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Major QTLs associated with green stem disorder insensitivity of soybean (*Glycine max* (L.) Merr.)

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Green stem disorder (GSD) is one of the most serious syndromes affecting soybean (*Glycine max*) cultivation in Japan. In GSD, stems remain green even when pods mature. When soybean plants develop GSD, seed surfaces are soiled by tissue fluid and seed quality is deteriorated during machine harvesting. We performed quantitative trait locus (QTL) analyses for GSD insensitivity using recombinant inbred lines (RILs; $n = 154$) derived from a cross between an insensitive line ('Touhoku 129') and a sensitive leading cultivar ('Tachinagaha') during a 6-year evaluation. Three effective QTLs were detected. The influences of these QTLs were in the following order: *qGSD1* (LG_H) > *qGSD2* (LG_F) > *qGSD3* (LG_L). At these three QTLs, 'Touhoku 129' genotypes exhibited more GSD insensitivity than 'Tachinagaha' genotypes. The lower incidence of GSD for 'Touhoku129' was attributable primarily to these three QTLs because RILs harboring a 'Touhoku 129' genotype at the three QTLs exhibited a GSD incidence similar to that of 'Touhoku 129.' Although a limitation of this study is that only one mapping population was evaluated, this QTL information and the flanking markers of these QTLs would be effective tools for resolving GSD in soybean breeding programs.

Key Words: *Glycine max* (L.) Merr., soybean, green stem disorder, *qGSD1*, maturity gene, QTL, DNA marker.

Introduction

During soybean (*Glycine max* (L.) Merr.) cultivation, green stem disorder (GSD) is a serious problem. In GSD, plant stems remain green even when pods mature. When soybean plants develop GSD, seed coat surfaces are soiled by tissue fluid and seed quality is deteriorated during machine harvesting (Hill *et al.* 2006, Morita *et al.* 2006).

In Japan, soybeans are used directly in foods such as *natto*, *nimame*, and *tofu* without processing, and not for oil extraction or feed. Thus, production of soybeans with high seed quality is essential. Deteriorated seed quality results directly in lower incomes for farmers. To avoid deterioration due to GSD, farmers tend to leave soybeans in the field until stem moisture decreases to <40%. However, because leading cultivars in Japan are mostly prone to shattering, yield loss can readily occur. Thus, farmers are in a dilemma between seed quality and soybean yield. Genetic improvement in GSD insensitivity is a promising approach for resolving this issue.

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Accurate trait evaluation is the basis of genetic analysis and reliable screening for breeding programs. The evaluation of GSD insensitivity and the screening of soybean plants for this trait in breeding programs would be facilitated by the development of DNA markers associated with and linked to GSD insensitivity.

Varietal differences in GSD insensitivity and related symptoms have been reported (Furuya and Umezaki 1993, Hill *et al.* 2006, Matsumoto *et al.* 1986, Mochizuki *et al.* 2005, Pierce *et al.* 1984). Indeterminate growth-type materials exhibit more GSD insensitivity than determinate growth-type materials (Hajika 2005, Pierce *et al.* 1984). However, most of the leading Japanese cultivars are of the determinate growth type. The indeterminate growth trait is controlled by a single gene (Woodworth 1933), which has been cloned and characterized by Tian *et al.* (2010). Hajika (2005) reported QTLs associated with GSD insensitivity in addition to the indeterminate growth QTL, although their explanation was variable in two experiments or was minor in effect compared to that of the indeterminate growth QTL. A precise, extensive QTL analysis is necessary for developing DNA markers to identify GSD insensitivity.

GSD of the leading cultivar 'Tachinagaha' has become a serious problem in recent years in the Kanto region of

Japan. However, a breeding line ('Touhoku 129') has been reported as green stem insensitive (Hajika 2005) and is a promising material for soybean breeding programs targeting GSD insensitivity.

The objectives of this study were to (1) conduct QTL analysis of GSD insensitivity- and maturity-related traits using recombinant inbred lines (RILs) derived from a cross between two determinate growth parents 'Tachinagaha' and 'Touhoku129' with different GSD sensitivity and (2) determine the effects of the detected QTL regions for several agronomic traits to assess their usefulness in soybean breeding programs.

Materials and Methods

Plant materials

The soybean breeding line 'Touhoku 129 (JP240542)' and the cultivar 'Tachinagaha (JP67666)' and their F_2 progeny were used for this study. 'Touhoku 129' has been used for its soybean mosaic virus (SMV) resistance, GSD insensitivity, and high yield. 'Satonohohoemi' (Kikuchi *et al.* 2011) was bred from the progeny of this line. 'Tachinagaha' is one of the leading Japanese cultivars well known for large seed size and good quality, but it is GSD sensitive. RILs (n = 154) derived by single-seed descent from F_2 plants of a cross between 'Touhoku 129' and 'Tachinagaha' were used for trait evaluations and QTL analysis. Both parents had determinate growth patterns. We considered that RILs were fully (>99%) inbred in the F_6 generation. Seed from each F_6 line was bulk harvested, and the F_6 bulks were used for trait evaluation and DNA extraction.

