory function, acting either in *cis* or in *trans* (Chen 2016). Their presence can be highly either

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tissue- or developmental stage-specific (Zhang et al. 2014). Since their production can, in some cases, be induced by external stimuli, the suggestion is they act as molecular signals in concert with transcription factors (Kim et al. 2009; Liu et al. 2010; Swiezewski et al. 2009; Wang and Chang 2011). Alternatively, they may act as a molecular decoy, by competitively binding to a specific protein (Wang and Chang 2011). They can target mimic miRNAs and splicing factors, thereby repressing their function (Bari et al. 2006; Chiou et al. 2006; Franco-Zorrilla et al. 2007; Hamburger et al. 2002). In *Arabidopsis*, a non-protein coding gene *IPS1* is induced under phosphate deficiency and acts as an lncRNA target mimics for miR399, to regulate phosphate homeostasis (Franco-Zorrilla et al. 2007). LncRNAs involvement in the regulation of the plant stress response has been also described by Wang et al. (2017). Here, the goal was to use the RNA-seq platform to reveal the extent to which exposure to a temperature high enough to induce endosperm chalkiness altered the lncRNA content of the developing rice endosperm, and to attempt to identify the genes with which they interact.

Materials and methods

Plant materials and growing conditions

The experiments were all based on the *japonica* rice cultivar NingJing1 (cv. NJ1). Plants raised to the booting stage in the field at the China National Rice Research Institute (Hangzhou, China) were removed to a growth chamber delivering a day/night temperature regime of either 36/33 °C (HT treatment) or 28/25 °C (NT treatment). In both treatments, the photoperiod was set to 12 h and the relative humidity to 75%. Ten days after fertilization, spikelets were snap-frozen in liquid nitrogen and stored at − 80 °C. The plants could reach maturity to analyze the resulting grain with respect to both their starch and protein content.

Characterization of grain set by high temperature stressed and non-stressed plants

The starch and amylose contents of flour milled from whole grains harvested from both HT and NT plants were measured using the commercial enzymatic kits K-TSTA and K-AMYL (Megazyme International Ireland Ltd., Bray, Co Wicklow, Ireland), following the protocols described by Wei et al. (2017). The protein content was measured using the micro-Kjeldahl pretreatment method (Guebel et al. 1991), protein was converted to ammonium nitrogen by sulfuric acid digestion and checked the absorbance value of the blue production of reaction with natrium salicylicum and hypochlorous acid at wavelength of 660 nm. The same instrument Rapid Flow Auto Analyzer (AA3, SEAL, Germany) was employed to determine nitrogen contents. A nitrogen conversion factor of 5.95 was used to estimate the protein content from brown rice flour samples with three repeats.

RNA library construction and sequencing

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Genomic DNA was removed from extracted total RNA by DNase I (Takara, Kyoto, Japan) treatment at 37 °C for 30 min. The RNAs obtained were quantified and their integrity checked using a Bioanalyzer 2100 device and an RNA 6000 Nano LabChip kit (Agilent, Santa Clara, CA, USA) with RIN number > 7.0 (Zhou et al. 2016). A ~ 10 ug of total RNA was used to deplete the ribosomal RNA fraction, using a Ribo-Zero® rRNA Removal Kit (Plant Seed/Root; Illumina, San Diego, CA, USA). Following purification, the RNA fractions were fragmented by the addition of divalent cations under elevated temperature (Yue et al. 2016). The resulting RNA fragments were reverse-transcribed to create a cDNA library, following the protocol supplied with an Illumina mRNA-Seq sample preparation kit; the average insert size was 300 ± 50 bp. Paired-end sequencing was performed by LC-Bio (Hangzhou, China) using an illumina Hiseq 4000 device. Three biological replicates were analyzed in both treatments DNA. The data for this article have been submitted to the NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/, GEO accession number GSE113067).

The identification of lncRNAs

Transcripts matching known mRNAs and those shorter than 200 nt were immediately discarded. Based on the programs CPC (Coding Potential Calculator; Kong et al. 2007) and CNCI (Coding-Non-Coding Index; Sun et al. 2013), those having both a CPC score of < -1 and a CNCI score of < 0 were retained. The remaining transcripts were considered to be potential lncRNAs.

