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ORIGINAL PAPER

Variation of *GmIRCHS* (*Glycine max* inverted-repeat *CHS* pseudogene) is related to tolerance of low temperature-induced seed coat discoloration in yellow soybean

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Abstract In yellow soybean, seed coat pigmentation is inhibited by post-transcriptional gene silencing (PTGS) of chalcone synthase (*CHS*) genes. A *CHS* cluster named *GmIRCHS* (*Glycine max* inverted-repeat *CHS* pseudogene) is suggested to cause PTGS in yellow-hilum cultivars. Cold-induced seed coat discoloration (CD), a commercially serious deterioration of seed appearance, is caused by an inhibition of this PTGS upon exposure to low temperatures. In the highly CD-tolerant cultivar Toyoharuka, the *GmIRCHS* structure differs from that of other cultivars. The aim of this study was to determine whether the variation of *GmIRCHS* structure among cultivars is related to variations in CD tolerance. Using two sets of recombinant inbred lines between Toyoharuka and CD-susceptible cultivars, we compared the *GmIRCHS* genotype and CD tolerance phenotype during low temperature treatment. The *GmIRCHS* genotype was related to the phenotype of CD tolerance. A QTL analysis around *GmIRCHS* showed that *GmIRCHS* itself or a region located very close to it was responsible for CD tolerance. The variation in *GmIRCHS* can serve as a useful DNA marker for marker-assisted selection for breeding CD tolerance. In addition, QTL analysis of the whole genome revealed a minor QTL that also affected CD tolerance.

Introduction

In yellow soybean, seed coat pigmentation is inhibited by post-transcriptional gene silencing (PTGS) of chalcone synthase (*CHS*) genes, which encode a key enzyme in the anthocyanin biosynthetic pathway (Todd and Vodkin 1996;

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Kasai et al. 2004; Senda et al. 2004; Kasai et al. 2009; Kurauchi et al. 2009; Tuteja et al. 2009). The PTGS of *CHS* genes in the seed coat is thought to be caused by the *I or* i^i allele at the *I* locus, which is suggested to be a complicated structure consisting of multiple copies of *CHS* genes (Senda et al. 2002a, b; Tuteja et al. 2004; Kasai et al. 2007; Kurauchi et al. 2009; Tuteja et al. 2009). In yellow-seed coat with yellow-hilum cultivars, an inverted-repeat of a *CHS* truncated sequence (*Glycine max* inverted-repeat *CHS* pseudogene, abbreviated to *GmIRCHS*) was suggested to be the *I* allele (Kasai et al. 2007). In yellow-seed coat with pigmented hilum cultivars, such as Williams 82, duplicated *CHS* gene clusters are believed to be the i^i allele (Clough et al. 2004).

Seed coat mottling caused by virus infection is a naturally occurring phenomenon that decreases seed quality. This virus-induced seed coat pigmentation results from the virus suppressing the PTGS of *CHS* genes (Senda et al. 2004). Cold-induced seed coat discoloration (CD) is partial seed coat pigmentation caused by low temperatures at early stages of seed development (Oka et al. 1989; Srinivasan and Arihara 1994; Morrison et al. 1998; Funatsuki and Ohnishi 2009). Kasai et al. (2009) demonstrated that CD was caused by a suppression of PTGS of *CHS* genes by low temperature. Thus, the appearance of yellow soybean seeds is frequently affected by the occurrence and extent of PTGS of *CHS* genes in the seed coat.

Exposure of young pods (5–10 days after flower opening) to low temperatures results in CD (Oka et al. 1989; Yumoto and Sasaki 1990; Takahashi and Abe 1994; Takahashi 1997). This is probably caused by accumulation of oxidized phenolic compounds (Takahashi and Akiyama 1993). This deterioration of seed quality frequently occurs in soybeans cultivated in subarctic zones, and decreases the value of the soybean crop markedly. Sensitivity to CD differs among cultivars (Srinivasan and Arihara 1994) and CD tolerance is one of the most important breeding objectives for cultivars that are grown for whole-processing use. An assay for CD tolerance was developed for use in soybean breeding programs (Yumoto and Sasaki 1990), and several cultivars and breeding lines, such as Tokei-924, were selected for CD tolerance. However, all of those cultivars (including Tokei-924) are not sufficiently tolerant to CD under more severe conditions, and therefore, there is a need for more highly tolerant lines for cultivation in areas with more severe conditions. Some maturity genes and QTLs have been reported to be involved in CD tolerance (Takahashi and Abe 1994, 1999; Benitez et al. 2004; Githiri et al. 2007) although those factors do not make sufficient contributions to achieve desirable levels of CD tolerance. Recently, plant breeders developed a very highly CD-tolerant cultivar, 'Toyoharuka' (abbreviated hereafter as TR). The structure of *GmIRCHS* in TR differs from that in other cultivars, including 'Toyomusume' (TM) which is CD sensitive. The *GmIRCHS* region of TR lacks one of the inversely repeated pseudo *CHS3* sequences, and instead contains an insertion consisting of the 5′ portion of *GmJ1*, which encodes a DnaJ-like protein (Kasai et al. 2009). This structure was named *GmASCHS* (*Glycine max* antisense *CHS* pseudogene) because of the presence of *CHS* antisense RNA, which is derived from this structure. Kasai et al. (2009) demonstrated that low temperature treatment reduced *CHS* siRNAs in the TM seed coat, leading to elevated levels of *CHS* mRNAs. In TR, however, low temperature did not affect the levels of *CHS* siRNAs and mRNAs. In addition, the *CHS* siRNAs in the seed coat is thought to be derived from *GmIRCHS* (Senda et al. 2004; Kasai et al. 2007; Kurauchi et al. 2009). From those observations, it was proposed that the variant structure of *GmIRCHS* (*GmASCHS*) in TR is responsible for the CD-tolerant phenotype. In this paper, we explored whether the structural changes in *GmIRCHS* in TR are related to CD tolerance. In addition, we confirmed that the variation in *GmIRCHS* is useful as a DNA marker for selection of CD tolerance.

