Breeding Science 71: 467–473 (2021)
doi: 10.1270/jsbbs.21035

Research Paper

A pubescence color gene enhances tolerance to cold-induced seed cracking in yellow soybean

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In yellow soybean, severe cold weather causes seed cracking on the dorsal side. Yellow soybeans carry the *I* or i^i allele of the *I* locus and have a yellow (*I*) or pigmented (i^i) hilum. We previously isolated an additional allele, designated as *Ic*, of the *I* locus, and reported that yellow soybeans with the *IcIc* genotype may be tolerant to cold-induced seed cracking. The *Ic* allele by itself, however, does not confer high tolerance. The association of a pubescence color gene (*T*) with suppression of low-temperature-induced seed coat deterioration has been previously reported. In the present study, we tested whether *T* is effective for the suppression of cold-induced seed cracking using two pairs of near-isogenic lines for the *T* locus in the i^ii^i or *IcIc* background. In both backgrounds, the cracked seed rate of the near-isogenic line with the *TT* genotype was significantly lower than that with the *tt* genotype, which indicates that *T* has an inhibitory effect on cold-induced seed cracking. Furthermore, we also showed that gene pyramiding of *Ic* and *T* can improve tolerance to cold-induced seed cracking. Our findings should aid the development of highly SC-tolerant cultivars in soybean breeding programs.

Key Words: yellow soybean, abiotic stress, seed cracking, tolerance, gene pyramiding.

Introduction

In soybean (*Glycine max*), seed coat pigmentation is mainly controlled by three independent genetic loci (*R*, *T* and *I*) (Bernard and Weiss 1973). The *R* and *T* loci are responsible for the following seed coat colors: black (*R*, *T*), brown (*r*, *T*), imperfect black (*R*, *t*), and buff (*r*, *t*). Anthocyanins and proanthocyanidins (PAs) are present in black and imperfect-black seed coats, whereas only PAs are found in brown and buff seed coats; these observations suggest that the *R* allele product is essential for anthocyanin synthesis (Todd and Vodkin 1993). In fact, the *R* allele encodes a R2R3 MYB transcription factor positively regulating a gene for a UDP-glucose:flavonoid 3-O-glucosyltransferase that acts in the final step of the anthocyanin biosynthesis pathway (Gillman *et al.* 2011, Yan *et al.* 2015, Zabala and Vodkin 2014). The *T* locus was originally identified on the basis of trichome hair (pubescence) color: tawny or brown (*T*) and gray (*t*) (Woodworth 1921). The *T* allele encodes a flavonoid 3′-hydroxylase (F3′H) (Nagamatsu *et al.* 2007, Toda *et al.* 2002, Zabala and Vodkin 2003). Seed coats of pigmented soybean with the *tt* genotype, which are imperfect black and buff, are prone to splits and cracks (Stewart and Wentz 1930). According to the results of histochemical staining, we previously proposed a model of seed coat cracking in buff-pigmented soybean (Senda *et al.* 2017).

In contrast to the functions of *R* and *T* loci, the inhibitor (*I*) locus controls the distribution of anthocyanins and PAs in the epidermal layer of the seed coat (Palmer *et al.* 2004, Todd and Vodkin 1993). Multiple alleles, such as *I*, i^i, i^k and *i*, are known at the *I* locus (Bernard and Weiss 1973). The *I* allele inhibits pigmentation of the hilum and the entire seed coat, whereas the *i* allele permits pigmentation of these regions. Pigmentation conferred by i^i and i^k alleles is limited to the hilum and the saddle-shaped region including the hilum, respectively. Yellow soybeans are either completely yellow (yellow hilum cultivars, *II* genotype) or with pigmentation restricted to the hilum (pigmented hilum cultivars, i^ii^i genotype). In yellow soybeans, inhibition of seed coat pigmentation results from naturally occurring RNA silencing of chalcone synthase (CHS) genes, which we refer to as CHS silencing (Senda *et al.* 2004, Tuteja *et al.* 2004). We previously isolated a candidate gene for the *I* allele, designated as *GmIRCHS* (*Glycine max* inverted repeat of *CHS* pseudogene), whose structure was predicted

Communicated by Toyoaki Anai
Received April 13, 2021. Accepted June 14, 2021.
First Published Online in J-STAGE on August 27, 2021.
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to induce CHS silencing (Kasai *et al.* 2007, Kurauchi *et al.* 2011).

