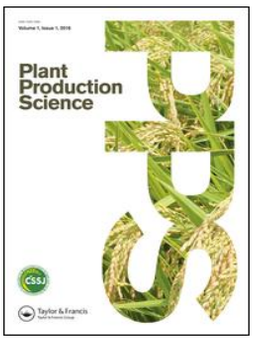

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Stability Verification of the Effects of Stem Determination and Earliness of Flowering on Green Stem Disorder of Soybean against Genetic Background and Environment

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Abstract: Green stem disorder (GSD) of soybean reduces harvesting efficiency and negatively impacts seed appearance when mechanical harvesting is employed. Two recombinant inbred populations were investigated for the effects of segregating stem determination and flowering time on GSD at two different locations, Kyoto and Akita, over two years. Although the severity of GSD of each line varied considerably with the location, the scores showed significant correlation with the environment. Quantitative trait locus (QTL) analysis revealed a strong and consistent QTL for GSD severity in one population of recombinant inbred lines (RILs) across the environments at the *Dt1* locus, which governs stem growth habits, and the determinate growth genotypes showed evident symptoms of GSD. However, QTLs were not detected near the *Dt1* locus in the other population. Thus, it was unclear if the responsible gene was identical to the stem determination gene. The early flowering genotype showed a more severe symptom of GSD in both populations, but this effect was dependent on the allele at the *Dt1* locus. The effect of another QTL detected in the latter population also depended on the allele at the *Dt1* locus. Our results indicated that the genetic factor at the *Dt1* locus and the factor controlling flowering time influenced the severity of GSD at each location and year and that their effects and interaction complicated the genetic control of the occurrence of GSD.

Key words: Flowering time, Green stem disorder, Quantitative trait locus, Recombinant inbred lines, Senescence, Soybean, Stem growth habit.

Soybean [*Glycine max* (L.) Merrill] is a typical monocarpic plant species that loses green color and stem moisture as the seeds mature. However, these plants occasionally maintain stem greenness, stem moisture and leaf color (green or yellow-green) at seed maturity. Phillips et al. (1984) described this phenomenon as "delayed leaf senescence", and Furuya et al. (1988) and Hobbs et al. (2006) termed this phenomenon as "inharmonious maturation" and "green stem disorder (GSD)", respectively, after unsynchronized senescence between vegetative and reproductive organs. The severity level in plants has been described from entirely green plants to leafless plants with a yellow-green stem. This phenomenon is regarded as a nuisance for farmers because GSD plants are difficult to cut, which places an extra load on combine harvesters (Malvick, 2001). Furthermore, GSD contaminates seeds with the sap of green and wet tissues during the threshing process, which reduces the appearance quality and storability of seeds (Ogiwara, 2002). Therefore, GSD is regarded as an unfavorable phenomenon that should be prevented.

Several studies have demonstrated the presence of a genetic variation in the severity of GSD (Pierce et al., 1984; Matsumoto et al., 1986; Furuya and Umezaki, 1993; Mochizuki et al., 2005; Hill et al., 2006), and identification of quantitative trait locus (QTL) is expected to contribute to effective breeding of soybean cultivars that normally senesce. Pierce et al. (1984) demonstrated the relationship between this phenomenon and stem growth habits dominated by the *Dt1* locus using near isogenic lines, and reported that determinate genotypes (D-type) showed severe GSD phenotypes in comparison with indeterminate

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Abbreviations: D-type, determinate stem growth habit; DSS, degree of synchronous senescence between vegetative and reproductive organs; GSD, green stem disorder; I-type, indeterminate stem growth habit; QTL, quantitative trait locus.

genotypes (I-type). These authors also reported that D-type lines with early flowering alleles exhibited more severe symptoms, and they suggested the involvement of the flowering time loci in the occurrence of GSD. However, Pierce et al. (1984) also noted that their results were conditioned on the genetic background and environment, and the mechanism of how stem growth habit and flowering time loci affect plant senescence remains unknown. Because these genetic factors influence important agronomical traits such as yield, maturity and lodging (Bernard, 1972; Foley et al., 1986; Curtis et al., 2000; Kilgore-Norquesta and Sneller, 2000; Cober and Voldeng, 2001), understanding of the effect of these genetic factors on GSD is necessary before the alleles of these loci can be modified.

To assess the stability of the effects of stem determination and earliness of flowering on GSD, we examined two sets of recombinant inbred populations segregating stem determination and earliness of flowering at two different locations (Kyoto and Akita). One population was derived from the Tachinagaha cultivar for the determinate parent, which is well-known for the frequent occurrence of GSD (Ookawa et al., 1999; Mochizuki et al., 2005). The GSD of the determinate parent of the other population, the Ohsuzu cultivar, has not been previously reported. QTLs involved in the severity of GSD were identified for both populations, and the genetic effects, including stem growth habit and earliness of flowering, were analyzed.

For the assessment of GSD severity, a 5-point scale evaluation was utilized. This method was developed by Furuya and Umezaki (1993) to describe the plant status of different GSD severities minutely and readily, and was therefore useful for the rapid assessment of a large number of samples. The score of the GSD severity refers to the change of plant greenness during the process of senescing, and was regarded as a continuous scale. This scale was expected to detect small genetic differences in GSD susceptibility as compared to the frequency of GSD occurrence.

Materials and Methods

1. Plant materials and linkage map construction

Two sets of recombinant inbred lines (RILs) were used in the present study. One population was derived from a cross between cvs. Stressland and Tachinagaha (ST RILs), and the other population was derived from a cross between cvs. Ohsuzu and Athow (OA RILs). The Stressland (MG V) and Athow (MG IV) cultivars are from the United States, and they have indeterminate stem growth habit (I-type). The Tachinagaha (MG V) and Ohsuzu (MG IV) cultivars are from Japan, and they have determinate stem growth habit (D-type). These two populations were developed via single seed descent to the F6 (ST RILs) or F7 (OA RILs) generation.

Total DNA was extracted from 10 mg of seed powder using an automated purification system (BioSprint 96 DNA Plant Kit, Qiagen, Germany). The DNA sample of ST and OA RILs was collected from five seeds of the single plant in their F7 and F6 generations, respectively. Genotyping was performed according to Sayama et al. (2011) because the methodology is systematic and rapid. Briefly, polymerase chain reaction (PCR) was performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA) in a reaction mixture containing multiple simple sequence repeat (SSR) primers labeled with different florescent dyes (6-FAM, VIC, NED and PET), and the SSR fragment length in the product were measured using a DNA analyzer (3730 DNA Analyzer, Applied Biosystems, USA) instead of general gel electrophoresis. The SSR markers used here were developed by USDA-ARS (United States Department of Agriculture, Agriculture Research Service), Chiba University and Kazusa DNA Research Institute (Cregan et al., 1999; Song et al., 2004; Hisano et al., 2007; Xia et al., 2007). Genetic linkage maps with 20 linkage groups were constructed with Kosambi's map function (Kosambi, 1943) using MAPMAKER/EXP VER. 3.0 software (Lincoln et al., 1993). A total of 169 SSR markers were used for ST RILs, and the map covered 2633.9 cM with an average distance between markers of 17.7 cM. The linkage map of OA RILs covered 2629.8 cM with an average distance of 15.1 cM using a total of 194 SSR markers.

