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ORIGINAL PAPER

qAC2, a novel QTL that interacts with *Wx* and controls the low amylose content in rice (*Oryza sativa* L.)

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Abstract

Key message **This manuscript reports the fine mapping of a novel QTL, *qAC2* controlling the low amylose in rice. The action mechanism of the *qAC2* is also investigated by the analysis of genetic interactions to Wx^a, Wx^b, *du1*, *du2* and *du3*.**

Abstract Amylose content of the rice (*Oryza sativa* L.) endosperm greatly affects starch properties and eating quality of cooked rice. Seeds of *japonica* rice cultivar Kuiku162 have low amylose content (AC) and good eating quality. Our analysis revealed a novel QTL, designated as *qAC2* that contributed to the low AC of Kuiku162. *qAC2* was fine mapped within a 74.9-kb region between two insertion and deletion markers, KID3001 and KID5101, on the long arm of chromosome 2. Seven genes are predicted in this region, but none of them is known to be related to the regulation of AC. The AC of a near-isogenic line (NIL110) carrying $qAC2^{Kuiku}$, the Kuiku162 allele of *qAC2*, in the genetic background of *japonica* cultivar Itadaki was lower by 1.1 % points than that of Itadaki. The chain length distributions of amylopectin were similar in NIL110 and Itadaki; therefore, the low AC of NIL110 was caused by a decrease in the actual AC, but not by a difference in the amylopectin structure. The interaction analyses revealed that $qAC2^{Kuiku}$ has epistatic interaction with Wx^a. The $qAC2^{Kuiku}$ has epistatic interactions with two loci, *du1* and *du2*, on Wx^b, whereas the genetic effect of $qAC2^{Kuiku}$ has additive to that of *du3* on Wx^b. Thus, similar to *du1* and *du2*, *qAC2* may have a function related to Wx^b mRNA splicing.

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Introduction

Starch is a major storage component of rice grains. The endosperm starch is composed of 20–30 % amylose and

70–80 % amylopectin (Juliano 1985). Amylose has a mainly linear structure with very few α-(1, 6) linkages, whereas amylopectin is highly branched (for review, see Preiss and Sivak 1996). In breeding, the amylose content (AC) needs to be taken into account, as it is the most important factor affecting the eating quality of cooked rice (Webb 1980; Juliano 1985). Rice cultivars with specific AC are required to match the preferences in different countries.

AC is a complex trait in rice (Ikeno 1914) and is controlled by many genes, such as *Waxy* (*Wx*) (Sano 1984), *Du1* (Satoh and Omura 1981), *Du2* and *Du3* (Satoh and Omura 1986), and *Du4* and *Du5* (Yano et al. 1988). The *Waxy* (*Wx*) gene encodes granule-bound starch synthase I (GBSSI), one of the enzymes involved in amylose synthesis, and is located on rice chromosome 6 (Sano 1984). In the rice, two functional alleles, Wx^a and Wx^b, are reported, Wx^b is mainly found in *japonica* cultivars, and Wx^a is found in *indica* cultivars and various wild rice species (Sano 1984; Sano et al. 1991). Wx^a and Wx^b were initially defined on the basis of the amount of their gene product (Sano 1984). The Wx^a allele produces about tenfold higher levels of mRNA and protein than those of Wx^b. As the result, AC of *japonica* cultivars is almost lower than 20 % while that of *indica* cultivars is higher than 20 %. Genetic regulation of the amylose synthesis pathway was also studied in the *dull* (*du*) mutants (Isshiki et al. 2000, 2008). The *Du1* gene encodes an mRNA splicing factor (Prp1; Zeng et al. 2007) and is located on chromosome 7; it is not allelic to *du2* (Yano et al. 1988). The *du1* and *du2* mutations reduce the Wx^b transcript levels in endosperm (Isshiki et al. 2000). The *Du3* gene encodes the rice homolog of cap-binding protein 20 kD subunit (CBP20) playing a role in pre-mRNA splicing, RNA nuclear export and nonsense-mediated decay (Isshiki et al. 2008), and is located in the middle of the long arm of chromosome 2. However, our knowledge of the functions of, and the relationships between, the genes controlling AC is still limited.

Recently, quantitative trait locus (QTL) mapping has contributed to the elucidation of the genetic basis of AC in rice. In addition to the mutant genes at the *wx* locus such as *Wx-mq* and *Wx1-1*, several other QTLs for AC have been detected (Sato et al. 2002; Ando et al. 2010). One QTL for AC each has been detected on chromosome 1 (Takeuchi et al. 2007), chromosome 3 (Li et al. 2003), chromosome 4 (Li et al. 2003), chromosome 5 (He et al. 1999; Li et al. 2003), chromosome 6 (Septiningsih et al. 2003) and chromosome 12 (Wan et al. 2004). Two QTLs each have been detected on chromosome 8 (Wan et al. 2003, 2004; Li et al. 2011) and chromosome 9 (Wan et al. 2004; Ando et al. 2010). These QTLs have not been confirmed by fine mapping, and no epistatic interactions between these genes have been reported.

Here we report a novel QTL, *qAC2*, in the near-centromere region of the long arm of chromosome 2, detected by QTL analysis using 191 BC_1F_4 lines derived from crosses between *japonica* cultivars Itadaki and Kuiku162 (a low AC line). We mapped this QTL within a 74.9-kb region by high-resolution mapping using backcross-derived progeny. The effect of *qAC2* was confirmed with the developed near-isogenic line (NIL) 110. The genetic interaction of *qAC2* was investigated with BC_3F_2 plants derived from crosses between NIL110 and four lines related to AC (Wx^a, *du1*, *du2* and *du3*).

