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Research Paper

Confirmation of the pleiotropic control of leaflet shape and number of seeds per pod by the *Ln* gene in induced soybean mutants

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Most soybean cultivars possess broad leaflets; however, a recessive allele on the *Ln* locus is known to cause the alteration of broad to narrow leaflets. The recessive allele *ln* has also been considered to increase the number of seeds per pod (NSP) and has the potential to improve yield. Recently, *Gm-JAG1* (*Glyma20g25000*), a gene controlling *Ln*, has been shown to complement leaf shape and silique length in *Arabidopsis* mutants. However, whether *Gm-JAG1* is responsible for those traits in soybean is not yet known. In this study, we investigated the pleiotropic effect of soybean *Ln* gene on leaflet shape and NSP by using two independent soybean *Gm-jag1* mutants and four *ln* near isogenic lines (NILs). The leaflet shape was evaluated using a leaf image analysis software, SmartLeaf, which was customized from SmartGrain. The leaflets of both the *Gm-jag1* mutants were longer and narrower than those of the wild-type plants. Interestingly, the image analysis results clarified that the perimeter of the mutant leaflets did not change, although their leaflet area decreased. Furthermore, one mutant line with narrow leaflets showed significantly higher NSP than that in the wild (or *Ln*) genotype, indicating that soybean *Ln* gene pleiotropically controls leaflet shape and NSP.

Key Words: *Ln* gene, *Glycine max* (L.) Merrill, pleiotropic effect, leaflet shape, seed number per pod.

Introduction

The genetic factor controlling broad and narrow shape of soybean leaflets was first reported by Takahashi and Fukuyama (1919). In heterozygous plants, the broad leaflet trait is inherited in an incompletely dominant manner, leading to the development of an intermediate leaf shape in heterozygous plants (Takahashi and Fukuyama 1919, Takahashi 1934). The gene responsible for this trait was named *Ln* and *ln* for broad and narrow leaflet shapes, respectively, on the *Ln* locus (Bernard and Weiss 1973). Interestingly, Takahashi (1934) first reported that the narrow leaflet trait (*ln*) is closely associated with the increase in the number of seeds per pod (NSP). Weiss (1970) suggested that it has a pleiotropic effect on increasing the NSP, which is also obviously influenced by the vigor and genetic background of plants. In this case, the genotype at *Ln* is important to control not only leaflet shape but also NSP, one of the seed yield components, in soybean.

Recently, the *Ln* locus was positioned into a soybean linkage map (Jeong *et al.* 2011) and *Gm-JAG1*, *Glyma20g25000* in the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax; Schmutz *et al.* 2010), was reported to be a responsible gene on the *Ln* locus (Jeong *et al.* 2012). The *JAGGED* (*JAG*) gene encodes a zinc finger protein family of plant transcription factors in *Arabidopsis thaliana*. The *jag* mutant line, which possesses a nonsense mutation on the *JAG* gene, leads to the development of jagged shape along the lateral margins of leaves and the narrowing of petals owing to the defects in both cell division and cell expansion (Ohno *et al.* 2004). Although narrow petals are known to contain elongated and round cells, the relationship between leaf and cell shapes is not yet completely known. Furthermore, Jeong *et al.* (2012) did not investigate the effect of *Gm-JAG1* on leaf shape and NSP in

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soybean. Thus, whether *Gm-JAG1* pleiotropically controls leaf shape and NSP as *Ln* in soybean is not yet known.

The leaflet size and shape are known to change with developing stages and environments; therefore, reliably evaluating these traits is difficult. Sawada (1992) reported that the central leaflet of the fifth main leaf from the base on the main stem has a distinctive ratio of length to width among soybean cultivars. Automatic image analysis allows the sequential measurement of many samples and could be used to measure the length and width of leaflets as well as to describe the features of leaflet shape, such as leaf area and perimeter. SmartGrain software, which allows easy manipulations, has been developed for high-throughput analysis of seed shape (Tanabata *et al.* 2012). SmartGrain might enable the analysis of leaflet shape, although this software is not suitable for the analysis of entire leaves.