Materials and methods

Plant materials

We used three soybean cultivars in these experiments: Toyoharuka (TR), Toyomusume (TM) and Tokei-924. These cultivars all have a yellow-seed coat and yellow-hilum with gray pubescence and were developed in northern Japan. In terms of cold-induced seed coat discoloration (CD), TR is highly tolerant, Tokei-924 is moderately tolerant, and TM is sensitive. A total of 144 recombinant inbred lines (RILs) designated as TR–TM were generated by crossing TR and TM, and another set of 118 RILs designated as TR-924 was derived from the cross between TR and Tokei-924. Both these RILs were derived from single seed descent to the F_4 generation. The CD tolerance and genotype evaluations were performed at the F_5 generation. For QTL analysis of the whole genome, we used the genotype data of the F_5 sister lines derived from the F_4 generation of the TR–TM RILs.

Genotyping of RILs

As described previously (Kasai et al. 2009), the structure of *GmIRCHS* in TR differs from that in TM (GenBank accession: AB480069 and AB480070 for TR and TM). Specific primer sets for amplifying this region of DNA in each cultivar were designed according to these variations. The primer pair used for TR-specific amplification was

GAG TTT GAA AAA TGT ATT CTT TCT CTT CC (forward) and GTA TCG CAG ATT CCT CCT GC (reverse). The primer pair used for TM-specific amplification was GCA AAC CAA ATC AAG TAA GAG CG (forward) and CCC ATT CCT TGA TTG CCT TA (reverse).

Evaluation of cold-induced seed coat discoloration

Yumoto and Sasaki (1990) reported that a low temperature treatment applied from 7 days after anthesis was suitable to evaluate CD sensitivity of cultivars. We used this assay to evaluate CD tolerance of cultivars and breeding lines. In brief, seeds were sown in May in pots (30-cm diameter) filled with low-humic andosols supplemented with ammonium sulfate (5.7 g) and potassium sulfate (10.8 g). Three plants per pot were grown outside under a rain cover in Memuro, Japan (42°54′N, 143°2′E). At 7 days after anthesis, plants were transferred into a phytotron and exposed to a 14-day low temperature treatment (18°C days/13°C nights). After the 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 days/20°C nights). Then, plants were returned to outdoor conditions until they reached maturity. The pigmentation of harvested seeds was evaluated by determining the percentage of seeds pigmented outside of the hilum (corresponding to 2–5 in Fig. 1a), and the percentage of hilum-pigmented seeds (corresponding to 1 in Fig. 1a). To minimize the effects of climatic variations, the pots were repositioned within the growth chamber every 3 days during the low temperature treatment, and every 7 days before and after the low temperature treatment. Two replications were carried out for each cultivar and each breeding line.

Takahashi (1997) reported that the most severe CD resulted from low temperature treatments that began 3–8 days after the opening of individual flowers. As a result of these observations, we conducted the assessments to determine CD tolerance in RILs. The TR–TM and TR-924 RILs (F_5 generation) were grown one plant per pot (19-cm diameter pot) in soil supplemented with ammonium sulfate (2.3 g) and potassium sulfate (4.3 g). A single plant of each RIL and 5–7 parent plants were evaluated. A set of 144 RILs for TR–TM and 118 RILs for TR-924 were grown in an unheated glasshouse. Plants were grown in the phytotron at 25°C (days) and 20°C (nights) from anthesis until maturity, except for the period of low temperature treatment. To evaluate TR–TM RILs, individual plants were exposed to a 12-day low temperature treatment (15°C days/10°C nights) from 10 days after anthesis. At the start of the low temperature treatment, the remaining flower buds were removed. After harvesting the seeds at maturity, each seed was scored according to the CD indices shown in Fig. 1a (0, not pigmented and 5, severely pigmented; modified indices of Takahashi and Abe 1994), and the mean CD index, which is the arithmetic mean of these indices, was used for the QTL analysis. For each line, 7–20 seeds were examined. In these