Recognition of lncRNA differential expressed

The StringTie program (Trapnell et al. 2010) was used to estimate the expression level of individual lncRNAs, based on the FPKM (fragments per kilobase of transcript per million mapped reads) statistic. The differentially expressed mRNAs and lncRNAs were selected with $\log_2$ (fold change) > 1 or < -1 and with statistical significance (P value < 0.05). To validate the differently expressed lncRNAs, quantitative real-time PCR (qRT-PCR) was performed. For lncRNAs confirmation, the *Ubiquitin* gene (LOC_Os03g13170) was a reference gene. Total RNA for

qRT-PCR was collected as described above. For lncRNA, cDNA synthesized by random primer from total RNA using the ReverTra Ac-α-® (TOYOBO, http://www.bio-toyobo.cn). Oligonucleotide primers used for qRT-PCR were designed at non-coding region of lncRNAs. Quantitative real-time PCR was performed on LightCycler® 480II (Roche, http://www.roche-applied-science.com) using following program: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 10 s, 72 °C for 10 s, and 95 °C for 15 s. Changes in lncRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Prediction of interacting miRNAs and the function of their target genes

The miRNA interacting with each differentially expressed lncRNA was predicted using the psRNATarget program (Dai and Zhao 2011). The neighbor's genes within 100 kb of their location of lncRNA were analyzed by gene ontology (GO), and GO terms were enrichment when $P \leq 0.05$ using Blast2GO (Conesa et al. 2005). The interaction network of starch metabolism-related lncRNAs and mRNAs was constructed by the software Cytoscape based on the Pearson correlation coefficients of lncRNAs and mRNAs. The potential functions of these genes in pathways were analyzed the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/).

Results

High temperature stress affects the quality of cv. NJ1 grain

The whole grains of cv. NJ1 set by plants subjected to HT manifested the typical chalky endosperm syndrome, but this was not the case for grains set by plants raised under NT (Fig. 1a). The stress also significantly reduced their thousand-grain weight (Fig. 1b). Quantification of the starch (Fig. 1c) and amylose (Fig. 1d) contents of the grain set by plants exposed to HT were lower than those of the control (NT plants), and their protein content was higher (Fig. 1e).

The population of lncRNAs in the developing grain

In all, some 57 million RNA reads were obtained from developing grains sampled from both the NT and HT plants (Table S1 in Supplementary material 1), from which 66,422 transcripts were assembled. Following the removal of short and known gene transcripts, the outcome of the filter based on the CPC program was a set of 1862 sequences (Fig. 2a), which was reduced to 578 following the application of the CNCI program (Fig. 2b). This final set of lncRNAs could be classified into five types, namely bi-directional lncRNAs (28 sequences, 4.8%), intronic lncRNAs (31, 5.4%), overlapping lncRNAs (17, 2.9%), intergenic lncRNAs (362, 62.6%) and antisense transcripts (140, 24.2%; Fig. 2c). The distribution of lncRNA types in the individual plants is depicted in Fig. S1 (Supplementary material 1).

Characteristics of the differentially expressed lncRNAs

The overall length, the number of exons and the open reading frame (ORF) length of members of the set of 578 lncRNAs were compared with those associated with the 40,129 protein-encoding transcripts generated from the RNA-seq analysis. The length range of ~78% of the lncRNAs laid in the range 200–1000 nt, leaving ~22% of transcripts longer than 1000 nt; in contrast, ~77% of the mRNAs were longer than 1000 nt (Fig. 3a). Around 80% of the lncRNAs comprised either one or two exons, while the exon number among the mRNAs was evenly distributed from one to nine,