Induced mutant libraries are a powerful tool for conducting genetic and functional analyses in soybean (Anai 2012, Cooper *et al.* 2008, Xia *et al.* 2012). The *Gm-jag1* mutant lines are attractive materials to determine the pleiotropic effect of *ln*. In this study, we evaluated the pleiotropic effects of *Ln* on leaflet shape and NSP by using the *Gm-jag1* mutant lines and an updated image analysis software of SmartGrain for leaf shape analysis. The leaflets of the two independent mutant lines were longer and narrower than those of the wild-type plants. Furthermore, one mutant line with narrow leaflets showed significantly increased NSP compared to that of the wild-type plants. To our knowledge, this is the first study to indicate that soybean *Ln* gene pleiotropically controls leaflet shape and NSP.

Materials and Methods

Characterization of 'Enrei' mutants for Gm-JAG1 *and development of backcrossed lines*

Tsuda *et al.* (2015) constructed a mutant library (M_2' generation) of broad leaflet cultivar 'Enrei' (EN) with a high mutation density by repeating chemical mutagen (ethyl methanesulfonate) treatment and obtained a total of eleven mutant types consisting of one null and ten missense mutations (named 'EnT lines') for *Gm-JAG1* (*Glyma20g25000*), although their phenotypes were not described. No M_3' seeds were obtained for two missense mutant lines. Fewer than nine M_3' plants per line were grown in the greenhouse controlled at 25°C under natural light condition with supplemental lighting of 79 W/m^2 by using metal-halide lamps (M360FCELSP-W/BUD; Iwasaki Electric, Tokyo, Japan) from 5:00 to 19:00. The leaflet shape and genotype of *Gm-JAG1* of respective plants were evaluated per the methods hereinafter described. The individuals showing narrow leaflets and mutant genotype were used for backcrossing to EN for evaluating the detailed leaflet shape and the relationship between genotypes and phenotypes to eliminate other mutation sites and to develop segregating progenies at the *Ln* locus.

For assessing the genotype of the mutants, DNA was extracted from a young leaf by using an automated purification system (BioSprint 96 DNA Plant Kit; Qiagen, Hilden, Germany), and the genotype for each mutation site was determined using high resolution melting (HRM) analysis according to Tsuda *et al.* (2015). The primer pairs for the HRM analysis are shown in **Table 1**.

NIL pairs carrying natural mutant and wild alleles at the Ln *locus*

In order to confirm that *Gm-JAG1* was the gene responsible for leaflet shape and had pleiotropic effects on NSP, we used several NIL lines, along with the abovementioned mutant lines. A narrow leaflet phenotype derived from 'Tachinagaha' (*ln* cultivar) was introduced to 'Sachiyutaka' (Norin No. 116; SC) and 'Fukuyutaka' (Norin No. 73; FK) to develop two NILs, 'Zenkei 101' (SC_*ln*) and 'Zenkei 102' (FK_*ln*) by backcrossing six times based on the leaflet shape in the Western Region Agricultural Research Center. These cultivars and NILs were registered and conserved in NARO Genebank. FK (JP29668) and EN (JP28862) in addition to 'Tachinagaha' (JP67666) is now available, and the others will be available soon. In addition, 'Clark' (CL) and 'Harosoy' (HR) and their NILs with the *ln* allele, CL_*ln* and HR_*ln* were registered as PI 548533, PI 548573, PI 547413, and PI 547690, respectively, in the Germplasm Resources Information Network (GRIN), USA. According to the pedigree of CL_*ln* and HR_*ln*, a narrow leaflet phenotype derived from 'T204' (*ln* cultivar) was introduced to CL and HR by backcrossing five times (Bernard *et al.* 1991).

DNA sequencing of the Glyma20g25000 *region*

The DNA sequence of the *Glyma20g25000* region for each mutant and near isogenic line has not been previously identified. Each sequence was determined by the following procedure.

The DNA was extracted from 10 mg seed powder by using an automated purification system (BioSprint 96 DNA Plant Kit). Next, the sequence of the *Glyma20g25000* region was amplified using the following primer pair: forward, 5′-AGCTTTAGTTTTATCCCTACCCACAC-3′ and

Table 1. Primers used for genotyping the nine EnT mutant lines by using high-resolution melting analysis

Target mutant lines	Forward primer	Reverse primer
EnT-0112, EnT-0160, EnT-0541	CTACACCTTCACACCCTTCTTTTCT	CAAGGAATATACCACAGGGATGG
EnT-0621, EnT-0685, EnT-0987 EnT-0634, EnT-1084 EnT-1619	GGTAATCGAGAATGGGGAGTT CATGACACTGTTTGATACTGCGA	CTTGTGAGAAGGAGTGGTAAAAGAA CTCTCATCTCTTTAGAAACCATCT

