In Hokkaido, the northernmost region of Japan, soybean \([\textit{Glycine max} (\text{L.}) \text{ Merr.}]\) crops are damaged by cold weather. Chilling temperatures negatively affect seed appearance by causing seed coat discoloration around the hilum region, which is called cold-induced discoloration (CD). An assay for CD tolerance using a phytotron was developed, and two quantitative trait loci (QTLs) associated with CD tolerance were identified. The major QTL was located in the proximal region of the \(I\) locus, and structural variation of this locus can serve as a useful DNA marker, called the \(Ic\) marker. To use this marker in breeding programs, the effects need to be assessed under field conditions because the \(Ic\) marker has been developed solely under phytotron conditions. The aim of this study was thus to assess the effect of the \(Ic\) marker under a cool field environment. We confirmed that the \(Ic\) allele was highly effective using 27 cultivars and breeding lines including a near-isogenic line grown in the field where severe cold-weather damage occurred. This allele had no negative influence on the agronomic traits in the near-isogenic line. Our results suggest that marker-assisted selection for the \(Ic\) allele is effective for improving CD tolerance in breeding programs.

**Key Words:** soybean, abiotic stress, cold-induced discoloration, field assessment, marker-assisted selection.
The frequency of cold-weather damage in Okhotsk is higher than that in Tokachi (Tanaka et al. 2003). The accumulated average temperatures over a recent 10-year period in July, the period of sensitivity to chilling temperature, in Abashiri and Kamishihoro were 560 and 579°C, respectively. In the years of cold weather, cold-induced cracking seeds also appear in addition to CD in the Okhotsk area (Senda et al. 2018, Yamaguchi et al. 2014b, 2015a).

The mechanism underlying the induction of CD has been clarified. Three genetic loci (I, R, and T) are known to control seed coat pigmentation in soybeans (Bernard and Weiss 1973, Palmer and Kilen 1987). In yellow soybean, seed coat pigmentation is inhibited by post-transcriptional gene silencing (PTGS) of chalcone synthase (CHS) genes (Kasai et al. 2009, Senda et al. 2004). CD is caused by the suppression of CHS PTGS by chilling temperature (Kasai et al. 2009). In yellow-hilum cultivars of yellow soybean, an inverted repeat of a CHS truncated sequence was suggested to be the I locus, and its double-stranded CHS RNA transcript is thought to induce CHS PTGS (Kasai et al. 2007, Kurauchi et al. 2011). Toyoharuka has a different allele at the I locus, which was later designated Ic (inhibitor of CD) (Kasai et al. 2009, Ohnishi et al. 2011, Senda et al. 2012, Yamaguchi et al. 2015b). In a previous study, Ohnishi et al. (2011) identified two quantitative trait loci (QTLs) associated with CD tolerance in Toyoharuka using the phytotron assay. The major QTL was located in the proximal region of the I locus on chromosome 8, and structural variation of the I locus can serve as a useful DNA marker, called the Ic marker (Ohnishi et al. 2011). The effect of the Ic marker was confirmed under phytotron conditions using breeding lines with various genetic backgrounds, but some breeding lines with the Ic allele showed lower CD tolerance than Toyoharuka (Ohnishi et al. 2011). It was unclear whether the Ic marker is sufficient to improve CD tolerance in field conditions. Tawny pubescence also darkens the entire seed coat, giving a dirty appearance to yellow-hilum seeds (Cober et al. 1998, Morrison et al. 1998). Previous studies reported that the Ic allele may control the intensity of seed coat discoloration in tawny-pubescent cultivars (Oyoo et al. 2011, Rodriguez et al. 2013).

To use the Ic marker for CD tolerance in breeding programs, the effects need to be assessed under field conditions where severe cold-weather damage frequently occurs because this marker has been developed solely under phytotron conditions. It is also necessary to confirm whether the Ic marker is sufficient to improve CD tolerance in field conditions. The aim of this study was thus to assess the effect of the Ic marker under a cool field environment. Severe cold-weather damage occurred in Abashiri in 2017 in particular. Thus, we assessed the effect of the Ic marker using 27 cultivars and breeding lines grown in Abashiri in 2017. Moreover, we confirmed the effect of the Ic marker using the near-isogenic line (NIL) and evaluated the agronomic traits of this line.

