Theor Appl Genet (2002) 104:1086–1091
DOI 10.1007/s00122-001-0801-4

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Analysis of the duplicated *CHS1* gene related to the suppression of the seed coat pigmentation in yellow soybeans

Received: 03 April 2001 / Accepted: 23 August 2001 / Published online: 20 February 2002

Abstract Seed coat color in soybean is controlled by the classically defined *I* (*Inhibitor*) locus. The seeds of most commercial soybean varieties are yellow due to the presence of a dominant allele of the *I* locus (*I*: yellow seed coat, or i^i : pigmented hilum and yellow seed coat), which inhibits seed coat pigmentation. Analysis of spontaneous mutations from *I* (yellow seed coat) to *i* (pigmented seed coat) has shown that these mutations are correlated with the deletion of a duplicated chalcone synthase gene-1 (*CHS1*) region. In the current study, we isolated the duplicated *CHS1* region from a soybean cultivar with a *I/I* genotype (cv Miyagi shirome) and determined its structure. The results showed that the duplicated *CHS1* contained intact regulatory and coding regions. We designated the duplicated *CHS1* as *ICHS1*. In the hypocotyls of Miyagi shirome, the cDNA derived from *ICHS1* mRNA was identified by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, whereas in the immature seed coats it was suggested that the amount of transcripts from *ICHS1* and/or another type of *CHS1* (*CHS1.1*) was very low. Interestingly, in the Miyagi shirome genome with a *I/I* genotype, *ICHS1* was closely linked to the truncated *CHS3*, and sequence comparison showed that this cluster probably arose from the *CHS1–CHS3* cluster by a 1.8-kb deletion event.

Communicated by P. Langridge

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Keywords Soybean · Seed coat pigmentation · *I* locus · *CHS* genes · Deletion

Introduction

Seed coat color in soybean is determined by the *I* (*Inhibitor*) locus. Four alleles are known at the *I* locus. The dominant *I* allele inhibits seed coat pigmentation, leading to possession of a yellow seed coat, whereas the recessive *i* allele confers a pigmented seed coat. Two other alleles, i^i and i^k, give rise to restricted pigmentation of the hilum and the saddle-shaped region, respectively. The dominance relationships between the four alleles are $I > i^i > i^k > i$. The molecular mechanism of suppression by the *I* allele in seed coat pigmentation is still unclear.

Analysis of spontaneous mutants from the *I* allele or the i^i allele to the *i* allele suggested that this mutation was correlated with polymorphism of the genes that encoded chalcone synthase (CHS) (Todd and Vodkin 1996). CHS is the first committed enzyme of the multibranched pathway of flavonoid/isoflavonoid biosynthesis. In genotypes with the *I* allele, steady state *CHS* mRNA and CHS enzyme activity levels are reduced at the stage of seed development compared with the levels in the *i/i* homozygous recessive genotype (Wang et al. 1994). Soybean *CHS* genes have been analyzed from cv Williams with the genotype i^i/i^i (Akada and Dube 1995). That study showed that soybean *CHS* genes were encoded by a multigene family that consisted of at least seven members, *CHS1–CHS7*. *CHS1* and *CHS3* are paired in a head-to-head orientation within an 8.0-kb *Hin*dIII region. Furthermore, *CHS4* or *CHS5* is also linked to *CHS3* in a tail-to-tail orientation (Akada and Dube 1995).

Todd and Vodkin (1996) reported that the dominant *I* allele possessed an extra *Hin*dIII fragment which hybridized with both of the *CHS1* promoter and coding-region probes, indicating the existence of an extra *CHS1*. They called this extra *CHS1* the duplicated *CHS1*. In mutants in which the dominant *I* allele had mutated to the recessive *i* allele, Southern-hybridization data suggested that

the deletion occurs at the duplicated *CHS1* region, and at least in the promoter region (Todd and Vodkin 1996). The deletion at the duplicated *CHS1* region restored a higher level of detection of total *CHS* mRNA in the seed coat (Todd and Vodkin 1996). These data indicated that the region of the duplicated *CHS1* gene was related to the suppression of seed coat pigmentation by the *I* allele. Although the existence of the duplicated *CHS1* has been shown by Southern-hybridization analysis, its detailed structure is still unknown. In the present study, we cloned and analyzed the duplicated *CHS1* region from a yellow seed coat cultivar with the *I/I* genotype (cv Miyagi shirome).

