Elucidation of Molecular Identity of the W3 Locus and Its Implication in Determination of Flower Colors in Soybean

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Abstract

The wide range of flower colors in soybean is controlled by six independent loci (W1, W2, W3, W4, Wm, and Wp). Among these loci, mutations in the W3 locus under the W4 allelic background (i.e., w3w4) produce near-white flowers, while the W3w4 genotype produces purple throat flowers. Although a gene encoding dihydroflavonol 4-reductase, DFR1, has been known to be closely associated with the W3 locus, its molecular identity has not yet been characterized. In the present study, we aimed to determine whether DFR1 is responsible for allelic variations in the W3 locus. On the basis of the sequence of a DFR probe, Glyma.14G072700 was identified as a candidate gene for DFR1, and nucleotide sequences of Glyma.14G072700 from cultivars with previously validated genotypes for the W3 locus were determined. As a result, a number of nucleotide polymorphisms, mainly single-base substitutions, between both coding and 5'-upstream region sequences of the W3 and w3 alleles were identified. Among them, an indel of 311-bp in the 5'-upstream region was noteworthy, since the Glyma.14G072700 in all the w3 alleles contained the indel, whereas that in all the W3 alleles did not; the former was barely expressed, but the latter was well expressed. These results suggest that Glyma.14G072700 is likely to correspond to DFR1 for the W3 locus and that its expression patterns may lead to allelic color phenotypes of W3 and w3 alleles under the w4 allelic background.

Introduction

Soybean [Glycine max (L.) Merr.] exhibits a wide range of flower colors, such as dark purple, purple, light purple, pink, magenta, near-white, and white. Sorting analysis of variations in flower color helps in understanding the evolutionary history of soybean cultivars [1]. Variations in flower colors in soybean are mainly ascribed to six different genetic loci (W1, W2, W3, W4, Wm, and Wp) [2]. Among them, W1, Wm, and Wp encode flavonoid 3'5'-hydroxylase,
flavonol synthase, and flavanone 3-hydroxylase, respectively; W2 corresponds to a MYB transcription factor that regulates the pH of vacuolar sap [3–11].

In soybean, anthocyanin pigments play a major role in flower color. In the anthocyanin biosynthesis pathway, dihydroflavonol 4-reductase (DFR) acts as an essential enzyme that catalyzes the production of leucoanthocyanidins, which are, at ensuing steps, converted to anthocyanins. The relationship between purple and white colors with DFR was studied in many ornamental and horticultural plants. DFR exhibited a low level of MYB and DFR transcripts in white rather than purple Phalaenopsis petals and sepals [12]. Similarly, in the orchid Dendrobium sonia, white tissues of petals rather than purple tissues showed repressed DFR expression [13]. In Nicotiana tabacum, the wild type flowers of which have pink petals, a white-flower mutant was characterized as a DFR-deficient one [14]. In soybean, genes that encode DFR enzymes cosegregate with two loci, namely, W3 and W4, and they act epistatic to each other under the W1 allelic background [15,16]. Soybean accessions with W3W4 produce dark purple flowers; w3W4, purple; W3w4, purple throat; and w3w4, near-white [17,18], indicating that allelic variations in the W3 locus under the w4 recessive allelic background are clearly distinguished by the color phenotypes (i.e., purple throat and near-white flowers).

As for other color phenotypes, low levels of DFR2 expression or aberrant transcripts of DFR2 were found to be associated with mutations in the W4 locus under the w3 recessive allelic background, leading to different shades of purple flowers, such as dilute purple, pale purple, and light purple [16,18,19].

To isolate the DFR gene responsible for anthocyanin biosynthesis in soybean, Wang et al. [20] developed a DFR probe, a 200-bp fragment from a genomic PCR clone (pDFR200) that contained the partial sequence of a DFR gene. Using the DFR probe, Fasoula et al. [15] performed restriction fragment length polymorphism (RFLP) analysis and revealed that the W3 locus was cosegregated with a DFR probe. However, these studies have not culminated in determining the molecular identity of the W3 locus. Herein, we tried to determine whether DFR1 is responsible for allelic variations in the W3 locus.

Results

Molecular identification of DFR1

Using RFLP analysis with the restriction enzyme HaeIII, Fasoula et al. [15] identified a DNA fragment of ~1.2-kb that covered the DFR probe in lines harboring the W3 allele (L70-4422) and found that the fragment was replaced by a longer one (i.e., ~1.7-kb) in the w3 alleles (Clark 63, L68-1774, L72-2181, and L69-4776). The study also revealed the presence of an invariant fragment of ~1.3-kb in all the lines examined, indicating that the ~1.3-kb fragment is independent of the W3 locus.

