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ORIGINAL PAPER

Major QTLs reduce the deleterious effects of high temperature on rice amylose content by increasing splicing efficiency of *Wx* pre-mRNA

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Abstract

Key message **We discovered four QTLs that maintain proper rice amylose content at high temperature by increasing the splicing efficiency of *Wx* gene.**

Abstract Amylose content mainly controlled by *Wx* gene is a key physicochemical property for eating and cooking quality in rice. During the grain filling stage, high temperature can harm rice grain quality by significantly reducing the amylose content in many rice varieties. Here, we provide genetic evidences between *Wx* gene expression and rice amylose content at high temperature, and identified several quantitative trait loci (QTLs) in this pathway. We performed a genome-wide survey on a set of chromosome segment substitution lines (CSSLs) which carried chromosomal segments from the heat resistant *indica* 9311 in the heat-sensitive *japonica* Nipponbare background. Four QTLs, *qHAC4*, *qHAC8a*, *qHAC8b* and *qHAC10*, which can reduce the deleterious effects of amylose content at high temperature, were identified and mapped to chromosome 4, 8, 8 and 10, respectively. The major QTL *qHAC8a*, with the highest LOD score of 6.196, was physically mapped to a small chromosome segment (~300 kb). The CSSLs carrying the *qHAC8a*, *qHAC8b* and/or *qHAC4* from 9311 have the high pre-mRNA splicing efficiency of *Wx* gene and likely lead to stable amylose content at high temperature. Thus, increasing pre-mRNA processing efficiency of *Wx* gene could be an important regulation mechanism for maintaining stable amylose content in rice seeds at high temperature. In addition, our results provide a theoretical basis for breeding heat-stable grain in rice.

Abbreviations

CSSL	Chromosome segment substitution line
DAF	Days after flowering
DBE	Starch debranching enzyme
D-value	Difference value
DD-value	Difference in the *D*-values
GBSS	Granule-bound starch synthase
HT	High temperature
HTC	High temperature condition
QTL	Quantitative trait locus
qHAC	QTLs for amylose content responsive to high temperatures
SBE	Starch branching enzyme
SSS	Soluble starch synthase
RT	Room temperature

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RTC	Room temperature condition
LT	Low temperature
LTC	Low temperature condition

Introduction

The Intergovernmental Panel on Climate Change (IPCC) has predicted that global surface temperatures will continue to increase in the twenty-first century (IPCC 2007). Rice is one of the world's major cereal crops, and its grain quality, which covers many physicochemical properties, is often adversely affected by high temperature (HT) at grain filling stage (Mitsui et al. 2013). Starch, the dominant form of carbohydrate present in the rice grain, is a key component to rice quality (Chen et al. 2012). There are two different types of starch in rice endosperm, amylose and amylopectin, and it is the amylose content that mainly affects the eating and cooking quality of rice (Juliano 1985). The effects of HT have been shown to result in a significant reduction in amylose content in many rice varieties, and thus negatively impact on rice quality (Jiang et al. 2003; Larkin and Park 1999; Yamakawa et al. 2007; Zhong et al. 2005).

Several enzymes, such as granule-bound starch synthase (GBSS), soluble starch synthase (SSS), starch branching enzyme (SBE), and starch debranching enzyme (DBE), are known to be involved in the starch biosynthesis (James et al. 2003; Tian et al. 2009) and many of them were shown to be regulated by temperature. Under HT, most of the isoforms of the *SBE* genes (*SBEI*, *SBEIIb* and *SBEIII*) were down-regulated, with only the expression of *SBEIV* being increased (Jiang et al. 2003; Yamakawa et al. 2007). In contrast, HT can induce the expression of most of the *SSS* isoforms (*SSSIIb*, *SSSIIc*, *SSSIIIb*, and *SSSIVa*) and some starch-consuming genes, α-amylase (*Amy1A*, *Amy3D*, and *Amy3E*), in developing seeds (Hakata et al. 2012; Wei et al. 2009).

GBSSI encoded by rice *Wx* gene is very important for amylose biosynthesis (Sano 1984; Wang et al. 1995), and *wx* mutant endosperm contains almost exclusively amylopectin (Hori et al. 2007). Genetic studies have shown that *Wx* is not only a major gene controlling amylose content, but also affecting gel consistency and gelatinization temperature of rice starch (Su et al. 2011). It was documented that *Wx* gene has several different alleles in different subspecies and cultivars of rice. Two major *Wx* alleles were identified in non-waxy rice varieties (Sano 1984). Wx^a is widely distributed in *indica* rice with high amylose content while Wx^b is mainly found in *japonica* rice with low or intermediate amylose content (Wang et al. 1995). The low level of both amylose content and mature transcript of Wx^b can be explained by a single nucleotide substitution (G-to-T) at the splice donor site of the first intron in Wx^b (Cai et al. 1998; Tian et al. 2009). The G to T variation could potentially alter the splicing site and decrease the splicing efficiency of the first intron of *Wx* (Wang et al. 1995; Cai et al. 1998; Larkin and Park 1999).