After the beginning of flowering, yellow seeds of soybean are damaged by low temperature, which leads to seed coat deterioration, including pigmentation and cracking around the hilum (Morrison *et al.* 1998, Srinivasan and Arihara 1994, Takahashi 1997, Takahashi and Asanuma 1996). This phenomenon is referred to as cold-induced seed coat discoloration (CD). CD is most likely due to the inhibition of CHS silencing by low temperature (Kasai *et al.* 2009, Senda *et al.* 2012). A CD-tolerant yellow hilum cultivar, Toyoharuka, possesses a polymorphic *GmIRCHS* structure, which has been designated as the *Ic* (Kasai *et al.* 2009, Yamaguchi *et al.* 2015b). *Ic*, a novel allele of the *I* locus, inhibits pigmentation of the hilum and the entire seed coat. The *Ic* allele is a major gene for CD tolerance, and a DNA marker distinguishing between *I* and *Ic* alleles, named the "Ic marker", has been shown to be effective for the selection of CD-tolerant soybean plants (Ohnishi *et al.* 2011). Cold-induced seed cracking (SC) is a type of seed damage that is even more severe than CD (Senda *et al.* 2018, Yamaguchi *et al.* 2014, 2015b). In typical SC-affected seeds, which also exhibit the CD phenotype, the seed coat splits on the dorsal side, and the two inside cotyledons are exposed and separated (**Fig. 1**). SC-damaged seeds are of lower quality than CD-affected ones and are discarded because they have no commercial value. As a consequence, SC hinders stable seed production. SC-tolerant cultivars are therefore needed in high latitude areas, where soybean plants are sometimes exposed to severe cold weather after the beginning of flowering. SC can be artificially induced using a phytotron by subjecting soybean plants to a 21-day low-temperature treatment 10 days after first flower opening (Yamaguchi *et al.* 2014). In a phytotron-based assay, we previously identified the cultivar Yukihomare (YH) and the breeding line Toiku 248 (T248) as susceptible and tolerant to SC, respectively, and detected two quantitative trait loci (QTLs) related to SC-tolerance, *qCS8-1* and *qCS11-1*, using recombinant inbred lines derived from a cross between T248 and YH (Yamaguchi *et al.* 2014, 2015b). We proposed the following model to explain the mechanism of SC in YH: under 21-day low-temperature treatment, PA accumulation expands to the dorsal side of the seed coat, and subsequent lignin deposition changes physical properties of the seed coat; as a result, a straight-line split occurs on the dorsal side of the seed coat at the full-sized seed stage, and SC takes place during seed maturation (Senda *et al.* 2018). In SC-tolerant T248, which possesses the *IcIc* genotype, PA accumulation is suppressed on the entire seed coat, even under low-temperature treatment, resulting in tolerance to SC (Senda *et al.* 2018). The *Ic* allele was thus suggested to be effective, not only for CD-tolerance, but also for SC tolerance. In fact, *qCS8-1*, a QTL for SC tolerance, was mapped near the *I* locus, which supports the possibility that the *Ic* allele is *qCS8-1* and contributes to SC tolerance (Yamaguchi *et al.* 2015b).

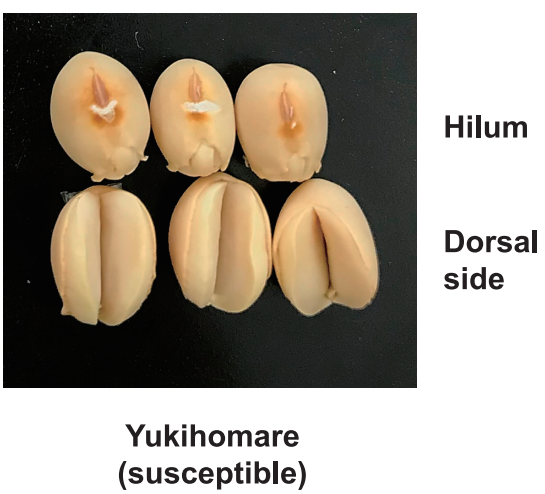

Fig. 1. Examples of cold-induced seed cracking. Yukihomare is a cultivar susceptible to seed cracking under low temperature. The seed coat on the dorsal side is split in a straight line, and the cotyledons are exposed and frequently separated.

