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Research Paper

A major gene for tolerance to cold-induced seed coat discoloration relieves viral seed mottling in soybean

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In yellow soybeans, inhibition of seed coat pigmentation by RNA silencing of *CHS* genes is suppressed by low temperature and a viral suppressor, resulting in 'cold-induced seed coat discoloration' and 'seed mottling', respectively. Differences exist in the degree of cold-induced seed coat discoloration among Japanese yellow soybean cultivars; for example, Toyomusume is sensitive, Toyohomare has some tolerance, and Toyoharuka is highly tolerant. In this study, we compared the degree of seed mottling severity due to soybean mosaic virus (SMV) among these three soybean cultivars. Obvious differences were found, with the order of severity as follows: Toyohomare > Toyomusume > Toyoharuka. RNA gel blot analysis indicated that *CHS* transcript abundance in the seed coat, which was increased by SMV infection, was responsible for the severity of seed mottling. Quantitative reverse transcription PCR analysis revealed why mottling was most severe in SMV-infected Toyohomare: the SMV titer in its seed coat was higher than in the other two infected cultivars. We further suggest that a major gene (*Ic*) for tolerance to cold-induced seed coat discoloration can relieve the severity of seed mottling in SMV-infected Toyoharuka.

Key Words: yellow soybean, *I* allele, cold-induced seed coat discoloration, soybean mosaic virus, seed mottling, *Ic* allele, residual heterozygous line.

Introduction

In pigmented soybean seeds, anthocyanins and proanthocyanidins accumulate in the epidermal layer of the seed coat (Palmer *et al.* 2004, Senda *et al.* 2017, Todd and Vodkin 1993). Seed coat pigmentation is mainly controlled by at least three independent genetic loci (Bernard and Weiss 1973, Palmer and Kilen 1987). Two of these loci (*R* and *T*) determine seed-coat pigments, while the third locus, *I* (inhibitor), controls the presence or absence of these pigments in the seed coat as well as their spatial distribution (Palmer and Kilen 1987, Palmer *et al.* 2004). At the *I* locus, four alleles (*I*, i^i, i^k and *i*) are known. The dominant *I* allele inhibits seed coat pigmentation in the whole area, whereas the recessive *i* allele permits pigmentation all over the seed coat. Two other alleles, i^i and i^k, are responsible for restricted pigmentation of the hilum and the saddle-shaped region containing the hilum, respectively. Yellow soybean cultivars have seed coats that are either completely yellow (*II* genotype) or with pigmentation restricted to the hilum (i^ii^i genotype). In yellow soybeans, inhibition of seed coat pigmentation by *I* and i^i alleles is the result of naturally occurring RNA silencing of chalcone synthase (CHS) genes, referred to as CHS silencing (Senda *et al.* 2004,

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Tuteja *et al.* 2004). A candidate for the *I* allele has been isolated and designated as *GmIRCHS* (*Glycine max* inverted repeat of *CHS* pseudogene) (Kasai *et al.* 2007). Because RNA silencing is suppressed by a viral suppressor (Kasschau and Carrington 1998) and low temperature (Szittya *et al.* 2003), these two factors can cause yellow soybeans to pigment seed coats: the former is called 'seed mottling' (Senda *et al.* 2004, 2012), and the latter is known as 'cold-induced seed coat discoloration' (CD) (Kasai *et al.* 2009, Senda *et al.* 2012). Seed coat pigmentation phenomena such as seed mottling and CD debase the seed quality of yellow soybeans; therefore, the breeding of tolerant cultivars is needed.

In Japan, CD damage has mainly been reported in Hokkaido, the northernmost region (Srinivasan and Arihara 1994, Yamaguchi *et al.* 2019). Genotypic differences have been reported in the degree of CD among yellow soybean cultivars with a yellow seed coat and hilum in Hokkaido. For example, the cultivar Toyomusume (TM) is sensitive, Toyohomare (TH) is somewhat tolerant, and Toyoharuka (TR) is highly tolerant to CD (Kasai *et al.* 2009, Yumoto *et al.* 1995). The order of CD severity is as follows: TM > TH > TR. Interestingly, the CD-tolerant cultivar TR possesses a different *GmIRCHS* structure and thus a different allele at the *I* locus (Kasai *et al.* 2009, Senda *et al.* 2012), which has been designated as the *Ic* (inhibitor of CD) allele (Yamaguchi *et al.* 2015). A distinguishable DNA marker between *I* and *Ic* alleles, the so-called 'Ic marker', has been shown to be effective for selecting CD-tolerant soybean plants (Ohnishi *et al.* 2011, Yamaguchi *et al.* 2019).