The expression of *Wx* gene was shown to be sensitive to temperature. Under cool temperature condition (18 °C), both the levels of the *Wx* gene transcript and Wx (named as GBSSI) protein have been found to increase significantly (Hirano and Sano 1998), while under the elevated temperature, the expression of *Wx* gene was down-regulated (Hirano and Sano 1998; Yamakawa et al. 2007). Proteomic analysis also showed that GBSSI protein level was reduced at HT condition (Lin et al. 2005). Furthermore, the G-to-T polymorphism at the splicing site in the leader intron was demonstrated to be associated with different sensitivities to temperature during filling stage (Larkin and Park 1999). Plants with Wx^b would accumulate more mature *Wx* mRNA (2.3 kb) and less premature *Wx* mRNA (3.4 kb) at cool temperature (18 °C) than at normal temperature (25 °C) or at HT (32 °C), whereas plants with Wx^a accumulate the same amount of mature *Wx* mRNA at different temperatures (Larkin and Park 1999). In addition, temperature was also found to affect the selection of first intron 5′ splicing site in these temperature-sensitive cultivars (Larkin and Park 1999). These results suggest that the down-regulation of *Wx* could be the major cause of the decrease in amylose content in the rice seed under conditions of HT. However, so far, none of the components (or loci) involved in the regulation of *Wx* gene expression and rice amylose content under HT was clarified.

The *Indica* variety 9311 and *Japonica* variety Nipponbare both carry the same *Wx* allele (Wx^b), and both have medium amylose content when grown at normal temperatures (Tian et al. 2009). However, it was reported that the apparent amylose content of Nipponbare but not 9311 was significantly decreased by raising the temperature (Yamakawa et al. 2007; Zhang et al. 2006). It appears that these two varieties may regulate grain amylose content in response to HT differently and there may be some factors in 9311 that inhibit the adverse effects of HT in reducing amylose content.

To isolate the major loci for regulating amylose content change in rice seeds under HT, we conducted a genome-wide survey. A set of chromosome segment substitution lines (CSSLs) that carry segments from 9311 in the Nipponbare genetic background (Zhang et al. 2011b) was used and their amylose content at HT and room temperature (RT) were phenotyped. To the end, we isolated four major loci from 9311, named *qHAC4*, *qHAC8a*, *qHAC8b* and *qHAC10*, with the positive effects on amylose content under HT. *qHAC4*, *qHAC8a* and *qHAC8b* from 9311 can increase the splicing efficiency of *Wx* pre-mRNA under HT and therefore ameliorate the deleterious effects of HT on rice amylose content. These loci will be the good

candidates of key components involved in the regulation of amylose content change at HT and can be used for breeding heat-stable rice varieties in the future.

Materials and methods

Plant materials

We used a set of 34 CSSLs and the two parental cultivars, 9311 (donor) and Nipponbare (recipient) in this study. The detailed genotypes of all CSSLs were determined by genome re-sequencing (Figs. S2, S3, Zhang et al. 2011b). Rice seeds were first sown in June 2012 in Haining of Zhejiang Province. Fifty days after sowing, eight plants of each line were transplanted into two pots. At the heading stage, spikelets were marked on the day of flowering. Three days after flowering (DAF), the plants were transferred to either a 35 °C, 12 h light/28 °C 12 h dark, or a 28 °C, 12 h light/22 °C 12 h dark chamber for HT or RT treatment until seed mature, respectively. Six or nine DAF, developing seeds were harvested, then immediately frozen in liquid nitrogen, and stored at −80 °C until use. Cooling treatment of Nipponbare was performed in an artificial climate chest at three DAF with 18 °C, 12 h light/12 h dark, and developing seeds were harvested at twelve DAF for GBSSI enzyme activity assay. Approximately 40 days after heading, ripen seeds were harvested for measurement of amylose content.

Starch isolation and amylose content determination

Since the storage protein content in rice seeds can also be affected by HT during the milky stage (Ma et al. 2009), the starch was purified from polished rice using the alkaline protease method (Lumdubwong and Seib 2000; Zhu et al. 2010) to eliminate storage protein contamination. Rice amylose content was measured using an iodine colorimetric method (Juliano 1971) with slight modification. The OD_{720} was measured according to the Standards of the Agricultural Department, People's Republic of China (2008). Four standard starch samples with various amylose contents, obtained from the China National Rice Research Institute, were used to define the standard curve.

QTL mapping for rice amylose content in response to HT

The genotypes of the CSSLs were reconstructed based on the re-sequencing results (Zhang et al. 2011a, b). The rice genome was separated into chromosomal segments with 164 tags, and each segment has two tags at the borders (Fig. 1a). Amylose content was obtained from the CSSLs and the parental line Nipponbare grown under either high temperature condition (HTC) or room temperature condition (RTC). For each condition, the amylose content value of Nipponbare was set to zero, and the amylose content of the CSSLs was represented by the difference value (*D*-value) between the CSSLs and Nipponbare. The *D*-values of the CSSLs at RTC were set as the control treatment, while the *D*-values of the CSSLs at HTC were designated as the experimental heat treatment. The difference in the *D*-values (DD-value) between the heat treatment and the control treatment of each line was defined as the phenotype.

Quantitative trait locus mapping was performed using the R/qtl package (Broman et al. 2003). LOD scores were calculated with a single-QTL model using the function "scanone" with the Haley–Knott regression method (Haleyand and Knott 1992). The LOD score significance threshold was established using 1,000 permutations. To raise reliability of the results and get the mainly contributed QTLs, the *p* value of 0.01 was selected in our study and the corresponding threshold of LOD score is 3.79. Using related CSSLs, QTLs were further physically mapped on the rice genome.

