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Research Paper

The *temperature sensitive hybrid breakdown 1* induces low temperature-dependent intrasubspecific hybrid breakdown in rice

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Hybrid breakdown (HB) is an important type of post-zygotic reproductive barrier that inhibits hybrid production during the process of cross-breeding. A novel low temperature-dependent HB was identified in a chromosomal segment substitution line (CSSL) library derived from a cross of two rice (*Oryza sativa* L. *japonica*) cultivars, Yukihikari and Kirara397. A set of weakness symptoms in a target CSSL was observed at 23°C, but was rescued at 27°C and/or 30°C. Genetic analysis of HB using an $F_{2:3}$ population of a cross between a target CSSL and Kirara397 found that a recessive *temperature sensitive hybrid breakdown1* (*thb1*) gene from Yukihikari caused HB in the genetic background of Kirara397. Molecular mapping showed that *thb1* was located within a 199-kb fragment on chromosome 6. A genetic study of F_2 populations of reciprocal crosses between Yukihikari and Kirara397 confirmed that this HB was induced by the interaction of two recessive genes. These results provide important clues to further dissect the mechanism of generation of a novel temperature sensitive HB in rice intrasubspecific crosses and suggest that these linked markers will useful in rice breeding.

Key Words: hybrid breakdown, intrasubspecific cross, temperature, rice, chromosome segment substitution line.

Introduction

Crop plants have been improved for consumption by selecting for useful traits and accumulating desirable genes from genetic resources. Hybridization breeding is a major approach for broadening genetic diversity in local gene pools. This process introduces agriculturally valuable traits/genes into existing cultivars, not only from breeding stocks or wild progenitors through inter-specific and inter-subspecific crosses but also from other elite cultivars by intrasubspecific crosses. During the process of hybridization breeding, however, reproductive barriers arise. The mechanisms of these barriers maintain the genetic integrity of species and/or prevent gene flow with other species, thereby reducing hybrid production. Alternatively, reproductive barriers may play an important role in speciation and maintaining species identity in many organisms (Coyne and Orr 2004, Dobzhansky 1937).

Reproductive barriers can be divided into two general categories: pre-zygotic and post-zygotic barriers. Hybrid breakdown (HB), a major type of post-zygotic barrier, is defined as sterility or weakness observed in the F_2 or later hybrid generations, in contrast to F_1 hybrids, which grow normally and show good fertility (Stebbins 1950). The genetic mechanisms of post-zygotic isolation have been theoretically explained by the Bateson-Dobzhansky-Muller (BDM) model (Bateson 1909, Dobzhansky 1937, Muller 1942), which postulates that deleterious interaction of two or more genes derived from different species or population causes post-zygotic isolation. In rice, HB reduces tiller numbers and retards growth, with short culms and panicles, late heading, chlorosis or necrosis of leaves, poor seed setting, and retarded root growth. The genetics of HB have been studied in rice, especially in interspecific (*Oryza. sativa* × *O. glumaepatula* and *O. sativa* × *O. nivara*) and intersubspecific (*japonica* and *indica*) hybridizations (Fukuoka *et al.* 1998, 2005, Ichitani *et al.* 2012, Jiang *et al.* 2008, Kubo and Yoshimura 2002, 2005, Li *et al.* 1997, 2015, Matsubara *et al.* 2006, 2007, 2015, Miura *et al.* 2008, Oka 1957, 1978, Oka and Doida 1962, Okuno 1986, Sato and Morishima 1988, Sobrizal *et al.* 2001, Wu *et al.* 1995, Yamamoto *et al.* 2007, 2010a, Yokoo 1984). A simple genetic mechanism based on complementary recessive genes has been proposed. To date, 19 loci have been genetically mapped using DNA markers, including *HWD1* and *HWD2* (Fukuoka *et al.* 1998); *HWF1* (Sobrizal *et al.* 2001); *HWE1*, *HWE2*, *HSA1*, *HSA2*, and *HSA3* (Kubo and

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Yoshimura 2002, 2005); *HWG1* and *HWG2* (Fukuoka *et al.* 2005); *HBD1*, *HBD2*, *HbBD3*, *HBD4*, and *HBD5* (Matsubara *et al.* 2006, 2007, 2015); *HBD1* (Miura *et al.* 2008); *HBD2* and *HBD3* (Yamamoto *et al.* 2007, 2010a); *HWH1* and *HWH2* (Jiang *et al.* 2008); and *HCA1* and *HCA2* (Ichitani *et al.* 2012). Among them, two genes have been associated with hybrid breakdown, *hybrid breakdown2* (*hbd2*) and *hbd3*, which encode casein kinase I and NBS-LRR, respectively, with hybrid breakdown attributed to an elevated autoimmune response (Yamamoto *et al.* 2010a). Complete understanding of the genetic mechanisms at the molecular level and overcoming problems in hybrid breeding require further studies to identify and characterize the novel loci causing HB.

