

Research Paper

Thermo-responsive allele of *sucrose synthase 3 (Sus3)* provides high-temperature tolerance during the ripening stage in rice (*Oryza sativa* L.)

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High-temperature stress during the ripening stage leads to quality deterioration due to an increase in chalky grains in brown rice (*Oryza sativa* L.). In a previous study, we identified a QTL for *Appearance quality of brown rice 1 (Apq1)* using chromosome segment substitution lines of the *indica* cultivar ‘Habataki’ in the *japonica* cultivar ‘Koshihikari’ background and narrowed down the locus to a 48-kb region on chromosome 7. To verify the function and mechanisms of this QTL in grain appearance, in this study, we fine-mapped the gene and conducted high-temperature tolerance tests. As a result of the genetic mapping, we narrowed down the candidate region of *Apq1* to a 19.4-kb region including three predicted genes. Among these, the temporal expression pattern of *sucrose synthase 3 (Sus3)* corresponded well with the high temperature-sensitive period during ripening, and expression of the ‘Habataki’ allele of *Sus3* was increased under high-temperature condition. In addition, we transformed the ‘Habataki’ *Sus3* gene into ‘Nipponbare’, and the transformants obtained high-temperature tolerance. Therefore, we conclude that the causal gene underlying the QTL *Apq1* is the thermo-responsive *Sus3* allele, and the increase in *Sus3* expression under high-temperature condition during ripening leads to high-temperature tolerance in rice.

Key Words: *Apq1*, appearance quality, high-temperature tolerance, *sucrose synthase 3*, rice (*Oryza sativa* L.).

Introduction

The higher average temperatures caused by global warming affect crop yields. Lyman *et al.* (2013) estimated that a 1°C increase in average growing season temperature reduces total rice milling revenue by 8.1% to 11.0%. The reduction in brown rice production caused by high temperatures is due to spikelet sterility and decreases in rice weight and quality (Folsom *et al.* 2014, Hakata *et al.* 2012, 2017, Morita *et al.* 2016, Peng *et al.* 2004). A high temperature during the rice ripening period causes an increase in grain chalkiness (Terashima *et al.* 2001). Chalky grain phenotypes include white back, basal white, milky white, white core, and white belly (Ebata 1961, Nagato and Ebata 1965, Tashiro and Ebata 1975). In Japan, the market value of brown rice is determined by grain appearance, particularly, the ratio of

chalky grains. Therefore, the development of novel cultivars with tolerance to high temperature during ripening is required.

Several studies have identified quantitative trait loci (QTLs) or genes related with chalky grain phenotypes. *Chalk5*, encoding a vacuolar H⁺-translocating pyrophosphatase, was detected as a major QTL for grain chalkiness (Li *et al.* 2014). QTLs for white-back, basal-white, and milky-white grains have also been reported (Ebitani *et al.* 2008, Kobayashi *et al.* 2013, Miyahara *et al.* 2017, Tabata *et al.* 2007, Wada *et al.* 2015, Wakamatsu *et al.* 2010). Yamakawa *et al.* (2007) and Hakata *et al.* (2012) observed increased expression of the α -amylase genes *Amy1A*, *Amy1C*, *Amy3A*, and *Amy3B* during ripening under high temperature and showed that the suppression of these genes decreases the chalky grain rate. Although several chalky grain-related genes have been identified, the molecular mechanism underlying chalky grains is still incompletely understood.

Previously, we identified a QTL for appearance quality of brown rice (*Appearance quality of brown rice 1, Apq1*) on chromosome 7 by using chromosome segment substitution

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lines of the *indica* cultivar ‘Habataki’ in the *japonica* cultivar ‘Koshihikari’ genetic background (Murata *et al.* 2014). We also demonstrated that the nearly isogenic line (NIL) of *Apq1* (*Apq1*-NIL) showed a significantly higher percentage of perfect grains (PPG) under field condition, and the candidate region of *Apq1* was delineated to a 48-kb region. Further, *Apq1*-NIL showed higher tolerance to high-temperature stress than ‘Koshihikari’ during the ripening period, with reduced ratios of basal-white and white-back grains. In the current study, we carried out further fine mapping to identify the causal gene of the QTL and we determined the high temperature-sensitive period during ripening to understand the mechanisms of high-temperature tolerance of *Apq1*.

