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Note

DNA marker-assisted evaluation of cooked bean hardness of three soybean progeny lines

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Cooked bean hardness is an important trait for the processing of soybean products such as nimame, natto, miso, and soy sauce. Previously, we showed that cooked bean hardness is primarily affected by the pectin methylesterase gene *Glyma03g03360*, and that calcium content has a secondary effect on this trait. To establish a simple and timely method for the evaluation of cooked bean hardness, primers of amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) were designed to detect a single-nucleotide polymorphism of *Glyma03g03360* and subsequently used to evaluate three soybean progeny lines. The determined genotypes were compared to those identified using the cleaved amplified polymorphic sequence (CAPS) method. Seven out of 284 lines presented different genotypes, which were determined using the two methods: A genotypes were incorrectly assigned as heterozygous by CAPS, suggesting that ARMS-PCR is more reliable. *Glyma03g03360* genotypes could be used to evaluate cooked bean hardness, except for intermediate values. Cooked bean hardness within the same genotype groups was significantly correlated with calcium contents. These findings indicate that ARMS-PCR is useful for a marker-assisted selection of soybean with soft-cooked beans and that calcium content may be used for additional selection.

Key Words: ARMS-PCR, calcium, cooked bean hardness, DNA marker-assisted selection, soybean.

Introduction

Soybean [*Glycine max* (L). Merr] is a major crop worldwide used in oil production, as a feed grain, and in processed foods. Soyfood has been consumed in Asian countries for over 1000 years and is becoming increasingly popular in other countries. In Japan, about 1 million tons of soybean, of which approximately 0.2 million tons are domestic, are used for food; about 0.5 million tons are used for tofu, and about 0.3 million tons are used for cooked beans (nimame), fermented steamed beans (natto), fermented steamed bean paste (miso), and as a soy source. Most domestic soybeans are used for these processed foods. Soybeans are first cooked and softened during the production of nimame, natto, miso, and soy sauce. Soft-cooked beans obtained with a shorter cooking duration are preferable for those four processed foods (Taira 1990, Yoshioka *et al.* 2009, Zhang *et al.* 2008). Harder beans require longer and stronger heating conditions, resulting in a darker color and unfavorable tastes (Mori and Taya 2008, Takemura 2001). Hard beans also result in an undesirable texture and an unfavorable ammoniac flavor in natto (Taira 1990). Therefore, the hardness of cooked beans is a main target of soybean breeding in Japan. Since measuring cooked bean hardness is both time and labor intensive, a simple evaluation method is required by breeders.

Previously, we demonstrated that the hardness of cooked soybeans is affected by genetic factors, and *Glyma03g03360* polymorphism, which was recently renamed as *Glyma.03g028900*, is associated with the cooked bean hardness (Hirata *et al.* 2014, Toda *et al.* 2015). *Glyma03g03360* encodes a pectin methylesterase (PME) of 490 amino acids in length (Toda *et al.* 2015, SoyBase, http://soybase.org). Pectin, which is a major component of primary cell walls and the middle lamella, is mainly constituted of linear chains of 1,4-linked alpha-D-galacturonic acid units. The carboxyl groups of the galacturonic acid units are partially esterified with methanol. During heating, the middle lamella pectin of the cell walls is depolymerized by β-elimination of methyl-esterified polygalacturonic acids, promoting an increase in cell separation, which softens the tissues (Pirhayati *et al.* 2011). PME converts the methoxyl groups into carboxyl groups on the polygalacturonic acid chain and releases both methanol and protons. β-elimination of pectins is primarily responsible for heat degradation at pH 6.1, and the rate of the β-eliminative cleavage of prepared pectin is dependent on the degree of

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esterification (Sajjaanantakul *et al.* 1989). *Glyma03g03360* is expressed in the cotyledon and seed coat during the later stage of seed development (Toda *et al.* 2015), which probably decreases the degree of esterification in those tissues, resulting in harder cooked beans.