RILs that segregated for a region containing the most effective QTL for GSD insensitivity were selected and used to construct heterogeneous inbred families (HIFs) (Tuinstra *et al.* 1997). Almost all genomic regions were believed to have been fixed to homozygosity during single-seed descent, with small regions remaining unfixed. Each individual in HIFs had a similar genetic background. Individual HIFs were used for evaluating the effects of QTL regions. The number of individuals in the HIFs in 2009 was 49 and included parental and heterozygous genotypes in the QTL regions. The seeds of each HIF plants were sown as lines in 2010. Only plants with the two parental genotypes were used for trait evaluation in 2009 and 2010. A line of the heterozygous genotype in 2009 was planted in 2010. Each plant in the line was separately harvested and grouped by genotype in the specific QTL regions in 2010. The newly grouped lines with parental genotypes were then increased and used for trait evaluation in 15 replications in 2011.

Growth conditions

Agronomic traits were evaluated from 2005 to 2011 at the Yawara experimental field, Miraidaira, Ibaraki, Japan (36°01′N, 140°05′E) and the Kannondai experimental field, Tsukuba, Ibaraki, Japan (36°00′N, 140°02′E). Sowing days and sampling generations are described in Table 1. The soil types were andosol (volcanic ash soil) at both sites. For RILs and HIFs, inter-row and -hill intervals were 0.7 and 0.13 m, respectively, at Yawara and 0.7 and 0.1 m, respectively, at Kannondai. RILs were planted in 1.5-m rows without replication. Two seeds were sown in every hill and plants were thinned to one plant after primary leaf expansion. HIFs were planted as individual plants in 2009, in 2.0-m rows in 2010, and in 0.65-m rows in 2011. Fertilizer was applied prior to planting with N : P_2O_5 : K_2O at 3 : 20 : 10 (g/m^2) at Yawara and 3 : 10 : 10 (g/m^2) at Kannondai. Herbicides (alachlor and linuron) were sprayed on the ground immediately after sowing. Inter-tillage and earthing-up were performed 1 month after sowing. Insecticides were sprayed every week after the first flower anthesis to the end of

Table 1. Experimental sites, growth conditions, and agronomical traits

Experimental sites	Year	Sowing date	Materials (generation)	GSD index[a] (0–5)	Number of days to flowering[b] (day)	Seed-filling period[c] (day)
Yawara Experimental Field	2005	14-Jun	RIL (F6)	○[d]		
	2006	27-Jun	RIL (F7)	○	○	○
	2007	26-Jun	RIL (F8)	○		
	2008	25-Jun	RIL (F9)	○		
	2009	25-Jun	RIL (F10)	○	○	○
	2009	26-Jun	HIF (F8)	○		
	2010	22-Jun	RIL (F11)	○	○	○
	2011	28-Jun	HIF (F10)	○	○	○
Kannondai Experimental Field	2006	13-Jun	RIL (F7)	○	○	○
	2007	12-Jun	RIL (F8)	○	○	○
	2008	11-Jun	RIL (F9)	○	○	○
	2009	5-Jun	RIL (F10)	○	○	○
	2010	3-Jun	HIF (F9)	○	○	○

[a] GSD index was classified into six levels; 0: GSD tolerant, 5: GSD intolerant.

[b] Number of days to flowering was defined as the number of days from the sowing date to the first flowering date.

[c] Seed-filling period was defined as a differentiation between the first flowering date and the maturity date. The maturity date was defined as a date of the day when 80% plants had matured pods in a plot.

[d] Circle indicates for evaluation conducted in the experiment.

September until injurious insects decreased. A miticide was sprayed at emergence, but bactericides or fungicides were not used.

Evaluation of GSD insensitivity

GSD insensitivity of plant materials was evaluated by visual inspections at pod maturation in the experimental fields. We used the GSD index to indicate GSD insensitivity during each experiment. Furuya and Umezaki (1993) reported evaluation standards for the GSD index. They described the GSD index by scoring non-uniformity of maturity between stems and pods at five levels.

In the expectation of handling many lines produced by the breeding program, we adapted these evaluation standards to evaluate the GSD index qualitatively at six levels with respect to stem and leaf conditions at pod maturity, as follows: 0: leaflets and leaf stems had dropped off, and the stem was dry and brown; 1: leaflets and leaf stems had dropped off, and the stem was moist and yellow; 2: leaflets and leaf stems had dropped off, and the stem was moist and faded green; 3: leaflets and leaf stems had dropped off, and the stem was vivid green; 4: most leaflets had dropped off, part of the leaf stems remained, and the stem was vivid green; and 5: most leaflets remained, and the stem was vivid green.