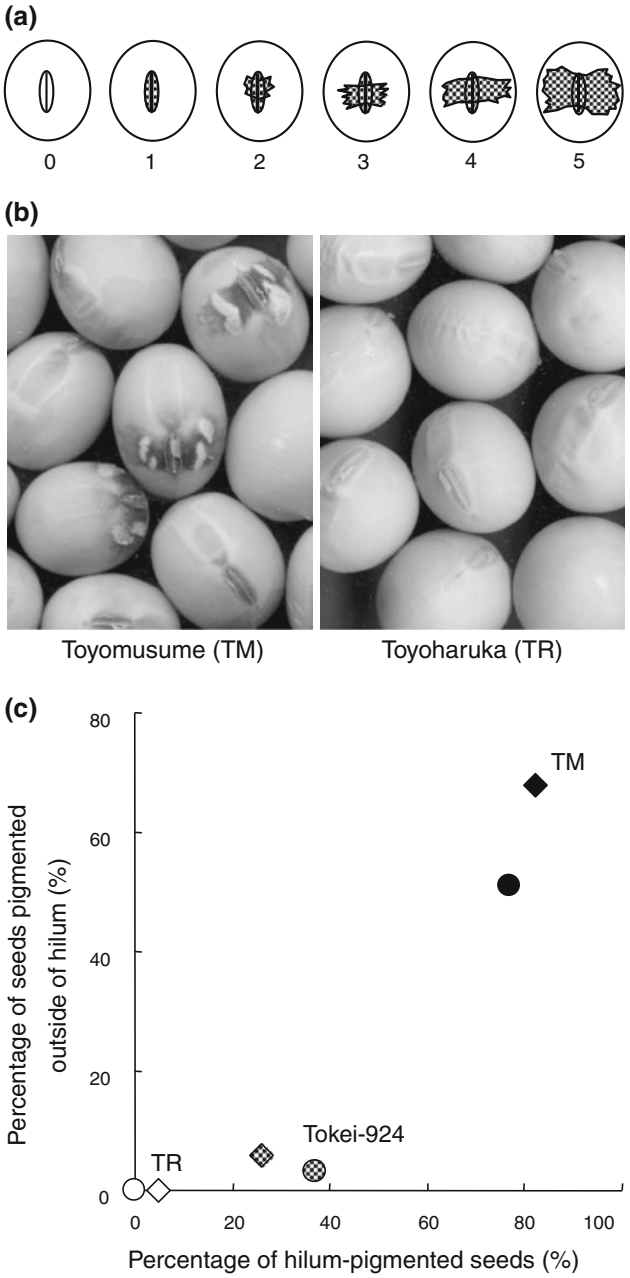

Fig. 1 Example of CD-tolerant phenotype. **a** Indices for CD for single seeds. **b** Example of CD in field-grown soybeans. Toyomusume (*TM*) is CD sensitive, Toyoharuka (*TR*) is CD tolerant. **c** Evaluations of CD tolerance by 14-day low temperature treatment applied from 7 days after anthesis. *Open circle* and *diamond* indicate TR, *halftone circle* and *diamond* indicate Tokei-924 (moderately CD-tolerant breeding line), *solid circle* and *diamond* indicate TM. *Circles* and *diamonds* represent evaluations carried out in 2000 and 2001, respectively. Hilum-pigmented seed corresponds to 1 in **a** and seed-pigmented outside corresponds to 2–5 in **a**

conditions, flowers were evaluated from 1 to 10 days after opening (1–10 DAO; Takahashi 1997). To evaluate TR-924 RILs, which were derived from a cross between CD-tolerant and CD moderately tolerant lines, a more severe cold treatment was applied. The 14-day low temperature treatment started 8 days after anthesis, and 1–8 DAO flowers were evaluated.

Map construction

For TR–TM RILs, we used linkage maps that were established previously (Ikeda et al. 2009). In brief, the maps were constructed from a set of 192 RILs consisting of F_5 sister lines derived from the F_4 generation of TR–TM RILs. To avoid redundancy, 105 polymorphic single sequence repeat (SSR) markers were chosen from previously placed SSR markers on integrated soybean genetic linkage maps (Song et al. 2004). For TR-924 RILs, the map around *GmIRCHS* in linkage group (LG) A2 in the integrated soybean genetic linkage maps (Song et al. 2004) was constructed as described previously (Ikeda et al. 2009).

QTL analysis around *GmIRCHS*

For TR-924 RILs, QTL interval analysis was performed using the mean CD index and the map around *GmIRCHS* as described above. Map Manager QTX Windows version (Manly et al. 2001) was used for interval mapping.

QTL analysis for whole genome

QTL analysis for the whole genome was performed using the mean CD index of TR–TM RILs and the genotype data of F_5 sister lines derived from the F_4 generation of TR–TM RILs. Composite interval mapping was performed using QTL Cartographer ver. 2.5 (Wang et al. 2005) as described previously (Ikeda et al. 2009).

QTL analysis for LG B2

Using Map Manager QTX, we carried out single marker analyses with the mean CD index and genotypes of SSR markers that showed LOD scores greater than 2.0 in the whole genome analysis described above. In the analysis for the second QTL in LG B2, *GmIRCHS* was set as the background. For this analysis, we used TR–TM RILs genotypes.

Evaluation of *GmIRCHS* as a DNA marker in elite breeding lines

We evaluated the CD tolerance of major cultivars and elite breeding lines developed in 2008 at the Hokkaido Central Agricultural Experiment Station and Hokkaido Prefectural Tokachi Experiment Station, Japan. The conditions to evaluate cultivars and breeding lines were as described above (18°C days, 13°C nights, for 14 days). The genotypes of *GmIRCHS* were determined using PCR-based DNA markers as described above.