Materials and Methods

Plant materials and growth conditions

Apq1-NIL was developed in our previous study (Murata *et al.* 2014) and it possesses less than a 2.8-Mb ‘Habataki’ chromosome segment (spanning 25.8–28.6-Mb on chromosome 7) of *Apq1* locus in the ‘Koshihikari’ genetic background. Two recombination lines were screened using two PCR markers, Tak6166-3 and RM21971, from approximately 10,000 F₂ plants derived from a cross between ‘Koshihikari’ and *Apq1*-NIL and the genotypes of these plants were determined by using markers ERF2-1, ERF2-2, and AP2-indel. The sequences of the primers used for genetic mapping are shown in **Supplemental Table 1**. The seedlings used for genetic mapping were transplanted to a paddy field at the Toyama Agricultural Research Institute in Toyama, Japan (36.4°N, 137.1°E) at the late April and were grown under natural climate condition until yielding. The average temperature of the ripening period was 27.1°C. Seedlings of ‘Koshihikari’ and *Apq1*-NIL were transplanted to a paddy field at the Research Center for Bioresources Development in Fukui, Japan (36.2°N, 136.2°E) at the beginning of May and were grown under natural climate condition. These plants were transferred from the paddy field into a low-temperature climate chamber (25°C from 07:00 to 19:00 and 23°C from 19:00 to 07:00) at 7 days before heading. Transgenic plants were grown in a closed climate chamber at 28°C from 09:00 to 18:00 and 25°C from 18:00 to 09:00 until one week before heading. To test high-temperature tolerance during the ripening stage for Koshihikari, *Apq1*-NIL and transgenic plants, plants were grown at low (25°C from 07:00 to 19:00 and 23°C from 19:00 to 07:00) and high temperatures (34°C from 07:00 to 19:00 and 26°C from 19:00 to 07:00) until yielding.

To delineate the high temperature-sensitive period, we subjected plants to three days of high-temperature treatment. At least 100 spikelets/plant were marked at the flowering day under the low-temperature condition. The start of the three-day treatment varied from 1 DAF to 30 DAF, and after treatment, the plants were returned to the low-temperature condition and grown until yielding. Three plants were used for each treatment.

Phenotype evaluation

Perfect grains were defined as non-chalky, with a normal shape. Grains with unusual shapes or colors were left out of the evaluation. Chalky grains were classified as basal-white kernels, white-back kernels, white-berry kernels, milky-white kernels, or white-core kernels as described by Ebitani *et al.* (2008). At least 100 grains per plant and three biological replicates were used for evaluation.

Production of transgenic plants

The genomic clone of *Sus3*^{Haba} was amplified using the primers Tak6166-3-Asc1-U (5'-ATGGCGGCCACATCCGATAATGGAGTCGA-3') and Asc1-Sus3-L (5'-GTAAGCGCCAAGCTCGCTGAGGCGCGCCAT-3') with PrimeSTAR Max DNA Polymerase (TAKARA Bio, Tokyo, Japan) and was cloned into the *AscI* site of the binary vector pYL-TAC7 (Liu *et al.* 1999), provided by the RIKEN BioResource Center. This clone contains a 6195-bp fragment upstream from the transcriptional start site of the *Sus3* gene and a 757-bp fragment downstream from the end of the *Sus3* 3'UTR. The binary vector was transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood 1993) by electroporation, and ‘Nipponbare’ plants were transformed as reported previously (Ashikari *et al.* 2005). ‘Nipponbare’ plants transformed with empty vector were used as controls. Transgenic plants carrying the transgene were selected by PCR using hygromycin resistance gene (*HPT*)-specific primers, HPT-L: 5'-CGTATATGCTCCGCATTGGT-3' and HPT-R: 5'-ATTTGTGTACGCCCGACAGT-3'.

RNA isolation and quantitative reverse-transcription (qRT)-PCR

Total RNAs were extracted from the ovary at 0 day after flowering (DAF), endosperm (immature grain includes endosperm and embryo) at 1, 4, 7, 10, 13, 16, 19 DAF, and flag leaf at 4 DAF, using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized from total RNA using SuperScript III (Invitrogen, Carlsbad, CA, USA). For quantification of mRNA, qRT-PCR was carried out using SYBR Premix Ex Taq™ II (TAKARA Bio). Primer sequences used for qRT-PCR are shown in **Supplemental Table 2**. *OsUbi1* was used as an internal control. The Thermal Cycler Dice Real Time System (TAKARA Bio) was used for qRT-PCR.