To determine whether DFR1 is responsible for allelic variations in the W3 locus, we first searched for candidate genes in soybean genome database (Phytozome version 10.3; http://phytozome.jgi.doe.gov/pz/portal.html) with nucleotide sequences that are highly similar to that of the DFR probe, the sequence of which was obtained from Wang et al. [20]. As a result, we found that Glyma.14G072700, actually its exon 3, showed 100% identity with the DFR probe sequence, indicating that it is the most probable candidate for DFR1 (Table 1). Glyma.14G072700 was annotated in the database as a gene that encodes a bifunctional DFR/flavanone 4-reductase (FNR). In addition, four genes were selected as possible candidates, since they showed >75% similarities to the DFR probe (Table 1). We then compared the HaeIII restriction fragment patterns of Fasoula et al. [15] with those of Glyma.14G072700 from W3 (L70-4422) and w3 alleles (Harosoy, L68-1774, and Williams 82). Sequence analysis revealed that Glyma.14G072700 from the W3 allele showed five restriction sites for HaeIII and that the
The length of the restriction fragment covering the DFR probe region was 1,097-bp (Fig 1A); all the w3 alleles showed only four restriction sites, since one was lost because of a single-base substitution (C to T) at position 2,029 in intron 3 (Fig 1B). Elimination of the HaeIII restriction site lengthened the restriction fragment covering the DFR probe to 1,594-bp. In brief, the restriction fragment lengths of Glyma.14G072700 from W3 and w3 alleles were ~1.1- and ~1.6-kb, respectively, which corresponded to the ~1.2- and ~1.7-kb fragments obtained by Fasoula et al. [15]. The result suggests that Glyma.14G072700 may be the DFR1 gene responsible for allelic variations in the W3 locus.

On the other hand, a gene positioned between Glyma.17G252300 and Glyma.17G252400, although not annotated in the database, had 92% similarity to the DFR probe, and produced a 1,237-bp-long restriction fragment (Table 1). Interestingly, the length is similar to that of the invariant fragment (~1.3-kb) described by Fasoula et al. [15]. Besides, three other candidate genes (Glyma.02G158700, Glyma.17G252200, and Glyma.14G072800) exhibited HaeIII restriction fragments whose lengths were widely different from those of Glyma.14G072700 as well as those described by Fasoula et al. [15]. The results altogether indicate that Glyma.14G072700 indeed corresponds to DFR1, which was previously proposed to be closely associated with the W3 locus.

### Table 1. List of genes selected as candidates for the W3 locus.

| Gene             | Gene annotation                | Identity with DFR probe | Size by HaeIII (bp)* |
|------------------|--------------------------------|-------------------------|----------------------|
| Glyma.14G072700  | Bifunctional DFR/FNR (DFR1)    | 100.0%                  | 1,594                |
| Glyma.17G252300  | Not annotated                  | 91.8%                   | 1,237                |
| Glyma.02G158700  | Bifunctional DFR/FNR           | 85.1%                   | 4,261                |
| Glyma.17G252200  | Bifunctional DFR/FNR (DFR2)    | 79.5%                   | 479 & 552            |
| Glyma.14G072800  | Bifunctional DFR/FNR           | 75.9%                   | 1,867 & 2,598        |

* HaeIII restriction fragment length covering the DFR probe.

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### Analysis of allelic variations in the coding region of DFR1

Next, we analyzed nucleotide sequences of the coding regions of DFR1 genes from both alleles (W3: L70-4422; w3: L68-1774, Harosoy, and Williams 82). Compared with DFR1 from the W3 allele, introns of the w3 allele had several single-base substitutions, one 12-bp deletion, and two insertions (Fig 1). In addition, the last exon of the w3 allele had a single-base substitution (G to C) at position 3,941, consequently substituting Ala for Gly at position 338 of the amino acid sequence of the DFR1 protein (Figs 1 and 2).

To infer whether the single-base substitution in the last exon could lead to changes in DFR1 protein activity, multiple alignment of amino acid sequences of DFR1-related proteins from 17 different plant species was constructed. The alignment showed that the C-terminal regions of DFR1 homologues were divergent between different species (Fig 2). Although whole amino acid sequences of W3 and w3 alleles of G. max are identical, except for the substitution at the last exon, C-termini of DFR1 homologues from other plant species are variable in length and composition of amino acid residues. It is, therefore, conceivable that the substitution in the last exon may not cause an alteration in DFR1 protein activity and, thus, may not be the cause for allelic variations in the W3 locus.