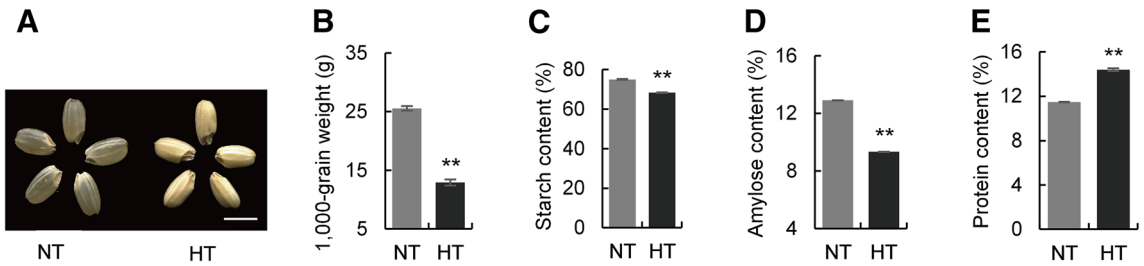

Fig. 1 Phenotype and physicochemical properties of Ningjing1 (NJ1) between normal temperature (NT) and high temperature (HT) condition. **a** Phenotype comparison of the NJ1 brown rice between NT and HT, bar = 5 mm. **b** Thousand-grain weight analyses of NJ1 brown rice between NT and HT. **c** Starch content comparison of NJ1 brown rice between NT and HT. **d** Amylose content comparison of NJ1 brown rice between NT and HT. **e** Protein content of NJ1 brown rice between NT and HT. Data as determined by a student's *t*-test in three replicates (**$P < 0.01$)

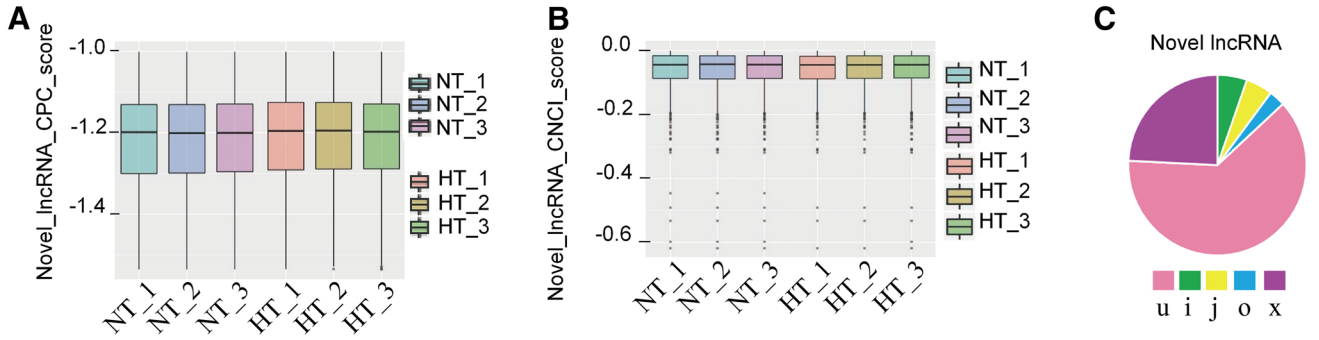

Fig. 2 Identify and distribution statistics of 578 rice lncRNAs. **a** Box plots of lncRNA transcripts prediction by CPC (Coding Potential Calculator) between NT and HT in development seeds (n = 3), CPC < − 1.0 were retained. **b** Box plots of lncRNA prediction by CNCI (Coding-Non-Coding Index) after CPC filtered between NT and HT in development seeds (n = 3), CNCI < 0 were consider to be novel lncRNAs. **c** Distribution statistics of predicted lncRNA transcripts, *u* represent intergenic transcript, namely intergenic lncRNA; *i* represent a transfrag falling entirely within a reference transcript, namely intronic lncRNA; *j* represent potentially novel isoform (fragment): at last on splice junction is shared with a reference transcript, namely bi-directional lncRNA; *o* represent generic exonic overlap with a reference transcript, namely overlapping lncRNA; *x* represent exonic overlap with reference on the opposite strand, namely antisense transcript lncRNA