Materials and methods

Japanese soybean cultivar Miyagi shirome (*I/I*) and its pigmented seed coat mutant line (*i/i*) were provided by Tohoku National Agricultural Experiment Station, Japan. Plants were grown in the field or in a green-house. Soybean genomic DNA was isolated from young leaves according the published protocol by Ausubel et al. (1987). A genomic DNA library of the Miyagi shirome was constructed in the phage vector lambda FIX II (Stratagene, USA) according to the manufacturer's method. Procedures for restriction enzyme digestion, agarose-gel electrophoresis and Southern hybridization have been described previously (Akada and Dube 1995). PCR amplification with LA *Taq* DNA polymerase was conducted according to the manufacture's recommendations (Takara Shuzo Co., Japan). RT-PCR analysis was performed according to Shimizu et al. (1999). The sequences of the primers used for the genomic PCR or RT-PCR experiments were: primer 1, 5′-CATCGACTTATCACCTGATCG-3′; primer 2, 5′-GCCGAATTCCATTATGCATTGCAATAAGATGGGGTCAGG-3′; primer 3, 5′-TCCAAACACTGATGCTTCCC-3′; primer 4, 5′-GGCAAGCTTAGCATGTGAGGGAAGCAGGG-3′; primer 5, 5′-GCCGAATTCACAATACGTTTTTTCAAACCGG-3′ and primer 6, 5′-CCAACATCCACCCCCATC-3′. The *Actin*-specific primers were used for the *Actin*-specific RT-PCR (Shimizu et al. 1999). Three-day old etiolated seedlings were exposed to white light for 6 h and subsequently the hypocotyl tissues of the seedlings were harvested. The hypocotyl RNA was extracted according to Shimizu et al. (1999). Seed coats were harvested from immature seeds (25–50 mg of fresh weight) and RNA was extracted according to Wang and Vodkin (1994).

Results

Southern-hybridization analysis

The restriction enzyme *Hin*dIII does not cut within the coding regions of *CHS* genes, and its pattern represents the individual *CHS* genes or *CHS* gene clusters (Akada et al. 1990; Akada and Dube 1995). A *CHS1* coding-region probe (probe 1, see Fig. 1A) was used to hybridize a Southern blot of *Hin*dIII-digested genomic DNAs from Miyagi shirome with the *I/I* genotype, and its pigmented seed coat mutant with the *i/i* genotype. This probe hybridized with all *CHS* genes and polymorphic fragments were identified between them (Fig. 1B). The Miyagi shirome displayed a *Hin*dIII fragment of 13-kb, not present in the mutant, which instead showed hybridization to a *Hin*dIII fragment of 15-kb. An Additional polymorphism was noted with a *CHS1* promoter probe (probe 2, see Fig. 1A) to the same blot. As shown in Fig. 1B, besides the 8.0-kb *Hin*dIII fragment in which *CHS1* and *CHS3* are clustered (Fig. 1A), the *CHS1* promoter probe also hybridized to the polymorphic 13-kb *Hin*dIII fragment in Miyagi shirome. On the other hand, the mutant possessed the 8.0-kb *Hin*dIII fragment in common with Miyagi shirome and Williams, but the polymorphic 15-kb *Hin*dIII fragment was not hybridized (Fig. lB). These results suggested that Miyagi shirome with the *I/I* genotype contained the duplicated *CHS1* in the 13-kb *Hin*dIII region, whereas in the genome of the mutant with the *i/i* genotype the duplicated *CHS1* was deleted, at least in the promoter region. This finding was similar to that reported by Todd and Vodkin (1996).