Materials and methods

Plant materials

For QTL detection, 191 BC_1F_4 lines were developed from crosses of Itadaki/Kuiku162//Itadaki (Supplementary Figure S1). Kuiku162 is a low AC line, which developed from *japonica* cultivar Kokuhorose. All plants and lines including the parental and the BC_1F_4 lines were grown at NARO National Institute of Crop Science (Tsukubamirai, Ibaraki, Japan). The lines were seeded on April 25 and transplanted on May 27, 2005. Seeds were harvested at maturity and used for AC measurement.

For high-resolution mapping of *qAC2* (Supplementary Figure S1), a BC_3F_1 plant from the advanced backcross progeny was selected on the basis of the genotype of the simple sequence repeat (SSR) markers. The selected BC_3F_1 plant was heterozygous for only one segment that included *qAC2* in the Itadaki background. The self-pollinated progeny of the BC_3F_1 plant (125 BC_3F_2 plants) and Itadaki were seeded on July 5 and transplanted on July 31, 2006. Seeds were harvested at maturity and used for AC measurement. Among 125 BC_3F_2 plants, 13 BC_3F_2 plants were selected in which recombination had occurred between the SSR markers flanking *qAC2,* RM1313 and RM3688. The BC_3F_3 lines were produced by self-pollination of the selected 13 BC_3F_2 plants. In 2009, three BC_3F_3 lines seeded on April 28 were transplanted on May 24 (early planting), and four BC_3F_3 lines seeded on May 25 were transplanted on June 19 (late planting). In 2010, six BC_3F_3 lines seeded on April 30 were transplanted on May 21. Five non-recombinant (control; C) and five recombinant homozygous plants (tester; T) were selected from each BC_3F_3 line by marker-assisted selection of 11 SSR and seven insertion and deletion (InDel) markers in the flanking region of the QTL. Seeds were harvested at maturity and used for AC measurements. The *qAC2* genotype of each BC_3F_2 plant was determined on the basis of the mean AC of the C and T selected from each BC_3F_3 line.

To further refine the position of *qAC2*, eight BC_3F_2 plants in which recombination occurred between SSR markers RM13268 and RM13276 were selected from 1,000 BC_3F_2 plants. The BC_3F_3 lines, produced by

self-pollination of the eight BC_3F_2 plants, were seeded on April 28 and transplanted on May 25, 2011. C and T were selected from each BC_3F_3 line as above. Seeds were harvested at maturity and used for AC measurements. The *qAC2* genotype of each BC_3F_2 plant was determined on the basis of the mean AC of the C and T of each BC_3F_3 line.

To verify the genetic effects of *qAC2*, we selected one near-isogenic plant from the BC_3F_3 lines on the basis of the genotype of 109 SSR markers covering all 12 chromosomes (McCouch et al. 2002) and seven InDel markers located in the *qAC2* flanking region. The NIL (designated NIL110) was produced by self-pollination of the selected one near-isogenic plant. The NIL110 was homozygous for the Kuiku162 allele of *qAC2* ($qAC2^{Kuiku}$) in the Itadaki background. The seeds of NIL110 and Itadaki were sown on May 1 and seedlings were transplanted on May 25, 2013. Seeds of each of the five plants of NIL110 and Itadaki were harvested at maturity and used for AC measurements, physical property measurements and amylopectin structure analyses.

To investigate the genetic interactions between $qAC2^{Kuiku}$ and four loci related to AC, we crossed NIL110 ($qAC2^{Kuiku}$) with a *japonica* cultivar Nipponbare NIL7 (Wx^a) and three *japonica* cultivar Kinmaze *du* mutant lines. All the *du* mutant lines (EM12, *du1*; EM2, *du2*; and EM23, *du3*) had the Wx^b allele and were provided by the Laboratory of Plant Genetic Resources, Kyushu University, Japan. Using the SSR markers flanking *qAC2* (RM13263 and RM1211) and the *du* phenotype (for the *du* mutants), we selected BC_3F_2 plants with four genotypes: $qAC2^{Kuiku}/Wx^a$; $qAC2^{Kuiku}/du1/Wx^b$; $qAC2^{Kuiku}/du2/Wx^b$; and $qAC2^{Kuiku}/du3/Wx^b$. The BC_3F_2 seeds were sown on May 1 and seedlings were transplanted on May 25, 2013. For each genotype, seeds of five plants were harvested at maturity and used for AC measurements. AC of five plants of each genotype was used for the calculation of the mean value and standard error.

Determination of apparent amylose content

Rice seeds were dehulled, and brown rice was polished to 90 % to remove the embryo, pericarp and seed coat. Polished rice was crushed using a motor mill. The powder (100 mg) was suspended in distilled water (5 mL) for 30 min, diluted with 10 mL of 0.5 M NaOH, and left for 24 h at room temperature. Apparent AC was determined by a colorimetric method (680 nm) (Juliano 1971; Nishi et al. 2001) using a Technicon Autoanalyzer II (Bran & Luebbe, Norderstedt, Germany). The calibration line was obtained using varying ratios of purified amylose from potato (Sigma, St. Louis, MO, USA) and rice amylopectin extracted from *japonica* glutinous cultivar Kogane-mochi in the iodine solution. In this paper, apparent AC was presented as AC.

Determination of physical property

Polished rice (10 g) was added with distilled water (16 g) in an aluminum cup (55 mm × 40 mm × 55 mm). After soaking for 1 h at room temperature, the rice was cooked in an electric rice cooker (RC183, Toshiba Corporation, Tokyo). To prevent moisture loss after cooking, cup was covered with an outer sheet of aluminum foil and an inner sheet of paper for the absorption of excess vapor, and sealed in a plastic and airtight vessel. The cooked rice was kept in the vessel for 2 h at room temperature. The physical property of stickiness in a surface layer of a single cooked rice grain was measured using a Tensipresser (Myboy System, Takemoto Electric Corporation, Osaka).

