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Research Paper

Quantitative trait locus mapping of soybean maturity gene *E5*

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Time to flowering and maturity in soybean is controlled by loci *E1* to *E5*, and *E7* to *E9*. These loci were assigned to molecular linkage groups (MLGs) except for *E5*. This study was conducted to map the *E5* locus using F_2 populations expected to segregate for *E5*. F_2 populations were subjected to quantitative trait locus (QTL) analysis for days to flowering (DF) and maturity (DM). In Harosoy-*E5* × Clark-*e2* population, QTLs for DF and DM were found at a similar position with *E2*. In Harosoy × Clark-*e2E5* population, QTLs for DF and DM were found in MLG D1a and B1, respectively. In Harosoy-*E5Dt2* × Clark-*e2* population, a QTL for DF was found in MLG B1. Thus, results from these populations were not fully consistent, and no candidate QTL for *E5* was found. In Harosoy × PI 80837 population, from which *E5* was originally identified, QTLs corresponding to *E1* and *E3* were found, but none for *E5* existed. Harosoy and PI 80837 had the *e2-ns* allele whereas Harosoy-*E5* had the *E2-dl* allele. The *E2-dl* allele of Harosoy-*E5* may have been generated by outcrossing and may be responsible for the lateness of Harosoy-*E5*. We conclude that a unique *E5* gene may not exist.

Key Words: *E5* gene, maturity gene, QTL mapping, soybean.

Introduction

Eight loci have been reported to control the time to flowering and maturity in soybean in temperate zones: *E1* and *E2* (Bernard 1971); *E3* (Buzzell 1971); *E4* (Buzzell and Voldeng 1980); *E5* (McBlain and Bernard 1987); *E7* (Cober and Voldeng 2001), *E8* (Cober *et al.* 2010); *E9* (Kong *et al.* 2014). Segregation for late maturity was reported in a backcross, Harosoy (6) × PI 80837 made to transfer *Pd1* (dense pubescence) to Harosoy (McBlain and Bernard 1987). A late BC_5F_4 progeny plant was designated as L64-4830. The F_2 population of L64-4830 × Harosoy segregated for a single locus for lateness. Test crosses of L64-4830 with Harosoy maturity-allele NILs that have different alleles of loci *E1*, *E2* and *e3* indicated that the maturity locus was not any of these loci. The new locus was designated as *E5*, and the genetic effect of *E5* on time to flowering and maturity was similar to that of *E2* (McBlain and Bernard 1987).

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Maturity loci, by controlling time to flowering and maturity, enable soybean cultivars to adapt to various regions with different daylengths. In addition, based on studies using NILs for maturity loci, maturity genes are involved in chilling tolerance in terms of quality and yield of seed. Chilling temperatures (about 15°C) during flowering period induce browning and cracking of the seed coats (Takahashi and Asanuma 1996). Genetic analysis suggest that a few major genes were involved in the tolerance to chilling temperatures and one of these genes was closely associated with maturity (Takahashi and Abe 1994). A QTL having a large effect on browning of the seed coat was identified at a similar position with *E1* (Githiri *et al.* 2007). To evaluate the separate effect of *E1* to *E5* on the intensity of seed coat browning and cracking, Harosoy (*e1 e2 E3 E4 e5 E7*) and its NILs for *E1* to *E5* were subjected to chilling treatments (Takahashi and Abe 1999). The intensity of browning was slightly reduced by *E2* and *e4*, and profoundly reduced by *E1* and *E5*, but it was not affected by *e3*. The degree of cracking was slightly increased by *e3* and drastically reduced by *e4*, *E1*, and *E5*. The *E7* allele had inhibitory effects for both browning and cracking (Benitez *et al.* 2004). Thus, *E5* had a large impact on the deterioration of seed

coats induced by low temperatures. Further, Harosoy NILs with an allelic combination of *E1e3e4* and NILs with *e1E3E4* had similar time to maturity and similar number of pods under control conditions (Takahashi *et al.* 2005). However, the NILs with *E1e3e4* had higher pod numbers than the NILs with *e1E3E4* under chilling treatments, resulting in a higher seed yields.

The linked loci *E1* and *E7*, *E2*, *E3*, *E4*, *E8* and *E9* loci were assigned to MLGs C2 (chromosome (chr) 6), O (chr 10), L (chr 19), I (chr 20), C1 (chr 4) and J (chr 16), respectively (Abe *et al.* 2003, Cober and Voldeng 2001, Cober *et al.* 2010, Cregan *et al.* 1999, Kong *et al.* 2014, Xia *et al.* 2012). Three cDNA copies for phytochrome A exist and two of them correspond to *E3* and *E4* (Liu *et al.* 2008, Watanabe *et al.* 2009). *E2* encodes a GIGANTEA-like protein (Watanabe *et al.* 2011). GIGANTEA is a nuclear-localized protein that functions upstream of CONSTANS and FLOWERING LOCUS T encoding a florigen (Lin *et al.* 2007, Mizoguchi *et al.* 2005). *E1* encodes a protein containing a bipartite nuclear localization signal and a region distantly related to the B3 domain, but its functional mechanism is uncertain (Xia *et al.* 2012). Based on nucleotide polymorphisms among *E1* to *E4* genes, amplicon length polymorphism (ALP) and derived cleaved amplified polymorphic sequence (dCAPS) markers were developed to classify alleles of these loci (Tsubokura *et al.* 2014). However, there is no map information for *E5* locus. This study was conducted to determine the location of *E5* using DNA markers.