Isolation of the duplicated *CHS1* region

One *CHS1*-containing clone was isolated by screening the Miyagi shirome genomic library using a *CHS1* promoter probe (probe 2, see Fig. 1A). Sequence analysis revealed that this clone (designated λ*Gm*MCHS1) contained the intact *CHS1* region. The 5′-flanking sequence of this clone was identical to that reported for soybean *CHS1* in the 8.0-kb *Hin*dIII fragment from cv Williams clone λ*Gm*W83 (Akada et al. 1991; Akada and Dube 1995). It is worth noting that the λ*Gm*MCHS1 sequence diverged from the *CHS1* sequence 673-bp upstream of the presumed initiation codon (Fig. 1C). To determine whether the λ*Gm*MCHS1 clone contains *CHS1* or the duplicated *CHS1*, we performed PCR analysis with primer 1, which was specific for the divergent sequence in λ*Gm*MCHS1 (Fig. 1A and C), and primer 2, which is located in the 5′ -flanking region of *CHS1* (Fig. 1A). As described above, in the mutant, Southern-hybridization analysis suggested that at least the promoter region was deleted in the duplicated *CHS1* region. If the *CHS1* region in the λ*Gm*MCHS1 clone is the duplicated *CHS1*, it is expected that PCR with primers l and 2 would amplify a 0.3-kb fragment specifically in Miyagi shirome, while a 0.3-kb fragment would not be amplified in the mutant due to the deletion of the promoter region in the duplicated *CHS1*. As seen in Fig. 1D, a 2.1-kb fragment was shared by Miyagi shirome and the mutant, and Miyagi shirome also contained a unique 0.3-kb fragment whose size is identical to the fragment amplified in pSC1.4, which is a subclone of the λ*Gm*MCHS1 clone (Fig. 1A). As a control, in PCR analysis with the primers located in the 5′ -flanking region of *CHS1* (primers 3 and 2, see Fig. 1A), a 0.9-kb fragment was amplified in both of them (Fig. 1D). From these results, we concluded that *CHS1* in the λ*Gm*MCHS1 clone was the duplicated *CHS1* and we designated this gene as *ICHS1* to distinguish it from *CHS1*.