Determination of the length distribution of α-1,4-glucan chains in α-polysaccharides by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The embryo, pericarp and seed coat were removed from three dehulled grains of average size and the endosperms were ground with a mortar and pestle. The resulting powder (5 mg) was suspended in methanol (5 mL) and boiled for 10 min. The homogenate was centrifuged at 2,500*g* for 5 min. The pelleted polyglucan was washed twice with 5 mL of 90 % (v/v) methanol, resuspended in 5 mL of distilled water, and boiled

Table 1 The list of InDel markers used in the fine mapping of *qAC2*

InDel marker	Position[a]	Left primer	Right primer
KID201	18134267	GGCATCAAAATATCAAATGCAA	CGGTTTTGATTGGCTTCTTC
KID3001	18294135	GAGACTACTATTCG CTAGAAAAAGACA	CAATGTTTGGATGATAACGACA
KID3401	18304760	CGTGTCCGCTACGAGGATAG	GTCTGCGACCTCTTCCACAG
KID3701	18311236	GTCCTTGCCTCGATCACG	TGACAGGAGGGAGAAGGAGA
KID4701	18342490	AACCCGTGCTGACTCAACTT	AGCCCTCCAACCGAGTATTT
KID5101	18369017	TCTAGAGCCGTGGCATAACC	TGCCAGCTAGGATATGAGAGTG
KID6101	18403523	TGATCGATCTTTTGCTTCCA	ATGACATGTTGATGCGATGG

[a] Position of InDel markers was determined using the primer sequence of against IRGSP Build 5.0 sequences

for 60 min. An aliquot (1.0 mL) of gelatinized polyglucan was added to 50 µL of 0.6 M sodium acetate buffer (pH 4.4) and 10 µL of 2 % (w/v) NaN_3, hydrolyzed with 10 µL of *Pseudomonas amyloderamosa* isoamylase (1,400 units; Seikagaku Corporation, Tokyo) at 37 °C for 24 h. The hydroxyl groups of the debranched glucans were reduced with 0.5 % (w/v) sodium borohydride under alkaline conditions for 20 h by the method of Nagamine and Komae (1996). The preparation was dried under vacuum, dissolved in 20 µL of 1.0 M NaOH for 60 min, and diluted with 180 µL of distilled water. An aliquot (25 µL) was injected into a BioLC System (model DX-500; Dionex, Sunnyvale, CA, USA) equipped with a PAD and a Carbopac PA-1 column (4-mm i.d. × 25 cm). Size fractionation of α-1,4-glucans was performed with a linear gradient of sodium acetate (50–500 mM) in 0.1 M NaOH at a flow rate of 1 mL/min.

Mapping quantitative trait loci

Linkage map

The genotypes of 191 BC_1F_4 lines and 125 BC_3F_2 plants were determined using 109 SSR markers covering all 12 chromosomes (Supplementary Table S1) (McCouch et al. 2002), and 11 SSR markers of the International Rice Genome Sequencing Project (IRGSP) genome sequence data (http://www.rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl) (Fig. 3a), respectively. The linkage and map distances were determined using MAPMAKER/EXP 3.0 software (Lander et al. 1987).

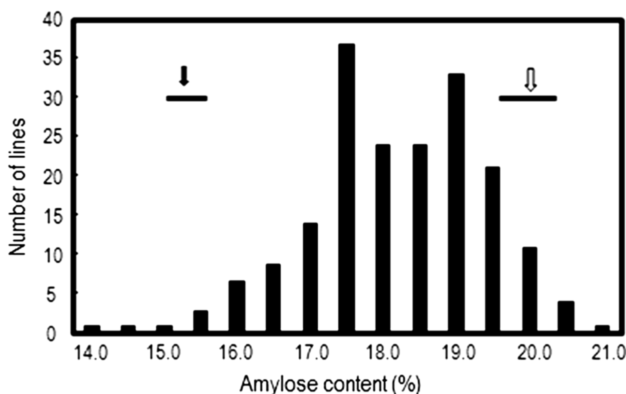

Fig. 1 Frequency distribution of amylose content in 191 BC_1F_4 (Itadaki/Kuiku162//Itadaki) lines. *Black* and *white arrows* indicate the mean values for Kuiku162 and Itadaki, respectively. *Horizontal lines* under the *arrows* indicate SD

Development of new markers

Seven InDel markers on the long arm of chromosome 2 were developed (Table 1) on the basis of the IRGSP genome sequence data. Primers were designed with the online primer design tool Primer 3 (http://frodo.wi.mit.edu/) to generate 100–300 bp amplicons at approximately 20-kb intervals across the region on the long arm of chromosome 2. The ability of primers to detect polymorphisms between Itadaki and Kuiku162 was verified.

QTL analysis

Quantitative trait locus analysis in the 191 BC_1F_4 lines and 125 BC_3F_2 plants was performed using genotype data for 109 and 11 SSR markers, respectively. Putative QTLs were detected by one-way ANOVA with SAS GLM PROC in a single-point analysis (SAS Institute, Cary, NC, USA). A threshold LOD score was determined at a significance level of 0.05 %. MAPMAKER/QTL software (the "f2 backcross" mode; Lander and Botstein 1989) was used to confirm the presence of a putative QTL and to estimate the additive effects and percentage of variance explained.