The GSD index of each line was assigned on the basis of the most common level observed for individual plants of the line and was increased by one level when a plant displaying a higher level than the most common one was included. Sterile plants or plants showing few pods and plants that had died from disease before first-pod maturity were omitted from the evaluation. We defined apparent GSD sensitivity as ≥3 GSD index. GSD incidence was defined as the percentage of experiments in each line displaying a GSD index of ≥3 in all experiments.

Evaluation of other agronomic traits

Plant materials were also evaluated for the dates of first flower anthesis and pod maturity. The first flowering date was defined as the date of first anthesis (R1; Fehr and Caviness 1977) for 50% of the plants in a plot. The number of days to flowering (NDF) was defined as the number of days from the sowing date to the first flowering date. The maturity date was defined as the date when 80% of the pods in a plot had matured. The number of days to maturity (NDM) was defined as the number of days from the sowing date to the maturity date. These definitions of NDF and NDM include the period before germination. The seed-filling period (FP) was defined as the difference between the first flowering date and maturity date. Total seed weight, 100-seed weight, and seed protein and oil contents were recorded for HIFs in some experiments, as shown in Table 4. Total seed weight of HIFs was evaluated for individual plants in 2009 and for whole plots in 2010 and 2011. Seed protein and oil contents were determined using a near-infrared spectrophotometer (Infratec 1241 Grain Analyzer; FOSS Tecator AB, Höganäs, Sweden). Estimated total seed number was calculated as total seed weight (g/m^2)/one seed weight (g)/number of plants ($plants/m^2$).

SSR marker detection

Total genomic DNA was extracted from young leaves of RILs in the F_9 generation using Biorobot EZ1 (Qiagen, Valencia, CA, USA) or Biosprint 96 kits (Qiagen). Marker panels covering the whole soybean genome (Sayama *et al.* 2011) were used to determine RIL genotypes. To determine a marker genotype, multiplex polymerase chain reaction (PCR) was performed using a 5.5-μl reaction mixture [50 nM of each fluorescent-labeled primer pair, 5 ng of total genomic DNA, and 2.5 μl of 2× Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany)] and a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Amplification and detection of the resulting amplicons using a fluorescence-based DNA sequencer were performed following the method of Sayama *et al.* (2011).

To fill gaps in the linkage map and to determine HIF genotypes, we used additive codominant markers (Supplemental Table 1; Hisano *et al.* 2007, Hwang *et al.* 2009, Xu *et al.* 2013). Genomic DNA was extracted from young leaves following the method of Mori *et al.* (2003). Unexpanded young leaves with a lamina length of 1 cm were crushed in 400 μl extraction buffer [0.1 M Tris-HCl (pH 8.0), 0.05 M EDTA (pH 8.0), 0.5 M NaCl, 0.043 M SDS, and 0.01 M dithiothreitol]. Samples were centrifuged (3000 × *g*, 10 min), and 200 μl of the supernatant was mixed with 100 μl of 5 M potassium acetate. The samples were centrifuged again (3000 × *g*, 10 min), and 200 μl of the supernatant was mixed with 500 μl of ethanol. The samples were centrifuged again (3000 × *g*, 10 min), and the pellets of genomic DNA were washed with 500 μl ethanol. Finally, genomic DNAs were diluted to 10 ng/μl. PCR was performed using sterilized distilled water (4.5 μl), dNTP (0.5 μl), 3.0 pmol/μl of a non-fluorescent labeled primer pair (1.5 μl), Takara Ex Taq buffer (1.0 μl; Takara Bio Inc., Tokyo, Japan), Takara Ex Taq (0.05 μl; Takara Bio Inc.), and template genomic DNA (2.5 μl). After an initial denaturation at 95°C for 2 min, we used 33 cycles of denaturation at 92°C for 1 min, annealing at 58°C for 1 min, and extension at 68°C for 1 min, followed by a final extension at 72°C for 7 min using Mastercycler ep 384 (Eppendorf, Hamburg, Germany). PCR products were detected in polyacrylamide gels following the method of Benitez *et al.* (2010).

Construction of a linkage map and QTL analysis

AntMap (Iwata and Ninomiya 2006) was used to construct a linkage map using the Kosambi map function. Linkage group (LG) nomenclature followed Song *et al.* (2004).

