JIPB Journal of Integrative Plant Biology

Journal of Integrative Plant Biology 2011, **53** (9): 756–765

Research Article

ALK, the Key Gene for Gelatinization Temperature, is a Modifier Gene for Gel Consistency in Rice

Zhenyu Gao[1], Dali Zeng[1], Fangmin Cheng[4], Zhixi Tian[2], Longbiao Guo[1], Yan Su[1], Meixian Yan[1], Hua Jiang[1], Guojun Dong[1], Yuchen Huang[3], Bin Han[3], Jiayang Li[2] and Qian Qian[1*]

[1]State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Science, Hangzhou 310006, China
[2]State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[3]National Center for Gene Research, Chinese Academy of Sciences, Shanghai 200233, China
[4]Department of Agronomy, Huajia Chi Campus, Zhejiang University, Hangzhou 310029, China
[*]Corresponding author
Tel: +86 571 6337 0537; Fax: +86 571 6337 0389; E-mail: qianqian188@hotmail.com
Articles can be viewed online without a subscription.
Available online on 29 June 2011 at www.jipb.net and www.wileyonlinelibrary.com/journal/jipb
doi: 10.1111/j.1744-7909.2011.01065.x

Abstract

Gelatinization temperature (GT) is an important parameter in evaluating the cooking and eating quality of rice. Indeed, the phenotype, biochemistry and inheritance of GT have been widely studied in recent times. Previous map-based cloning revealed that GT was controlled by *ALK* gene, which encodes a putative soluble starch synthase II-3. Complementation vector and RNAi vector were constructed and transformed into Nipponbare mediated by *Agrobacterium*. Phenotypic and molecular analyses of transgenic lines provided direct evidence for *ALK* as a key gene for GT. Meanwhile, amylose content, gel consistency and pasting properties were also affected in transgenic lines. Two of four nonsynonymous single nucleotide polymorphisms in coding sequence of *ALK* were identified as essential for GT. Based on the single nucleotide polymorphisms (SNPs), two new sets of SNP markers combined with one cleaved amplified polymorphic sequence marker were developed for application in rice quality breeding.

Keywords: *ALK*; gelatinization temperature; gel consistency; rice.

Gao Z, Zeng D, Cheng F, Tian Z, Guo L, Su Y, Yan M, Jiang H, Dong G, Huang Y, Han B, Li J, Qian Q (2011) *ALK*, the key gene for gelatinization temperature, is a modifier gene for gel consistency in rice. *J. Integr. Plant Biol*. **53**(9), 756–765.

Introduction

As one of the most important crops, rice provides the staple food for more than 50% of the population in the world. With the increase in grain yield and the improvement of living conditions, grain quality has drawn more and more attention. However, preference for cooking properties of rice varies among consumers around the world. As an important parameter for rice cooking and eating quality, gelatinization temperature (GT) is the critical temperature at which about 90% of the starch granules have swelled irreversibly in hot water and start to lose crystallinity and birefringence (Khush et al. 1979). Instead of hot water, 1.7% potassium hydroxide solution may be used to gelatinize the starch. The digestibility of the milled rice starch, as indicated by the degree of spreading, termed alkali spreading value (ASV), is inversely related to GT, which can be measured directly by differential scanning calorimetry (DSC).

The inheritance of GT in rice has been widely studied since the 1990s (Xu and Mo 1996). Studies have demonstrated

© 2011 Institute of Botany, Chinese Academy of Sciences

that *acl(t)*, a determinator of the profile for amylopectin chain-length of *japonica* and *indica* rice, *alk(t)* and *SSSIIa* were all mapped to the same locus on chromosome 6 (Umemoto et al. 2002). Three single nucleotide polymorphisms (SNPs) were reported to be associated with GT through haplotype analysis (Umemoto et al. 2004). With shuffling experiments, Nakamura et al. (2005) further analyzed in detail the effect of amino acid replacement caused by these SNPs on enzyme activity of SSSIIa, amylopectin structure and GT, and suggested two of the SNPs were essential for SSSIIa activity, which proved to play a specific role in the synthesis of the long B1 chains by elongating short A and B1 chains. A study by Bao et al. (2006) provided further support for the utilization of the GC/TT polymorphism via association mapping. It was reported that negative correlation was significant between amylose content (AC) and gel consistency (GC), two important characters influencing the cooking and eating quality of rice (Sun et al. 2005). But GT has not yet been found to be correlated with AC, although it was affected by *Waxy* (*Wx*) locus (Fan et al. 2005; Wang et al. 2007).

We have fine mapped the *ALK* gene via map-based cloning strategy (Gao et al. 2003). In the present study, the genomic sequence of *ALK* involving promoter region and coding sequence of Shangke Zao, an *indica* rice variety with high GT, and RNA interference (RNAi) vector containing gene-specific cDNA fragments of *ALK* of Nipponbare, a *japonica* rice variety with low GT, were transferred into Nipponbare for the first time, respectively. Polished seeds of T_2 were used for genetic and physico-chemical analysis. Transgenic rice offered an ideal material with the same background for association analysis among GT, AC and GC. Compared with Nipponbare, AC and GC decreased in T_2 with *ALK* transgene from Shangke Zao. Likewise, AC and GC increased in T_2 with inhibited *ALK* expression. Comparison of GT and alignment of coding sequences among 15 rice varieties varying in GT demonstrated two of four nonsynonymous SNPs of *ALK* were identified as essential for GT. Two sets of SNP markers and one cleaved amplified polymorphic sequence (CAPS) marker for marker assisted selection (MAS) of GT were also developed in this study.

Results

GC/TT and G/A are key SNPs in rice *ALK* gene

Alignment of genomic sequences from the start codon (ATG) to stop codon (TGA) between Shuangke Zao (GenBank accession number: AY423717) and Nipponbare showed 19 SNPs in introns and four SNPs in exons (data not shown). Coding sequences of *ALK* from 15 rice varieties were obtained from DNA fragments amplified with separate polymerase chain reaction (PCR) reactions. Combined with ASV of 15 rice varieties, SNP4 (GC/TT) together with SNP3 (A/G) showed highest association with ASV ($R = 0.936$, $P = 0.0001$, Table 1). Varieties with a combination of $G_{4327}C_{4328}$ and G_{4196} in *ALK* appeared to have low ASV. That means, valine and leucine residues at corresponding sites located in predicted glucose transfer domain (amino acid $_{610–785}$) were essential for SSSIIa function, as reported by Nakamura et al. (2005). Either methionine or phenylalanine resulted in medium or high ASV.