Gene expression and splicing efficiency analysis

Total RNAs were extracted from rice endosperm using TRIzol reagent (Invitrogen) (Zhang et al. 2005), and contaminating gDNA was digested with TURBO DNA-free™ (Invitrogen). The isolated RNA was reverse transcribed into cDNA with the GoScript™ Reverse Transcription System (Promega). The rice *actin* gene (She et al. 2010) was used as an internal standard to normalize the expression of tested genes. The *Wx* gene-specific primers, 484, 466 (Larkin and Park 1999), EMS10 and EMS21 (Table S1) were used for the semi-quantitative PCR analysis (Fig. 3b). qRT-PCR assays were performed with iQ™ SYBR® Green Supermix (Bio-Rad) on a C1000 real-time thermal cycler (Bio-Rad) with 18S rRNA (Jain et al. 2006) as the reference gene. Primers 484, 466, and qWx-2F&R were used to identify the *Wx* transcript without the first intron, while EMS10, EMS21, and Wx-10F&R were used to identify the *Wx* transcripts with and without the first intron in the semi-quantitative PCR assays. Primer pairs qWx-2F&R and qWx-10F&R were used to specifically identify mature and premature mRNAs of the *Wx* gene in the qRT-PCR assays (Fig. 3b; Table S1). The splicing efficiency for *Wx* is defined as the ratio of the amplification products of primer pair qWx-10F&R to those of primer pair qWx-2F&R.

GBSSI enzymatic activity assays and protein quality analysis

Enzyme activity analysis was performed as described previously (Liu et al. 2013). The plants were transferred

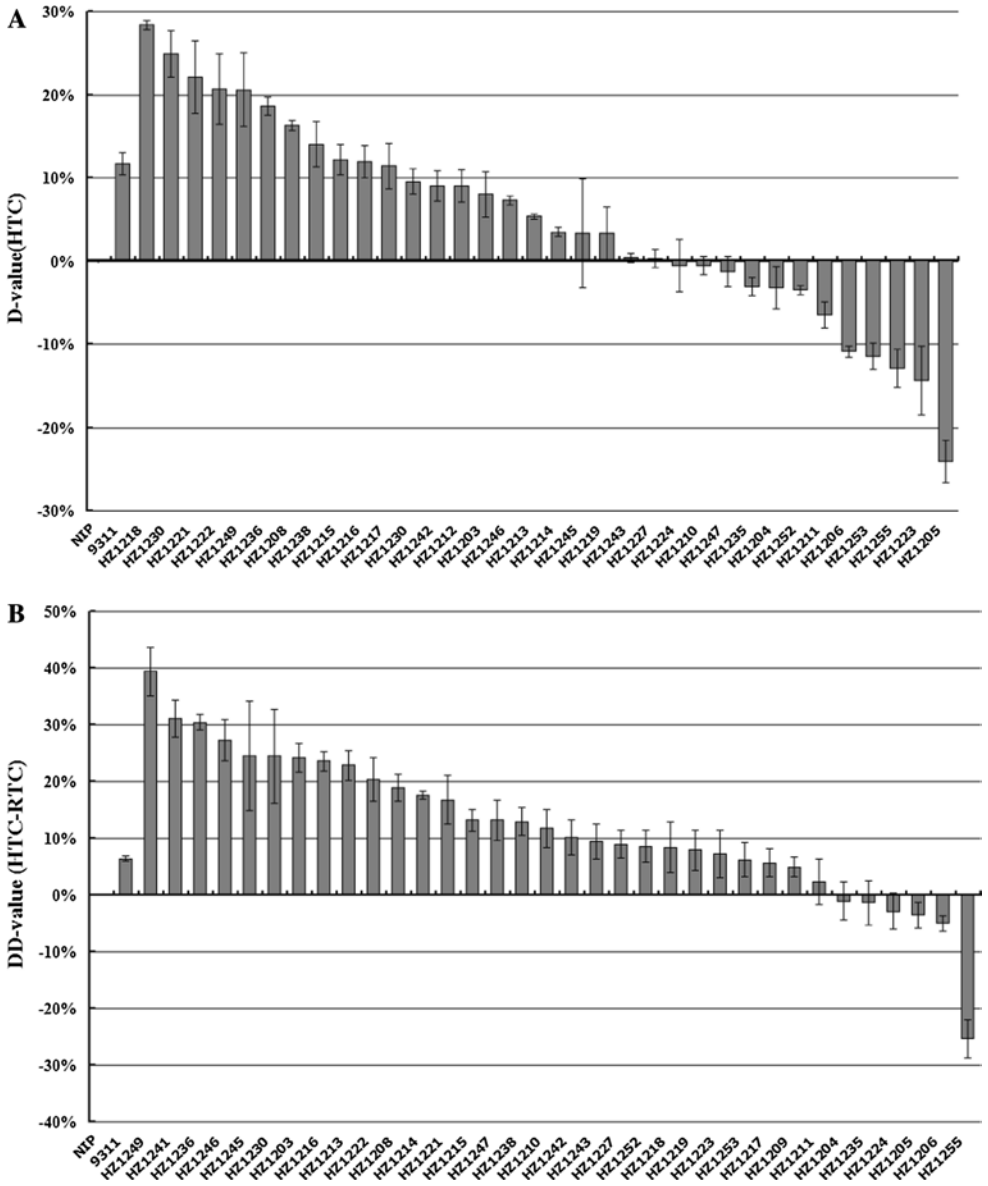

Fig. 1 *D*-values and DD-values for amylose content of CSSLs. **a** Differences in the amylose content (*D*-values) between the CSSLs and Nipponbare (NIP) under HTC. **b** DD-values for amylose content in the CSSLs between HTC and RTC

to different growth chambers (35 °C/HT, 28 °C/RT and 18 °C/LT) at 3 DAF, respectively. The endosperm from HT and RT was collected at 9 DAF, while the endosperm from LT was collected at 12 DAF, based on their different grain filling rates at different temperatures (Larkin and Park 1999). The GBSSI protein was extracted from rice endosperm (Nakamura 1989). The activity of GBSSI was measured with a Lumi-nometer (Promega GloMax-20/20) by quantifying the amount of ATP that was converted from ADP (Sigma-Aldrich, St. Louis, USA), which was produced by GBSSI. ELISA was performed to quantify the concentration of GBSSI protein in the gross enzyme extract using anti-GBSSI antibody, and the OD_{450} was measured. The seed enzymeactivity, seed protein amount and specific activity of GBSSI in Nipponbare at HT were set to 1.

