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Review

Suppressive mechanism of seed coat pigmentation in yellow soybean

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In soybean seeds, numerous variations in colors and pigmentation patterns exist, most of which are observed in the seed coat. Patterns of seed coat pigmentation are determined by four alleles (I, i^i, i^k and i) of the classically defined *I* locus, which controls the spatial distribution of anthocyanins and proanthocyanidins in the seed coat. Most commercial soybean cultivars produce yellow seeds with yellow cotyledons and nonpigmented seed coats, which are important traits of high-quality seeds. Plants carrying the *I* or i^i allele show complete inhibition of pigmentation in the seed coat or pigmentation only in the hilum, respectively, resulting in a yellow seed phenotype. Classical genetic analyses of the *I* locus were performed in the 1920s and 1930s but, until recently, the molecular mechanism by which the *I* locus regulated seed coat pigmentation remained unclear. In this review, we provide an overview of the molecular suppressive mechanism of seed coat pigmentation in yellow soybean, with the main focus on the effect of the *I* allele. In addition, we discuss seed coat pigmentation phenomena in yellow soybean and their relationship to inhibition of *I* allele action.

Key Words: *CHS* genes, dsRNA, pigmentation, RNA silencing, seed coat, siRNA, soybean.

Introduction

In soybean (*Glycine max*), seed coat pigmentation is controlled by three independent genetic loci (*I*, *R* and *T*) (Bernard and Weiss 1973). The *R* and *T* loci determine the type of anthocyanin and proanthocyanidin synthesized, by which a specific seed coat color is determined as follows: black (*R*, *T*), imperfect black (*R*, *t*), brown (*r*, *T*) and buff (*r*, *t*). The *T* locus encodes a flavonoid 3′-hydroxylase (F3′H) responsible for synthesis of the cyanidin-based anthocyanins and proanthocyanidins (Nagamatsu *et al.* 2007, Toda *et al.* 2002, Zabala and Vodkin 2003). The molecular nature of the *R* locus remains to be elucidated, although its product is speculated to act after leucoanthocyanidin production but before the formation of anthocyanins (Todd and Vodkin 1993). In contrast to the *R* and *T* loci, the *I* locus (inhibitor), which has four alleles (I, i^i, i^k and i), determines the spatial distribution of pigments in the epidermal layer of the seed coat. The *I* allele inhibits the production and accumulation of pigments over the entire seed coat, resulting in uniformly yellow-colored seeds, whereas the *i* allele leads to completely pigmented seeds by allowing the production and accumulation of pigments over the entire seed coat (Fig. 1). The remaining two alleles, i^i and i^k, inhibit pigmentation except in the hilum and a saddle-shaped region, respectively (Fig. 1). Yellow soybean cultivars carry the *I* allele for a light (nonpigmented) hilum or the i^i allele for a dark (pigmented) hilum. The dominance relationships among the four alleles are $I > i^i > i^k > i$. Inhibition of seed coat pigmentation by the *I* locus, at least the *I* and i^i alleles, is the result of RNA silencing of chalcone synthase (*CHS*) genes (Senda *et al.* 2004, Tuteja *et al.* 2004).

Inhibition of seed coat pigmentation in yellow soybean occurs via naturally occurring RNA silencing of *CHS* genes

In the seed coat of yellow soybean with either the *I* allele or i^i allele, the steady-state *CHS* mRNA level is markedly reduced compared with that in the pigmented soybean seed coat with the *i*/*i* genotype (Senda *et al.* 2002b, Wang *et al.* 1994). The reduction in the *CHS* mRNA level occurs only in the seed coat tissue throughout seed development (Tuteja *et al.* 2004). Concomitant with the decrease in *CHS* mRNA level, CHS activity is reduced (Wang *et al.* 1994). CHS is a key enzyme in the flavonoid pathway leading to the biosynthesis of anthocyanins and proanthocyanidins, and therefore reduction of the *CHS* mRNA level is likely to be the basis for the inhibition of seed coat pigmentation (Wang *et al.* 1994). Indeed, using a plant virus vector based on *Cucumber*

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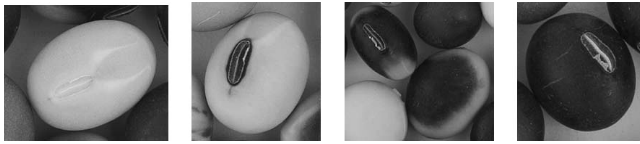

Fig. 1. Soybean seed pigmentation patterns determined by the four alleles at the *I* locus (from left to right): *I*, light (non-pigmented) hilum; i^i, dark (pigmented) hilum; i^k, saddle-shaped pigmented region; *i*, full pigmentation.

mosaic virus (CMV), the *CHS* mRNA level was reduced in the pigmented soybean plant, and consequently the pigmentation of the seed coat was clearly inhibited (Nagamatsu *et al.* 2007, see review in this issue by Kasai and Kanazawa).