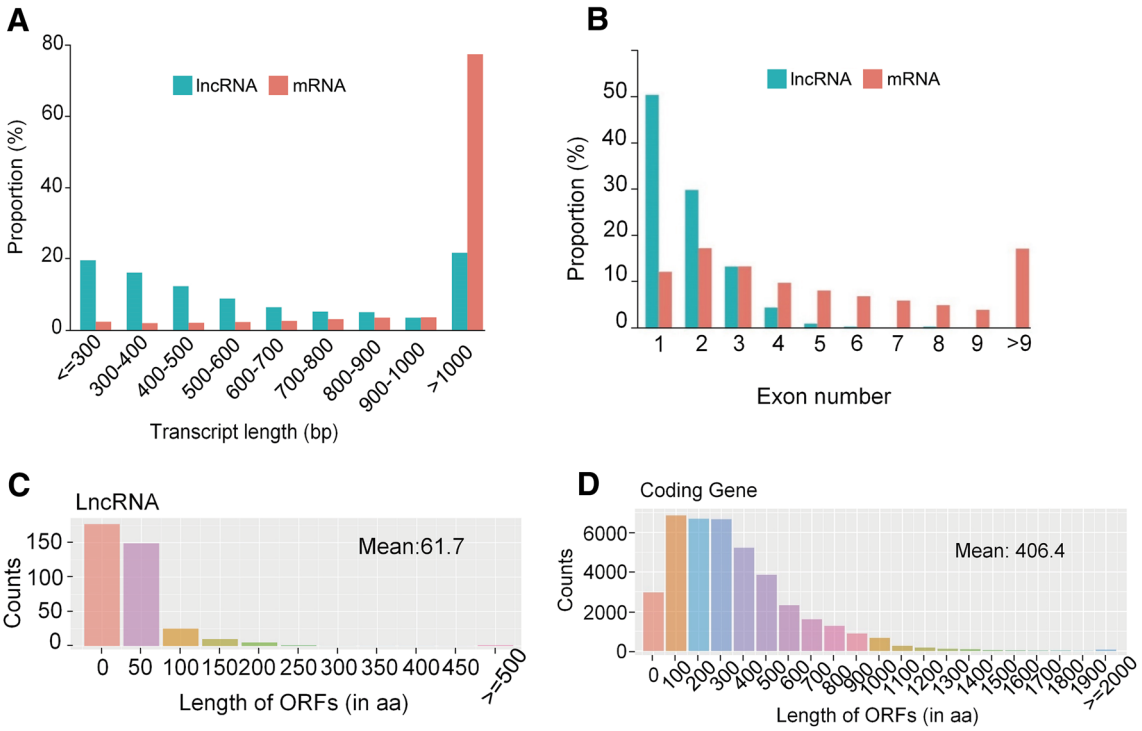

Fig. 3 Comparison of structural features of lncRNAs and mRNAs. **a** Comparison of transcript lengths of lncRNAs and mRNAs, blue represent lncRNA, red represent mRNA. **b** Comparison of exon numbers of lncRNAs and mRNAs, blue represent lncRNA, red represent mRNA. **c** ORF (open reading frame) length of lncRNAs. **d** ORF length of mRNAs

with ~ 17% of them harboring ten or more exons (Fig. 3b). Nearly half (48%) of the lncRNAs lacked an ORF, ~ 41% included a short (< 50 residues) ORF, and their mean ORF length was 61 residues (Fig. 3c); in contrast, 73.5% of the mRNAs included a 100–600 residue ORF (mean length 406 residues; Fig. 3d).