QTL analysis was performed with R/QTL (Broman *et al.* 2003) using parametric interval mapping for NDF and FP. Non-parametric interval mapping was used for the GSD index and GSD incidence because their respective frequency

distributions were ordinal and non-normally distributed. These trait values were recorded as discontinuous values and percentages, respectively. QTL analyses were performed separately for each of the experiments conducted over two locations and 6 years (Table 1) for all traits, except for GSD incidence, which was only one evaluated value for each RIL calculated from multiple scores over all experiments.

To confirm the effects of the detected QTLs, RILs were grouped by the genotypes of the markers closest to these QTLs, and differences in GSD incidence between the groups was analyzed. To evaluate the influences of the most significant QTL on agronomic traits, HIFs were also grouped by the genotypes of the marker closest to the QTL peak position. Average values among genotypes for the GSD index and other agronomic traits were compared. Statistical comparisons between groups were performed by the Wilcoxon test for discontinuous variables and by *t* test for continuous GSD using SPSS 17.0 (SPSS 2008; SPSS Inc., Tokyo, Japan). A p value of <0.05 was considered significant.

Results

QTLs for GSD insensitivity and maturity-related traits

A total of 220 markers including 217 SSR markers, two morphological markers (flower color and leaflet shape), and an allele-specific DNA marker for *E3* (Xu *et al.* 2013) were mapped. By comparison with a reference map (Song *et al.* 2004), we found that five genomic regions had no polymorphic markers, resulting in splitting of single chromosomes into different linkage groups.

GSD incidence and the average value of the GSD index for each RIL in Yawara and Kannondai are shown in Fig. 1. GSD incidence and the average value of the GSD index for each experiment were higher for Yawara than for Kannondai.

Two QTL regions were detected for GSD incidence and were designated *qGSD1* (LG_H) and *qGSD2* (LG_F) (Table 2), and six QTL regions were detected for the GSD index (Table 2). Among QTLs detected for the GSD index, a QTL region in LG_L was repeatedly detected in two experiments and was designated *qGSD3* (LG_L) (Table 2). To exclude the detection of false-positive QTLs, QTLs other than these three QTLs were not named or further analyzed because they were not detected repeatedly and their effects on GSD insensitivity were assumed to be lower than those of the three major QTLs.

The effects of the three major QTLs for GSD incidence were evaluated in a subsequent analysis for comparisons between the groups classified by these QTL genotypes. The strength of the QTL effects for GSD incidence were in the