Promoter-swap analysis

Promoter sequences of *Sus3* were amplified with the primers BamHI-pSus3-U (5'-ggatccCAGTTTAAACCGCTAATAGTCA-3') and pSus3-L (5'-AGAAGACAGCCACAGCTCA-3') using PrimeSTAR Max DNA Polymerase and genomic DNA of ‘Koshihikari’ and ‘Habataki’ as templates. Coding sequences of *Sus3* were amplified with the primers *Sus3*-mRNA-5UTR-U (5'-GCATCCATCGGTTCTCTGCT-3') and *Sus3*-mRNA-3UTR-NosT-L (5'-ttgaacgacCGGGTAAGGCCAGATCATT-3') using PrimeSTAR Max DNA Polymerase and cDNA of ‘Koshihikari’ and

‘Habataki’ as templates. The Nos terminator sequence was amplified with the primers Sus3-Nos-T-U (5′-GCCTTAC CCGgatcgttcaaacatttgca-3′) and NosT-Not1-L (5′-GCG GCCGCCCCgatctagtaacatagat-3′) using PrimeSTAR Max DNA Polymerase and pYL-TAC7 DNA as a template. The primers Sus3-mRNA-3UTR-NosT-L and Sus3-Nos-T-U were designed to overlap each other by 20 bp. The promoter sequences were cloned into the pCR4 vector using Zero Blunt TOPO PCR Cloning Kit for Sequencing (Life Technologies, CA, USA). The coding sequences and Nos terminator sequence were fused by PCR using PrimeSTAR max DNA Polymerase and the concatenated product was cloned into the pCR4 vector using Zero Blunt TOPO PCR Cloning Kit for Sequencing. Promoter and coding + Nos terminator sequences were digested using the restriction enzymes *Bam*HI and *Not*I, respectively. The fragments obtained were cloned into *Bam*HI- and *Not*I-digested pYL-TAC7 vector. Transgenic plants carrying the transgene were selected by PCR using the *HPT*-specific primers described above.

Results

Apq1 reduces the basal-white and white-back grain ratios

To confirm the function of *Apq1* in grain appearance, we compared the appearance ratios of perfect grains and each type of chalky grains between ‘Koshihikari’ and *Apq1*-NIL plants grown under low and high temperatures during ripening. Both ‘Koshihikari’ and *Apq1*-NIL showed high PPG under low-temperature condition (89% and 98%, respectively) (Fig. 1A). In line with our previous report, the PPG of ‘Koshihikari’ decreased to 25%, while that of *Apq1*-NIL remained high (81%), under high temperature (Fig. 1A). Consistently, the basal-white and white-back grain ratio was restored in *Apq1*-NIL (Fig. 1A). To delineate the high temperature-sensitive period, we subjected plants to three

days of high-temperature treatment throughout ripening. As a result, we observed the highest percentage of basal-white and white-back (PBW) kernels in ‘Koshihikari’ exposed to high temperature at 3 DAF (Fig. 1B). *Apq1*-NIL showed significantly lower PBW at 3 DAF. This result indicates that PBW in ‘Koshihikari’ is the most sensitive to high temperature at 3–5 DAF, and the QTL *Apq1* might provide high-temperature tolerance in this period.

Genetic mapping of Apq1

To determine the causal gene of *Apq1*, we selected two plants possessing chromosome recombination in the previously delineated candidate 48-kb region between PCR markers Tak6166-3 and RM21971 from F₂ plants derived from a cross between ‘Koshihikari’ and *Apq1*-NIL (Fig. 2A). For finer gene mapping, we selected the homozygous genotypes from each F₃ line. By comparing the PPG of the two genotypes, the candidate region of *Apq1* was narrowed down to a 19.4-kb region between Tak6166-3 and ERF2-2, and this region harbors three predicted genes in the ‘Nipponbare’ genome (Fig. 2A, 2B, Rice Genome Annotation Project, <http://rice.plantbiology.msu.edu/>). According to gene prediction, LOC_Os07g42490, LOC_Os07g42500, and LOC_Os07g42510 encode sucrose synthase 3, FYVE zinc finger domain-containing protein, and AP2 domain-containing protein, respectively. As a result of DNA sequencing of this 19.4-kb candidate region of ‘Koshihikari’ and ‘Habataki’ and gene prediction using FGENISH (<http://www.softberry.com/berry.phtml>, Solovyev *et al.* 2006), we revealed that there are no variety specific genes, and detected amino-acid substitutions in the coding regions of LOC_Os07g42490 and LOC_Os07g42510 between the varieties. In LOC_Os07g42500, only a synonymous substitution was detected.

