Identification of quantitative trait loci associated with boiled seed hardness in soybean

Kaori Hirata*1,2), Ryoichi Masuda2), Yasutaka Tsubokura3), Takeshi Yasui3), Tetsuya Yamada2), Koji Takahashi2), Taiko Nagaya2), Takashi Sayama4), Masao Ishimoto4) and Makita Hajika2)

1) NARO Tohoku Agricultural Research Center, 297 Uenodai, Kariwano, Daisen, Akita 019-2112, Japan
2) NARO Institute of Crop Science (NICS), 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan
3) Snow Brand Seed Company, Limited, 634 Naganumahara, Inage, Chiba 263-0001, Japan
4) National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

Boiled seed hardness is an important factor in the processing of soybean food products such as nimame and natto. Little information is available on the genetic basis for boiled seed hardness, despite the wide variation in this trait. DNA markers linked to the gene controlling this trait should be useful in soybean breeding programs because of the difficulty of its evaluation. In this report, quantitative trait locus (QTL) analysis was performed to reveal the genetic factors associated with boiled seed hardness using a recombinant inbred line population developed from a cross between two Japanese cultivars, ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’, which differ largely in boiled seed hardness, which in ‘Natto-shoryu’ is about twice that of ‘Hyoukei-kuro 3’. Two significantly stable QTLs, qHbs3-1 and qHbs6-1, were identified on chromosomes 3 and 6, for which the ‘Hyoukei-kuro 3’ alleles contribute to decrease boiled seed hardness for both QTLs. qHbs3-1 also showed significant effects in progeny of a residual heterozygous line and in a different segregating population. Given its substantial effect on boiled seed hardness, SSR markers closely linked to qHbs3-1, such as BARCSOYSSR_03_0165 and BARCSOYSSR_03_0185, could be useful for marker-assisted selection in soybean breeding.

Key Words: boiled seed hardness, soybean, DNA markers, QTL, SSR, marker-assisted selection.

Introduction

Soybean [Glycine max (L.) Merr.] is a major crop worldwide and is globally used for oil production and feed grain, as well as locally for processing into a variety of traditional foods, such as tofu, boiled green soybeans (edamame), boiled beans (nimame), fermented steamed beans (natto), and fermented steamed bean paste (miso). In Japan and other East Asian countries where soybean is used commonly for traditional foods, the processing properties of soybean seeds, including size, color, texture, and degree of water absorption, are important for preparation of such food products. Cooked seed hardness is one of the most important factors for the processing of these food products, though the cooking methods for softening beans differ depending on the end product, for example, for edamame and nimame beans are boiled, and for natto and miso beans are steamed (Taira 1990, Yoshioka et al. 2009, Zhang et al. 2008). In this report, the terms ‘boil’, ‘steam’ and ‘cook’ are distinguished on the basis of the heating method used to soften soybean seeds; ‘boil’ is used for heating in boiling water, ‘steam’ for heating under pressure, and ‘cook’ in a broad sense for various heating methods including steaming and boiling.

Several studies have indicated that cooked seed hardness is positively correlated with quantity of calcium ions in the cooking solution, and negatively correlated with amount of dissolved polysaccharides or boron during cooking of the seeds, but the seed component that determines cooked seed hardness remains to be identified (Makabe 2006, Yoshioka et al. 2009). Moreover, negative correlations between cooked seed hardness and 100-seed weight or degree of water absorption have been reported, but the underlying genetic mechanisms have not been elucidated (Hirota et al. 2005, Motoki et al. 1999).

A Japanese soybean cultivar ‘Natto-shoryu’ (Fig. 1) was developed for natto by pure-line selection at Ibaraki Agricultural Experiment Station in 1976. This cultivar, with small seeds and yellow seed coat, has been used extensively as a parent to breed a number of cultivars for natto, including ‘Suzuotome’, ‘Suzukomachi’, and ‘Suzuroman’ (Matsunaga et al. 2003, Sakamoto et al. 2002, Yagasaki et al. 2008). The appropriate texture is required for natto because the hardness of steamed seeds correlated with the amount of unfavorable ammonia in natto (Taira 1990, Zhang et al. 2008). On the other hand, the Japanese landrace, ‘Tanbaguro’ (Nagata 1953), is popular for nimame because of the remarkably soft
texture of the cooked seeds and its distinctive large seeds and black seed coat. ‘Hyoukei-kuro 3’ (Fig. 1) is a cultivar of relatively recent origin developed from ‘Tanbaguro’ by pure-line selection at the Hyogo Prefectural Research Center of Agriculture, Forestry and Fisheries, and is the dominant cultivar grown in Hyogo, Japan (Hirota et al. 2012). ‘Hyoukei-kuro 3’ has almost the same properties as the ancestral landrace in terms of cooked seed hardness (Hirota et al. 2005). For nimame, a soft texture is generally preferable because it directly affects eating quality. Therefore, identification of factors that control cooked seed hardness in ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’ would be useful to improve processing suitability both for natto and nimame.

DNA marker-assisted selection (MAS) has been successfully incorporated into soybean breeding programs in order to select diverse traits such as soybean pod dehiscence and maturity time (Suzuki et al. 2010, Yamada et al. 2012). Zhang et al. (2008) performed quantitative trait locus (QTL) mapping for cooked (steamed) seed hardness using a population derived from cultivars developed for natto, and identified two stable QTLs with logarithm of the odds ratio (LOD) scores ranging from 5.1 to 6.2. DNA markers linked to genes that determine cooked seed hardness would be useful because it is otherwise time consuming and laborious to evaluate these traits. In this report, to reveal the genetic factors that control cooked seed hardness, QTL analysis was performed using a recombinant inbred line (RIL) population derived from a cross between ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’. Identification of QTLs and their flanking marker information in this report could contribute to development of an MAS system to discriminate boiled seed hardness.