The pubescence color gene *T* prevents seed coat deterioration due to low temperature, such as pigmentation and cracks, in yellow soybean (Takahashi 1997, Takahashi and Asanuma 1996). In yellow hilum soybean plants with the *II* genotype, however, *T* darkens the entire seed coat, resulting in dull yellow seeds with a dirty appearance (Cober *et al.* 1998, Morrison *et al.* 1998, Oyoo *et al.* 2011, Takahashi 1997, Takahashi and Asanuma 1996). In contrast, the *Ic* allele is likely responsible for minimizing *T*-induced dulling of the seed coat and leads to yellow seeds with an acceptable external appearance (Oyoo *et al.* 2011, Rodriguez *et al.* 2013). In the present study, we investigated whether *T* can contribute to SC tolerance using two pairs of near-isogenic lines (NILs). As demonstrated by our results, *T* can enhance the SC tolerance of yellow soybeans possessing $i^i i^i$ and *IcIc* genotypes, with the allelic combination of *T* and *Ic* conferring the highest SC tolerance.

Materials and Methods

Plant materials

All cultivars, breeding lines and NILs were bred at the Tokachi Agricultural Experiment Station (TAES), Memuro, Hokkaido, Japan. The two cultivars and five breeding lines used for Experiment 1 are listed in **Table 1**. The standard cultivar YH (Tanaka *et al.* 2003), which is susceptible to SC under low temperature, was used as a reference (**Fig. 1**).

A pair of To-NILs for the *T* locus with brown hilums ($i^i i^i$), To7B (tawny pubescence, *TT*) and To7G (gray pubescence, *tt*), were used for Experiment 2 (**Fig. 2**). To7B and To7G were respectively registered as JP nos. 53420 and 53421 in Genebank, NARO, Japan (https://www.gene.affrc.go.jp/index_en.php). A pair of 2304-NILs for the *T* locus possessing yellow hilums (*IcIc*), 2304-9-B (*TT*) and 2304-9-G (*tt*), were developed in this study from a

Table 1. Cracked seed rates of seven soybean cultivars and breeding lines subjected to phytotron-based assays in 2010 to 2012

Cultivar or line	Genotype		Cracked seed rate (%)			Note
	Ic/I	*T/t*	2010	2011	2012	
Yukihomare	*II*	*tt*	21.9	45.9	29.3	Susceptible cultivar (reference)
Toyomusume	*II*	*tt*	66.1	23.4	53.3	
Toiku 251	*II*	*tt*	21.5	–	–	Parental line of 2304-NILs
Toiku 238	*IcIc*	*tt*	2.2***	5.4**	6.0**	
Toiku 248	*IcIc*	*tt*	7.4**	–	8.4*	
Toiku 254	*IcIc*	*TT*	–	0.0***	0.0***	Parental line of 2304-NILs
Tokei 1105	*IcIc*	*TT*	–	1.4**	–	

*,** and *** denote values significantly lower than Yukihomare at the 5%, 1% and 0.1% levels, respectively. Dunnett's test was performed in each year using Yukihomare as a reference.