Total enzyme activity of GBSSI = enzyme activity (total quantity of ATP)/seed weight.

Protein amount of GBSSI = total amount of GBSSI protein/seed weight.

Specific activity of GBSSI = total enzyme activity of GBSSI/total GBSSI protein.

Results

Amylose content in CSSL seeds grown at different temperatures

The CSSLs and their two parental varities, Nipponbare (recipient) and 9311 (donor) were grown under HTC and RTC and their seeds were used for amylose content

Table 1 Descriptive statistics for grain amylose content in Nipponbare and the CSSLs under HTC and RTC

Environment site	Mean	STD	Skewness	Kurtosis	Min	Max	*W* test
HTC	13.69	1.61	−0.20	−0.57	9.92	16.76	0.98
RTC	16.21	1.74	0.76	0.23	13.81	21.12	0.94

measurement. The mean values (±SD) of amylose content from the CSSLs grown at HTC (13.69 ± 1.61 %) is much lower than these grown at RTC (16.21 ± 1.74 %), indicating that rice amylose content was negatively affected by HT (Table 1; Fig. S1). The amylose content value of Nipponbare was set to zero, and the difference of amylose content between the CSSLs and Nipponbare was represented by *D*-value. Under HTC, the *D*-value of most CSSLs and the donor parent variety 9311 (*D*-value = 11.7 %) was positive (Fig. 1a), indicating that Nipponbare is more sensitive to high temperatures than 9311, and that chromosome segments from 9311 could inhibit the decrease of amylose content under HT. DD-value for each CSSL was obtained by subtracting the *D*-values of RTC from HTC. Almost all of CSSLs had a positive DD-value (>0) except for six lines: HZ1204, HZ1205, HZ1206, HZ1224, HZ1235 and HZ1255 (Fig. 1b). From the re-sequencing results of CSSLs (Fig S3), it implied that multiple loci distributed across the 9311 genome contribute to the HT tolerance with respect to seed amylose content and the trait is quantitative.

Genome-wide survey for QTLs regulating rice amylose content under HT conditions

To define the QTL for regulation of amylose content in response to HT, a genome-wide survey was carried out. The difference of amylose content (DD-values) between HTC and RTC of each line was used as the phenotype for QTL mapping. A major locus, *qHAC8a*, with the highest LOD score of 6.196 (Table S2), was detected on chromosome 8 (Fig. 2a). Based on the re-sequencing results of HZ1216, HZ1249, and HZ1246, we were able to map *qHAC8a* to a small chromosomal region between positions 0.7 Mb and 1 Mb on chromosome 8 (Fig. 2b). The other loci, *qHAC4*, *qHAC8b* and *qHAC10*, with LOD scores of 4.933, 5.591 and 5.2 (Table S2), respectively, were mapped to chromosome 4, 8 and 10 (Fig. 2b). HZ1249 contains all the four loci based on the re-sequencing results and shows the highest DD-value in our experiment. In addition to the *qHAC8a*, the CSSL HZ1216 also carries the *qHAC4* and HZ1246 also carries *qHAC8b*, respectively. However, the CSSL HZ1203 only takes the QTL *qHAC10*. The high DD-values for CSSLs HZ1203, HZ1216, HZ1246, and HZ1249 (Fig. 2b), indicated *qHAC4*, *qHAC8a*, *qHAC8b* and *qHAC10* from 9311 are major loci having a positive effect on seed amylose content at HT.

CSSLs carrying qHAC8a, qHAC8b and/or qHAC4 have a higher efficiency of Wx pre-mRNA processing than Nipponbare at HT

Wx gene is responsible for amylose biosynthesis in rice endosperm and its expression can be suppressed by HT in Nipponbare at grain filling stage (Yamakawa et al. 2007). The CSSLs HZ1203, HZ1216, HZ1246 and HZ1249 and the donor line 9311 inhibit the decrease of amylose content at HT may be due to the stable level of *Wx* transcript at different temperatures.

To verify this possibility, developing seeds were harvested at 6 and 9 DAF to analyze the expression of the *Wx* from CSSLs and their parent lines under different temperatures. Gene-specific primers EMS10 and EMS21 were used for RT-PCR analysis (Table S1, Fig. 3b). All the CSSLs and 9311 produce lower level of *Wx* transcript at HTC than at RTC (Fig. 3c, d). These results indicated that the transcription of *Wx* gene is inhibited under HT in both parent lines.