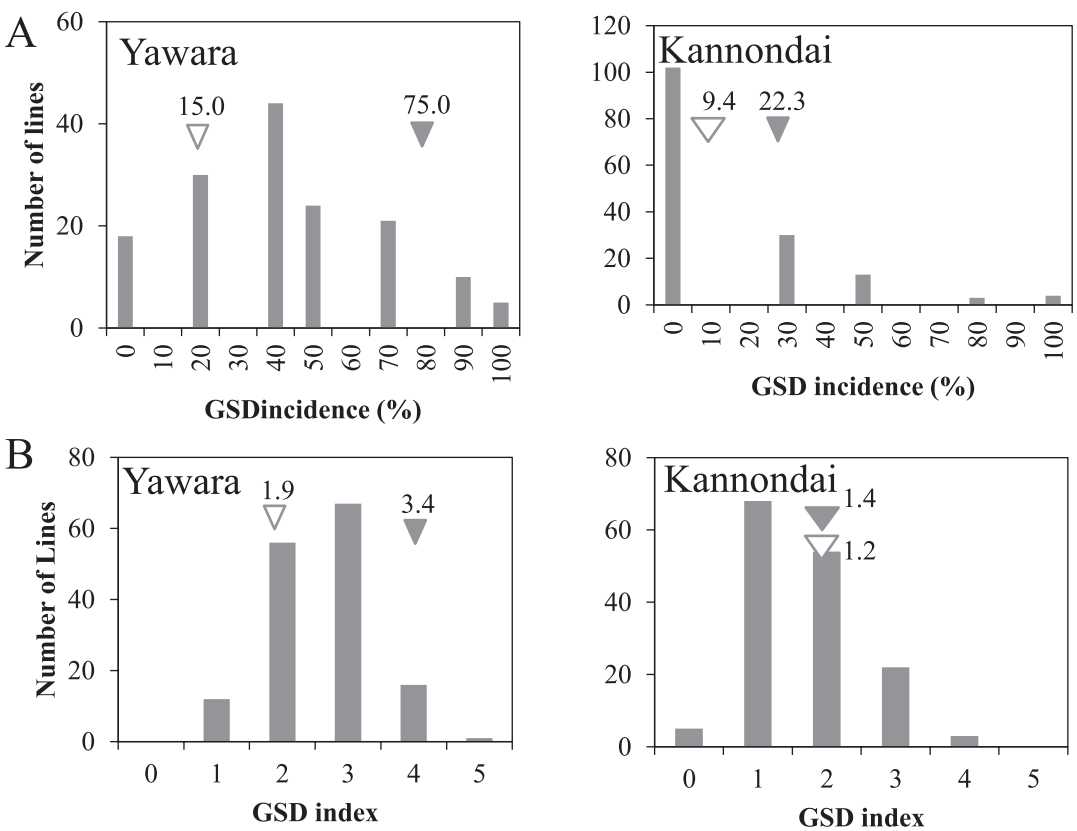

Fig. 1. Green stem disorder (GSD) incidence and average values of the GSD index for each RIL derived from a cross between the soybean breeding line 'Touhoku 129' and the leading cultivar 'Tachinagaha' in Yawara (n = 6 years) or Kannondai (n = 4 years). A: GSD incidence, B: Average values of the GSD index, ▽: 'Touhoku 129', ▼: 'Tachinagaha'. Numbers above triangles indicate parental values for each trait.

Table 2. QTLs detected for the GSD index, GSD incidence, number of days to flowering, and seed-filling period for RILs derived from a cross between the soybean breeding line 'Touhoku 129' and the leading cultivar 'Tachinagaha'

Traits	Experimental sites (Year)	Linkage groups	DNA markers closest to the peak position	Peak position (cM)	LOD	QTL	Additive effect[e]	R^2
GSD incidence[a] (%)		F	Satt114	89	3.2	*qGSD2*	+	
		H	GMES6355	70	9.8	*qGSD1*	+	
GSD index[b] (0–5)	Kannondai (2009)	F	Flower color	30	4.4		–	
	Yawara (2009)	F	Satt114	80	2.9	*qGSD2*	+	
	Yawara (2005)	H	GMES6355	70	3.6	*qGSD1*	+	
	Kannondai (2006)	H	GMES6355	70	5.2	*qGSD1*	+	
	Kannondai (2007)	H	GMES6355	71	7.5	*qGSD1*	+	
	Yawara (2007)	H	Satt253	72	2.9	*qGSD1*	+	
	Kannondai (2008)	H	GMES6355	70	7.8	*qGSD1*	+	
	Kannondai (2009)	H	GMES6355	70	6.0	*qGSD1*	+	
	Yawara (2009)	H	GMES6355	71	8.2	*qGSD1*	+	
	Yawara (2010)	H	GMES6355	71	6.3	*qGSD1*	+	
	Kannondai (2007)	K_2	GMES1010	2	5.6		–	
	Kannondai (2009)	L	*E3*	167	5.4	*qGSD3*	+	
	Yawara (2010)	L	*E3*	155	6.0	*qGSD3*	+	
Number of days to flowering[c] (days)	Kannondai (2006)	L	*E3*	162	34.7	*qGSD3*	–2.8	0.83
	Yawara (2006)	L	*E3*	156	24.6	*qGSD3*	–2.9	0.70
	Kannondai (2007)	L	*E3*	156	38.1	*qGSD3*	–2.9	0.81
	Kannondai (2008)	L	*E3*	156	39.7	*qGSD3*	–2.4	0.81
	Kannondai (2009)	L	*E3*	156	42.6	*qGSD3*	–3.4	0.81
	Yawara (2009)	L	*E3*	156	28.4	*qGSD3*	–1.9	0.64
	Yawara (2010)	L	*E3*	155	37.9	*qGSD3*	–2.8	0.73
Seed-filling period[d] (days)	Kannondai (2007)	F	Sat_375	107	3.6	*qGSD2*	1.9	0.03
	Kannondai (2006)	H	GMES6355	71	7.5	*qGSD1*	2.1	0.18
	Yawara (2006)	H	Sat_401	69	3.7	*qGSD1*	1.5	0.13
	Kannondai (2007)	H	Sat_206	77	5.2	*qGSD1*	2.4	0.26
	Kannondai (2008)	H	GMES6355	70	5.3	*qGSD1*	1.9	0.17
	Yawara (2010)	H	Satt253	73	3.7	*qGSD1*	1.4	0.06
	Kannondai (2009)	L	*E3*	156	11.0	*qGSD3*	6.8	0.28
	Yawara (2009)	L	*E3*	168	5.8	*qGSD3*	–1.3	0.15
	Yawara (2010)	L	*E3*	156	17.2	*qGSD3*	2.6	0.54
Number of days to maturity[d] (days)	Yawara (2009)	B1_1	GMES2543	61	3.5		–1.4	0.10
	Kannondai (2007)	F	Sat_375	108	3.4	*qGSD2*	2.2	0.01
	Yawara (2010)	F	Satt516	58	4.0		0.9	0.14
	Kannondai (2007)	H	Sat_206	78	3.0	*qGSD1*	2.4	0.22
	Kannondai (2008)	H	GMES6355	70	3.3	*qGSD1*	1.4	0.15
	Kannondai (2009)	H	GMES6355	70	3.0	*qGSD1*	3.1	0.08
	Yawara (2010)	H	Sat_401	68	3.5	*qGSD1*	0.8	0.08
	Yawara (2006)	J_2	Sat_224	35	3.0		1.6	0.09
	Kannondai (2007)	J_2	Sctt011	21	3.0		2.3	0.07
	Kannondai (2006)	L	*E3*	164	10.3	*qGSD3*	–2.7	0.36
	Yawara (2006)	L	*E3*	165	8.0	*qGSD3*	–2.2	0.46
	Kannondai (2007)	L	*E3*	167	11.6	*qGSD3*	–3.7	0.39
	Kannondai (2009)	L	*E3*	156	3.6	*qGSD3*	3.2	0.08
	Yawara (2009)	L	*E3*	165	18.1	*qGSD3*	–2.9	0.46