Materials and Methods

Plant materials and experimental condition

A population of 126 RILs was developed using a single seed descent procedure from F2 segregants derived from a cross between two Japanese soybean cultivars, ‘Natto-shoryu’ (NIAS Genebank JP29161) and ‘Hyoukei-kuro 3’ at the NARO Institute of Crop Science (NICS). ‘Natto-shoryu’ was selected as a typical cultivar for natto, and ‘Hyoukei-kuro 3’ was selected as a typical cultivar for nimame. The F5 and F6 generations of the RIL population were sown in the experimental field at NICS, Ibaraki, Japan (36°01′N, 140°05′E), on 22 June, 2010 and 28 June, 2011. The plot was fertilized with 30 kg N ha⁻¹, 200 kg P2O5 ha⁻¹, and 100 kg K2O ha⁻¹ before sowing; each line was planted in a single-row plot of 3.0 and 1.5 m in length in 2010 and 2011, respectively, with a row spacing of 0.7 m and plant separation within each row of 0.13 m without replication in both years. The parental cultivars were planted with two replications in the same conditions. The parental cultivars and each line were harvested and threshed in bulk within each row.

To survey the candidate genetic region and validate the effect of a major QTL, we selected a heterozygous F5 plant from the RIL population using DNA markers in the region proximal to the QTL. The residual heterozygous line (RHL) (Yamanaka et al. 2005) in the F5 generation was selfed to produce the F6 generation, and the resultant individuals were harvested in bulk and segregating F5:7 seeds were obtained in 2011. Another F2 population derived from a cross between the Japanese breeding line ‘Kanto-115’ and ‘Hyoukei-kuro 3’ was used to confirm the effect of a major QTL detected in this report. ‘Kanto-115’ is a breeding line with small seeds and black seed coat derived from a cross between the Japanese cultivars ‘Kuro churyu’ (NIAS Genebank JP27680) and ‘Natto-shoryu’ at NICS. Ninety-six F5:7 progeny plants of the RHL and the F2 population comprised of 51 plants were planted in the experimental filed at NICS on 26 June, 2012. Each population was grown in rows 3.0 m in length and plants were harvested individually. The fertilizer application, row spacing, and individual plant spacing within each row were the same as those applied in 2010 and 2011.

Phenotypic measurements of RIL population

Approximately 30 seeds of each line or cultivar were soaked in 100 mL deionized water at 20°C for 22 ± 2 h. Unimbibed seeds were removed and the water was discarded after soaking. Imbibed seeds were boiled for 10 min with a gas burner in 200 mL deionized water in a 500-mL tall glass beaker (HARIO Ltd., Tokyo, Japan). Seeds were packed in a tea bag made from plastic fiber (Daisanshigyo Ltd., Aichi, Japan) to avoid bubbling damage during boiling. After cooking, the water was drained well and seeds stood for approximately 15 min at room temperature. The seed coat and embryo axis was removed from the boiled seeds, and then the cotyledons were separated from each other. The puncture strength was measured using a Rheoner RE-3305S texture analyzer (Yamaden Corp., Tokyo, Japan) fitted with a cylindrical probe (3 mm outside diameter) and a 2-kg-capacity load cell. The adaxial (flat) side of a piece of cotyledon was placed facing downward on the stage of the analyzer, and the hardness was measured. The probe was operated at a distance of 2.45 mm and a speed of 1 mm/s. Puncture strength was determined as maximum force (Pa). The average score calculated from the maximum force for more than 20 cotyledons was used as an index of boiled seed hardness for each sample. Hereafter, this average score...
is referred to as ‘boiled seed hardness’. Boiled seed hardness and the coefficient of variation for each RIL and the parental cultivars are presented in Supplemental Table 1 together with the numbers of soaked, unimbibed and measured seeds. Boiled seed hardness was determined from less than 20 cotyledons in the three (RIL_36, 68, 81) and two (RIL_13, 58) recombinant inbred lines in 2010 and 2011, respectively, because many seeds exhibited low water absorption in these lines. Boiled seed hardness was not determined in the eight lines (RIL_9, 29, 38, 71, 81, 93, 102, 105) in 2011 because these lines did not produce a sufficient number of seeds for measurement of boiled seed hardness (Supplemental Table 1).

The 100-seed weight was calculated from the number and weight of air-dried seeds before soaking, and water absorption ratio was calculated as the ratio of the seed weight after soaking to that before soaking.

**DNA isolation and marker analysis**

Genomic DNA was isolated from young leaves from one of the F2 generation plants for each RIL by the method described previously (Mori et al. 2003) with some modifications. Leaves were crushed in 400 μL DNA extraction buffer (0.1 M Tris–HCl, pH 8.0; 0.05 M EDTA, pH 8.0; 0.5 M NaCl; 43.3 mM SDS; 10 mM dithiothreitol) with a SHAKE MASTER ball mill (Inter Bio Techno, Ltd., Tokyo, Japan), and homogenates were centrifuged at 2750 × g in a Hitachi CF 5RX centrifuge (Hitachi, Ltd., Tokyo, Japan) for 10 min. An aliquot of the supernatant was mixed with 100 μL of 5 M potassium acetate buffer and incubated at 5°C for 10 min. After centrifugation, 300 μL of the supernatant was gently mixed with 300 μL isopropyl alcohol and incubated at 5°C for 10 min. The total DNA was precipitated and segregated by centrifugation at 2750 × g at 5°C for 10 min, then washed with 500 μL of 99.5% ethyl alcohol, and suspended in distilled water.