Results

CD tolerance of cultivars

Cold-induced seed coat discoloration (CD) is a naturally occurring change in seed coat pigmentation caused by low temperature inhibition of PTGS of *CHS* genes in soybean seed coats. TR is highly tolerant to CD, and shows little seed pigmentation under conditions in which the CD-sensitive cultivar TM shows severely decreased seed quality (Fig. 1b). When plants were exposed to a 14-day low temperature treatment (18°C days/13°C nights) from 7 days after anthesis, TR showed almost no seed coat pigmentation, while more than 40% of TM seeds were pigmented around the outside of the hilum and 70% showed a pigmented hilum (Fig. 1c). In these conditions, the moderately CD-tolerant breeding line Tokei-924 showed a phenotype that was intermediate between those of TR and TM (Fig. 1c).

Genotyping of *GmIRCHS*

GmIRCHS is a candidate for the *I* allele at the *I* locus which is located at linkage group (LG) A2 and is responsible for seed coat color. Although both TR and TM are yellow-seed coat/yellow-hilum cultivars, Kasai et al. (2009) reported that the structure of *GmIRCHS* in TR differed from that of TM, and named this structure *GmASCHS*. Based on the sequence data reported previously (Kasai et al. 2009), we developed specific PCR primer sets to amplify the different genotypes of *GmIRCHS* in TR and TM (Fig. 2a). The two primer sets specifically amplify a part of *GmIRCHS* from TR or TM (Fig. 2a, b), and the different fragments can be distinguished by their length. The PCR amplification pattern and Southern hybridization patterns with a *CHS* probe indicated that the structure of *GmIRCHS* in Tokei-924 is identical to that in TM (data not shown).

Relationship between *GmIRCHS* genotype and CD tolerance in RILs

Kasai et al. (2009) showed that CD is related to PTGS and suggested that *GmIRCHS* structure was related to CD tolerance. To verify whether differences in *GmIRCHS* structure are related to variations in CD tolerance among cultivars, we evaluated the genotype of *GmIRCHS* and the phenotype of

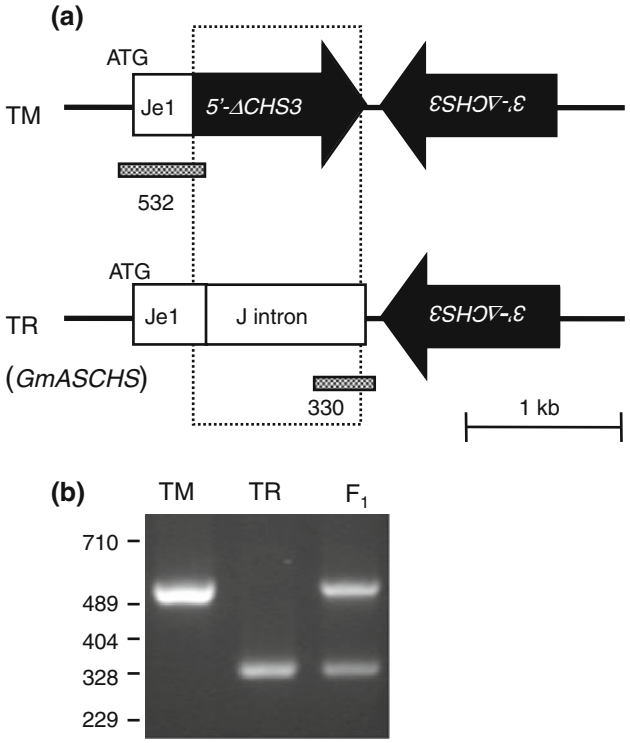

Fig. 2 Structure of *GmIRCHS* in TR and TM, and specifically amplified fragments obtained using specific primer sets. **a** Structure of *GmIRCHS* in TM and TR (*GmASCHS*) as reported by Kasai et al. (2009). Positions and orientations of *CHS* pseudogenes shown by *black arrows*. Coding sequence of exon 1 and intron (Je1 and J intron, respectively) in *GmJ1* (*Glycine max* DnaJ-like protein) shown in *white boxes*. The location of start codons is indicated. *Dotted frame* shows region that differs between TM and TR. Regions specifically amplified from TM or TR using specific primers shown by *hatched bars*. Predicted nucleotide lengths shown *below bars*. **b** Fragments amplified by PCR using mixture of TM- and TR-specific primer sets. F_1, F_1 plant derived from TM × TR cross

CD tolerance for two sets of RILs. The RILs TR–TM were derived from the cross between TR (CD tolerant) and TM (CD sensitive). The RILs TR-924 were derived from the cross between TR (CD tolerant) and Tokei-924 (moderately CD tolerant). The genotype of *GmIRCHS* was evaluated by PCR with the primer sets described above. For the TR–TM RILs, the phenotype of CD tolerance was evaluated by applying a 12-day low temperature treatment. We used a more severe low temperature treatment (14 days) to assess differences in CD tolerance among TR-924 RILs. The relationship between *GmIRCHS* genotype and CD tolerance phenotype is shown in Fig. 3. These experiments show that the genotype of *GmIRCHS* affected CD tolerance; lines with the TR genotype at *GmIRCHS* exhibited comparatively low CD indices and lines with the TM genotype exhibited high CD indices. Moreover, almost all lines with CD indices as high as that of TR (lines with index 0.0 in Fig. 3a–c, and 0.0–0.9 in Fig. 3d–f) had the TR genotype at *GmIRCHS*. However, the *GmIRCHS* genotype could not explain all CD tolerance phenotypes, i.e. lines with the TR genotype exhibiting indices from 0.6 to 3.3 in Fig. 3a and lines with indices from 1.2 to 5.0 in Fig. 3d. There are several possible explanations for these lines showing high indices; first, another locus aside from *GmIRCHS* might partially affect CD tolerance. Second, it could be that the variation in *GmIRCHS* is not the cause of CD tolerance, but the locus that controls CD tolerance is linked to *GmIRCHS*, and recombination between *GmIRCHS* and that locus occurred in these lines.

