Whole-Genome Sequencing Revealed a Late-Maturing Isogenic Rice Koshihikari Integrated with *Hd16* Gene Derived from an Ise Shrine Mutant

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Received 27 May 2021; Revised 24 July 2021; Accepted 26 July 2021; Published 6 January 2022

Academic Editor: Vijay Gahlaut

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We identified the key genes controlling the late maturation of the Japonica cultivar Isehikari, which was found at Ise Jingu Shrine and matures 6 days later than Koshihikari. We conducted a genetics-based approach through this study. First, the latest mature plants, which flowered later than Isehikari, were segregated in the F2 and F3 generations of Koshihikari × Isehikari. Next, the linkage relationship of a single late-maturing gene with the SSR markers on the long arm of chromosome 3 was inferred by using late-maturing homozygous F2 segregants. Moreover, genetic analyses of late maturity were conducted through the process of six times of continuous backcross with Koshihikari as a recurrent parent by using the late-maturing homozygous F3 line as a nonrecurrent parent, thus developing a late-maturing isogenic Koshihikari (BC6F2). As a result, we elucidated a single late-maturing gene with incomplete dominance that caused the 14-day maturation delay of Koshihikari. The whole-genome sequencing was conducted on both of Koshihikari and the late-maturing isogenic Koshihikari. Then, the SNP call was conducted as the reference genome of Koshihikari. Finally, a single SNP was identified in the key gene *Hd16* of the late-maturing isogenic Koshihikari.

1. Introduction

Rice is cultivated worldwide, particularly in Asia, and is one of the world’s top three grains with an annual yield of 680 million tons, alongside corn (1.73 billion tons) and wheat (680 million tons) [1]; therefore, stable production is crucial. The earth has warmed by approximately 1.0°C from preindustrial levels, and temperatures are predicted to rise another 1.5°C between 2030 and 2052 [2]. In the IPCC’s 6th evaluation report, global warming is expected to bring about an increase in the frequency of strong tropical cyclones, and there are concerns that damage from heavy rains will be magnified [3, 4]. Rice has suffered from lodging damage, yield reduction, lowered grain quality by ear germination, and lowered production efficiency, which are brought by serious strong winds and rain [5]. Furthermore, if the average daily temperature exceeds 23°C–24°C during the 20 days after heading, a white immature grain arises [6]. The white immature grain occurs when warm nights lead to increased respiration, causing a reduction in nitrogen content, and consequently reducing the transportation of photosynthetic products (sugars), which serve as a substrate for starch, to the panicles; thus, it results in an imbalance in nutrient supply and demand [7–9]. At 27°C, both white-back immature grains and milky-white immature grains arise; at 30°C, white-back immature grains occur frequently; and at 33°C, milky-white immature grains frequently happen [10]. Climate changes over recent years have resulted in record-breaking heat waves, with a spread in the deterioration of rice quality. In 2011, 170,000 tons of rice, or 21% of the total

Hindawi
International Journal of Genomics
Volume 2022, Article ID 4565977, 12 pages
https://doi.org/10.1155/2022/4565977

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production volume, suffered high-temperature damage [11]. The proportion of 1st-grade quality rice has been greatly reduced over the last 4 years from 78.8%–85.7% to 62.4% [12]. This is because the leading variety Koshihikari, which comprises 37.3% of rice acreage in Japan [13], heads and ripens in the high-temperature phase in August. In 2010, when the average temperature in August was approximately 2.25°C higher than the yearly average, the grain quality noticeably degraded, and the proportion of 1st-grade rice was 23.1% lower than in 2009 [14]. In 2018, disastrous rainfall represented as “Heavy Rain in July, Heisei 30”, large typhoons, and heat wave over 40°C caused poor filling and widespread yield reduction in Japan [15–19]. Koshihikari is globally valued and produced including in the United States and Australia. To continue the commercial trend of Koshihikari, it is necessary to avoid heading and ripening during the high temperature phase by genetically altering rice maturation to the early or late phase. Rice production industries also request late-maturing varieties instead of Koshihikari to avoid high temperature ripening. Modifications in day length responsiveness enable selecting regionally adaptive new genotypes and dispersing the current overconcentration on Koshihikari.