[a] GSD incidence was defined as an incidence ratio of ≥3 on the GSD index among experiments.

[b] GSD index was classified into six levels; 0: GSD tolerant, 5: GSD intolerant.

[c] Number of days to flowering was defined as the number of days from the sowing date to the first flowering date.

[d] Seed-filling period was defined as a differentiation between the first flowering date and the maturity date. The maturity date was defined as a date of the day when 80% plants matured in a plot.

[e] Direction of the additive effect, where "+" and "–" indicate the increasing and decreasing effects of the allele from 'Tachinagaha', respectively, for the traits. Values of additive effect and R^2 for GSD incidence and GSD index could not be calculated because of non-parametric method applied for QTL analysis for these traits.

following order: *qGSD1* > *qGSD2* > *qGSD3* (Table 3). A 'Touhoku 129' genotype for these QTLs reduced the GSD index. RILs harboring 'Touhoku129' genotypes at these three effective QTL regions exhibited a GSD incidence similar to that of the GSD insensitive parental line, 'Touhoku129' (Table 3). In turn, RILs harboring 'Tachinagaha' genotypes in these three QTL regions exhibited a GSD incidence similar to the GSD sensitive parental cultivar,

Table 3. Comparisons of GSD[a] incidence between genotypes of detected *qGSD1*, *qGSD2*, and *qGSD3* in RILs derived from a cross between the soybean breeding line 'Touhoku 129' and the leading cultivar 'Tachinagaha'

Genotype of DNA markers closest for each QTL[b]	QTLs					Parental line and cultivar
	qGSD3	*qGSD2*	*qGSD1*	*qGSD1* and *qGSD2*[c]	*qGSD1*, *qGSD2*, and *qGSD3*	
Touhoku 129	28.0 ± 3.0	22.5 ± 1.9	17.2 ± 1.8	14.0 ± 1.8	13.0 ± 3.0	12.5
Tachinagaha	34.0 ± 3.0	33.9 ± 2.7	39.8 ± 2.4	45.2 ± 3.6	52.0 ± 6.0	51.6
p value[d]	0.1272	0.0058	0.0000	0.0000	0.0000	–

[a] GSD incidence was defined as an incidence ratio of ≥3 on the GSD index among experiments. GSD index was classified into six levels; 0: GSD tolerant, 5: GSD intolerant.

[b] GMES1506, Satt114, and *E3* were used for genotyping for *qGSD1*, *qGSD2*, and *qGSD3*, respectively.

[c] Two groups of RILs harboring maternal or paternal genotypes at QTLs were compared.

[d] *p* values of the Wilcoxon rank sum test between genotypes.

'Tachinagaha' (Table 3). A QTL for NDF was detected only in a region similar to *qGSD3* (Table 2). In addition, the 'Touhoku129' genotype was associated with later flowering (Table 2).

NDF, FP, and NDM were significantly correlated with GSD incidence (ρ = –0.243, 0.615, and 0.287, respectively). The correlation coefficient of FP was higher than that of NDM or NDF, and NDF was negatively correlated with GSD incidence. Subsequent analysis was performed only for FP and NDF because the results for NDM were a summation of those for NDF and FP and analysis of NDF and FP was sufficient. QTLs for FP and NDM were detected primarily in a region similar to those of *qGSD1* and *qGSD3* (Table 2). In addition, the 'Touhoku129' genotype in the *qGSD1* region was associated with shorter FP (Table 2). The effects of the *qGSD3* region on FP were variable and did not always correspond to the effects on NDF (Table 2). When RILs were grouped by their *qGSD3* genotypes, the 'Touhoku129' genotype exhibited significantly lower GSD index values in four (Kannondai in 2008 and 2009, Yawara in 2006 and 2010) of ten experiments (Table 4).