To test whether the splicing efficiency of the first intron of *Wx* gene is different between 9311 and Nipponbare under HTC, gene-specific primers (484 and 466) flanking the first intron splice sites were utilized for RT-PCR as (Larkin and Park 1999) mentioned (Fig. 3b). Two major PCR products (~210 and ~120 bp) from mature *Wx* mRNA and one PCR product (~1.3 kb) from premature *Wx* mRNA can be amplified using these primers (Fig. 3c). More mature *Wx* transcripts produced in 9311 than in Nipponbare under HTC (Fig. 3c). These results indicated that 9311 may have higher splicing efficiency of *Wx* gene under HT than Nipponbare, which is correlated with their grain amylose contents. The splicing efficiency of *Wx* gene under HT was further investigated in all CSSLs. Line HZ1246 and HZ1249 showed higher splicing efficiency than most of the lines (Fig. 3d). In addition, qRT-PCR analysis also showed that HZ1216, HZ1246, HZ1249 had the higher *Wx* splicing efficiency than Nipponbare but similar to 9311 (Fig. 3e). Genomic re-sequencing of the CSSLs showed that all these three lines contained *qHAC8a* (Fig. 2b), which is the major locus identified with a positive effect for amylose content under HT conditions (Fig. 2a). Besides the *qHAC8a*, the CSSL HZ1216 carries the *qHAC4* and HZ1246 carries *qHAC8b*, respectively. However, the *Wx* splicing efficiency in the line HZ1203, which only takes the QTL *qHAC10*, was slightly lower than that of Nipponbare. These data suggest the *qHAC4*, *qHAC8a* and *qHAC8b* from 9311 might

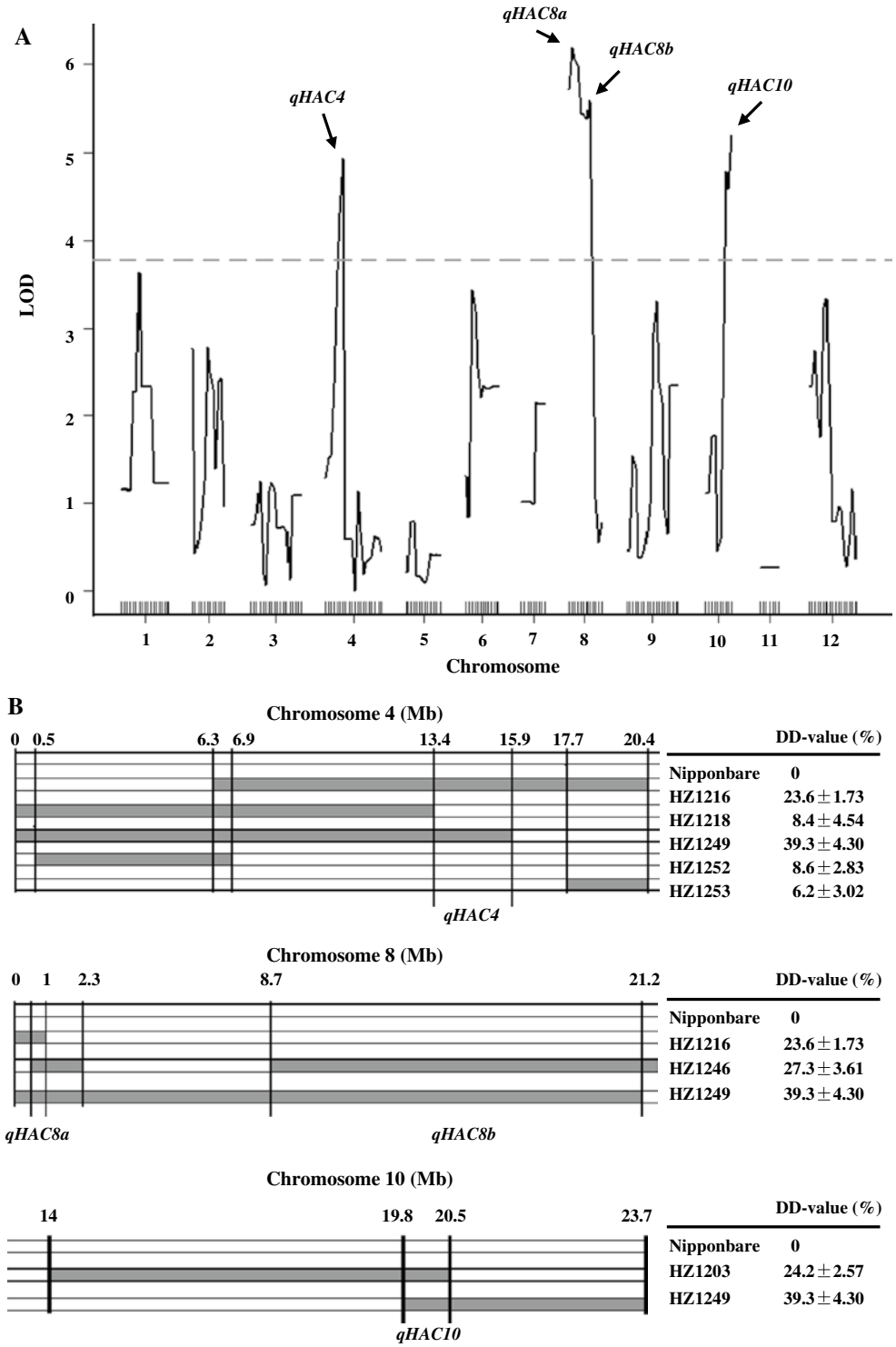

Fig. 2 QTL mapping for rice amylose content in response to HT. **a** QTL mapping for amylose content in response to HT with a single-QTL model. The LOD score significance threshold was established using 1,000 permutations (LOD = 3.79, $p = 0.01$); *tick marks* indicate tag positions. **b** Physical mapping of the QTLs in the rice genome. *Gray bars* represent substituted segments from the donor parent, and *open bars* represent chromosomal contributions from the parent line Nipponbare. The DD-values of chromosome segment substitution lines (CSSLs) were defined as the phenotype