We identified four *Glyma03g03360* polymorphisms, named as A, A′, B, and B′. Polymorphism A′ contains a single base substitution in exon 3, resulting in an amino acid substitution from threonine to serine; this amino acid is not predicted to be important for enzymatic activity. Polymorphism B contains a single-base substitution in exon 3, and polymorphism B′ contains a single-base deletion in exon 1 (**Supplemental Figs. 1**, **2**). These polymorphisms are summarized in **Supplemental Table 1**. A stop codon is inserted in the B and B′ genotypes, resulting in amino-acid sequences lacking amino acids that are catalytically important (Bosch *et al.* 2005, Jimenez-Lopez *et al.* 2012). The cooked bean hardness of soybean carrying the A or A′ genotypes was found to be significantly higher than that of soybean carrying the B or B′ genotypes (Toda *et al.* 2015), which supports the suggested mechanism of softening tissues by the β-elimination of pectins.

DNA marker-assisted selection (MAS) has been successfully incorporated into soybean breeding programs to select diverse traits such as soybean pod dehiscence and maturity time (Suzuki *et al.* 2010, Yamada *et al.* 2012). Cooked bean hardness can also be evaluated by DNA markers, because our previous studies indicated a primary effect of *Glyma03g03360* on this trait (Hirata *et al.* 2014, Toda *et al.* 2015).

Dozens of single nucleotide polymorphism (SNP)-genotyping techniques have been described (Isobe *et al.* 2016, Jungerius *et al.* 2003). A real-time polymerase chain reaction (PCR) using fluorescent probes, high-resolution melting (HRM) post PCR analysis, and digital PCR (dPCR), which are simple and time-saving, have become more popular, but they require expensive equipment and/or expensive reagents. Sequencing-based methods have recently become popular too, but their costs are still high. Hybridization-based assays for SNP genotyping have been developed as low-cost and high-throughput methods, but they hold the risk for cross-hybridization and unspecific bindings (Hacia 1999, Li *et al.* 2012). PCR-electrophoresis-based methods would be preferable for many researches and breeders who require robust and simple SNP genotyping methods with low costs. In the present study, to establish MAS for cooked bean hardness, two sets of primers were designed for amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) to detect the B and B′ genotypes of *Glyma03g03360*, although the cleaved amplified polymorphic sequence (CAPS) method has been established to detect the B genotype (Toda *et al.* 2015). CAPS is a two-step method in which DNA is first amplified by PCR, and then PCR products are digested with restriction enzymes. In contrast, ARMS-PCR is a one-step method; it allows genotyping solely by inspection of reaction mixtures after electrophoresis (Newton *et al.* 1989). In addition, ARMS-PCR is more cost-effective than CAPS.

In the present study, ARMS-PCR was performed to genotype *Glyma03g03360* and the correctness of genotyping and the relationship between the genotype and cooked bean hardness were considered. Since calcium content was positively correlated with cooked bean hardness (Saio and Watanabe 1972, Toda *et al.* 2015), the effects of calcium content were also considered. Our data are useful for soybean breeders and manufacturers who have an interest in cooked bean hardness and plan to evaluate this trait using a simple method.

Materials and Methods

Plant materials

The soybean recombinant inbred line (RIL) population in the F_8 generation derived from a cross between ‘Satonohohoemi’ and ‘Fukuibuki’, and parental cultivars were planted in fields at Ishinazaka (39°32′N, 140°23′E) in the Kariwano Branch of the Daisen Research Station of Tohoku Agricultural Research Center, NARO, on 23th May, 2018.

Two soybean progeny lines in the F_5 generation derived from crosses between ‘Satonohohoemi’ and ‘Sachiyutaka’, and those between ‘Karikei-920’ and ‘Karikei-898’, and parental cultivars were planted in the same fields on the same day. These two progeny lines have been selected for good agricultural traits such as pest resistance and plant shape, but not for cooked bean hardness.