Isehikari was discovered in 1989 in Ise Jingu Shrine in Ise City in Mie Prefecture, when a typhoon caused the large-scale lodging of Koshihikari in the shrine’s rice fields, and only Isehikari remained standing [20, 21]. This cultivar can withstand typhoon wind speeds exceeding 50 m/s, and it matures 10 days later than Koshihikari [22, 23]. In terms of yield, Isehikari was used in Yamaguchi Prefecture’s Yamaguchi Active Aging City “Environmental Future City Concept,” in which 70% of farmers gained a yield of over 500 kg per 10 acres and heavily fertilized areas displayed a potential high yield of 700 kg [24]. Thus, Isehikari is an extremely beneficial genetic resource; however, its nature of genetic alteration is unclear. Therefore, molecular genetic analyses for useful variations such as late maturation and short culms are extremely essential for efficient application in practical rice breeding.

In this study, we focused on the late-maturation trait of “Isehikari,” and we conducted a genetics-based genomics approach. We firstly conducted genetic analyses for late maturity in F2 and F3 between Koshihikari×Isehikari. Because this is a hybrid between Japonica cultivars, there are only 66 SSR markers, which show DNA polymorphisms between Japonica cultivars. As polymorphic DNA markers were limited, first, we roughly mapped the late-maturation gene in the 30–33 Mb region of chromosome 3 by using polymorphic F2 generations. We then proceeded six times of continuous backcrossing with Koshihikari to develop late-maturing isogenic Koshihikari, which was introduced with the late-maturing gene from Isehikari. Then, we conducted a whole-genome sequencing analysis of the late-maturing Koshihikari, and finally, we identified only a single SNP in Hd16 among the coding sequences in the 30–33 Mb region of chromosome 3. The developed 14-day late-maturing Koshihikari with Hd16 was registered as a new cultivar “Koshihikari Suruga Hd16” under Japanese varietal protection, which is useful to avoid flowering and maturity in the hottest August.

2. Materials and Methods

2.1. Genetic Analysis and Development of Late-Maturing Isogenic Line. First, a genetic analysis on heading date was conducted by using 149 individuals in the F2 of Koshihikari×Isehikari. Because the genetic background between Koshihikari and Isehikari was divergent, heading-date distribution in the F2 of Koshihikari×Isehikari was continuous, and late-maturing individuals that matured 7 days later than Isehikari were segregated. Then, progeny tests were conducted on 70 randomly selected F2 plants by using self-propagated 70 F3 lines consisting of 570 individuals derived from each 70 F2 individuals to identify the genetic mode of heading date. The self-propagated F3 lines were cultivated within the Field Science Center’s fields, and the heading date, culm length, and phenotype were examined. Late-maturing homozygote in the F3 of Koshihikari×Isehikari was utilized as a nonrecurrent parent; six times of continuous backcross with Koshihikari as a recurrent parent were conducted. Finally, the late-maturing isogenic Koshihikari, Koshihikari×6/[(Koshihikari×Isehikari) F2 late-maturing type] BC6F2, was developed. In each BC6F2 generation through the backcrossing process, the heading date was genetically analyzed, and the latest mature segregants were isolated and used as pollen parents for the backcross with Koshihikari as a recurrent parent.

Cultivation of genetic materials was carried out in a paddy field at Shizuoka University, Shizuoka, Japan, from 2013 to 2019. Genetic materials were sown in late June, and then, seedlings were individually transplanted into a paddy field in mid-July with a transplanting density of 22.2 seedlings/m2 (one seedling per 30 × 15 cm). The paddy field was fertilized by 4.0 kg of basal fertilizer containing nitrogen, phosphorus, and potassium (weight ratio, nitrogen : phosphorus : potassium = 2.6 : 3.2 : 2.6) at a rate of 4.3 g/m2 nitrogen, 5.3 g/m2 phosphorus, and 4.3 g/m2 potassium across the field. The heading date was recorded as the date the first panicle had emerged from the flag leaf sheath for each plant. Culm length was measured as the length between the ground surface and the panicle base.