Three combinations with two sets of SNP markers were applied in GC/TT screening in *ALK* among 26 varieties with

Table 1. Alkali spreading value (ASV) and single nucleotide polymorphism (SNP) genotypes in the *ALK* gene of 15 rice varieties

Variety	ASV	ALK-SNP1-264	ALK-SNP2-3797	ALK-SNP3-4196	ALK-SNP4-4327, 4328
Nipponbare	6.1 (0.2)	C	A	A	GC
Suyu Nuo	6.5 (0.4)	C	A	A	GC
C Bao	5.7 (0.5)	C	A	G	TT
Wuyun Jing 7	6.8 (0.0)	C	A	G	TT
Chunjiang 06	6.9 (0.1)	C	A	G	TT
Jingxi 17	7.0 (0.0)	C	A	G	TT
Yangdao 6	6.0 (0.0)	C	A	G	TT
Longtefu	7.0 (0.0)	A	A	G	TT
TN1	7.0 (0.0)	C	A	G	TT
Shuangke Zao	1.9 (0.3)	G	G	G	GC
Zhaiye Qing 8	4.0 (0.2)	G	G	G	GC
Teqing	2.0 (0.0)	G	G	G	GC
Minghui 63	2.2 (0.4)	G	G	G	GC
Guanglu Ai 4	3.2 (0.5)	G	A	G	GC
Zhefu 802	4.0 (0.0)	C	A	G	GC

ASV value is mean (*SD*), $n = 18$; number after dash corresponds to genomic site from A_{+1}TG in *ALK*.

different ASV, including 15 *indica* and 11 *japonica* with four glutinous rice (**Figure 1A**). As shown in **Figure 1B**, the abundance of PCR product was distinguishable between GC and TT haplotype. For further detection of A/G in GC haplotype, a 756 bp PCR product amplified with ALKCAPSF and ALKCAPSR was cut with NlaIII (**Figure 1C**). With two sets of SNP markers and one CAPS marker, 26 rice varieties with high GT (ASV < 4.5) and low or intermediate GT (ASV$\geq$4.5) could be differentiated (**Figure 1D**). Therefore, the reliable molecular markers can be used for MAS of rice cooking and eating quality in the majority of rice varieties.

ALK gene is a determinate for GT in rice

Phenotype in T_1 transformed with pCAMBIA1300-*ALK* showed segregation in ASV with Shuangke Zao-type (S-type): Nipponbare-type (N-type) ≅ 3:1 ($P > 0.5$). ASV in four T_2 lines with *ALK* transgene from Shuangke Zao decreased markedly and close to that of Shuangke Zao; meanwhile, ASV in three T_2 lines transformed with pTCK303-*ALKi* increased by 2.9–12.0% compared with that of Nipponbare (**Figure 2B,C**; **Figure 3B,F,G**). Thermal properties of T_2 with *ALK* transgene confirmed *ALK* is a key gene for GT (**Figure 2D**). A significant difference was found in the DSC parameters between Shuangke Zao and Nipponbare, with the former showing higher transition temperature (To, Tp, Tc) and ΔH. DSC curves of all T_2 with *ALK* transgene were similar with that of Shuangke Zao, although ALKC-T1 and ALKC-T3 hold the highest Tp and highest ΔH in DSC, respectively. ALKC-CK, which was transformed with pCAMBIA1300-*alk* had similar DSC curve to Nipponbare. All hygromycin-positive T_2 had *ALK* transgene by sequencing and the CAPS marker except for ALKC-CK, which was transformed with pCAMBIA1300-*alk* as a control (**Figure 2G**). T_2 plants representative of five transgenic lines (ALKC-T1, ALKC-T2, ALKC-T3, ALKC-T4, and ALKC-CK) were subjected to genomic blot analysis to determine copy number of transgene. Because the sequence between LB and RB of pCAMBIA1300-*ALK* or pCAMBIA1300-*alk* has a single EcoRV site, the hybridizing band reflects integration of the gene in the plant genome, and band intensity provides an estimate of the copy number of transgene integrated in the transgenic plants. Of the five transgenic lines examined, the number of hybridization bands varied from one to two copies (ALKC-T1 had two copies) in different transgenic lines (**Figure 2H**).

Grain shape including grain length, grain width and ratio of length : width in T_2 changed little. But surprisingly, compared with Nipponbare, 1000-grain weight increased by 6.6% on average in T_2 with *ALK* transgene, and decreased by 6.2% on average in T_2 with inhibited *ALK* expression (data not shown).

ALK functions as a minor gene for GC in rice

Besides GT, AC and GC in T_2 with *ALK* transgene also decreased by 12.2–24.9% and 4.8–9.5% compared with those of Nipponbare, respectively (**Figure 2E,F**). Interestingly, AC ($R = 0.844$) and GC ($R = 0.801$) in T_2 had high correlation with ASV, while starch content was basically unchanged (Supplementary Figure S1). On the contrary, when *ALK* gene was inhibited in T_2 transformed with pTCK303-*ALKi*, AC and GC increased by 0.1–10.4% and 0.8–13.9% compared with those of Nipponbare, respectively (**Figure 3C,D**). GC in T_2 had high correlation with ASV ($R = 0.999^*$) as well. Thereby, GT and AC were negatively correlated in T_2 with *ALK* transgene; GT and GC were negatively correlated both in T_2 with additional *ALK* gene and in T_2 with inhibited *ALK* expression, which reflect that GC was influenced by GT under the same background except for *ALK*. Pasting properties were also affected in T_2 with *ALK* transgene (**Table 2**). It is believed that GC is significantly and positively correlated with peak viscosity (Hu et al. 2004; Wan et al. 2006). Consistent with previous reports, GC in T_2 had high correlation with peak viscosity (0.817); that is, with the increment in GT, rice pasting properties changed with decreasing peak viscosity.

Expression of rice starch synthase genes in transgenic lines

ALK was expressed specifically in rice endosperm in the mid to later stage of grain filling (Hirose and Terao 2004). We extracted RNA from T_2 endosperm with *ALK* transgene at 12 d after flowering (DAF) for expression analysis of *ALK* and two related starch synthase genes: granule-bound starch synthase I (*GBSSI*), a late expresser for amylose synthesis and soluble starch synthase I (*SSSI*), a steady expresser expressed relatively constantly during grain filling. Expression level of *GBSSI* and *SSSI* were relatively unchanged in T_2 (**Figure 2J**). *ALK* transgene (*SSSII-3*) expressed at the same level in Shuangke Zao and T_2 except in ALKC-T1 and ALKC-T3, which has two copies of transgene and holds the highest ΔH in DSC, respectively. They appeared to express abundantly in *ALK* (**Figure 2I**). Therefore, a gene-dosage effect of the *ALK* transgene on transcription level and GT may exist in transgenic rice. Real-time reverse transcription (RT)-PCR for T_2 transformed with pTCK303-*ALKi* showed a sharp decline in RNA transcripts of *ALK*, in accordance with changes in GT and GC (**Figure 3E**).