Each plot consisted of a single row with row spacings of 0.75 m, and 0.12 m between plants within each row. Starter fertilizers included 24 kg ha^{-1} N, 120 kg ha^{-1} P_2O_5, 80 kg ha^{-1} K_2O, and no compost. Soils of the experimental fields were andosol. The parental cultivars and each line were harvested in bulk.

20 lines of A and B types each were randomly selected for analyses from the RIL population derived from a cross between ‘Satonohohoemi’ and ‘Fukuibuki’. Six lines of the A′ type and five lines of the B type were used from the progeny lines derived from a cross between ‘Satonohohoemi’ and ‘Sachiyutaka’, and three lines of the A type and five lines of the B type were used from the progeny lines derived from a cross between ‘Karikei-920’ and ‘Karikei-898’ for analyses, because only progeny lines with good agricultural traits were harvested.

DNA isolation and marker analysis

Total DNA was extracted from leaves using a BioSprint 96 automatic DNA isolation system (QIAGEN) as described previously (Taguchi-Shiobara *et al.* 2019). RIL populations derived from a cross between ‘Satonohohoemi’ and ‘Fukuibuki’ were descended from a single F_5 plant and the leaves for DNA extraction were collected from the F_5 plant. For two progeny lines derived from crosses between

Table 1. Primers used for ARMS-PCR and CAPS

Primer sequence	Description	Purpose
AGCAGTGGCACTCAAGGTAAG	A forward outer primer	
TACTTCCTCCAAGGCCTACC[a]	A reverse outer primer	For detecting polymorphism B of *Glyma03g03360* by ARMS-PCR
CTACCGCGACTGCTATGTATAC	A forward primer having a mismatch for B	
CAAAGATGAAATCAATGGTGCCC	A reverse primer having a mismatch for A, A′, and B′	
GCCACACAATCCTAGTGCC	A forward outer primer	
CCTTTCCCTTTGCCTTTATTATTCTTTG	A reverse outer primer	For detecting polymorphism B′ of *Glyma03g03360* by ARMS-PCR
CAAACAAGTGCTACAATAGCCT	A forward primer having a mismatch for A, A′, and B	
TGGCACAATCACTAAGGGC	A reverse primer having a mismatch for B′	
TTTTCAAAAAGTAATCCAAGATTTTTC	A forward primer upstream of the SNP of B	For detecting polymorphism B of *Glyma03g03360* by CAPS
TACTTCCTCCAAGGCCTACC[a]	A reverse primer downstream of the SNP of B	

[a] Same DNA sequence.

'Satonohohoemi' and 'Sachiyutaka', and between 'Karikei-920' and 'Karikei-898' in the F_5 generation, the leaves for DNA extraction were collected from three individuals out of 25 per line in the field in 2018 and bulked these into a single one.

Two sets of ARMS-PCRs and a set of CAPS primers are described in **Table 1**. ARMS-PCR was performed in a 10 µL reaction volume using GoTaq Green Master Mix according to the manufacturer's instructions (Promega KK, Tokyo, Japan). Samples were amplified with an initial incubation for 1 min at 94°C, then 40 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C. PCR for CAPS was performed in a 10 µL reaction volume using LA Taq Hot Start Version according to the manufacturer's instructions (TAKARA, Shiga, Japan). Amplifications involved an initial incubation for 2 min at 95°C, then 33 cycles of 1 min at 92°C, 1 min at 58°C, and 1 min at 68°C, followed by a final incubation for 5 min at 72°C. Next, 2.5 µL of PCR product was digested with 1 U of *Bst1107* I (TAKARA, Shiga, Japan) at 37°C overnight. DNA fragments were separated using TAE agarose gel electrophoresis or the LabChip GX electrophoresis system (PerkinElmer, MA, US).