### Expression patterns of DFR1 in W3 and w3 alleles

We performed reverse transcription–polymerase chain reaction (semi-quantitative and quantitative RT-PCR) analyses to determine the expression level of DFR1 in standard petals of W3.
and w3 plants (Fig 3). In both analyses, DFR1 expression was high in the W3 allele with purple throat flowers, whereas it was barely detected in the w3 allele with near-white flowers (Fig 3B and 3C). We also analyzed expression levels of two other genes, Glyma.14G072800, which is located at the position subsequent to DFR1 and annotated as a DFR, and Glyma.14G197600, which is positioned at the same chromosome as that with DFR1 and has 52.3% amino acid sequence similarity to DFR1. As a result, both genes (Glyma.14G072800 and Glyma.14G197600) were expressed independently of the allelic variations of the W3 locus (Fig 3B). The results indicate that the expression patterns of DFR1 are tightly correlated with purple throat and near-white flowers.

Analysis of the DFR1 5′-upstream region

We observed a significant difference in the expression levels of DFR1 between W3 and w3 alleles. Herein, we compared the nucleotide sequences of the 5′-upstream region (up to -1.5-kb from the start codon) of DFR1 from both alleles to search for variations that could be responsible for DFR1 differential expression. Compared with the DFR1 5′-upstream region of the W3 allele, that of the w3 alleles contains four single-base substitutions, a 32-bp deletion, and an indel of 311-bp (325-bp insertion and 14-bp deletion) at position -230 (Fig 4A). PCR using primers flanking the indel amplified a DNA fragment of ~200-bp in the W3 allele and a longer one (~500-bp) in all the w3 alleles because of the presence of the indel (Fig 4B). The results are consistent with the DFR1 expression patterns and, thus, with the color phenotypes observed in W3 and w3 alleles.

Fig 1. DFR1 gene structure and polymorphism between W3 and w3 alleles. (A) The W3 allele (GenBank accession number for DFR1 of L70-4422; KT721361) shows five HaeIII restriction sites and a 1.1-kb restriction fragment covering the DFR probe. Boxes and solid lines represent exons and introns, respectively. The white box indicates the DFR probe region. H denotes HaeIII restriction sites (GGCC). (B) The w3 allele (GenBank accession numbers for DFR1 of Harosoy and L68-1774; KT721359 and KT721360, respectively) shows several differences when compared with the W3 allele: a number of single-base substitutions, a deletion, and three insertions in introns as well as a single-base substitution in exon 6. Note that no polymorphism was detected between three cultivars with the w3 allele. The asterisk indicates deletion of the HaeIII restriction site caused by a single-base substitution (T to C) in intron 3. Ins and del denote insertion and deletion.

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To confirm the correlation between the indel and DFR1 expression, we extended the analysis to more soybean accessions (four purple throats with W3 and seven near-whites with w3 from United States Department of Agriculture–Germplasm Resource Information Network database (USDA-GRIN; http://www.ars-grin.gov/). We selected those accessions, as they harbor W3 or w3 alleles under the w4 allelic background (Fig 5A). Otherwise, it would be difficult to phenotypically distinguish between W3 and w3 because under the W4 allelic background, W3 and w3 alleles produce purple and dark purple flowers, respectively. As a result, all purple throat accessions showed ~200-bp amplification products in the indel analysis and were accompanied by normal expression of DFR1; this was in accordance with the results for the W3 allele (L70-4422) (Fig 5B). In contrast, all near-white accessions showed ~500-bp products and barely expressed for DFR1, which was consistent with the results of the w3 allele (L68-1774). It should be noted that three accessions (i.e., PI 437570, PI 437918, and PI 550733) were described as purple throat accessions in the USDA-GRIN database. However, those accessions developed near-white flowers under our field conditions (see Materials and Methods), and indel and expression analyses indicated that those three accessions actually fitted better into the category of the w3 allele. These results indicate that the indel of the 5′-upstream region is tightly correlated with the expression level of DFR1.