2. Experimental sites and design

In 2009, Stressland, Tachinagaha and 78 lines of ST RILs were planted at the Kyoto Experimental Farm of the Graduate School of Agriculture, Kyoto University, Kyoto, Japan (Lat. 35°2′N, Long. 135°47′E and 65 m altitude; Fluvic Endoaquepts soil type). In 2010, 118 lines of both ST and OA RILs along with parental cultivars were planted at the Kyoto field, and the 118 lines of ST RILs, 229 lines of OA RILs and their parental cultivars were planted at the Kariwano branch of the Daisen research station of NARO Tohoku Region Agricultural Research Center, Daisen, Akita (Lat. 39°32′N, Long. 140°22′E and 30 m altitude; Melanudand soil type). At Kyoto, the sowing dates were June 19 in 2009 and June 28 in 2010. At Akita in 2010, ST and OA RIL seeds were sown on June 15 and June 23, respectively. To ensure the heritability of the degree of GSD, 18 selected lines from each population and parental variety, consisting of early, middle and late flowering lines with different stem growth habits, were tested at Kyoto in 2011, and the seeds were sown on June 23. In all experiments, plots were single row with two replications in a randomized complete block design. The row and plant spacing distances were 0.7 and 0.15 m, respectively. For each plot, samples were composed of 8 (in 2009) and 10 (in 2010 and 2011) randomly selected non-sequential

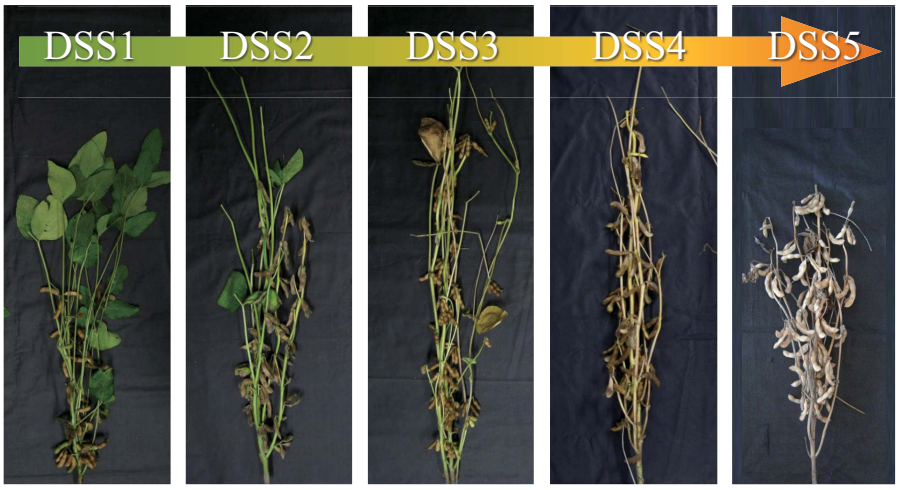


Fig. 1. Degree of synchronous senescence (DSS) of vegetative and reproductive organs proposed by Furuya and Umezaki (1993) and its plant status at full maturity (R8).

plants in a single row.

3. Trait evaluations and data recording

The dates of the beginning of flowering (R1 stage) and full maturity (R8 stage) were recorded according to Fehr et al. (1971). The node number of the main stem was counted at R1 and R8 at Kyoto in 2010 to certify the accurate chromosomal position of the *Dt1* locus, which dominates stem determination. At R8, the severity of GSD was recorded by using a simple scoring method proposed by Furuya and Umezaki (1993), which evaluates the degree of synchronous senescence (DSS) between vegetative and reproductive organs with a 5-point scale from DSS1 to DSS5 (Fig. 1). At DSS1, the stem is green and green or yellow-green leaves remain at more than one-third of the nodes of the plant. At DSS2, the stem is green or green-yellow and green or yellow-green leaves remain at less than one-third of the nodes of the plant. At DSS3, the stem is light green and contains some moisture and chlorophyll, and several leaves contain some moisture or several petioles without leaves remain at R8. At DSS4, the stem is yellow and retains some moisture, and occasionally a few leaves are yellow to yellow-green. At DSS5, the stem is dry with gray or brown color, and all leaves have abscised. Plants exhibiting intermediate symptoms were assigned intermediate scores. Thus, a severe symptom of GSD corresponds to a low value. In general, we regard the plant status categorized into less than DSS3 as GSD.

For each trait, the average values for the plants in the plot, excluding border plants at both ends of the plot were recorded, and the mean of the replications was adopted as the representative value of each line.

4. Statistical and QTL analysis

Analysis of variance (ANOVA) was conducted to test the differences of trait values among location, year and genotype. The Tukey's test at $P < 0.05$ was used for the comparison of means. The Steel-Dwass test was adopted when the Tukey's test was not applicable. The correlation of DSS for the location and year and the relationship between the traits were statistically analyzed by Pearson's correlation test. Spearman's rank correlation coefficient was also shown for fear that parametric approach was not applied to DSS. QTL analysis was performed using Windows QTL Cartographer 2.5 (Wang et al., 2007; http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). The composite interval mapping (CIM) method with a walk speed of 1.0 cM was adopted for the detection of chromosomal positions and additive effects of QTLs for each trait. The threshold value of the logarithm of odds (LOD) for the detection of QTL was calculated by the permutation test (1000 runs, $P = 0.05$).

Results

1. Variations and distributions of DSS

Wide variations of DSS were observed in ST RILs with the location and year (Table 1 and Fig. 2). The DSS ranged from 1.0 to 4.5, with an average of 2.9, at Kyoto in 2009. The scores in Stressland (maternal parent) and Tachinagaha (paternal parent) were 3.1 and 2.2, respectively. At Kyoto in 2010, the DSS range was from 1.3 to 4.0, with an average of 2.8, and the scores in Stressland and Tachinagaha were 3.1 and 2.0, respectively. At Akita in 2010, the two latest lines did not reach R8 due to frost, so the lines were not included in the analyses. The DSS range was from 1.0 to 5.0, but the average value was as high as 4.4 because the frequency did not exhibit normal distribution. The scores in maternal and pollen parents were 5.0 and 3.8, respectively. When the selected 20 genotypes were tested at Kyoto in 2011, the range of DSS was between 1.4 and 3.9, and the scores in Stressland and Tachinagaha were 3.9 and 2.8, respectively. The score in Tachinagaha was lower than

Table 1. General statistics for DSS of ST and OA RILs across the environments.