2.2. Mapping of Late-Maturation Gene by DNA Markers. In the F3 progeny test, 23 F3 lines were fixed in late-maturation headings between 8/31 and 9/8, which derived from late-maturing F2 plants that head from 8/23–9/2. Namely, the progeny from late-maturing F3 plants was confirmed to be fixed to late-maturing homozygotes. The late homozygous 23 F3 plants fixed in the F3 were used to roughly map a late-maturation gene linkage by using 66 SSR markers. F3 leaves were placed in a 2 mL tube with ceramic beads, immersed in liquid nitrogen and frozen, and pulverized with a high-speed bead mill homogenizer, the Precellys 24 high-throughput bead-mill homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), by 6500 rotations with two periods of 20 s with a 5 s interval. Genome DNA was extracted by using the cetyltrimethylammonium bromide (CTAB) method [25]. Utilizing the 66 SSR markers that show DNA polymorphism between Koshihikari and Nipponbare, which dispersed on 12 rice chromosomes, a linkage analysis was
conducted with a late-maturation gene. For SSR markers, using 20 ng each of rice genomic DNA as a template, 50 μL of a reaction solution containing 200 mmol/L each primer (33 ng), 100 μmol/L dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 1.5 mmol/L MgCl₂, and 1 U TaKaRa LA Taq (Takara Bio Inc., Kyoto, Japan) was prepared. Using the Thermal Cycler CFX96 (Bio-Rad Laboratories, Hercules, CA), the reaction solution was subjected to 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and primer extension at 72°C for 1 min. The first denaturation at 94°C and the last extension at 72°C were set for 5 min. The SSR polymorphisms in the PCR products were analyzed by electrophoresis using a cartridge QIAxcel DNA Screening Kit (2400) in a QIAxcel electrophoresis apparatus (QIAGEN, Hilden, Germany) at 5 kV for 10 min. Similarly, late-maturing homozygous segregants isolated from BC₆F₂ were used for mapping.

Late-maturing homozygous segregants in BC₆F₂ and BC₇F₂ were also confirmed by SSR marker RM16089 or TaqMan probe both tightly linked to SNP in Hd16 identified by NGS. TaqMan probes specific to chromosome 3’s SNP alleles in Hd16 were designed as 5′-CTAGCTATCTAATGTTCCTCTGTGAAA-3′ for hd16 in Koshihikari and 5′-CTAGGTATCTAATTGTTCCCCTGTAA-3′ for Hd16 in late-maturing isogenic Koshihikari (Isehikari) and labeled with fluorescent dyes FAM or HEX, respectively. The forward and reverse primers for the real-time PCR were 5′-TGCTCAATTATACCTACGGACCTAAAG-3′ and 5′-CTACCTGTACGGACCTACAGCTGAAA-3′, respectively. The real-time PCR reaction was conducted to amplify the allele-specific fluorescence, by first heating the material to 95°C for 30 seconds to denature the DNA, then submitting it to 40 cycles of denaturing at 95°C for 15 seconds, and annealing at 48°C for 30 seconds.