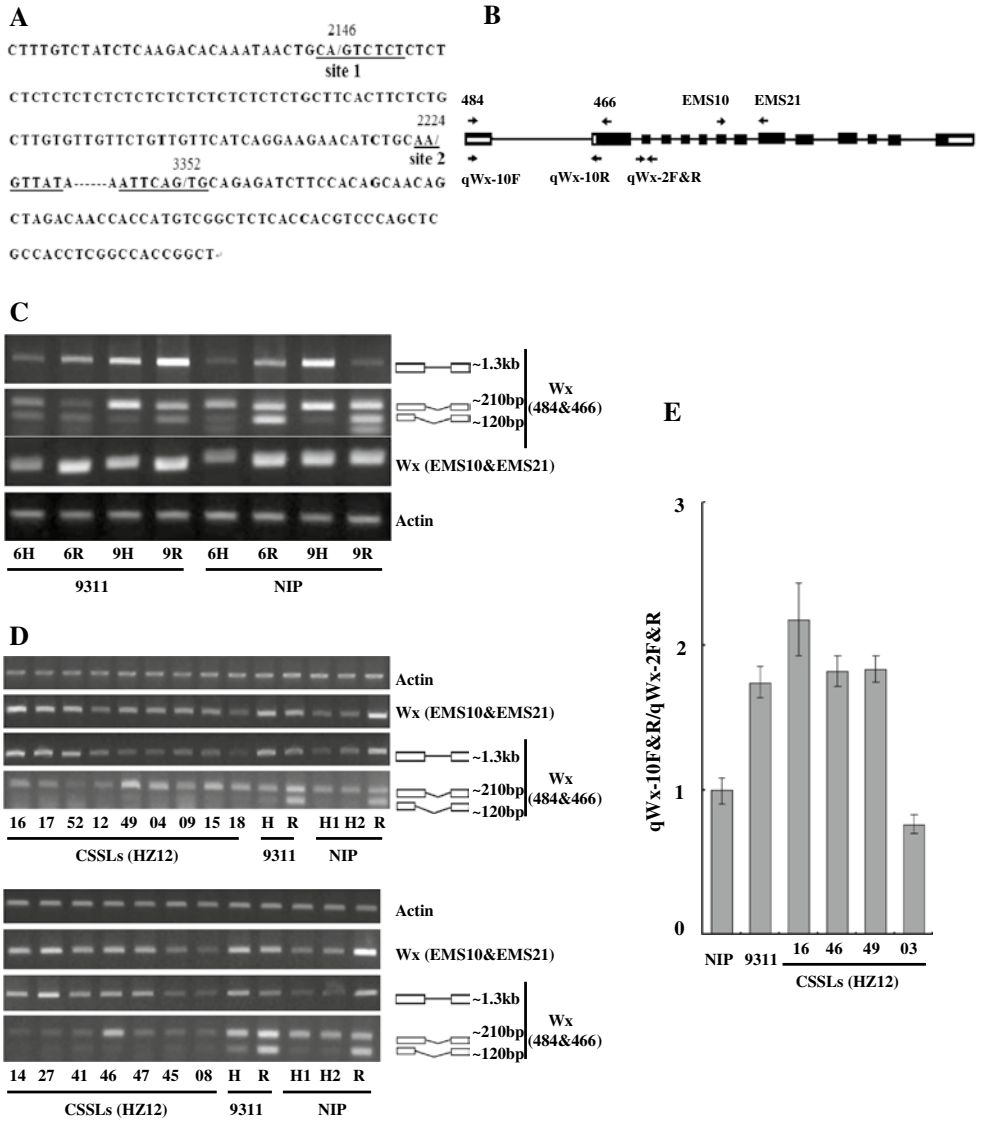

Fig. 3 Gene expression analysis of *Wx* in 9311, Nipponbare (NIP) and the CSSLs. **a** Two alternative splicing sites (site 1 and site 2) in the leader intron of the *Wx* gene. **b** Location of *Wx* gene Primers used in gene expression analysis. *Black bars*, *white bars*, and *black lines*, respectively, represent coding region, untranslated regions, and introns. *Black arrows* denote primer locations. **c** Semi-quantitative PCR analysis of *Wx* gene expression under different temperature conditions at different filling stages. The numbers 6 and 9 indicate six and nine DAF, respectively, H and R indicate HT and RT conditions, respectively. **d** Semi-quantitative PCR analysis of the *Wx* gene in 9311, Nipponbare (NIP) and the CSSLs under HT at 9 DAF. The RNA from the CSSLs (HZ), 9311H and NIPH were isolated from rice endosperm of plants grown under HT; the RNA from 9311R and NIPR were isolated from endosperm of plants grown at RT. **e** qRT-PCR analysis of *Wx* gene splicing efficiency in 9311, Nipponbare (NIP) and three CSSLs (HZ1203, HZ1216, HZ1246, and HZ1249) grown at HT. The splicing efficiency of *Wx* is represented by the ratio of the amplification products of qWx-10F&R to qWx-2F&R. The data are mean ± SD for three individual samples

have the effects to increase the splicing efficiency of Wx^b, and thus inhibit the decrease of rice amylose content at HT.

We further sequenced two major PCR products (~210 and ~120 bp) from mature *Wx* mRNA. There are two major mRNA splicing donor sites in the first intron of Wx^b. One is just before the CT repeat in the sequence CA/GTCTCT (site 1), and the other site is near the G/T single nucleotide polymorphism (SNP) in the sequence AA/GTTATA (site 2) (Fig. 3a). Larkin and Park (1999) showed that the selections of alternative splicing site are temperature dependent. Site 1 is utilized frequently at cool temperature (e.g. 18 °C) while site 2 is selected often at the elevated temperature (25 or 32 °C) (Larkin and Park 1999). Here, we further confirmed that at HT, site 2 is utilized preferentially and more 210 bp products could be generated than that at RT (Fig. 3c).