Population	Location	Year	I/D-type	n	mean ± s.d. [a),b)]		Range	Maternal [c)]	Paternal [c)]
ST RILs			Both	80	2.89 ± 0.69	CD	1.00 – 4.50	3.09	2.24
	Kyoto	2009	I-type	48	3.24 ± 0.49	d	2.13 – 4.50	3.09	–
			D-type	32	2.36 ± 0.61	e	1.00 – 3.33	–	2.24
	Kyoto	2010	Both	120	2.76 ± 0.58	D	1.25 – 4.00	3.13	2.00
			I-type	67	3.12 ± 0.38	d	2.25 – 4.00	3.13	–
			D-type	53	2.30 ± 0.45	e	1.25 – 3.50	–	2.00
	Kyoto	2011	Both	20	2.71 ± 0.77	BCD	1.38 – 3.88	3.88	2.75
			I-type	9	3.36 ± 0.48	cd	2.50 – 3.88	3.88	–
			D-type	11	2.17 ± 0.50	e	1.38 – 2.88	–	2.75
	Akita	2010	Both	118[d)]	4.35 ± 0.87	A	1.00 – 5.00	5.00	3.75
			I-type	65[d)]	4.65 ± 0.40	a	3.50 – 5.00	5.00	–
			D-type	53	3.98 ± 1.12	bc	1.00 – 5.00	–	3.75
OA RILs			Both	120	3.20 ± 0.45	B	1.75 – 4.63	3.81	3.69
	Kyoto	2010	I-type	61	3.21 ± 0.41	d	2.38 – 4.38	–	3.69
			D-type	59	3.18 ± 0.49	d	1.75 – 4.63	3.81	–
	Kyoto	2011	Both	20	3.17 ± 0.78	BC	1.25 – 4.50	3.50	3.75
			I-type	9	3.57 ± 0.50	cd	2.75 – 4.50	–	3.75
			D-type	11	2.84 ± 0.84	de	1.25 – 3.88	3.50	–
	Akita	2010	Both	231[e)]	4.54 ± 0.56	A	2.00 – 5.00	4.75	5.00
			I-type	141	4.60 ± 0.51	a	2.00 – 5.00	–	5.00
			D-type	85	4.45 ± 0.62	ab	2.00 – 5.00	4.75	–

ANOVA [f)]						
Kyoto and Akita in 2010	Population	***	Population × Location	**		
	Location	***	Population × I / D-type	***		
	I/D-type	***	Location × I / D-type	NS		
			Population × Location × I / D-type	NS		
Kyoto in 2010 and 2011	Population	***	Population × Year	NS		
	Year	NS	Population × I / D-type	***		
	I/D-type	***	Year × I / D-type	***		
			Population × Year × I / D-type	NS		

a) s.d. represents standard deviation.

b) Values with different letters are significantly different at $P < 0.05$ by Steel-Dwass test, and small and capital letters are applied to the comparisons with and without classification by stem determination, respectively.

c) Maternal and paternal represent the seed and pollen parents of each populations.

d) Two I-type lines did not reach R8 due to frost.

e) Stem growth habits of five lines were not identified.

f) ** and *** represent statistical significance at $P < 0.01$ and 0.001, respectively, and NS means not significant.

that in Stressland in every test.

Variations of DSS were also observed in OA RILs (Table 1 and Fig. 3). DSS ranged from 1.8 to 4.6 with an average of 3.2 at Kyoto in 2010, and the scores in Ohsuzu and Athow were 3.8 and 3.7, respectively. At Akita in 2010, the DSS range was from 2.0 to 5.0, and the average value was 4.5. The distribution of OA RILs showed the same trend as that of ST RILs at Akita. The scores of Ohsuzu and Athow were 4.8 and 5.0, respectively. At Kyoto in 2011, the DSS range of the selected 20 genotypes tested was from 1.3 to 4.5, and the scores in Ohsuzu and Athow were 3.5 and 3.8, respectively.

Correlation coefficients of the DSS of each line for the location and year were analyzed by Pearson's correlation test and Spearman's rank correlation test (Table 2 and 3). The relationships were close even between the different locations except for the relationship in OA RILs between 2010 and 2011 at Kyoto (Table 3). Excluding the data of a line, OA_156, however, the correlation coefficient was statistically significant at $P < 0.001$.

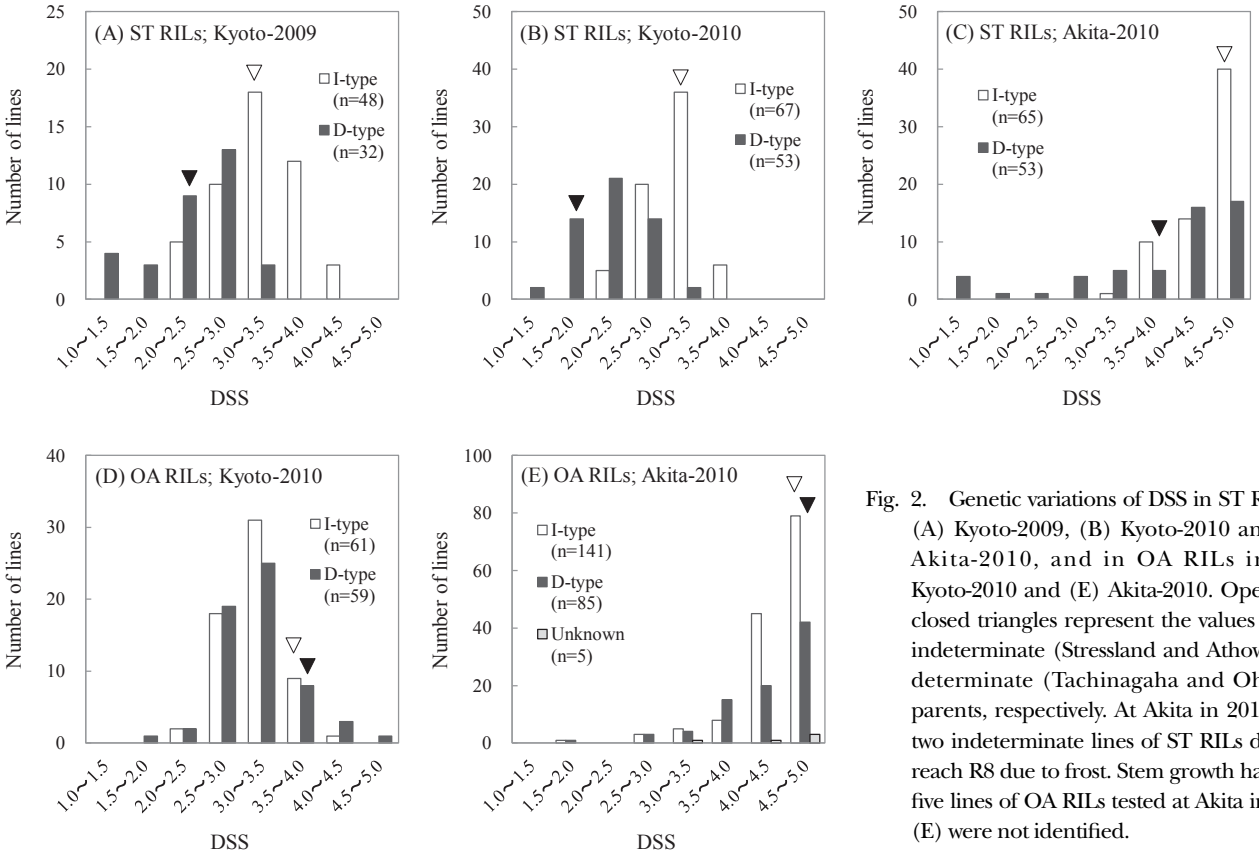


Fig. 2. Genetic variations of DSS in ST RILs in (A) Kyoto-2009, (B) Kyoto-2010 and (C) Akita-2010, and in OA RILs in (D) Kyoto-2010 and (E) Akita-2010. Open and closed triangles represent the values of the indeterminate (Stressland and Athow) and determinate (Tachinagaha and Ohsuzu) parents, respectively. At Akita in 2010 (C), two indeterminate lines of ST RILs did not reach R8 due to frost. Stem growth habits of five lines of OA RILs tested at Akita in 2010 (E) were not identified.