We have developed a series of chromosome segment substitution lines (CSSLs), Yukihikari-Kirara397 CSSLs (YK3CSSLs), derived from crosses and back-crosses of two rice (*O. sativa* L. *japonica*) cultivars, the donor Yukihikari and the recipient Kirara397 (Kato and Hirayama 2021). Both parental cultivars have been cultivated in Hokkaido, the northernmost island of Japan, located at one of the northernmost limits of rice cultivation in the world. These CSSLs were developed to clarify the genetic basis of the agronomic traits of Yukihikari and its ability to ameliorate atopic dermatitis (Yanagihara unpublished results). A field trial of the target CSSL, YKCSSL-6.1, showed poor growth with extremely late heading (Kato and Hirayama 2021). The weak plants were short with short panicles and weak culm and root growth (Kato unpublished data). Because these weak plants were rescued by higher temperature in the greenhouse, we were able to harvest seeds obtained from self-pollination and cross hybridization of these plants grown at higher temperature. In the present study, we clarified the mechanism by which YKCSSL-6.1 caused a low temperature dependent HB and identified a single recessive gene, *temperature sensitive hybrid breakdown1* (*thb1*) on chromosome 6 of Yukihikari, as causing HB on a genetic background of Kirara397. Furthermore, we found that this HB was induced by the interaction of two recessive genes, *thb1* and *thb2*, in F_2 populations derived from reciprocal crosses between Yukihikari and Kirara397.

Materials and Methods

Plant materials

The *O. sativa* L. ssp. *japonica* cultivars Yukihikari and Kirara397 and the progeny of crosses between these two cultivars were used throughout this study. A CSSL library was derived from the cross between Kirara397 as the recipient parent and Yukihikari as the donor parent (Kato and Hirayama 2021). Plants used for phenotypic characterization included a target CSSL, YK3CSSL-6.1 (BC_3F_7), which exhibited weakness syndrome, and YK3CSSL-5.4 (BC_3F_7) as a positive control for normal plant and parental cultivars (**Fig. 1**). Genetic analysis and molecular mapping were performed using F_2 (BC_4F_2) and F_3 (BC_4F_3) generations, which were generated by backcrossing Kirara397 to YK3CSSL-6.1, and F_1 and F_2 populations of the reciprocal crosses between Yukihikari and Kirara397.

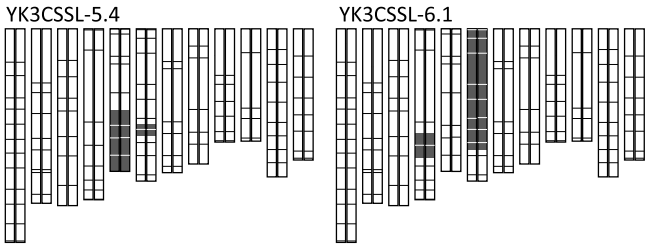

Fig. 1. Graphic genotypes of YK3CSSL-5.4 and YK3CSSL-6.1. The *white* and *gray* boxes represent chromosomal segments from Kirara397 and Yukihikari, respectively, and the *horizontal lines* indicate the positions of markers used for genotyping (Kato and Hirayama 2021).

Phenotypic characterization

The effects of temperature and photoperiod on plant phenotype were evaluated by measuring days to heading, plant height, culm length, panicle length, panicle number, spikelet number per panicle, and seed fertility. Seeds sterilized by treatment with 0.2% benomyl hydrate solution for 24 h at 30°C were germinated in reverse osmosis (RO) water for 2 d in the dark at 30°C. The germinated seeds were planted in a cell plug tray (each genotype per treatment, cell count, 4 × 4; cell size, 3 × 3 cm; cell depth, 4.4 cm) filled with soil and allowed to grow in the dark for 2 d at 30°C. Four cell plug trays (4 genotypes × 16 individuals) were placed in a plastic container (44.6 cm × 29.8 cm with a depth of 5.8 cm). The seedlings were grown in the growth chamber under six growth conditions, consisting of a combination of two day-length conditions and three temperature conditions; 14.5 h light (350 μmol/m^2/s)/9.5 h dark at 23°C, 27°C, or 30°C, or for 11.5 h light (350 μmol/m^2/s)/12.5 h dark at 23°C, 27°C, or 30°C. One month after planting and every two weeks thereafter, the plants were supplied with 0.2% Hyponex solution [N-P-K 6-10-5 (%) HYPONeX JAPAN, Osaka, Japan, 0.8 L per container]. Because panicles did not fully emerge in plants with the weakness phenotype, the heading date was defined as the date at emergence of the tip of primary panicle. Seed fertility was scored as filled spikelets per total number of spikelets on the main culm of each individual.