Analysis of SSR and InDel markers

Total DNA was extracted from small leaf pieces of BC_1F_4, BC_3F_2, and BC_3F_3 plants, and NIL110, and BC_2F_2 plants with four genotypes ($qAC2^{Kuiku}/Wx^a$; $qAC2^{Kuiku}/du1/Wx^b$; $qAC2^{Kuiku}/du2/Wx^b$; and $qAC2^{Kuiku}/du3/Wx^b$). Each leaf piece was crushed in a 2.0-mL tube, incubated with 1.0 mL of a solution containing 1.5 % CTAB, 75 mM Tris–HCl (pH 8.0), 1.05 M NaCl and 20 mM EDTA at 65 °C for 20 min, and centrifuged. The supernatant was mixed with 1 mL chloroform:isoamylalcohol (24:1) for 20 min and

Table 2 QTL for AC in 191 BC_1F_4lines

Chromosome	Nearest marker	1-way ANOVA		MAPMAKER/QTL		
		Probability	PVE[a]	AE[b]	PVE[a]	LOD[c]
2	RM1211	0.0001	14.0	−0.51	13.9	6.201
8	RM1235	0.0001	9.9	0.39	9.5	2.118

[a] LOD, log-likelihood value

[b] PVE, percentage of total phenotypic variance explained by the QTL

[c] AE, additive effect of Kuiku162 allele

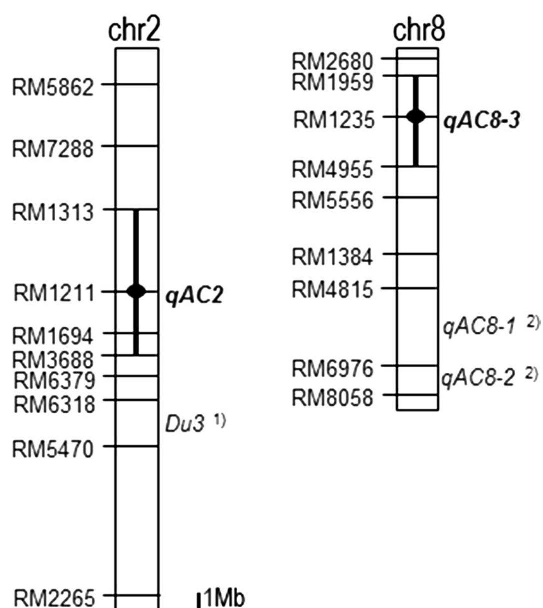

Fig. 2 Chromosomal positions of putative QTLs for amylose content detected in 191 BC_1F_4 lines. SSR markers are shown on the *left* of the map. *Black circles* indicate the nearest SSR marker loci revealed by one-way ANOVA. *Bold lines* indicate the most likely chromosomal regions for the putative QTLs within a certain confidence interval (defined by a decrease of 1.0 from the peak LOD values). [1)] Position of *Du3* as reported by Isshiki et al. (2008), [2)] Positions of *qAC8-1* and *qAC8-2* as reported by Li et al. (2011)

then centrifuged. The aqueous phase was transferred to a new 2.0-mL tube. DNA was precipitated with 1.5 mL of a buffer containing 1.0 % CTAB, 50 mM Tris–HCl (pH 8.0) and 10 mM EDTA. The pellet was washed with ethanol and redissolved in 50 µL of a buffer of 0.1 × TE containing 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. For PCR, 1 µL DNA (50 ng/µL) was combined with 0.7 µL 10 × PCR buffer (Promega, Madison, WI, USA), 0.7 µL of a solution containing 2 mM each dNTP (Boehringer Mannheim, Mannheim, Germany), 0.1 µL of 5 U *Taq* DNA polymerase (Promega), 0.3 µL of a 20 pM solution of each primer, and 3.2 µL H_2O. Amplification was performed for 30 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; followed by a final cycle at 72 °C for 7 min. Amplified fragments were separated by electrophoresis in 3.5 % agarose gel, and stained with ethidium bromide.

Results

QTL mapping for AC in BC_1F_4 lines

In 2005, AC of the parental lines was 20.2 % for Itadaki and 15.3 % for Kuiku162. The AC frequency distribution for the 191 BC_1F_4 lines is shown in Fig. 1. The BC_1F_4 lines showed a continuous AC distribution from 14.4 to 21.4 %. We detected two QTLs for AC in the 191 BC_1F_4 lines. One QTL was detected near RM1211 in the near-centromeric region of the long arm of chromosome 2 (Table 2; Fig. 2). This QTL explained 13.9 % of the total phenotypic variance (Table 2). The Kuiku162 allele at this QTL decreased AC. Previously, the *Du3* locus was mapped between the SSR marker RM3515 and the CAPS marker 5300 in the middle of the long arm of chromosome 2 (Isshiki et al. 2008). Because the QTL on chromosome 2 detected in this study was located at a different position from the *Du3* locus, we designated it as *qAC2*.

The other QTL was detected near RM1235 on the short arm of chromosome 8 (Table 2; Fig. 2). The Kuiku162 allele of this QTL increased AC. This QTL explained 9.5 % of the total phenotypic variance (Table 2). Previously, *qAC8* was also mapped on chromosome 8 between the restriction fragment length polymorphism markers G1149 and R727 (Wan et al. 2004). *qAC8* was further resolved into *qAC8-1*, mapped between the SSR markers RM7356 and RM7756, and *qAC8-2*, mapped between the SSR markers RM23510 and RM23479 (Li et al. 2011). The QTL on chromosome 8 detected in this study differed from both *qAC8-1* and *qAC8-2*, and was designated as *qAC8-3*.