To determine when these three genes are expressed, qRT-

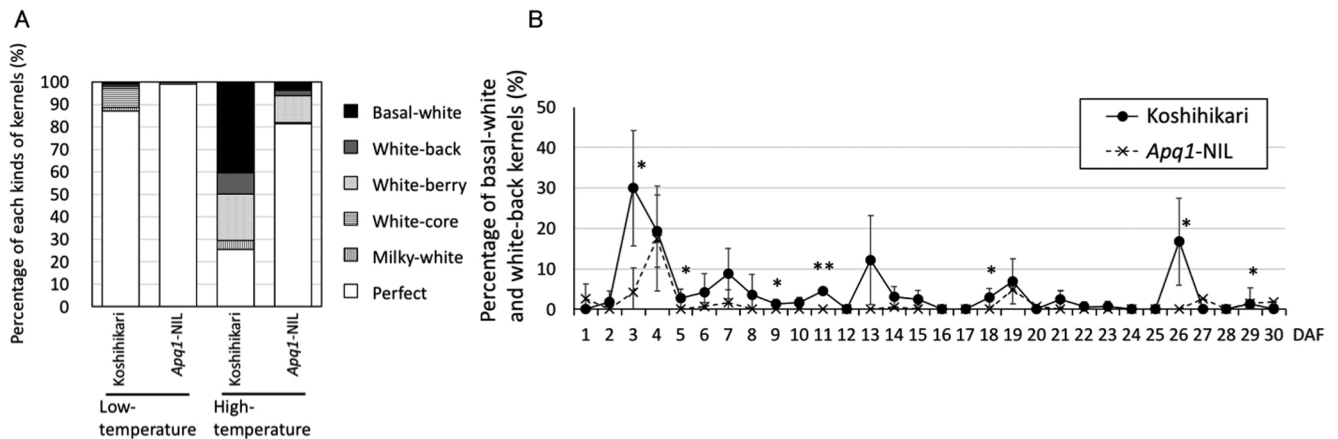


Fig. 1. Confirmation of the role of *Apq1* in high-temperature tolerance. (A) Percentages of indicated kernel phenotypes of ‘Koshihikari’ and *Apq1*-NIL grown under low- and high-temperature conditions during the ripening stage. (B) Percentages of basal-white and white-back kernels, measured to compare high-temperature tolerance. Each spikelet was marked at opening and fertilization day under low-temperature condition and incubated for three days under high temperature. The numbers in the X-axis indicate initial day of three days high-temperature treatment. Error bars indicate standard deviation. ** $p < 0.01$, * $p < 0.05$ (Student’s *t*-test).