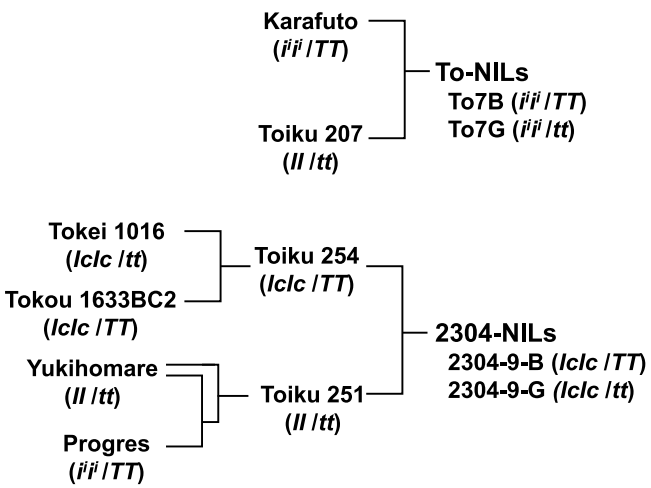

Fig. 2. Pedigrees of the two pairs of near-isogenic lines (NILs) for the *T* locus used in this study. Genotypes are shown in parentheses.

cross between breeding lines Toiku 254 (*IcIc*/*TT*) and Toiku 251 (*II*/*tt*) (Yamaguchi *et al.* 2015a) (**Fig. 2**). The 2304-NILs were bred as described below. The cross was conducted at TAES, and 58 plants were selected from the F_2 population. Line selection was then carried out in the F_3 to F_6 generations according to the pedigree method. The six F_6 sister lines selected from the F_5 line were segregated into *IcIc*/*TT*, *IcIc*/*Tt* and *IcIc*/*tt* genotypes and were found to have similar maturity dates and plant shapes according to field observations. F_6 bulked progeny with *IcIc*/*TT* and *IcIc*/*tt* genotypes were designated as 2304-9-B and 2304-9-G, respectively (**Fig. 2**).

Genotype determination of I and T loci

PCR determination of the *Ic*/*I* genotype was carried out using Ic markers as described previously (Yamaguchi *et al.* 2019). Total genomic DNA was extracted from young leaves of three plants. Sequences of primers used for Ic-specific PCR amplification were 5′-GAGTTTGAAAAATG TATTCTTTCTCTTCC-3′ and 5′-GTATCGCAGATTCCTC CTGC-3′, and those used for I-specific PCR amplification were 5′-GCAAACCAAATCAAGTAAGAGCG-3′ and 5′-CCCATTCCTTGATTGCCTTA-3′ (Ohnishi *et al.* 2011). The *T*/*t* genotype was determined by phenotypic observation of pubescence color. The *R*/*r* genotype of all materials used in this study was estimated to be *rr*.

Evaluation of SC tolerance in a phytotron-based assay (SC tolerance test)

An SC tolerance test was conducted according to Yamaguchi *et al.* (2014). Ten seeds per pot were planted in plastic pots (25L) on 17 to 28 May in 2010 to 2018. Two or three pots were prepared for each line. Two weeks after seedling emergence, two (in 2010 to 2014) or three (in 2017 to 2018) plants per pot were selected. These plants were grown in an experimental facility under a plastic roof without walls. At 10 days after the flowering date, the pots were transferred to a phytotron and grown for 21 days under the following chilling-temperature conditions: 18°C day (08:00–18:00) and 13°C night (18:00–08:00), with 55% shading. After this treatment, the pots were returned to the experimental facility, and the plants were grown to maturity. Seeds were harvested from each pot, and the cracked seed rate was calculated as follows: cracked seed rate (%) = (number of cracked seeds/total number of seeds) × 100. Each individual pot was considered as a replicate. After arcsine transformation, the cracked seed rate per pot was subjected to statistical analysis using the JMP 10 statistical package (SAS, Cary, NC, USA). Differences in the cracked seed rate among cultivars and breeding lines in each year (Experiment 1) were assessed by one-way analysis of variance (ANOVA), and Dunnett's test was performed using YH as the reference.

Comparison of SC tolerance between NILs

Two-way ANOVA was used to test for differences in the cracked seed rate between NILs (Experiment 2). "Year" and "cultivar" were considered as two factors. The standard cultivar YH, which is susceptible to SC, was used as a control to check the results of the SC tolerance test (Yamaguchi *et al.* 2014). The average cracked seed rate of YH in 2013 and 2014, used for comparisons between To-NILs, was 20.6%, and that in 2017 and 2018, used for comparisons between 2304-NILs, was 28.8%.