Fine mapping of qAC2

In 2006, the 125 BC_3F_2 plants showed a continuous AC distribution from 19.4 to 22.7 % (Supplementary Fig. S2). One QTL *qAC2* was detected a near RM13329 in the long arm of chromosome 2. Thirteen BC_3F_2 plants were selected in which recombination had occurred between the SSR markers RM1313 and RM3688 flanking *qAC2*. The position of *qAC2* was refined using BC_3F_3 lines derived from self-pollination of 13 BC_3F_2 plants. Seven BC_3F_3 lines derived from self-pollination of seven BC_3F_2 plants recombinant in the region between RM1313 and RM3688 were grown in 2009, and six such BC_3F_3 lines were grown in 2010. In 2009, four of the BC_3F_3 lines were planted early and three were planted late. Among the seven BC_3F_2 plants with progeny grown in 2009, 09-IK5 and 09-IK6 were homozygous for the Kuiku162 allele of *qAC2*, 09-IK9 was homozygous for the Itadaki allele and four (09-IK10, 09-IK22, 09-IK7 and 09-IK20) were heterozygous (Table 3, Supplementary Table S2). Among the six BC_3F_2 plants with progeny grown in 2010, 10-IK22 was homozygous for the Kuiku162 allele of *qAC2*, 10-IK25 and 10-IK29 were homozygous for the Itadaki allele, and three (10-IK33, 10-IK35 and 10-IK36) were heterozygous. These results showed that *qAC2* is located in the region between SSR markers RM13268 and RM13276 on chromosome 2 (Table 3; Fig. 3a).

Table 3 Genotypes of DNA markers and *qAC2* of BC_aF_2plants

BC_3F_2 plants	Genotype of SSR markers[a]																		Genotype of *qAC2*[b]
	RM1313	RM13091	RM13226	RM13228	RM13268	KID201	KID3001	KID3401	KID3701	KID4701	KID 5101	KID 6101	RM 13276	RM13279	RM1211	RM13329	RM1694	RM3688	
2009 early planting																			
09-IK5	IK	IK	K	K	K	K	K	K	K	K	K	K	K	K	K	K	IK	IK	K
09-IK10	I	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	IK
09-IK22	K	K	K	K	K	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	K	IK
2009 late planting																			
09-IK6	IK	IK	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
09-IK7	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	K	IK
09-IK9	K	K	IK	IK	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
09-IK20	I	I	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	IK
2010 early planting																			
10-IK22	I	I	I	K	K	K	K	K	K	K	K	K	IK	IK	IK	IK	IK	I	K
10-IK25	I	I	I	I	I	I	I	I	I	I	I	I	I	IK	IK	I	I	I	I
10-IK29	I	I	I	I	I	I	I	I	I	I	I	I	I	IK	IK	IK	K	K	I
10-IK33	I	I	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	K	IK
10-IK35	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	K	IK
10-IK36	I	I	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	K	IK
2011 early planting																			
11-IK4	I	I	I	I	IK	IK	IK	IK	IK	IK	IK	K	K	K	K	I	I	I	IK
11-IK5	I	I	I	I	IK	IK	IK	IK	IK	IK	K	K	K	K	K	I	I	I	IK
11-IK6	I	I	I	I	IK	IK	IK	IK	IK	IK	K	K	K	K	K	I	I	I	IK
11-IK7	I	I	I	I	IK	IK	K	K	K	K	K	K	K	K	K	I	I	I	K
11-IK8	I	I	I	I	IK	IK	K	K	K	K	K	K	K	K	K	I	I	I	K
11-IK9	I	I	I	I	K	K	K	IK	IK	IK	IK	IK	IK	IK	IK	I	I	I	IK
11-IK13	I	I	I	I	K	K	K	K	K	K	IK	IK	IK	IK	IK	I	I	I	K
11-IK14	I	I	I	I	K	K	K	IK	IK	IK	IK	IK	IK	IK	IK	I	I	I	IK

[a] Genotypes of SSR and InDel markers are represented by K(Kuiku162 homozygous), I (Itadaki homozygous) and IK (heterozygous)

[b] Genotypes of *qAC2* are determined by AG in the BC_3F_3 lines (Supplementary Table S2)

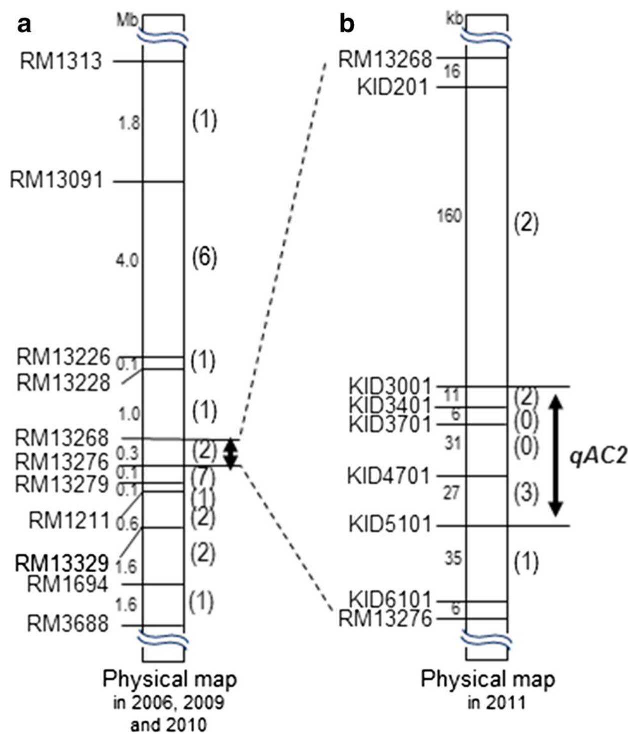

Fig. 3 Physical maps of the region surrounding *qAC2* on the long arm of chromosome 2. **a** Physical map of the region surrounding *qAC2*. Physical positions of the markers, based on the build 5.0 rice genome sequences (International Rice Genome Sequencing Project) are shown on the *left* side of the map. *Numerals in parentheses* indicate the number of recombinant BC_3F_2 plants in 2009 and 2010. **b** High-resolution physical map of the region surrounding *qAC2*. *qAC2* was localized within the 74.9-kb region between two InDel markers, KID3001 and KID5101. *Numerals in parentheses* indicate the number of recombinant BC_3F_2 plants in 2011

In 2011, the position of *qAC2* was further refined using BC_3F_3 lines derived from self-pollination of eight BC_3F_2 plants recombinant between RM13268 and RM13276. Among eight BC_3F_2 plants, 11-IK7, 11-IK8 and 11-IK13 were homozygous for the Kuiku162 allele of *qAC2*, and 11-IK4, 11-IK5, 11-IK6, 11-IK9 and 11-IK14 were heterozygous (Table 3, Supplementary Table S2). These results showed that *qAC2* is located in a 74.9-kb region between two InDel markers, KID3001 and KID5101 (Fig. 3b). This region included at least seven genes, detected using Knowledge-based *Oryza* Molecular biological Encyclopedia (KOME: http://cdna01.dna.affrc.go.jp/cDNA/). In the *qAC2* region, three genes annotated to encode glucose/ribitol dehydrogenase family, glycosyl transferase, and auxin responsive proteins. The other genes were hypothetical proteins. None of these genes encoded proteins related to starch-synthesizing enzymes.