$BC_4F_{2:3}$ population of a cross between YK3CSSL-6.1 and Kirara397

YK3CSSL-6.1 was backcrossed with Kirara397. The 313 BC_4F_2 seeds derived from BC_4F_1 plants by self-pollination and 40 seeds each of Yuklihikari, Kirara397, and YK3CSSL-6.1 plants were individually sterilized on 15 April 2019 by treatment with 0.2% benomyl hydrate solution for 24 h at 30°C. The seeds were germinated in RO water for 2 d in the dark at 30°C, planted in a cell plug tray (cell count, 8 × 16; tray size, 52 cm × 25 cm; cell size, 3 cm × 3 cm with cell

depth, 4.4 cm) filled with soil on 18 April 2019, and allowed to grow in a greenhouse. On 25 May 2019, 37 days after sowing, the plants were moved outdoors and allowed to grow under natural light and temperature at Obihiro, Hokkaido, Japan (latitude 42.9°N). Each cell plug tray was placed in a plastic container (53 cm × 34.8 cm with a depth of 15.6 cm) and water maintained at a depth of 3–10 cm, depending on plant size. One month after planting and every two weeks thereafter, the plants were supplied with 0.2% Hyponex solution [N-P-K 6-10-5 (%) HYPONeX JAPAN, Osaka, Japan, 10 L per container]. Phenotype data for HB of each F_2 individual were collected on 3 June 2019, 46 days after sowing. The heading date of the individual plants was defined as the date of appearance of the tip of the first panicle.

The precise position of causal gene was assessed by progeny tests. Recombinant BC_4F_2 plants with recombination between the markers YKOInDel-17328 and YJInDel-206 were selected. The growth habits of 8–44 BC_4F_3 plants derived from self-pollinated BC_4F_2 plants, as well as the parental cultivars and YK3CSSL-6.1, were evaluated. Seeds were sterilized and germinated as described above, and sown in soil compost. The plants were grown at about 25°C in the greenhouse or glass house, depending on outside temperatures, from December 2019 to April 2020 and outdoors from May to August in 2020. Plants were cultivated as described for the BC_4F_2 population.

The mean daily air temperatures at Obihiro during the growing seasons in 2019 and 2020 were obtained from the Japan Meteorological Agency (**Supplemental Fig. 1**).

F_2 populations of the reciprocal crosses between Yukihikari and Kirara397

Individual F_2 seeds derived from each of three F_1 plants of the reciprocal crosses between Yukihikari and Kirara397 by self-pollination and 3–10 seeds each of Yuklihikari, Kirara397, and a target CSSL were sterilized on 9 April 2020 by treatment with 0.2% benomyl hydrate solution for 24 h at 30°C. The seeds were germinated in RO water for 3 d in the dark at 30°C, planted in a cell plug tray (cell count, 8 × 16; tray size, 52 cm × 25 cm; cell size, 3 cm × 3 cm with cell depth 4.4 cm) filled with soil on 13 April 2020, and grown in a greenhouse. On 23 May 2020, 40 days after sowing, the plants were moved outdoors and grown under natural light and temperature and cultivated as described above. The height of each F_2 individual was measured on 27–30 July 2020, 75–78 days after sowing.

Genotyping of InDel markers

Nine previously described InDel markers (Kato and Hirayama 2021, Kinoshita *et al.* 2016) and three new InDel markers were used in the present study (**Supplemental Table 1**). DNA was extracted from fresh young leaves of parental plants and each BC_4F_2. For InDel marker analysis, amplification reactions were performed in a total volume of 10 µL, containing 40 ng template DNA, 1× PCR buffer, 0.2 mM of each dNTP, 1U *Taq* DNA polymerase (GoTaq® Green Master Mix, Promega) and 10 pmol of each forward and reverse primer. The amplification protocol consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, amplification at 50–60°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1–4% agarose gels, which were stained with ethidium bromide and viewed under UV light.

Linkage analysis and QTL mapping

Linkage analysis between the molecular markers and *thb1* locus were performed using JoinMap® 4 (van Ooijen 2006), with a limit of detection (LOD) of 3.0. Genetic distances were estimated using the Kosambi map function (Kosambi 1943). Putative QTLs for heading date were identified by the simple interval mapping (SIM) and multiple QTL model (MQM) mapping functions of MapQTL® 6 (van Ooijen 2002). The LOD threshold for a significant QTL ($p < 0.05$) was based on the results of 1,000 permutations (Churchill and Doerge 1994). Genetic parameters, including the percentage of phenotypic variance explained and the additive effect of each QTL, were estimated using MapQTL.

Results

Phenotypic characterization

When grown at 23°C, YK3CSSL-6.1 showed a weak phenotype, with plant height at 31 days after imbibition (DAI), culm length, panicle length, panicle number, heading rate, spikelet number per panicle, and seed sterility being independent of photoperiod (**Table 1**). Heading rates (HR) at 130 DAI under SD and LD were 50% and 43.8%, respectively. No YK3CSSL-6.1 plant headed between 87 DAI and 130 DAI. These characteristics of YK3CSSL-6.1 were all inferior to those of Kirara397, Yukihikari, and YK3CSSL-5.4. The set of weakness symptoms in YK3CSSL-6.1 disappeared when these plants were grown at 27°C or 30°C, independent of photoperiod (**Table 1**, **Fig. 2**). Because the parental cultivars and another CSSL had normal phenotypes at 23°C, we concluded that the appearance of weakness syndrome in YK3CSSL-6.1 resulted from a temperature-dependent HB.