Discussion

Since GT has significant correlation with To, Tp and ΔH, DSC has been commonly used for measuring the degree of gelatinization of starch. In our study, correlations between ASV and To (–0.913**), ASV and Tp (–0.915*) were also significant. It is generally accepted that the increase in viscosity observed during the heating of starch in water with Rapid

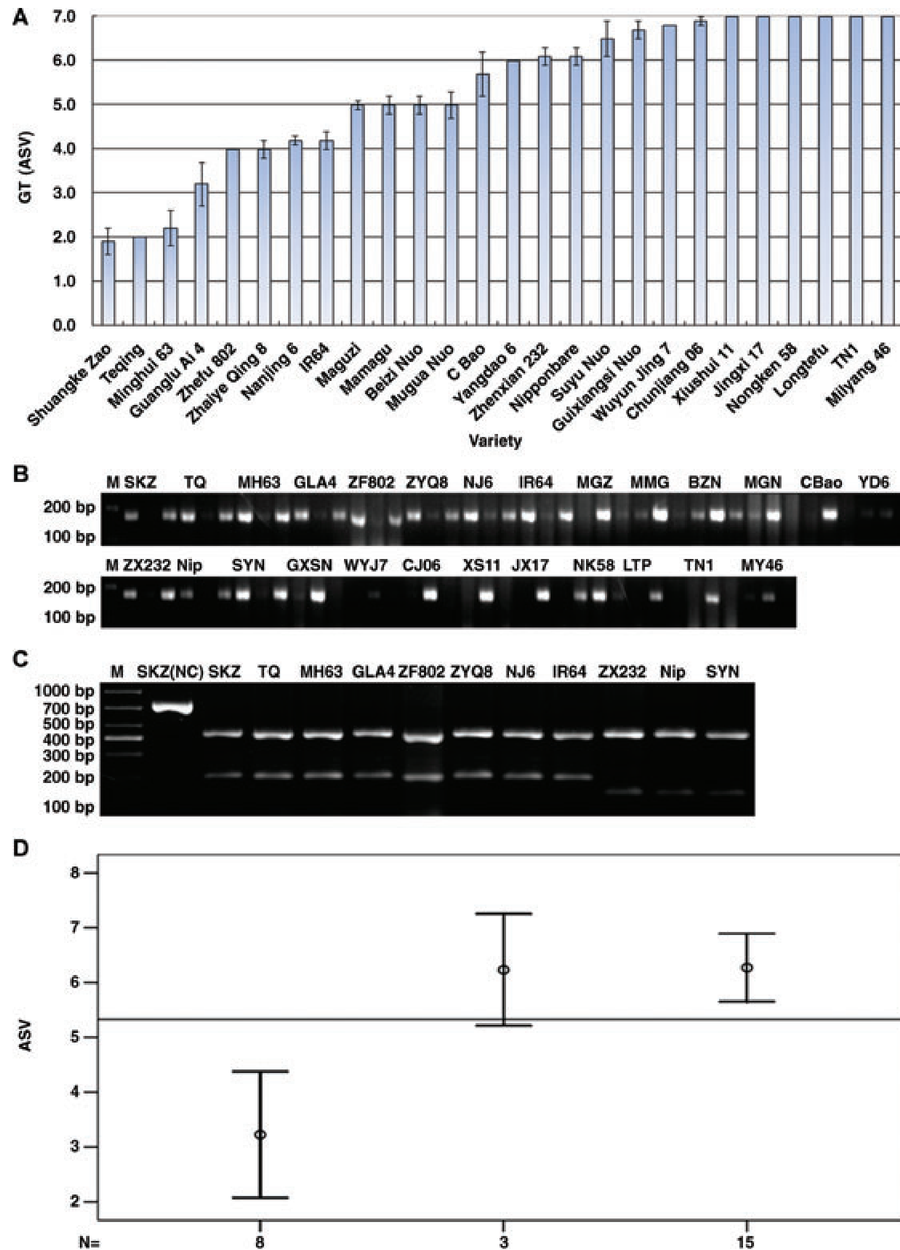


Figure 1. Relationship between haplotype in *ALK* and alkali spreading value (ASV) of different rice genotypes.

(**A**) Gelatinization temperature (GT) (ASV) of 26 rice varieties. The bar for each value represents mean ± *SD*. $n = 18$.

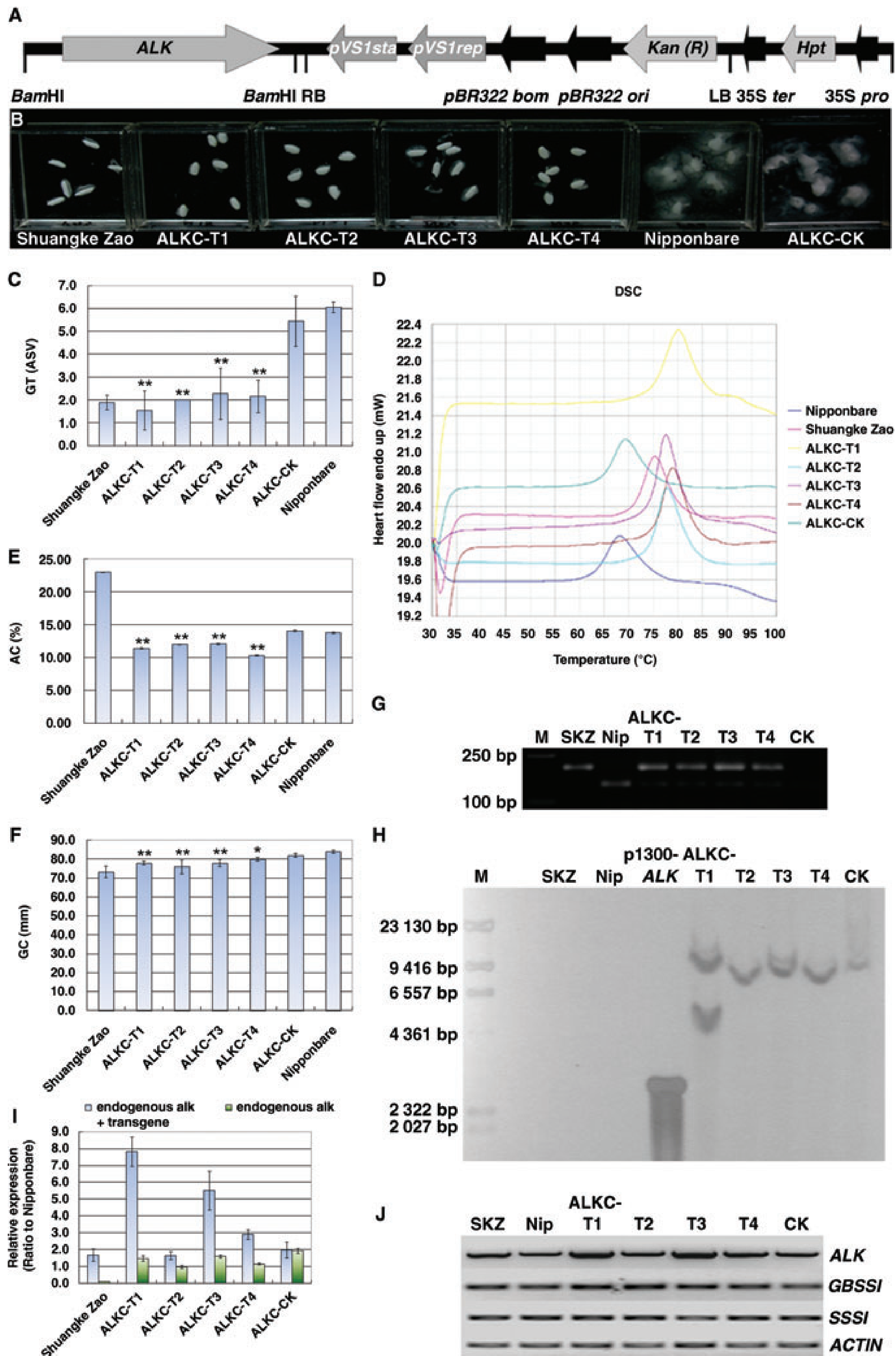
(**B**) Genotyping of 26 rice varieties with the single nucleotide polymorphism (SNP) markers in *ALK*.