Materials and Methods

Plant materials

Canadian cultivar Harosoy, NILs with genetic background of Harosoy or US cultivar Clark, and PI 80837 were used (**Table 1**). PI 80837 is a plant introduction collected from Akita Prefecture, Japan (landrace Mejiro). Germplasm information is detailed in the Germplasm Resources Information Network (GRIN) website at https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1118475. Harosoy has gray normal pubescence (*t pd1*), Clark has tawny normal pubescence (*T pd1*) and PI 80837 has gray dense pubescence (*t Pd1*). Harosoy and Clark (*e1 E2 E3 E4 e5 E7*) differ only at the *E2* locus at the known maturity loci. Three cross combinations were made by pollinating Harosoy (or Harosoy NIL) with pollen from Clark NIL to develop F_2 populations that, among maturity genes, were expected to differ in only *E5* (**Table 2**). Another F_2 population Harosoy × PI 80837 that is identical with the original cross from which *E5* was identified (McBlain and Bernard 1987), was included in the test. Seeds of the NILs and PI 80837 were obtained from the USDA Soybean Germplasm Collections. The NILs were produced by crossing donor cultivars with lines having the respective alleles and backcrossing the progeny up to BC5 (Bernard *et al.* 1991). Flowers of Harosoy or Harosoy NILs were emasculated one day before opening and were pollinated with Clark NILs or PI 80837. Hybridity of the F_1 plants was ascertained by either color or density of pubescence.

Plant cultivation

Pot experiments were conducted from June to October at the National Institute of Crop Science, Tsukuba, Japan (36°06′N, 140°05′E). Five seeds from each of the parents and three F_2 seeds were planted in pots (12.5 cm diameter) filled with 2.5 kg soil (low-humic andosols) supplemented with ammonium sulfate (0.8 g), monocalcium phosphate (1.6 g), fused magnesium phosphate (3.2 g) and potassium sulfate (0.8 g). One week after emergence, seedlings were thinned to one per pot and grown in an unheated vinyl plastic greenhouse. Pots were distributed at random in the greenhouse and repositioned twice a week. Field experiments were carried out from May to October at Hokkaido University, Sapporo, Japan (43°25′N, 143°32′E). Seeds were sown in paper pots on May 25 and the seedlings were transplanted to the field on June 5 in 2011. N, P, and K were applied at 3.0, 4.4, and 8.3 g m^{-2}, respectively. Plants were

Table 1. Soybean materials used in this study

Line name	Line designation	Genotype	Pedigree
Harosoy	–	*e1 e2 E3 E4 e5 E7 dt2 t pd1*	–
Harosoy-*E5*	L64-4830	*e1 e2 E3 E4 E5 E7 dt2 t pd1*	Harosoy (6) × PI 80837
Harosoy-*E5Dt2*	L62-812	*e1 e2 E3 E4 E5 E7 Dt2 t pd1*	Harosoy (6) × PI 80837
Clark	–	*e1 E2 E3 E4 e5 E7 dt2 T pd1*	–
Clark-*e2*	L62-1392	*e1 e2 E3 E4 e5 E7 dt2 T pd1*	Clark (6) × PI 86024 (*e2*)
Clark-*e2E5*	L94-1110	*e1 e2 E3 E4 E5 E7 dt2 T pd1*	L63-3117 (Clark-*e2*) (6) × L64-4830
PI 80837	–	*E5 Dt2 t Pd1*	–

Table 2. F_2 populations used in this study

Cross combination	Year of crossing	Date of planting	Cultivation method	Location	Number of	
					Parents	F_2 plants
Harosoy-*E5* × Clark-*e2*	2007	June 20, 2008	Pot	Tsukuba	4	98
Harosoy × Clark-*e2E5*	2010	June 16, 2011	Pot	Tsukuba	8	110
Harosoy-*E5Dt2* × Clark-*e2*	2010	May 25, 2011	Field	Sapporo	4	119
Harosoy × PI 80837	2012	June 27, 2013	Pot	Tsukuba	8	104

individually planted 25 cm apart within rows that were spaced 60 cm apart. The number of parents and F_2 plants used in each experiment are listed in **Table 2**. Number of days from planting to opening of the first flower (R1) (Fehr *et al.* 1971) was recorded for individual plants. In pot experiments, days from planting to maturity (R8), when 95 percent of pods had mature color, were also recorded.

Seeds of the parental lines were planted on June 7, 2012 in the field at the National Institute of Crop Science. N, P and K were applied at levels similar to those in the field of Hokkaido University. Eleven to 14 plants for each line were individually grown at a spacing of 70 cm between rows and 10 cm between plants. Days to flowering (R1) were individually recorded and subjected to analysis of variance. Means of days to flowering among lines were compared with Tukey's HSD test using the Statistica software 03J (StatSoft).