Enzyme activity of GBSSI protein varied at different temperatures

To test the enzyme activity of the two major splice variants of *Wx*, we conducted an additional cooling (18 °C) treatment for the Nipponbare. Rice endosperm at 12 DAF was harvested at low temperature (LT), while the endosperm from RT and HT was collected at 9 DAF, based on their grain filling rate (Larkin and Park 1999). RT-PCR results indicated that the expression of *Wx* (total *Wx* transcript) at cool temperature condition was much higher than that at RT and HT (Fig. 4a). Using primers qWx2-F&R, qRT-PCR results further confirmed this conclusion. Compared to the expression of *Wx* at RT, the total transcript of *Wx* reduced to less than half at HT, but increased to more than threefold at LT (Fig. 4b). Moreover, our data clearly showed that the small product (~120 bp) was produced much more than the large one (~210 bp) (Fig. 4a) at cool temperature. These results indicated that the site 1 is also utilized frequently at cool temperature in Nipponbare like other rice varieties, such as Panda, Toro-2 and Nato (Larkin and Park 1999).

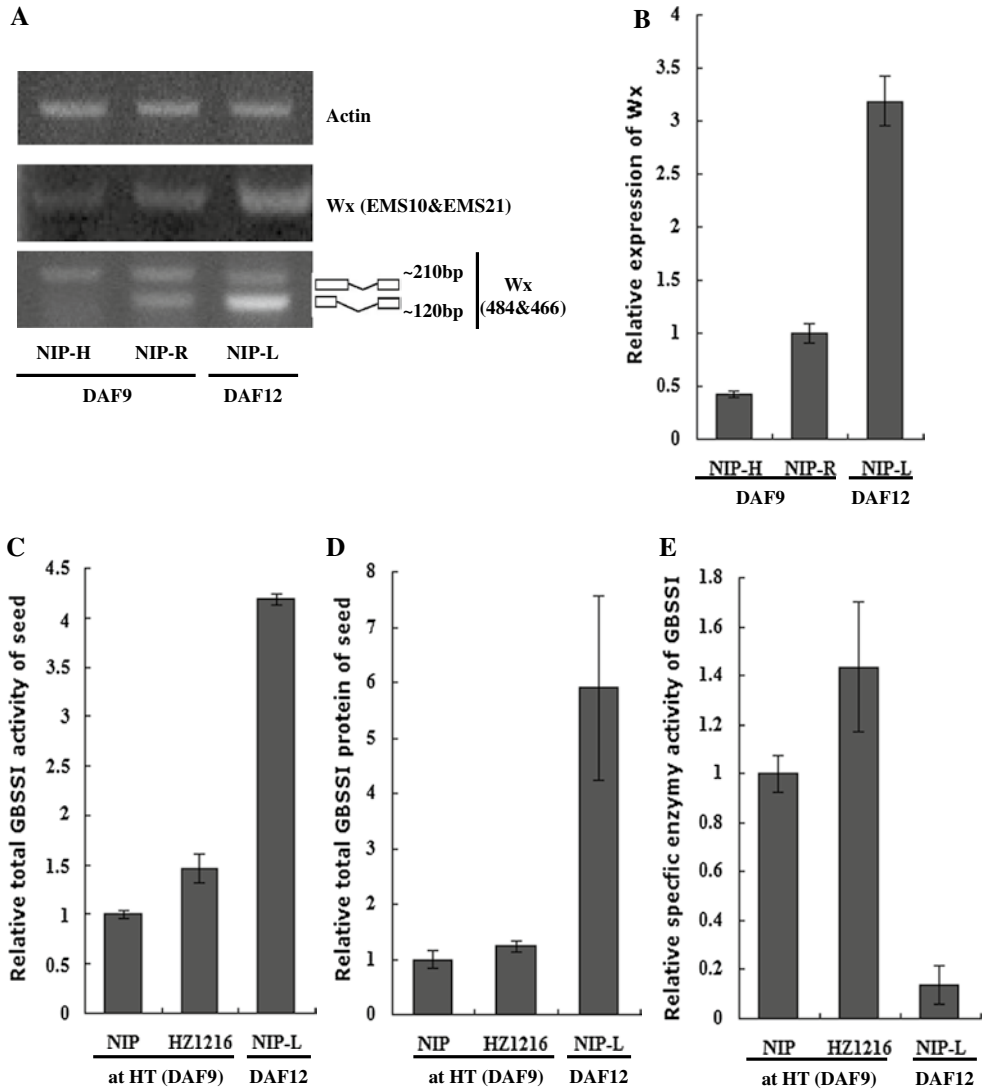

Fig. 4 Enzymatic activity assay of GBSSI protein in Nipponbare and CSSL HZ1216. **a** RT-PCR analysis of *Wx* gene expression in Nipponbare (NIP) at different temperatures. The total RNA from the NIP-H, NIP-R and NIP-L were isolated from rice endosperm of Nipponbare grown at HT, RT and cool temperature condition, respectively. **b** qRT-PCR analysis of *Wx* gene expression in Nipponbare (NIP) at different temperatures. The data are mean ± SD for three individual samples. **c**, **d** Total enzyme activity and protein amount of GBSSI in rice seeds from CSSL HZ1216 (HZ1216-H) grown at HT (DAF9) and Nipponbare (NIP-L) grown at cool temperature condition (DAF12) relative to that from Nipponbare (NIP-H) grown at HT (DAF9). The data are mean ± SD for three individual samples. **e** Specific enzyme activity of GBSSI in HZ1216 (HZ1216-H) at HT (DAF9) and in Nipponbare (NIP-L) at cool temperature condition (DAF12) relative to that in Nipponbare (NIP-H) at HT (DAF9). The data are mean ± SD for three individual samples