### Plant materials

All 31 cultivars and breeding lines were developed at the Tokachi Agricultural Experiment Station (TAES), Memuro, Hokkaido, Japan (Tables 1, 2). These are determine and have a gray pubescence with a yellow hilum. Tokei 1284, an NIL derived from the cultivar Toyomizuki (recurrent parent, Ic allele) × the breeding line Tokei 1091 (donor parent, Ic allele), was bred by the backcrossing method using the Ic marker (Table 1). The Ic allele of Tokei 1091 originated from Toyoharuka. Tokei 1153 and Tokei 1179, the NILs derived from the cultivar Toyomusume (recurrent parent, Ic allele) × Toyoharuka (donor parent, Ic allele), were bred by the backcrossing method using the Ic marker (Table 2).

### Molecular marker analysis

Total genomic DNA was extracted from young leaves of three plants by a modified version of the CTAB method (Suzuki et al. 2012). The Ic/Ic alleles were determined by PCR for Ic markers as described previously (Ohnishi et al. 2011). The Ic marker sequences were 5′-GAG TTT GAA AAA TGT ATT CCT TCT CCT CC-3′ and 5′-GTA TCG CAG ATT CCT CCT GC-3′ for the Ic-specific amplicon and 5′-GCA AAC ATC AAG TAA GAG GAG CG-3′ and 5′-CCC ATT CCT TGA TTG CCT TA-3′ for the I-specific amplicon (Ohnishi et al. 2011).

### Evaluation of CD tolerance in the cool field environment

Two yield trials were performed in the field in Abashiri, Hokkaido, Japan (43°90′ N, 144°30′ E). Fertilizer was applied in accordance with Hokkaido fertilization standards (6 N–48 P₂O₅–6 K₂O–8 MgO kg ha⁻¹). In total, 15 and 12 soybeans were evaluated for trials A and B, respectively (Table 1). Seeds were sown on 22nd May, 2017. Each plot consisted of two (trial B) and four (trial A) rows with lengths of 2.5 m; these were spaced 66 cm apart with an 18-cm inter-hill distance with two plants per hill. This gave a plant population density of 16.6 plants m⁻². A randomized complete block design with two replicates was used for each test. Fifty seeds were prepared from the bulk seeds of each plot, and the degree of pigmentation of each seed was visually classified using a pigment index as follows: 0: no pigmentation; 1: pigmented only in the hilum; 2: pigmented in the hilum and slightly around it (pigment extending <1 mm outside of the hilum); 3: pigmented in the hilum and around it on both sides (pigment extending ≥1 mm outside of the hilum); and 4: pigmented in the hilum and around it severely on both sides (pigment extending ≥3 mm outside of the hilum) (Fig. 1). This is a modified version of the index reported in previous studies (Ohnishi et al. 2011, Takahashi and Abe 1994). Pigment indices of 50 seeds from each plot were averaged (average pigment index, API). The JMP 10 statistical package (SAS, Cary, NC, USA) was used for statistical analysis. Two-way analysis of variance (ANOVA) was used to test differences of API among cultivars in each
Table 1. Average pigment index (API) of 27 soybean cultivars and breeding lines in the field test and phytotron assay in 2017