2.3. Next-Generation Sequencing (NGS) Analysis. Whole-genome sequencing was conducted on both Koshihikari and the late-maturing homozygous isogenic lines (BC₆F₂, BC₇F₂), which were integrated with a late-maturing gene derived from Isehikari through continuous backcrossing into the genetic background of Koshihikari. The leaves were powdered using a mortar and pestle while being frozen in liquid nitrogen. The genomic DNA was then extracted from each cultivar by the CTAB method. Genomic DNA was fragmented and simultaneously tagged so that the peak size of the fragments was approximately 500 bp using the Nextera® transposome (Illumina Inc., San Diego, CA). After purification of the transposome by DNA Clean & Concentrator™-5 (Zymo Research, Irvine, CA), adaptor sequences, including the sequencing primers for fixation on the flow cell, were synthesized at both ends of each DNA fragment via a limited time of polymerase chain reaction, and then, size selection of DNA fragments was conducted by using AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Finally, to prepare a DNA library for NGS, qualitative check by using a Fragment Analyzer™ (Advanced Analytical Technologies, Heidelberg, Germany) and quantitative measurements by a Qubit® 2.0 Fluorometer (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA) were conducted. Sequencing was conducted by using the prepared DNA library in paired-end 2 × 100 bp on a HiSeq 2500 next-gen sequencer, according to the manufacturer’s protocols (Illumina Inc., San Diego, CA). The sequencing data were gained with paired-end reads. The gained Illumina reads were trimmed using Trimmomatic (version 0.39) [26]. The sequencing adapters and sequences with low quality scores on 3′ ends (Phred score [Q], <20) were trimmed. Raw Illumina WGS reads were quality checked by performing a quality control with FastQC (version 0.11.9; Babraham Institute). Mapping of reads from Koshihikari to the Nipponbare genome as a reference was conducted with Burrows-Wheeler Aligner (BWA) software (version bwa-0.7.17.tar.bz2) [27], duplicated reads were removed using Picard (version 2.25.5) (http://broadinstitute.github.io/picard), and secondary aligned reads were removed by using SAMtools (version 10.2) [28], to construct the consensus sequence of the Koshihikari genome. Next, the read sequences obtained from the late-maturing isogenic Koshihikari line were mapped by using the “consensus genome” of Koshihikari as a reference. To identify genetic variations among strains, single nucleotide variant (SNV) detection (variant calling) and SNV matrix generation were conducted using GATK version 4.1.7.0 [29].

3. Results

3.1. Inheritance and Phenotypic Expression of a Late-Maturing Gene in an Isogenic Background. In the F₂ of Koshihikari×Isehikari, late-mature individuals that matured later than Isehikari were segregated. Progeny tests were conducted on 70 randomly selected F₂ plants by using self-propagated 70 F₃ lines from each F₂ plant. The F₂ genotype was determined by examination of the distribution of the heading date and culm length in the F₃ lines. Twenty-three F₃ lines were fixed in late-maturation headings between 8/31 and 9/8, which derived from late-maturing F₂ plants that head from 8/23 to 9/2. Namely, the progeny from late-maturing F₂ plants was confirmed to be fixed to late-maturation homozygotes. Twenty-one F₃ lines derived from early-maturing F₂ plants were fixed late-maturation headings between 8/26 and 9/2, which derived from early-maturing F₂ plants that head from 8/14 to 8/20. Namely, the progeny from early-maturing F₂ plants were confirmed to be fixed with early maturation homozygotes. On the other hand, 26 F₃ lines, which derived from midmaturing F₂ plants that head 8/18–8/28, segregated in heading dates from 8/26 to 9/8 straddling their parents’ heading dates. These segregating F₂ lines were thought to be derived from heterozygous F₂ plants. Thus, the genotype of the heading date was determined according to the ratio of 23 late-maturing homozygous lines:26 heterozygous lines:21 early maturing homozygous lines (Figure 1(a)), which fit to a theoretical single gene ratio of 1:2:1 (χ² = 4.74, 0.05 < p < 0.10). These results suggested that Isehikari has a single late-maturing gene.

We then used the latest maturing F₃ (confirmed late-maturing homozygote in F₃, Figure 1(a)) as the first nonrecurrent parent for six times of continuous backcross with
Koshihikari as a recurrent parent. BC$_1$F$_1$ was directly backcrossed with Koshihikari, and the most late-maturing segregant of BC$_2$F$_2$ was used for the third backcross with Isehikari. In the BC$_3$F$_2$, early maturation-type plants heading like Koshihikari during 8/12–8/17, midmaturation-type plants heading during 8/19–8/23, and late-maturation-type plants heading during 8/26–8/28, which was 7 days later than Isehikari, were segregated as a histogram with 3 tops (Figure 1(b)). The segregation ratio was 12 early maturation : 28 midmaturation : 10 late maturation, which was well consistent with the theoretical single gene ratio of 1 : 2 : 1 ($\chi^2 = 4.74$, 0.05 < $p < 0.10$). BC$_3$F$_2$ was also segregated in a ratio of 8 early maturation : 11 midmaturation : 3 late maturation, which was well consistent with the theoretical 1 : 2 : 1 ratio ($\chi^2 = 2.27$, 0.30 < $p < 0.50$). Thus, we identified a single major late-maturing gene derived from Isehikari, that is, incomplete dominance and that causes a 14-day delay in the flowering time in the genetic background of Koshihikari. To map the late-maturing gene, a linkage analysis was conducted by using 23 F$_2$ plants of Koshihikari × Isehikari by using 66 SSR markers distributed on 12 rice chromosomes, which showed polymorphism between Japonica cultivars Koshihikari and Isehikari. The results showed that the late-maturing gene was linked to the SSR markers RM2593, RM1038, and RM1373 on chromosome 3 (Figure 2(b)), with recombination values of 21.4, 11.7, and 20.2, respectively. Because this is a hybrid between Japonica cultivars, polymorphic DNA markers were limited and roughly linked. Then, we completed six times of continuous backcrossing with Koshihikari, and finally, a late-maturing isogenic Koshihikari was developed up to BC$_6$F$_2$, which was introduced with the target late-maturing gene derived from Isehikari, for the next-generation sequencing survey.