Table 2. Plant materials and planting design

Plant materials	Phenotype of leaflet shape (narrow leaflet origin)	Genotype	Cultivar or Generation	Planting design	
				in Tsukuba	in Tsukubamirai
EN	Broad	*Ln/Ln*	Enrei	7 plants, 4 reps.	4 plants, 3 reps.
685w	Broad	*Ln/Ln*	BC_2F_3	7 plants, 4 reps.	4 plants, 3 reps.
685h	Intermediate	*Ln/L10F*	BC_2F_3	7 plants, 4 reps.	4 plants, 3 reps.
685m	Narrow (EnT-0685)	*L10F/L10F*	BC_2F_3	7 plants, 4 reps.	4 plants, 3 reps.
541w	Broad	*Ln/Ln*	M_4'	(Not planted)	4 plants, 3 reps.
541h	Intermediate	*Ln/M1null*	M_4'	(Not planted)	4 plants, 3 reps.
541m	Narrow (EnT-0541)	*M1null/M1null*	M_4'	(Not planted)	4 plants, 3 reps.
SC	Broad	*Ln/Ln*	Sachiyutaka	7 plants, 4 reps.	(Not planted)
SC_*ln*	Narrow (Tachinagaha)	*ln/ln*	BC_6	7 plants, 4 reps.	(Not planted)
FK	Broad	*Ln/Ln*	Fukuyutaka	7 plants, 4 reps.	(Not planted)
FK_*ln*	Narrow (Tachinagaha)	*ln/ln*	BC_6	7 plants, 4 reps.	(Not planted)
CL	Broad	*Ln/Ln*	Clark	4 plants, 3 reps.	(Not planted)
CL_*ln*	Narrow (T204)	*ln/ln*	BC_5	4 plants, 3 reps.	(Not planted)
HR	Broad	*Ln/Ln*	Harosoy	4 plants, 3 reps.	(Not planted)
HR_*ln*	Narrow (T204)	*ln/ln*	BC_5	4 plants, 3 reps.	(Not planted)

Reps. in planting design means replications; EnT lines are derived from M1′ plants (the generation after two mutagen treatments), and their genotypes indicate deduced amino acid mutations in the *Gm-JAG1* (*Glyma20g25000)* gene; EnT-0685 (the tenth leucine changed to phenylalanine: *L10F*) and EnT-0541 (the start codon was altered: *M1null*).

reverse, 5′-CCACATAACATAACAGAAACATACCA-3′; the sequence was then determined using the primer-walking method by using BigDye Terminator v3.1 Cycle Sequencing Kit and 3500xl Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, USA).

Planting design

Seeds were sown on June 26, 2015 in a field in Tsukuba (Institute of Crop Science, NARO; 36°01′N, 140°06′E), the soil type of which was Andosol, and on July 14, 2015 in a field in Tsukubamirai (Institute of Crop Science, NARO; 36°00′N, 140°01′E), which has gray lowland soil. Chemical fertilizer (N:P:K) was applied at the rate of 20:280:60 (kg/ha) and 30:100:100 (kg/ha) in Tsukuba and Tsukubamirai, respectively. Each line or cultivar was planted in single-row plots following a randomized block design with 3–4 replications. Each plot consisted of a row of 4–7 plants that were spaced 0.2 m apart, and each row was 0.8 or 0.7 m apart (**Table 2**).

Evaluation of leaflet shape-related traits by using image analysis

A fully expanded central leaflet of the fifth leaf from the base on the main stem was collected according to Sawada (1992). The image of the leaflet was captured using a scanner GT-X970 (EPSON, Tokyo, Japan) and saved as a jpeg image file. The leaflet image was analyzed using SmartLeaf software (http://phenotyping.image.coocan.jp/smartleaf/), which was an updated image analysis software of SmartGrain (Tanabata *et al.* 2012) adapted for leaf shape analysis. SmartLeaf can automatically measure the length, width, perimeter, and area of a leaf and calculate the length/width ratio (L/W ratio), centroid position, circularity, and L*a*b* colorimetric values.