DNA extraction and SSR analysis

Total DNA was extracted from trifoliolate leaves of the parents and the F_2 population by the CTAB method (Murray and Thompson 1980). A total of 94 F_2 plants were randomly selected and used for analysis, because PCR reaction plates and the electrophoresis apparatus were designed for multiples of 96 samples (94 F_2 plants and two parents). SSR markers developed by USDA (Song *et al.* 2004) or by the Kazusa DNA Research Institute (Hisano *et al.* 2007) were used for screening of polymorphisms between the parents. The PCR mixture contained 20 ng of genomic DNA, 2.25 pmol of primer, 625 pmol of nucleotides, and 0.125 unit of ExTaq in 1 × ExTaq Buffer supplied by the manufacturer (Takara Bio, Ohtsu, Japan) in a total volume of 5 μL. An initial 4 min denaturation at 95°C was followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 49°C, and 1 min extension at 68°C. PCR was performed in an Applied Biosystems 9700 thermal cycler (Applied Biosystems, Foster City, CA). PCR products were separated in 8% nondenaturing acrylamide gels, and the fragments were visualized by staining with ethidium bromide. To investigate the possibility of outcrossing, SSR marker genotypes of Harosoy-*E5* (5 markers for each MLG) were compared with Harosoy (recurrent parent) and PI 80837 (donor parent).

Linkage mapping and QTL analysis

Markers were tested by Chi-square analyses for segregation in 1:2:1 or 3:1 ratio. A linkage map was constructed using MAPMAKER/EXP. ver. 3.0 (Lander *et al.* 1987) with a threshold LOD score of 3.0. Designation of MLGs followed Cregan *et al.* (1999). QTL analysis was performed by composite interval mapping (Zeng 1993) using the QTL Cartographer version 2.5 (Wang *et al.* 2007). The threshold LOD score was determined by permutation test with 1000 repetitions corresponding to genome-wide 5% level of significance.

Genotyping of E1 to E4 genes

Total DNA was extracted from trifoliolate leaves of genotypes listed in **Table 1** along with reference cultivars having specific maturity alleles (Bay for *E1* and *E2-dl* alleles, Tachinagaha for *e3-tr* allele, Moshidou Gong 503 for *e3-Mo* allele and Tokei 780 for *e4-SORE-1* allele) (Liu *et al.* 2008, Tsubokura *et al.* 2014). Alleles of *E1* to *E4* were determined by ALP or dCAPS analyses following previous reports (Liu *et al.* 2008, Tsubokura *et al.* 2014). Alleles of *E1* gene (*E1* or *e1-as*) were determined by PCR (primers: G33snpTaqcutF and G33snpTaqcutR1) and *Taq*αI digestion (marker designation: E1_*Taq*αI). Allele of *E2* gene (*e2-ns* or other alleles) was determined by PCR (primers: SoyGI_dCAPaMs19300FW and SoyGI_dCAPa19440RV) and *Dra*I digestion (marker designation: E2_*Dra*I). Allele of *E2* gene (*E2-in* or *E2-dl*) was determined by PCR amplicon size (primers: E2_15345FW and E2_15856RV) (marker designation: E2_InDel). Allele of *E3* gene (*e3-tr* or other alleles) was determined by PCR amplicon size (primers: E3_08557FW, E3_09908RV, E3Ha_1000RV, and e3tr_0716RV) (marker designation: E3_Mix). Allele of *E3* gene (*E3-Ha* or *e3-Mo*) was determined by PCR (primers: E3_08094FW and E3_08417RV) and *Mse*I digestion (marker designation: E3_*Mse*I). Allele of *E4* gene (*E4* or *e4-SORE-1*) was determined by PCR amplicon size (primers: PhyA2-for, PhyA2-Rev/E4, PhyA2-Rev/e4) (marker designation: E4_Mix). PCR products for E2_InDel and E4_Mix were separated in agarose gels of 2 and 1%, respectively. The other products were separated in 8% nondenaturing acrylamide gels. A diagnostic marker for the *E2* gene (E2_*Dra*I) was taken for the Harosoy-*E5* × Clark-*e2* population. Diagnostic markers for *E1* (E1_*Taq*αI) and *E3* genes (E3_Mix) were taken for the Harosoy × PI 80837 population.

Results

Distribution of days to flowering and maturity

The frequency distribution of DM and DF of F_2 populations as well as their mean parental values are presented in **Fig. 1**. The Harosoy-*E5Dt2* × Clark-*e2* population was grown in field at Sapporo. The other populations were grown in pots at Tsukuba. In the Harosoy-*E5* × Clark-*e2* population, Clark-*e2* flowered 6 days earlier and matured 10 days earlier than Harosoy-*E5*. DF and DM of F_2 plants were distributed without apparent transgression. DM is generally subject to environmental factors, and the appearance of late-maturing plants may not be attributable to transgressive effects. In the Harosoy × Clark-e*2E5* population, Harosoy flowered 3 days earlier and matured 20 days earlier than Clark-*e2E5*. Transgressive segregation was observed in F_2 plants; 3 plants flowered 4 days later than Clark-*e2E5* and one plant matured 8 days earlier than Harosoy. In the Harosoy-*E5Dt2* × Clark-*e2* population, Clark-*e2* flowered 4 days earlier than Harosoy-*E5Dt2*. Transgressive segregation was observed in F_2 plants; two plants flowered 4 days earlier than Clark-*e2* and three plants flowered 3 days later than Harosoy-*E5Dt2*. In the Harosoy × PI 80837 population, Harosoy flowered 4 days earlier and matured 5 days