Table 4. Comparisons of GSD index values between *qGSD3* genotypes on RILs derived from a cross between soybean the breeding line 'Touhoku 129' and the leading cultivar 'Tachinagaha'

Experimental sites (Year)	*qGSD3* genotypes[a]		*p* value[b]
	A	B	
Yawara (2005)	1.0 ± 0.1	1.2 ± 0.2	0.3737
Kannondai (2006)	1.2 ± 0.1	1.0 ± 0.1	0.3855
Yawara (2006)	2.1 ± 0.1	2.6 ± 0.1	0.0088
Kannondai (2007)	0.9 ± 0.1	0.8 ± 0.1	0.9453
Yawara (2007)	2.4 ± 0.1	2.1 ± 0.1	0.0663
Kannondai (2008)	1.0 ± 0.1	1.4 ± 0.1	0.0105
Yawara (2008)	1.7 ± 0.1	1.7 ± 0.1	0.7678
Kannondai (2009)	1.4 ± 0.1	2.3 ± 0.1	0.0000
Yawara (2009)	2.1 ± 0.1	2.0 ± 0.1	0.3399
Yawara (2010)	3.1 ± 0.2	4.1 ± 0.1	0.0000

[a] Primer set reported by Xu *et al.* 2013 for identifying the genotype of *E3* was used. "A" indicates the 'Touhoku 129' genotype and "B" indicates the 'Tachinagaha' genotype. The gray cell indicates the genotypes more insensitive to GSD.

[b] *p* values of the Wilcoxon rank sum test between *E3* genotypes.

Confirmation and evaluation of the influence of the qGSD1 region by HIFs

HIFs of *qGSD1* were produced and segregated only for a genomic region adjacent to *qGSD1* in LG_H from Satt469 to Sat_206 (>14.8 cM and <64.8 cM). When HIFs were grouped by their *qGSD1* genotypes, significant differences were found for their GSD index values, FP, 100-seed weight, total seed weight, and estimated number of seeds in every experiment in which these traits were also evaluated (Table 5). For seed protein and oil contents, significant differences were found in 2011, but not in 2009 ($p = 0.08$ and 0.07, respectively) (Table 5). NDF was not significantly different between the genotypes in 2010 or 2011 (Table 5). The 'Tohoku129' genotype exhibited lower GSD index values, smaller seeds, lower protein contents, higher oil contents, higher total seed weights, and larger estimated numbers of seeds (Table 5).

Discussion

Effects of major QTLs associated with GSD insensitivity

The lower GSD incidence for 'Touhoku129' than for 'Tachinagaha' was attributable primarily to the three QTLs detected in this study: *qGSD1*, *qGSD2*, and *qGSD3*. This result was obtained because RILs harboring a given parental genotype at these three QTLs exhibited a GSD incidence similar to the insensitive parent. These QTLs have not been previously reported in studies of association with GSD.

Varietal differences in GSD insensitivity have been reported, as described in the Introduction. Various degrees of insensitivity among the cultivars could be explained by multiple loci and alleles of small effects. However, the multiple experiments conducted over 6 years and two locations in this study revealed far fewer major QTLs controlling a large part of the GSD index. Marker-assisted selection is a powerful tool for improving such traits.

The main diagnostic feature of GSD is the presence of mature pods and seeds with green stems (Hill *et al.* 2006). Although the definitions of GSD index in this report were similar, the definitions in this report did not omit symptoms caused by stink bug feeding, which were omitted by Hill

Table 5. Agronomic traits of HIFs derived from a cross between the soybean breeding line 'Touhoku 129' and the leading cultivar 'Tachinagaha' for each *qGSD1* genotype[a]

Experimental sites (Year)	HIF	Genotypes[b]	GSD index (0–5)	Number of days to flowering (days)	Seed-filling period (days)	100-seed weight (g)	Seed protein content (%)	Seed oil content (%)	Total seed weight (kg/a)	Estimated number of seeds[e] (seed/plant)
Yawara (2009)	(F8, individual)	A (n = 12)	2.1 ± 0.2	–[d]	–	31.2 ± 0.4	42.9 ± 0.1	19.7 ± 0.1	–	–
		B (n = 6)	4.0 ± 0.3	–	–	34.2 ± 0.4	43.5 ± 0.4	19.3 ± 0.2	–	–
		p value[c]	0.0010			0.0001	0.0809	0.0667		
Kannondai (2010)	(F9, line)	A (n = 18)	0.9 ± 0.1	48.3 ± 0.6	106.0 ± 2.8	–	–	–	–	–
		B (n = 5)	1.8 ± 0.4	48.6 ± 1.3	121.0 ± 4.4	–	–	–	–	–
		p value	0.0220	0.8009	0.0181					
Yawara (2011)	(F10, line)	A (n = 15)	1.9 ± 0.3	41.5 ± 0.2	71.5 ± 0.2	34.2 ± 0.2	42.9 ± 0.2	20.7 ± 0.1	375.8 ± 13.6	100.1 ± 3.6
		B (n = 15)	3.5 ± 0.2	41.1 ± 0.1	72.1 ± 0.2	37.4 ± 0.2	43.9 ± 0.2	20.4 ± 0.1	302.7 ± 11.8	73.8 ± 3.0
		p value	0.0002	0.1178	0.0436	0.0000	0.0001	0.0255	0.0000	0.0000