A whole-genome simple sequence repeat (SSR, also referred to as microsatellite) panel, consisting of 304 SSR loci, was used for mapping in the RIL population (Sayama et al. 2011). The amplification and detection of the resulting amplicons using a fluorescence-based DNA sequencer followed the method described by Sayama et al. (2011). To increase the marker density and fill several gaps in the linkage map, additional 72 SSR markers (Hwang et al. 2009, Song et al. 2010, Soybase [http://soybase.org/]), two insertion/deletion (In/Del) markers (Sca184, 394k and SRM1; Suzuki et al. 2010), and a dCAPS marker (E2_Dral; Tsubokura et al. 2014) were analyzed.

Fifteen additional SSR markers were selected according to their location between the proximal markers of a major QTL (Sct_195 and Satt530) and polymorphism between parental cultivars (Supplemental Table 2) (Song et al. 2010, Soybase [http://soybase.org/]) in order to survey the candidate genetic region and validate the effect of the major QTL using progeny of the RHL and another F2 population derived from a cross between ‘Kanto-115’ and ‘Hyoukeikuro 3’. Among the markers, two polymorphic markers, BARCSOYSSR_03_0172 and BARCSOYSSR_03_0175, were used to compare boiled seed hardness between the ‘Natto-shoryu’-type and the ‘Hyoukei-kuro 3’-type in the progeny of the RHL, and a different combination of two polymorphic markers, BARCSOYSSR_03_0165 and BARCSOYSSR_03_0185, was used to compare boiled seed hardness between the ‘Kanto-115’-type and the ‘Hyoukei-kuro 3’-type in the F2 population. The allele genotype at the major QTL was also investigated using four polymorphic markers, BARCSOYSSR_03_0165, BARCSOYSSR_03_0172, BARCSOYSSR_03_0175, and BARCSOYSSR_03_0185, for ‘Kanto-115’ derived from a cross between ‘Kuro churyu’ and ‘Natto-shoryu’. To determine the genotypes of the progeny of the RHL, the F2 population and ‘Kanto-115’, PCR was performed in a 10 μL reaction volume prepared with 450 nM of each primer, 37.5 ng total genomic DNA, and 5 μL of 2× KAPA2G Robust HS RM with dye (NIPPON Genetics Co, Ltd., Tokyo, Japan). Amplification was performed with an initial incubation for 2 min at 95°C, then 33 cycles of 1 min at 92°C, 1 min at 58 or 60°C (depending on the primers used), and 1 min at 68°C, followed by a final incubation for 5 min at 72°C. The PCR products were electrophoresed on 8% (w/v) non-denaturing polyacrylamide gels, and the fragments were stained with GelRed Nucleic Acid Gel Stain 3× in water (Nakalai Tesque, Kyoto, Japan).

**Construction of a linkage map and QTL analysis**

The linkage map of the RIL population was constructed with MAPMAKER/EXP version 3.0b software (Lander et al. 1987). Linkage distances were estimated using the Kosambi mapping function (Kosambi 1943). Analysis of QTLs was performed using Windows QTL Cartographer version 2.5 software (Wang et al. 2012), employing composite interval mapping (CIM) with the Model 6: Standard Model (5 control markers, 10.0 cM window size, and Forward and Backward Method [3]). The threshold LOD score for QTL significance was estimated with a 1000-fold permutation test at 3.0 for boiled seed hardness in both years, 2.9 and 3.1 for 100-seed weight in 2010 and 2011, respectively, and 2.9 and 3.0 for water absorption ratio in 2010 and 2011, respectively.

**Results**

**Phenotypic variation of boiled seed hardness**

Boiled seed hardness determined by the texture analyzer differed significantly (ANOVA, p < 0.01) between the two parental cultivars, with that for ‘Natto-shoryu’ being more than twice the strength of that for ‘Hyoukei-kuro 3’ (Table 1). Boiled seed hardness in the RIL population showed a continuous distribution with some lines showing values beyond the range of the parental cultivars (Table 1, Fig. 2). There was a significant positive correlation (r = 0.87, p < 0.01)
Genetic analysis of boiled seed hardness

between the values of boiled seed hardness for each RIL in 2010 and 2011 (Fig. 3). These results suggested that boiled seed hardness is controlled genetically by multiple QTLs.

**Construction of a linkage map and QTL analysis for boiled seed hardness**

A genetic linkage map was constructed for the RIL population using a whole-genome SSR panel composed of 304 SSR loci in addition to 75 additional markers. A total of 220 marker loci, comprising 145 SSRs from the whole-genome panel and the 75 additional markers, exhibited obvious polymorphism in the RIL population. The resultant linkage map comprised 27 linkage groups and covered a total of 1505.1 cM. The marker order and distance between the loci of the newly constructed linkage maps showed good accordance with previously reported maps (Hwang et al. 2009, Sayama et al. 2011) (Supplemental Table 3).