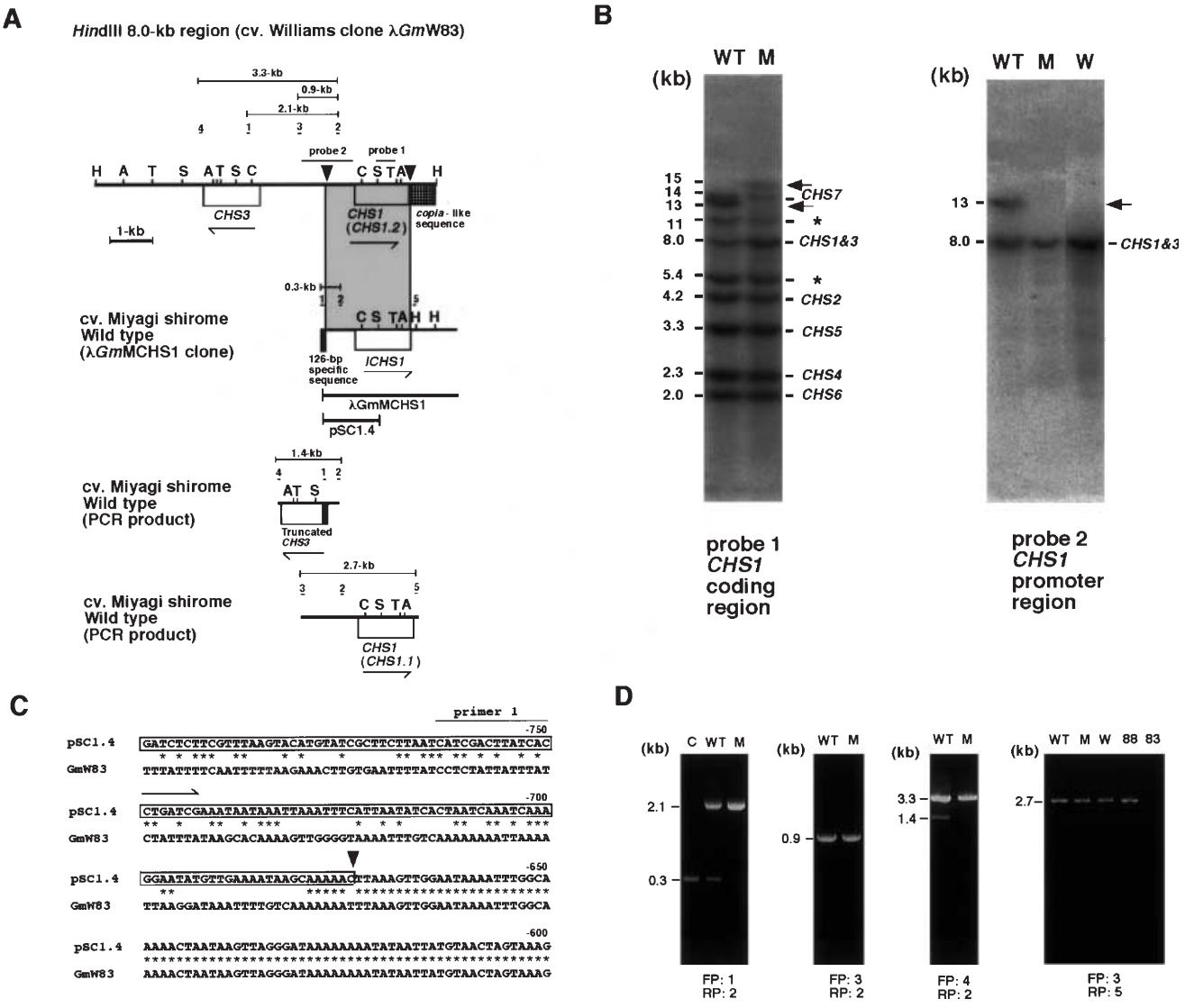

Fig. 1 **A** Schematic representation of the *CHS3–CHS1* (*CHS1.2*) cluster in an 8.0-kb *Hin*dIII region from the cv Williams λ*Gm*W83 clone, a *ICHS1* region from the cv Miyagi shirome λ*Gm*MCHS1 clone, and the PCR products of the truncated *CHS3* and *CHS1* (*CHS1.1*) regions from cv Miyagi shirome. The homologous region between two clones is *shaded*. The points of divergence between two clones are denoted by *arrowheads*. The restriction mapping data of the *CHS3–CHS1* (*CHS1.2*) cluster were taken from Akada et al. (1990, 1991). Restriction sites are: *A Ava*II, *C Sac*I, *H Hin*dIII, *S Sal*I, *T Sty*I. The *open boxes*, *hatched box* and *black box* represent the coding regions, the sequence homologous to the *copia*-like retrotransposon and the 126-bp λ*Gm*MCHS1-specific sequence, respectively. The relative positions of part of the λ*Gm*MCHS1 clone and its plasmid subclone pSC1.4 are shown. The relative orientations of *CHS* genes are indicated by *horizontal arrows*. The locations of probes used for Southern-blot analyses in Fig. 1B are shown. Positions of primers 1, 2, 3, 4 and 5 used for the PCR analyses in Fig. 1D and the predicted sizes of amplified fragments are indicated. **B** Southern-blot analysis of *Hin*dIII-digested genomic DNAs with the promoter or coding region of *CHS1* as a probe (probe 1 or 2, see Fig. 1A). Genomic DNAs from Miyagi shirome, the mutant and Williams are denoted by WT, M and W, respectively. The polymorphic *Hin*dIII fragments between Miyagi shirome and the mutant are shown by *horizontal arrows*. The probe-hybridized *Hin*dIII fragments which have not been yet analyzed are indicated by *asterisks*. **C** Nucleotide-sequence comparison of the pSC1.4 and λ*Gm*W83 clones. *Asterisks* indicate identical nucleotides. The point of divergence between two clones is denoted by a *arrowhead*. A 126-bp specific sequence of the pSC1.4 clone is *boxed*. Numbering of the nucleotides is from the presumed initiation codon. The location of primer 1 is also presented. **D** PCR analyses with several primers. The forward and reverse primers used are abbreviated as *FP* and *RP*, and the numbering of the primers is as indicated in Fig. 1A. DNA of the Miyagi shirome genomic clone pSC1.4, genomic DNAs from Miyagi shirome, the mutant and Williams, and DNAs of the Williams genomic clones λ*Gm*W88 and λ*Gm*W83 used as the templates, are denoted by C, WT, M, W, 88 and 83, respectively.

