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 \( I, i, \beta \) 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 \( i \) 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 (\textit{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 \((R, i)\), brown \((r, T)\) and buff \((r, i)\). 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, Zahala 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 \((I, i, \beta\) 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, \(i\) and \(\beta\), 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 \(i\) allele for a dark (pigmented) hilum. The dominance relationships among the four alleles are \(I > \beta > i > i\). Inhibition of seed coat pigmentation by the \(I\) locus, at least the \(I\) and \(i\) 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 \(i\) 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
mosaic virus (CMV), the \( CHS \) mRNA level was reduced in the pigmented soybean plant, and consequently the pigmentation in 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 1 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 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-suppressing (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) pseudogene 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 (\( ii \)) 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 1 allele or \( \tilde{r} \) 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 \( 1 \) 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 \( GmiIRCHS \) (Glycine max inverted repeat of \( CHS \) pseudogene) (Kasai et al. 2007). \( GmiIRCHS \) 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 \( GmiIRCHS \) 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 \( GmiIRCHS \) 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 \( GmiIRCHS \) 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.
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 soybean 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 pseudoCHS3 in GmIRCHS and most of the gene copies in the CHS gene family (Kurauchi et al. 2009). If CHS siRNAs are generated from the pseudoCHS3 dsRNA formed in the GmIRCHS transcripts, sequences of all CHS siRNAs should be matched with sense or antisense sequences of pseudoCHS3, 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).
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 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.
Suppressive mechanism of seed coat pigmentation in yellow soybean

Full seed coat pigmentation by gene mutation in yellow soybean

Among the four alleles comprising the I locus, at least the I and i alleles trigger RNA silencing of CHS genes in the seed coat (Senda et al. 2004, Tutteja et al. 2004). In most commercial yellow-seeded soybeans with the I or 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 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 HindIII band is specifically detected using a CHS1-specific probe, but this HindIII 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 HindIII band in which CHS3 and CHS1 are clustered, but the 8.0-kb HindIII 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 HindIII 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, GmIRCHS1, 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 pseudoCHS3 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 pseudoCHS3, 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 pseudoCHS3 (pseudoCHS3 and its complementary sequence). In TR, the 5' portion of GmJ1 extends to the middle of the intron and the extended region replaces pseudoCHS3, hence the IR of pseudoCHS3 characteristic of GmIRCHS is missing and only a complementary sequence of pseudoCHS3 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 pseudoCHS3 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 pseudoCHS3 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-Hagedíss 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 search Center for Tohoku Region, Japan) for the gift of soybean seeds. We thank Dr. Setsuzo Yumoto (National Agricultural Research Center for Tohoku Region, Japan) for the gift of soybean seeds. We also wish to thank all of the past and present students in our laboratory who contributed to our study. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 20380001 to M.S.), and from Hirosaki University, Japan (Priority Area 2008–2009 and Institutional Research 2010–2011 to M.S.).

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