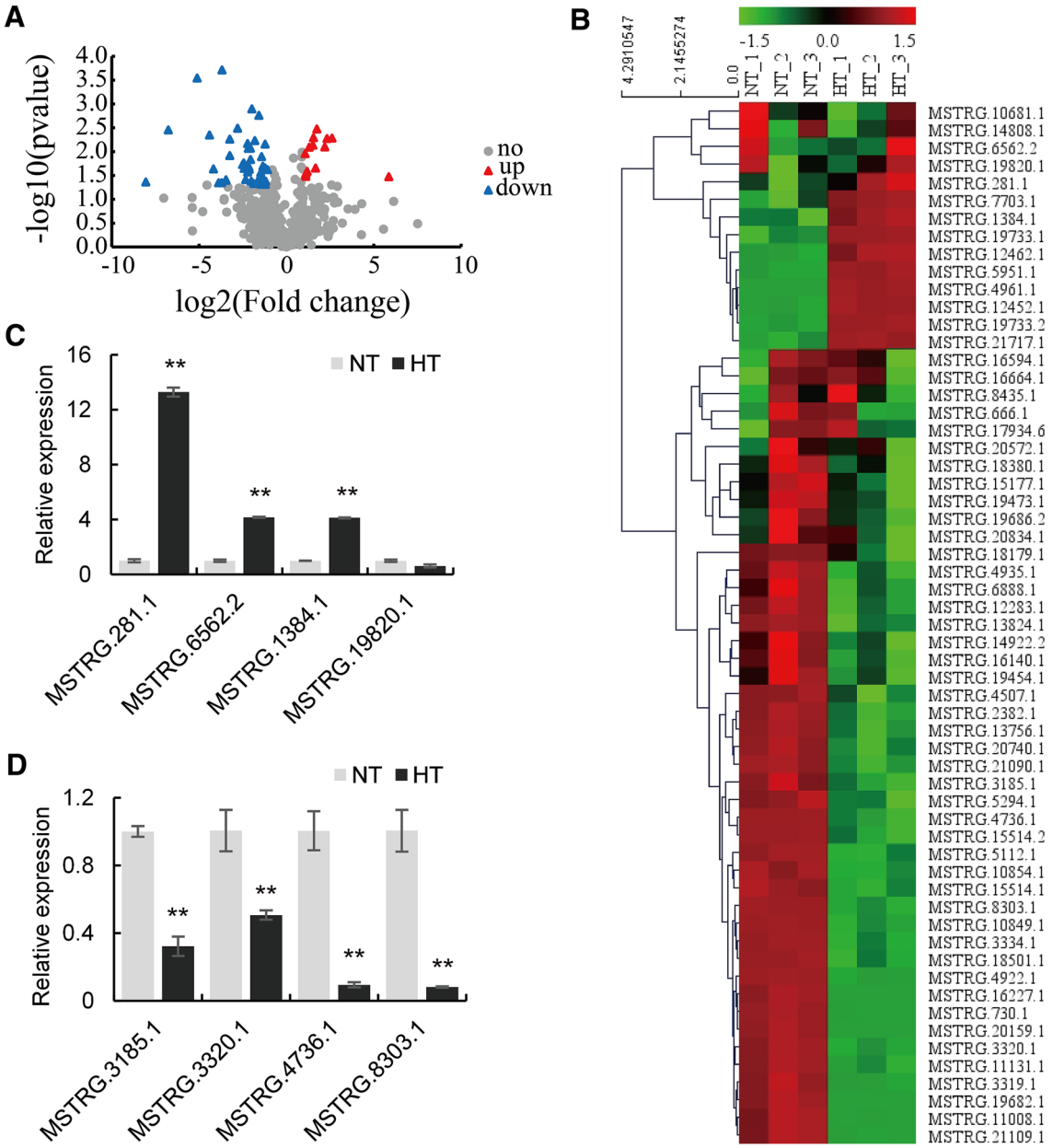

Fig. 4 LncRNAs differential expression analysis. **a** Differentially expressed volcano plots of lncRNA at high temperature and normal temperature conditions, red triangles represent significantly up-regulated expressed lncRNAs, blue triangles represent significantly down-regulated expressed lncRNA, gray circles represent insignificantly expressed lncRNAs. **b** A cluster heat map illustrating the expressed of the lncRNAs in the three replicate plants of each treatment (HT1–HT3 and NT1–NT3). **c**, **d** Validation of differential expressed lncRNAs using quantitative real-time PCR (qRT-PCR): four lncRNA randomly selected as **c** up-regulated expressed and **d** down-regulated expressed validation. Data as determined by a student's *t*-test in three replicates between NT and HT, (**$P < 0.01$)

Differential expressed of lncRNAs between NT and HT plants

Based on the FPKM statistic, of the 578 lncRNAs, 14 were concluded to be up-regulated expressed in the developing grain set by plants exposed to HT than in the grain of NT plants, and 45 down-regulated expressed (Fig. 4a). A cluster heat map of these 59 lncRNAs was displayed as Fig. 4b, 14 significantly up-regulated expressed lncRNAs and 45 significantly down-regulated expressed lncRNAs can be accurately clustered into two groups. For validation purposes based on qRT-PCR, four lncRNAs were randomly selected from each of the two groups. The result indicated that expression trends of seven lncRNAs were consistent with RNA-Seq (Fig. 4c, d).