Truncated *CHS3* located upstream from *ICHS1*

In contrast to the 5′ -flanking region of *CHS1*, the λ*Gm*MCHS1 clone contained only a 126-bp *ICHS1*-specific region (Fig. 1A and C). We searched for sequences homologous to the specific 126-bp region in the DDBJ/EMBL/GenBank databases. Surprisingly, this 126-bp sequence showed high homology with the complemen-

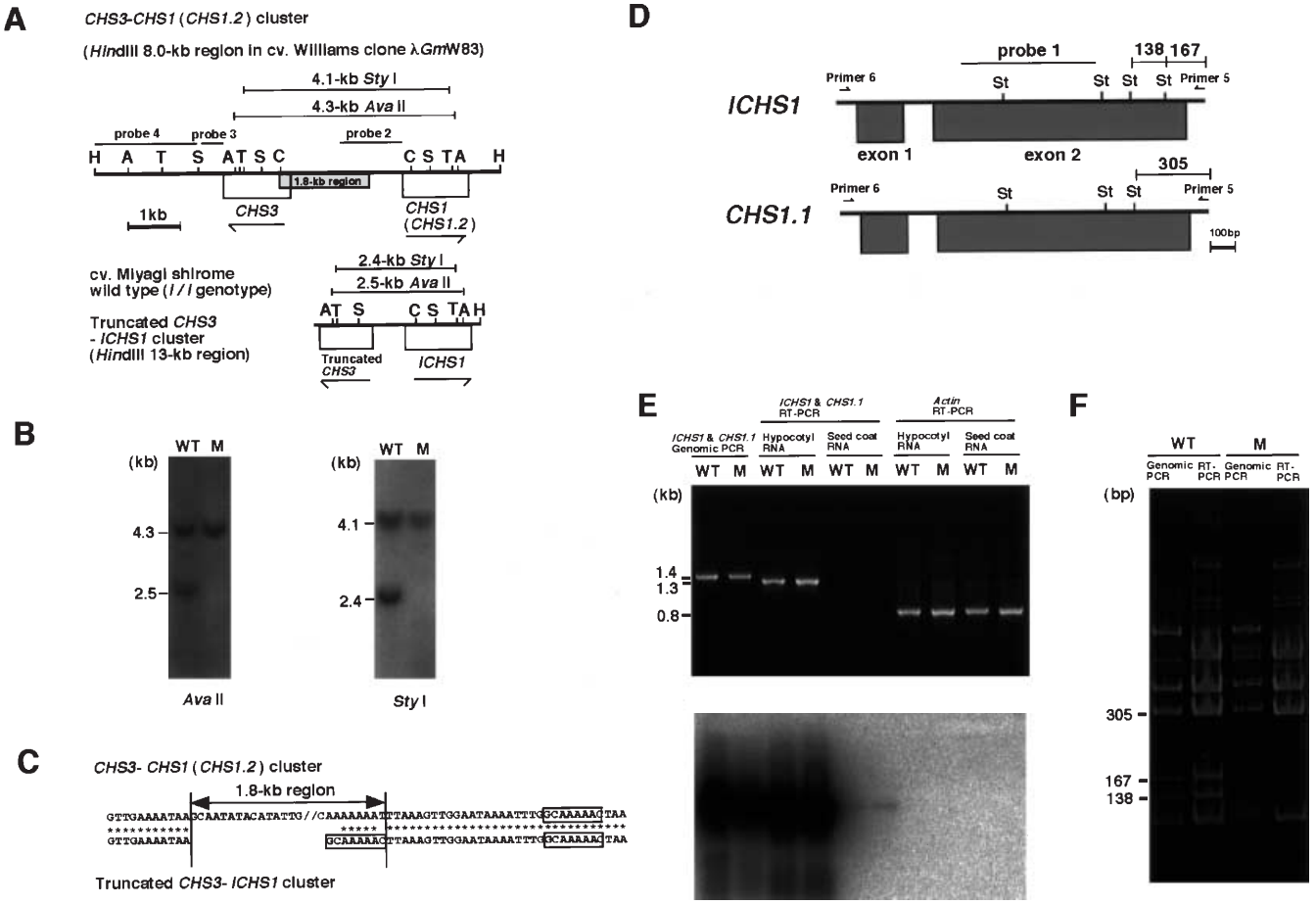

Fig. 2 **A** Schematic representation of the *CHS3–CHS1* (*CHS1.2*) cluster in the *Hin*dIII 8.0-kb region from cv Williams and the truncated *CHS3–ICHS1* cluster in the 13-kb *Hin*dIII region from cv Miyagi shirome. The *open boxes* and *shaded box* represent the coding regions and the 1.8-kb region which does not exist in the truncated *CHS3–ICHS1* cluster, respectively. The relative orientations of *CHS* genes are indicated by *horizontal arrows*. The locations of probes used for Southern-blot analyses are shown. The predicted sizes of the probe 2-hybridized restriction fragments are indicated. Restriction sites are: *A Ava*II, *C Sac*I, *H Hin*dIII, *S Sal*I, *T Sty*I. The sequence data of the truncated *CHS3–ICHS1* cluster of Miyagi shirome has been submitted to the DDBJ/EMBL/GenBank databases under accession number AB052783. **B** Southern-blot analysis of *Ava*II- or *Sty*I-digested genomic DNAs from Miyagi shirome (WT) and the mutant (M) with a *CHS1* promoter probe (probe 2, see Fig. 2A). **C** Alignment of the sequences surrounding the 1.8-kb region (*shaded box*, see Fig. 2A) between the *CHS3–CHS1* (*CHS1.2*) and truncated *CHS3–ICHS1* clusters. *Asterisks* indicate identical nucleotides. The 8-bp duplicated sequences are *boxed*. **D** Schematic representation of the *ICHS1* and *CHS1.1* regions. The *shaded boxes* represent exons. Primers 5 and 6 used for RT-PCR analysis are shown by *horizontal arrows*. *Stu*I sites are denoted by *St*. Sizes are indicated in bp. The location of probe 1 used for Southern-hybridization analysis in Fig. 2E is indicated. **E** Genomic