3.2 Next-Generation Sequencing Analysis of Late-Maturing Isogenic Line. We conducted continuous backcrossing with Koshihikari to develop the late-maturing isogenic Koshihikari, which was introduced with the late-maturing gene of Isehikari into the Koshihikari genome. A whole-genome analysis by next-generation sequencing was conducted on Koshihikari and the late-mutation isogenic Koshihikari (BC$_6$F$_2$), which was integrated with the late-maturity gene derived from Isehikari. First, the read sequences gained by NGS from the Koshihikari genome were mapped by using the Nipponbare genome as a reference sequence. As a result, a 372,912,445 bp long consensus sequence of the Koshihikari genome was constructed at the average depth of 35.68 with...
isogeneic lines (BC 4F2 and BC 6F2) integrated with the late-maturing gene in the Koshihikari genome.

(c) Development of late-maturity isogenic Koshihikari by continuous backcrossing for NGS analysis. (d) Whole-genome analysis of markers distributed on 12 rice chromosomes. The late-maturing gene was linked to the SSR markers on the long arm of chromosome 3.

99.0% cover ratio (Table 1 and Figure 3). We then conducted a whole-genome sequencing of the late-maturing isogenic Koshihikari with high coverage (average 61.52). By using the consensus sequence of Koshihikari as a reference, read sequences gained from late-maturation isogenic Koshihikari were assembled at 99.1% coverage ratio with the average depth of 61.52 (Table 1), and SNP calling was conducted using the vcf file. The results showed that a large portion of the 12 rice chromosomes were substituted to the same sequence as Koshihikari (black) (Figure 4), and SNPs and INDELS (red) derived from Isehikari were found to be concentrated around the vicinity of 33 Mb in the long arm of chromosome 3 (Figures 2 and 4). Namely, SNPs derived from Isehikari were found to be concentrated and remained within chromosome 3, which was also linked with SSR markers. Others were almost replaced by the Koshihikari genome. In the region linked to SSR markers, only a single SNP was detected in several annotated coding sequences (Figure 5). The SNP was situated in the Hdi6 gene (Os03g0793500) encoding casein kinase I, which was located at 32,993,321-33,000,717 (SNP: 32,996,608, A→G), and it caused a single amino acid change from threonine to alanine, at the distal end of the short arm of chromosome 3 in Koshihikari (Supplementary file 1). All 22 read sequences from the late-maturing isogenic Koshihikari showed the SNP (A→G at 32,996,608) in the Hdi6 gene (Os03g0793500) of Koshihikari. This result was reliable than sequencing of artificially amplified products by PCR. On the other hand, the DNA sequence of Hdi6 (Os03g0762000) in the late-maturing isogenic Koshihikari, which was also located at 31,496,180-31,490,533 from the distal end of the short arm of chromosome 3 near to Hdi6, was completely identical to that of Hdi6 in the Koshihikari genome (Supplementary file 1). Therefore, the responsible gene for the late maturation of isogenic Koshihikari derived from Isehikari was identified as Hdi6 (Figure 5). DNA sequences of photo-periodic genes Hda5, RFTI, and Ehd1, which are related with Hdi6, in the late-maturing isogenic Koshihikari were confirmed to be identical to that of Koshihikari.