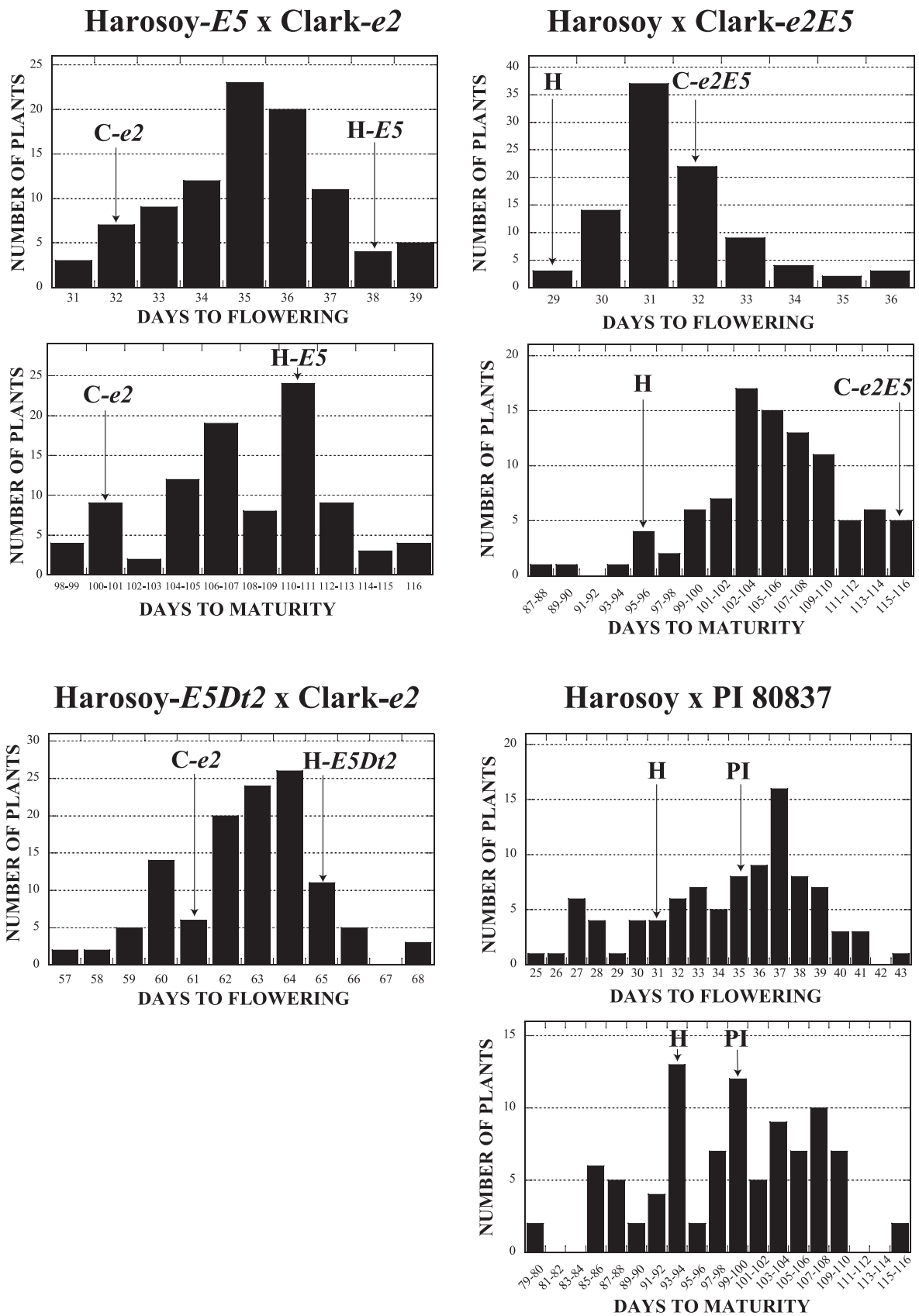

Fig. 1. Frequency distribution of days to flowering and maturity in four soybean F_2 populations. Mean values of days to flowering and maturity of the parents are shown by arrows. The Harosoy-*E5Dt2* × Clark-*e2* population was grown in field at Sapporo. The other populations were grown in pots at Tsukuba.

Table 3. Days to flowering of soybean lines at Tsukuba, Japan in 2012

Line	Days to flowering
Harosoy	35.3a[a]
Harosoy-*E5*	45.2c
Harosoy-*E5Dt2*	36.0ab
Clark-*e2*	37.1ab
Clark-*e2E5*	37.8b
PI 80837	47.2c

[a] Means within a column followed by the same letter are not significantly different (P = 0.05) according to Tukey's HSD test.