It was shown that the *CHS* mRNA reduction in the yellow soybean seed coat is the result of RNA silencing of *CHS* genes (Senda *et al.* 2004, Tuteja *et al.* 2004). RNA silencing, otherwise known as RNA interference (RNAi), refers collectively to diverse RNA-based processes resulting in sequence-specific inhibition of gene expression either at the transcriptional level (inhibition of transcription) or posttranscriptional level (mRNA degradation or inhibition of translation) (Brodersen and Voinnet 2006). On this basis, RNA silencing can be divided into two categories, namely transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). In the seed coat of yellow soybean carrying the *I* allele, expression of *CHS* genes is suppressed by PTGS, i.e. endogenous *CHS* mRNAs are degraded after transcription (Senda *et al.* 2004). Similar dominant inhibitory phenotypes were found in a colorless mutant of maize and a star-type petunia cultivar, in both of which *CHS* expression was also suppressed mainly at the posttranscriptional level (Della Vedova *et al.* 2005, Koseki *et al.* 2005). One of the natural roles of PTGS is as a defense mechanism against invading RNAs such as RNA viruses, so that PTGS in plants is usually elicited by viral pathogens (Voinnet 2005). However, PTGS suppressing *CHS* expression found in soybean, maize and petunia is naturally occurring and is gained as a consequence of mutations, not of transgenic techniques as reported in co-suppression (Napoli *et al.* 1990, Van der Krol *et al.* 1990), and is called 'naturally occurring RNA silencing' (Frizzi and Huang 2010, Kanazawa 2008). Another example of naturally occurring RNA silencing is the rice LGC-1 (low glutelin content-1) produced by mutagenesis. In LGC-1, expression of *Glutelin* genes is posttranscriptionally suppressed by *Lgc1* generated with a dominant mutation (Kusaba *et al.* 2003).

Soybean *CHS* gene family comprises at least nine members

In soybean, CHS is encoded by a multigene family composed of at least nine members (*CHS1–CHS9*) (Akada and Dube 1995, Tuteja and Vodkin 2008), for which expression patterns are variable in different tissues (Tuteja *et al.* 2004). The nine *CHS* members have two exons (exon1 and exon2) separated by one intron, and exhibit high nucleotide similarity in the open reading frame (ORF). By contrast, nucleotide sequences of the 5′ upstream regions and the 3′ untranslated regions are not conserved in most *CHS* members (Tuteja and Vodkin 2008). Based on phylogenetic analysis of ORF nucleotide sequences, the nine *CHS* members are classified into two subfamilies, comprising *CHS7*/*CHS8* and other *CHS* members (*CHS1–CHS6*, *CHS9*) (Kurauchi *et al.* 2009, Tuteja and Vodkin 2008).

In the seed coat of pigmented soybean (*i*/*i*) in which RNA silencing of *CHS* genes does not occur, the *CHS7*/*CHS8* transcripts are abundant and constitute most *CHS* transcripts, whereas those of other *CHS* members are less abundant (Kasai *et al.* 2004, Tuteja *et al.* 2004). Compared with pigmented soybean, in the seed coat of yellow soybean with either the *I* allele or i^i allele, the mRNA levels of most *CHS* members are significantly decreased by RNA silencing, in particular those of *CHS7*/*CHS8* are markedly reduced (Kasai *et al.* 2004, Tuteja *et al.* 2004).