In yellow soybeans, seed mottling is caused by soybean mosaic virus (SMV) because of suppression of CHS silencing (Senda *et al.* 2004). In the present study, we compared the severity of seed mottling due to SMV among three cultivars (TM, TH and TR) having different degrees of CD tolerance. Obvious differences were found among the three cultivars, but the order of severity of SMV mottling, TH > TM > TR, was different than the order of CD severity. Given these results, we examined why TH suffers from severe SMV mottling and why this symptom is relieved in TR.

Materials and Methods

Plant materials

The yellow soybean cultivars (TH, Yukihomare [YH], TM and TR) and breeding lines (Toiku 238 [T238] and Toiku 239 [T239]) used in this study were bred at the Tokachi Agricultural Experiment Station, Hokkaido, Japan. All these cultivars and breeding lines possess a yellow seed coat and hilum. The yellow soybean cultivars TH, YH and TM have the *II* genotype. TM is sensitive to CD. TH and YH have some tolerance to CD, and the extent of the pigmented region around the hilum is smaller than that of TM (Yumoto *et al.* 1995). TR, T238 and T239 are CD-tolerant and possess the *IcIc* genotype (Ohnishi *et al.* 2011). Two residual heterozygous lines (RHLs) of the *I* locus, namely, RHL nos. 22 and 34, were selected from recombinant inbred lines derived from a TR × TM cross; these two RHLs were heterozygous at the *I* locus, with the other genomic regions being fixed (Ikeda *et al.* 2009, Tuinstra *et al.* 1997). Seeds obtained from each RHL were genotyped using the Ic marker and thereby sorted into TR (*IcIc*), TM (*II*) and heterozygous genotypes. A pair of near isogenic lines (NILs) bred from the progeny of RHL no. 22 and having TR and TM genotypes were named as NIL22R and NIL22M, respectively. In the same way, two NILs were bred from the progeny of RHL no. 34 and named as NIL34R and NIL34M.

SMV materials

SMV isolates from Japan were previously grouped into five strains, A to E, with strain A later subdivided into strains A_1 and A_2 (Nakano *et al.* 1982, Saruta *et al.* 2005, Takahashi *et al.* 1980). In the present study, we used an SMV isolate and two SMV strains. The SMV isolate used for the experiments in **Figs. 1**, **2** and **3** was isolated from an experimental field belonging to Hirosaki University in Aomori Prefecture, Tohoku, northeastern Japan (Senda *et al.* 2004). This SMV isolate was designated as the Aomori isolate. Although the Aomori isolate was not identified to strain based on observations of phenotypic reactions with a series of different cultivars developed by Takahashi *et al.* (1980) and Nakano *et al.* (1982), the nucleotide sequence of the coat protein (CP) gene was highly similar (ca. 99%) to that of SMV-D (DNA database accession AB100447). The SMV strains used for the experiments in **Figs. 4** and **5** were SMV-A_2 and SMV-C, respectively.

SMV inoculation and detection

SMV inoculation was carried out according to Saruta *et al.* (2012). SMV-infected soybean leaf tissue frozen at –80°C was homogenized in 0.1 M sodium phosphate buffer (pH 7.0) using a ratio of approximately 1 g of infected tissue per 10 mL of buffer. The homogenate was then rub-inoculated on fully expanded primary leaves of soybean seedlings dusted with carborundum. Seven to ten days after inoculation, infection was verified by observation of leaf mosaic symptoms and/or by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to the protocol of Clark and Adams (1977). Leaf disks (ϕ = 1.0 cm) were ground with 2 mL of grinding buffer (0.02 M PBS, 0.05% Tween 20, and 1% PVP K-30), and the suspension was transferred to a tube. After centrifugation at 10,000 rpm for 3 min, the supernatant was used for DAS-ELISA.