Effect of qAC2

To verify the genetic effects of *qAC2*, we selected a near-isogenic plant from the BC_3F_3 lines by genotype analysis using 109 SSR and seven InDel markers (Fig. 4a). The NIL110 produced by self-pollination of the near-isogenic plant had the Kuiku162 allele of *qAC2* ($qAC2^{Kuiku}$) in the Itadaki genetic background. The culm length, flowering date and grain yield of NIL110 were similar to those of Itadaki (Supplementary Table S3). AC of NIL110 (18.2 %) was lower than that of Itadaki (19.3 %) ($p < 0.01$) (Fig. 4b), confirming the effect of $qAC2^{Kuiku}$. The stickiness in a surface layer of a single cooked rice grain of NIL110 ($27.3 \times 10^2 N/m^2$) was higher than that of Itadaki ($20.3 \times 10^2 N/m^2$) ($p < 0.01$) (Fig. 4c). On the other hand, chain length distribution of amylopectin in NIL110 and Itadaki was almost identical (Fig. 5).

Genetic interaction between qAC2 and other loci related to AC

To study the genetic interaction between $qAC2^{Kuiku}$ and five alleles of four other loci related to AC, we developed BC_3F_2 plants derived from crosses between NIL110 and each of four lines: Nipponbare NIL7 (Wx^a), and Wx^b lines EM12 (*du1*), EM2 (*du2*) and EM23 (*du3*). AC was similar in $qAC2^{Kuiku}/Wx^a$ (30.7 %) and Nipponbare NIL7 (30.7 %), $qAC2^{Kuiku}/du1/Wx^b$ (6.1 %) and EM12 (6.2 %), and $qAC2^{Kuiku}/du2/Wx^b$ (7.5 %) and EM2 (7.3 %), respectively (Fig. 6). AC in $qAC2^{Kuiku}/du3/Wx^b$ (11.4 %) was remarkably lower than in EM23 (12.1 %) ($p < 0.01$). These results indicate that the $qAC2^{Kuiku}$ has epistatic interaction with Wx^a. The $qAC2^{Kuiku}$ has epistatic interactions with two loci, *du1* and *du2*, on Wx^b, whereas the genetic effect of $qAC2^{Kuiku}$ has an additive to that of *du3* on Wx^b. These results indicate that the expression of Wx^a would be not affected by $qAC2^{Kuiku}$, whereas the expression of Wx^b affected.

Discussion

Identification of the novel QTL controlling low amylose content

Amylose synthesis is catalyzed by GBSSI, encoded by the *Wx* gene located on chromosome 6. AC is determined by the GBSSI level (Sano 1984; Umemoto and Terashima 2002). Wide variations in AC have been reported (Morishima et al. 1992; Juliano and Villareal 1993). Several other QTLs related to AC have also been reported. To clarify the details of the genetic control of amylose synthesis, we carried out fine mapping of a novel QTL for AC, and analyzed its genetic interaction with five other loci related to amylose synthesis.