Molecular structure of the *I* allele and *CHS* dsRNA formation in its transcripts

Although the processes of RNA silencing are diverse, three common features are shared (Brodersen and Voinnet 2006): (1) formation of double-stranded RNA (dsRNA); (2) processing of dsRNA into small (approximately 21–25 nucleotides [nt]) RNAs (sRNAs) including short interfering RNAs (siRNAs) and microRNAs (miRNAs); and (3) a selected sRNA strand within effector complexes guides them to partially or fully complementary target sites of RNA or DNA for inhibitory action. The soybean dominant *I* allele induces RNA silencing of *CHS* genes to inhibit seed coat pigmentation. A candidate for the *I* allele has been identified in the yellow soybean genome and designated *GmIRCHS* (*Glycine max* inverted repeat of *CHS* pseudogene) (Kasai *et al.* 2007). *GmIRCHS* consists of the 5′-portion of *GmJ1* (from the promoter region to part of exon1) and a perfect inverted repeat (IR) of the *CHS* pseudogene (pseudo*CHS3*) (Fig. 2). *GmJ1* encodes a type III DnaJ-like protein with only a J-domain (Cheetham and Caplan 1998, Miernyk 2001), but its function is still unknown. The IR of pseudo*CHS3* is very closely spaced (a distance of only 78 bp) and arranged tail-to-tail (Fig. 2). The structure of *GmIRCHS* raises the possibility that its transcripts may lead to *CHS* dsRNA formation and trigger RNA silencing of *CHS* genes (Kasai *et al.* 2007). A 1087-bp *CHS* dsRNA-forming region of the *GmIRCHS* transcripts was identified in the seed coat of soybean carrying the *I* allele, indicating that the complete read-through transcription occurs from the pseudo*CHS3* to its complementary sequence (Fig. 3A) (Kurauchi *et al.* 2011). Interestingly, *CHS* dsRNA formation in the *GmIRCHS* transcripts was observed not only in the seed coat but also in the cotyledon and leaf (Kurauchi *et al.* 2011). RNA silencing of *CHS* genes is restricted to the seed coat (Tuteja *et al.* 2004), and its tissue-specificity may be determined in the step(s) after *CHS* dsRNA formation.

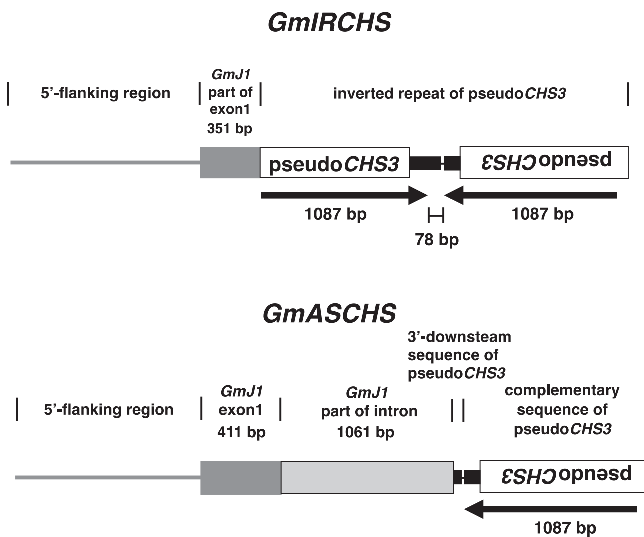

Fig. 2. Schematic representation of the structure of *GmIRCHS* and *GmASCHS*. A 5′-flanking region, exon1 and intron of *GmJ1* are represented by a gray line, gray box and stippled box, respectively. A pseudo*CHS3* and its 3′-downstream region are indicated by an open box and a broad black line, respectively. The position and relative orientations of a pseudo*CHS3* inverted repeat are indicated by black horizontal arrows. The 78 bp distance between a pseudo*CHS3* (1087 bp) and its complementary sequence (1087 bp), which comprise the inverted repeat of pseudo*CHS3*, is indicated. The 16-bp unique sequence is denoted by a thin black line.

Characterization of *CHS* siRNAs in the seed coat

sRNA generated from the long dsRNA precursors are called siRNAs (Shiomi and Shiomi 2009). siRNAs are produced by RNase-III-type enzymes called Dicers with distinctive dsRNA binding, RNA helicase, RNase III and PAZ (Piwi/Argonaute/Zwille) domains (Brodersen and Voinnet 2006). In *Arabidopsis*, different Dicer-like enzymes (DCLs), such as DCL2, DCL3 and DCL4, produce siRNAs of distinctive size: 22 nt, 24 nt and 21 nt, respectively (Voinnet 2008). These different-sized siRNAs in *Arabidopsis* have distinct functions: 21- and 24-nt siRNAs are believed to guide PTGS and TGS, respectively, whereas 22-nt siRNAs of DCL2 are considered to substitute for 21-nt siRNAs in case of loss or suppression of DCL4 activity in antiviral defense (Bouché *et al.* 2006, Deleris *et al.* 2006, Dunoyer *et al.* 2010).