Composite interval mapping for boiled seed hardness was conducted, and identified QTLs that included the same

### Table 1. Distribution of phenotypic traits in the parental cultivars and the RIL population in 2010 and 2011

| Trait                           | Year | Parents       | RILs          | CV (%) |
|---------------------------------|------|---------------|---------------|--------|
|                                 |      | Natto-shoryu  | Hyoukei-kuro 3|        |
|                                 |      | Range         | Mean          |        |
| Boiled seed hardness ($\times 10^5$ Pa) |      |               |               |        |
| 2010                            | 14.6 | 4.9           | 4.7–23.2      | 37.8   |
| 2011                            | 13.2 | 5.2           | 5.1–23.0      | 37.0   |
| Difference                       | **   |               |               |        |
| 100-seed weight (g)              |      |               |               |        |
| 2010                            | 10.6 | 66.6          | 13.8–41.5     | 19.4   |
| 2011                            | 11.2 | 61.1          | 15.6–33.3     | 17.2   |
| Difference                       | **   |               |               |        |
| Water absorption ratio           |      |               |               |        |
| 2010                            | 2.3  | 2.6           | 2.1–2.8       | 4.6    |
| 2011                            | 2.2  | 2.5           | 2.2–2.5       | 3.4    |
| Difference                       | **   |               |               |        |

CV coefficient of variation.

** indicates that the values of the two parental cultivars were significantly different at the 1% level (ANOVA).

**Fig. 2.** Frequency distribution of boiled seed hardness in the RIL population planted in 2010 and 2011. White and black arrows indicate the mean of boiled seed hardness for ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’, respectively.

**Fig. 3.** Correlation of boiled seed hardness for the RIL population in two cultivation years (2010 and 2011). ‘r’ is the correlation coefficient value; values that are significant according to a t-test at the 1% significance level are indicated by **.
Table 2. Detection of QTLs associated with boiled seed hardness, 100-seed weight and water absorption ratio in the RIL population

| Trait                      | Year | Chromosome | Marker interval flanking QTL peak (marker position cM) | LOD score | Contribution (%) | Additive effect | QTL name     |
|----------------------------|------|------------|--------------------------------------------------------|-----------|-----------------|----------------|--------------|
| Boiled seed hardness       | 2010 | 3          | Sct_195 (0.0)–Satt530 (25.9)                           | 25.0      | 47.4            | 2.94           | qHbs3-1      |
|                            | 2011 | 6          | Sat_062 (0.0)–Sat_153 (17.9)                           | 4.0       | 5.5             | –1.01          | qHbs6-1      |
|                            | 2011 | 6          | Satt277 (49.7)–Satt307 (67.4)                          | 11.2      | 15.8            | 1.72           | qHbs3-1      |
|                            | 2011 | 3          | Sct_195 (0.0)–Satt530 (25.9)                           | 20.7      | 44.3            | 2.55           | qHbs3-1      |
|                            | 2011 | 6          | Satt277 (49.7)–Satt307 (67.4)                          | 6.8       | 11.6            | 1.32           | qHbs6-1      |
| 100 seed-weight            | 2010 | 17         | Sat_284 (0.0)–CSSR172 (16.8)                           | 4.6       | 16.5            | –1.93          | qHbs3-1      |
|                            | 2011 | 6          | Sat_213 (36.7)–Sat_076 (43.9)                          | 3.2       | 7.8             | –1.15          | qHbs3-1      |
|                            | 2011 | 6          | Satt277 (49.7)–Satt100 (54.8)                          | 3.2       | 7.8             | –1.15          | qHbs3-1      |
|                            | 2011 | 17         | Sat_284 (0.0)–CSSR172 (16.8)                           | 7.3       | 23.4            | –2.01          | qHbs3-1      |
| Water absorption ratio     | 2010 | 5          | Satt545 (88.7)–Satt211 (110.4)                         | 5.0       | 14.2            | –0.04          | qHbs3-1      |
|                            | 2011 | 12         | Sat_218 (4.2)–Satt442 (7.6)                            | 3.0       | 8.3             | –0.03          | qHbs3-1      |
|                            | 2011 | 12         | Satt666 (0.0)–GMES0816 (9.5)                           | 3.2       | 8.0             | 0.02           | qHbs3-1      |

a LOD scores were calculated by CIM. QTL significance was estimated with a 1000-fold permutation test at 3.0 for boiled seed hardness in both years, 2.9 and 3.1 for 100-seed weight in 2010 and 2011, respectively, and 2.9 and 3.0 for water absorption ratio in 2010 and 2011, respectively.
b Additive effect of ‘Natto-shoryu’ allele.

adjacent markers were regarded as identical. From data in 2010, one QTL was mapped to chromosome (Chr) 3 (Sct_195–Satt530) with LOD score 25.0 and two QTLs were mapped to Chr 6 (Sat_062–Sat_153 and Satt277–Satt307) with LOD scores 4.0 and 11.2, respectively (Table 2). Two QTLs were mapped to Chr 6, but these QTLs were not regarded as identical because the QTL intervals beyond significant LOD scores were well separated from each other. From data in 2011, three QTLs were mapped to Chrs 2, 3, and 6 (Sat_135–Satt141, Sct_195–Satt530, and Satt277–Satt307) with LOD scores 3.1, 20.7, and 6.8, respectively (Table 2). Among these six QTLs identified from data in 2010 and 2011, two QTLs mapped to Chrs 3 (Sct_195–Satt530) and 6 (Satt277–Satt307) were identified consistently across years with substantial LOD score ranges of 20.7–25.0 and 6.8–11.2, respectively. These QTLs were temporarily named qHbs3-1 (QTL controlling hardness of boiled soybean) and qHbs6-1, respectively (Table 2). The contributions to total phenotypic variance of qHbs3-1 were 47.4% and 44.3% for 2010 and 2011, respectively, and those of qHbs6-1 were 15.8% and 11.6% for the two years, respectively. The alleles derived from ‘Hyoukeikuro 3’ contributed to decrease boiled seed hardness at both loci, qHbs3-1 and qHbs6-1.

