QTL Mapping for Photoperiod Insensitivity of a Japanese Soybean Landrace Sakamotowase

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The insensitivity of flowering to long daylength is an important characteristic which soybeans have used to adapt to environments at higher latitude. The objective of this study was to map the novel gene(s) for photoperiod insensitivity in the Japanese soybean landrace Sakamotowase. A previous study suggested that Sakamotowase possessed the genotype e1e1e3e3E4E4. The progeny of testcrosses with the Harosoy isolate for e3 (L62-667) produced the roughly expected segregation pattern for the monogenic inheritance, suggesting the major involvement of a single gene in photoperiod insensitivity of Sakamotowase. By mapping analysis for 6 linkage groups (LGs) harboring the known major genes and quantitative trait loci (QTLs) for flowering, we detected a major QTL for the insensitivity near a simple sequence repeat marker (Satt577) in LG C2 and a minor QTL in LG L. Our results therefore suggest that a novel gene for photoperiod insensitivity of Sakamotowase was located in LG C2. It was estimated from the position of the tagging marker that the novel gene may be an allele at the E1 or E7 loci or a novel gene tightly linked to the E1 locus.

Key words: Glycine max, soybean, photoperiod insensitivity, QTL

Most soybean (Glycine max) cultivars have a short-day requirement for floral induction and flowering is normally suppressed under long daylength conditions. The insensitivity of flowering to long daylength, an ability of soybean plants to flower under long daylength, is thus an important characteristic in the development of cultivars adapted to the long daylength conditions of high-latitude regions. Eight loci have been reported to control the time of flowering and maturity in soybean: E1 and E2 (Bernard 1971), E3 (Buzzell 1971), E4 (Buzzell and Voldeng 1980), E5 (McBlain and Bernard 1987), E6 (Bonato and Vello 1999), E7 (Cober and Voldeng 2001), and J (Ray et al. 1995). Of these, E1, E3, E4, and E7 control the response of flowering to artificially induced long daylength (Buzzell 1971; Buzzell and Voldeng 1980; Saindon et al. 1989; Cober et al. 1996a; Cober and Voldeng 2001). The E3 locus was identified under fluorescent long daylength (FLD) by extending natural daylength to 20 h using cool white fluorescent lamps; e3 singly controls the insensitivity to FLD (Buzzell 1971). On the other hand, the E4 locus was identified under incandescent long daylength (ILD) by extending the natural daylength period to 20 h using incandescent lamps; e4 combines with e3 to control the insensitivity to ILD (Buzzell 1971; Buzzell and Voldeng 1980). Recent molecular studies revealed that E3 and E4 encode 2 phytochrome A (phyA) proteins, GmPHYA3 and GmPHYA2, respectively, and a phyA-regulated pathway is involved in the photoperiod insensitivity in soybean (Liu et al. 2008; Watanabe et al. 2009).

There are 2 cultivar groups in Japan, which possess the ILD insensitivity (Abe et al. 1991). One group consists of landraces that have been adapted to the cool summer of northern and northeastern Hokkaido and Sakhalin, where the frost-free season is limited to less than 130 day. Another group consists of landraces that have been cultivated as a short-season crop in widespread regions of Japan and the Korean peninsula. The F2 progeny of the cross between landraces of the 2 cultivar groups, Miharudaizu and Sakamotowase, exhibited a transgressive pattern of segregation toward ILD-sensitive late flowering (Abe et al. 1998). Based on the results obtained from the genetic analyses for 2 segregating F8 families for ILD insensitivity, Abe et al. (2003) determined the genotypes at the 3 loci, E1, E3, and E4, of Miharudaizu and Sakamotowase to be E1E1e3e3e4e4 and e1e1e3e3E4E4, respectively. The genotype of Miharudaizu was therefore the same as the double recessive genotype for E3 and E4, which was determined in previous studies to condition the ILD insensitivity (Buzzell 1971; Buzzell and Voldeng 1980). In contrast, the genotype of Sakamotowase was the same as a Harosoy near-isogenic line (NIL) for the e3 allele, which develops no flower buds under ILD treatment. Therefore, novel gene(s) may be needed for Sakamotowase to initiate flowering under ILD. The
objective of this study was to identify the novel gene(s) for ILD insensitivity in Sakamotowase using testcrosses with the ILD-sensitive Harosoy NIL for the e3 allele.