Discussion

We demonstrated that Glyma.14G072700 corresponds to DFR1 and thus the W3 locus and that there are many nucleotide polymorphisms in its introns, exon 6, and 5′-upstream region.
**Fig 3. Gene expression profiles of W3 and w3 alleles from soybean cultivars.** (A) Photographic images of soybean genotypes *W1W13W3w4w4* (L70-4422) with purple throat flowers, *W1W1w3w3W4W4* (Harosoy) with purple flowers, *W1W1w3w3w4w4* (L68-1774) with near-white flowers, and *w1w1w3w3W4W4* (Williams 82) with white flowers. (B) RT-PCR analysis of Glyma.14G072700 (*DFR1*) and its related genes (Glyma.14G072800 and Glyma.14G197600) of the indicated lines. GmActin expression was used as the loading control. (C) qRT-PCR analysis of Glyma.14G072700 (*DFR1*). Values are means and standard deviations from three biological replicates. *DFR1* expression was normalized with constitutive gene 7 (Cons7) as the reference gene.

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Among them, the indel of the 5’-upstream region is noteworthy for not only distinguishing W3 from w3 but also characterizing their expression patterns and resulting in different shades of flower colors. Therefore, it is a tempting speculation that the indel of DFR1 may be a cause for allelic variations in the W3 locus.

In plants, cis-elements control phenotypes by regulating gene expression so that their mutations or modifications can lead to dramatic changes in tissue-specific expression patterns [21,22,23]. A previous study showed that modifications in cis-regulatory elements of DFR genes caused limited expression and thus resulted in aberrant anthocyanin synthesis in Caryophyllales [24]. The nucleotide polymorphisms exhibited by the DFR1 5’-upstream region, notably the 311-bp indel, may compromise DFR1 expression due to interference of the binding of certain transcription factors to their target sites.

Structural genes involved in the anthocyanin biosynthesis are tightly regulated by transcription factors [25]. In petals of Phalaenopsis amabilis, for instance, absence of the expression of an MYB transcription factor gene was responsible for the absence of DFR expression and lack of anthocyanin pigments [12]. On the basis of the cis-element finding tool (www.dna.affrc.go.jp/PLACE), we analyzed the indel sequence. The indel sequence harbors putative cis-elements, such as ARR1AT (NGATT), CACTFTPPCA1 (YACT), and CAAT box (CAAT). ARR1AT is a cytokinin response regulator that acts as transcriptional activator in Arabidopsis and rice [26]. YACT is a tetranucleotide motif responsible for mesophyll-specific gene expression in C4 plants [27]. CAAT box is important for the tissue-specific promoter activity of LegA in pea.

Fig 4. Sequence analysis of the 5’-upstream region of DFR1. (A) Diagrams of 5’-upstream sequences up to -1.5-kb of DFR1 (upper: W3, lower: w3). Note that no polymorphism was detected between three cultivars with the w3 allele. (B) A correlation between the indel and expression of DFR1. The shorter (~200-bp) and longer fragments (~500-bp) result from DFR1 of W3 and w3 alleles, respectively.

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Thus, the 311-bp indel of \textit{w3} allele may inhibit the expression of \textit{DFR1} by binding of certain transcription factors to the \textit{cis}-element. However, we cannot rule out that the 14-bp deletion or other polymorphisms may also influence \textit{DFR1} expression.

Interestingly, coding and 5'-upstream region sequences of all three \textit{w3} accessions (Harosoy, L68-1774, and Williams 82) analyzed in this study showed no difference in sequences, which makes us envisage the possibility that the origin of \textit{w3} alleles may be the same. However, we need to check whether the other seven \textit{w3} alleles tested also have the same sequences, which

\textbf{Fig 5.} \textit{Indel and expression analysis of \textit{DFR1} in 11 soybean accessions.} (A) Photographs of standard petals of purple throat (upper row) and near-white accessions (middle and bottom rows). Asterisks indicate the accessions that were described as purple throat flowers in the USDA-GRIN database. (B) Determination of genotypes of purple throat and near-white phenotypes with the \textit{indel} marker in the 5'-upstream region of \textit{DFR1}. \textit{GmActin} expression was used as the loading control.

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polymorphism critically affects the expression of DFR1, and how the polymorphism affects its expression.

**Materials and Methods**

**Plant material**

The following soybean cultivars were analyzed: L70-4422 with purple throat flowers ($W^1 W^3 W^1 W^4$), L68-1774 with near-white flowers ($W^1 W^3 W^1 W^4$), Harosoy with purple flowers ($W^1 W^3 W^3 W^4$), and Williams 82 with white flowers ($w^1 w^3 w^3 W^4$) [29]. In addition, PI accessions from USDA-GRIN (four purple throat and seven near-white flower accessions) were used for the PCR experiments (Table 2). All soybean accessions used in this study were grown in the experimental fields of Kyungpook National University (Gunwi, 36°07’ N, 128°38’ E, Republic of Korea).