Relationships of identified QTLs with seed weight and water absorption ratio

Relationships of 100-seed weight and water absorption ratio with boiled seed hardness were investigated for qHbs3-1 and qHbs6-1. The 100-seed weight and water absorption ratio differed significantly (ANOVA, p < 0.01) between the parental cultivars, and the RIL population showed a continuous distribution in these traits that approximately spanned the values for the two parents (Table 1).

For 100-seed weight, one QTL was mapped to Chr 17 (Sat_284–CSSR172) with LOD score 4.6 in 2010. From data in 2011, two QTLs were mapped to Chr 6 (Sat_213–Sat_076 and Satt277–Satt100) with LOD scores 3.2, 3.2, and one QTL was mapped to Chr 17 (Sat_284–CSSR172) with LOD score 7.3 (Table 2). The QTL mapped to Chr 17 was identified consistently across years. Two QTLs were mapped to Chr 6 in 2011, but these QTLs were not regarded as identical because the QTL intervals beyond significant LOD scores differed from each other.

For water absorption ratio, two QTLs were mapped to Chrs 5 and 12 (Satt545–Satt211 and Sat_218–Satt442) with LOD scores 5.0 and 3.0 in 2010, and two QTLs were mapped to Chrs 5 and 12 (Satt545–Satt211 and Satt666–GMES0816) with LOD scores 7.2 and 3.2 in 2011 (Table 2). The QTL mapped to Chr 5 was identified consistently across years. Two QTLs were mapped to Chr 12 across years, but these QTLs were not regarded as identical because the QTL intervals beyond significant LOD scores differed from each other.

Among the QTLs identified consistently across years for 100-seed weight and water absorption ratio, no QTL was identified in similar chromosomal regions to those of qHbs3-1 and qHbs6-1 (Table 2).

Localization and validation of qHbs3-1

To validate the effect of qHbs3-1 correctly and scan the candidate genetic region of qHbs3-1 accurately, we selected one line as an RHL according to the genotype for additional 15 SSR markers in this QTL region (Supplemental Table 2). These 15 SSR markers were selected to localize the candidate qHbs3-1 region because of the large linkage distance between the proximal markers of qHbs3-1 (Sct_195–Satt530, 25.9 cM). To select the most effective RHL for validation and scanning the candidate genetic region of qHbs3-1, rough mapping using information of RIL population was...
Genetic analysis of boiled seed hardness

performed. The mean values of boiled seed hardness of ‘Natto-shoryu’-type and ‘Hyoukei-kuro 3’-type RILs that showed ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’ alleles over the proximal markers of \( qHbs3-1 \) (Sct_195 and Satt530) were 13.9 and 7.7 (\( \times 10^5 \) Pa), respectively. To estimate rough candidate genetic region of \( qHbs3-1 \), 27 RILs that showed the recombinant genotypes between the proximal markers of \( qHbs3-1 \) (Sct_195 and Satt631 or Satt631 and Satt530) were selected. The genotypes at \( qHbs3-1 \) of the 27 RILs were estimated on the basis of their mean values of boiled seed hardness from tested two years (Supplemental Table 4), and genotypes of additional 15 SSR markers were tested for these RILs. The marker order was estimated from information of Soybase [http://soybase.org/] and graphical genotypes of the 27 RILs (Supplemental Table 4). From the graphical and estimated genotypes at \( qHbs3-1 \) of the 27 RILs, it was estimated that \( qHbs3-1 \) might be located around BARCSOYSSR_03_0175 (Supplemental Table 4). From this estimation, RIL_78 that showed segregation for only BARCSOYSSR_03_0172 and BARCSOYSSR_03_0175 among the 15 SSR markers was selected as the most effective RHL for validation and scanning the candidate genetic region of \( qHbs3-1 \) (Supplemental Table 4). The 96 progeny plants of the RHL were grouped according to their genotypes for the BARCSOYSSR_03_0172 and BARCSOYSSR_03_0175 loci, and were classified into ‘Natto-shoryu’-type (42 plants), ‘Hyoukei-kuro 3’-type (39 plants), and heterozygous-type (14 plants). One plant showed the recombinant genotype between the two tested markers. Boiled seed hardness was not determined in two plants classified as ‘Kanto-115’-type because these plants did not produce a sufficient number of seeds for measurement of boiled seed hardness. Among 16 analyzed individuals classified into the homozygous-type groups, the ‘Hyoukei-kuro 3’-type individuals showed significantly lower boiled seed hardness compared with the ‘Kanto-115’-type individuals (Table 3).