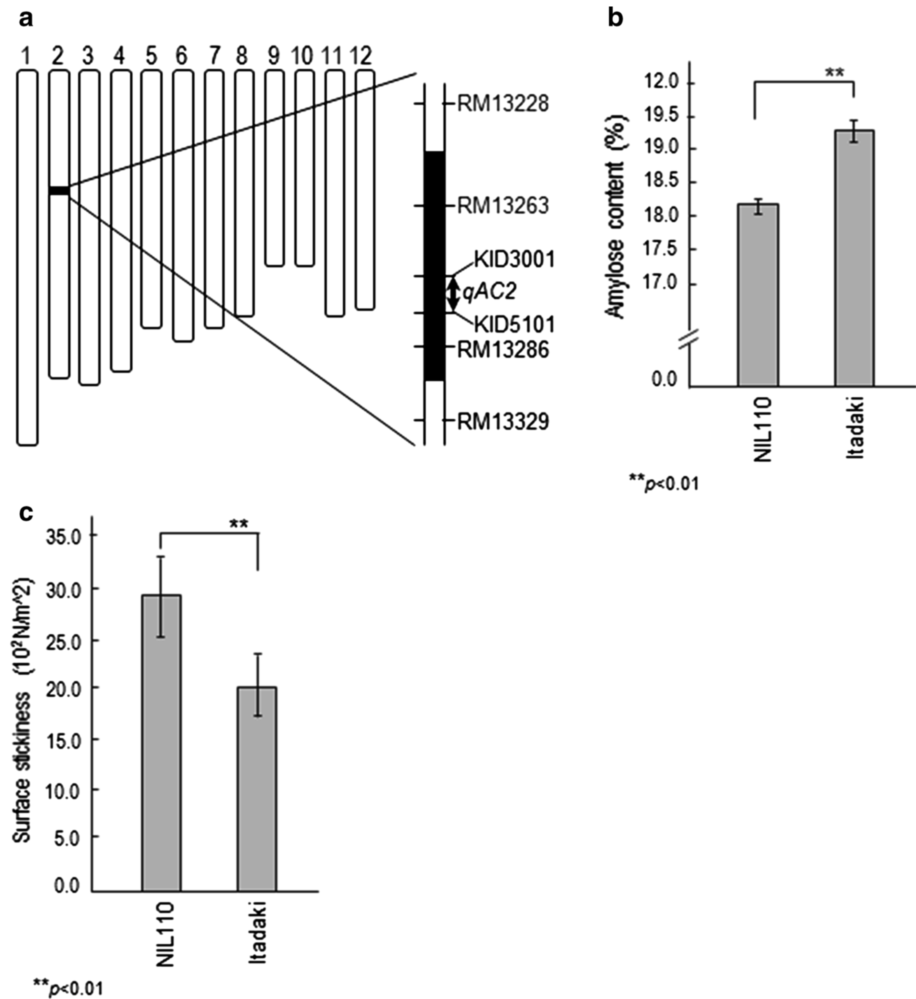

Fig. 4 Genotype and amylose content of NIL110, carrying $qAC2^{Kuiku}$. **a** Graphical genotype of NIL110. *Black block* denotes a region derived from Kuiku162; *white blocks* denote those from Itadaki. **b** Amylose content of NIL110 in comparison with Itadaki. Amylose content of NIL110 and Itadaki represent the mean score of each five plants. *Error bars* indicate SD. ** indicate significance at $p < 0.01$. **c** The surface stickiness of NIL110 in comparison with Itadaki. The surface stickiness of NIL110 and Itadaki represent the mean score of each of the 15 cooked rice grains. *Error bars* indicate SD. ** indicate significance at $p < 0.01$

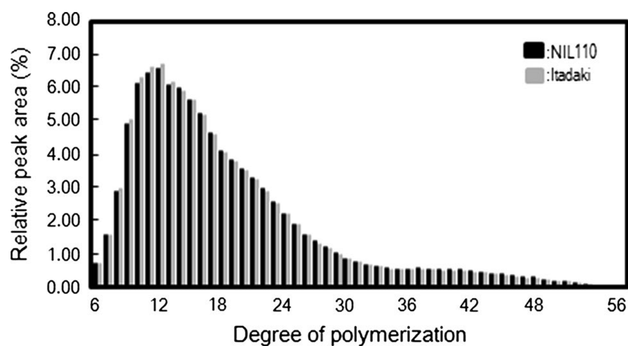

Fig. 5 Comparison of amylopectin structure between NIL110 and Itadaki. The chain length distributions of amylopectin of NIL110 and Itadaki were measured using high-performance anion exchange chromatography with pulsed amperometric detection. The chain length distributions of amylopectin of NIL110 and Itadaki represent the mean score of each of two replication

Kuiku162, used in this study, has low AC. We identified a QTL for low AC (designated *qAC2*) in this cultivar, located in a 74.9-kb region between the InDel markers KID3001 and KID5101 on the near-centromeric region of the long arm of chromosome 2. This position differed from that of the *Du3* locus, which is located in the middle of the long arm of chromosome 2 (Isshiki et al. 2008), suggesting that the *qAC2* is a novel QTL for AC. In the *qAC2* region, three annotated genes to encode glucose/ribitol dehydrogenase family, glycosyl transferase, and auxin responsive proteins and four genes annotated to encode conserved hypothetical proteins unrelated to starch biosynthesis were found using KOME.

We also identified a QTL for high AC (designated *qAC8-3*) in Kuiku162 on the short arm of chromosome 8. This position of the *qAC8-3* differed from those of the *qAC8-1* and *qAC8-2*, which are located in the long arm of chromosome 8 (Li et al. 2011). This suggests that the *qAC8-3* is a novel QTL for AC.

In this study, 109 SSR markers were mapped onto all 12 chromosomes, while a few markers were mapped on the short arm of chromosomes 4 and 10. To detect all QTLs involved in AC, more high density markers on these chromosomal regions would be required.

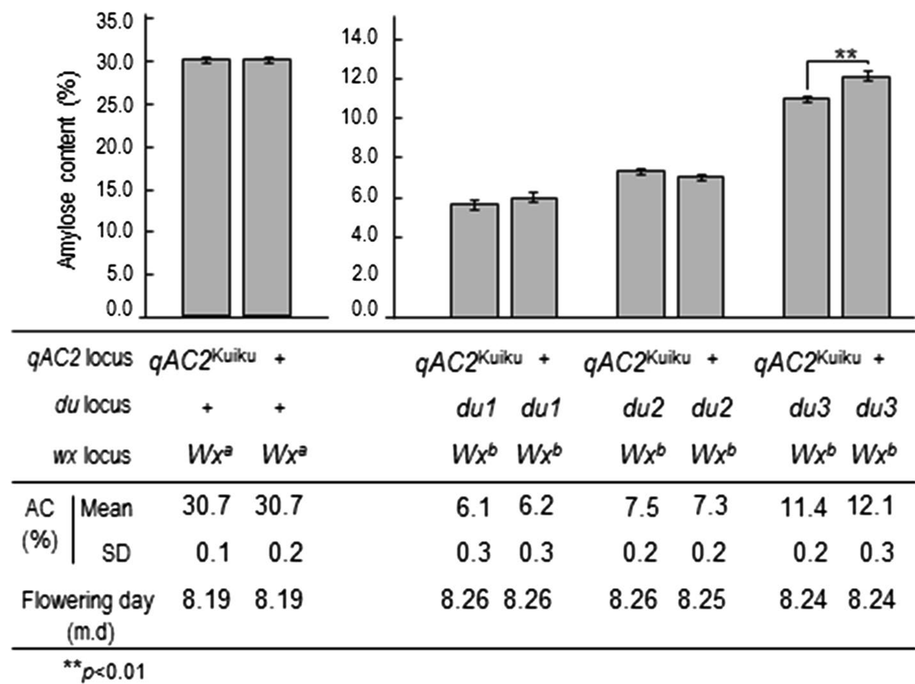

Fig. 6 Amylose content of BC_3F_2 plants derived from crosses between NIL110 and each of four lines: Nipponbare NIL7 (*Wx*^{a}), EM12 (*du1*), EM2 (*du2*) and EM23 (*du3*). Each of the progeny genotypes tested is shown below the graph. For each genotype, five plants were used, which had the same date of flowering as the parental line. *Error bars* indicate SD. ** indicate significance at $p < 0.01$