DNA sequencing

DNA fragments were amplified by PCR using primers designed for CAPS (**Table 1**) and genomic DNA as a template. PCR was performed under the same conditions as the CAPS method. For DNA sequence analysis, DNA fragments were purified with a MonoFas DNA purification kit I (GL Sciences Inc., Tokyo, Japan) following electrophoresis on agarose gel. Sequencing was performed by Eurofin Genomics Inc. (Tokyo, Japan).

Measurements of cooked bean hardness

Cooked beans were prepared, and cotyledon breaking stress was measured as described previously (Hirata *et al.* 2014, Toda *et al.* 2015). Briefly, 30 seeds were soaked in 100 mL of deionized water at 20°C for 22 ± 2 h. Imbibed seeds were boiled for 10 min in 200 mL of deionized water in a 500 mL tall glass beaker (HARIO Ltd., Tokyo, Japan) using a hot stirrer (IKA RCT basic, IKA Japan K.K., Osaka, Japan). After cooking, the seeds were cooled in water at room temperature and the water was drained. The seed coat and embryo axis were removed from the boiled seeds; then, the two cotyledons were separated. 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 down on the analyzer stage, and the hardness was measured. The probe was operated at a distance of 2.45 mm with a speed of 1 mm/s. Puncture strength was determined as maximum force (pascals). The average score for more than 40 samples was used as an index of cooked bean hardness per sample.

The 100-seed weight and the water absorption ratio were calculated as described previously (Toda *et al.* 2015).

Calcium measurements

Seed calcium concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7700x, Agilent Technologies, Tokyo, Japan) according to a previously described method with slight modification (Takagi *et al.* 2015). More than 5 g of seeds were used for analysis. Seeds were milled into flour using a vibrating mill (TI-100, Cosmic Mechanical Technology Co. Ltd., Fukushima, Japan). Soybean flour was preliminarily dried at 70°C for 2 h. Then, 20 mg ground samples were transferred into 15 mL metal-free centrifuge tubes (INA·OPTIKA, Osaka, Japan) and digested in 0.3 mL concentrated nitric acid (1.38) at 95°C for 2 h. The digested volumes were then brought up to 10 mL with ultrapure water. ICP-MS was performed using 5 µg/L ^{115}In as an internal standard. The average concentration of each sample from two measurements was converted into the amounts of mineral elements (mg kg^{-1} dry weight) in each sample. Moisture contents were calculated by weighing flour samples before and after heating at 105°C for 24 h.

Statistical analysis

Statistical analyses were performed using the SPSS software package (version 22, IBM Japan, Tokyo, Japan). Data

from parental lines, the RIL population, and the progeny lines were used (n = 65).