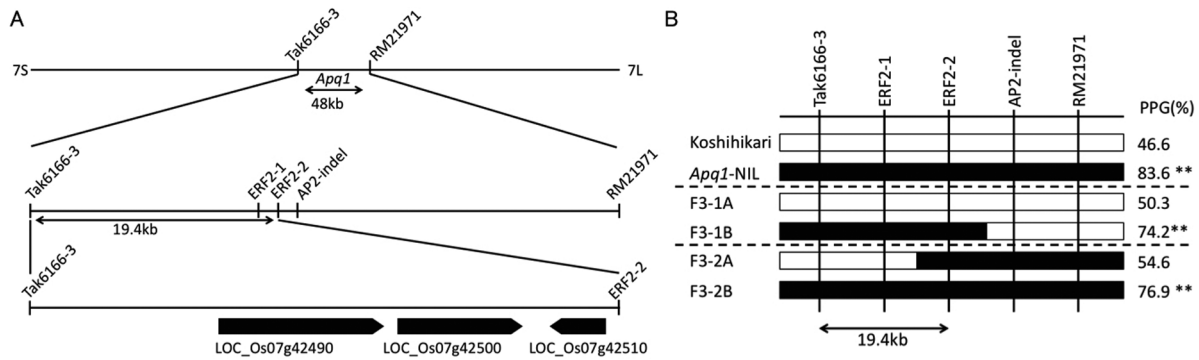


Fig. 2. Genetic mapping of *Apq1*. (A) Location of *Apq1* on the genetic map. Box arrows indicate the predicted gene locus. (B) Genotypes and phenotypes of the recombinants. White and black bars indicate homozygous chromosomes of ‘Koshihikari’ and ‘Habataki’, respectively. ** $p < 0.01$, * $p < 0.05$ (Student’s *t*-test).

PCR was performed using RNA extracted from ovaries and endosperms at various developmental stages. LOC_Os07g42490 was highly expressed from the early endosperm developmental stages and its expression reached a peak at 4 DAF (Fig. 3A). Although the expression level of LOC_Os07g42490 was not different between ‘Koshihikari’ and *Apq1*-NIL grown under low-temperature condition, significantly higher expression was detected in *Apq1*-NIL than in ‘Koshihikari’ under high temperature at 4 and 7 DAF (Fig. 3A). LOC_Os07g42500 and LOC_Os07g42510 showed no significant differences at any developmental stage (Fig. 3B, 3C). Because LOC_Os07g42490 expression peaked at 4 DAF, which corresponds with the high-temperature sensitive period, and was promoted by high

temperature, we speculated that LOC_Os07g42490, *sucrose synthase 3* (*Sus3*), is the causal gene of *Apq1*.

Complementation test of *Apq1*

Based on genome sequence comparison, we detected 21 polymorphisms around the *Sus3* gene between ‘Koshihikari’ and ‘Habataki’ (Fig. 4A). Six single nucleotide polymorphisms were detected in the coding sequence of *Sus3*, four of which result in amino-acid substitutions. These four amino-acid changes are not located in any conserved domain of sucrose synthase (Huang *et al.* 2016). Six polymorphisms were detected in the region upstream from the first ATG, including the UTR, and in an intron of *Sus3*. To confirm that *Sus3* is the causal gene for *Apq1*, we cloned a 12.5-kb fragment of the ‘Habataki’ allele of *Sus3* (*Sus3*^{Haba}), containing all polymorphisms, into the binary vector pYL-TAC7 and transformed the construct into plants of the *japonica* cultivar ‘Nipponbare’ by the *Agrobacterium*-mediated method. The *Sus3* gene sequence of ‘Nipponbare’ is identical to that of ‘Koshihikari’. All *Sus3*^{Haba}-transgenic plants showed 0% PBW after high-temperature treatment during ripening, while basal-white and white-back kernels appeared in most empty-vector control plants (Fig. 4B). Therefore, we concluded that *Sus3* is the causal gene of *Apq1*, and polymorphisms detected in the ‘Habataki’ allele of *Sus3* offer tolerance to high temperature during the ripening stage. Furthermore, we measured 1,000-grain weight of these transgenic plants because we observed that it was slightly decreased in *Apq1*-NIL (Murata *et al.* 2014). As a result, *Sus3*^{Haba}-transgenic plants showed significantly lower 1,000-grain weight than that of Empty vector plants (Fig. 4C).

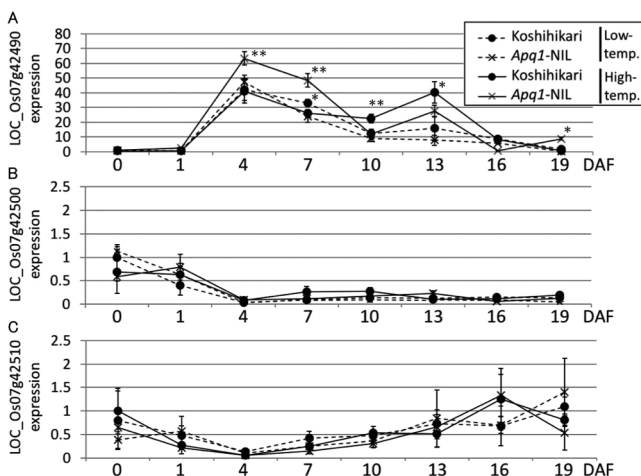


Fig. 3. Expression analysis of *Apq1* candidate genes. Relative expression of three candidate genes of *Apq1* in endosperms grown under low- and high-temperature conditions was calculated using *OsUbi1* as an internal control and was normalized to the expression level in samples of ‘Koshihikari’ at 0 DAF. LOC_Os07g42490, LOC_Os07g42500, and LOC_Os07g42510 are shown in (A), (B), and (C), respectively. Error bars indicate standard deviations. Student’s *t*-test was performed between ‘Koshihikari’ and *Apq1*-NIL under the same temperature condition. ** $p < 0.01$, * $p < 0.05$.