Seed mottling evaluation (field)

Seeds were sown individually in small plastic pots on May 17, 2006. Plants of the three soybean cultivars were inoculated with SMV (Aomori isolate) on May 26, 2006, and infection was confirmed on the basis of leaf mosaic

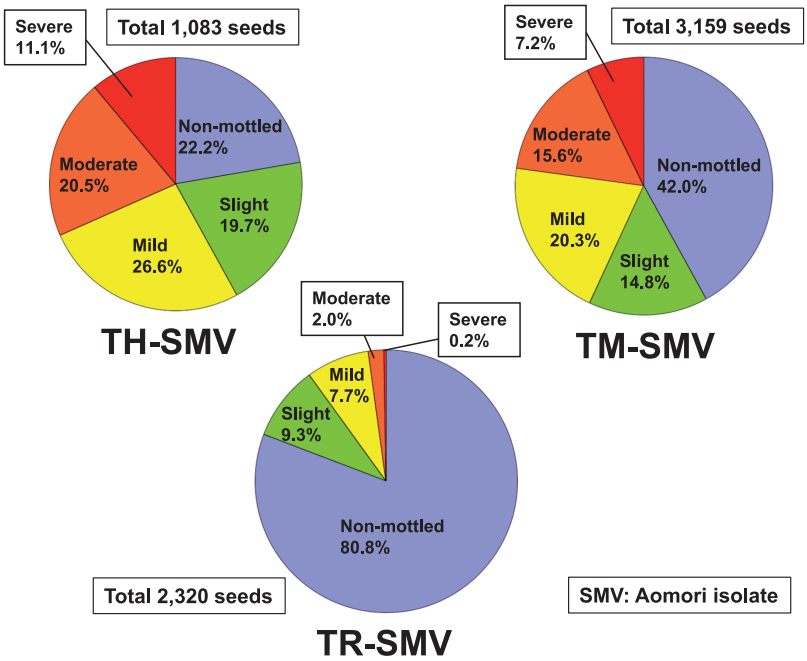

Fig. 1. Percentages of seeds categorized by degree of mottling in soybean mosaic virus (SMV)-infected soybean cultivars.

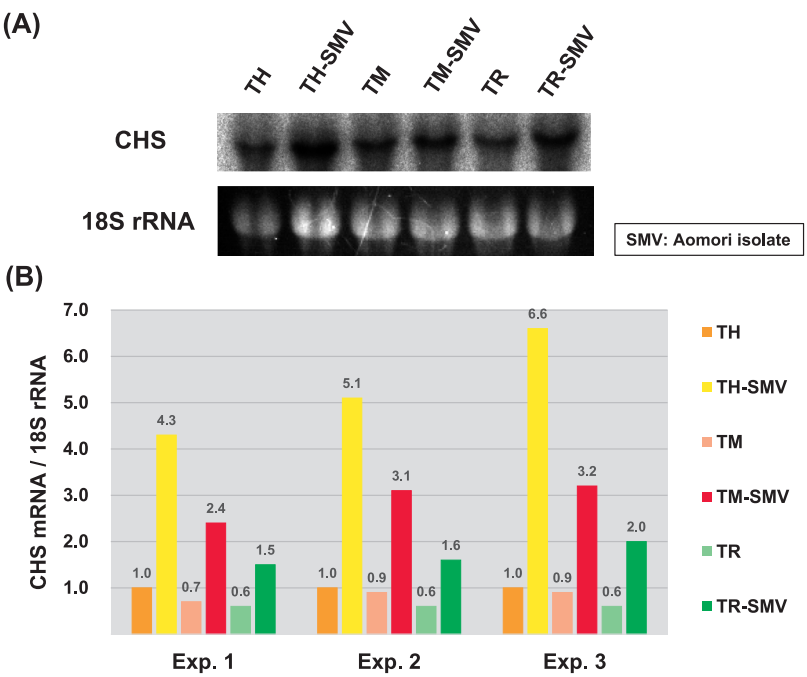

Fig. 2. Analysis of CHS mRNA levels in soybean seed coats. (A) RNA gel blot analysis of CHS mRNA in seed coats from healthy and SMV-infected plants of three soybean cultivars. RNA gel blots were hybridized with a soybean CHS probe (first panel). Ethidium bromide-stained 18S rRNA, loaded in equal amounts as an internal control, is shown in the second panel. (B) Relative level of CHS mRNA in seed coats from healthy and SMV-infected plants of three soybean cultivars. The relative level of CHS mRNA was calculated by dividing the level of CHS-specific radioactivity by the 18S rRNA level. The level of CHS mRNA in healthy Toyohomare (TH) plants was set to 1.0.