Evaluation of NSP and other seed yield components

Single seed weight (SSW), seed weight per plant (SWP), number of pods per plant (NP), and NSP were measured for individual plants. First, pods were separated from the stem. They were classified based on the number of seed cavity per pod by visual inspection based on the method described by Jeong *et al.* (2011), and then the pod number was recorded for each plant. After the pods were threshed, the seed number was counted. Sufficiently air-dried seed samples were weighed, and the water content of the seeds was immediately measured using a grain moisture tester (PM-830-2; Kett, Tokyo, Japan). SSW was determined by weighing all seeds except pest-damaged ones from each plant. SWP was calculated as the potential ability of plants to produce seeds by multiplying SSW and the total number of seed cavities per plant. The SSW and SWP were adjusted to reflect a moisture content of 15%. NSP was calculated by dividing the total number of seed cavities per plant by NP.

Statistical analysis

Statistical analysis was performed using the statistical package R version 3.1.2 (http://www.r-project.org). The statistical significance was evaluated using analysis of variance followed by Student's *t*-test for comparisons between two groups or by using the Tukey–Kramer HSD test for comparisons among more than two groups. Significance was determined using an alpha level of 5%.

Results

Identification of ln *mutants by using the reverse genetic approach*

Of the nine M_3' mutant lines with one null (altered initiation codon) and eight missense (amino acid substitution)

mutations for *Gm-JAG1* (*Glyma20g25000*; **Fig. 1**), only two mutant lines, 'EnT-0541' (the start codon was altered: M1null) and 'EnT-0685' (tenth leucine changed to phenylalanine: L10F), exhibited narrow leaflet characteristics. Whether the narrow leaflet phenotypes of these mutants were controlled by *Gm-JAG1* was confirmed by genetically analyzing the relationship between the mutations and leaflet shape in the segregating progenies of 'EnT-0541' (M_4') and 'EnT-0685' (BC_2F_3; **Fig. 2**). The two sets, including the wild, heterozygous, and mutant genotypes derived from 'EnT-0541' and 'EnT-0685', were named as follows: 541w, 541h, 541m, 685w, 685h, and 685m, respectively (**Table 2**). According to genomic sequences in the *Glyma20g25000* regions of 541m and 685m (respective DDBJ accession numbers were LC203763 and LC203764), no mutations were confirmed except for the respective base substitutions detected by HRM analysis (**Supplemental Fig. 1**). The mutation of 685m (L10F) existed in the ethylene response factor-associated amphiphilic repression (EAR) motif (Ohta *et al.* 2001) like the mutation of *ln* cultivars (ninth aspartic acid changed to histidine: D9H) reported by Jeong *et al.* (2012). Conversely, the genomic sequences for the four *ln* NILs, SC_*ln*, FK_*ln*, CL_*ln*, and HR_*ln*, showing narrow leaflet phenotype (**Table 2**), contained the same base substitution (D9H) in the *Glyma20g25000* region (**Supplemental Fig. 1**). The five *Ln* cultivars, EN, SC, FK, CL, and HR, yielded sequences identical to 'Williams 82' in the Phytozome. All the cultivars showed broad leaflet phenotype.

High-throughput analysis of leaflet shape by using the enhanced SmartGrain software

The leaflet shape was resolved into length, width, L/W ratio, perimeter, and leaf area by using SmartLeaf, an updated image analysis software of SmartGrain (**Table 3**) adaptable for leaf shape analysis. Unlike manual measurement, SmartLeaf automatically recognizes the leaflet area only from images and successively reports various parameters characterizing the leaflet (**Fig. 3**). This automation reduced the measuring time, and personal equation could be eliminated at any time if leaf images were obtained.

The 685m and 541m leaflets were longer, narrower, and had larger L/W ratio and smaller area than those of the wild-type and EN leaflets (**Table 3**). These values for the two heterozygous mutant lines, 685h and 541h, were intermediate between the narrow and broad leaflet types. The L/W ratio characterizing the leaflet shape stably reflected the genotypes independently of growth conditions (**Table 3a**, **3b**). Similarly, four *ln* NILs had similar leaf shape parameter values as those of the mutants. The two mutant lines, 685m and 541m, and two NILs, CL_*ln* and HR_*ln*, did not show significant differences in leaf perimeters from those of the wild (or *Ln*) and heterozygous genotypes, whereas SC and FK *ln* NILs had slightly longer leaf perimeter than that of the *Ln* genotypes.