Inheritance mode and linkage mapping of a causal gene on chromosome 6

To examine the mode of inheritance of the gene responsible for HB, we assessed the growth habits of 313 BC_4F_2 plants derived from a cross between YK3CSSL-6.1 and Kirara397. A total of 231 F_2 plants were scored as normal, whereas 82 showed poor growth as short plants and fewer tillers (**Fig. 3**). The segregation ratio of normal to poor-growth phenotypes fit a 3:1 ratio ($\chi^2 = 0.24$, $p = 0.62$), indicating that poor growth was controlled by a single recessive gene, named *temperature sensitive hybrid breakdown1*

Table 1. Morphological and physiological characteristics of Kirara397, Yukihikari, YK3CSSL-5.4 and YK3CSSL-6.1 plants

Trait	Condition	Kirara397	Yukihikari	YK3CSSL-5.4	YK3CSSL-6.1	Trait	Condition	Kirara397	Yukihikari	YK3CSSL-5.4	YK3CSSL-6.1
PL (cm)	SD 23°C	62.3 a	52.1 c	57.6 b	25.0 d	SPN	SD 23°C	23.0 b	31.0 a	32.7 a	3.7 c
	SD 27°C	73.1 a	61.6 b	64.5 b	61.2 b		SD 27°C	21.3 ab	24.3 a	21.3 ab	12.3 b
	SD 30°C	69.6 ab	64.2 b	69.6 ab	72.4 a		SD 30°C	16.0 a	21.0 a	17.0 a	17.7 a
	LD 23°C	49.7 a	43.9 b	49.4 a	21.1 c		LD 23°C	42.0 a	41.0 a	33.7 a	6.3 b
	LD 27°C	63.9 a	55.9 b	62.7 a	54.9 b		LD 27°C	31.0 a	26.0 ab	22.7 b	12.0 c
	LD 30°C	68.9 a	58.9 c	64.1 b	69.8 a		LD 30°C	26.0 a	16.0 a	19.7 a	20.0 a
CL (cm)	SD 23°C	44.8 a	45.0 a	40.0 a	12.8 b	SF (%)	SD 23°C	100.0 a	95.1 a	99.0 a	11.1 b
	SD 27°C	49.3 a	39.7 b	42.0 b	39.5 b		SD 27°C	78.2 b	95.8 a	93.3 a	100.0 a
	SD 30°C	48.3 a	35.0 c	39.7 b	44.1 a		SD 30°C	65.0 a	75.8 a	76.5 a	70.9 a
	LD 23°C	45.7 a	43.5 ab	42.2 b	16.9 c		LD 23°C	86.6 a	91.7 a	81.2 a	18.5 b
	LD 27°C	46.0 a	40.3 a	43.8 a	40.3 a		LD 27°C	85.3 a	85.8 a	89.2 a	86.1 a
	LD 30°C	44.9 ab	36.5 c	37.8 bc	45.2 a		LD 30°C	66.4 a	65.5 a	58.5 a	54.2 a
PAL (cm)	SD 23°C	12.1 a	13.7 a	11.8 a	6.4 b	HR (%)	SD 23°C	100.0	100.0	100.0	50.0
	SD 27°C	11.1 a	12.3 ab	12.0 a	10.4 b		SD 27°C	100.0	100.0	100.0	100.0
	SD 30°C	9.7 b	11.8 a	10.8 ab	9.7 b		SD 30°C	100.0	100.0	100.0	100.0
	LD 23°C	16.0 a	13.7 b	12.8 b	8.8 c		LD 23°C	100.0	100.0	100.0	43.8
	LD 27°C	13.7 a	12.1 ab	12.4 ab	11.3 b		LD 27°C	100.0	100.0	100.0	100.0
	LD 30°C	10.9 b	12.6 a	11.4 ab	11.5 ab		LD 30°C	100.0	100.0	100.0	100.0
PN	SD 23°C	6.7 a	3.3 b	3.3 b	1.0 c	DTH	SD 23°C	58.7 c	62.9 b	64.1 b	67.3 a
	SD 27°C	3.7 a	3.7 a	3.7 a	3.0 a		SD 27°C	48.3 b	53.7 a	53.3 a	46.9 b
	SD 30°C	2.7 a	3.0 a	2.7 a	4.7 a		SD 30°C	48.8 c	56.1 a	53.5 b	48.5 c
	LD 23°C	3.7 a	3.7 a	3.0 a	1.0 b		LD 23°C	77.7 a	68.1 b	68.4 b	79.7 a
	LD 27°C	2.7 a	3.7 a	3.7 a	3.3 a		LD 27°C	56.0 a	51.4 b	50.5 bc	49.9 c
	LD 30°C	1.7 a	2.3 a	3.3 a	3.3 a		LD 30°C	56.9 a	57.6 a	53.0 b	54.1 b

Data for heading characteristics represent the mean for 16 plants, whereas data for other characteristics represent the mean for eight plants.
Abbreviations: PL, plant length at 31 days after imbibition; CL, culm length; PAL, panicle length, PN, panicle number; SPN, spikelet number per panicle; SF, seed fertility; HR, heading rate, DTH, days to heading.
Means denoted by letters indicate significant differences between genotypes by Tukey–Kramer multiple comparison tests ($p < 0.05$).