Materials and Methods

Plant Materials and Evaluation of ILD Sensitivity

A photoperiod insensitive landrace in Hokkaido, Japan, Sakamotowase, and an F0 ILD-insensitive line (no. 9-I) were used in this study. No. 9-I was developed by repetitive heterozygous selection from an F2 plant of the cross between Sakamotowase and the ILD-insensitive landrace Miharudaizu; it possessed the same genotype as Sakamotowase at the maturity loci, E1, E3, and E4 (Abe et al. 2003). Sakamotowase and no. 9-I were crossed with the ILD-sensitive Harosoy NIL for e3 (L62-667; Harosoy-e3), the genotype of which was e1e1e3e3E4E4 (Voldeng and Saindon 1991; Voldeng et al. 1996). Seeds of the parents and F1 plants were sown on paper towels on 28 May 2004, and the 3-day-old seedlings were individually transplanted into Jiffypot strips (JIFFY A/S, Denmark) filled with nursery soil (N: 200 mg; P: 1000 mg; K: 200 mg per liter). Pots were placed in an experimental field with outdoor lighting at Hokkaido University, Sapporo, Japan (43°04′, 141°21′E), until the seedlings were transplanted 2 weeks later. ILD was generated using 500-W incandescent lamps placed 2 m above the soil surface at intervals of 4 m. Lights were turned on from 0200 h to 0600 h and from 1800 h to 2200 h till the end of treatment (July 31). The natural daylength period in Sapporo, including twilight, reached a maximum of 16.5 h. Under incandescent lamps, the red to far-red quantum ratio (R:FR; 660:730) was 0.72, and the average photosynthetic photon flux at the canopy surfaces was 1 μmol photon sec⁻¹ m⁻², as measured at night using a LI-COR quantum sensor (Model LI-1800C, LI-COR Inc, Lincoln, NE). The plants were checked every other day for the date when the first flower opened (stage R1; Fehr et al. 1971). The progeny test was carried out for randomly selected F2 plants in 2005. Seeds were directly sown in rows in the ILD field on 28 May. The responses to ILD were evaluated individually for each F2 plant. ILD was rated as insensitive, those that reached stages R1 to R2 (a flower at the nodes immediately below the uppermost nodes) were classified as intermediate, and those that remained vegetative were classified as ILD sensitive.

DNA Isolation and SSR Analysis

DNA was extracted individually from leaves of the parents and F2 plants, as described by Doyle JI and Doyle JL (1990). Thirty-seven simple sequence repeat (SSR) markers and a derived cleaved amplified polymorphic sequence (dCAPS) marker were used to construct the maps for 5 linkage groups (LGs), C2, G, I, J, and L, in which the known maturity loci and quantitative trait loci (QTLs) controlling ILD insensitivity are reported to exist. SSR analysis was carried out with 6% denatured polyacrylamide gel electrophoresis with fluorescent-labeled primers. The polymerase chain reaction (PCR) mixture contained 30 ng of total genomic DNA, 0.25 μM of 5’- and 3’-end primers, 200 μM of each dNTP, 0.5 units of Taq polymerase (TaKaRa, Otsu, Japan), and 1X PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂) for a total volume of 20 μl. The PCRs were performed with a GeneAmp PCR System 9700 (Perkin Elmer/Applied Biosystems, Foster City, CA) using the following program: 32 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. After the amplifications, up to 4 PCR products were combined and brought to a total volume of 20 μl by adding distilled water. An aliquot (1.5 μl) of the mixed PCR products combined with a loading buffer (1.5 μl) was denatured at 95 °C for 5 min and then loaded and separated using an ABI 377 sequencer (Perkin Elmer/Applied Biosystems). GeneScan software (version 3.1) was used to score marker polymorphisms. Genotypes at the T locus for individual F2 plants were determined using the dCAPS marker developed by Toda et al. (2002), which revealed that the T gene encoded a flavonoid 3’-hydroxylase protein (g3’h).