symptoms and DAS-ELISA. For the experiment in **Fig. 1**, 50 SMV-infected plants were randomly transplanted to a plot in the experiment field (40°34′ N, 140°28′ E) on June 21, 2006, and carefully cultivated to prevent co-infection with other viruses until harvest on September 12, 2006. Seed mottling was evaluated using the harvested mature desiccated seeds because seed mottling was first visible at the desiccated stage. The mottled seed rate (%) was calculated as 100 × (number of mottled seeds/number of total seeds). The degree of seed mottling was classified

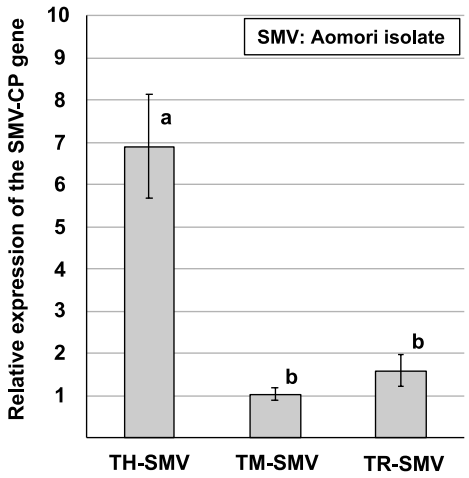

Fig. 3. Relative expression levels of the SMV-CP gene in SMV-infected cultivars. The data represent means of three samples, with SE indicated by error bars. The expression level of the SMV-CP gene in SMV-infected Toyomusume (TM-SMV) sample No. 2 was set to 1.0. Different letters indicate significant differences ($P < 0.01$) according to a Tukey's test.

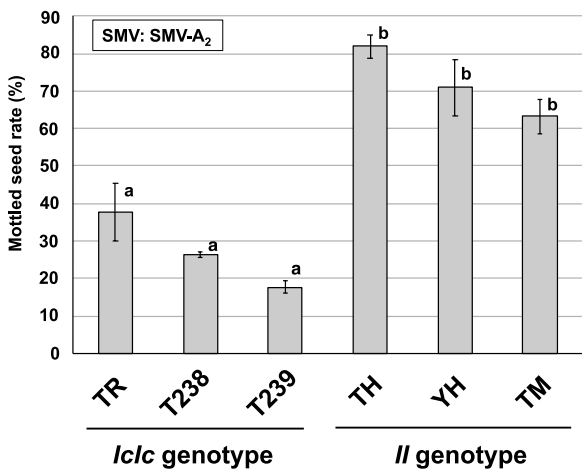

Fig. 4. Comparison of mottled seed rates of SMV-infected soybean cultivars (TR: Toyoharuka, TH: Toyohomare, YH: Yukihomare and TM: Toyomusume) and breeding lines (T238: Toiku 238 and T239: Toiku 239). Vertical bars represent means ± SE ($n = 3$). Different letters indicate significant differences ($P < 0.05$) according to a Tukey's test.

according to Takahashi *et al.* (1980) as follows: severe, pigmented areas constitute more than half of the whole seed area; moderate, pigmented areas constitute from more than a quarter to half of the whole seed area; mild, pigmented areas constitute less than a quarter of the whole seed area; slight, seeds are slightly pigmented around the hilum (**Supplemental Fig. 1**).

Seed mottling evaluation (pot)

Three 1/2000 a Wagner pots were prepared per cultivar and line for the experiments in **Figs. 4** and **5**. Four seeds were sown in each Wagner pot (July 7, 2006, in **Fig. 4**; July