Interval analysis around *GmIRCHS*

We performed interval analysis to examine whether there was a higher likelihood region than *GmIRCHS* located near *GmIRCHS*. There were very few polymorphisms around *GmIRCHS* in the parents of TR–TM RILs; therefore, we used TR-924 RILs. The sequence of *GmIRCHS*, a candidate for the *I* allele at the *I* locus, was not found in the Williams 82 genome sequence (Schmutz et al. 2010) nor in the BAC clone 104J7, which is reported to harbor the i^i allele of Williams 82 (Clough et al. 2004; Tuteja and Vodkin 2008). This is probably because Williams 82 is a black-hilum cultivar with the i^i allele, and *GmIRCHS* is the candidate for the *I* allele, which is allelic with i^i. We can confirm that *GmIRCHS* is mapped between Sat_400 and Satt187, where the BAC clone 104J7 harboring the i^i allele is also located (Fig. 4). For interval analysis with the mean CD index, as indicated in Fig. 4, the LOD score was highest at the exact location of *GmIRCHS*. This observation suggests that the variation in *GmIRCHS* is itself responsible for CD tolerance, or that this region is very tightly linked to the locus responsible for CD tolerance.

Effects of *GmIRCHS* genotype on CD in various genetic backgrounds

We examined the effects of the *GmIRCHS* genotype on CD phenotype in various genetic backgrounds, including breeding materials, to evaluate whether *GmIRCHS* variations could serve as a DNA marker. The relationship between the *GmIRCHS* genotype and the CD tolerance phenotype was investigated for elite breeding lines developed in northern Japan (Fig. 5). Most CD-tolerant lines had the TR genotype at *GmIRCHS*, especially the highly tolerant lines that showed low percentages of pigmentation at and around the hilum (<20% pigmentation at the hilum, and no pigmentation around the hilum). It is clear that the *GmIRCHS* genotype strongly affects CD tolerance even among breeding lines with different genetic backgrounds. Thus, the genetic variation in *GmIRCHS* and the primer sets we developed are useful for marker-assisted selection of CD tolerance.

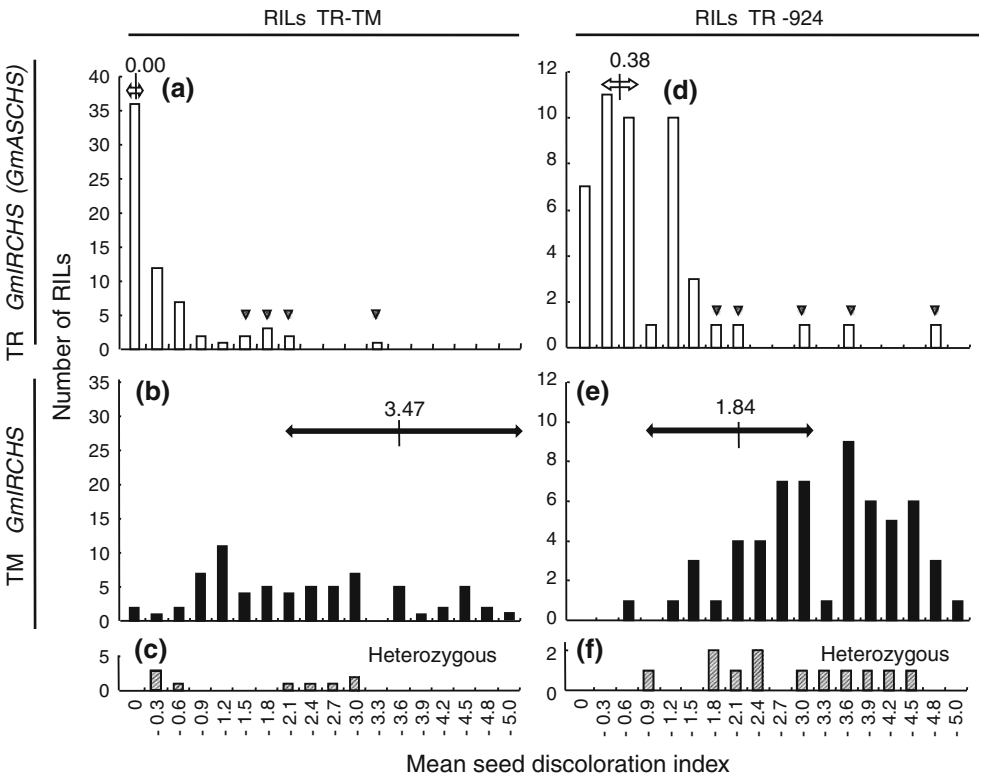

Fig. 3 Effect of *GmIRCHS* genotype on frequency distribution for seed discoloration indices in F_5 RILs derived from TR × TM (**a**–**c**) or TR × Tokei-924 (**d**–**f**). **a**, **d** Lines with TR *GmIRCHS* (*GmASCHS*), **b**, **e** TM *GmIRCHS*, and **c**, **f** heterozygous. *Triangles* represent lines poorly explained by *GmIRCHS* genotype. *Horizontal arrows* and *values* beside them indicate ranges (max–min) and averages of parents ($n = 7$ for TR–TM, $n = 5$ for TR-924)