In the seed coat of yellow soybean with the *I* allele, RNA gel blot analysis with *CHS* sense- or antisense-specific RNA probes revealed that two size classes of sRNAs, corresponding to both sense and antisense strands of *CHS* genes, are migrated in the gel at a rate similar to 22- and 26-nt DNA oligomers (Kurauchi *et al.* 2009, Senda *et al.* 2004). The shorter sRNAs were specifically detected only in the seed coat of yellow soybean, and not in that of pigmented soybean or in other tissues (cotyledon and leaf) of both soybean types. In contrast, the longer sRNAs were detected in all tissues (seed coat, cotyledon and leaf) of yellow and pigmented soybeans. RNA silencing of *CHS* genes occurs only in the seed coat of yellow soybean (Tuteja *et al.* 2004), and the detection of the shorter sRNAs agrees well with its tissue specificity (Kurauchi *et al.* 2009). Therefore, detected sRNAs near the 22-nt oligomer were likely to be siRNAs of *CHS* genes (*CHS* siRNAs), whereas those near the 26-nt oligomer were unlikely to be *CHS* siRNAs (Kurauchi *et al.* 2009). Deep sequencing and bioinformatic analyses of sRNAs in the yellow soybean seed coat enabled *CHS* siRNAs to be characterized at the sequence level (Kurauchi *et al.* 2009, Tuteja *et al.* 2009). *CHS* siRNAs are derived from either sense or antisense strands of *CHS* genes, especially in the central and 3′-regions of exon2. The predominant size classes of *CHS* siRNAs are 21 and 22 nt, with the 21-nt size class being the most abundant. It remains unclear whether different-sized siRNAs in soybean have distinct functions similar to those in *Arabidopsis*.

Because of the high degrees of nucleotide sequence identity of the ORF among *CHS* members (*CHS1–CHS9*), it is difficult to identify a single *CHS* gene from which each *CHS* siRNA is derived. Fortunately, some *CHS* siRNAs could be mapped on the sense or antisense strand of a single *CHS* gene, demonstrating that *CHS* siRNAs are derived from pseudo*CHS3* in *GmIRCHS* and most of the gene copies in the *CHS* gene family (Kurauchi *et al.* 2009). If *CHS* siRNAs are generated from the pseudo*CHS3* dsRNA formed in the *GmIRCHS* transcripts, sequences of all *CHS* siRNAs should be matched with sense or antisense sequences of pseudo*CHS3*, not other *CHS* members. To explain this discrepancy, involvement of RNA-dependent RNA polymerases (RDRs) in *CHS* siRNA amplification is suggested as follows. (1) From *CHS* dsRNA-forming regions in the *GmIRCHS* transcripts, primary *CHS* siRNAs are produced by DCL (Fig. 3A). (2) Primary *CHS* siRNAs guide an effector complex named RISC (RNA-induced silencing complex) to the complementary or near-complementary mRNAs of most *CHS* members, which are then cleaved by the slicer activity of Argonaute protein (AGO) present within the RISC (Fig. 3B). (3) RDR synthesizes *CHS* dsRNAs from the cleaved mRNAs of most *CHS* members, although the mechanism whereby RDR recognizes the cleaved mRNA remains poorly understood (Fig. 3C). In *Arabidopsis*, studies of transacting siRNA pathway suggested that the function of sRNAs is influenced by size, and a 22-nt sRNA (miRNA and siRNA) may be a trigger of secondary siRNA production: AGO1-bound 22-nt sRNA cleaves a target transcript and recruits RDR6 to convert the 3′ cleavage fragment into dsRNA (Chen *et al.* 2010, Cuperus *et al.* 2010, Schwab and Voinnet 2010). Thus, in the yellow soybean seed coat, the 22-nt *CHS* siRNAs may be important for *CHS* dsRNA synthesis. (4) *CHS* dsRNAs are processed by DCL, and secondary *CHS* siRNAs, which are derived from most *CHS* members, are generated (Fig. 3C). (5) Secondary *CHS* siRNAs are amplified by repeating the third and fourth steps (Fig. 3B, 3C). (6) *CHS* siRNAs guide RISCs to *CHS* mRNAs, which are cleaved and subsequently rapidly degraded (Fig. 3B, 3D).