The BC4F2 segregant that matured 15 days later than Koshihikari was used for the 5th backcross with Koshihikari. The resultant 35 BC4F2 plants were genotyped by using the SSR marker RM16089, which is closely linked to Hdi6 on chromosome 3. The ratio of [Hdi6 homozygotes+heterozygotes]: Hdi6 homozygotes was 26:9, which was consistent with a 3:1 ratio ($\chi^2 = 0.0095, 0.90 < p < 0.95$). Hdi6 homozygous segregants from BC4F2 were selected and used for the 6th backcross to Koshihikari, and whole-genome sequencing
A whole-genome analysis by next-generation sequencing was conducted on Koshihikari and the late-maturation isogenic Koshihikari (BC4F2), which was integrated with the late-maturation gene derived from Isehikari. First, the read sequences gained by NGS from Koshihikari genome were mapped by using the Nipponbare genome as a reference sequence. As a result, a 372,912,445 bp long consensus sequence of the Koshihikari genome was constructed at the average depth of 35.68 with 99.0% cover ratio. Next, by using the consensus sequence of Koshihikari as a reference, the read sequences gained from late-maturation isogenic Koshihikari were assembled at 99.1% cover ratio with the average depth of 61.52.

![Consensus genome of Koshihikari](image)

**Table 1: Summary of resequencing data by next-generation sequencing.**

| Library name | Seq by Illumina HiSeq 4000 | Mapping to the ref. genome | Mapping status |
|--------------|----------------------------|-----------------------------|----------------|
|              | Reads obtained (bp) | Bases generated (bp) | Reads unmapped/mapped ratio (%) | Ref. bases cover ratio (%) | Q median | Q mean | Depth median | Depth mean |
| Koshihikari_1 | 66,135,729 | 6,679,708,629 | 0.005884119 | 99.03155997 | 153 | 113.783 | 30 | –35.67571 |
| Koshihikari_2 | 66,135,729 | 6,679,708,629 | 0.003406305 | 99.18284484 | 184 | 160.232 | 30 | –61.51913 |
| Koshihikari Hd-i_1 | 114,244,160 | 11,538,660,160 | 0.005884119 | 99.03155997 | 153 | 113.783 | 30 | –35.67571 |
| Koshihikari Hd-i_2 | 114,244,160 | 11,538,660,160 | 0.003406305 | 99.18284484 | 184 | 160.232 | 30 | –61.51913 |

A whole-genome analysis by next-generation sequencing was conducted on Hd16 homozygous late-maturing segregant from BC4F2, which was also confirmed by a TaqMan probe for the SNP in the exon 9 of Hd16. Read sequences gained from the late-maturing gene derived from Isehikari. First, the read sequences gained by NGS from Koshihikari genome were mapped by using the Nipponbare genome as a reference sequence. As a result, a 372,912,445 bp long consensus sequence of the Koshihikari genome was constructed at the average depth of 35.68 with 99.0% cover ratio. Next, by using the consensus sequence of Koshihikari as a reference, the read sequences gained from late-maturation isogenic Koshihikari were assembled at 99.1% cover ratio with the average depth of 61.52.

**Figure 3: Construction of consensus genome of Koshihikari by whole-genome sequencing by next-generation sequencing.** Read sequences gained from Koshihikari genome were mapped by using the Nipponbare genome as a reference sequence. As a result, a complete length bp of Koshihikari consensus sequence was determined with 99.0% cover ratio at the average depth of 35.68. Polymorphisms found among Nipponbare and Koshihikari contained 47.0 + 20.8 = 60.8% SNPs and 38.6% Indel.