The lane from left to right for each variety is the polymerase chain reaction (PCR) product amplified with primers ALKSNP3-GF and ALKSNP4-GCR, ALKSNP3-GF and ALKSNP4-TTR, ALKSNP3-AF and ALKSNP4-GCR, respectively.

(**C**) Genotyping of 26 rice varieties with a cleaved amplified polymorphic sequence (CAPS) marker cut with NlaIII in *ALK*. M, DL1000 MARKER; SKZ, Shuangke Zao (*indica*); TQ, Teqing (*indica*); MH63, Minghui 63 (*indica*); GLA4, Guanglu Ai 4 (*indica*); ZF802, Zhefu 802 (*indica*); ZYQ8, Zhaiye Qing 8 (*indica*); NJ6, Nanjing 6 (*indica*); IR64 (*indica*); MGZ, Maguzi (*indica*); MMG, Mamagu (*indica*); BZN, Beizi Nuo (*japonica*); MGN, Mugua Nuo (*japonica*); C Bao (*japonica*); YD6, Yangdao 6 (*indica*); ZX232, Zhenxian 232 (*indica*); Nip, Nipponbare (*japonica*); SYN, Suyu Nuo (*japonica*); GXSN, Guixiangsi Nuo (*japonica*); WYJ7, Wuyun Jing 7 (*japonica*); CJ06, Chunjiang 06 (*japonica*); XS11, Xiushui 11 (*japonica*); JX17, Jingxi 17 (*japonica*); NK58, Nongken 58 (*japonica*); LTF, Longtefu (*indica*); TN1 (*indica*); MY46, Milyang 46 (*indica*). SKZ (NC), Shuangke Zao (not cut).

(**D**) Relationship between haplotype and ASV for 26 rice genotypes.

Mean (M) ASV and *SD* for each haplotype (from left to right) are as follows: haplotype 1 (G/GC), M = 3.2, *SD* = 1.0; haplotype 2 (A/GC), M = 6.2, *SD* = 0.2; haplotype 3 (G/TT), M = 6.3, *SD* = 0.9. Bars represent the 95% confidence interval for mean of each haplotype.



A
ALK
pVS1sta
pVS1rep
Kan (R)
Hpt
BamHI
BamHI RB
pBR322 bom
pBR322 ori
LB 35S ter
35S pro
B
Shuangke Zao
ALKC-T1
ALKC-T2
ALKC-T3
ALKC-T4
Nipponbare
ALKC-CK
C
GT (ASV)
D
DSC
Heart flow endo up (mW)
Temperature (°C)
Nipponbare
Shuangke Zao
ALKC-T1
ALKC-T2
ALKC-T3
ALKC-T4
ALKC-CK
E
AC (%)
F
GC (mm)
G
ALKC-
M SKZ Nip T1 T2 T3 T4 CK
250 bp
100 bp
H
p1300- ALKC-
M SKZ Nip ALK T1 T2 T3 T4 CK
23 130 bp
9 416 bp
6 557 bp
4 361 bp
2 322 bp
2 027 bp
I
endogenous alk + transgene
endogenous alk
Relative expression (Ratio to Nipponbare)
J
SKZ Nip ALKC- T1 T2 T3 T4 CK
ALK
GBSSI
SSSI
ACTIN

Visco Analyzer (RVA) is mainly caused by the swelling of granules, while breakdown of viscosity is caused by breakdown of gelatinized starch granules (Whistler and BeMiller 1997). Therefore, pasting characteristics of defatted rice starches are determined with RVA.

It was reported that AC of rice positively correlated with the level of GBSSI in endosperm encoded by *Wx* gene (Sano 1985). *qAC-6*, located in the 9.9–32.1 cM region of chromosome 6, was identified for AC of milled rice with chromosome segment substitution lines (Hao et al. 2009). It is understandable that the relationship between GT and AC becomes confused in separate studies using different rice genotypes since *Wx* displays genetic linkage to *SSSI* and *ALK*. The correlation was reported to be significantly negative or null in previous studies (Nakamura et al. 2002). Recently, GT became significantly greater than that of the wild type in two Longtefu-derived transgenic rice with antisense *Wx* gene (Yu et al. 2009). In the study, there was no correlation between ASV and AC among the 26 different rice varieties studied (R = –0.314). However, with the decrease of ASV in T_2 with *ALK* transgene, AC decreased significantly compared to that of Nipponbare, in agreement with a recent report by Tian et al. (2009). This may be as a result of competition for ADP-glucose, the raw material for both amylose and amylopectin synthesis, considering the unchanged starch content in rice T_2. The hypothesis requires further testing.

Gel consistency is believed to be controlled by a single gene with major effects along with several minor genes and modifiers. It was reported that the *Wx* locus showed major effects on AC and GC (Fan et al. 2005). Two QTLs on chromosome 6 and one on chromosome 7 were detected for GC by using a recombinant inbred line (RIL) population derived from the cross KDML105/CT9993 (Lanceras et al. 2000). Recently, Wang et al. (2007) observed that the *Wx* locus also affected ASV while the *ALK* locus made minor contribution to GC and some parameters of paste viscosity, too. Our results provided evidence for the *ALK* gene as a modifier gene negatively controlling GC.