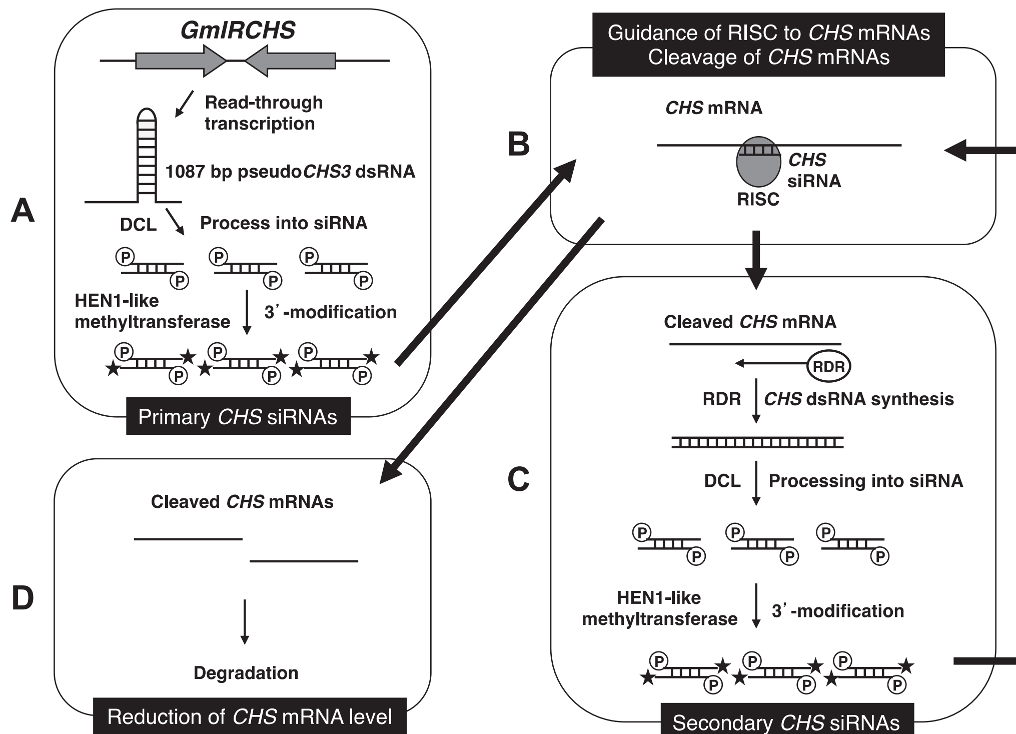

Fig. 3. Model for induction and maintenance of RNA silencing of *CHS* genes in the seed coat of yellow soybean. The 5′-monophosphorylated ends and modified 3′-ends of *CHS* siRNAs are indicated by a circled ‘P’ and star, respectively. A: production of primary *CHS* siRNAs from *GmIRCHS* transcripts. B: guidance of RISC to *CHS* mRNAs and cleavage of *CHS* mRNAs. C: *CHS* dsRNA synthesis and production of secondary *CHS* siRNAs. D: degradation of cleaved *CHS* mRNAs and reduction of *CHS* mRNA level.

Characterization of both 5′- and 3′-ends revealed that *CHS* siRNAs are modified at the 3′-ends and bear 5′-monophosphorylated ends (Kurauchi *et al.* 2011). *In vitro* systems using *Drosophila* embryo lysate and wheat germ extract showed that siRNAs generated from dsRNA by Dicer have a 5′ monophosphate at the 5′-ends (Elbashir *et al.* 2001, Tang *et al.* 2003). In *Arabidopsis*, miRNAs and siRNAs were demonstrated to be 2′-*O*-methylated at their 3′-ends, for which the RNA methyltransferase HEN1 is responsible (Li *et al.* 2005, Yang *et al.* 2006, Yu *et al.* 2005); 2′-*O*-methylation by HEN1 was suggested to protect small RNAs from 3′-end oligouridylation and subsequent degradation, leading to the stability of small RNAs (Li *et al.* 2005). This modification at the 3′-ends is also likely to be important in small RNAs of other plants including soybean. The structure of the 5′- and 3′-ends led to the conclusion that *CHS* siRNAs in soybean are actually processed by DCL from *CHS* dsRNA and probably 2′-*O*-methylated by HEN1-like methyltransferase for stability (Fig. 3A, 3C).