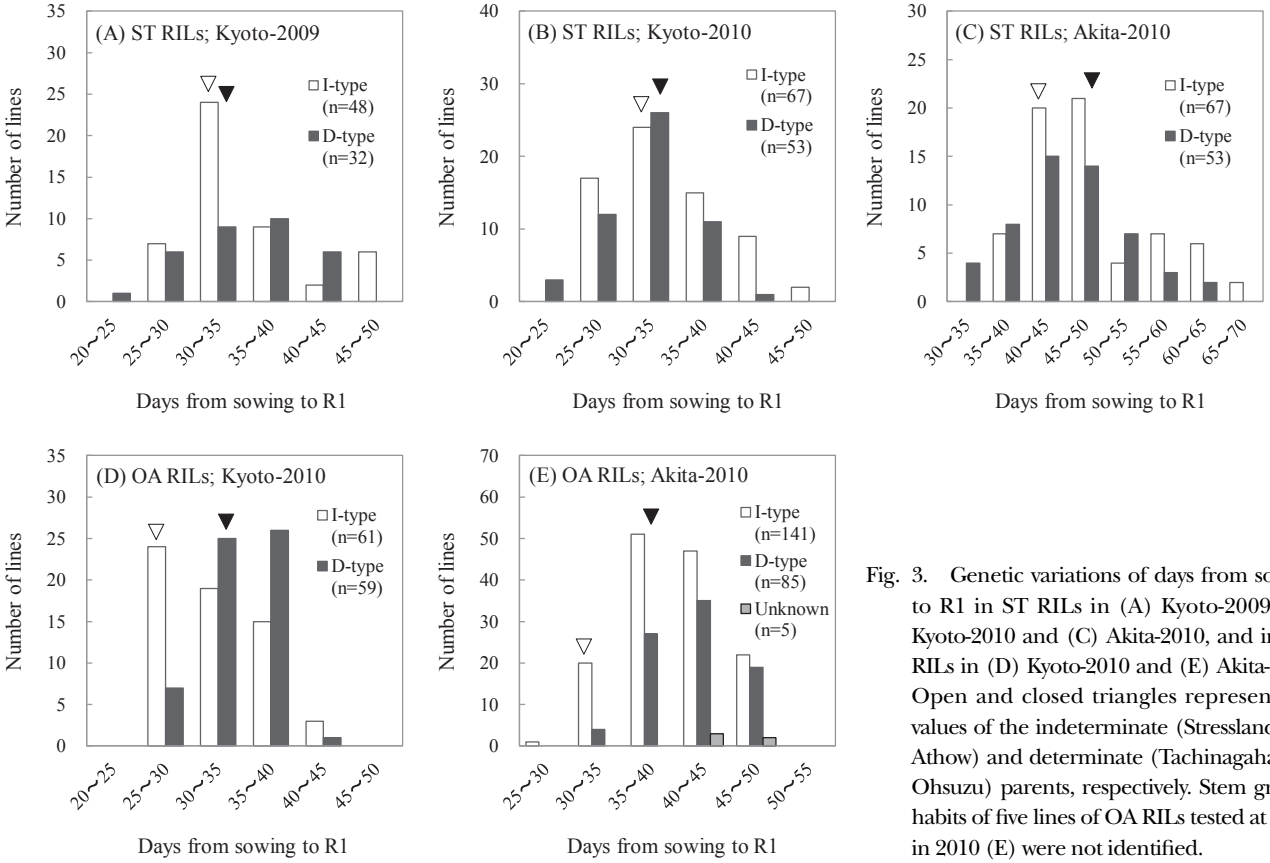


Fig. 3. Genetic variations of days from sowing to R1 in ST RILs in (A) Kyoto-2009, (B) Kyoto-2010 and (C) Akita-2010, and in OA RILs in (D) Kyoto-2010 and (E) Akita-2010. Open and closed triangles represent the values of the indeterminate (Stressland and Athow) and determinate (Tachinagaha and Ohsuzu) parents, respectively. Stem growth habits of five lines of OA RILs tested at Akita in 2010 (E) were not identified.

Table 2. Correlation coefficients for DSS of ST RILs in different environments. Upper and lower tables show Pearson's correlation coefficients and Spearman's rank-correlation coefficients, respectively.

Location	Year	Kyoto			Akita
		2009	2010	2011	2010
Kyoto	2009	–	0.78 ***	0.74 ***	0.54 ***
Kyoto	2010	$n = 80$	–	0.86 ***	0.61 ***
Kyoto	2011	$n = 19$	$n = 20$	–	0.56 **
Akita	2010	$n = 79$	$n = 118$	$n = 20$	–
Kyoto	2009	–	0.80 ***	0.78 ***	0.49 ***
Kyoto	2010	$n = 80$	–	0.86 ***	0.51 ***
Kyoto	2011	$n = 19$	$n = 20$	–	0.56 **
Akita	2010	$n = 79$	$n = 118$	$n = 20$	–

** and *** represent statistical significance at $P < 0.01$ and 0.001, respectively.

Table 3. Correlation coefficients for DSS of OA RILs in different environments. Upper and lower tables show Pearson's correlation coefficients and Spearman's rank-correlation coefficients, respectively.

	Location	Year	Kyoto		Akita
			2010	2011	2010
All lines	Kyoto	2010	–	0.30 n.s.	0.27 **
	Kyoto	2011	$n = 20$	–	0.54 *
	Akita	2010	$n = 120$	$n = 20$	–
	Kyoto	2010	–	0.50 *	0.31 ***
	Kyoto	2011	$n = 20$	–	0.61 **
	Akita	2010	$n = 120$	$n = 20$	–
Excluding OA_156 in Kyoto –2010	Kyoto	2010	–	0.82 ***	0.28 **
	Kyoto	2011	$n = 19$	–	0.54 *
	Akita	2010	$n = 119$	$n = 20$	–
	Kyoto	2010	–	0.75 ***	0.31 ***
	Kyoto	2011	$n = 19$	–	0.61 **
	Akita	2010	$n = 119$	$n = 20$	–

*, ** and *** represent statistical significance at $P < 0.05$, 0.01 and 0.001, respectively, and n.s. represents not significant.

Table 4. Putative QTL, map position and genetic contribution for DSS in ST and OA RILs.

Population	Location	Year	Chromosome number	Linkage group	Peak position (cM)	Flanking[a)] markers	LOD score	Additive[b)] effect	R^2
ST RILs	Kyoto	2009	19	L	95.4	**CSSR116** – Sat_286	10.35	0.42	0.35
			19	L	96.8	**Sat_286** – Satt229	9.91	0.42	0.35
		2010	13	F	155.3	**Sat_197** – Sat_417	3.20	−0.21	0.13
			19	L	95.4	**CSSR116** – Sat_286	20.03	0.40	0.44
	Akita	2010	12	H	68.6	**Sat_206** – Satt302	3.01	0.24	0.08
			19	L	95.4	**CSSR116** – Sat_286	5.11	0.33	0.14
OA RILs	Kyoto	2010	3	N	77.5	**Satt521** – Satt237	4.86	0.18	0.15
	Akita	2010	3	N	45.3	**Sat_208** – Sat_033	3.59	0.16	0.08

a) Bold font represents a marker closer to peak position.

b) Additive effect represents the effect of allele of maternal parent against that of paternal parent.