Positional cloning of *ALK* in rice showed that it encodes a putative SSSII (Gao et al. 2003). The expression of *SSSII-3* in rice was almost specific to the panicles at the grain-filling stage, while *SSSII-2* was expressed highly in leaf blades and leaf sheaths, and weakly in the panicles. Meanwhile, *SSSII-1* was expressed in a variety of tissues, including roots and germinating seeds (Hirose and Terao 2004). Nakamura et al. (2005) indicated that the failure of *japonica* SSSIIa in elongating short chains of DP $\leq$ 10 to DP $\leq$ 24 resulted in the formation of S-type amylopectin. Based on the expression of *ALK* and phenotype in T_2, it was confirmed indirectly that GT was controlled by both enzymatic activity of SSSII-3 and expression level of *ALK*. Therefore, combining previous reports with our data, a hypothetical mechanism can be advanced, in which base substitutions in *ALK* cause amino acid changes in SSSII-3 and result in the alteration of enzymatic activity, and thereafter alter the fine structure of crystalline lamellae of amylopectin, and are finally reflected in physicochemical properties, especially GT.

Through statistical analysis of GT in 70 rice genotypes, Waters et al. (2006) believed two continuous SNPs could classify them into either high GT or low GT type, which differed in GT by 8°C. However, in our report, the corresponding SNPs could only differentiate rice varieties with high GT and low or intermediate GT. Not all *indica* exhibited high GT, for example, TN1 and Longtefu, suggesting these two SNPs may have been introgressed from *japonica* and selected for their desirable cooking qualities. Some molecular markers have been used in rice breeding programs for a range of traits including AC. The application of SNPs in plant breeding involves the analysis of

Figure 2. Phenotypic analysis and molecular detection of T_2 lines with *ALK* transgene.

(A) Schematic structure of the complementation construct pCAMBIA1300-*ALK*. pCAMBIA1300-*ALK* contained the entire *ALK* gene, the 1 766 bp upstream sequence, and the 677 bp downstream sequence; Alkali digestion test **(B)** and gelatinization temperature (GT) (alkali spreading value [ASV]) **(C)** of Shuangke Zao, Nipponbare and T_2. The bar for each value represents mean $\pm$ *SD*. n = 18. **(D)** Differential, scanning calorimetry (DSC) profiles of Shuangke Zao, Nipponbare and T_2. To = Onset temperature, Tp = Peak temperature, Tc = Conclusion temperature. amylose content (AC) **(E)** and gel consistency (GC) **(F)** of Shuangke Zao, Nipponbare and T_2. The bar for each value represents mean $\pm$ *SD*. n = 3. * and ** indicate the least significant difference at 0.05 and 0.01 probability level compared with Nipponbare, respectively. **(G)** Cleaved amplified polymorphic sequence (CAPS) with NlaIII for *ALK* transgene detection in T_2. **(H)** DNA gel blot analysis with *hpt* probe in T_2. M: λ-HindIII MARKER; p1300-*ALK*: pCAMBIA1300-*ALK*. ALKC-T1, ALKC-T2, ALKC-T3 and ALKC-T4 are T_2 transformed with pCAMBIA1300-*ALK*. ALKC-CK is the T_2 transformed with pCAMBIA1300-*alk* as a control. **(I)** Transcriptional level of endogenous *alk* and total *ALK* (endogenous *alk*+transgene) in transgenic lines revealed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The error bar for each value represents mean $\pm$ *SD*. (n = 3). **(J)** Expression at transcript level for three rice starch synthase genes in T_2 endosperm. *Actin* acts as an internal control. M: DL2000 MARKER; Nip, Nipponbare; SKZ, Shuangke Zao.

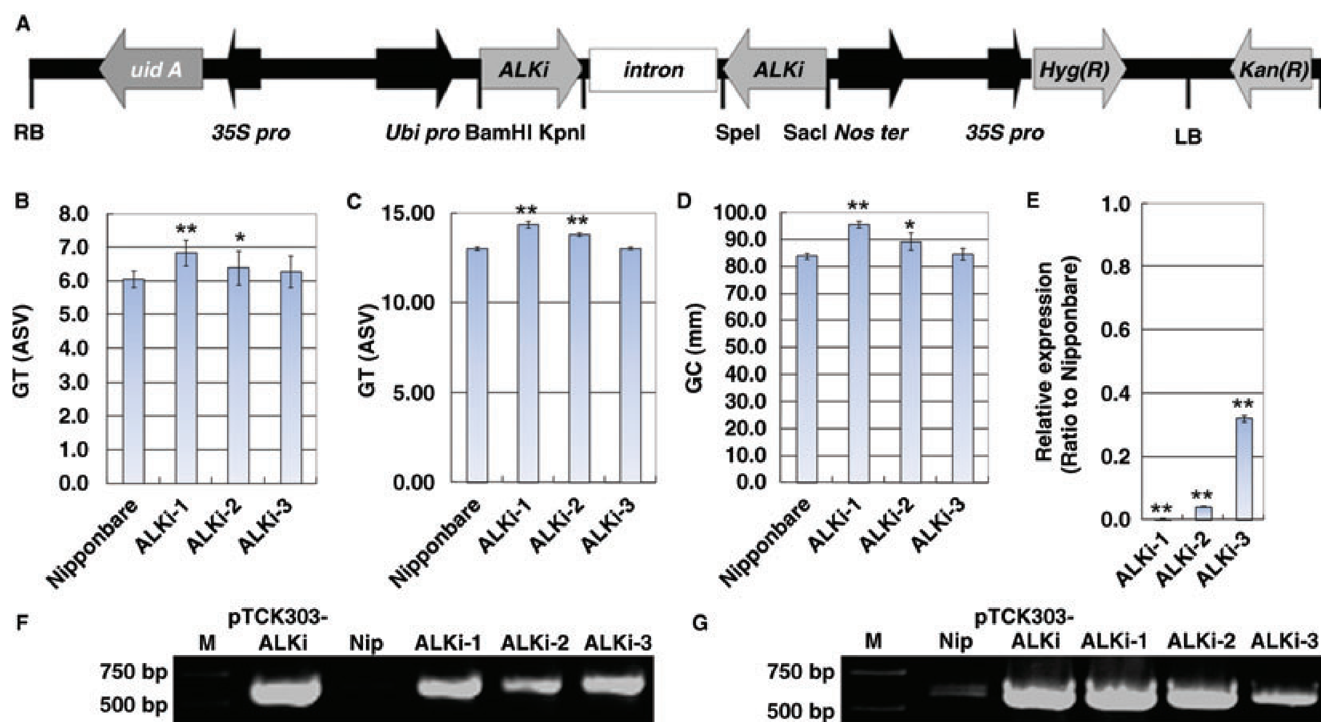


Figure 3. Phenotypic analysis and molecular detection of T_2 lines with RNAi.