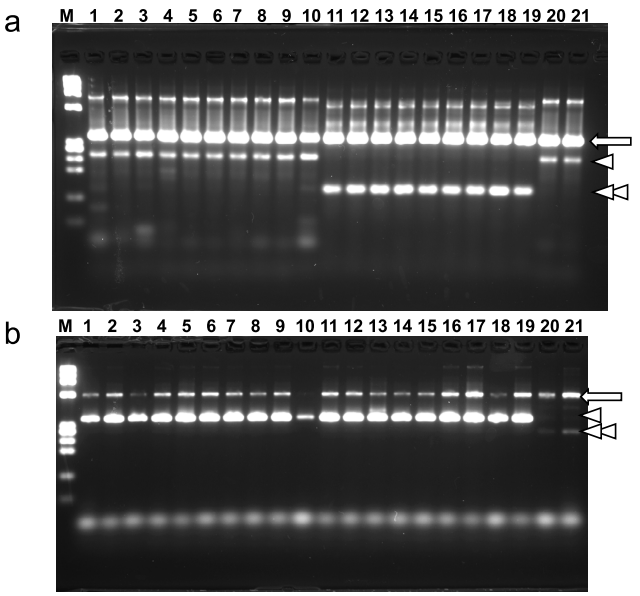
Results and Discussion

DNA fragment pattern obtained by ARMS-PCR

The ARMS-PCR primer sets used to detect the B and B′ polymorphisms of *Glyma03g03360* are described in **Table 1**. Positions of primers for ARMS-PCR are explained in **Supplemental Figs. 1** and **2**. Polymorphism B-specific DNA fragments of 143 bp were detected using B-type cultivars, while larger DNA fragments of 252 bp were detected using A-, A′-, or B′-type cultivars by ARMS-PCR to detect polymorphism B (**Fig. 1a**). Polymorphism B′-specific DNA fragments of 271 bp were detected using B′-type cultivars, while larger DNA fragments of 353 bp were detected using A-, A′- or B-type cultivars by ARMS-PCR to detect polymorphism B′ (**Fig. 1b**). The results of ARMS-PCRs using mixed genomic DNA from different cultivars indicated that these methods can detect heterozygous genotypes (**Fig. 2**).

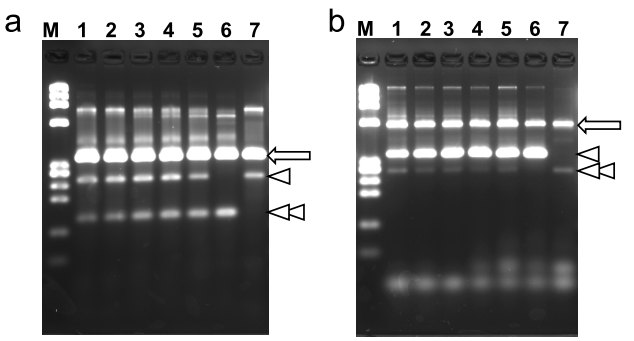
Genotyping soybeans using ARMS-PCR and CAPS

To verify the usefulness of ARMS-PCR, *Glyma03g03360* genotype and cooked bean hardness were analyzed using



Lane	Cultivar	Genotype
1	Suzukari	A
2	Suzuyutaka	A
3	Kosuzu	A
4	Fukuibuki	A
5	Tamahomare	A
6	Suzumaru	A
7	Fukuyutaka	A′
8	Suzukaren	A′
9	Sachiyutaka	A′
10	Fukuminori	A′
11	Tachiyutaka	B
12	Satonohohoemi	B
13	Enrei	B
14	Tachinagaha	B
15	Oosuzu	B
16	Akishirome	B
17	Ryuuhou	B
18	Kotoyutaka	B
19	Himeshirazu	B
20	Shuurei	B′
21	Tamadaikoku	B′

Fig. 1. Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) analysis of 21 soybean cultivars. (a) ARMS-PCR to detect polymorphism B of *Glyma03g03360*. An arrow indicates the common bands produced from the genomic DNA of all cultivars. An arrowhead indicates bands specific for A-, A′-, and B′-type cultivars. A double arrowhead indicates the band specific for B-type cultivars. (b) ARMS-PCR to detect polymorphism B′ of *Glyma03g03360*. An arrow indicates the common bands produced from the genomic DNA of all cultivars. An arrowhead indicates the bands specific for A-, A′-, and B-type cultivars. A double arrowhead indicates the band specific for B′-type cultivars. Information on lanes, cultivars and genotypes is shown on the right. M: DNA marker (ϕX174 DNA digested with HaeIII).



Lane	Cultivar	Genotype
1	Hyoukei-kuro 3 (H) / Tamadaikoku (T) (1:4)	
2	H / T (1:2)	
3	H / T (1:1)	
4	H / T (2:1)	
5	H / T (4:1)	
6	Hyoukei-kuro 3 (H)	B
7	Tamadaikoku (T)	B′