Table 5. Putative QTL, map position and genetic contribution for days from sowing to R1 and node number produced after R1 in ST and OA RILs.

Trait	RIL	Location	Year	Chromosome number	Linkage group	Peak position (cM)	Flanking [a)] markers	LOD score	Additive [b)] effect	R^2
Days to R1	ST RILs	Kyoto	2009	6	C2	75.0	Satt277 – **Satt557**	23.17	−5.13	0.68
				10	O	116.5	**GMES4019** – Satt243	4.90	1.57	0.08
				19	L	95.4	**CSSR116** – Sat_286	10.05	2.80	0.19
			2010	6	C2	66.1	Satt457 – **Satt277**	26.82	−3.81	0.59
				6	C2	75.0	Satt277 – **Satt557**	46.96	−3.99	0.65
				10	O	116.5	**GMES4019** – Satt243	21.41	1.96	0.16
				19	L	104.8	Sat_286 – **Satt229**	19.90	1.99	0.16
		Akita	2010	6	C2	67.1	Satt457 – **Satt277**	23.58	−6.09	0.57
				6	C2	75.0	Satt277 – **Satt557**	45.61	−6.55	0.65
				10	O	115.2	Satt592 – **GMES4019**	25.28	3.72	0.22
				19	L	104.8	Sat_286 – **Satt229**	14.89	2.73	0.12
				19	L	108.4	**Satt229** – Satt373	14.52	2.64	0.11
	OA RILs	Kyoto	2010	6	C2	109.4	Satt322 – **Satt277**	7.67	2.73	0.41
				6	C2	126.8	**Satt557** – Satt307	20.81	3.99	0.65
				10	O	111.6	Satt592 – **GMES4019**	5.58	−1.50	0.12
				10	O	114.0	**GMES4019** – Sat_038	5.41	−1.54	0.12
		Akita	2010	6	C2	109.4	Satt322 – **Satt277**	8.58	2.21	0.24
				6	C2	122.8	**Satt557** – Satt307	18.27	2.62	0.28
				8	A2	49.7	**AW132402** – CSSR420	4.13	1.20	0.07
				10	O	102.4	Satt477 – **Satt592**	4.19	−1.17	0.06
				10	O	111.6	Satt592 – **GMES4019**	9.99	−1.84	0.15
				10	O	114.0	**GMES4019** – Sat_038	9.18	−1.78	0.14
Node production after R1	ST RILs	Kyoto	2010	19	L	95.4	**CSSR116** – Sat_286	72.29	5.37	0.89
	OA RILs	Kyoto	2010	19	L	112.3	**Sat_286** – Sat_184	75.05	−4.85	0.82
				19	L	114.7	**Sat_184** – Satt229	55.72	−4.74	0.79

a) Bold font means a marker closest to peak position.
b) Additive effect means the effect of allele of maternal parent against that of paternal parent.

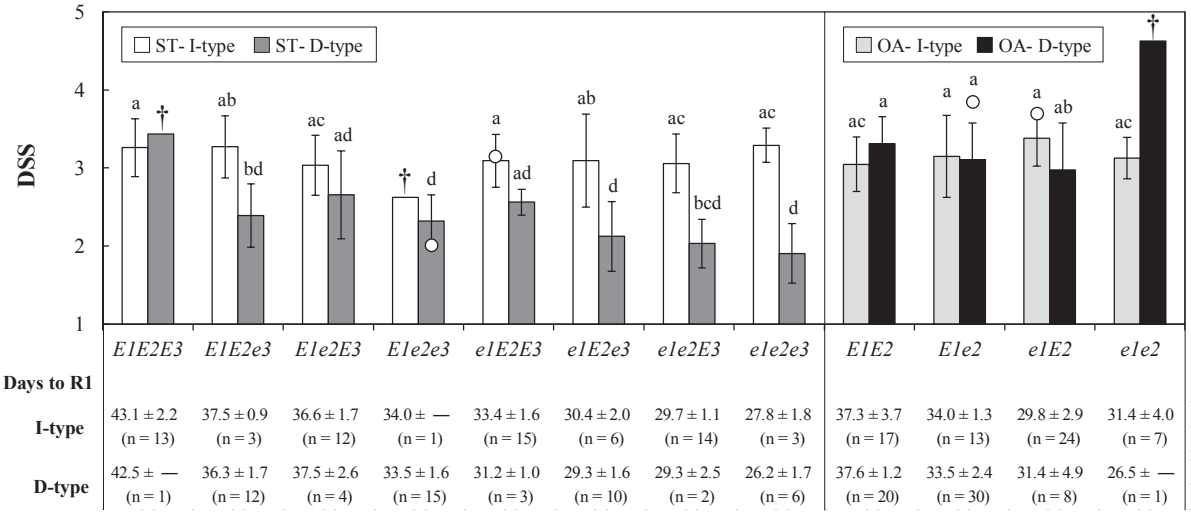
2. Variations of duration from sowing to flowering

At Kyoto, the durations from sowing to R1 were relatively constant in both years and ranged among lines from 25 to 48 days and from 24 to 42 days in ST and OA RILs, respectively (Table 1 and Fig. 3). At Akita, the durations were longer than those at Kyoto and ranged from 32 to 66 days and from 30 to 49 days in ST and OA RILs, respectively. The difference in the duration between the locations was larger in ST RILs in comparison with OA RILs. The durations from sowing to R1 in Stressland and Tachinagaha were 34 and 33 days at Kyoto in 2009, 33 and 34 days at Kyoto in 2010, 43 and 50 days at Akita in 2010, and 30 and 33 days at Kyoto in 2011, respectively. In Ohsuzu and Athow, the duration was 32 and 29 days, at Kyoto in 2010, 39 and 35 days, at Akita in 2010, and 33 and 27 days, at Kyoto in 2011, respectively.

3. QTL analyses for DSS, days from sowing to R1 and node production after R1

Two QTLs for DSS were detected in every test in ST RILs (Table 4) although not the same two QTLs in each test. However, one QTL for DSS was consistently mapped at the chromosomal position of 95.4 cM on chromosome (Chr.) 19, which was coincident with the *Dt1* locus. The Stressland (I-type) cultivar had a higher value of DSS. The other QTL mapped close to the *Dt1* locus found at Kyoto in 2009 was assumed to be due to the effect of the QTL at the *Dt1* locus and the recombination pattern of the lines used. Other QTLs were detected on Chr. 13 and 12 at Kyoto and Akita, respectively, in 2010, but they were inconsistent between experiments and explained only minor proportions of the total variance. In OA RILs, one QTL for DSS were mapped on Chr. 3 in each test at Kyoto and Akita in 2010. These QTLs did not co-locate, but their additive effects were similar.