Evaluation of agronomic traits of NILs

Yield trials of 2304-NILs were performed in the field at TAES (42°89′ N, 140°07′ E) in 2017 and 2018. Seeds were sown on 23 May 2017 and 22 May 2018. Each plot consisted of four 3.5-m long rows spaced 60 cm apart, with a 20-cm inter-hill distance and two plants per hill, resulting in a plant population density of 16.7 plants m^{-2}. A randomized complete block design with two replicates was used. Maturity was defined as the time when >80% of plants were yellow and defoliated and their pods rattled when shaken. Before harvesting, the main stem length (distance from cotyledonary node to terminal node) of five consecutive plants in the center of each plot was recorded. Seed yield was assessed by manual harvest of individual plots and adjusted to 15% moisture. The content of seed protein was determined using a near-infrared spectrophotometer (Infratec 1241 Grain Analyzer, FOSS Tecator AB, Höganäs, Sweden). Combined ANOVA was carried out using the mixed model procedure. "Year" and "replication within year" were considered as random effects, while "cultivar" was considered as a fixed effect.

Evaluation of the seed coat color of NILs

The seed coat color of 2304-NILs was determined according to previous studies (Kato *et al.* 2000, Yousif 2014). Three color components were measured with a CM5 spectrophotometer (Konica Minolta, Osaka, Japan): L^*, a measure of lightness; a^*, a red–green color coordinate; and b^*, a yellow–blue color coordinate. The color of 20 seeds collected from the 2-year yield trial was measured through a small aperture (3 mm diameter). The standard index for assessing color differences, ΔE^*ab, was calculated as follows: $\Delta E^*ab = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$. ΔE^*ab values and corresponding visual color differences were as follows (Yousif 2014): 0–0.5, trace difference; 0.5–1.5, slightly discernible; 1.5–3.0, noticeable; 3.0–6.0, appreciable; 6.0–12.0, large; and >12, extreme.

Results

Evaluation of SC tolerance using cultivars and breeding lines (Experiment 1)

Seven cultivars and breeding lines were tested in a phytotron-based assay from 2010 to 2012 (**Table 1**). No cultivars or breeding lines with the *I*/*TT* genotype were housed in the TAES collection because this allelic combination darkens the seed coat color, resulting in a dirty seed appearance (Cober *et al.* 1998, Morrison *et al.* 1998, Oyoo *et al.* 2011, Takahashi 1997, Takahashi and Asanuma 1996) and rendering them unsuitable as breeding materials. We therefore were unable to test soybeans possessing the *I*/*TT* genotype for SC tolerance. In contrast to the *I*/*TT* genotype, the allelic combination of *IcIc*/*TT* minimizes seed coat dullness, resulting in an appearance similar to that of yellow hilum cultivars (Rodriguez *et al.* 2013). Two SC-susceptible cultivars (YH and Toyomusume) and a breeding line (Toiku 251), all possessing the *I*/*tt* genotype, had high cracked seed rates (**Table 1**). The two breeding lines with the *IcIc*/*tt* genotype (Toiku 238 and Toiku 248) had lower cracked seed rates than YH (*I*/*tt*), thus indicating that the *Ic* allele can suppress SC (Senda *et al.* 2018) (**Table 1**). The cracked seed rates of the two breeding lines (Toiku 254 and Tokei 1105) with the *IcIc*/*TT* genotype were even lower than those of Toiku 238 and Toiku 248 (*IcIc*/*tt*); in particular, the cracked seed rate of Toiku 254 was 0.0% in 2 years, i.e., SC was totally suppressed (**Table 1**).