PCR and RT-PCR analyses of *ICHS1* and *CHS1.1* in the hypocotyls and immature seed coats from Miyagi shirome (WT) and the mutant (M). The *Actin*-specific RT-PCR with *Actin*-specific primers was also carried out as a control. Gel electrophoresis is in 2% agarose (upper panel) and autoradiography of the gel with the probe 1 (lower panel). **F** Gel electrophoresis of *Stu*I-digested genomic PCR products and *Stu*I-digested RT-PCR products from hypocotyl RNAs in a 5% acrylamide gel

tary sequence of the intron and the 3′ -exon of soybean *CHS* genes, indicating that the sequence of primer 1 was a part of the coding sequence of the soybean *CHS* genes. It has been shown that *CHS3* is located upstream from *CHS1* (Fig. 1A, Akada and Dube 1995). As described above, in PCR with primers 1 and 2, a 2.1-kb fragment was shared by Miyagi shirome and the mutant (Fig. 1D). It is reasonable to assume that the 2.1-kb fragment was the PCR product from the *CHS3–CHS1* cluster region (Fig. 1A). We designed primer 4 located in the 3′ -flanking region of *CHS3* (Fig. 1A) and carried out PCR with primers 4 and 2. As expected, a 3.3-kb fragment was amplified from the *CHS3–CHS1* cluster in both the Miyagi shirome and mutant genomes (Fig. 1A and D). Interestingly, a 1.4-kb fragment was also amplified specifically in the Miyagi shirome genome (Fig. 1D). This 1.4-kb fragment was directly sequenced. The sequence data revealed that in the region upstream from *ICHS1*, truncated *CHS3* was located in the opposite orientation as in the *CHS3–CHS1* cluster (Fig. 2A). To confirm this result, the *CHS1* promoter probe (probe 2, see Fig. 2A) was used to hybridize Southern blots of *Ava*II- or *Sty*I-digested genomic DNA from Miyagi shirome (*I/I*) and the mutant (*i/i*). A fragment of the size predicted for the polymorphic fragment was specifically detected in Miyagi shirome (Fig. 2A and B). Sequence comparison between the truncated *CHS3–ICHS1* and the *CHS1–CHS3* clusters suggested that the truncated