Map Construction and QTL Mapping

A total of 38 markers were mapped in the 2 F2 populations. Marker order and distance were determined by the Map Manager program QTXb17 using the Kosambi function and a criterion of 0.001 probability (degrees of freedom = 4), equivalent to a logarithm of the odds (LOD) score of 2.4. Marker order and distance inferred by QTXb17 were used to find the candidate QTL by composite interval mapping (CIM) implemented by MapQTL 5 (Van Oojen 2004). We performed 1000 permutations to establish the empirical LOD threshold at 0.05 probability (Churchill and Doerge 1994). QTLs were considered to exist only at positions where a LOD score exceeded the corresponding significance threshold. CIM was performed by using the markers nearest to the significant QTL detected by interval mapping in advance as cofactors and were repeated by adding the markers nearest to the significant QTLs newly detected by CIM as cofactors until additional QTL were no longer detected. The magnitude and direction of the additive and dominant effects, and the proportion of the phenotypic variation explained for each detected QTL, were obtained from the CIM output. One-LOD support limits for the position of each QTL were calculated from the CIM results.

Genotyping at the E3 Locus Using Allele-Specific Markers

The genotype at the E3 locus was determined for Sakamotowase and no. 9-I, using the allele-specific markers developed by Watanabe et al. (2009). The E3 locus is one of the PhyA homologues, GmPHYA3, and the e3 allele lacks a region of 13.33 kb covering exon 4 (Watanabe et al. 2009).
Thirty nanograms of total genomic DNA was used as a template and the PCR was performed using Ex-Taq polymerase (TaKaRa) with 30 cycles at 96°C for 30 s, 58°C for 30 s, 72°C for 2 min. The primers used were TGGGTCTTCAGTTCAGTTGG (F), AGGAAAGGTGG-GAAGGCGTAT (RV2), and CCTGATGCTATCAATGCCTG (P5). PCR products were separated by 1.5% (w/v) agarose gel and visualized with ethidium bromide. A fragment of 1904 bp was amplified in Harosoy (E3E3), but not in Harosoy-e3, with the primers F and RV2, whereas a fragment of 829 bp was amplified in Harosoy-e3, but not in Harosoy, with the primers F and P5.

Results and Discussion

Segregation of ILD Insensitivity

Sakamotowase and no. 9-I are ILD insensitive (Abe et al. 2003). On average, they flowered at 50 and 51 days after sowing (July 17 and 18) under ILD conditions (Figure 1) and produced pods of 2 cm or more (stages R4 to R5; Fehr et al. 1971) at the end of ILD treatment (July 31). In contrast, Harosoy-e3 developed no flower buds till the end of ILD treatment and flowered at 77 days after sowing (Figure 1).

Flowering time in the F2 populations exhibited almost continuous variation from 46 to 79 days after sowing, although there was a tendency for F2 plants to fall into 3 classes, early flowering (46–57 days), intermediate (58–71 days), and late flowering (74–79 days) (Figure 1A,B). The segregation patterns of the flowering time observed in the 2 populations were in sharp contrast to a sib family of no. 9-I tested in our previous study (Abe et al. 2003); it produced only 2 distinctive phenotypic classes, “ILD insensitive” in which plants initiated flowering at the middle of July and “ILD sensitive” in which plants remained vegetative at the end of ILD treatment, with a monogenic segregation ratio.

The progeny test was carried out for 86 F2 plants in the cross with Sakamotowase and for 88 plants in the cross with no. 9-I (Table 1). Based on the segregation patterns in the F3 progeny, we classified F2 plants into 3 groups; “fixed for ILD insensitivity,” in which all the F3 plants reached stages R4 to R5, “fixed for ILD sensitivity,” in which all the F3 plants remained vegetative, and “segregating,” in which plants ranged from “vegetative” to stage R5. Of the 86 F2 plants tested in the cross with Sakamotowase, 20 were classified as fixed for ILD insensitivity, 17 as fixed for ILD sensitivity, and 49 as segregating. Of the 88 F2 families tested in the cross with no. 9-I, 21 were classified as fixed for ILD insensitivity, 22 as fixed for ILD sensitivity, and 45 as segregating. The observed ratio for the 3 groups fit the expected ratio from the monogenic inheritance in the cross with Sakamotowase ($\chi^2 = 1.884, P = 0.390$) and the cross with no. 9-I ($\chi^2 = 0.068, P = 0.967$). Taken together, the ILD insensitivity of Sakamotowase and no. 9-I was mainly controlled by a single major gene and possibly influenced by a few minor genes.