The biological significance of the differentially expressed lncRNAs

For further understanding the function of lncRNAs, lncRNAs target mRNAs have been predicted based on positional prediction. And, the target differential expressed mRNAs of lncRNAs were performed on GO analysis and KEGG pathway analysis. The outcome of the GO analysis was that the genes affected were active in every cellular component, and were involved mainly in transcription, protein phosphorylation and oxidation/reduction. Their molecular functions spanned protein binding, ATP binding and DNA binding (Fig. 5a). In the 59 differentially expressed lncRNAs under high temperature stress, 24 significantly up-regulated and 8 significantly down-regulated expression lncRNAs interacted with genes encoding starch metabolism-related proteins, namely *OsAGPS1* (LOC_Os09g12660, *AGP small subunit 1*), *OsAmy3D* (LOC_Os08g36910, *Alpha-amylase isozyme 3D*), *SSIIa* (LOC_Os06g12450, *Starch synthases IIa*), *OsAGPS2a* (LOC_Os08g25734, *AGP small subunit 2a*) and *α-Amy3E* (LOC_Os08g36900, *Alpha-amylase isozyme 3E*) (Fig. 5b). The expression levels of these target genes were analyzed by qRT-PCR, and the result showed that *OsAGPS1, OsAGPS2a* and *SSIIa* significantly down-regulated expression, whereas *α-Amy3E* significantly up-regulated expression under high temperature stress. *OsAmy3D* was induced expression by high temperature, but no expressed under normal temperature condition (Fig. 5c). The lncRNAs target genes *OsAGPS1* and *OsAGPS2a* are mainly involved in the catalytic synthesis of ADPglucose from glucose 1-phosphate (G1P), *SSIIa* play a distinct role in the synthesis of starch, *α-Amy3E* and *OsAmy3D* were up-regulated by high temperature induction, resulting in degradation of starch to maltodextrin and maltose (Fig. 5d). In addition, the KEGG pathway analysis was showed that the lncRNAs interacted with genes involved in spliceosome activity, ribosomal activity, in protein processing in the endoplasmic reticulum, in plant hormone signal transduction, in endocytosis and in carbon metabolism (Fig. S2 in Supplementary material 1).

In recent years, lncRNAs were involved in the regulation of plant development as competitive endogenous RNA (ceRNA) or endogenous mimics (eTMs) (Franco-Zorrilla et al. 2007; Wang et al. 2015b; Wu et al. 2013). An analysis of the full set of 578 lncRNAs based on the psRNATarget program predicted 9033 miRNA–lncRNA interactions (Attached data1 in Supplementary material 2); three of the lncRNAs shared full sequence complementarity with a corresponding miRNA (Fig. 5e). Among them, the lncRNA MSTRG.8267.1 likely interacted with miR827 which is a miRNA expressed in the rice grain (Xue et al. 2009) and targets the two Pi deficiency inducible mRNAs *SPX-MFS1* and *SPX-MFS2* (Lin et al. 2010; Wang et al. 2009).

Discussion

Too much chalkiness is a restricting factor for the popularity of one rice variety, and high temperature can induce chalky formation. The rice endosperm chalkiness syndrome is induced by exposure after fertilization to ambient temperatures above 28 °C (Miyahara et al. 2017). The syndrome is accompanied by a loss in grain weight and a reduction in the amylose content of the starch (Tashiro 1991a, b). The rice quality also declines with increase in temperature (Thitisaksakul et al. 2012). In recent years, high temperature weather is frequent in rice areas in southern China, the reduction of rice grain weight and shortening of the grain filling stage by high temperature has a serious impact on rice quality (Tashiro 1989). Here, the HT stress was effective in inducing the formation chalky grains of cv. NJ1; the grains set under these conditions exhibited a reduction in their content of both starch and amylose, while their protein content was increased, as similarly observed by Tashiro (1991b). These changes to the composition of the grain are detrimental to their market value.