(**A**) Schematic structure of the RNAi construct pTCK303-*ALKi*. pTCK303-*ALKi* contained nucleotide sequence 3746 to 4295 in *ALK*. Gelatinization temperature (GT) (alkali spreading value [ASV]) (**B**) ($n = 18$), amylose content (AC) (**C**) ($n = 3$) and gel consistency (GC) (**D**) ($n = 3$) of Nipponbare and T_2 transformed with pTCK303-*ALKi*. The bar for each value represents mean $\pm$ *SD*. (**E**) Transcriptional level of *ALK* in transgenic lines revealed by quantitative reverse transcription-polymerase chain reaction (RT-PCR). The error bar for each value represents mean $\pm$ *SD*. $n = 3$. * and ** indicate the least significant difference at 0.05 and 0.01 probability level compared with Nipponbare, respectively. Primers for identification of transgenic lines are Hyg-2F and Hyg-2R (**F**), Hi438F and A4297R (**G**).

large numbers of samples, and therefore requires rapid, reliable and inexpensive methods to genotype the sequence variants. Herein, two sets of SNP markers together with one CAPS marker were developed for further differentiating rice varieties with high GT from those with low or intermediate GT. They can be used in rice varieties selection concerning GT, one of the important indices for rice cooking and eating quality because low and intermediate GT was preferred by most consumers.

Table 2. Rapid Visco Analyzer (RVA) profile characteristic values in Shuangke Zao, Nipponbare and T_2

Variety/Line	Peak viscosity	Holding strength	Final viscosity	Breakdown	Consistency	Setback
Shuangke Zao	314.58 (1.70)	192.42 (3.96)	398.42 (10.04)	122.16 (2.26)	206.00 (6.08)	83.84 (8.34)
ALKC-T1	402.75 (0.71)	178.67 (4.31)	224.08 (3.14)	280.08 (3.61)	101.41 (1.17)	–122.67 (2.43)
ALKC-T2	410.58 (3.68)	192.50 (2.90)	289.67 (5.29)	218.08 (0.78)	97.17 (2.39)	–120.91 (1.61)
ALKC-T3	416.33 (4.53)	187.73 (1.90)	228.60 (5.69)	281.50 (2.63)	93.77 (3.79)	–134.83 (1.16)
ALKC-T4	426.42 (0.12)	188.67 (3.66)	280.50 (2.94)	237.75 (3.54)	91.84 (0.71)	–145.92 (2.82)
ALKC-CK	430.63 (3.12)	212.29 (8.19)	331.21 (3.71)	218.33 (11.31)	118.92 (4.48)	–99.42 (6.83)
Nipponbare	431.58 (1.77)	204.29 (3.13)	320.08 (12.02)	227.29 (1.36)	115.79 (8.90)	–111.5 (6.25)

Breakdown is equal to the difference between peak viscosity and holding strength; Consistency is equal to the difference between final viscosity and holding strength; Setback is equal to the difference between final viscosity and peak viscosity. Data are means (*SD*) and duplicate measurements were performed for each sample.

Materials and Methods

Plant materials

Plant materials including transgenic lines were cultivated in genetic modified (GM) fields in Hangzhou, Zhejiang province and Lingshui, Hainan province of China.

Evaluation of starch content, AC, GC and GT

Starch content was determined according to Zou (1995). AC was measured following the procedure of Perez and Juliano (1978) with some modifications. Portions of the starch solution were transferred into AutoAnalyzer cups and the amylose-iodine blue color was determined at 608 nm. GC was evaluated according to Cagampang et al. (1973). Triplicate measurements were performed for each sample. GT was indirectly estimated via the alkali digestion test (Little et al. 1958). Six whole-grain, milled rice samples were placed in triplicate square plastic boxes containing 10 mL 1.7% potassium hydroxide (KOH). The boxes were incubated for 23 h at 30 °C. Grain appearance and disintegration were visually rated after incubation based on the standard numerical scale. Statistical analyses and correlation analysis were conducted with SAS 9.1 and SPSS 13.0 for Windows, respectively.

Vector construction and plant transformation

ALK in Shuangke Zao, an *indica* rice variety, and *alk* in Nipponbare, a *japonica* rice variety, were assembled from three fragments via high fidelity PCR using primers as follows: C1F (5′-TCTAGATTGCTACACGTGAGAGG-3′) and C1R (5′-GGAGCCACCTGTAAAGCGTG-3′), C2F (5′-GGAGCGTTGC GGATTGCCAC-3′) and C2R (5′-CCGCGTAATCACCGTACC TTGG-3′), C3F (5′-AACGGGGACTCTCGGTGACTTC-3′) and C3R (5′-GTTCCCGCAATGGCAAATGTC-3′), respectively. pCAMBIA1300-*ALK* and pCAMBIA1300-*alk* were constructed with a total length of 15.855 kb (including 1 766 bp upstream of start codon ATG, 4 420 bp *ALK* gene and 677 bp downstream of stop codon TGA) (**Figure 2A**). The schematic diagram of RNA interference (RNAi) vector pTCK303-*ALKi* is shown in **Figure 3A**. The 564 bp and 563 bp gene-specific cDNA fragments of the *ALK* gene in Shuangke Zao were amplified by PCR using the primer pairs ASpeF (5′-ATACTAGTCACCTGCAGTCCGACGGCTACG-3′) and ASacR (5′-AAGAGCTCTCGAGGCAGTGGCCGAGCG-3′), AKpnF (5′-ATGGTACCACCTGCAGTCCGACGGCTACG-3′) and ABamHR (5′-AAGGATCCTCTCGAGGCAGTGGCCGAG CG-3′), respectively. After being double digested with *Spe*I-*Sac*I and *Kpn*I-*Bam*HI, respectively, the two fragments were then successively inserted into the two sides of pTCK303 in opposite orientation and sequenced.

The constructs were introduced into the *Agrobacterium tumefaciens* strain, EHA105, and the positive clones were selected to infect callus of *japonica* rice variety Nipponbare according to Hiei et al. (1994). The primary transformants (T_0) were self-pollinated, the resulting seeds (T_1) were then self-pollinated and seeds (T_2) were harvested. Primers for molecular detection of transgene in RNAi inhibition were Hyg-2F (5′-GGAGCATATACGCCCGGAGT-3′) and Hyg-2R (5′-GTTTATCGGCACTTTGCATCG-3′), Hi438F (5′-ACCTAATGATTGACTATGACACGGCTG-3′) and ALKiR (5′-TCTCGAGGCAGTGGCCGAGCG-3′), respectively.