| Cultivar or line         | Generation | Cross combinationa | Ic/I allele | API (0–4) |
|-------------------------|------------|--------------------|-------------|-----------|
|                         |            | P1                 | P2          | Field test | Phytotron assay |
| Trial A (2nd preliminary yield trial) |            |                    |             |           |                |
| Yukihomare               | –          | Tokei 783          | Tokei 780   | I          | 1.04 de<sup>b</sup> | 1.77 b |
| Toyomizuki               | –          | Yukihomare         | Tokei 930   | I          | 1.05 de         | 2.88 a |
| Tokei 1266               | F<sub>2</sub> | Tokei 842          | Toyomizuki  | I          | 1.44 cd         | ND<sup>c</sup> |
| Tokei 1283               | F<sub>8</sub> | Tokei 253          | Tokei 1125  | I          | 1.55 c          | 1.97 bc |
| Tokei 1287               | F<sub>5</sub> | Chukei 508         | Tokei 253   | I          | 2.27 b          | 2.94 a  |
| Tokei 1299               | F<sub>8</sub> | Tokei 1117         | Tokei 1128  | I          | 1.01 e          | 1.25 c  |
| Tokei 1292               | F<sub>8</sub> | Tokei 1120         | Chukei 566  | I          | 3.50 a          | 2.85 a  |
| Tokei 1295               | F<sub>8</sub> | Chukei 552         | Toyomizuki  | I          | 0.08 f          | 1.45 c  |
| Tokei 1296               | F<sub>7</sub> | Chiku 66           | Tokei 254   | I          | 3.16 a          | 3.50 a  |
| Tokei 266                | F<sub>7</sub> | Tokei 248          | Tokei 253   | I<sub>c</sub> | 0.01 f  | 1.05 cd |
| Tokei 1284               | BC<sub>3</sub>F<sub>6</sub> | Toyomizuki(4)     | Tokei 1091  | I<sub>c</sub> | 0.00 f  | 0.10 de |
| Tokei 1286               | F<sub>5</sub> | Tokei 248          | Chukei 552  | I<sub>c</sub> | 0.00 f  | 0.05 e  |
| Tokei 1289               | F<sub>5</sub> | Toyomizuki         | Tokei 1125  | I<sub>c</sub> | 0.04 f  | 0.01 e  |
| Tokei 1298               | F<sub>8</sub> | Mazowia            | Toyoharuka  | I<sub>c</sub> | 0.00 f  | 0.00 e  |
|                         |            |                    |             | F-value    | 1085.49 |
|                         |            |                    |             | Significance | ***   |

*** indicates significant at the 0.1% level.
<sup>a</sup> Underlined cultivars or lines have the Ic allele. Tokei 1284 was derived from Toyomizuki (recurrent parent) × Tokei 1091 (donor parent) by the backcrossing method using the Ic marker. All Chukei and Chiku lines were bred at Central Agricultural Experiment Station in Naganuma, Hokkaido, Japan. The cultivar Mazowia was bred in Poland. The other cultivars and lines were bred at Tokachi Agricultural Experiment Station in Memuro, Hokkaido, Japan.

<sup>b</sup> Means followed by a common letter in each trial are not significantly different according to Tukey’s HSD test (P ≥ 0.05).

<sup>c</sup> ND, no data.

Table 2. Average pigment index (API) of the near isogenic lines in the Toyomusume background in the phytotron assay in 2014

| Cultivar or line | Generation | Cross combinationa | Ic/I allele | API (0–4) in the phytotron assay |
|------------------|------------|--------------------|-------------|---------------------------------|
| Toyoharuka       | –          | Tokei 793          | Ic           | 0.00 e<sup>a</sup>               |
| Toyomusume       | –          | Toyosuzu           | I           | 2.75 a                           |
| Tokei 1153       | BC<sub>3</sub>F<sub>5</sub> | Toyoharuka     | Ic           | 0.95 b                           |
| Tokei 1179       | BC<sub>3</sub>F<sub>3</sub> | Toyomusume(3)    | Toyoharuka  | Ic                               |
|                  |            |                    | F-value     | 36.08                           |
|                  |            |                    | Significance | ***                             |

*** indicates significant at the 0.1% level.
<sup>a</sup> Underlined cultivars or lines have the Ic allele. Tokei 1153 and Tokei 1179 were derived from Toyomusume (recurrent parent) × Toyoharuka (donor parent) by the backcrossing method using the Ic marker.

Means followed by a common letter are not significantly different according to Tukey’s HSD test (P ≥ 0.05).
Results

Occurrence of CD in the field test

The average temperatures in Abashiri in 2017 are shown in Fig. 2. The standard cultivar Yukihomare started flowering on 23rd July. The average temperatures from 2nd to 20th Aug were much lower than those of the 30-year average: the plants were exposed to an 18-day chilling temperature from 10 days after flowering (Fig. 2). This period would correspond approximately to chilling temperature conditions in the phytotron assay, in which the plants were exposed to a 14-day chilling temperature from 7 days after flowering (Yumoto and Sasaki 1990). The seeds of Tokei 1296 (Table 1), a CD-sensitive breeding line grown in the field test, were pigmented in the hilum and also severely around it (Fig. 3).