(*thb1*). The three genotypes at each of the nine DNA marker loci fit a 1:2:1 Mendelian ratio, demonstrating that there was no segregation distortion in the BC_4F_2 population (**Supplemental Table 2**). Linkage analysis showed that *thb1* was mapped to the 10.3 cM interval between YKOInDel-17328 and YJInDel-206 on chromosome 6 (**Fig. 4A**).

To map *thb1* precisely, progeny tests were performed to determine the BC_4F_2 genotype at *thb1* using the 40 BC_4F_3 families derived from the self-pollination of each individual BC_4F_2 carrying recombinant chromosomes between YKOInDel-17328 and YJInDel-206. In addition, we used three new InDel markers in a target chromosomal region. Finally, *thb1* was mapped to a 199 kb interval between YK3InDel06-646046 and YK3InDel06-845078_2 (**Fig. 4B**).

Mapping QTLs for heading date

To clarify the genetic basis for the extremely late heading of YKCSSL-6.1 observed in a field trial (Kato and Hirayama 2021), we performed QTL analysis for heading date using the BC_4F_2 population derived from a cross between YK3CSSL-6.1 and Kirara397. The days to heading of Kirara397 and Yukihikari plants were 86.2 ± 2.9 DAI and 101.5 ± 3.3 DAI, respectively. Assessment of 40 YK3CSSL-6.1 plants showed that 10 (25%) headed between 118 DAI (11 August) and 141 DAI (3 September) (mean 132.3 ± 7.8 DAI (**Fig. 5A**); 24 (60%) did not head before 142 DAI (4 September), and six (15%) had dead.

Frequency distributions of DTH for the BC_4F_2 population showed a broad range, from 86 DAI (10 July) to 137 DAI (31 August) and continuous distribution (**Fig. 5B**). Of the 81 poor growly F_2 plants, 21 (25.9%) died before heading and 37 (45.7%) failed to head before 142 DAI (4 September). QTL analysis was performed to compensate for the missing DTH data of these 58 plants. We detected two large QTLs for DTH, one between YKOInDel-17328 (0.2 Mb) and YJInDel-206 (2.0 Mb) (PVE 42.6%) and the other between YJIndel-207 (5.2 Mb) and YJInDel-230 (11.7 Mb) (PVE 28.2%) (**Table 2**). The former QTL was matched to *thb1*, whereas the latter, located close to YJInDel-230, could be *Hd1* (**Supplemental Fig. 2**).

Further evidence for the genetic basis of HB

To confirm if the complementary recessive genes are associated with the HB, we genetically analyzed F_1 and F_2 individuals of the reciprocal crosses between Yukihikari and Kirara397. Because weak plants were short plant, we measured the plant length of 973 F_2 individuals (**Supplemental Fig. 3**). Both parental plants and F_1 plants of the

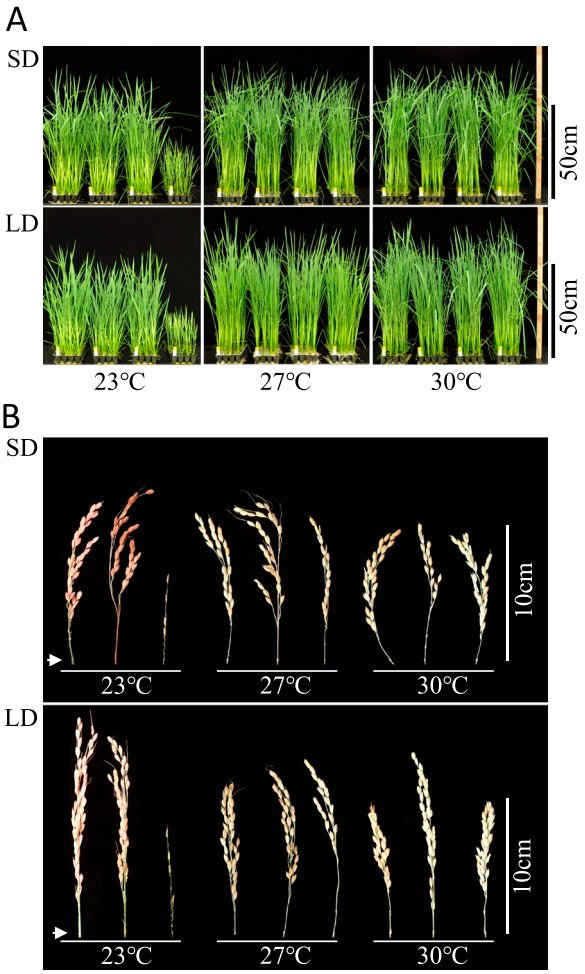

Fig. 2. (A) Plant types of Kirara397, Yukihikari, YK3CSSL-5.4 and YK3CSSL-6.1 (*left* to *right*) on DAI 31 grown at 23°C, 27°C and 30°C under short day (SD: 11.5 h light, *upper*) and long day (LD: 14.5 h light, *lower*) conditions. (B) Panicle morphologies of Kirara397, Yukihikari and YK3CSSL-6.1 (*left* to *right*) grown at 23°C, 27°C and 30°C under short day (SD: 11.5 h light, *upper*) and long day (LD: 14.5 h light, *lower*) conditions. *White arrows* indicate panicle nodes.