![Fig. 4. Schematic illustration of genotypes in the qHbs3-1 region of the RHL selected from the RIL population and the RHL progeny. White, black and gray bars indicate ‘Natto-shoryu’-type, ‘Hyoukei-kuro 3’-type and heterozygous genotypes, respectively.](image)

Fifty-one individuals in the \( F_2 \) population derived from a cross between ‘Kanto-115’ and ‘Hyoukei-kuro 3’ were classified into ‘Kanto-115’-type (11 plants), ‘Hyoukei-kuro 3’-type (seven plants), and heterozygous-type (32 plants) on the basis of the genotype of the BARCSOYSSR_03_0165 and BARCSOYSSR_03_0185 loci. One plant showed the recombinant genotype between the two tested markers. Boiled seed hardness was not determined in two plants classified as ‘Kanto-115’-type because these plants did not produce a sufficient number of seeds for measurement of boiled seed hardness. Among 16 analyzed individuals classified into the homozygous-type groups, the ‘Hyoukei-kuro 3’-type individuals showed significantly lower boiled seed hardness compared with the ‘Kanto-115’-type individuals (Table 3).

The genotypes of BARCSOYSSR_03_0165, BARCSOYSSR_03_0172, BARCSOYSE_03_0175, and BARCSOYSSR_03_0185 for ‘Kanto-115’ were the ‘Natto-shoryu’-type, which indicated that the ‘Kanto-115’ allele in \( qHbs3-1 \) was identical to the ‘Natto-shoryu’ allele.

Discussion

In this report, we analyzed boiled seed hardness in the RIL population derived from a cross between the two Japanese soybean cultivars, ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’. AACC International published an approved method for evaluation of boiled seed hardness in 2012 (AACC International 2012). Following this method, boiled seed hardness
was evaluated after boiling for 40 min, and 40-min-cooked soybean seeds had softness comparable to commercial cooked soybean products (Yasui et al. 2014); They also reported that a highly positive correlation was present between the hardness of samples cooked for 40 min and that for 10 min. The boiled seed hardness measured by the present report’s method were positively correlated ($r = 0.92$, $p < 0.001$) with the hardness of embryo (cotyledons plus embryo axis) measured by the AACC approved method after 40-min cooking (Yasui, personal communication).

The significant difference in boiled seed hardness between the cultivars, ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’ was confirmed in two subsequent cultivation years, with the boiled seed hardness for ‘Natto-shoryu’ being more than twice that for ‘Hyoukei-kuro 3’. We considered that the RIL population derived from a cross between the two cultivars, ‘Natto-shoryu’ and ‘Hyoukei-kuro 3’, twice that for ‘Hyoukei-kuro 3’. We considered that the RIL population in this report were ‘Natto-shoryu’ (used for natto) and ‘Hyoukei-kuro 3’ (used for nimame). Thus, the newly identified QTLs, $qHbs3-1$ and $qHbs6-1$, might be useful in the breeding of soybean cultivars both for natto and nimame.

Negative correlations between cooked seed hardness and 100-seed weight or degree of water absorption have been reported (Hirata et al. 2005, Motoki et al. 1999), and several QTLs associated with seed weight have been reported near the regions of $qHbs3-1$ and $qHbs6-1$, respectively (Hyten et al. 2004, Li et al. 2008). In addition, QTLs associated with seed coat permeability and seed cracking have been reported near the $qHbs3-1$ region (Ha et al. 2012, Keim et al. 1990), and QTLs associated with water absorption ratio have been reported near the $qHbs6-1$ region (Watanabe et al. 2004). Therefore, we determined the 100-seed weight and water absorption ratio in the RIL population and performed QTL analysis for these traits. A total of eight QTLs for 100-seed weight and water absorption ratio were detected in two cultivation years. Among these QTLs, a QTL mapped to Chr 17 (Sat_284–CSSR172) for 100-seed weight and a QTL mapped to Chr 5 (Satt545–Satt211) for water absorption ratio were identified in both years. The QTL mapped to Chr 17 (Sat_284–CSSR172) for 100-seed weight was anchored to the same proximal molecular marker (CSSR172) as the previously described QTL, $qSw17-1$, for seed weight (Kato et al. 2014), supporting the hypothesis that this QTL controls seed weight in a multiple genetic background. Two QTLs mapped to Chr 6 (Sat_213–Sat_076 and Satt277–Sat100) for 100-seed weight in 2011 had similar chromosomal regions to $qHbs6-1$, but the LOD scores and the contributions to total phenotypic variance of these QTLs were low, and these QTLs were identified in only one cultivation year. From these results, we considered that these QTLs were not significant for 100-seed weight, and that no significant QTL associated with 100-seed weight and water absorption ratio was identified in similar regions to $qHbs3-1$ and $qHbs6-1$, which indicated that $qHbs3-1$ and $qHbs6-1$ are independent of 100-seed weight, as well as of water absorption ratio.

Two significant QTLs were identified for boiled seed hardness in this report, of which $qHbs3-1$ showed a high significant effect with contribution to total phenotypic variance being more than three-fold that of $qHbs6-1$. The progeny of an RHL selected from the RIL population, and an F2 population derived from a cross between ‘Kanto-115’ and ‘Hyoukei-kuro 3’ were developed and used to validate the effect of $qHbs3-1$. The progeny of the RHL would show simple phenotypic segregation for the target QTL in a uniform genetic background (Yamanaka et al. 2005). ‘Kanto-115’ has a black seed coat and shares the same allele in the $qHbs3-1$ region as ‘Natto-shoryu’, thus the segregating population derived from a cross between ‘Kanto-115’ and ‘Hyoukei-kuro 3’ is useful for further validation of the $qHbs3-1$ genotype for boiled seed hardness. From these analyses, $qHbs3-1$ was found to contribute to the variation.