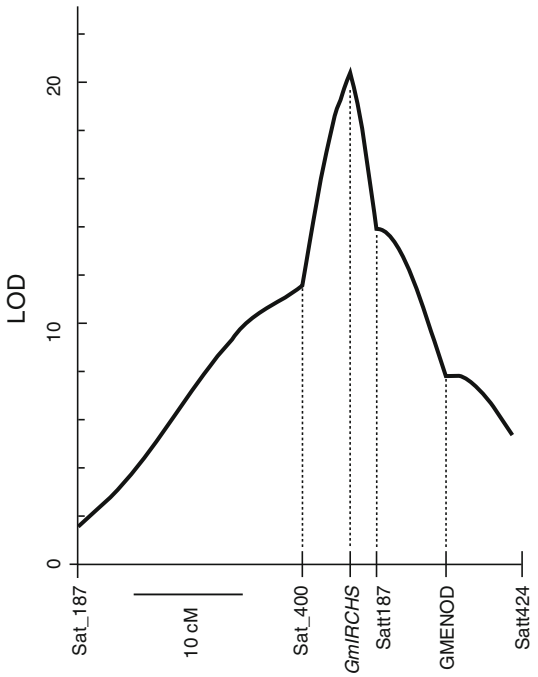

Fig. 4 Interval analysis of QTL for CD tolerance around *GmIRCHS* in TR-924 RILs. CD tolerance was evaluated by determining mean CD index

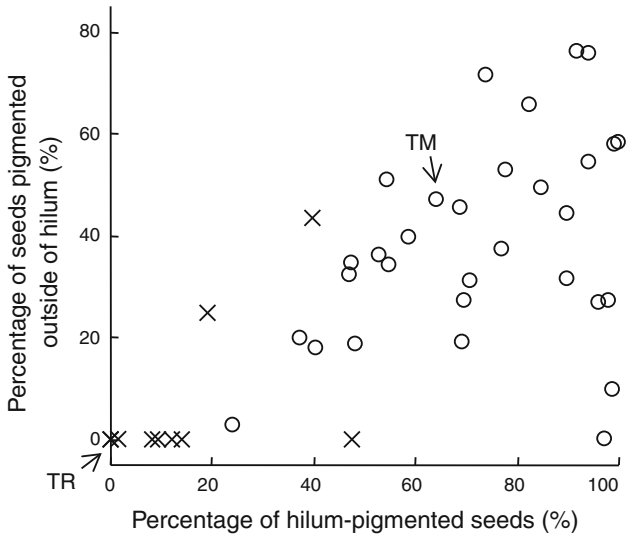

Fig. 5 Effects of *GmIRCHS* genotype on CD tolerance among elite breeding lines. Low temperature treatment and axis labels are the same as in Fig. 1c. *Open circles* and *crosses* represent breeding lines with TM and TR genotype, respectively, at *GmIRCHS*. Plot of TM and TR cultivars indicated with *arrows*

Effects of a second QTL

We searched the entire genome for other QTLs associated with CD tolerance. For this purpose, we first conducted a QTL analysis with the mean CD index of TR–TM RILs and previously genotyped data of sister lines of TR–TM RILs. A composite interval mapping with 105 markers over the whole genome revealed two additional QTLs with LOD scores greater than 2.0, located at LG A2 and B2 (Fig. 6). We repeated the QTL analysis around the candidate

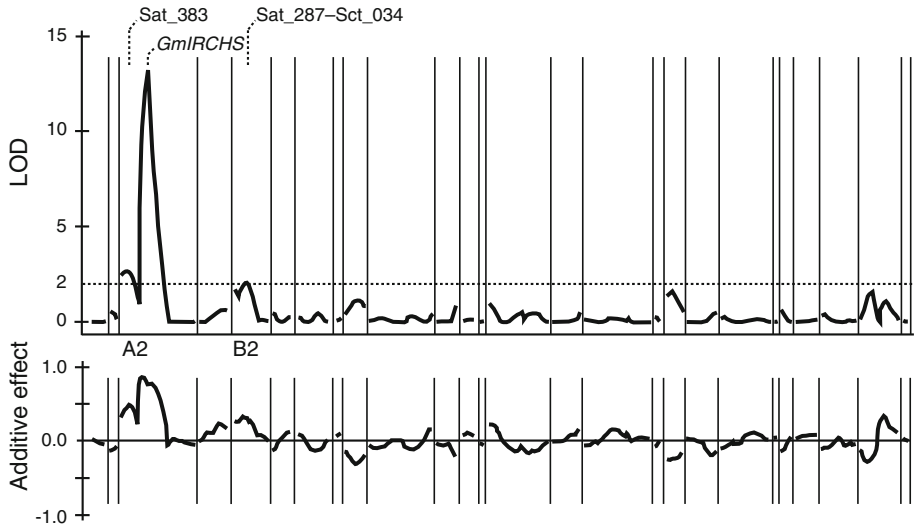

Fig. 6 Composite interval analysis of CD tolerance QTL for whole genome using a set of TR–TM RILs. CD tolerances were evaluated by determining mean CD index. Regions partitioned by two *vertical lines* represent each linkage group. *Dotted vertical lines* show regions with LOD scores >2.0. Additive effect indicates effect of TR alleles in comparison to TM