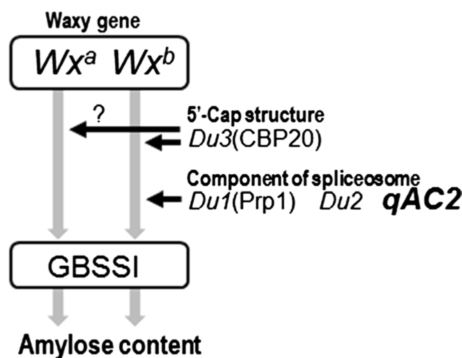

Fig. 7 Hypothetical function of *qAC2*. The *vertical arrows* show the pathway of amylose synthesis. The analysis of genetic interactions indicated the possibility that the function of *qAC2* is related to the regulation of *Wx*^{b} (like *du1* and *du2*)

Characterization of qAC2

The genetic effect of *qAC2*^{Kuiku} was verified by the development of an NIL110 with a significantly decreased AC (Fig. 4a, b). AC in the NIL110 was determined using a colorimetric method (Fig. 4b). In the *ae* mutant, AC determined by a colorimetric method is affected by altered amylopectin structure (Nishi et al. 2001; Takeda and Hizukuri 1987). As NIL110 and Itadaki in our study had similar chain length distributions of amylopectin (Fig. 5), low AC in NIL110 would be not related to the amylopectin structure. Low AC in NIL110 was probably caused by the decrease in actual AC.

Genetic control of amylose synthesis

To understand the function of *qAC2*^{Kuiku} in the amylose synthesis pathway, we investigated the genetic interaction between *qAC2*^{Kuiku} and five alleles of four loci related to amylose synthesis: *Wx*^{a}, *Wx*^{b}, *du1*, *du2* and *du3*. Our interaction analysis indicates that *Wx*^{a} has epistatic interaction with *qAC2*^{Kuiku} (Fig. 6). This result indicates that the *Wx*^{a} expression and GBSSI protein level would be not affected by *qAC2*^{Kuiku}. On the other hand, AC in NIL110 with *qAC2*^{Kuiku}/*Wx*^{b} was lower than that of Itadaki with *Wx*^{b} (Fig. 4b). This result indicates that the *Wx*^{b} expression would be affected by *qAC2*^{Kuiku}. *qAC2*^{Kuiku} also has epistatic interactions with two loci, *du1* and *du2*, on *Wx*^{b} (Fig. 6). Isshiki et al. (2000) reported similar genetic interaction between *Wx* alleles and both *du1* and *du2*; the level of the GBSSI protein in the *du* mutants decreased because of inefficient splicing of *Wx*^{b} pre-mRNA, but neither of these mutants affected the expression of the *Wx*^{a} allele. The *Du1* gene encodes the Prp1 protein, which is a component of the U4/U6 snRNP required for spliceosome assembly (Zeng et al. 2007); *du2* may be also related to mRNA splicing (Isshiki et al. 2000). The decrease in AC by *qAC2*^{Kuiku} may be caused by the spliceosome factors similar to *Du1* and *Du2* (Fig. 7). Our results also indicate that *qAC2*^{Kuiku} has an additive effect with *du3* on *Wx*^{b} (Fig. 6). *Du3* encodes the rice homolog of CBP20, which plays a role in pre-mRNA splicing (Isshiki et al. 2008). We think that the function of *qAC2* in the amylose synthesis pathway would be different from that of *Du3* (Fig. 7).

Further studies such as map-based cloning, functional analysis of *qAC2* and the analysis of its interaction with other loci related to starch synthesis would clarify the role of *qAC2* in amylose synthesis.

qAC2 as a genetic resource for breeding

Wx^a, Wx^b and *du* loci have been used as genetic resources for modification of AC. These genes strongly affect AC and starch properties, and considerable modification of AC may result in undesirable eating qualities. The effect of $qAC2^{Kuiku}$ on AC is moderate (1.1 % points). Cooked NIL110 rice is stickier than Itadaki (Fig. 4c). Thus, $qAC2^{Kuiku}$ is a useful genetic resource for the improvement of eating quality of cooked rice.

Author contribution statement Yoko Takemoto-Kuno, Keitaro Suzuki, Hideyuki Hirabayashi, Takuro Ishii, Ikuo Ando, Tokio Imbe, and Yoshinobu Takeuchi designed research; Yoko Takemoto-Kuno, Hiroki Mitsueda, Keitaro Suzuki, Hideyuki Hirabayashi, Osamu Ideta, Noriaki Aoki, Takayuki Umemoto, and Yoshinobu Takeuchi performed research; Yoko Takemoto-Kuno, Hideyuki Hirabayashi, Takayuki Umemoto, and Yoshinobu Takeuchi contributed new reagents/analytic tools; Yoko Takemoto-Kuno, and Yoshinobu Takeuchi analyzed data; Yoko Takemoto-Kuno, Keitaro Suzuki, Takayuki Umemoto, Ikuo Ando, Hiroshi Kato, Hiroshi Nemoto, Tokio Imbe, and Yoshinobu Takeuchi wrote the paper.

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Conflict of interest The authors declare that they have no conflict of internet.

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