Table 4. Linkage groups obtained from four soybean F_2 populations

Cross combination	Number of polymorphic marker	Number of linked marker	Number of linkage group	Total map length (cM)
Harosoy-*E5* × Clark-*e2*	162	142	36	1524
Harosoy × Clark-*e2E5*	155	154	31	1741
Harosoy-*E5Dt2* × Clark-*e2*	185	177	27	1982
Harosoy × PI 80837	209	199	35	2555

earlier than PI 80837. Transgressive segregation of F_2 plants was most evident in this population; one plant flowered 6 days earlier than Harosoy and one plant flowered 8 days later than PI 80837. Further, two plants matured 15 days earlier than Harosoy and two plants matured 17 days later than PI 80837. In every population, it was difficult to classify the F_2 plants into early and late categories for either DF or DM. Therefore, QTL analysis was performed to map the maturity genes. Days to flowering of the parental lines are shown in **Table 3**. Harosoy, Harosoy-*E5Dt2* and Clark-*e2* composed an early-flowering group. Harosoy-*E5Dt2*, Clark-*e2* and Clark-*e2E5* composed an intermediate group that partially overlapped with the early-flowering group. Harosoy-*E5* and PI 80837 were classified into the late-flowering group. Days to flowering of Harosoy-*E5* was 10 d later than Harosoy. In contrast, days to flowering of Clark-*e2E5* and Harosoy-*E5Dt2* were not significantly different from Clark-*e2* and Harosoy, respectively.

Linkage groups

In the four F_2 populations, 155 to 209 markers that were polymorphic between the parents and distinctly segregated in the F_2 population were used for linkage mapping (**Table 4**). A total of 142 to 199 markers were linked to comprise 27 to 36 linkage groups spanning 1524 to 2555 cM (**Supplemental Fig. 1**).

QTLs for days to flowering and maturity

In the Harosoy-*E5* × Clark-*e2* population, large QTLs for DF (qDF_01) and DM (qDM_01) were found in the vicinity of E2_*Dra*I around the end of MLG O (chr 10) (**Table 5**, **Fig. 2**). The qDF_01 locus had a LOD score of 26.44, accounting for 51.2% of phenotypic variation. The qDM_01 locus had a LOD score of 19.51, accounting for 29.5% of phenotypic variation. The alleles from Harosoy-*E5* increased DF and DM at the QTLs.

In the Harosoy × Clark-*e2E5* population, a QTL for DF (qDF_02) was found between Satt198 and Satt077 in MLG D1a (chr 1). The locus had a LOD score of 5.71, accounting for 14.6% of phenotypic variation. The allele from Clark-*e2E5* increased DF at the QTL. Further, a QTL for DM (qDM_02) was found between Satt509 and Sat_247 in MLG B1 (chr 11). The locus had a LOD score of 8.48, accounting for 32.3% of phenotypic variation. The allele from Clark-*e2E5* increased DM at the QTL. In the Harosoy-*E5Dt2* × Clark-*e2* population, a QTL for DF (qDF_03) was found between Sat_149 and Sat_348 in MLG B1. The locus had a LOD score of 6.46, accounting for 25.5% of phenotypic variation. The allele from Harosoy-*E5Dt2* increased DF at the QTL.

In the Harosoy × PI 80837 population, large QTLs for DF (qDF_04) and DM (qDM_03) were found in the vicinity of E1_*Taq*αI in MLGs C2 (chr 6). The qDF_04 locus had a LOD score of 37.15, accounting for 69.5% of phenotypic variation. The qDM_03 had a LOD score of 6.76 accounting for 56.2% of phenotypic variation. The alleles from PI 80837 increased DF and DM at the QTLs. In addition, QTLs for DF (qDF_05) and DM (qDM_04) were found in the vicinity of E3_Mix in MLG L (chr 19). The qDF05 locus had a LOD score of 15.87, accounting for 19.5% of

Table 5. QTLs responsible for days to flowering and maturity observed in four soybean F_2 populations

Cross combination	Phenotype (days to)	QTL name	Linkage group[a]	Proximal marker	Position (cM)[b]	LOD score	Additive effect[c]	Dominance effect	Variance explained (%)
Harosoy-*E5* × Clark-*e2*	Flowering	qDF_01	O (10)	E2_*Dra*I	146.5	26.44	2.38	0.43	51.2
	Maturity	qDM_01	O (10)	E2_*Dra*I	145.5	19.51	4.74	1.64	29.5
Harosoy × Clark-*e2E5*	Flowering	qDF_02	D1a (1)	Satt468	29.0	5.71	–0.98	–0.20	14.6
	Maturity	qDM_02	B1 (11)	Satt509	30.0	8.48	–4.26	0.35	32.3
Harosoy-*E5Dt2* × Clark-*e2*	Flowering	qDF_03	B1 (11)	Sat_149	73.4	6.46	1.59	–0.18	25.5
Harosoy × PI 80837	Flowering	qDF_04	C2 (6)	E1_*Taq*I	72.2	37.15	–4.48	2.07	69.5
		qDF_05	L (19)	E3_Mix	24.3	15.87	2.90	0.27	19.5
	Maturity	qDM_03	C2 (6)	E1_*Taq*I	69.3	6.76	–5.71	4.45	56.2
		qDM_04	L (19)	E3_Mix	25.3	4.47	5.14	1.14	14.3

[a] Linkage group name is followed by chromosome number in parenthesis.

[b] Distance from top of linkage group.

[c] Additive effects of each QTL are those of Harosoy or Harosoy-NIL allele in contrast to pollen-parent allele.