There were several QTLs for days from sowing to R1



	E1E2E3	E1E2e3	E1e2E3	E1e2e3	e1E2E3	e1E2e3	e1e2E3	e1e2e3	E1E2	E1e2	e1E2	e1e2
Days to R1												
I-type	43.1 ± 2.2 (n = 13)	37.5 ± 0.9 (n = 3)	36.6 ± 1.7 (n = 12)	34.0 ± — (n = 1)	33.4 ± 1.6 (n = 15)	30.4 ± 2.0 (n = 6)	29.7 ± 1.1 (n = 14)	27.8 ± 1.8 (n = 3)	37.3 ± 3.7 (n = 17)	34.0 ± 1.3 (n = 13)	29.8 ± 2.9 (n = 24)	31.4 ± 4.0 (n = 7)
D-type	42.5 ± — (n = 1)	36.3 ± 1.7 (n = 12)	37.5 ± 2.6 (n = 4)	33.5 ± 1.6 (n = 15)	31.2 ± 1.0 (n = 3)	29.3 ± 1.6 (n = 10)	29.3 ± 2.5 (n = 2)	26.2 ± 1.7 (n = 6)	37.6 ± 1.2 (n = 20)	33.5 ± 2.4 (n = 30)	31.4 ± 4.9 (n = 8)	26.5 ± — (n = 1)

Fig. 4. DSS (upper figure) and days from sowing to R1 (lower table) of genotypes of ST and OA RILs segregating the alleles at the E1, E2, E3 and *Dt1* (I/D-type) loci at Kyoto in 2010. Genotype of each line was determined based on the alleles of SSR markers closest to the loci. The values of DSS and days from sowing to R1 are shown as mean ± standard deviation. Circles represent DSS values of the parental cultivars. †; Only one line belonged to the genotype group. The DSS values with different letters are significantly different at $P < 0.05$ by Tukey's test.

detected in both RILs (Table 5). In ST RILs, the consistent QTLs across the environments were located on Chr. 6, 10 and 19, corresponding to linkage groups (LG) C2, O and L, respectively, and these QTLs explained 65.9, 15.1 and 15.6% on average, respectively, of the total phenotypic variation in days from sowing to R1. In OA RILs, the consistent QTLs were mapped on Chr. 6 and 10, which accounted for 64.8 and 11.8%, respectively, of the total phenotypic variation at Kyoto, and 27.7 and 14.7%, respectively, of total phenotypic variation at Akita. The additive effects of the Tachinagaha and Ohsuzu alleles were positive for the QTL on Chr. 6 and negative for the QTL on Chr.10. The additive effect of the QTL on Chr. 19 was positive with the Stressland-derived allele in ST RILs. The QTLs of both RILs on Chr. 6 and Chr. 10 were commonly linked to markers, Satt557 and GMES4019, respectively. The QTL of ST RILs on Chr. 19 was mapped beside Satt229. The QTLs on Chr. 6, 10 and 19 were co-located with the *E1*, *E2* and *E3*, respectively (Bernard, 1971; Buzzell, 1971).

A QTL for node production after R1 was detected on Chr. 19 in both RIL populations, which indicated the accurate chromosomal positions of the *Dt1* locus (Table 5). The peak position of LOD for the unique QTL was at 95.4 cM in ST RILs, and this was in accordance with the LOD peak of a QTL for DSS. Moreover, 2 contiguous peaks were detected in OA RILs, but the LOD peaks were shaped by the effect of a single locus according to the directions and the additive effects of these QTLs. These QTL were closely linked to CSSR116 and Sat_286 for ST and OA RILs, respectively, and the markers were located close to the tagging marker (Satt006) for the *Dt1* locus by Molnar et al. (2003).

4. Relationship between days from sowing to R1 and DSS

After the classification of the genotype of each line on the *E1* (*E1/e1*), *E2* (*E2/e2*), *E3* (*E3/e3*) and *Dt1* (*Dt1/Dt1*, I/D-type) loci, DSS in each genotype group at Kyoto in 2010, where the frequency exhibited normal distribution, was compared (Fig. 4). The genotype of each line was determined according to the alleles of Satt557, GMES4019 and Satt229 for the *E1*, *E2* and *E3* loci, respectively. The alleles with positive effects were represented with capital letters, and the alleles with negative effects were with lower-case letters. Stem determination type was determined based on the phenotypic value of node number produced after R1, namely the lines produced more than 5 nodes after R1 were classified into I-type. In ST RILs, I-type lines had large values of DSS compared with D-type lines, and the difference was evident in early flowering genotype. On the other hand, the difference of DSS between the stem determination types was not significant in OA RILs. DSS of OA RILs with D-type growth habit was larger than that of ST RILs with D-type growth habit even with the same flowering time.

The correlation between days from seed sowing to R1 and DSS was examined (Table 6 and 7). These analyses showed positive correlations between the traits in D-type lines in both RIL populations in most of the tests. The stem growth habit of each line was determined based on the phenotypic value of node production after R1, or the SSR allele linked to this QTL for the lines that were not tested at Kyoto in 2010. Although the correlation in D-type lines of OA RILs was not statistically significant at Kyoto in

Table 6. Pearson's correlation coefficients and Spearman's rank correlation coefficients between days from seed sowing to R1 and DSS in ST RILs.

Location	Year	I/D-type	*n*	Pearson's [a] correlation coefficient	Spearman's [a] rank correlation coefficient
Kyoto	2009	Both	80	0.27 *	0.18 n.s.
		I-type	48	0.22 n.s.	0.26 n.s.
		D-type	32	0.42 *	0.38 *
Kyoto	2010	Both	120	0.36 ***	0.31 ***
		I-type	67	0.15 n.s.	0.16 n.s.
		D-type	53	0.51 ***	0.39 **
Kyoto	2011	Both	20	0.16 n.s.	0.17 n.s.
		I-type	9	−0.41 n.s.	−0.51 n.s.
		D-type	11	0.19 n.s.	0.19 n.s.
Akita	2010	Both	118[b]	0.29 **	0.16 n.s.
		I-type	65[b]	−0.08 n.s.	−0.03 n.s.
		D-type	53	0.45 ***	0.36 **

a) *, ** and *** represent statistical significance at $P < 0.05$, 0.01, 0.001, respectively, and n.s. represents not significant.
b) Two indeterminate lines did not reach R8.

Table 7. Pearson's correlation coefficients and Spearman's rank correlation coefficients between days from seed sowing to R1 and DSS in OA RILs.

Location	Year	I/D-type	*n*	Pearson's [a] correlation coefficient	Spearman's [a] rank correlation coefficient
Kyoto	2010	Both	120	0.01 n.s.	0.01 n.s.
		I-type	61	−0.19 n.s.	−0.19 n.s.
		D-type	59	0.23 n.s.	0.31 *
Kyoto (Excluding OA_156)	2010	Both	119	0.06 n.s.	0.04 n.s.
		D-type	58	0.39 **	0.38 **
Kyoto	2011	Both	20	0.55 *	0.52 *
		I-type	9	0.06 n.s.	−0.10 n.s.
		D-type	11	0.82 **	0.78 **
Akita	2010	Both	231	0.28 ***	0.33 ***
		I-type	141	0.25 **	0.28 ***
		D-type	85	0.46 ***	0.50 ***

a) *, ** and *** represent statistical significance at $P < 0.05$, 0.01, 0.001, respectively, and n.s. represents not significant.

2010, the relationship became evident when the OA_156 line data was removed. In contrast, the I-type lines of both RIL populations showed indistinct relations in most of the tests.