Our results revealed that the protein amount of GBSSI (Fig. 4d) and its total enzyme activity (enzyme activity/seed weight) in rice seeds (Fig. 4c) were very high at cool temperature, which was correlated well with the high amylose content of rice grain at that condition. However, we found that the total enzyme activity of GBSSI was dependent on the protein amount, and the specific enzyme activity of GBSSI (enzyme activity/protein amount of GBSSI) at cool temperature was significantly lower than that at HT (Fig. 4e). It is suggested that average enzyme activity of protein translated from the larger product (~210 bp) should be much higher than that generated from the smaller one (~120 bp).

Meanwhile, our results also showed that rice seeds from CSSL HZ1216, which taken the QTLs *qHAC8a* and *qHAC4*, produced more GBSSI protein (Fig. 4d) and with higher total and also higher specific enzyme activities (~1.5-fold, Fig. 4c, e) than Nipponbare at HT. This is consistent well with the transcript level of *Wx* and amylose content in rice seed at HT. And it is also suggested that major QTLs *qHAC8a* or *qHAC4* might have the potential to increase the GBSSI enzyme activity at HT.

Discussion

High temperature damages the crop production frequently in the past decades due to the accelerated global warming (Peng et al. 2004). Amylose content is an important quality criterion in rice. Several studies have shown that amylose content in some varieties can decrease significantly by exposure to elevated temperatures during the grain filling stage (Yamakawa et al. 2007; Zhong et al. 2005). However, the molecular mechanisms still unknown.

A number of important genes are involved in the starch biosynthesis pathway (James et al. 2003). *Wx* (*GBSSI*) is known to be a key gene responsible for amylose biosynthesis although several other starch biosynthesis genes also have been detected to associate with rice amylose content at normal temperature condition (Sano 1984; Wang et al. 1995; Tian et al. 2009). Using a set of CSSLs, we identified several loci which could reduce the deleterious effects of amylose content at HT in this study. The *qHAC8a* is the largest QTLs with positive effect on amylose content change at HT, and it was physically mapped to a small chromosome segment (~300 kb) on chromosome 8. However, there is no known starch biosynthesis genes are present in this region. Besides *qHAC8a*, three other QTLs, *qHAC4*, *qHAC8b* and *qHAC10* were also detected above the threshold and mapped on chromosome 4, 8 and 10, respectively.

There are two major *Wx* alleles identified in the Asian cultivated rice. The abundant of mature *Wx* transcripts are tenfold lower in Wx^b than that in Wx^a due to the G/T SNP in the leader intron of Wx^b and inefficient splicing of this intron (Wang et al. 1995; Cai et al. 1998). The splicing efficiency of Wx^b was found to be temperature-sensitive (Hirano and Sano 1998; Larkin and Park 1999). The higher levels of *Wx* tanscripts and Wx protein are consistent well with the higher amylose content at LT (Hirano and Sano 1998). However, there is no such correlation under HT in different varieties (Zhong et al. 2005; Cheng et al. 2005). Here, for the first time we reported that different varieties even with the same *Wx* alleles show different splicing efficiency of *Wx* (*GBSSI*) which might be a major cause of amylose content variation at HT. Our results demonstrated that CSSLs HZ1216, HZ1246 and HZ1249 have higher splicing efficiency of *Wx* gene than Nipponbare at HT. And three major loci, *qHAC8a*, *qHAC8b* and *qHAC4* with positive effects on amylose content change at HT were detected in these lines. However, the splicing efficiency of *Wx* in CSSL HZ1203, which contains the fourth major locus *qHAC10*, was lower than Nipponbare. These data suggested that besides the increase of *Wx* splicing efficiency, there might be other ways inhibiting the amylose content reduction in 9311 at HT.

Besides splicing efficiency, temperature could affect the selection of splicing donor site of Wx^b (Cai et al. 1998; Larkin and Park 1999; Wang et al. 1995). At LT (18 °C), 120 bp mature *Wx* mRNA PCR-product is predominant while at elevated temperature (25 and 32 °C) 210 bp mature *Wx* mRNA PCR-product accumulates to higher levels. Consistent with these reports, two different types (~120 and ~210 bp) of mature *Wx* mRNA from 9311 and Nipponbare were detected at RT, while the smaller band (~120 bp) was greatly suppressed at HT. Our data further confirmed that the splicing donor site near G/T SNP is preferentially utilized at HT.

Enzymatic activity assay showed that although the total enzyme activity of GBSSI in rice seeds at cool temperature is much higher than that at HT, whereas the specific enzyme activity is significantly lower. It is implied that the more GBSSI protein might be generated from the small transcript (~120 bp) at cool temperature, but the higher enzyme activity are produced from the GBSSI encoded by the large product (~210 bp).

In summary, our results demonstrated the splicing patterns of Wx^b varied under different temperatures. The increased splicing efficiency of *Wx* might be a major cause of keeping amylose content stable at HT in some heat-tolerant varieties (i.e. 9311). The major QTLs, *qHAC8a*, *qHAC8b* and *qHAC4*, with positive effects on amylose content responsive to HT, could be good candidate loci responsible for this pathway. Incorporation of these QTLs from heat-tolerant rice into heat-sensitive rice might benefit breeding of high quality rice cultivars in the regions that experience HT.

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