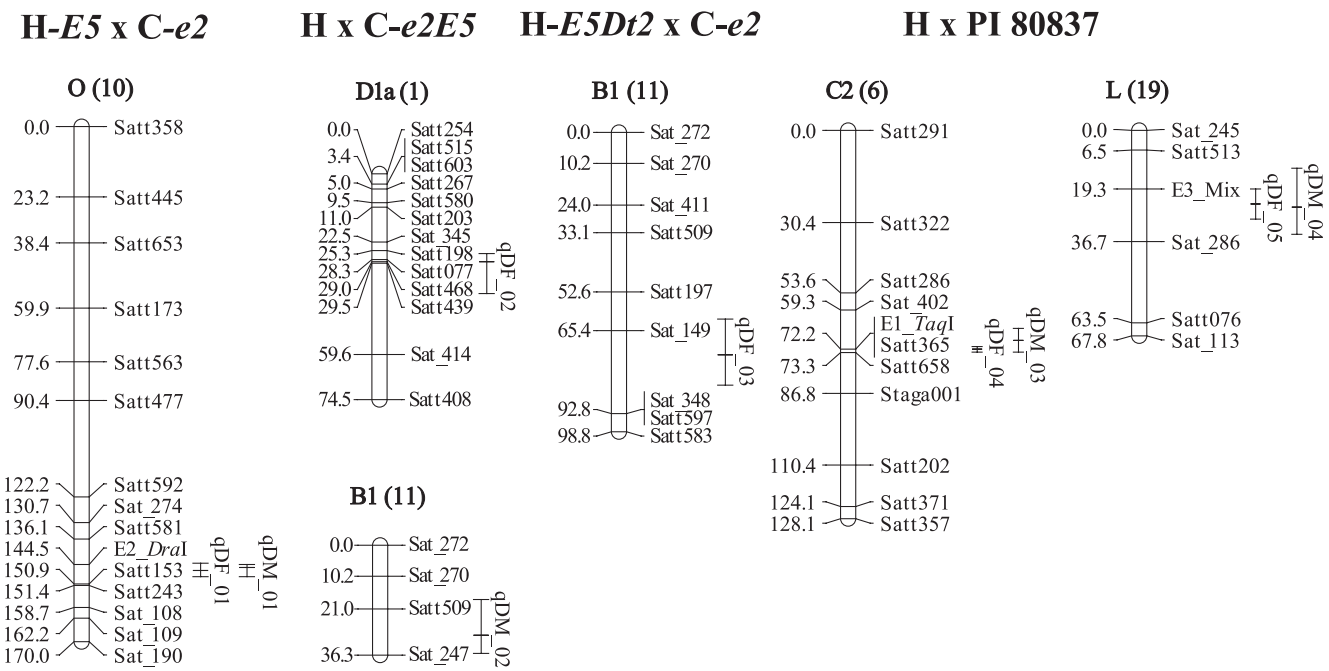

Fig. 2. Linkage groups containing QTLs for days to flowering and maturity found in four soybean F_2 populations. The name of the linkage group followed by the chromosome number in parenthesis is indicated at the top. Distances of markers (cM) from the top of each linkage group are shown on the left. Length of vertical bars is equal to the one-LOD likelihood confidence interval. Horizontal lines in the middle of the bars indicate the positions of the QTL peak.

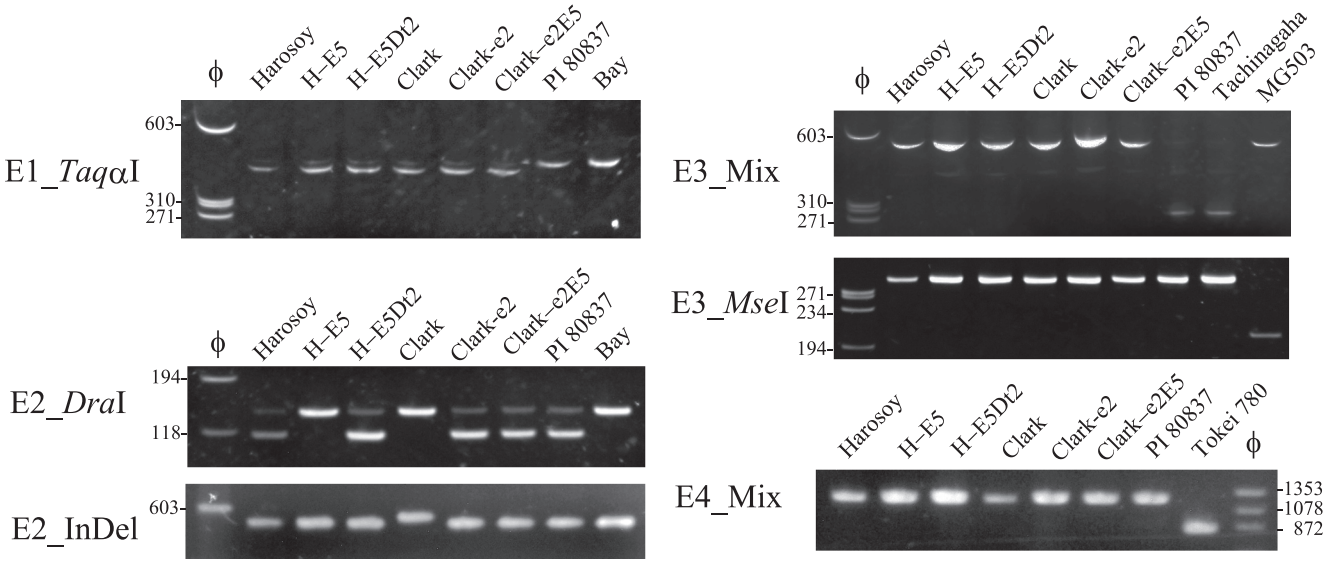

Fig. 3. Results of ALP and dCAPS analyses to determine genotype of soybean maturity genes *E1* to *E4*. Marker designations are indicated to the left of each panel. ϕ, molecular marker ϕx174/*Hae*III; H-E5, Harosoy-*E5*; H-E5Dt2, Harosoy-*E5Dt2*. The migration of size markers (bp) is shown to the left or right of the gel.

phenotypic variation. The allele from PI 80837 decreased DF. The qDM_04 locus had a LOD score of 4.47, accounting for 14.3% of phenotypic variation. The allele from PI 80837 increased DM.