The average flowering dates in F2 were different among the 3 groups in both crosses (Table 1). In particular, the F2 plants whose families were classified as segregating exhibited a wider range of flowering times.

Table 1. Progeny test for randomly selected F2 plants in the testcrosses of photoperiod insensitive cultivar (Sakamotowase) and line (no. 9-I) with the Harosoy NIL for e3 (Harosoy-e3)

<table>
<thead>
<tr>
<th>Cross combination/families</th>
<th>Number of F2 plants (families)</th>
<th>Flowering time in F2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Sakamotowase × Harosoy-e3</td>
<td>Fixed for ILD insensitivity</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Fixed for ILD sensitivity</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Segregating</td>
<td>49</td>
</tr>
<tr>
<td>No. 9-I × Harosoy-e3</td>
<td>Fixed for ILD insensitivity</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Fixed for ILD sensitivity</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Segregating</td>
<td>45</td>
</tr>
</tbody>
</table>
the average flowering times closest to the intermediate values between the mean values for the F2 plants fixed for ILD insensitivity and those for ILD sensitivity (64.2 days in both crosses), suggesting a partially dominant nature of the gene involved.

QTL Mapping

In order to map the novel gene(s) for the ILD insensitivity of Sakamotowase, we performed QTL analysis, by constructing 6 LGs where the major genes and QTLs controlling ILD insensitivity were located; \(E1\) and \(E7\) in LG C2 (Molnar et al. 2003), \(E4\) in LG I (Abe et al. 2003; Molnar et al. 2003; Liu et al. 2008), \(E3\) in LG L (Molnar et al. 2003; Watanabe et al. 2009), and the QTLs in the LGs G and J (Tasma et al. 2001). All the LGs except for LG J were constructed along the order of the SSR markers expected from the consensus map (Cregan et al. 1999) (Figure 2). LG J was separated into 2 parts, J-1 and J-2, under the conditions used for map construction.

Figure 2. Linkage maps for 6 LGs where the known major genes and QTLs for flowering under long daylength are located and the position of the QTL for the ILD insensitivity of Sakamotowase. The box delineates the one-LOD support interval.
The QTL analysis revealed that a major QTL was located near Satt557 in LG C2 in both of the 2 populations tested (Table 2 and Figure 2). The QTL exhibited high LOD scores (32.4 and 32.3) and accounted for 67.2% and 74.8% of the observed variations in flowering time under ILD. The peak of the LOD scores suggests that the QTL was located in proximity to Satt557 (Figure 3). The allele from Harosoy-e3 at the QTL exhibited an additive effect of 10.6 days in both populations (Table 2), but the degree of dominance, the ratio of the dominance effect to the additive effect, was negligible (0.11 and 0.28). In addition, a minor QTL with a LOD score of 3.1 was detected near Satt099 in LG L in the cross between Sakamotowase and Harosoy-e3. It accounted for only 3.5% of the observed variation. The results of the QTL analyses thus suggest that a major QTL in LG C2, and at least one minor QTL, are involved in the difference in ILD sensitivity between Sakamotowase and Harosoy-e3.

Genotype at the E3 Locus as Revealed by Allele-Specific Markers

The E3 locus was identified under FLD by extending the natural daylength to 20 h using cool white fluorescent lamps; e3 by itself controlled the insensitivity to FLD (Buzzell 1971). In our previous study, we assumed the genotype at the E3 locus for Miharudaizu and Sakamotowase as e3e3, based on their responses to FLD. By a genome walking approach, Watanabe et al. (2009) identified the E3 locus as one of PhyA homologues, GmPHYA3, and the e3 allele lacks a region of 13.33 kb covering exon 4, which resulted in an incomplete His-Kinase domain. We determined the genotype at the E3 locus for Sakamotowase and no. 9-I by a pair of allele-specific markers. As shown in Figure 4, both produced the expected 829-bp fragment on PCR which exclusively amplified the e3-specific fragment but did not produce any fragment on PCR which amplified the Harosoy-specific 1904-bp fragment. Therefore, Sakamotowase and no. 9-I possessed the recessive genotype at the E3 locus, as predicted previously.