4. Discussion

Flowering time of rice (O. sativa L.) is among the most important agronomic traits for regional adaptation and grain yield. Heading date is one of the most important traits in rice breeding, because it defines where rice can be cultivated and influences the expression of various agronomic traits. Rice, a facultative short-day plant, flowers early in short-day and late in long-day conditions. To date, more than 40 genes or quantitative trait loci (QTLs) controlling flowering time have been identified in rice, and diverse allelic variations for these flowering genes have been revealed [30].
depends on the circadian gene OsGI, which is induced under long-day conditions [31]. The OsGI-heading date 1 signaling pathway is common to all plants, including Arabidopsis thaliana [32, 43, 44]. OsGI transcripts are phosphorylated by genes downstream of Hd6, which increases photosensitivity and causes late maturation. The expression of RFT1 is promoted by Ehd1 and DTH2, but it is suppressed via Se14. The expression of Ehd1 is promoted via Ehd2 and Ehd4 but is suppressed via DTH8 and Ghd7. The expression of Ghd7 is induced by Ehd3 and Hd17. Ghd7 activity increases via phosphorylation by Hd6. Furthermore, OsPRR37/Hd2 always suppresses blooming in both short-day and long-day conditions, independent of these transcription pathways [45]. Above findings indicated that the underlying regulation mechanism of flowering time in rice is very complicated. Moreover, the alteration of photoperiod sensitivity has let breeders diversify flowering time in rice and develop cultivars adjusted to a range of growing season periods.

In this study, we identified Hd16 as a responsible gene for late maturation of Isehikari, which was discovered in the field of Ise Jingu Shrine. First, 149 F2 plants of
Koshihikari × Isehikari and a total of 570 plants of 70 F3 lines self-propagated from 70 F2 plants were used for genetic analyses on heading date. The progeny test in F3 lines was in accordance with the theoretical single gene ratio of 1 late-maturing homozygous line : 2 heterozygous lines : 1 early maturing homozygous line. To map the late-maturation gene of Isehikari, a linkage analysis was conducted utilizing 66 SSR markers that distributed on 12 rice chromosomes that

**Figure 5:** Identification of a single nucleotide substitution in *Hd16* (Os03g0793500) responsible for the late maturity of isogenic Koshihikari derived from Isehikari. In the region between linked SSR markers, only a single SNP in *Hd16* gene encoding casein kinase I was detected in several annotated coding sequences. The SNP was the same as that of *Hd16* gene in Nipponbare. TaqMan probe specific to *Hd16* allele labeled with FAM detected the SNP. The TaqMan probe for the SNP is useful for practical MAS breeding.
show polymorphism among Japonica rice. The late-maturation gene was mapped as a single Mendelian factor based on the linkage with the SSR markers located around 30–33 Mb on the short arm of chromosome 3. Furthermore, we developed a late-maturing isogenic line (BC₆F₂), which was integrated with the late-maturation gene of Isehikari in the genetic background of Koshihikari, via six times of continuous backcross. The existence of a single incomplete dominant late-maturation gene from Isehikari, which causes 14-day delay in maturation in the genetic background of Koshihikari, was identified. A next-generation sequencing analysis with high coverage was conducted, and 99.5% of the whole-genome sequence of Koshihikari and late-maturing isogenic Koshihikari were determined. Although the whole-genome sequence of Koshihikari has not been published, we have created a consensus sequence for Koshihikari. Finally, the cause of late maturation in Isehikari was identified as a single SNP in exon 9 of the Hd16 gene in chromosome 3, which encodes casein kinase I.

The SNP found in the Hd16 gene of Isehikari was the same as that of Nipponbare. Hd16 of Nipponbare encodes a casein kinase I protein [36, 46]. Casein kinases (CKs) generally act as positive regulators in many signaling pathways in plants. In floral induction, Hd16 acts upstream of the floral repressor Ghd7, and phosphorylates the transcripts of the photosensitive gene Ghd7, which is located upstream of the flowering gene Ehd1, in long-day conditions. It is thought to strengthen photosensitivity and delay maturation [36, 47]. The rice Ghd7-Ehd1-Hd3a/RFT1 pathway modulated by Hd16 is not present in Arabidopsis [37]. In this study, one nonsynonymous substitution in Hd16 with Koshihikari allele resulted in decreased photoperiod sensitivity and a function to delay flowering time. On the other hand, Hd6 encoding CK2, which is located at 31.5 Mb on chromosome 3, phosphorylates the transcripts of Hdl under long-day conditions and increases photosensitivity to suppress flowering [48]. DTH8, which is located at 4.3 Mb on chromosome 8 and encodes the NF-Yb subunit of the trimeric NF-Y transcription factor, forms a complex with Hd1, suppressing the expression of Ehd1 and delaying blooming [43, 49, 50]. In this study, gene diagnosis by DNA resequencing showed that the photoperiodic genes Ghd7, Ehd1, Hd3a, RFT1, Hd6, and DTH8 in the late-maturing isogenic Koshihikari were confirmed to be identical to those in Koshihikari.