Genotype of maturity genes

PCR with E1_*Taq*αI primers produced a 444 bp fragment in all materials including the reference cultivar Bay. *Taq*αI digestion generated a 412 bp fragment in Harosoy, Harosoy-*E5*, Harosoy-*E5Dt2*, Clark, Clark-*e2*, Clark-*e2E5* whereas products of PI 80837 and Bay were undigested (**Fig. 3**). The results suggest that PI 80837 and Bay have the dominant allele (*E1*) whereas the other materials have the recessive allele (*e1-as*).

PCR with E2_*Dra*I primers produced a 142 bp fragment in all materials. *Dra*I digestion generated a 115 bp fragment

Table 6. Alleles of maturity genes in soybean lines

Line	*E1*	*E2*	*E3*	*E4*
Harosoy	*e1-as*	*e2-ns*	*E3-Ha*	*E4*
Harosoy-*E5*	*e1-as*	*E2-dl*	*E3-Ha*	*E4*
Harosoy-*E5Dt2*	*e1-as*	*e2-ns*	*E3-Ha*	*E4*
Clark	*e1-as*	*E2-in*	*E3-Ha*	*E4*
Clark-*e2*	*e1-as*	*e2-ns*	*E3-Ha*	*E4*
Clark-*e2E5*	*e1-as*	*e2-ns*	*E3-Ha*	*E4*
PI 80837	*E1*	*e2-ns*	*e3-tr*	*E4*

in Harosoy, Harosoy-*E5Dt2*, Clark-*e2*, Clark-*e2E5* and PI80837 whereas products of Harosoy-*E5*, Clark and Bay were undigested. These results suggest that Harosoy-*E5* had the dominant allele (*E2*) similar to Clark and Bay, whereas Harosoy, Harosoy-*E5Dt2*, Clark-*e2*, Clark-*e2E5* and PI 80837 had the *e2-ns* allele. PCR with E2_InDel primers generated a 544 bp fragment only in Clark whereas the other materials had a 512 bp fragment. The results suggest that Clark had the *E2-in* allele whereas Harosoy-*E5* and Bay had the *E2-dl* allele.

PCR with E3_Mix primers produced a 274 bp fragment in PI 80837 and Tachinagaha whereas the primers generated a 558 bp fragment in other materials. The results suggest that PI 80837 and Tachinagaha have the *e3-tr* allele. PCR with E3_MseI primer generated a 324 bp fragment in all materials. *Mse*I digestion generated a 223 bp fragment only in Moshidou Gong 503 whereas products of the other materials were unaffected. The results suggest that Moshidou Gong 503 had the *e3-Mo* allele and the other materials had the *E3-ha* allele. PCR with E4_Mix primers produced a 837 bp fragment in Tokei 780 whereas a 1,229 bp fragment was generated from the other materials. The results suggest that Tokei 780 had the *e4-SORE1* allele whereas the other materials had the *E4* allele. Alleles of the maturity genes in the soybean lines are summarized in **Table 6**.

Genotype of SSR markers

Out of 100 SSR markers polymorphic between Harosoy and PI 80837, 95 markers of Harosoy-*E5* had a genotype of Harosoy and one marker (Satt148 in MLG I) had a genotype of PI 80837. The results indicate that Harosoy-*E5* is a NIL of Harosoy. However, four markers of Harosoy-*E5* (Sat_183 in MLG D1b, Satt049 in MLG I, Satt406 in MLG J and Satt592 in MLG O) had genotypes different from Harosoy or PI 80837 (**Supplemental Table 1**), suggesting that an outcrossing event may have occurred during the development of this NIL.

Discussion

In the Harosoy-*E5* × Clark-*e2* population, QTLs having high LOD scores, qDF_01 and qDM_01, were found in the vicinity of the *E2* gene (Watanabe *et al.* 2011). The results suggest a possibility that *E5* is located in the close vicinity of *E2*, or *E5* is an allele of the *E2* locus. However, ALP and dCAPS analyses revealed that Harosoy and PI 80837 had the *e2-ns* allele whereas Harosoy-*E5* had the *E2-dl* allele. Harosoy-*E5* was originated from a cross between PI 80837 and Harosoy so it must have the *e2-ns* allele. We suspected seed contamination during propagation and obtained another batch of seed samples from the USDA germplasm collection. However, the analytical results were identical to the previous batch.