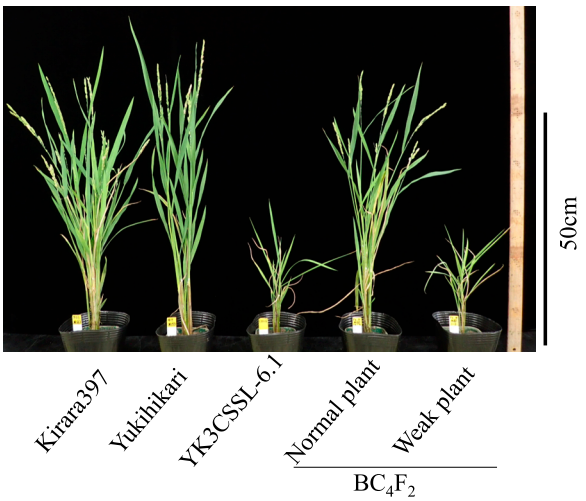

Fig. 3. Sizes of Kirara397, Yukihikari, YK3CSSL-6.1, normal and weak plants segregated in the BC_4F_2 population of the cross between YK3CSSL-6.1 and Kirara397 at 149 DAI.

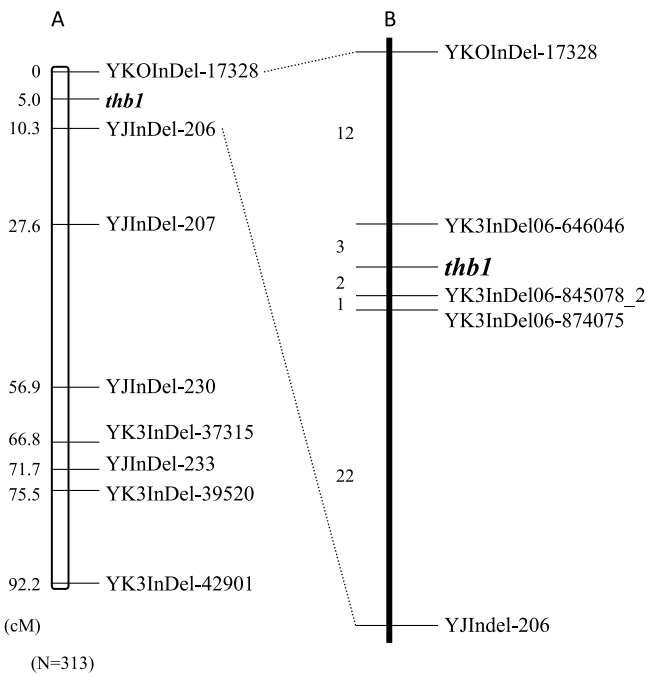

Fig. 4. Linkage map showing the *thb1* locus for low temperature dependent hybrid breakdown. (A) Initial map of *thb1* constructed from the BC_4F_2 population of a cross between YK3CSSL-6.1 and Kirara397 (n = 313). Markers are indicated to the right of the chromosome, and genetic distance (cM) is shown on the left. (B) Narrowed map of *thb1* constructed using 40 recombinant BC_4F_2 plants between YKOInDel-17328 and YJInDel-206. Markers are indicated to the right of the chromosome, and the number of recombinants between marker positions is shown on the left.

reciprocal crosses were normal. Individual F_2 plants from the cross #K19-01 (Yukihikari × Kirara397) ranged in height from 9.0 cm to 75.1 cm (average 58.3 cm) and those from the cross #K19-04 (Kirara397 × Yukihikari) ranged in height from 10.2 to 76.0 cm (average 55.0 cm). To determine whether the weak plants were genetically regulated, we calculated segregation ratio of normal (≥40 cm) versus weak (<40 cm) plants in each F_2 population. The segregations ratios of normal to weak plants were 465:19 in the cross #K19-01 and 451:38 in the cross #K19-04 (**Supplemental Table 3**). These correspond to a ratio of 15:1 ($\chi^2 = 4.46$, $p = 0.035$ and $\chi^2 = 1.93$, $p = 0.165$) for two recessive genes rather than 3:1 ($\chi^2 = 114.64$, $p < 0.001$ and $\chi^2 = 77.42$, $p < 0.001$) for single recessive genes or 63:1 ($\chi^2 = 17.57$, $p < 0.001$ and $\chi^2 = 122.55$, $p < 0.001$) for triplicate recessive genes. The HB was present in both F_2 populations derived from the reciprocal crosses between Yukihikari and Kirara397, suggesting that the HB was due to an interaction between *thb1* from Yukihikari and a second gene, named *thb2*, from Kirara397, rather than to a cytoplasmic effect.