Evaluation of SC tolerance using two pairs of NILs (Experiment 2)

We examined the effect of *T* on SC tolerance using the To-NILs, the pair of NILs for the *T* locus in the $i^i i^i$ background (**Fig. 2**). The cracked seed rate of To7B (12.8%) was significantly lower than that of To7G (29.7%) in the 2-year phytotron-based assay (**Fig. 3**). Next, we conducted a phytotron-based assay of 2304-NILs during 2 years (**Fig. 4**). The cracked seed rates of the 2304-NILs were apparently lower than those of the To-NILs, which implies that the *Ic* allele can suppress SC (**Figs. 3**, **4**) (Senda *et al.* 2018). The cracked seed rate of 2304-9-B (0.2%) was significantly lower than that of 2304-9-G (5.1%). 2304-9-B exhibited the highest level of SC tolerance, nearly equal to Toiku 254 (**Table 1**, **Fig. 4**). These results indicate that *T* enhances SC tolerance in both $i^i i^i$ and *IcIc* backgrounds.

Comparison of agronomic traits between NILs for the T locus in the IcIc background

We examined the agronomic traits of the two 2304-NILs, i.e., the NILs for the *T* locus in the *IcIc* background (2304-9-B: *IcIc*/*TT*; 2304-9-G: *IcIc*/*tt*). No significant

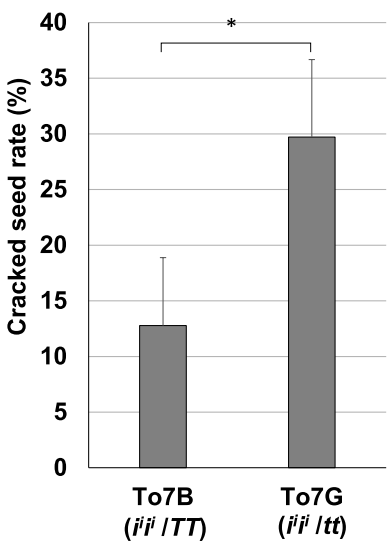

Fig. 3. Comparison of cracked seed rates of two near-isogenic lines for the *T* locus with brown hilums ($i^i i^i$ genotype) based on averages from 2013 and 2014. Error bars indicate standard error. *, significant at the 5% level.

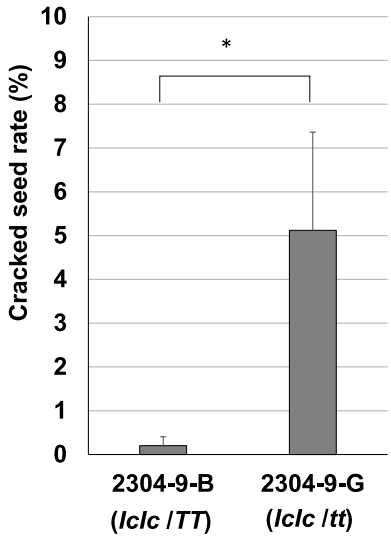

Fig. 4. Comparison of cracked seed rates of two near-isogenic lines for the *T* locus with yellow hilums (*IcIc* genotype) based on averages from 2017 and 2018. Error bars indicate standard error. *, significant at the 5% level.

Table 2. Comparison of agronomic traits of two near-isogenic lines (NILs) for the *T* locus (averages from 2017 and 2018)

NIL	Genotype		Days to maturity (days)	Main stem length (cm)	Seed yield (t ha^{-1})	100-seed weight (g)	Protein content (%)
	I	*T*					
2304-9-B	*IcIc*	*TT*	124	63	3.28	36.1	43.2
2304-9-G	*IcIc*	*tt*	123	59	3.25	34.9	43.0
p-value			0.215	0.208	0.689	0.234	0.362

differences were observed in maturity date, main stem length, seed yield, 100-seed weight or seed protein content between the two 2304-NILs in the 2-year field test (**Table 2**). According to these results, *T* has no negative effect on agronomic traits in the *IcIc* background.