Introgression of Hd16, DTH2, and DTH8 into Koshihikari has been reported to cause a 10-day delay in maturation [36, 51–54]. In this study, we constructed a comprehensive isogenic genome via backcrossing, and we clarified the genome structure in which almost all sequences are substituted by the Koshihikari genome except for the vicinity around the Hd16 gene on chromosome 3, by whole-genome sequencing. A 14-day delay of maturation is considered a noticeable effect of Hd16 expression in the highly isogenic background. Fourteen-day late-maturing Koshihikari due to Hd16 will avoid ripening in the high-temperature season in summer. Furthermore, the selection of a more regionally adaptive genotype is possible, instead of overuse of Koshihikari all over Japan. MAFF have registered the late-maturing isogenic Koshihikari integrated with Hd16, designated as “Koshihikari Suruga Hdl” as a new plant variety [55], which is a genetic resource to avoid heat damage when ripening during high-temperature summer periods by late maturation. Yield merit underpinning Hdl6 was also reported [56]. We also developed the late-maturing and semidwarf isogenic Koshihikari, which was integrated with both Hdl6.
and semidwarf gene $d60$ [57–59], designated as “Koshihikari Suruga $d60\text{Hd16}$” [60].

By using the methods described above, we conducted genetics-directed genomics approach through this study. We firstly genetically mapped the late-maturation gene utilizing a polymorphic population in early generations of a hybrid. Furthermore, we conducted genetic analyses in each BCF$_2$ generation, which was aimed at integrating with a late-maturation gene through six times of continuous backcrossing with Koshihikari. Then, NGS analysis of the late-maturing isogenic Koshihikari identified only a single SNP in $Hd16$ among the coding sequences in the 30–33 Mb region of the short arm of chromosome 3. This late-maturing Koshihikari with $Hd16$ was registered as a new cultivar “Koshihikari Suruga $Hd16$” under Japanese varietal protection, which is useful to avoid flowering and maturity in the hottest August. The late-maturing Koshihikari is desired in the rice industry.

5. Conclusions

Genetic analyses of late maturity were conducted through the process of six times of continuous backcross with Koshihikari as a recurrent parent by using the late-maturing homozygous $F_2$ line of Koshihikari$x$Isehikari as a nonrecurr-ent parent, thus developing a late-maturing isogenic Koshihikari ($\text{BC}_6F_2$). As a result, we identified a single late-maturing gene with incomplete dominance that caused the 14-day maturation delay of Koshihikari. The whole-genome sequencing was conducted on both of Koshihikari and the late-maturing isogenic Koshihikari. Finally, a single SNP was identified in the key gene $Hd16$ of the late-maturing isogenic Koshihikari.

Data Availability

The authors confirm that all the supporting data and protocols have been provided within the article. Sequence data that support the findings of this study are given in Supplementary file 1.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This work is funded by the Adaptable and Seamless Technology Transfer Program (A-STEP), Industry-Academia Joint Promotion Stage, High Risk Challenge grant by the Japan Science and Technology Agency (JST) to Motonori Tomita, project ID14529973, entitled “Development of Super High-Yield, Large-Grains, and Early/Late Flowering Rice Cultivars Suitable to the Era of Globalization and Global Warming by Next-Generation Sequencer Oriented Genome-Wide Analysis,” since 2014 to 2018. The author thanks Yudai Iwase and Koki Muramatsu for their assistance on the phenotypic investigation.

Supplementary Materials

Supplementary file 1 shows the sequence data of $Hd16$ and $Hdb$. (Supplementary Materials)

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