Evaluation of CD tolerance by the phytotron assay

The CD tolerances of 14 cultivars and breeding lines in 2017 (Table 1) and four cultivars and NILs in 2014 (Table 2) were evaluated by the phytotron assay, as described previously (Ohnishi et al. 2011). Seeds were sown on 22nd May, 2014, and 17th May, 2017 in Wagner pots (30-cm diameter; Fujiwara Scientific) filled with low-humic andosols. Two pots were prepared for each breeding line. Three plants per pot were grown in an experimental facility under a plastic roof without walls in Memuro, Hokkaido, Japan (42°89′ N, 140°07′ E). Seven days after flowering, plants were transferred into the phytotron and exposed to the following chilling-temperature conditions for 14 days: 18°C day (08:00–18:00) and 13°C night (18:00–08:00), with 55% shading to avoid excessive heating from sunlight. After this 14-day treatment, the remaining flower buds were removed and plants were grown for a further 7 days in the phytotron at higher temperatures (25°C day/20°C night). After this treatment, the pots were returned to the experimental facility and the plants were grown to maturity. The pigment indices of all harvested seeds from each plant were evaluated, and APIs were calculated according to the above (Fig. 1). One-way ANOVA was used to test differences of API among cultivars. Tukey’s HSD test with a threshold for significance of $P < 0.05$ was used to confirm the differences.

Evaluation of agronomic traits of near-isogenic line

The yield trials of Toyomizuki and Tokei 1284 were performed in the field in Abashiri in 2015 to 2017. The generations of Tokei 1284 were BC3F4, BC3F5, and BC3F6 in 2015, 2016, and 2017, respectively. Seeds were sown on 25th May, 2015, 24th May, 2016, and 22nd May, 2017. Each plot consisted of two (in 2015 and 2016) and four (in 2017) rows with lengths of 2.5 m, which were spaced 66 cm apart with an 18-cm inter-hill distance with two plants per hill. This gave a plant population density of 16.6 plants m$^{-2}$. A randomized complete block design with two replicates was used for the experiments. Maturity was defined as the time when >80% of the plants were defoliated and had turned yellow, with pods that rattled when shaken. Before harvesting, main stem length (distance from cotyledonary node to terminal node) was recorded from ten central consecutive plants from each plot. Seed yield was assessed by hand harvest per plot and adjusted to 15% moisture. Combined ANOVA was carried out using the mixed model procedure. “Years” and “replications within year” were considered as random effects, while “cultivars” was considered as a fixed effect.
Field assessment of a QTL for cold-induced discoloration in soybean

**Evaluation of CD tolerance in the breeding lines**

In the field test of trial A ($n = 15$), the APIs of the five lines with the \( Ic \) allele were significantly lower than those of the nine cultivars and lines with the \( I \) allele, with the exception of breeding line Tokei 1295 (Table 1). Only the API of Tokei 1295 was similar to those of the five lines with the \( Ic \) allele (API = 0.08). In the field test of trial B ($n = 12$), the APIs of the ten lines with the \( Ic \) allele were significantly lower than those of the two lines with the \( I \) allele (Table 1). ANOVA revealed that there was a significant difference between the \( Ic/I \) alleles in the field test through the two trials (Table 3). The API of the \( Ic \) allele was significantly lower than that of the \( I \) allele (Fig. 4).

In the phytotron assay ($n = 14$), the APIs of the four lines with the \( Ic \) allele, with the exception of the breeding line Toiku 266, were significantly lower than those of the nine cultivars and lines with the \( I \) allele (Table 1). Only the API of Toiku 266 was slightly higher than those of the other four lines with the \( Ic \) allele (API = 1.05). The APIs in the phytotron assay were positively correlated with the APIs in the field test (Fig. 5).