As described above, *CHS* dsRNA derived from the *GmIRCHS* transcripts was detected not only in the seed coat but also in other tissues such as the cotyledon and leaf (Kurauchi *et al.* 2011). In contrast, RNA silencing of *CHS* genes occurs only in the seed coat, not in the cotyledon and leaf (Tuteja *et al.* 2004). If primary *CHS* siRNAs are produced from the *CHS* dsRNA of the *GmIRCHS* transcripts by DCL, it is expected that these siRNAs may also exist in the cotyledon and leaf where RNA silencing of *CHS* genes does not occur. Although this possibility has yet to be determined by deep sequencing analysis, RNA gel blot analysis showed that *CHS* siRNA signal is not detected in the cotyledon and leaf, suggesting the presence of very few primary *CHS* siRNAs and their negligible influence on *CHS* mRNA cleavage. Thus, seed coat specificity of RNA silencing of *CHS* genes may be determined in amplification step(s) of secondary *CHS* siRNAs rather than *CHS* dsRNA formation from *GmIRCHS* transcripts.

Full and partial seed coat pigmentation in yellow soybean

Soybean cultivars possessing the *I* or i^i allele produce yellow seeds by RNA silencing of *CHS* genes. However, sometimes in the seed production of yellow soybean cultivars, fully or partially pigmented seeds are found (Fig. 4). Yellow seed color is one of the most important characters in yellow soybean, and undesirable full or partial seed coat pigmentation debases the yellow seeds. These seed coat pigmentation phenomena in yellow soybean are classified into three categories based on the cause of inhibition of RNA silencing of *CHS* genes: (1) gene mutation, (2) viral suppressor proteins, and (3) low temperature.

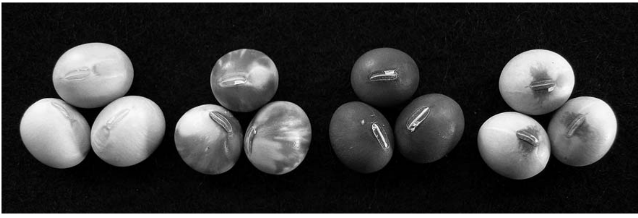

Fig. 4. Pigmented seeds produced by the yellow soybean (cv. Toyohomare). Left to right: yellow seeds with light hilum, mottled seeds induced by *Soybean mosaic virus* infection, fully pigmented seeds produced by recessive mutation of allele *I* to allele *i*, and discoloration induced by low-temperature treatment.

Full seed coat pigmentation by gene mutation in yellow soybean

Among the four alleles comprising the *I* locus, at least the *I* and i^i alleles trigger RNA silencing of *CHS* genes in the seed coat (Senda *et al.* 2004, Tuteja *et al.* 2004). In most commercial yellow-seeded soybeans with the *I* or i^i allele, undesirable fully pigmented seeds are found in the harvested seeds (Fig. 4). Although the percentage is usually quite low, their appearance has created some concern among farmers and seedsmen (Bernard and Weiss 1973). This phenomenon is thought to be derived from a spontaneous mutation either from the *I* or i^i allele to the *i* allele. Such mutations are associated with the deletion of *CHS1* or *CHS4*, at least in the promoter region, respectively (Todd and Vodkin 1996). Only in yellow soybeans with the *I* allele, an approximately 12.5-kb *Hin*dIII band is specifically detected using a *CHS1*-specific probe, but this *Hin*dIII band is affected by the mutation from *I* to *i* (Kasai *et al.* 2007, Senda *et al.* 2002a, 2002b, Todd and Vodkin 1996). Regardless of the *I* locus genotype, the *CHS1*-specific probe generally detects an 8.0-kb *Hin*dIII band in which *CHS3* and *CHS1* are clustered, but the 8.0-kb *Hin*dIII band is not affected by the mutation from *I* to *i* (Akada and Dube 1995, Senda *et al.* 2002a). *CHS1* in the 12.5-kb *Hin*dIII region specific to the *I* allele was regarded as a duplicated *CHS1* (Todd and Vodkin 1996) and was later designated *ICHS1* to distinguish it from *CHS1* in the *CHS3*–*CHS1* cluster (Senda *et al.* 2002a). A candidate for the *I* allele, *GmIRCHS*, is located 680 bp upstream of *ICHS1* (Kasai *et al.* 2007). In three mutants showing mutation of allele *I* to allele *i*, each of which was found from a different cultivar or strain, the whole *ICHS1* region was retained in one mutant, whereas the IR structure of pseudo*CHS3* in *GmIRCHS* was missing in all of the mutants (Kasai *et al.* 2007, Senda *et al.* 2002b). Although analysis of greater numbers of soybean mutants showing mutation of allele *I* to allele *i* is required, this result supports the possibility that the IR region of pseudo*CHS3*, not *ICHS1* region, is essential for the function of the *I* allele.