Recent evidence has implicated the involvement of certain lncRNAs in the plant stress response (Wang et al. 2017), as well as in reproductive growth in rice (Zhang et al. 2014). As yet, their contribution, if any, to endosperm development in plants exposed to high temperature stress has not been reported. Zhang et al. (2014) revealed that the lncRNA content of a variety of vegetative and reproductive structures in rice, identifying over 2000 sequences, many of which were classified as either organ- or developmental stage-specific. A subsequent study has described over 11,000 lncRNAs, many of which were suggested to participate in the regulation of development and the stress response (Wang et al. 2015a). In the model plant *Arabidopsis thaliana*, the expressed of an lncRNA localized to the nucleus has been reported to respond to both drought and salinity stress (Qin et al. 2017), while in rice have demonstrated the differential expressed of over 100 lncRNAs in response to drought stress (Shin et al. 2016); similarly, 31 lncRNAs were induced to increase in expressing upon the plants' exposure to drought, and 67 which became less expressed (Chung et al. 2016). According to Ding et al. (2012), in a photoperiod-sensitive male sterile rice plant, pollen development under long photoperiod conditions requires the presence of a specific lncRNA. The current studies indicating that only a few lncRNAs are functional and most are unknown (Ulitsky and Bartel 2013). In general, lncRNAs are involved in the regulation of gene expression and exert their biological effects individually, such as *IPS1, HID1* (Franco-Zorrilla et al. 2007; Wang et al. 2014). In this study, 32 lncRNAs were found involved in starch synthesis and metabolism (Fig. 5b), in which, 24 were significantly down-regulated and 8 were significantly