CHS3–ICHS1 cluster had arisen from the *CHS1–CHS3* cluster by a 1.8-kb deletion event (Fig. 2A and C). As shown in Fig. 2C, the sequence data also indicated that an 8-bp sequence was duplicated in the DNA that underwent the 1.8-kb deletion event. It has been reported that *CHS4* or *CHS5* is linked to *CHS3* in a tail-to-tail orientation (Akada and Dube 1995). To further investigate the region upstream of the truncated *CHS3–ICHS1* cluster, Southern-hybridization analyses were done with probes covering the spacer region between *CHS4/CHS5* and *CHS3* (probe 3 and 4, see Fig. 2A). Neither of these probes hybridized with the 13-kb *Hin*dIII fragment harboring the truncated *CHS3–ICHS1* cluster (data not shown). This result indicated that the sequence diverged in the region immediately upstream of the truncated *CHS3–ICHS1* cluster as compared to the upstream region of the *CHS3–CHS1* cluster.

Sequence comparison between *ICHS1* and *CHS1.1* in the Miyagi shirome genome

Sequence diversity occurred between *ICHS1* and *CHS1* in the 3′ -flanking region as well as the 5′ -flanking region (Fig. 1A). Interestingly, using a *CHS1*-specific primer (primer 3) and an *ICHS1*-specific primer (primer 5), a 2.7-kb fragment was amplified in Miyagi shirome, the mutant and Williams (Fig. 1D). This result indicates that another type of *CHS1* that includes the *CHS1*-specific upstream and the *ICHS1*-specific downstream regions exists in these soybean genomes. In fact, this possibility was proven to be true by direct sequencing of the 2.7-kb amplified fragment of Miyagi shirome. Akada and Dube (1995) reported that two types of *CHS1* clones were isolated from the Williams genome, λ*Gm*W83 and λ*Gm*W88, which differed from each other in the 3′-flanking regions. As shown in Fig. 1A, they determined the nucleotide sequence of the *CHS3–CHS1* cluster from the λ*Gm*W83 clone (Akada and Dube 1995), and in the 3′-flanking region of *CHS1* a sequence homologous to the *copia*-like retrotransposon was found (White et al. 1994). PCR with primers 3 and 5 amplified the 2.7-kb fragment from the λ*Gm*W88 clone DNA, but not the λ*Gm*W83 clone DNA (Fig. 1D). Thus we named another type of *CHS1* in Miyagi shirome and the Williams λ*Gm*W88 clone, and the previously reported *CHS1* in the Williams λ*Gm*W83 clone as "*CHS1.1*" and "*CHS1.2*", respectively (Fig. 1A). Sequence comparison between the *ICHS1* and *CHS1.1* coding regions in the Miyagi shirome genome revealed four nucleotide differences and, notably, one *Stu*I-site specific to the *ICHS1* coding region was gained (Fig. 2D).