Table 1 Results of single marker regression for mean CD index

LG[a]	Marker	TR–TM RILs			TR-924 RILs		
		LOD	Variance (%)	Additive effect[b]	LOD	Variance (%)	Additive effect[b]
A2	*GmIRCHS*	16.6	41	0.91	20.2	56	1.19
	Sat_383	1.5	5	0.31	n.d.	n.d.	n.d.
B2	Sat_342	1.1	3	0.26	0.6	2	0.24
	Sat_287	1.4	4	0.29	0.4	2	0.19
	Sct_034	1.8	6	0.36	1.8	7	0.44

n.d. not determined

[a] Linkage groups designated according to Song et al. (2004)

[b] Effect of Toyoharuka (TR) allele

regions in LG A2 and B2 with the genotype of the RILs that we used to evaluate CD tolerance. The results without or with the *GmIRCHS* background are shown in Tables 1 and 2, respectively. A QTL detected around Sat_383 (Fig. 6) appeared to be a false positive, because its LOD score was 0.8 in the *GmIRCHS* background (Table 2). However, a QTL around Sat_342 to Sct_034 did affect CD tolerance, and showed LOD scores of 2.1–4.0 (Table 2), although we could not precisely map the location of this QTL. To confirm the effect of the second QTL, we analyzed the relationship between CD tolerance and the genotype of *GmIRCHS* and Sat_287; a SSR marker in the center of the candidate region. In both sets of RILs, the genotype of Sat_287 affected CD tolerance (Fig. 7). Lines with the TR genotype at *GmIRCHS* (Fig. 7a, c) that showed high CD indices did not show the TR genotype at Sat_287 (lines indicated with triangles in Fig. 7a, c). Some lines were not tolerant to CD despite their TR genotype at *GmIRCHS* (indicated with triangles in Fig. 3a, d), but the second QTL can partly explain these anomalies. The lines with the TM genotype at *GmIRCHS* (Fig. 7b, d) that showed comparatively high CD indices did not have the TR genotype at Sat_287 (lines with indices 0–0.9 in Fig. 7b, 1.2–1.8 in Fig. 7d). Thus, the second QTL appears to affect CD tolerance independently of the *GmIRCHS* genotype.

Discussion

Relationship between *GmIRCHS* and CD tolerance

Our results indicate that the variations in *GmIRCHS* are strongly correlated with the CD tolerance phenotype. Kasai et al. (2009) reported that CD tolerance was correlated with the amount of siRNA for *CHS* gene in the seed coat under low temperature conditions. In addition, *GmIRCHS* or the region including *GmIRCHS* appears to be the unique trigger of siRNA for *CHS* in the seed coat, because spontaneous mutants that lack the *GmIRCHS* structure do not accumulate siRNAs for *CHS* genes in their seed coat (Senda et al. 2004; Kasai et al. 2007; Kurauchi et al. 2009).

Table 2 Results of single marker regression for mean CD index with *GmIRCHS* background

LG[a]	Marker	TR–TM RILs			TR-924 RILs		
		LOD	Variance (%)	Additive effect[b]	LOD	Variance (%)	Additive effect[b]
A2	Sat_383	0.8	1	0.17	n.d.	n.d.	n.d.
B2	Sat_342	4.0	7	0.38	1.4	2	0.24
	Sat_287	3.0	6	0.32	1.4	2	0.23
	Sct_034	2.7	5	0.33	2.1	4	0.32

n.d. not determined

[a] Linkage groups designated according to Song et al. (2004)