Fig. 2. ARMS-PCR analysis using mixed genomic DNAs. (a) ARMS-PCR to detect polymorphism B of *Glyma03g03360*. An arrow indicates the common bands produced from the genomic DNA of all cultivars. An arrowhead indicates the bands specific for A-, A′-, and B′-type cultivars. A double arrowhead indicates the band specific for B-type cultivars. (b) ARMS-PCR to detect polymorphism B′ of *Glyma03g03360*. An arrow indicates the common bands produced from the genomic DNA of all cultivars. An arrowhead indicates the bands specific for A-, A′-, and B-type cultivars. A double arrowhead indicates the band specific for B′-type cultivars. Information on lanes, genomic DNA, and genotypes is shown on the right. A total of 25 ng DNA template was used for all lanes. The amount of DNA was calculated from the absorbance at 260 nm. M: DNA marker (ϕX174 DNA digested with HaeIII).

the soybean RIL population and progeny lines described above. Genotypes of 284 lines were determined by ARMS-PCR and CAPS. As only Shuurei and Tamadaikoku were determined as B′ genotypes, no breeding lines derived from a B′-type cultivar were obtained. Using ARMS-PCR, 113 lines were determined as A or A′, 134 lines were determined as B, and 37 lines were determined as heterozygous. Seven of 284 lines presented different genotype results between ARMS-PCR and CAPS. These seven lines were found to carry polymorphism A by ARMS-PCR and as heterozygous by CAPS (**Supplemental Table 2**). These lines were found to carry polymorphism A by sequencing analysis. Using the CAPS method, PCR products from A- or A′-type lines were digested by *Bst1107* I; however, this restriction site was lost by a single base substitution in B-type lines. Seven polymorphism A lines were incorrectly identified as heterozygous due to the incomplete digestion of *Bst1107* I in the CAPS method. It has been pointed out that incomplete enzyme digestion is a problem inherent to the CAPS for genotyping (Duraisingh *et al.* 1998, Johnson *et al.* 2004).

A prerequisite of ARMS-PCR is the absence of 3′-exonucleolyric proofreading activity associated with the DNA polymerase (Newton *et al.* 1989). Another requirement in the application of ARMS-PCR is that 3′-OH terminal mismatched primers are refractory to extension. Single mismatches have been reported to instigate a broad variety of effects, including those that have minor (eg, A–C, C–A, T–G, G–T) and severe (eg, A–A, G–A, A–G, C–C) impacts on PCR amplification (Stadhouders *et al.* 2010). The use of ARMS-PCR to detect polymorphism B of *Glyma03g03360* showed high credibility, probably because it meets the conditions described above. C–C mismatches occur between A-, A′-, or B′-type genomes and the primer "CAAAGATG AAATCAATGGTGCCC", and between the B-type genome and the primer "CTACCGCGACTGCTATGTATAC" at the 3′ end (**Table 1**, **Supplemental Fig. 1**).

Use of ARMS-PCR to detect polymorphism B′ of *Glyma03g03360*, results in a T–G mismatch between A-, A′- or B-type genomes and the primer "CAAACAAGTGC TACAATAGCCT", which has a minor effect. In contrast, G–G and A–C mismatches occur between the B′-type genome and the primer "TGGCACAATCACTAAGGGC" at the two bases of the 3′ end (**Table 1**, **Supplemental Fig. 2**). A G–G mismatch has been reported to have a major refractory effect on PCR amplification (Stadhouders *et al.* 2010). In addition, the Tm value on the calculation is severely reduced due to a single base deletion between the B′-type genome with the primer "TGGCACAATCACTAA GGGC". For those reasons, it is expected that ARMS-PCR can be used to accurately detect polymorphism B′.

Relationship between Glyma03g03360 genotype and cooked bean hardness, and the effect of calcium

Cooked bean hardness was examined using the soybean RIL population and progeny lines described above and their parental cultivars, 'Satonohohoemi', 'Fukuibuki', 'Sachiyutaka', 'Karikei-920', and 'Karikei-898'. Calcium contents were also analyzed to investigate their effects on cooked bean hardness.