RT-PCR of *ICHS1* and *CHS1.1* transcripts from Miyagi shirome and the mutant

Since the DNA sequence including the coding and flanking regions of *ICHS1* is almost identical to the corresponding *CHS1.1* sequence, it is difficult to identify the *ICHS1*-specific transcript by Northern-blot analysis. However, the *Stu*I-site specific to *ICHS1* enabled us to distinguish between cDNAs derived from the *ICHS1* and *CHS1.1* mRNAs. Total RNA was extracted from hypocotyls and seed coats of Miyagi shirome and the mutant. RT-PCR was carried out with primers common to *ICHS1* and *CHS1.1* (primers 6 and 5, see Fig. 2D), and genomic DNA controls were also carried out simultaneously to eliminate the possibility of contaminating DNA in mRNA samples. Both *ICHS1* and *CHS1.1* contain one intron (122 bp), and the amplified cDNA fragment derived from transcripts of these genes (1.3 kb) is smaller than the amplified genomic PCR fragment (1.4 kb). In hypocotyls, as shown in Fig. 2E, a single 1.3-kb fragment was amplified from the RNAs of both Miyagi shirome and the mutant. Each RT-PCR product was digested with *Stu*I and electrophoresed on a 5% acrylamide gel. As a result, a 305-bp fragment derived from *CHS1.1* was detected in Miyagi shirome and the mutant, whereas 138- and 167-bp fragments specific to *ICHS1* were specifically visible in samples from Miyagi shirome (Fig. 2F). Thus *ICHS1* is apparently transcribed in the hypocotyls of Miyagi shirome. By contrast, in samples from the mutant, *ICHS1* specific-sized fragments were not detected in the *Stu*I- digested genomic and RT-PCR product (Fig. 2F), probably due to the deletion of the *ICHS1* promoter region containing the primer-6 site. In contrast to the high level of detection in hypocotyls, Southern-hybridization analysis of RT-PCR blot suggested that the amount of transcripts from *ICHS1* and/or *CHS1.1* was very low in the immature seed coats (Fig. 2E).

Discussion

The soybean *CHS* genes are encoded by a multigene family that is composed of at least seven members (Akada and Dube 1995). Even in one member, such as *CHS1*, three types of *CHS1*, *CHS1.1*, *CHS1.2* and *ICHS1*, were characterized. In the yellow soybean cultivars with a *I/I* genotype, the mutation of the seed coat pigmentation from *I* to *i* correlated with deletion of the *ICHS1* region, and it has been suggested that the *ICHS1* region was related to suppression of the seed coat pigmentation by the *I* allele. In the present study, we have isolated and analyzed the *ICHS1* region from cv Miyagi shirome with a *I/I* genotype. The remarkable features of the *ICHS1* region are as follows: (1) *ICHS1* contains intact regulatory and coding regions, and is apparently transcribed in the hypocotyl, while the amount of the transcripts from *ICHS1* and/or *CHS1.1* appeared to be very low in the immature seed coat. (2) Truncated *CHS3* is closely linked to *ICHS1* in a head-to-head orientation, and it is possible that this cluster region was generated via a 1.8-kb deletion from the *CHS3–CHS1* cluster. In the 3′ -flanking region of *CHS1.2*, a sequence homologous to the *copia*-like retrotransposon was found, indicating an ancient insertion in *CHS1* (White et al. 1994).

These findings suggest the possibility that, firstly, the *CHS1.1* region was duplicated and, secondly, the 1.8-kb deletion at the 5′ -upstream region, or the *copia*-like retrotransposon insertion at the 3′ -downstream region, produced the *ICHS1* or *CHS1.2* regions, respectively. (3) In the pigmented seed coat mutant of Miyagi shirome with a *i*/*i* genotype, although we have not identified the deleted region, Southern-hybridization analysis indicated that the truncated *CHS3–ICHS1* cluster was deleted at least in the *ICHS1* promoter region.

Todd and Vodkin (1996) suggested that the suppression mechanism of the seed coat pigmentation by the dominant *I* allele was due to homology dependent *CHS* gene-silencing. Homology dependent gene-silencing mechanisms are generally classified into two types, transcriptional gene-silencing (TGS) and post-transcriptional gene-silencing (PTGS) (Vaucheret et al. 1998). Recently, in both types of gene-silencing, evidence of the importance of closely spaced inverted repeat (IR) sequences has accumulated (Bender and Fink 1995; Stam et al. 1998; Jacobsen 1999; Smith et al. 2000). The truncated *CHS3* was highly homologous (98%) and close to *ICHS1* in a head-to-head orientation, so that they may constitute a closely spaced IR. Further investigations are needed to elucidate the relationship between the truncated *CHS3–ICHS1* cluster and the suppression of the seed coat pigmentation by the dominant *I* allele.

Acknowledgements We thank S. Katayama and K. Kasai for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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