Comparison of seed appearance between NILs for the T locus in the IcIc background

Seeds of 2304-9-B (*IcIc*/*TT*) and 2304-9-G (*IcIc*/*tt*) in the 2-year yield trial were visually evaluated, and their appearances were found to be similar (**Fig. 5**). Three color components (L^*, a^* and b^*) of the 2304-NILs (2304-9-B and 2304-9-G) were measured spectrophotometrically, and values of ΔE^*ab in 2017 and 2018 were calculated as 3.55 and 2.91, respectively (**Table 3**). A value of 1.5 to 3.0 in this color difference index corresponds to a level detectable by trained observers (Yousif 2014). The value of ΔE^*ab was within this range in 2018 and close to it in 2017, which indicates that the seed colors of the two 2304-NILs are actually similar to each other. Next, the seed dullness of 2304-9-B (*IcIc*/*TT*) and 2304-9-G (*IcIc*/*tt*) in the 2-year yield trial was compared by visual observation; neither line appeared to have dull seeds (**Fig. 5**). L^* is an index of lightness, with dull seeds exhibiting lower L^* values (Oyoo *et al.* 2011). The L^* value of 2304-9-B (*TT*) in 2017 and 2018 was 72.33 and 72.31, respectively, whereas that of 2304-9-G (*tt*) in the 2 years was 75.63 and 75.08, respectively. During both years, the L^* value of 2304-9-B (*TT*) was lower than that of 2304-9-G (*tt*) (**Table 3**). In 2304-9-B seeds, slight dullness in the seed coat, if any exists, could be obscured by the high level of lightness and would thus not be visually detectable.

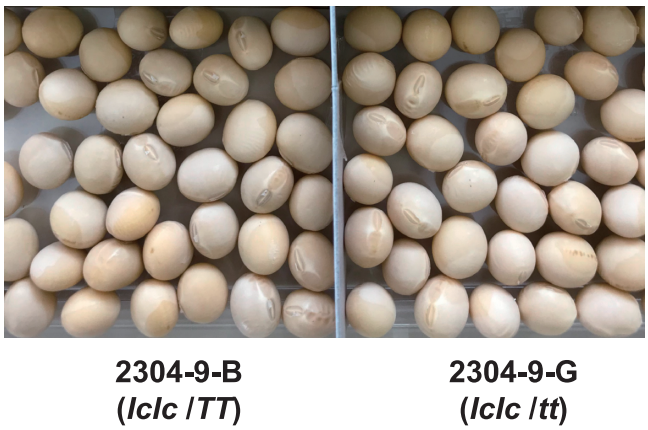

Fig. 5. Seed appearances of two near-isogenic lines (NILs) for the *T* locus. Genotypes of NILs are shown in parentheses. Seeds were harvested in field tests at the Tokachi Agricultural Experiment Station in 2018.

Table 3. Comparison of seed coat colors of near-isogenic lines (2304-NILs) for the *T* locus

NIL	Genotype		Year	L^*	a^*	b^*	ΔE^*ab
	I	*T*					
2304-9-B	*IcIc*	*TT*	2017	72.33	3.97	25.27	3.55
			2018	72.31	4.50	25.78	2.91
2304-9-G	*IcIc*	*tt*	2017	75.63	4.14	26.59	
			2018	75.08	4.36	26.67	
Yukihomare (control)	*II*	*tt*	2018	74.98	4.23	31.18	

Discussion

Our previous comparative analysis of SC-susceptible and SC-tolerant soybean cultivars/lines suggested that the *Ic* allele participates in SC tolerance (Senda *et al.* 2018). The *Ic* allele alone, however, was insufficient to confer high SC tolerance (**Table 1**). To develop highly SC-tolerant soybean cultivars, the identification of another SC tolerance-related gene and its pyramiding with the *Ic* allele was thus required. The pubescence color gene, *T* is effective against low-temperature-induced seed coat deterioration, such as pigmentation and cracks, in yellow soybean (Takahashi 1997, Takahashi and Asanuma 1996). In the present study, we first demonstrated that *T* is effective in suppressing SC (**Figs. 3**, **4**, **Table 1**). A major cause of SC is a straight-line split on the dorsal side of the seed coat induced by low

temperature (Senda *et al.* 2018). This seed coat split may be suppressed by *T*. In Japan, yellow hilum cultivars (*II* genotype) are preferred over pigmented hilum cultivars ($i^i i^i$ genotype). The introduction of *T* into yellow hilum soybeans (*II* genotype) should therefore be useful for the breeding of SC-tolerant soybeans, but the combination of *I* and *T* alleles darkens the yellow seed coat, leading to a dirty seed appearance—a major problem when *T* is introduced into yellow hilum cultivars (Cober *et al.* 1998, Morrison *et al.* 1998, Oyoo *et al.* 2011, Takahashi 1997, Takahashi and Asanuma 1996).