[a] GMES1506 was used for genotyping and represented the *qGSD1* genotype.
[b] "A" indicates the 'Touhoku 129'genotype and "B" indicates the 'Tachinagaha' genotype.
[c] *p* values of the Wilcoxon rank sum test for the GSD index and t test for the other traits between genotypes.
[d] Trait values lacking in Table 5 were not measured.
[e] Estimated total seed number was calculated as "total seed weight (g/m^2)/1 seed weight (g)/number of plants ($plants/m^2$)".

et al. (2006), because the causes of symptoms could not be identified in our study. For this reason, the QTLs detected in this study may include QTLs for avoiding or recovering from insect damage to pods.

Multiple effects of the qGSD1 region

The multiple effects of a QTL region caused by linkage drag and the pleiotropic effects of a causal QTL gene are serious problems for marker-assisted selection in breeding programs. The GSD insensitivity of 'Touhoku129' could include the multiple effects of the *qGSD1* region of 'Touhoku129' compared with that of 'Tachinagaha'. The *qGSD1* region of the 'Touhoku129' genotype was believed to confer a shorter FP, lower 100-seed weight, lower protein content, higher oil content, and higher total seed weight as well as lower GSD index values than that of the 'Tachinagaha' genotype (Table 5). Given that in the present study we evaluated these traits for individual plants or small line plots, seed productivity could not be evaluated accurately. Further experiments are needed to determine whether the multiple effects of *qGSD1* were caused by linkage drag or pleiotropy.

Relationship between qGSD3 and E3

Among QTLs for GSD index values, only *qGSD3* was detected in the vicinity of *E3*, which has been reported to be a maturity gene (McBlain *et al.* 1987). The positive effects of the *E3* locus on FP and NDF in the same direction were also noted (McBlain *et al.* 1987). The causal gene of *E3*, *GmPhyA3*, has been identified by a map-based cloning strategy using flowering time evaluation by Watanabe *et al.* (2009).

Because the 'Touhoku 129' genotype exhibited later flowering, it was considered a later flowering genotype presented as *E3*. However, the 'Touhoku 129' genotype did not always exhibit a longer FP; rather, the opposite case was frequently observed (Table 2). Thus, the reason for the shorter FP caused by the 'Touhoku 129' genotype in this region, which presumably contained *E3* in contrast to the earlier flowering genotype, denoted as *e3*, remains to be determined.

Insect damage has been reported to be a promoting factor of delayed maturity (Boethel *et al.* 2000) and green stem (Lustosa *et al.* 1999). Drought stress has also been reported to be a promoting factor of delayed stem maturation (Sakashita *et al.* 2003). Because these symptoms were similar to GSD and expressed when sink potential is lower than source potential (Egli and Bruening 2006, Shiraiwa *et al.* 2005), earlier flowering and earlier maturing lines are believed to express higher GSD index values in response to insect damage and drought stress during midsummer, both of which could be limiting factors for sink potential.

In view of the previous findings described above and because a shorter FP almost always coincided with GSD insensitivity owing to the 'Touhoku 129' genotype at this region, the presence of GSD may be one result of longer FP, and *E3* may be associated with *qGSD3*. Humid conditions under a canopy would delay pod dehydration. Thus, the relationship between maturity and GSD insensitivity should be investigated.

Considerations for breeding improvement for GSD insensitivity of soybeans

In conclusion, we detected three major effective QTLs for GSD insensitivity using cumulative data from multiyear and multilocation experiments. Although only one mapping population was evaluated in the present study, these QTLs and the flanking markers may be effective tools for lowering GSD risk in similar populations using 'Touhoku129' and 'Tachinagaha' or their descendants as a crossing parent because 'Tachinagaha' is a leading cultivar and is frequently used as a crossing parent for breeding programs. DNA marker-assisted selection is particularly useful for selection

of the trait like GSD insensitivity in a breeding practice because appropriate evaluation of the GSD index in a single experiment is difficult. Further studies on fine mapping of these QTLs will result in increasingly precise marker-assisted selection and aid in identifying the responsible genes.

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