Genetic Basis for the Photoperiod Insensitivity of Sakamotowase

Miharudaizu and Sakamotowase possess different genotypes at the E1 and E4 loci (Abe et al. 2003). The genotype of Miharudaizu is considered to be E1E1e3e3e4e4, of which the doubly recessive genotype at the E3 and E4 loci is known to condition the ILD insensitivity (Buzzell 1971; Buzzell and Voldeng 1980; Saindon et al. 1989; Cober et al. 1996a). Both of these recessive alleles are loss-of-function alleles for 2 phyA genes, GmPHYA2 and GmPHYA3, which enable soybean plants to initiate flowering under long daylength condition (Liu et al. 2008; Watanabe et al. 2009). The genotype of Sakamotowase, on the other hand, has been considered to be e1e1e3e3E4E4 as the Harosoy NIL for e3, which did not develop any flower buds during the ILD treatment. The e1 allele could not by itself induce the ILD insensitivity in the presence of the E4 allele, as has been

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>LG</th>
<th>Nearest marker</th>
<th>LOD</th>
<th>Phenotypic variation explained</th>
<th>Additive effect (days)</th>
<th>Dominance effect (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakamotowase × Harosoy-e3</td>
<td>C2</td>
<td>Satt557</td>
<td>32.4</td>
<td>67.2</td>
<td>10.6</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>Satt099</td>
<td>3.1</td>
<td>3.5</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>No.9-I × Harosoy-e3</td>
<td>C2</td>
<td>Satt557</td>
<td>32.3</td>
<td>74.8</td>
<td>10.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

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Figure 3. LOD score curves on the linked DNA markers in LG C2. Both crosses, Sakamotowase × Harosoy-e3 and no. 9-I × Harosoy-e3, exhibited the highest LOD score at Satt557, a tagging marker for the E1 locus (Molnar et al. 2003).

Figure 4. Genotyping of the E3 locus for ILD-insensitive Sakamotowase and no. 9-I by PCR with allele-specific primers. M1; OX174/HaeIII digest, M2; λ/HindIII digest.
suggested by Cober et al. (1996a). These results have suggested that an unknown gene which, singly or in combination with the e1 allele, induces plants homozygous for the e3 and E4 alleles to flower under ILD and is involved in the ILD insensitivity of Sakamotowase (Abe et al. 2003).

The testcrosses between Sakamotowase and no. 9-I with Harosoy-e3 revealed that a major QTL located in LG C2 accounted for a large part of the observed variation in flowering time under ILD. Molnar et al. (2003) successfully determined the possible positions in the LGs for each of the maturity and determinate habit genes, by analyzing SSR marker polymorphisms among the NILs, and found that Satt557 is the closest marker for the E1 locus. Another gene controlling ILD insensitivity, E7, is located at a genetic distance of 6.2 cM from the E1 locus (Cober and Voldeng 2001). The tagging markers for the E7 locus are Satt100, Satt319, and Satt460 (Molnar et al. 2003), which are not in accordance with the position of the highest LOD score for the QTL detected in this study (Figure 3). Therefore, a gene for the ILD insensitivity of Sakamotowase may be a novel allele at the E1 locus or may be a novel gene tightly linked to the E1 locus, although the possibility that the novel gene was the e7 allele could not be excluded. The E1 locus has the largest effect on flowering among soybean maturity genes compared (Bernard 1971; McBlain et al. 1987; Saindon et al. 1990; Cober et al. 1996b). The results obtained from the present study suggest that the E1 locus controls a wide range of flowering by allelic variation or by combination with tightly linked genes. Molecular dissection of the QTL in the future will facilitate our understanding of diverse mechanisms involved in photoperiod response of soybean.


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