Sus3 promoter-swap analysis

To determine whether polymorphisms detected in the region upstream of the first ATG of *Sus3* are involved in tolerance to high temperature, we performed a promoter-swap analysis using four constructs. First ATG-upstream regions were cloned from genomic DNA of ‘Koshihikari’ (pKoshi) and ‘Habataki’ (pHaba) and fused to coding regions cloned

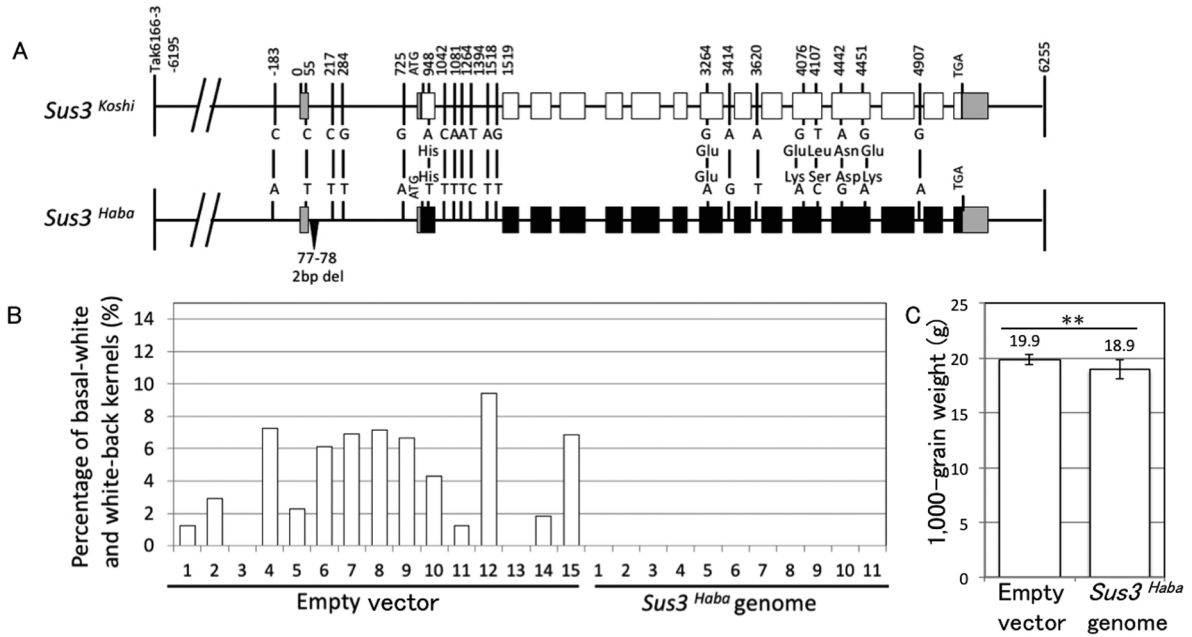


Fig. 4. Complementation test of *Apq1*. (A) DNA sequence polymorphisms of *Sus3* in 'Koshihikari', *Sus3*^{Koshi}, and 'Habataki', *Sus3*^{Haba}. White and black boxes indicate protein-coding regions of 'Koshihikari' and 'Habataki', respectively. Gray boxes indicate UTRs. Numbers indicate positions of polymorphism from the transcription start site of *Sus3*. (B) High-temperature treatment test of *Sus3*^{Haba}-transformed plants. Empty vector- and *Sus3*^{Haba}-transformed 'Nipponbare' were grown under high-temperature condition during ripening stage. The percentages of basal-white and white-back kernels were measured to compare high-temperature tolerance. Independently obtained empty vector-transformed ($n = 15$) and *Sus3*^{Haba}-transformed ($n = 11$) plants were tested. (C) Average of 1,000-grain weight (dry-weight) of transgenic plants. ** $p < 0.01$ (Student's *t*-test).