**Isolation of DNA and nucleotide sequence analysis**

Genomic DNA of the soybean accessions was isolated from trifoliolate leaves by using the CTAB method [30]. To amplify the exon, intron, and 5'-upstream region of DFR1, PCR was performed using the following profile: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 40 s, and extension at 72°C for 1 min; a final extension was performed at 72°C for 5 min. PCR products were separated using 1.2% agarose gel, stained with ethidium bromide and visualized under UV light, and finally subjected to sequencing (Solgent, Korea). The primers used for the amplification and sequencing of DFR1 are listed in Table 3.

**Isolation of RNA and cDNA synthesis**

Total RNA was isolated from freeze-dried standard petals of soybean accessions by using the phenol-chloroform and lithium chloride precipitation methods [31]. RNA samples were

| Phenotype     | Accession                  |
|---------------|----------------------------|
| Purple throat | PI 547524 (L70-4422)       |
|               | PI 315701                  |
|               | PI 417579                  |
|               | PI 547524                  |
|               | PI 547750                  |
| Near-white    | PI 547498 (L68-1774)       |
|               | PI 81763                   |
|               | PI 81765                   |
|               | PI 81767                   |
|               | PI 437570 *                |
|               | PI 437918 *                |
|               | PI 547751                  |
|               | PI 55073 *                 |
| Purple        | PI 548573 (Harosoy)        |
| White         | PI 518671 (Williams 82)    |

*Accessions were described as purple throat flowers in observations in the USDA-GRIN soybean database. However, we observed their petals to be near-white.

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treated with DNaseI to remove contaminating DNA (TaKaRa, Japan). First-strand cDNA was synthesized by reverse transcription of total RNA with an oligo-dT(20) primer and Superscript III, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Semi-quantitative RT-PCR analysis

To determine the transcript level of DFR1 (Glyma.14G072700), PCR was performed using the first-strand cDNA. Two more candidate genes (Glyma.14G072800 and Glyma.14G197600) and a housekeeping gene (GmActin) were also analyzed, and the primers used are listed in Table 3.

qRT-PCR analysis

qRT-PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche, Germany). qRT-PCR (20 μl) required 2 μl of first-strand cDNA, 10 pmol of forward and reverse primers, and 10 μl of SYBR green I Master (Roche, Germany). The soybean gene Cons7 was used as the reference gene [32]. Experiments were performed in triplicate. The following PCR cycle was used: 95°C for 5 min, followed by 45 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 20 s. qRT-PCR data and PCR efficiencies were analyzed using the LightCycler® 480 software (Roche, Germany). The primers used in this analysis are listed in Table 3.

Indel analysis

To distinguish between W3 and w3 alleles, genetic markers were developed on the basis of the indel found in the 5′-upstream region. PCR was performed using the following profile: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 5 min. The upstream and downstream primers used are listed in Table 3. PCR products for the w3 allele were longer than those for the W3 allele because of the existence of the 311-bp indel (325-bp insertion and 14-bp deletion) in the 5′-upstream region.

Author Contributions

Conceived and designed the experiments: GTP JS JTS. Performed the experiments: GTP JS. Analyzed the data: JDL JHK HSS JTS. Contributed reagents/materials/analysis tools: GTP JS JDL JTS. Wrote the paper: GTP JS JHK HSS JTS.

Table 3. List of primers used in this study.

| Gene           | Forward primers (5′ to 3′) | Reverse primers (5′ to 3′) |
|----------------|---------------------------|---------------------------|
| Glyma.14G072700 | AAATGGGTTCAGCATCCGAAA    | AGCAAGTTGCAACGCCGATCA     |
| Glyma.14G072800 | CTCAGAGAGAAGCTTTGATG     | TCAGATTTGCGCTTCACG       |
| Glyma.14G197600 | CAACAGCAACGGTGACCA       | GCAGCACATATTAAAGA         |
| GmActin        | GTTTGCCGCAATGGAACAGGATAAG | TAATCTTCATGTCTACTTG       |
| Glyma.14G072700 | GTTTCGGTGGTCCCTTTCTGAT  | TACCTCCCCCTCCACTCTTG      |
| Cons7          | ATGAAATTGCGGTTCCATGTA    | GCCATTAAGCCAAGCTCATC      |
| Glyma.14G072700 | CCCCCTAAAAACTGCTCCCATT  | GGGACCAAAGAAATTACTAGTGA  |

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