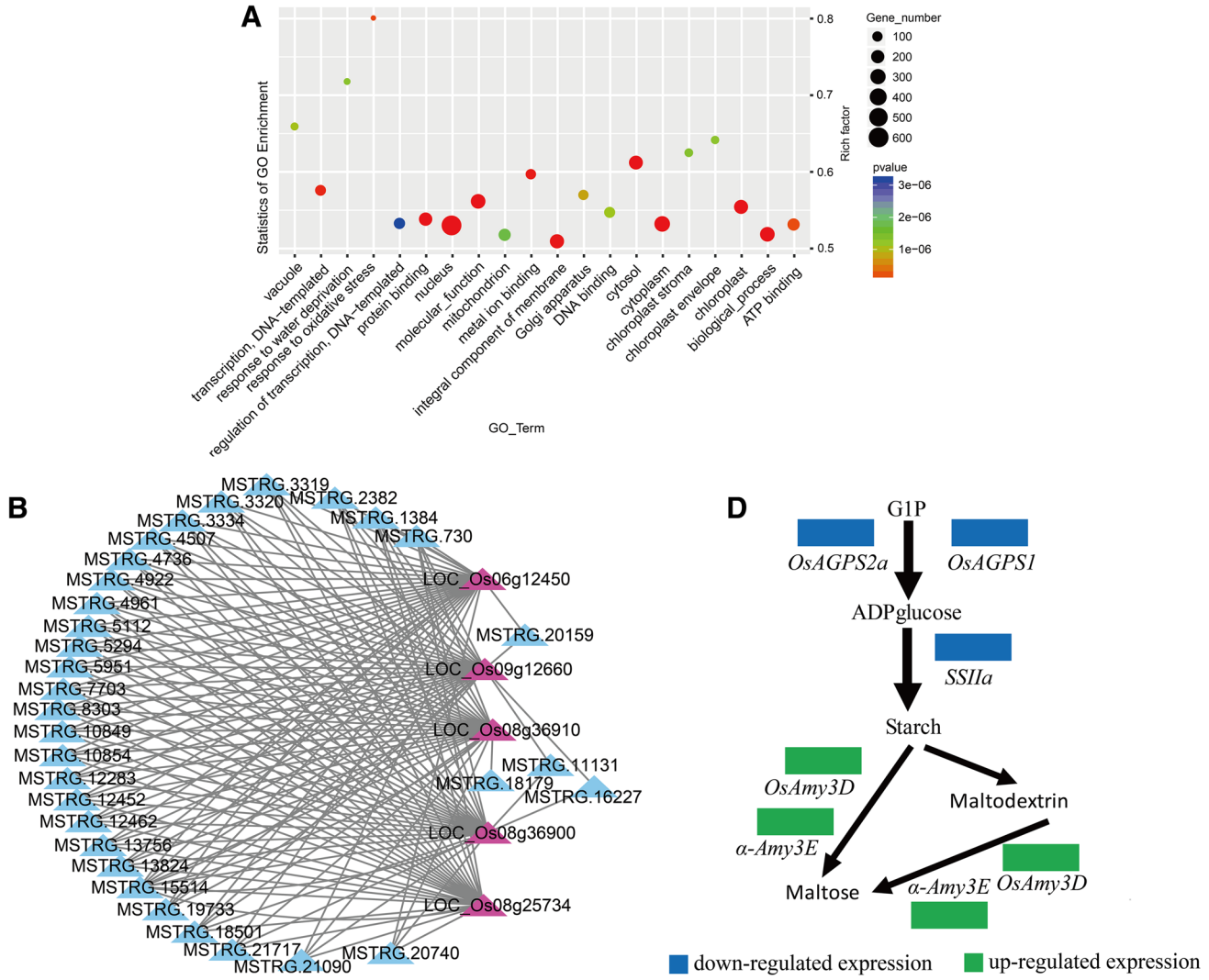

Fig. 5 LncRNAs potential functional analysis. **a** The GO enrichment of lncRNAs targeted mRNAs, circle size represents the number of differential genes, colors represents significance of difference. **b** The network of lncRNAs and these target mRNAs related to starch metabolism, blue triangle represent starch synthesis-associated lncRNAs, red triangle represent those lncRNAs targeted mRNAs. **c** The relative expression level of lncRNAs target genes, *OsAGPS1* (LOC_Os09g12660, *AGP small subunit 1*), *OsAmy3D* (LOC_Os08g36910, *Alpha-amylase isozyme 3D*), *SSIIa* (LOC_Os06g12450, *Starch synthases IIa*), *OsAGPS2a* (LOC_Os08g25734, *AGP small subunit 2a*) and *α-Amy3E* (LOC_Os08g36900, *Alpha-amylase isozyme 3E*). Data as determined by a student's *t*-test in three replicates between NT and HT, (**$P < 0.01$), n.d. represent not dectected. **d** Regulatory pathway of lncRNAs target genes, the blue rectangle represents down-regulated expression, the green rectangle represents up-regulated expression. **e** Predicted miRNAs crosstalking with lncRNAs

up-regulated under high temperature stress. The expression level of *OsAGPS1, OsAGPS2a* and *SSIIa* involved in starch synthesis were down-regulated, and the expression level of *OsAmy3D* and *α-Amy3E* involved in starch degradation were up-regulated (Fig. 5c, d). It has been reported that lncRNAs could regulate gene expression in *cis* or *trans* (Fatica and Bozzoni 2014). *Cis*-acting lncRNAs regulate expression of neighboring genes (Chen 2016). We speculate that the up- or down-expression of the five genes involved in starch synthesis and metabolism under high temperature stress may be caused by the *cis* or *trans* regulation by these differentially expressed lncRNAs. Then the disorder expression of genes involved in starch synthesis and degradation lead to the chalkiness syndrome and lower starch contents endosperm under high temperature stress (Fig. 1).

In contrast to our understanding of the mRNAs and miRNAs, the little is known about functional and regulatory mechanisms of lncRNAs. Recently, lncRNAs communicate with and regulate corresponding miRNA and target genes have been reported. Two reproduction-related lncRNAs can target miRNA160 and miRNA164, respectively. And miRNA160 is highly expressed in seeds after pollination, while miRNA164 is specially expressed in pistil and anther, and then regulate floral and/or seed development (Zhang et al. 2014). Interestingly, 9033 lncRNA–miRNA interaction pairs were predicted in this study (Attached data1 in Supplementary material 2). Among them, osa-miR2872, osa-miR5505 and osa-miR827 can perfectly match with lncRNAs MSTRG.10809.1, MSTRG.13794.1 and MSTRG.8267.1, respectively (Fig. 5e). These lncRNAs may regulate the seed development and respond to high temperature stress by the interaction with corresponding miRNA and subsequent target genes.

Here, a set of 578 lncRNAs was identified in the developing rice grain, 14 of which became significantly up-regulated expressed and 45 became significantly down-regulated expressed when the plants after fertilization were exposed to high temperature stress. Out of these 59 transcripts, 32 interacted with mRNAs encoding key enzymes involved in starch metabolism or catabolism. Our research provides new ideas for the regulation of endosperm development under high temperature stress, and related transgenic trials will be continued in the future.

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Author contributions RL, RC and GJ performed the experiments. RL and XW analyzed the data. PH and XW designed the project. RL and XW draft the manuscript. YL, MZ, and ST performed a critical revision of the article. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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