In the Harosoy × Clark-*e2E5* population, DF differed by only 3 days but DM differed by 12 days among the parents. In this population, QTLs for DF (qDF_02) and DM (qDM_02) were found. Consistent with the parental differences, LOD score of qDM_02 was larger than qDF_02. In the Harosoy-*E5Dt2* × Clark-*e2* population, a QTL for DF (qDF_03) was observed in MLG B1. A gene for type of stem termination (*Dt2*) that has a potential effect on maturity (Bernard 1972) is expected to segregate in the population. However, qDF_03 was mapped to a different MLG from *Dt2* located in MLG G (chr 18) (Ping *et al.* 2014). Though the three populations are expected to segregate for the same gene, results of QTL analysis were not consistent. Harosoy-*E5* flowered 11 days later and matured 13 days later compared with Harosoy at Tsukuba, suggesting that the effects of *E5* were comparable to those of *E1* and *E2* at this location (Takahashi and Abe 1999). In these populations, however, QTLs with large effects (qDF_01 and qDM_01) corresponded to *E2*. The effects of the other QTLs were too small to consider them as *E5*.

The Harosoy × PI 80837 population must segregate for *E5* if a unique *E5* gene exists. Two QTLs for DF (qDF4 and qDF5) and two QTLs for DM (qDM_03 and qDM_04) were found in this population. The QTLs qDF_04 and qDM_03 were found in the vicinity of the *E1* gene (Xia *et al.* 2012). dCAPS analysis revealed that PI 80837 has the *E1* allele whereas Harosoy had the *e1-as* allele. Further, a large LOD score of qDF_04 was consistent with a large effect of the *E1* gene (Takahashi and Abe 1999). The results suggest that qDF_04 and qDM_03 may correspond to *E1*. The qDF_05 and qDM_04 loci were found in the vicinity of *E3* gene (Watanabe *et al.* 2009). ALP and dCAPS analyses revealed that Harosoy has the *E3-ha* allele and PI 80837 has the *e3-tr* allele. The results suggest that qDF_05 and qDM_04 may correspond to *E3*. The effect of *E3* was not evident compared to *E1* and *E2*, probably because of the short daylength at the location of experiments (Takahashi and Abe 1999). Harosoy and PI 80837 probably have allelic combinations of *e1 e2 E3 E4* and *E1 e2 e3 E4*, respectively. Transgressive segregation observed in their F_2 population may have been caused by appearance of plants with double-recessive and double-dominant alleles of *E1* and *E3* loci. Thus, no candidate QTL corresponding to the *E5* gene was found in this population. Furthermore, all of the QTLs found in the other populations (Harosoy-*E5* × Clark-*e2*, Harosoy × Clark-*e2E5* and Harosoy-*E5Dt2* × Clark-*e2*) may not correspond to *E5*, because the *E5* gene was expected to segregate in the Harosoy × PI 80837 population.

Harosoy-*E5* has an allelic combination of *e1-as E2-dl*

E3-Ha E4. Harosoy-*E5* may have been generated by outcrossing with pollen having the *E2-dl* allele. The finding that four SSR markers of Harosoy-*E5* had genotypes different from Harosoy or PI 80837 strongly suggests outcrossing. Lateness of Harosoy-*E5* may be attributable to the *E2-dl* allele. Test crosses for *E2* locus were made by crosses between Harosoy-*E5* and Harosoy-*E2* (L74-21 or L74-27) (McBlain and Bernard 1987). L74-21 and L74-27 were developed by crossing Harosoy six times with Clark (Bernard *et al.* 1991) which has the *E2-in* allele. The test crosses may have generated plants having a heterozygous *E2-in E2-dl* genotype. Crossing experiments between plants with *E2-dl* and *E2-in* allele should be performed to check if transgressive late segregants would appear as reported previously (McBlain and Bernard 1987). The two dominant alleles *E2-in* and *E2-dl* have many nucleotide polymorphisms in the 5′ upstream region, exons, introns and 3′ downstream region (Tsubokura *et al.* 2014). Harosoy-*E5* flowered 3d later and matured 9 d later than Harosoy-*E2* in Tsukuba (Takahashi and Abe 1999). The frequency of seeds with low-temperature induced seed coat deterioration of Harosoy-*E5* (browning: 29.4%, cracking: 38.9%) was lower than Harosoy-*E2* (browning: 55.9%, cracking: 69.2%). The different effects of Harosoy-*E2* (L64-4584) and Harosoy-*E5* are possibly caused by allelic differences between *E2-in* and *E2-dl*, or differences in unidentified genetic backgrounds. Under the same environments, days to flowering of Harosoy-*E5* were substantially later than Harosoy, whereas days to flowering of Clark-*e2E5* and Harosoy-*E5Dt2* were similar to Clark-*e2* and Harosoy, respectively. Clark-*e2E5* and Harosoy-*E5Dt2* may have been developed by introducing other genomic regions affecting time to flowering and maturity, partially because backcrossing was done without using genetic markers.

In conclusion, Harosoy-*E5* may have been generated by unexpected outcrossing with pollen having an *E2-dl* allele. The allele may be responsible for lateness of Harosoy-*E5*. Thus, we conclude that a unique *E5* gene does not exist, though the existence of a maturity locus in the gap of the linkage groups cannot be excluded. Some of the QTLs found in this study have not yet been reported. Analysis of the pertinent genes may be useful to further understand the control mechanism for time to flowering and maturity in soybean.

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