[b] Effect of Toyoharuka (TR) allele

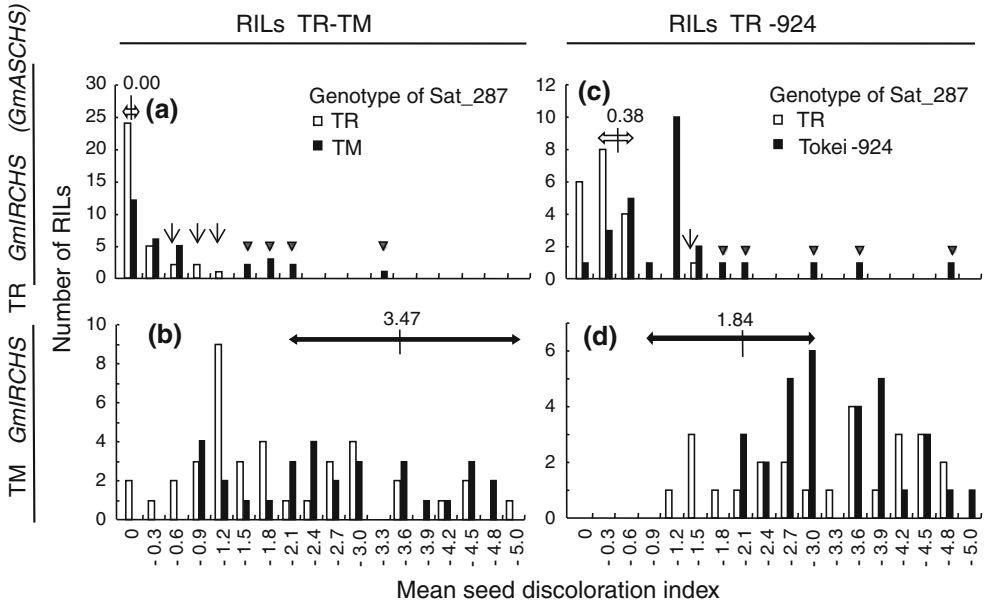

Fig. 7 Effect of second QTL (Sat_287) on frequency distributions of CD indices in different *GmIRCHS* genotype backgrounds. **a** Relationship between CD indices and Sat_287 genotype in TR–TM RILs with TR *GmIRCHS*. **b** TR–TM RILs with TM *GmIRCHS*. **c** TR-924 RILs with TR *GmIRCHS*. **d** TR-924 RILs with Tokei-924 (=TM) *GmIRCHS*. *Open bars* represent lines with TR genotype at second QTL. *Solid bars* represent RILs with TM or Tokei-924 genotype at second QTL. *Triangles* represent the same lines indicated with *triangles* in Fig. 3 (i.e. those poorly explained by *GmIRCHS* genotype). *Vertical arrows* indicate lines poorly explained by both *GmIRCHS* genotype and second QTL. *Horizontal arrows* and *values* are the same as in Fig. 3

Together with these observations, our results suggest that *GmIRCHS* is responsible for the differences in CD tolerance between TR and TM, although we cannot completely exclude the possibility that another gene tightly linked to *GmIRCHS* may be responsible. Further research is required to determine whether *GmIRCHS* itself or another tightly linked gene is responsible for CD tolerance.

In general, low temperatures suppress PTGS (Kalantidis et al. 2002; Szittya et al. 2003). Sós-Hegedus et al. (2005) reported an example of low temperature-resistant antisense-mediated RNA silencing. As discussed previously (Kasai et al. 2009), the CD tolerance of TR will be related to the fact that *GmIRCHS* of TR (*GmASCHS*) lacks the inverted repeat, but contains a structure that will transcribe antisense *CHS* RNA. In addition, *GmIRCHS* of TR has an intron adjacent to the *CHS* sequence. When PTGS is artificially induced by transformation, addition of an intron between the inverted repeats of target sequences enhances PTGS efficiency (Smith et al. 2000). It will be interesting to determine whether the intron adjacent to the *CHS* sequence in *GmASCHS* affects the PTGS of *CHS* genes.

Soybean maturity genes have been reported to affect CD tolerance (Takahashi and Abe 1999; Benitez et al. 2004). However, we did not find any QTLs for CD tolerance connected to previously reported maturity genes. This is because TR, TM, and Toke-924 are highly adapted to conditions in Northern Japan, and they have the same set of alleles with respect to maturity genes.

Githiri et al. (2007) reported QTLs for CD located at different regions from those of *GmIRCHS* and the second

QTL we found. This inconsistency is a result of the different tolerant parents used in each study. The CD tolerance of TR is much higher than that of Koganejiro, the tolerant cultivar used by Githiri et al. (2007).

Application of *GmIRCHS* variation as a DNA marker

We demonstrated that the TR genotype of *GmIRCHS (GmASCHS)* exhibited a CD-tolerant phenotype among breeding lines with various genetic backgrounds. Therefore, marker-assisted selection targeting *GmIRCHS* is an effective strategy for breeding CD-tolerant lines. If the second QTL was mapped precisely, the combination of *GmIRCHS* and the second QTL could achieve a more reliable marker-assisted selection for CD tolerance.

Second QTL for CD tolerance

The *GmIRCHS* genotype did not explain all CD phenotypes (e.g. TR-genotyped RILs with indices >0.3 in Fig. 3a and >0.9 in Fig. 3d). This result suggests that there are other QTLs for CD tolerance in addition to *GmIRCHS*. We detected a second QTL for CD tolerance that was located around Sat_342 to Sct_034 in LG B2. This QTL made a smaller contribution to CD tolerance, but helped in explaining the CD tolerance of some lines that were not well explained by the *GmIRCHS* genotype (Fig. 7a, c). However, even the combination of the *GmIRCHS* genotype and the second QTL cannot completely explain all the CD-tolerant phenotypes (e.g. Fig. 7, the lines indicated with vertical arrows and in Table 1, only 47–63% phenotypic variation was explained by the two QTLs). This was partially due to an experimental error. Further, we cannot rule out the effect of other QTLs. The moderately CD-tolerant phenotype of Tokei-924 may be explained by these minor QTLs. In our experimental conditions, we might not have sufficient markers to cover the whole genome, and hence, we may not be able to discover all QTLs involved in CD tolerance.

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