### Table 3. Comparison of boiled seed hardness for the RHL progeny and the F2 population showing segregation of the $qHbs3-1$ genotype

| Parents or population | Genotype at $qHbs3-1$* | Number of plants | Boiled seed hardness ($\times 10^3$ Pa) | Mean ± SD |
|-----------------------|------------------------|------------------|---------------------------------------|-----------|
| RHL progeny           |                        |                  |                                       |           |
| P1: Natto-shoryu      | 8                      | 17.1 ± 0.5       |                                       |           |
| P2: Hyoukei-kuro 3    | 8                      | 8.5 ± 0.3        |                                       |           |
| P1: Natto-shoryu      | 20                     | 20.8 ± 0.3       |                                       |           |
| P2: Hyoukei-kuro 3    | 20                     | 13.1 ± 0.3       |                                       |           |
| Difference            |                        |                  | ***                                   |           |
| F2 population         |                        |                  |                                       |           |
| P1: Kanto-115         | 1                      | 14.5             |                                       |           |
| P2: Hyoukei-kuro 3    | 2                      | 6.4 ± 0.3        |                                       |           |
| P1: Kanto-115         | 9                      | 16.8 ± 0.7       |                                       |           |
| P2: Hyoukei-kuro 3    | 7                      | 9.3 ± 0.9        |                                       |           |
| Difference            |                        |                  | ***                                   |           |

* Allelic type for $qHbs3-1$ is based on the genotype of markers BARCSOYSSR_03_0172 and BARCSOYSSR_03_0175 for the RHL progeny, and BARCSOYSSR_03_0165 and BARCSOYSSR_03_0185 for the F2 population.

*** indicates that the values for the P1-genotype group differed significantly from those of the P2-genotype group at the 0.1% level (t-test).
in boiled seed hardness and the allele derived from ‘Hyoukei-kuro 3’ contributes substantially to the soft texture of boiled seeds. The results for the F2 population and the location of \( qHbs3-1 \) indicated that boiled seed hardness was independent of seed coat color.

The marker interval distance proximal to \( qHbs3-1 \) was large (Set_195–Sat530, 25.9 cM) in the present QTL analysis, and therefore we delimited the range of \( qHbs3-1 \) by using the RHL and additional DNA markers between Set_195 and Sat530. Delimiting the candidate region of a target QTL is important to develop DNA markers linked tightly to the gene controlling the target trait and enables efficient selection in a MAS system. In addition, determining an accurate position for \( qHbs3-1 \) would enable isolation of the gene responsible for boiled seed hardness. The analysis using the progeny of the RHL delimited the candidate region for \( qHbs3-1 \) between BARCSOYSSR_03_0165 and BARCSOYSSR_03_0185, a physical distance of approximately 330 kb according to information from Phytozome (http://www.phytozome.net/) and 28 annotated putative genes are located in this region. Additional research such as fine-scale mapping will be required to identify the gene on \( qHbs3-1 \), though the present information for the QTLs and their flanking DNA markers is useful for application in a MAS system to improve cooked seed hardness in soybean breeding programs.

Acknowledgments

The authors are grateful to the Hyogo Prefectural Research Center of Agriculture, Forestry and Fisheries for providing ‘Hyoukei-kuro 3’ seeds.

Literature Cited

AACC International (2012) Approved methods of analysis, 11th Ed. Method 56-36.01. Firmness of cooked pulses. Approved August, 2012. AACC International, St. Paul, MN, USA. (http://dx.doi.org/10.1094/AACCIntroMethod-56-36.01)

Ha, B.K., H.K. Kim and S.T. Kang (2012) Mapping QTLs with epistatic effects and QTL-by-environment interactions for seed coat cracking in soybeans. Euphytica 186: 933–942.

Hirota, T., K. Takahata, T. Ogawa, M. Iwai and Y. Inoue (2005) Quality of soybean seeds grown in Hyogo prefecture. Bull. Hyogo Pre. Tech. Cent. Agr. Forest. Fish. (Agriculture) 53: 6–12.

Hirota, T., T. Sayama, M. Yasumasa, H. Sasama, T. Sugimoto, M. Ishimoto and S. Yoshida (2012) Diversity and population structure of black soybean landraces originating from Tanba and neighboring regions. Breed. Sci. 61: 593–601.

Hwang, T.Y., T. Sayama, M. Takahashi, Y. Takada, Y. Nakamoto, H. Funatsuki, H. Hisano, S. Sasamoto, S. Sato, S. Tabata et al. (2009) High-density integrated linkage map based on SSR markers in soybean. DNA Res. 16: 213–225.

Hyten, D.L., V.R. Pantalone, C.E. Sams, A.M. Saxton, D. Landau-Ellis, T.R. Stefaniak and M.E. Schmidt (2004) Seed quality QTL in a prominent soybean population. Theor. Appl. Genet. 109: 552–561.

Kato, S., T. Sayama, K. Fuji, S. Yumoto, Y. Kono, T.Y. Hwang, A. Kikuchi, Y. Takada, Y. Tanaka, T. Shiraia et al. (2014) A major and stable QTL associated with seed weight in soybean across multiple environments and genetic backgrounds. Theor. Appl. Genet. 127: 1365–1374.

Keim, P., B.W. Diers and R.C. Shoemaker (1990) Genetic analysis of soybean hard seedlessness with molecular makers. Theor. Appl. Genet. 79: 465–469.