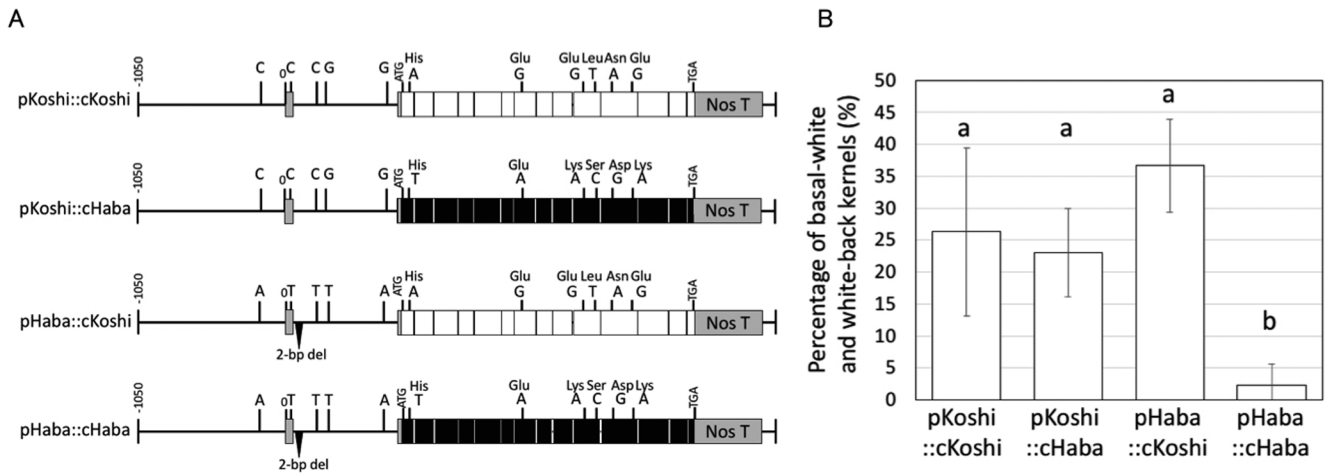


Fig. 5. Promoter-swap analysis of *Sus3*. (A) Vector constructs for promoter-swap analysis. Promoter sequences of *Sus3*^{Koshi} (pKoshi) and *Sus3*^{Haba} (pHaba) were fused to the coding cDNA sequences of *Sus3*^{Koshi} (cKoshi) and *Sus3*^{Haba} (cHaba). All four constructs were fused with the Nos terminator at the end of the coding sequence. (B) High-temperature treatment test of promoter-swap plants. The four constructs indicated in (A) were transformed into 'Nipponbare'. Transformants were grown under high-temperature condition during the ripening stage. The percentages of basal-white and white-back kernels were measured to compare high-temperature tolerance. Error bars indicate standard deviations. Different letters above the bars indicate a significant difference at 5% (Tukey's HSD test), $n > 3$.

from cDNA of 'Koshihikari' (cKoshi) and 'Habataki' (cHaba) (Fig. 5A). Interestingly, only pHaba::cHaba plants showed low PBW among the four types of transgenic plants (Fig. 5B). This result indicated that both the promoter and the coding region of the 'Habataki' allele of *Sus3* are re-

quired for high-temperature tolerance during ripening. From these results, we concluded that *Apq1* provides high-temperature tolerance via higher expression of *Sus3* at the early stage of endosperm development.