Discussion

1. DSS variation and environmental effects

This study evaluated DSS, a measure of GSD severity, for two RIL (ST and OA RILs) at two different locations, Kyoto and Akita. The results showed that DSS of each line differed with the location and that most of the lines of both RILs had higher values at Akita compared to those at Kyoto (Table 1 and Fig. 1). Because many lines reached the maximum value of DSS (DSS5) at Akita and thus the frequency of DSS showed a skewed distribution, it was assumed that it was difficult to detect the genotypic differences of DSS there. In addition, the fact that many lines normally senesced at Akita was considered to cause the interaction between location and population, and between location and I/D-types. Konaka and Takahashi

(1965) and Furuya et al. (1988) reported that cultivars developed in the northern region of Japan showed GSD symptoms when they are cultivated in the southern region of Japan. The experimental site at Akita (lat. 39°32′N) is located approximately 4°1′ north of Kyoto (lat. 35°2′N). The results of the present study agreed with these previous results because the genotypes adapted to the latitude of Akita, as shown by the reduced DSS scores at Kyoto. However, the susceptibility to GSD of each line was consistent according to the correlation and rank-correlation analyses for location and year (Table 2 and 3). These results suggested that the genetic approach was effective for the improvement of resistance to GSD.

DSS of the I-type parent of ST RILs, Stressland, was higher than that of the D-type parent, Tachinagaha, in every test (Table. 1). In OA RILs, DSS values of the I-type parent, Athow, and the D-type parent, Ohsuzu, were similar, and were as high as that of Stressland. D-type plants are known to exhibit more severe symptom of GSD than I-type plants (Pierce et al., 1984). If the effect of stem determination, which was governed by the *Dt1* locus, on DSS was consistent, it was presumed that the lower DSS of Tachinagaha was caused by the allele, and Ohsuzu possessed the genetic factors that modified the adverse effect of the D-type allele. The variations of DSS in both ST and OA RILs were considerably wide in every test as compared with the differences of DSS between the parental cultivars, thus both populations showed transgressive segregation. Therefore, it is likely that the susceptibility to GSD is governed by multiple loci and that the parental cultivars carried alleles that both positively and negatively affect DSS.

2. QTL mapping for flowering time and node number produced after R1

Three consistent QTLs for days from sowing to R1 were detected on Chr. 6, 10 and 19 in ST RILs, and two consistent QTLs for days from sowing to R1 were detected on Chr. 6 and 10 in OA RILs (Table 5). QTLs mapped on the same chromosomes were assumed to be common loci between the two RIL populations. Another consistent QTL was mapped on Chr. 19 in ST RILs. Several QTLs for days to R1 have been previously reported (Cober et al., 1996 ; Mansur et al., 1996; Yamanaka et al., 2000; Tasma et al., 2001; Abe et al., 2003). The QTLs detected on Chr. 6, 10 and 19 corresponded to *E1*, *E2* and *E3* loci, respectively (Bernard, 1971; Buzzell, 1971). The genes on these loci have been genetically and molecularly identified. *E1* and *E3* gene are involved in photoperiod response, and a clock-related protein is encoded at the *E2* locus (Watanabe et al., 2009; Watanabe et al., 2011; Xia et al., 2012). Thus, phenotypic polymorphism was recognized for the *E1*, *E2* and *E3* in ST RILs and for the *E1* and *E2* in OA RILs. These QTLs explained 96.6% of the total phenotypic variance on average in ST RILs, and 76.6 and 42.4% in Kyoto and Akita in OA RILs. The additive effects of the *E1* and *E2* hardly differed with the location in OA RILs, while all of the consistent QTLs of ST RILs had larger additive effects at Akita. This result is in accord with the significant interaction between population and location by ANOVA (Table 1). A major difference between the populations was the segregation at the *E3* locus and this segregation might provide the cause of the interaction.

Stem growth habits segregated into I- and D-types in both RIL populations. A QTL for node number produced after R1 was clearly mapped in each population on Chr. 19 with an R^2 value of 0.8 or higher, and this QTL corresponded to the *Dt1* locus (Table 5). At this locus, an ortholog of Arabidopsis *TERMINAL FLOWER, GmTfl1b*, is present, which controls the emergence of the terminal raceme at the shoot apical meristem (Liu et al., 2010).

3. Stem determination and DSS

A strong QTL for DSS was mapped at the *Dt1* locus in ST RILs, and the DSS scores of the I-types were higher than those of the D-types both at Kyoto and Akita (Table 4 and 5), which agreed with the result reported by Pierce et al. (1984). In OA RILs, however, no QTLs were detected near the *Dt1* locus even though this population showed segregation in stem growth habits. These results were also observed as an interaction between population and I/D-type by ANOVA (Table 1). Regarding these inconsistent results, three different possibilities can be hypothesized. The first possibility is that the responsible gene is not *GmTfl1b* but rather another closely linked gene, which would suggest that stem growth habit does not affect GSD and that the GSD trait of Tachinagaha can be improved by recombination of this allele without conversion to I-type. The second possibility is that *GmTfl1b* interacts with other genetic factor(s) in relation to the occurrence of GSD. The third possibility is that *GmTfl1b* has an additional function other than the segregation of stem growth habit. The *Dt1* locus is also known to influence various traits such as leaf morphological traits (Tanaka et al., 2009), and expression of *GmTfl1b* is also observed in the root (Liu et al., 2010). Therefore, it is possible that some mutation within this gene acts on DSS without the conversion of stem growth habit. Progeny of a cross between Tachinagaha and Ohsuzu or plants transgenic for *GmTfl1b* should be analyzed to verify these hypotheses. These results suggest that the parental cultivars of the OA RILs carried the effective genetic factor(s) and it was possible to improve resistance to GSD without conversion of stem growth habit even if the responsible gene at the *Dt1* locus was identical to *GmTfl1b*.

4. Earliness of flowering and DSS

Pierce et al. (1984) reported that D-type genotypes with

early flowering alleles (*e1e1/e2e2/dt1dt1*) show severe symptoms of GSD. In the present study, DSS was used as a measure of the severity of GSD, but we did not detect any QTLs for DSS at flowering-time loci, such as *E1, E2* and *E3*, in any test. However, the classification by the alleles at the *E1, E2, E3* and *Dt1* (I/D-type) loci, which was based on the alleles of the flanking markers, showed the trend that D-type with early flowering allele had lower DSS although the difference of DSS between early and late flowering genotypes was not significant by the Tukey's test (Fig. 4). Moreover, our results illustrated the successive change of DSS along with earliness of flowering in D-type lines, although this trend was not general in I-type lines (Table 6 and 7). This result indicated that each locus controlling flowering time would have a weak effect on the total variance of DSS and that other weak QTLs and/or environmental factors were concealed. Furthermore, it is likely that the responsible gene at the *Dt1* locus and earliness of flowering interact with each other in relation to the occurrence of GSD. Interestingly, this particular relationship was observed even in OA RILs even though no QTLs were detected at the *Dt1* locus. Therefore, the OA RILs population was assumed to have a similar genetic factor influencing the variation of DSS at this locus. Although this interactive effect was observed in both RIL populations, we could not determine whether *GmTfl1b* itself or a closely linked gene is involved in the occurrence of GSD.

The date of R1 in Ohsuzu, D-type parent of OA RILs, was not very different from that of Tachinagaha, D-type parent of ST RILs, but DSS in Ohsuzu was markedly greater than that in Tachinagaha (Table 1). Similarly, OA RILs tended to have higher values of DSS compared to ST RILs with the same flowering period though earliness of flowering influenced DSS of the determinate genotypes in both ST and OA RILs (Fig. 4). These results indicated that the effective genetic factor(s) of OA RILs was independent of flowering time, and thus it enabled enhancement of the DSS of D-type genotypes without altering flowering time.