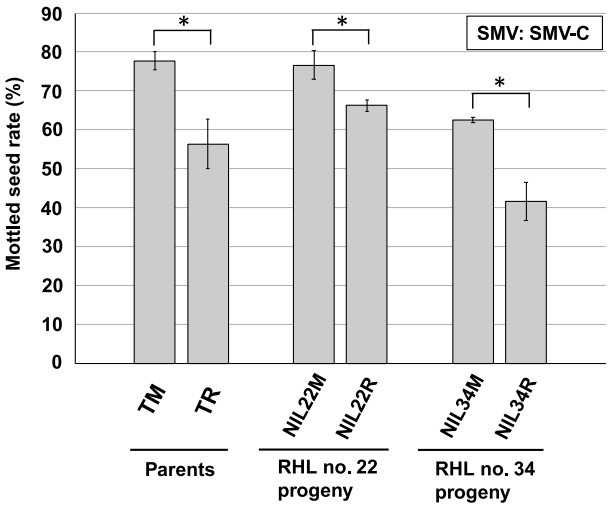

Fig. 5. Comparison of mottled seed rates between soybean plants with *II* (TM type) and *Iclc* (TR type) genotypes at the *I* locus. Comparisons of the two genotypes in parental cultivars (left), NIL22M/NIL22R (center) and NIL34M/NIL34R (right) are shown. Vertical bars represent means ± SE ($n = 3$). Asterisks indicate significant differences ($P < 0.05$) according to a *t*-test.

14, 2008, in **Fig. 5**). Four plants per pot were inoculated with SMV (SMV-A_2 on July 14, 2006, in **Fig. 4**; SMV-C on July 21, 2008, in **Fig. 5**), and infection was verified by observation of leaf mosaic symptoms. SMV-infected plants were cultivated until harvest in a greenhouse (34°13′ N, 133°47′ E) at 18°C to 25°C. Mature desiccated seeds were harvested in early October and mottled seed rates were calculated for each pot.

RNA gel blot analysis and quantitative reverse transcription PCR (qRT-PCR)

Soybean plants were cultivated in a greenhouse. Seed coats were collected from immature seeds having fresh weights below 50 mg. RNA was extracted from the seed coats according to the protocol of Wang and Vodkin (1994). RNA gel blot analysis, including soybean CHS probe preparation, was performed as described previously (Senda *et al.* 2002), and mRNA band intensities were quantified as described by Senda *et al.* (2004). qRT-PCR was conducted as described previously (Kasai *et al.* 2004). Reverse transcription (RT) from SMV RNA was carried out using the reverse primer SMV-RP1 (5′-ATTCTCGACAATGGGCTTCA-3′), with the annealing site located in the SMV CP region. Real-time PCR of SMV cDNA was conducted using forward primer SMV-FP1 (5′-ATGATGAGCAGATGGGTGTG-3′), located in the CP region, and the same reverse primer used for RT (SMV-RP1). The soybean actin gene *SAc3* was used as a control for normalization of total RNA transcript abundance (Shah *et al.* 1982). Forward and reverse primer sequences used for RT-PCR of *SAc3* mRNA are detailed in Kasai *et al.* (2004). The expression level of the *SAc3* gene was not substantially different among the three SMV-infected cultivars, thus indicating that the

expression level of *SAc3* was unaffected by SMV infection (data not shown).

Results

Comparison of the severity of seed mottling among SMV-infected soybean cultivars

The three cultivars used in this study, TH, TM and TR, have different degrees of CD tolerance. After field harvesting of mature desiccated seeds, the severity of seed mottling was compared among SMV-infected cultivars based on the mottled seed rate, and the percentage of seeds was categorized by the degree of mottling (severe, moderate, mild or slight; see Materials and Methods: Seed mottling evaluation [field]). As shown in **Fig. 1**, obvious differences in seed mottling severity were observed among SMV-infected cultivars. In addition, we found that a higher mottled seed rate corresponded to a more severe degree of seed mottling, which indicated that the severity of seed mottling could be approximated by the mottled seed rate. Among the three infected cultivars, SMV-infected TH (TH-SMV) had the highest mottled seed rate (77.8%) and produced the highest percentage of severely mottled seeds (11.1%), which was followed by SMV-infected TM (TM-SMV) having a mottled seed rate of 58.0% and a severely mottled seed percentage of 7.2%. Finally, the mottled seed rate of SMV-infected TR (TR-SMV) was 19.2%, while the severely mottled seed percentage was only 0.2%. The order of severity of SMV mottling in the three infected cultivars was TH-SMV > TM-SMV > TR-SMV. This experiment was repeated in 2007 and similar results were obtained (**Supplemental Fig. 2**).

Comparison of seed-coat CHS transcript abundance among SMV-infected soybean cultivars

Seed-coat RNAs were isolated from healthy and SMV-infected plants of the three soybean cultivars. According to RNA gel blot analysis using a soybean CHS probe, SMV infection increased CHS transcript abundance in all cultivars (**Fig. 2**). Among the three infected cultivars, CHS transcripts were most abundant in TH-SMV and least abundant in TR-SMV. Noteworthily, CHS transcript abundance in the three infected cultivars was directly linked to the severity of seed mottling (**Figs. 1**, **2**).