**Evaluation of CD tolerance and agronomic traits in the near-isogenic line**

The APIs of Tokei 1153 and Tokei 1179, the NILs of Toyomusume for the \( Ic \) allele, were significantly lower than that of Toyomusume and higher than that of Toyoharuka in the phytotron assay (Table 2). The API of Tokei 1284, the NIL of Toyomizuki for the \( Ic \) allele, was significantly lower than that of Toyomizuki in both the field test and the phytotron assay (Table 1). The seed appearance in the field test is shown in Fig. 6. It is clear that the number of seeds pigmented in the hilum in Tokei 1284 was lower than that in

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### Table 3. Analysis of variance for the effects of the \( Ic \) allele on average pigment index in the field test in 2017

| Source                   | Sum of squares | Degrees of freedom | Mean squares | F-value | P-value | Significance |
|--------------------------|----------------|--------------------|--------------|---------|---------|--------------|
| Model                    | 35.145         | 4                  | 8.786        | 19.983  | <0.0001 | ***          |
| \( Ic/I \) allele        | 27.606         | 1                  | 27.606       | 62.786  | <0.0001 | ***          |
| Trial                    | 0.062          | 1                  | 0.062        | 0.142   | 0.708   | ns           |
| Replication within trial | 0.004          | 2                  | 0.002        | 0.043   | 0.996   | ns           |
| Error                    | 21.545         | 49                 | 0.440        | 0.027   | 0.059   | 0.981        |
| Lack of fit              | 0.082          | 3                  | 0.027        | 0.059   | 0.981   | ***          |
| Pure error               | 21.463         | 46                 | 0.467        |         |         |              |
| Total                    | 56.689         | 53                 |              |         |         |              |

*** and ns indicate significant at the 0.1% level and not significant, respectively.

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Fig. 4. Comparison of average pigment index in the field test in 2017. Gray boxes represent least square means with standard errors. *** indicates significant at the 0.1% level.

Fig. 5. Correlation between average pigment index (API) in the field test and API in the phytotron assay ($n = 14$). *** indicates significant at the 0.1% level.

Fig. 6. The seeds of Toyomizuki and Tokei 1284, the near isogenic line derived from Toyomizuki (recurrent parent, \( I \) allele) \( \times \) Tokei 1091 (donor parent, \( Ic \) allele), in the field test in Abashiri in 2017. The average pigment indices of Toyomizuki and Tokei 1284 were 1.05 and 0.00, respectively.
In summary, we confirmed the effect of a major QTL for CD tolerance in field conditions. The Ic allele had no negative influence on the agronomic traits in the NIL. Our results

| Cultivar or line | Ic/I allele | Maturity (days) | Main stem length (cm) | Seed yield (t/ha⁻¹) | 100-seed weight (g) |
|-----------------|-------------|----------------|-----------------------|---------------------|-------------------|
| Toyomizuki I    | 133.3       | 62.3           | 2.82                  | 32.7                |
| Tokei 1284 Ic   | 132.5       | 62.0           | 2.88                  | 33.4                |
| P-value         | 3.049       | 0.861          | 0.179                 | 4.349               |
| F-value         | 0.141       | 0.902          | 0.090                 | 0.091               |

The near-isogenic line derived from Toyomizuki (recurrent parent, I allele) × Tokei 1091 (donor parent, Ic allele) by the backcrossing method using the Ic marker.

Table 4. Comparison of the agronomic traits of Toyomizuki and Tokei 1284 (average in 2015 to 2017)

Table 4. Comparison of the agronomic traits of Toyomizuki and Tokei 1284 (average in 2015 to 2017)
suggest that marker-assisted selection for the \textit{Ic} allele is effective for improving CD tolerance in breeding programs.

**Author Contribution Statement**

NY designed the research, conducted the phytotron experiment, analyzed the data, and wrote the manuscript. SH conducted the field experiment. DH conducted the DNA marker experiment. All authors read and approved the manuscript.

**Acknowledgments**

We thank Edanz (www.edanzediting.co.jp) for editing the English text of a draft of this manuscript. This work was supported by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics-based Technology for Agricultural Improvement, SFC1006, and Research Program on Development of Innovative Technology, 26098C).

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