Table 2. QTLs for days to heading in the BC_4F_2 population of a cross between YK3CSSL-6.1 and Kirara397

Marker interval	LOD	PVE[a] (%)	Additive effect	Dominance effect	Donor of positive allele
YKOIndel-17328–YJIndel-206	59.1	42.6	11.1	–9.4	Yukihikari
YJIndel-207–YJInDel-230	47.7	28.2	8.6	–3.1	Yukihikari

[a] Phenotypic variance explained.

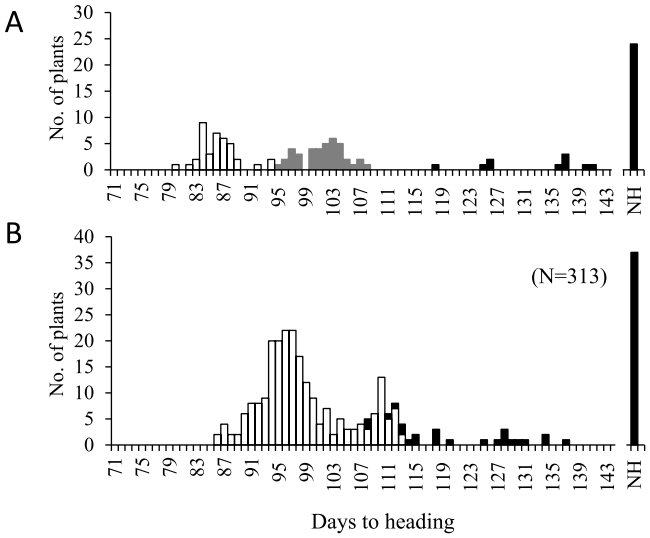

Fig. 5. Frequency distribution of days to heading. (A) Frequency distribution of days to heading of Kirara397, Yukihikari and YK3CSSL-6.1. *White*, *gray* and *black bars* indicate Kirara397, Yukihikari and YK3CSSL-6.1, respectively. (B) Frequency distribution of days to heading in the BC_4F_2 population derived from a cross between YK3CSSL-6.1 and Kirara397. *White* and *black* bars indicate normal and weak plants, respectively. *NH*, no heading before 144 DAI.

Discussion

The map of HB genes provides valuable information for gene cloning, for understanding the mechanisms at the molecular level, and for overcoming problems in cross breeding. The present study identified a novel HB caused by the interaction between two recessive genes, *thb1* and *thb2*, in the cross between Yukihikari and Kirara397, grown in Hokkaido, one of the northernmost limits of rice cultivation in the world. Of these two loci, *thb1*, from Yukihikari, was located between YK3InDel06-646046 (0.6 Mb) and YK3InDel06-845078_2 (0.8 Mb) on chromosome 6. To date, *hwg1*, was mapped between RM7193 (20.3 Mb) and C214 (21.6 Mb) on chromosome 6 (Fukuoka *et al.* 2005), demonstrating that *thb1* was nonallelic to *hwg1*. Because the chromosomal position of *thb1* did not match that of previously reported genes, *thb1* is likely a novel gene involved in rice HB. The present genetic study was based on the phenotype data of the BC_4F_2 population and the F_2 population of the crosses between Yukihikari and Kirara397 grown outside at Obihiro. Mean air temperature during the vegetative stage, from May to July, was less than 25°C during both seasons. Therefore, the phenotype of the temperature sensitive HB was accurately evaluated.

Two molecular mechanisms underlying the BDM type of HB and hybrid weakness have been reported in *Arabidopsis*: an elevated autoimmune response associated with an NBS-LRR gene (Alcázar *et al.* 2009, Bomblies *et al.* 2007) and reciprocal silencing or loss of duplicated genes (Bikard *et al.* 2009, Vlad *et al.* 2010). In rice, HB caused by an autoimmune response has been reported in a cross between *japonica* and *indica* cultivars (Yamamoto *et al.* 2010a). The HB in F_2 plants of this cross occurred when *hbd2*, a gene encoding casein kinase I derived from the *indica* cultivar, combined with an NBS-LRR gene, *hbd3*, derived from the *japonica* cultivar (Yamamoto *et al.* 2010a).

The reciprocal silencing or loss of duplicated genes has also been proposed as a genetic basis for hybrid incompatibility (Lynch and Conery 2000, Lynch and Force 2000, Werth and Windham 1991). In rice, two cases of reciprocal silencing have been demonstrated in F_1 sterility caused by *DOPPELGANGER1* on chromosome 1 and *DOPPELGANGER2* on chromosome 6 (Mizuta *et al.* 2010) and duplicated *mtRPL27* at *S27* on chromosome 8 and *S28* on chromosome 4 (Yamagata *et al.* 2010). Both whole genome and segmental duplication have been observed in rice (Thiel *et al.* 2009, Wang *et al.* 2007, Yu *et al.* 2005). The *thb1* region, located at 0.2–2.0 Mb on chromosome 6, is at least partly duplicated on rice chromosome 2 (Thiel *et al.* 2009). Because the genomic position of *thb2* remains undetermined, we could not exclude the possibility that the HB identified in the present study derived from the reciprocal silencing or loss of duplicated genes on chromosomes 6 and 2. At present, we are attempting to molecularly map *thb2*. Furthermore, molecular cloning of *thb1* and *thb2* is needed to clarify the mechanism.