Comparison of seed-coat SMV titers among SMV-infected soybean cultivars

We compared seed-coat SMV titers, measured by qRT-PCR analysis of the SMV CP gene among the three SMV-infected cultivars. SMV was most abundant in TH-SMV, which suggests that the degree of seed mottling severity is related to the SMV titer (**Fig. 3**). Compared with its level in TH-SMV, SMV was less abundant in TM-SMV and TR-SMV; however, the SMV titers of these latter two cultivars were not significantly different from each other even though that of TM-SMV was slightly lower (**Fig. 3**). This result suggests that the different degrees of seed mottling severity between TM-SMV and TR-SMV were unrelated to the SMV titer (**Figs. 1**, **3**).

Comparison of SMV titers in leaf tissue among SMV-infected soybean cultivars

After inoculation, we measured SMV titers in leaf tissues (third, fifth and seventh leaves) by DAS-ELISA. Absorbance values (A_{405}) representing the SMV titers were compared among the three SMV-infected cultivars. At each leaf position, TH-SMV leaf tissue tended to have a slightly higher SMV titer than that of TM-SMV and TR-SMV, even though no significant difference was detected (**Supplemental Fig. 3**).

Comparison of mottled seed rates among SMV-infected soybean cultivars and lines possessing II or IcIc genotypes

We compared mottled seed rates among SMV-infected soybean cultivars and breeding lines possessing an *II* or *IcIc* genotype at the *I* locus. In particular, TH, YH and TM have the *II* genotype, while TR, T238 and T239 possess the *IcIc* genotype. Three pots were prepared per cultivar and line for evaluating mottled seed rates (see Materials and Methods: Seed mottling evaluation [pot]). As shown in **Fig. 4**, a significant difference was found between the two groups, namely, the mottled seed rate was significantly lower in soybeans harboring the *IcIc* genotype than in the group with the *II* genotype. Within the latter group, TH appeared to have the highest mottled seed rate, although no significant difference was detected compared with YH and TM (**Fig. 4**).

Comparison of mottled seed rates between NILs of I or Ic alleles

SMV was inoculated into NILs having different alleles (*II* genotype [TM type] or *IcIc* genotype [TR type]) at the *I* locus. Two pairs of different NILs, NIL22M/NIL22R and NIL34M/NIL34R, were used for the comparison of mottled seed rates, with three pots prepared per cultivar and line (see Materials and Methods: Seed mottling evaluation [pot]). As shown in **Fig. 5**, the mottled seed rate of the TR type (*IcIc* genotype) was significantly lower than that of the TM type (*II* genotype) in both NIL22M/NIL22R and NIL34M/NIL34R, which suggests that *Ic* is responsible for the suppression of SMV-induced seed mottling.

Discussion

In yellow soybeans, inhibition of seed coat pigmentation is caused by a marked decrease in CHS transcript abundance due to CHS silencing (Senda *et al.* 2004, Tuteja *et al.* 2004). CHS silencing is the naturally-occurring RNA silencing of *CHS* genes induced by the presence of *I* or i^i alleles (Cho *et al.* 2019, Clough *et al.* 2004, Kasai *et al.* 2007, Senda *et al.* 2004). Seed mottling results from the suppression of CHS silencing by a viral suppressor: when

CHS mRNA accumulates beyond a certain threshold, the seed coat cells synthesize and accumulate pigments, leading to mottling of the yellow seeds (Senda *et al.* 2004). SMV belongs to the genus *Potyvirus*, and its helper component-protease (HC-Pro) is considered to be the viral suppressor (Gao *et al.* 2015).