The hardness of cooked whole beans has shown a significant positive correlation with that of the cooked embryo (cotyledon and embryonic axis, $r = 0.976$, $p < 0.001$). A highly positive correlation was observed in the hardness of whole beans ($r = 0.874$, $p = 0.010$) and embryos ($r = 0.832$, $p = 0.020$) between materials cooked for 10 and 40 min (Yasui *et al.* 2014). Shorter cooking time is preferred because it allows reducing industrial processing costs (da Silva *et al.* 2009) and longer cooking time or higher cooking temperature result in a darker color and unfavorable tastes (Mori and Taya 2008, Takemura 2001). For those reasons, cotyledon breaking stress was measured after 10 min cooking for evaluation of cooked bean hardness.

The cooked bean hardness of RILs and progeny lines ranged from 682 to 1734 kPa (**Supplemental Table 3**). The cooked bean hardness of 26 of 29 A- or A′-type lines was greater than 1100 kPa, and the cooked bean hardness of 26 of 30 B-type lines was less than 1100 kPa.

The independent sample t-test detected a significant difference between cooked bean hardness of A or A′-type lines and that of B-type lines ($p < 0.05$). Correlation among *Glyma03g03360* genotype, calcium content, 100-seed weight, water absorption ratio, and cooked bean hardness were analyzed. Motoki *et al.* (1999) reported that 100-seed weight and water absorption ratio showed a significant negative correlation with cooked bean hardness ($r = -0.478$ and -0.416, respectively). Hirota *et al.* (2005) also reported a similar result ($r = -0.582$ and -0.680, respectively). *Glyma03g03360* genotype, calcium content, and 100-seed weight were significantly related with cooked bean hardness (**Table 2**). A correlation coefficient of the *Glyma03g03360* genotype was negative, because A (or A′) and B genotypes were assigned as 1 and 2, respectively, for analysis. The value –0.73 indicated that the contribution ratio of the *Glyma03g03360* genotype to the cooked bean hardness was 0.52. It was close to the contributions of *Glyma03g03360* using the RIL population derived from a cross between two Japanese soybean cultivars, 'Nattoshoryu' and 'Hyoukei-kuro 3' in 2010 and 2011, which was previously reported (Hirata *et al.* 2014).

Table 2. Correlation among *Glyma03g03360* genotype, Ca content, 100-seed weight, water absorption ratio, and cooked bean hardness

	Ca content	100-seed weight	Water absorption ratio	Hardness of cooked bean
Genotype	–0.08	0.15	–0.03	–0.73**
Ca content	—	–0.48**	–0.03	0.40**
100-seed weight	—	—	–0.39**	–0.25*
Water absorption ratio	—	—	—	–0.05

n = 65. * significant at 0.05 level. ** significant at 0.01 level.

A multiple linear regression model of the cooked bean hardness was obtained using *Glyma03g03360* genotype, calcium content, and 100-seed weight as three independent variables (**Table 3**). Collinearity was also checked using the tolerance values and the variance inflation factors (VIFs). Small tolerance values (much below 0.1) or large VIF values (above 10) indicate high collinearity (Hair *et al.* 1995). In the present study, tolerance values were more than 0.7 and VIF values were less than 2 (**Table 3**). Standardized partial regression coefficients of *Glyma03g03360* genotype, calcium content, and 100-seed weight were –0.702, 0.350, and 0.025, respectively, indicating high contribution of the *Glyma03g03360* genotype. Adjusted R-squared of the multiple linear regression was 0.62 ($p < 0.001$), which was not reduced when 100-seed weight was removed. We concluded that *Glyma03g03360* genotype and calcium content mainly affect the cooked bean hardness among factors examined in this study.

Cooked bean hardness within the same genotype group was significantly correlated with calcium content (**Fig. 3**). The cooked bean hardness of A-type lines with low calcium content, such as SHxFK_060 and SHxFK_065, and the parental cultivar 'Sachiyutaka' was less than 1100 kPa, likely due to the low calcium content (**Supplemental Table 3**). The line K920xK898_14 presented a higher calcium content (2337 mg/kg), which may result in greater cooked bean hardness (1203 kPa), than the other B-type lines (**Supplemental Table 3**).