Kosambi, D.D. (1943) The estimation of map distances from recombination values. Ann. Eugen. 12: 172–175.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln and L.A. Newburg (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage map of experimental and natural populations. Genomics 1: 174–181.

Li, W., D.H. Zheng, K. Van and S.H. Lee (2008) QTL mapping for major agronomic traits across two years in soybean (Glycine max L. Merr.). J. Crop Sci. Biotech. 11: 171–190.

Makabe, Y. (2006) Effect of salts on cooked beans. Bull. Soc. Sea Water Sci., Jpn. 60: 342–347.

Matsunaga, R., M. Takahashi, K. Komatsu, M. Haji, S. Sakai, K. Igita and Y. Nakazawa (2003) New soybean cultivar ‘Suzukomachi’. Bull. Natl. Agric. Res. Cent. Kyushu Okinawa Reg. 42: 31–48.

Mori, K., K. Komura and K. Hosaka (2003) DNA marker-assisted selection in potato breeding using a one-minute DNA extraction method. Breed. Res. 5 (Suppl. 2): 191.

Motoki, S., N. Yamada, N. Tanaka, M. Takamatsu and N. Takahashi (1999) Studies on selection of soybean for high processing suitability in bean paste (miso). The Hokuriku Crop Sci. 34: 118–119.

Nagata, T. (1953) Some considerations on the culture of a black soybean, Tamba-Kuro-Daizu. Sci. Reports of Hyogo Univ. Agr. 1: 9–12.

Sakamoto, H., M. Takamatsu, N. Yamada and K. Yagasaki (2002) Breeding of a new soybean variety “Suzukomachi”. The Hokuriku Crop Sci. 37: 82–84.

Sayama, T., T.Y. Hwang, K. Komatsu, Y. Takada, M. Takahashi, S. Kato, H. Sasama, A. Higashi, Y. Nakamoto, H. Funatsuki et al. (2011) Development and application of a whole-genome simple sequence repeat panel for high-throughput genotyping in soybean. DNA Res. 18: 107–115.

Song, Q., G. Jia, Y. Zhu, D. Grant, R.T. Nelson, E.Y. Hwang, D.L. Hyten and P.B. Cregan (2010) Abundance of SSR motifs and development of candidate polymorphic SSR markers (BARCSOYSSR_1.0) in soybean. Crop Sci. 50: 1950–1960.

Suzuki, M., K. Fujino, Y. Nakamoto, M. Ishimoto and H. Funatsuki (2010) Fine mapping and development of DNA markers for the \( qPDH1 \) locus associated with pod dehiscence in soybean. Mol. Breed. 25: 407–418.

Taira, H. (1990) Quality of soybeans for processed foods in Japan. JARQ 24: 224–230.

Tsukubayama, Y., S. Watanabe, Z. Xia, H. Kamomori, H. Yamagata, A. Kaga, Y. Katayose, J. Abe, M. Ishimoto and K. Harada (2014) Natural variation in the genes responsible for maturity loci \( E1, E2, E3 \) and \( E4 \) in soybean. Ann. Bot. 113: 429–441.

Wang, S., C.J. Basten and Z.B. Zeng (2012) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm)

Watanabe, S., T. Tajuddin, N. Yamana, M. Hayashi and K. Harada (2004) Analysis of QTLs for reproductive development and seed quality traits in soybean using recombinant inbred lines. Breed. Sci. 54: 399–407.

Yagasaki, K., H. Sakamoto, T. Taniguchi, N. Yamada, E. Sodeyama, M. Takamatsu, N. Takahashi, S. Motoki, T. Ushiyama, I. Shigemori et al. (2008) New soybean [Glycine max] variety ‘Suzuroman’.
Bull. Nagano Chushin Agric. Exp. Stn. 18: 71–88.
Yamada, T., M. Hajika, N. Yamada, K. Hirata, A. Okabe, N. Oki, K. Takahashi, K. Seki, K. Okano, Y. Fujita et al. (2012) Effects on flowering and seed yield of dominant alleles at maturity loci E2 and E3 in a Japanese cultivar, Enrei. Breed. Sci. 61: 653–660.
Yamanaka, N., S. Watanabe, K. Toda, M. Hayashi, H. Fuchigami, R. Takahashi and K. Harada (2005) Fine mapping of the FT1 locus for soybean flowering time using a residual heterozygous line derived from a recombinant inbred line. Theor. Appl. Genet. 110: 634–639.
Yasui, T., N. Yamada, K. Hirata, A. Okabe, N. Oki, K. Takahashi, K. Seki, K. Okano, Y. Fujita et al. (2012) Effects on flowering and seed yield of dominant alleles at maturity loci E2 and E3 in a Japanese cultivar, Enrei. Breed. Sci. 61: 653–660.
Yasui, T., T. Sasaki, K. Kohyama and M. Hajika (2014) Variation in firmness of whole beans, embryos, and testas of cooked soybean (Glycine max) cultivars. Cereal Chem. 91: 419–424.
Yoshioka, K., M. Sekine, M. Suzuki and K. Otobe (2009) Influence of soaking and steaming conditions on the hardness of steamed soybeans and fermented soybeans (natto). Nippon Shokuhin Kagaku Kogaku Kaishi 56: 40–47.
Zhang, B., P. Chen, C.Y. Chen, D. Wang, A. Shi, A. Hou and T. Ishibashi (2008) Quantitative trait loci mapping of seed hardness in soybean. Crop Sci. 48: 1341–1349.