In the current study, we first compared the severity of seed mottling caused by SMV among TH, TM and TR, which have different degrees of tolerance to CD (order of CD severity: TM > TH > TR). Differences were apparent among the three cultivars: TH-SMV exhibited the most severe mottling, while the severity of mottling was relieved in TR-SMV (**Fig. 1**, **Supplemental Fig. 2**); consequently, the order of severity of SMV mottling was TH-SMV > TM-SMV > TR-SMV. Next, RNA gel blot analysis indicated that the severity of seed mottling was directly mirrored by an increase in CHS mRNA abundance (**Fig. 2**). Finally, qRT-PCR analysis revealed that SMV was most abundant in the seed coat of TH-SMV, which exhibited the most severe seed mottling, thus suggesting that the severe mottling in TH-SMV is due to the abundance of SMV (**Fig. 3**). Genetic factor(s) controlling the accumulation of SMV in the seed coat may vary among these cultivars. Another consideration is that TH-SMV leaf tissue tended to have a slightly higher SMV titer (as measured by DAS-ELISA) than that of TM-SMV and TR-SMV, even though no significant difference was detected (**Supplemental Fig. 3**). We thus cannot exclude the possibility that the slightly higher SMV titer in leaf tissue of TH-SMV is responsible for the higher level in the seed coat; in other words, differences of SMV susceptibility among soybean cultivars may reflect differences in seed coat SMV titers.

At the same time, the severity of seed mottling was reduced in the remaining two SMV-infected cultivars, slightly in TM-SMV and strongly in TR-SMV (**Fig. 1**); however, their SMV titers were almost same (**Fig. 3**), which suggests that their different degrees of seed mottling severity cannot be explained only by the abundance of SMV. In regard to the relief of seed mottling severity and the genotype at the *I* locus, we noted that CD-tolerant TR, unlike TM with the *II* genotype, possesses the *IcIc* genotype. Relief of the severity of seed mottling was observed not only in TR but also in other CD-tolerant breeding lines (T238 and T239) with the *IcIc* genotype (**Fig. 4**). These results suggest that *Ic* has a function that relieves the seed mottling symptom. In fact, mottled seed rates of NILs with the *IcIc* genotype were significantly lower than those with the *II* genotype (**Fig. 5**). Furthermore, three types of SMV (the Aomori isolate, SMV-A_2 and SMV-C) were used in this study. In each case, mottled seed rates in soybeans with the *IcIc* genotype were lower than those with the *II* genotype (**Figs. 1**, **4**, **5**), which suggests that the *Ic* allele can relieve seed mottling symptoms regardless of the specific SMV isolate/strain (**Figs. 1**, **4**, **5**).

A candidate for *Ic* has been isolated and designated as *GmASCHS* (*Glycine max* antisense *CHS* pseudogene) (Kasai *et al.* 2009). Unlike *GmIRCHS*, from which pseudo*CHS* double-stranded RNA (dsRNA) can be transcribed, pseudo*CHS* antisense RNA can be transcribed from *GmASCHS* (Kasai *et al.* 2007, 2009, Kurauchi *et al.* 2011, Senda *et al.* 2012). Antisense pseudo*CHS* RNA possibly forms dsRNA by hybridization with endogenous *CHS* transcripts, and triggers CHS silencing (Kasai *et al.* 2009, Senda *et al.* 2012). The abundance of CHS mRNA in seed coats of TR-SMV with the *IcIc* genotype was lower than that in seed coats of TM-SMV possessing the *II* genotype (**Fig. 2**). The abundance of CHS mRNA in the TR seed coat is lower than that in the TM seed coat, probably as a result of the antisense action of *Ic* (**Fig. 2**). The lowered abundance of CHS mRNA in the TR seed coat may contribute to the suppression of the elevated CHS mRNA in the TR-SMV seed coat; however, the mechanism responsible for the relief of seed mottling by *Ic* remains to be elucidated. In Japan, SMV is one of the most prevalent viral pathogens, and seed mottling caused by SMV decreases soybean seed value. Our study suggests that *Ic* is potentially useful in soybean breeding programs for the relief of SMV seed mottling severity.

Other viruses producing mottling on yellow soybean seeds in addition to SMV include cucumber mosaic virus (CMV), southern bean mosaic virus (SBMV) and peanut stunt virus (PSV). The pattern of seed mottling is virus-specific; for example, mottling caused by SMV appears as a patch, blotch or band, generically referred to as radial-type mottling, while mottling caused by CMV is in a concentric ring (Koshimizu and Iizuka 1963). Future research is required to verify whether *Ic* also affects the relief of seed mottling severity caused by viruses such as CMV, SBMV and PSV.

Author Contribution Statement

M. Saruta and M. Senda designed the research. M. Saruta, HA, TM, HO, MH, AK and TS conducted experiments. M. Saruta, SO and HF bred RHLs and NILs of the *I* locus. M. Saruta, MK and M. Senda analyzed the data. M. Senda wrote the manuscript. All authors read and approved the manuscript.

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