Seed mottling in response to viral infection

In yellow soybean infected with certain viruses such as *Soybean mosaic virus* (SMV) or CMV, pigments often appear on seed coats in irregular streaks and blotches, usually called 'mottling' (Bernard and Weiss 1973; Fig. 4). Mottling was considered to be a mysterious phenomenon until it was demonstrated that the yellow-seeded phenotype is a result of RNA silencing of *CHS* genes. RNA silencing has diverse biological roles, one of which is host defense against viruses (Baulcombe 2004, Voinnet 2005). As a counter-defense mechanism, many plant viruses including SMV and CMV produce proteins that suppress RNA silencing (Kasschau and Carrington 1998, Voinnet 2005). In yellow soybean, these viral suppressor proteins are also able to interfere with endogenous RNA silencing of *CHS* genes, leading to the scattered distribution of pigmented cells in the non-pigmented seed coat tissue and formation of mottling (Senda *et al.* 2004).

Cold-induced seed coat discoloration

In yellow soybean, low temperature (≤15°C) at early stages of seed development causes the expression of pigmentation around the hilum region (Fig. 4) (Morrison *et al.* 1998, Oka *et al.* 1989, Srinivasan and Arihara 1994). This phenomenon, referred to as 'cold-induced seed coat discoloration' (CD), is a severe problem in the northernmost island of Japan, Hokkaido, where soybean plants are frequently exposed to low temperatures even in summer. The CD reduces the commercial value of yellow soybean. Moreover, cracks occur easily in the pigmented seed coat region and further reduce the seed value. In Hokkaido, CD-tolerant soybean cultivars are desired for stable production of high-quality yellow seeds. Genotypic differences are reported in the degree of CD (Srinivasan and Arihara 1994). A Japanese cultivar, 'Toyomusume' (TM), shows high susceptibility to CD, whereas the cultivar 'Toyoharuka' (TR) is highly CD-tolerant (Kasai *et al.* 2009, Takahashi and Abe 1999). As the suppressive mechanism of seed coat pigmentation became clear, the molecular mechanism of CD was revealed (Kasai *et al.* 2009). In seed coats of TM plants treated with low temperature, the level of *CHS* siRNAs was markedly reduced compared with that of non-treated plants (Kasai *et al.* 2009). Temperature regulates RNA silencing through siRNA production in transgenic tobacco (Kalantidis *et al.* 2002). In *Nicotiana benthamiana*, *Arabidopsis* and potato, the levels of virus- or transgene-derived siRNAs are reduced markedly at low temperature, resulting in inhibition of RNA silencing (Szittya *et al.* 2003). Thus, low-temperature treatment of yellow soybean plants severely inhibits accumulation of *CHS* siRNAs in the seed coat, and consequently CD occurs owing to deficiency in suppression of seed coat pigmentation. In plants, siRNAs are generated and amplified for the initiation and maintenance of RNA silencing (Voinnet 2008). The marked decrease in siRNA level caused by low temperature is probably because of inhibition in enzymatic activity and/or gene expression of the protein(s) that plays important roles in siRNA generation and/or amplification. In transgenic tobacco, expression of a gene encoding RDR is down-regulated at low temperature (Wu *et al.* 2008). Unlike TM, in the highly CD-tolerant cultivar TR, accumulation of *CHS* siRNAs is weakly inhibited by low temperature, and