Physicochemical properties analysis of starch granules

Thermal properties were determined with a differential scanning calorimeter Model DSC-7 (Perkin-Elmer, Norwalk, CA, USA) equipped with an intra-cooling system. The onset temperature of gelatinization (To), peak temperature of gelatinization (Tp), conclusion temperature of gelatinization (Tc) and enthalpy (ΔH) were determined from DSC thermograms, as described by the method of Fujita et al. (1993). Pasting properties of starch granules were analyzed by Rapid Visco Analyzer (RVA) model 3-D (Newport Scientific, Sydney, Australia) as described by Chen et al. (1999). Duplicate measurements were performed for each sample.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from leaves following the method of Murray and Thompson with modifications (1980). Southern blot analysis was performed with enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Primers used for amplification of the probe for *hpt* are listed as follows: HygF: 5′-GCTTTCAGCTTCGATGTAGGAG-3′ and HygR: 5′-CTACACAGCCATCGGTCCAGA-3′. The 862 bp PCR product was recovered from the agarose gel and purified with the QIAquick gel extraction kit (QIAGEN, Hilden, Germany).

Semi-quantitative RT-PCR and quantitative real-time RT-PCR analysis

Total RNA was isolated from rice endosperm at 12 DAF using the methods of Zheng et al. (1993) with modifications. After treatment with RQ1 RNase-free DNase (Promega, Madison, WI, USA), RNA ($\cong$ 4 μg) was reverse-transcribed by SuperScript II (Invitrogen, Carlsbad, CA, USA) using an oligo-dT15 primer. The gene-specific primers used for semi-quantitative PCR are listed in **Table 3** with *Actin* as an internal control. The PCR program consisted of 25–30 cycles of 30 s at 94 °C, 40 s at 57 °C, 1 min at 72 °C, and a final extension at 72 °C

Table 3. Primers for semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and real time RT-PCR

Gene	Forward primer	Reverse primer
ALK	5′-GCACTCCTGCCTGTTTATCTGAAG-3′	5′-CCTTGTACTTGCGGTACGTC-3′
GBSSI	5′-AGGCATCGAGGGTGAGGAG-3′	5′-CCATCTGGCCCACATCTCT- 3′
SSSI	5′-GGGCCTTCATGGATCAAC-3′	5′-CCGCTTCAAGCATCCTCATC-3′
ACTIN	5′-CATGCTATCCCTCGTCTCG-3′	5′-CGCACTTCATGATGGAGTTG-3′
OsACTIN1	5′-CCATTGGTGCTGAGCGTTT-3′	5′-CGCAGCTTCCATTCCTATGAA-3′
ALKR	5′-ACAATGGCATGATGCAGTACACT-3′	5′-CGGCCCTGGTAAGCGATAT-3′
ALKR-N	5′-TCGGCGGGCTGAGGGACACCA-3′	5′-TCGATGAGCTTGTGCGGCTCG-3′

In real time RT-PCR, *ALKR* was used for amplification of total *ALK* (endogenous *alk*+transgene) and *ALKR-N* was designed as specific primers for endogenous *alk* detection.

for 7 min. The amplified DNA fragments were separated on a 1.2% (w/v) agarose gel and amplified bands were then cloned and sequenced to confirm identity.

For real-time RT-PCR, the cDNAs were assayed using 2× SYBR Green PCR Master Mix on the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression level of each transcript was obtained by normalization to the *OsActin1* gene. PCR was carried out as follows: preheating at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The primers are listed in Table 3.

DNA sequencing analysis

The genomic sequences of *ALK* in different rice varieties were amplified by high fidelity PCR. Primers are listed as follows: A1F (5′-ATGTCGTCGGCCGTCGTC-3′) and C2R, C3F and A2R (5′-AGAAAAACAAAACCTGGTAAGCG-3′), A3F (5′-ACAATGGCATGATGCAGTACACTCG-3′) and A3R (5′-CTCC GGGTTCCACTCCCGG-3′), A4F (5′-CATCATACGGGAGAA CGACTGGA-3′) and A4R (5′-TCACCATTGGTACTTGGCCT TGA-3′). DNA of interest was sequenced by Sanger's dideoxy method with 3730xl DNA Sequencer (Applied Biosystems). Multi-sequence alignment analysis was performed with Clustal W program (http://www.genome.ad.jp).

Molecular markers developed for the *ALK* gene

Single nucleotide polymorphism markers for SNP3 and SNP4 are ALKSNP3-AF 5′-TCGGCGGGCTGAGGGACACCA-3′ and ALKSNP4-GCR 5′-ACATGCCGCGCACCTGGAGC-3′, ALKSNP3-GF 5′-TCGGCGGGCTGAGGGACACCG-3′ and ALKSNP4-TTR 5′-ACATGCCGCGCACCTGGAAA-3′. A CAPS marker, PCR amplified with primers ALKCAPSF 5′-CAT CATACGGGAGAACGACTGGA-3′ and ALKCAPSR 5′-TCA CCATTGGTACTTGGCCTTGA-3′ and then digested with restriction endoenzyme NlaIII, was used for genotyping *ALK* in different rice varieties and detecting *ALK* transgene in T_2.

Acknowledgements

The authors thank Professor Kang Chong at the Institute of Botany, CAS, for kindly supplying vector pTCK303. This work is supported by grants from the Hi-Tech Research and Development (863) Program of China (2006AA10A102), Transform Program (2008ZX08001-006), Science and Technology Project, Zhejiang Province (2009C32047) and CNRRI foundation (2009RG002-1).

Received 24 Apr. 2011 Accepted 25 Jun. 2011

References

Bao JS, **Corke H**, **Sun M** (2006) Nucleotide diversity in starch synthase IIa and validation of single nucleotide polymorphisms in relation to starch gelatinization temperature and other physicochemical properties in rice. *Theor. Appl. Genet*. **113**, 1171–1183.

Cagampang GB, **Perez CM**, **Juliano BO** (1973) A gel consistency test for the eating quality of rice. *J. Sci. Food Agric.* **24**, 1589–1594.

Chen JJ, **Lu S**, **Li CY** (1999) Effect of milling on the physicochemical characteristics of waxy rice in Taiwan. *Cereal Chem*. **76**, 795–799.

Fan CC, **Yu XQ**, **Xing YZ**, **Xu CG**, **Luo LJ**, **Zhang QF** (2005) The main effects, epistatic effects and environmental interactions of QTLs on the cooking and eating quality of rice in a doubled haploid line population. *Theor. Appl. Genet.* **110**, 1445–1452.