We next investigated whether pyramiding of *Ic* and *T* alleles, both contributing to SC tolerance, can lead to high SC tolerance. We found that Tokei 1105 and Toiku 254, both with the *IcIc*/*TT* genotype, exhibited high tolerance to SC. Among the seven cultivars and lines tested in this study, the highest SC tolerance was that of Toiku 254, in which the occurrence of SC was completely suppressed (**Table 1**). Comparative analysis of the 2304-NILs in the *Ic*/*Ic* background revealed that 2304-9-B with the *IcIc*/*TT* genotype is more SC-tolerant than 2304-9-G with the *IcIc*/*tt* genotype and that the SC tolerance of 2304-9-B is the same as that of Tokei 1105 and Toiku 254, both with the *IcIc*/*TT* genotype (**Fig. 4**, **Table 1**). These results strongly indicate that gene pyramiding of *Ic* and *T* contributes to high SC tolerance. In addition, the *IcIc* genotype suppresses the degree of seed coat dulling to an acceptable external level, thus resulting in a better appearance than that of the *II* genotype in the *TT* background (Oyoo *et al.* 2011, Rodriguez *et al.* 2013). In fact, seeds harvested from 2304-9-B (*IcIc*/*TT*) and 2304-9-G (*IcIc*/*tt*) looked similar to each other and did not have a dull appearance (**Table 3**, **Fig. 5**). The problem arising from the introduction of *T* into yellow hilum cultivars may be overcome by the use of the *Ic* allele.

Previous studies have found that the degree of chilling tolerance of the *TT* genotype is higher than that of the *tt* genotype (Kurosaki *et al.* 2004, Takahashi and Asanuma 1996, Takahashi *et al.* 2005). Toda *et al.* (2011) performed a comparative analysis of second trifoliate leaves of To7B (*TT*) and To7G (*tt*). They reported that chilling injury was more severe in To7G, whereas enhancement of antioxidant activity by low temperature was more prominent in To7B. After chilling treatment, the contents of quercetin glycoside and isorhamnetin glycoside, which are 3′,4′-dihydroxylated flavonol derivatives, increased in To7B; in contrast, the content of kaempferol glycoside, which is a 4′-monohydroxylated flavonol derivative, increased in To7G. Taken into account these results, the authors concluded that the chilling tolerance conferred by *T* (soybean *F3′H*) is likely caused by the increasing antioxidant activity of 3′,4′-dihydroxylated flavonol derivatives (Toda *et al.* 2011). A QTL associated with chilling tolerance has been mapped near the *Ic* allele (Ikeda *et al.* 2009). Gene pyramiding of *Ic* and *T* may contribute not only to high SC tolerance but also to high levels of chilling tolerance. Phytotron-based assays are required for the evaluation of chilling tolerance in soybeans with *IcIc*/*TT* genotypes.

In summary, we have validated the effect of *T* on SC tolerance. Gene pyramiding of *Ic* and *T* increases SC tolerance without discoloring seeds. The selection of *Ic* and *T* alleles using an Ic marker and pubescence color, respectively, may be effective for the breeding of highly SC-tolerant soybean.

Author Contribution Statement

NY bred the NILs and conducted the phytotron-based assay. NY and CS performed the field experiments. YY genotyped the breeding lines. NY and MS designed the research, analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

We thank Edanz Group (https://www.edanz.com/) for editing the English text of a draft of this manuscript. This work was supported in part by the Japan Society for the Promotion of Science [KAKENHI, grant number 18K05589 (to MS and NY)] and Grants-in-Aid for the Regional R&D Proposal-Based Program from the Northern Advancement Center for Science & Technology of Hokkaido Japan (to NY).

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