Table 3. Summary of regression analysis on relationship between *Glyma03g03360* genotype, calcium content, and 100-seed weight, and cooked bean hardness

Independent variable	Standardized coefficient beta	Tolerance value	VIF
Glyma03g03360 genotype	–0.702	0.977	1.024
Ca content	0.350	0.771	1.297
100-seed weight	0.025	0.758	1.319

n = 65.

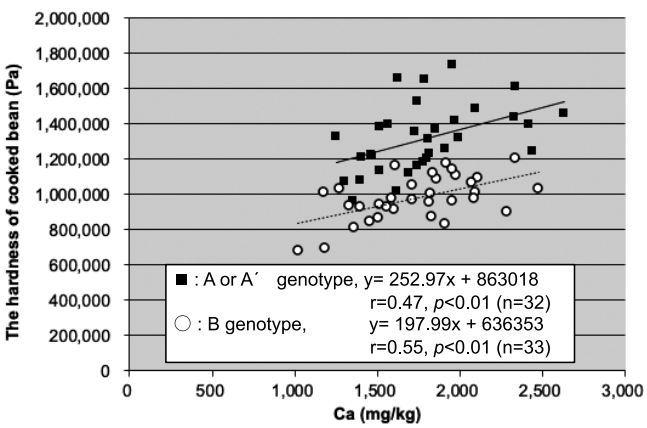


Fig. 3. Correlation between calcium content and the cooked bean hardness of soybean lines with an A or A′ genotype (black squares), and a B genotype (white circles), including 6 parental data. Approximate lines, expressions for each group, correlation coefficients, and sample numbers are shown in the graph.

Calcium pretreatment has been shown to improve the firmness of thermally processed vegetables (Siliha *et al.* 1996, Smout *et al.* 2005, Stanley *et al.* 1995). Makabe (2006) reported that cooked seed hardness is positively correlated with the quantity of calcium ions in the cooking solution. Furthermore, endogenous calcium contents were also found to be positively correlated with cooked bean hardness (Saio and Watanabe 1972, Toda *et al.* 2015). Free carboxyl groups produced by PME activity on the pectin molecules can cross-link by calcium bridges, which provides structural rigidity to the cell wall (Goldberg *et al.* 1986, Holdaway-Clarke and Hepler 2003). Calcium ions bind to protein or phytate, which may also explain the effect of calcium on cooked bean hardness. The relationship between calcium content and cooked bean hardness obtained using soybeans with polymorphism B (**Fig. 3**) indicates that other PMEs may affect the cotyledon, or mechanisms other than calcium binding with pectin.

In conclusion, in this study, primers for ARMS-PCR were designed to detect B and B′ genotypes of *Glyma03g03360*. Comparison of the results obtained using ARMS- PCR, CAPS, and sequencing suggests that ARMS-PCR accurately detects B-type genotypes. Cooked bean hardness of RILs and progeny lines was assessed by genotyping *Glyma03g03360*. RILs or progeny lines with polymorphism A or A′ presented cooked bean hardness greater than 1000 kPa, while those with polymorphism B presented cooked bean hardness less than 1300 kPa. Overall, 88% (52 out of 59) of RILs or progeny lines were considered to be at the border of 1100 kPa by genotyping in this study. Previously, we showed that *Glyma03g03360* significantly affected cooked bean hardness in many soybean cultivars and F_2 populations (Toda *et al.* 2015). The ARMS-PCR method established in the present study is expected to be useful for the selection of other breeding lines. Since calcium contents were positively correlated with cooked bean hardness, this may be used for additional selection.

Author Contribution Statement

KT, SK, KH, AK, and MH planned the research. SK and AK developed the breeding lines. KT and YN performed the experiments and analyzed the data. KT prepared the manuscript.

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