Fujita S, **Morita T**, **Fujiyama G** (1993) The study of melting temperature and enthalpy of starch from rice, barley, wheat, fortail- and proso-millets. *Starch/Starke* **45**, 436–441.

Gao ZY, **Zheng DL**, **Cui X**, **Zhou YH**, **Yan MX**, **Huang DN**, **Li JY**, **Qian Q** (2003) Map-based cloning of the *ALK* gene, which controls the gelatinization temperature of rice. *Sci. China* (*Ser. C*) **46**, 661–668.

Hao W, **Zhu MZ**, **Gao JP**, **Sun SY**, **Lin HX** (2009) Identification of quantitative trait loci for rice quality in a population of chromosome segment substitution lines. *J. Integr. Plant Biol.* **51**, 500–512.

Hiei Y, **Ohta S**, **Komari T**, **Kumashiro T** (1994) Efficient transformation of rice mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hirose T, **Terao T** (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta* **220**, 9–16.

Hu PS, **Zhai HQ**, **Tang SQ**, **Wan JM** (2004) Rapid evaluation of rice coking and palatability quality by RVA profile. *Acta Agr. Sin.* **30**, 519–524.

Khush GS, **Paule CM**, **de La Cruz NM** (1979) Rice grain quality evaluation and improvement at IRRI. In: Brady NC, ed. *Proceedings of the Workshop on Chemical Aspects of Rice Grain Quality*. International Rice Research Institute, Los Banos, Laguna, Philippines. pp. 20–31.

Lanceras JC, **Huang ZL**, **Naivikul O**, **Vanavichit A**, **Ruanjaichon V**, **Tragoonrung S** (2000) Mapping of genes for cooking and eating qualities in Thai jasmine rice (KDML105). *DNA Res.* **7**, 93–101.

Little RR, **Hilder GB**, **Dawson EH** (1958) Differential effect of dilute alkali on 25 varieties of milled white rice. *Cereal Chem.* **35**, 111–126.

Murray MG, **Thompson WF** (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.

Nakamura Y, **Francisco PB Jr**, **Hosaka Y**, **Sato A**, **Sawada T**, **Kubo A**, **Fujita N** (2005) Essential amino acid of starch synthase IIa differentiate amylopectin structure and starch quality between *japonica* and *indica* rice varieties. *Plant Mol. Biol.* **58**, 213–227.

Nakamura Y, **Sakurai A**, **Inaba Y**, **Kimura K**, **Iwasawa N**, **Nagamine T** (2002) The fine structure of amylopectin in endosperm from Asian cultivated rice can be largely classified into two classes. *Starch/Starke* **54**, 117–131.

Perez CM, **Juliano BO** (1978) Modification of the simplified amylose test for milled rice. *Starch/Starke* **30**, 424–426.

Sano Y (1985) Gene regulation at the *waxy* locus in rice. *Gamma Field Symposia*. Vol. 24. Institute of Radiation Breeding, NIAR, Ibaraki, Japan. pp. 63–79.

Sun YY, **Yan LB**, **Dong CL**, **Wang PR**, **Huang XQ**, **Deng XJ** (2005) Genetic relationship among *Wx* gene, AC, GC and GT of rice. *Acta Agr. Sin.* **31**, 535–539.

Tian ZX, **Qian Q**, **Liu QQ**, **Yan MX**, **Liu XF**, **Yan CJ**, **Liu GF**, **Gao ZY**, **Tang SZ**, **Zeng DL**, **Wang YH**, **Yu JM**, **Gu MH**, **Li JY** (2009) Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. *Proc. Natl. Acad. Sci. USA* **106**, 21760–21765.

Umemoto T, **Yano M**, **Satoh H**, **Shomura A**, **Nakamura Y** (2002) Mapping of a gene responsible for the difference in amylopectin structure between *japonica*-type and *indica*-type rice varieties. *Theor. Appl. Genet*. **104**, 1–8.

Umemoto T, **Aoki N**, **Lin HX**, **Nakamura Y**, **Inouchi N**, **Sato Y**, **Yano M**, **Hirabayashi H**, **Maruyama S** (2004) Natural variation in rice starch synthase IIa affects enzyme and starch properties. *Func. Plant Biol.* **31**, 671–684.

Wan JM, **Huo ZY**, **Gong ZK**, **Zhang HC**, **Lin ZC**, **Dai QG**, **Xu K** (2006) Effect of nitrogen application on main quality and RVA profile characters of hybrid rice. *Acta Agr. Sin.* **32**, 1491–1497.

Wang LQ, **Liu WJ**, **Xu Y**, **He YQ**, **Luo LJ**, **Xing YZ**, **Xu CG**, **Zhang QF** (2007) Genetic basis of 17 traits and viscosity parameters characterizing the eating and cooking quality of rice grain. *Theor. Appl. Genet.* **115**, 463–476.

Waters DL, **Henry RJ**, **Reinke RF**, **Fitzgerald MA** (2006) Gelatinization temperature of rice explained by polymorphisms in starch synthase. *Plant Biotec. J.* **4**, 115–122.

Whistler L, **BeMiller JN** (1997) Carbohydrate chemistry for food scientists. In: Whistler L, BeMiller JN, eds. *American Association of Cereal Chemists*. Inc., St. Paul, MN, USA. pp. 117–151.

Xu CW, **Mo HD** (1996) Qualitative quantitative analysis for inheritance of gelatinization temperature in *Indica* rice. *Acta Agr. Sin.* **22**, 385–391.

Yu HX, **Liu QQ**, **Xu Li**, **Lu MF**, **Yang XJ**, **Gong ZY**, **Cai XL**, **Zhang YS**, **Zhang CQ**, **Wang ZY**, **Gu MH** (2009) Quality characteristics and field performance of selectable marker-free transgenic rice with antisense *Wx* gene and improved quality derived from the elite parents of hybrid *indica* rice. *J. Cereal Sci.* **50**, 370–375.

Zheng Z, **Kawagoe Y**, **Xiao S**, **Li Z**, **Okita TW**, **Hau TL**, **Lin A**, **Murai N** (1993) 5′ distal and proximal cis-acting regulator elements are required for developmental control of a rice seed storage protein glutelin gene. *Plant J*. **4**, 357–366.

Zou Q (1995) *A Laboratory Guide for Plant Physiology and Biochemistry*. China Agricultural Press, Beijing, China. pp. 53–56.

(Co-Editor: Lixin Zhang)

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Starch content of Shuangke Zao, Nipponbare and T_2 transformed with pCAMBIA1300-*ALK*. The bar for each value represents mean $\pm$ *SD*. $n = 3$.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.