The flowering date in short-day plant species is delayed in higher latitude areas. At Akita, the pre-flowering period in each line was longer compared with that at Kyoto because Akita is located at a higher latitude than Kyoto. The delay of flowering date might explain the difference in DSS in D-type lines with the location, but it is unlikely that this effect can be applied to I-type lines. Because early planting is likely to cause GSD (Isobe et al., 2011), geographical or environmental effects, such as planting date and day-length, may have impacts on the occurrence of GSD.

5. OA_156 is a unique line in OA RILs

The OA_156 line was considered a unique line in this study because it had an unusually high DSS at Kyoto in 2010 even though it was a D-type line with an early flowering genotype (*e1e2dt1*, Fig. 4). However, the DSS score of the OA_156 line was only 1.3 in an additional test in 2011, which was much lower than the score at Kyoto in 2010. Lethal yellowing diseases, such as Phytophthora stem rot, can blight the soybean plant (Dorrance et al., 2003). Thus, it is likely that the score in 2010 may have been increased by some undetected disease. Excluding this line, the correlation coefficients of DSS of each line with the location and year were higher, and the relationship between DSS and earliness of flowering became more apparent (Table 3 and 7). Although this irregular procedure did not affect the QTL analysis result for DSS, attention should be paid to the influence of diseases to avoid overlooking important signals that lead to improvement of the GSD trait.

6. Effects of other QTLs on DSS

In ST RILs, QTLs were detected on Chr. 13 (Kyoto-2010) and Chr. 12 (Akita-2010) other than the *Dt1* locus, and they were linked to the marker loci, Sat_197 and Sat_206, respectively (Table 4). In these regions, several QTLs for plant height and seed yield have been previously reported (Specht et al., 2001; Reinprecht et al., 2006; Abdel-Haleem et al., 2012). In OA RILs, QTLs found on Chr. 3 were mapped near with Satt521 and Sat_208. Several researchers have reported QTLs related to seed yield in these regions (Kabelka et al., 2004; Chen et al., 2007). Leopold et al. (1959) demonstrated that the removal of reproductive organs delayed plant senescence, and many researchers reported similar results (Wittenbach, 1982; Crafts-Brandner et al., 1984). Furthermore, Noodén (1980) suggested the presence of senescence factor produced in the seed. Sinclair and de Wit (1975) related the high nitrogen demand of seeds to 'self-destruction' of vegetative organs to produce translocatable nitrogen. Because the QTLs for DSS found in the present study co-located with yield-related QTLs, seed or pod production might have affected the degree of GSD in a similar way.

The effect of a QTL for DSS detected at Kyoto in 2010 in OA RILs was analyzed after classification based on the allele of the Satt521 marker, which was tightly linked to this QTL. The effect of the QTL was evident only in D-type lines though the interaction between stem determination and this QTL allele was not significant ($P = 0.11$ without OA_156) by ANOVA (Fig. 5). Although the effect of the genetic factor at the *Dt1* locus was not confirmed in OA RILs by the QTL analysis for DSS, the QTL on Chr. 3, as well as flowering time, may interact with the genetic factor at the *Dt1* locus. Moreover, the segregation pattern of DSS by this marker allele had a commonality with the difference between ST and OA RILs. Such a QTL might explain the difference of DSS between ST and OA RILs as well as between Tachinagaha and Ohsuzu, and might

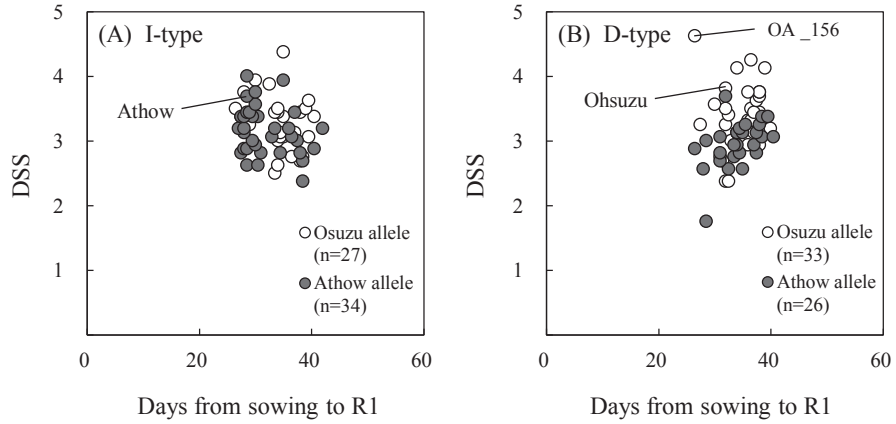


Fig. 5. Relationship between days from seed sowing to R1 and DSS in (A) I-type and (B) D-type of OA RILs at Kyoto in 2010. Open and closed circles represent the genotypes with Ohsuzu and Athow-derived allele at Satt521, respectively.

influence the effect of the stem determination gene on DSS. In the case where *GmTfl1b* certainly engages in the occurrence of GSD, this kind of genetic factor may explain the inconsistent results of the QTL analyses between the populations segregating stem growth habits.

The QTL analysis for DSS separately for I- and D-types within each population was incrementally conducted (data not shown). Several QTLs were detected except for the I-type of ST RILs at Kyoto in 2010 and D-type of OA RILs at Akita in 2010. However, no QTLs were co-located between the stem determination types. A QTL in the D-type of ST RILs found at Kyoto in 2010 and at Akita in 2010 was located near the *E1* locus. A QTL from the OA RILs was identified on Chr. 3 at Kyoto in 2010 using all lines tested but was only found in D-type lines. These results suggested the interactions between the genetic factor at or near the *Dt1* locus and other genetic factors in other places in the genome affect the occurrence of GSD. Some QTLs detected using all lines were not found in both I- and D-types. Possibly, the reduction of population size by separating the stem determination types was the reason for this. On the other hand, in the D-type ST RILs, a consistent QTL at Kyoto and Akita was detected on Chr. 13, which was not found by the normal QTL analysis at Akita in 2010. Therefore, to investigate the genetic factors that influence DSS of both I- and D-types and that involve complex interactions, a greater number of lines need to be evaluated. However, in populations not segregating for stem determination and flowering time, evaluation of fewer lines may be effective in detecting QTLs for the resistance to GSD.

Conclusions

The effects of stem determination and earliness of flowering on the severity of GSD under different genetic backgrounds and environments were investigated using two recombinant inbred lines. For the assessment of severity of GSD, the degree of synchronous senescence (DSS) of vegetative and reproductive organs proposed by Furuya and Umezaki (1993) was employed. Although the DSS of each line differed with the location, genetic improvement of resistance to GSD was considered of value because of close correlations of DSS with the location and year. A series of analyses did not confirm the effect of stem determination on the occurrence of GSD, because the advantage of I-type, which has been previously reported, was not observed in one population (OA RILs). Earliness of flowering clearly influenced DSS but the effect was dependent on the allele at or near the *Dt1* locus, which governs stem determination. The genetic factor at the *Dt1* locus and earliness of flowering interacted with other genetic factor(s), which suggested the presence of complex genetic control of the occurrence of GSD. The D-type lines of one population tended to have higher values of DSS as compared to the D-type lines of the other population and the difference was independent of earliness of flowering. Therefore, it may be possible to improve resistance to GSD without altering stem growth habit and flowering time.

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