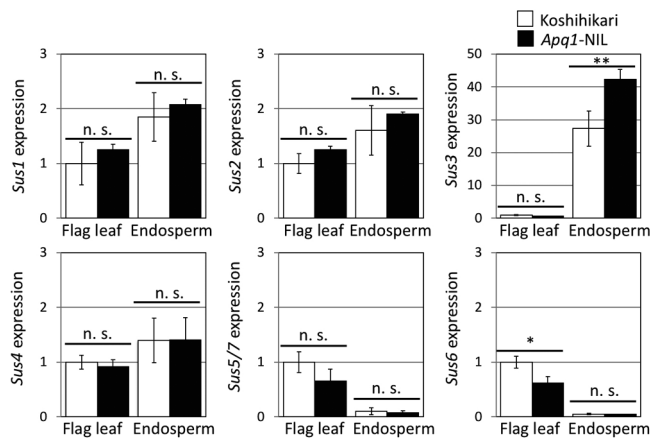


Fig. 6. Expression analysis of *Sus* genes in rice. Expression levels of all seven *Sus* genes in flag leaf and endosperm under the high-temperature condition were compared at 4 DAF. White and black bars indicate ‘Koshihikari’ and *Apq1*-NIL, respectively. Because of the highly similar mRNA sequences of *Sus5* and *Sus7*, the expression was measured using a common sequence primer set. Gene expression was calculated using *OsUbi1* as an internal control and was normalized to the expression level in the flag leaf of ‘Koshihikari’. Error bars indicate standard deviations. ** $p < 0.01$, * $p < 0.05$ (Student’s *t*-test), $n > 3$.

Expression analysis of other *Sus* genes

The rice genome contains seven conserved *Sus* genes, which are suspected to have compensatory functions (Cho *et al.* 2011, Hirose *et al.* 2008). We analyzed the expression of all *Sus* genes under high-temperature condition at 4 DAF. While *Sus6* was significantly lower expressed in the flag leaf of *Apq1*-NIL than in that of ‘Koshihikari’, the *Sus* genes did not show significant differences in expression in the endosperm between ‘Koshihikari’ and *Apq1*-NIL at 4 DAF stage, except for *Sus3* (Fig. 6). This result indicated that only *Sus3*^{Haba} is responsive to high temperature and substantiated that this gene is responsible for the high-temperature tolerance during ripening conferred by *Apq1*.

Discussion

Sus3 confers high-temperature tolerance during the ripening stage in rice

In this study, *Sus3*^{Haba} was demonstrated to provide high-temperature tolerance during the ripening stage in the *japonica* genetic background. *Sus* is a key enzyme that catalyzes the first step of starch synthesis, namely, the conversion of sucrose and uridine diphosphate (UDP) into fructose and UDP-glucose, in the rice endosperm (Huang *et al.* 2016). *Sus* enzyme activity was reported to lower under high-temperature conditions in rice and maize (Chaturvedi *et al.* 2017, Wilhelm *et al.* 1999). The higher expression of *Sus3*^{Haba} might compensate for the reduced activity of *Sus* under high temperature. In a previous study, we observed slightly decreased 1,000-grain weight and culm length (Murata *et al.* 2014). We detected smaller 1,000-grain weight of *Sus3*^{Haba}-transgenic plants and this is consistent

with the previous study (Fig. 4C). Hence, *Apq1* seems to slightly decrease grain weight also under high-temperature stress conditions. Although we did not focus to culm length or other agronomic traits of *Apq1* in this study, the information on the pleiotropic effect is also important. We will clarify this point in future studies.

Sus3 polymorphisms underlying high-temperature tolerance

In this study, we detected 21 polymorphisms around the *Sus3* gene and we found that both the promoter and the coding region are required for high-temperature tolerance (Fig. 5B). Although four amino-acid substitutions were detected, we could not conclude which substitution is important for high-temperature tolerance because none of the four substitutions are in the conserved domain of *Sus*. However, since pHaba::cHaba plants acquired high-temperature tolerance, at least polymorphisms in the intron regions might not be important. We identified five single nucleotide polymorphisms and a 2-bp deletion upstream of the first ATG of *Sus3* between ‘Koshihikari’ and ‘Habataki’ (Fig. 4A). One of these polymorphisms might be involved in the high expression of *Sus3* in response to high temperature.

Application of *Apq1* in breeding programs

Apq1 is predicted to be an economically important QTL to increase the PPG of the elite rice variety ‘Koshihikari’. Because its effect was confirmed in ‘Nipponbare’ background, *Apq1* might be applicable in a wide range of *japonica* rice varieties. Although *Apq1* was originally identified from ‘Habataki’, an *indica* variety, ‘Habataki’ shows low PPG (Murata *et al.* 2014). Thus, the effect of *Apq1* seems to depend on the genetic background. In this study, although we succeeded in identifying the thermo-responsive *Sus3* as the causal gene of *Apq1*, the underlying mechanism is still unclear. Clarifying this mechanism should further contribute to the application of high-temperature tolerance in future breeding programs.

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