Alternatively, the present *thb1-thb2* temperature sensitive HB may be associated with immune responses. In plants, conditionally expressed reproductive barriers have been observed. Hybrid necrosis or weakness studied to date are temperature sensitive (Alcázar *et al.* 2009, Bomblies *et al.* 2007, Chen *et al.* 2014, Fu *et al.* 2013, Saito *et al.* 2007), only one low temperature-dependent HB has been reported in *Arabidopsis* (Alcázar *et al.* 2009). Usually, low temperature promotes the expression of inferior symptoms in hybrid necrosis or weakness, consistent with the notion that high temperature inhibits plant immunity (Alcázar and Parker 2011, Hua 2013, Traw and Bergelson 2010). For HB, *Hwi1/Hwi2*-induced hybrid weakness in rice is suppressed by low temperature, with this suppression thought

to be an overactivation of defense responses through pattern-triggered immunity (PTI) at high temperatures (Chen *et al.* 2014). Effector-triggered immunity (ETI) is preferentially activated in plants at low temperatures, whereas PTI is preferentially activated at high temperatures (Cheng *et al.* 2013), supporting the idea that the genes involved in ETI are more likely to be recruited for establishing hybrid incompatibility conditioned at low temperature. Molecular cloning of the two recessive genes is needed to determine whether the *thb1-thb2* system is due to over-activation of ETI.

Because the HB genes are pre-existing in nature, not created *de novo*, domestication and breeding programs may comprehensively change the maintenance and distribution of these genes (Chen *et al.* 2016). The breeding of temperate *japonica* rice for growth during the summer monsoon season at higher latitudes has a long history in Japan. Hokkaido, which is located at 45–42°N latitude, is the northernmost region of rice paddy cultivation in Japan and one of the northernmost limits of rice cultivation in the world. The alternative breeding history of rice cultivars grown in Hokkaido starts in the late 1800s and is relatively short. After rice production was improved so that Japan was almost completely self-sufficient, the main breeding objective was changed from high yield to good eating quality (Horie *et al.* 2005). Eating quality has been improved by inclusion of the elite Japanese cultivar Koshihikari, released for cultivation on Honshu, the main island of Japan, and related modern Japanese rice cultivars (Yamamoto *et al.* 2010b). In Hokkaido, the first good eating quality rice cultivar Yukihikari, released in 1981, was derived from the progeny of crosses between Hokkaido landraces, without crossing with exotic germplasms such as Koshihikari. Therefore, the eating quality of Yukihikari is achieved by pyramiding spontaneous mutation(s) and/or pre-existing gene(s) in the Hokkaido gene pool (Fujino *et al.* 2019, Kato and Hirayama 2021, Kinoshita *et al.* 2017, Shinada *et al.* 2014). In contrast, Kirara397 was derived from a cross between the cultivars Shimahikari and Kitaake, which were derived from the progeny of crosses between Hokkaido cultivars and two exotic cultivars, Koshihikari from Honshu, Japan and Cody from the United States, respectively. Thus, these two exotic cultivars are likely candidate donors of *thb2*. In contrast, the pedigree of Yukihikari suggested that Hokkaido landraces were the most likely donors of *thb1*. In addition, we could not exclude the possibility that the causative gene for *thb1* emerged during the breeding programs in Hokkaido.

Cross breeding is important for controlling and broadening genetic diversity in local gene pools. Breeders use hybridization to combine desirable phenotypic characters from two or more cultivars or species into a single cultivar. Exotic germplasm as a parental cultivar can be used to explore the genetic diversity of local gene pools and may break the balance of elite phenotypes from the combination of desirable traits/genes. In some cases, genetic recombination results in the production of new and desirable characteristics not found in either parent. HB caused by a complementary effect of different chromosomal regions would be a serious problem for hybridization breeding. Close linkage between a favorable allele and a deleterious allele would result in their co-segregation (Lynch and Walsh 1998). For example, if a deleterious allele, such as *thb1* in Yukihikari or *thb2* in Kirara397, were linked closely to an allele favorable to rice breeding, the favorable allele could not be transferred to the progeny or may be eliminated when breeders select out plants with a weakness phenotype. Therefore, to avoid such problems in future breeding programs, it is important to recognize those cultivars that carry such deleterious *thb1* and *thb2* alleles and determine the precise chromosomal positions of these genes.

The biochemical and molecular mechanisms underlying HB, as well as the relationships between causal genes, are unclear. A complete understanding of the process underlying the development of for low temperature dependent HB requires further characterization of the mechanisms by which gene products induce hybrid dysfunction at the molecular, cellular and organ levels. These studies will not only improve understanding of HBs but also help improve crop cross-breeding.

Author Contribution Statement

KK designated and managed the project and wrote the initial draft of the manuscript. YY and TW performed the experiments.

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