consequently *CHS* siRNAs are accumulated in the seed coat. Furthermore, as shown in Fig. 2, TR possesses a different *GmIRCHS* structure. *GmIRCHS* generally consists of the 5′ portion of *GmJ1* (from the promoter region to part of exon1) and an IR of pseudo*CHS3* (pseudo*CHS3* and its complementary sequence). In TR, the 5′ portion of *GmJ1* extends to the middle of the intron and the extended region replaces pseudo*CHS3*, hence the IR of pseudo*CHS3* characteristic of *GmIRCHS* is missing and only a complementary sequence of pseudo*CHS3* remains. Thus the TR-specific *GmIRCHS* region was designated *GmASCHS* (*Glycine max* antisense *CHS* pseudogene) (Kasai *et al.* 2009). The structure of *GmASCHS* suggested that antisense RNA of pseudo*CHS3* may be transcribed in the seed coat of TR, and such RNA was detected by RT-PCR (Kasai *et al.* 2009). It is possible that the antisense pseudo*CHS3* RNA forms dsRNA by hybridization with the endogenous *CHS* transcripts or by the action of RDR, subsequently triggering RNA silencing of *CHS* genes. Interestingly, in some potato knockdown lines of an endogenous gene or two endogenous genes silenced by expressing the corresponding antisense RNA, RNA silencing was not inhibited by low temperature, suggesting that the antisense-mediated gene silencing (ASGS) may be temperature-independent although not in all cases (Sós-Hagedűs *et al.* 2005). The temperature-independent mechanism of ASGS remains to be elucidated.

For breeding of highly CD-tolerant cultivars, large phytotron growth chambers are often needed to carry out stable low-temperature treatment. However, a large phytotron has high construction and running costs. In addition, the number of soybean plants available for selection is limited because of limited space in the phytotron. Therefore, a DNA marker has been sought to aid breeding of highly CD-tolerant cultivars. A quantitative trait locus (QTL) analysis suggested that *GmASCHS*, or another gene tightly linked to it, may be responsible for CD tolerance, and a DNA marker discriminating between *GmIRCHS* and *GmASCHS* can be useful for marker-assisted selection of CD-tolerant plants (Ohnishi *et al.* 2011). However, variation of *GmIRCHS* genotypes cannot necessarily explain all CD phenotypes, suggesting the existence of other QTLs for CD tolerance. Indeed, a second QTL has been identified, which made a smaller contribution to CD tolerance than *GmIRCHS* (Ohnishi *et al.* 2011). If other QTLs are detected and mapped precisely, more reliable marker-assisted selection of CD tolerance could be achieved by the combination of DNA markers for *GmIRCHS* and other QTLs.

Conclusion

In this review, we described the role of the *I* allele, rather than the i^i allele, in triggering RNA silencing of *CHS* genes. We also described the seed coat pigmentation phenomena in yellow soybean mainly possessing the *I* allele. A candidate for the i^i allele was previously reported and suggested to consist of a 5.87-kb separated perfect IR of the entire 10.91 kb *CHS1–CHS3–CHS4* cluster, termed *CHS* Cluster A and *CHS* Cluster B (Clough *et al.* 2004). In seed coat pigmentation mutants derived from mutation of allele i^i to allele *i*, a region ranging from part of *CHS* Cluster A (at least the promoter region of *CHS4*) to the whole of *CHS* Cluster B is thought to be deleted (Todd and Vodkin 1996, Tuteja *et al.* 2004, 2009). The precise function of the i^i candidate in triggering RNA silencing of *CHS* genes remains unclear, in particular how *CHS* dsRNA is produced in the i^i candidate region. Sequences of *CHS* siRNAs in the seed coat of plants possessing the i^i allele were characterized, and were generally similar to those carrying the *I* allele, indicating that both *I* and i^i alleles effectively silence the targeted *CHS* genes by producing siRNAs that mostly map to exon2 of both strands of the individual *CHS* members (Tuteja *et al.* 2009). At present, the molecular structure of the i^k allele and its suppressive mechanism in seed coat pigmentation remains to be elucidated. In contrast to the *I* allele, which inhibits seed coat pigmentation completely, the i^i and i^k alleles permit seed coat pigmentation in the hilum and a saddle-shaped region, respectively. The molecular mechanism regulating the spatial distribution of seed coat pigmentation in the presence of the i^i and i^k alleles remains unknown, and the reason for seed coat pigmentation occurring only in the hilum and a saddle-shaped region, respectively, also requires elucidation.

Most commercial soybean cultivars have yellow seeds, which is an important characteristic of high-quality seeds. More detailed elucidation of the mechanism responsible for the yellow seed phenotype will be valuable for future breeding of yellow soybean cultivars.

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