Effects on NSP and other seed yield components

The mutant line 685m showed 10%–20% greater NSP than that of 685w and 685h, but had 7%–9% smaller SSW under both the growing conditions. No significant difference in NP and SWP was found among the three genotypes

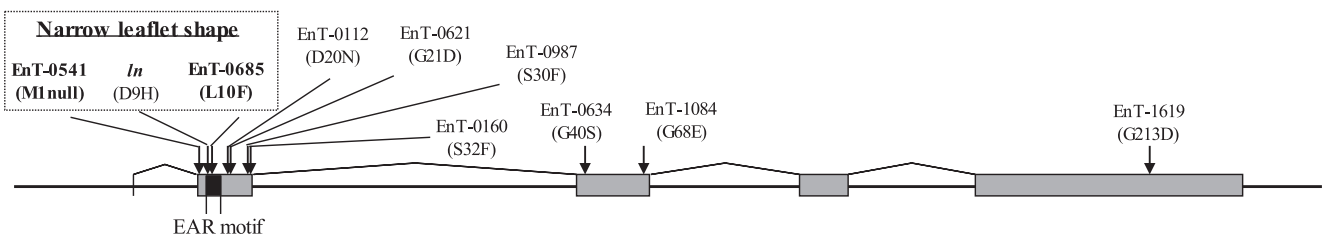

Fig. 1. Schematic diagram of mutations of *Gm-JAG1* (*Glyma20g25000*) in the high-density mutant library of 'Enrei'. *Glyma20g25000* consists of 2,436 bp (black line), including 771 bp coding sequences connected by 5 exons. The first exon consists of a sequence of 2 bases, and the remaining exons are included in 4 boxes. The mutant line 'EnT-0685' and a natural variation of *ln* (ninth aspartic acid changed to histidine: D9H) have a respective unique mutation on the ERF-associated amphiphilic repression (EAR) motif (8aa-LDLNNLP-14aa; black box). Only the progenies of 'EnT-0541' and 'EnT-0685' with the mutant genotype showed narrow leaflet shape like the *ln* genotype. The detailed information of 1 null and 8 missense mutant lines and screening method has been described in Tsuda *et al.* (2015).

Fig. 2. Representative leaflet shapes of EN (a), 541m (b), and 685m (c) planted in Tsukubamirai. These plant materials are shown in **Table 2**.

Table 3. Traits related to leaflet shape

a) Experiment conducted in Tsukuba

Plant materials	Genotype	Length (L) [cm]	Width (W) [cm]	L/W ratio	Perimeter [cm]	Area [cm^2]
EN	*Ln/Ln*	9.53	6.09	1.57	25.16	39.82
685w	*Ln/Ln*	8.74^c	5.46^a	1.61^c	22.80	32.22^a
685h	*Ln/L10F*	9.60^b	4.81^b	2.00^b	23.96	31.67^a
685m	*L10F/L10F*	10.25^a	3.65^c	2.82^a	24.00	26.12^b
SC	*Ln/Ln*	9.26^b	6.32^a	1.47^b	25.21^b	38.94^a
SC_*ln*	*ln/ln*	11.41^a	4.55^b	2.51^a	27.32^a	34.19^b
FK	*Ln/Ln*	9.10^b	6.54^a	1.39^b	24.89^b	39.26^a
FK_*ln*	*ln/ln*	11.28^a	4.53^b	2.50^a	27.12^a	33.85^b
CL	*Ln/Ln*	8.87^b	5.65^a	1.56^b	23.22	32.82^a
CL_*ln*	*ln/ln*	10.44^a	3.57^b	2.93^a	24.83	25.18^b
HR	*Ln/Ln*	8.52^b	4.93^a	1.73^b	21.86	27.69^a
HR_*ln*	*ln/ln*	9.82^a	3.55^b	2.78^a	23.23	23.23^b

b) Experiment conducted in Tsukubamirai

Plant materials	Genotype	Length (L) [cm]	Width (W) [cm]	L/W ratio	Perimeter [cm]	Area [cm^2]
EN	*Ln/Ln*	12.31	8.75	1.41	33.88	75.09
685w	*Ln/Ln*	10.77	7.83^a	1.38^c	30.23	59.74
685h	*Ln/L10F*	11.37	6.81^b	1.68^b	30.51	56.38
685m	*L10F/L10F*	11.74	6.11^b	1.93^a	29.98	48.80
541w	*Ln/Ln*	11.61^b	7.79^a	1.50^c	31.19	62.54^a
541h	*Ln/M1null*	12.20^b	7.33^a	1.67^b	31.73	62.36^a
541m	*M1null/M1null*	13.38^a	5.53^b	2.43^a	32.34	49.37^b

^{a,b,c} Different letters indicate significant difference at the 5% level between respective NILs.

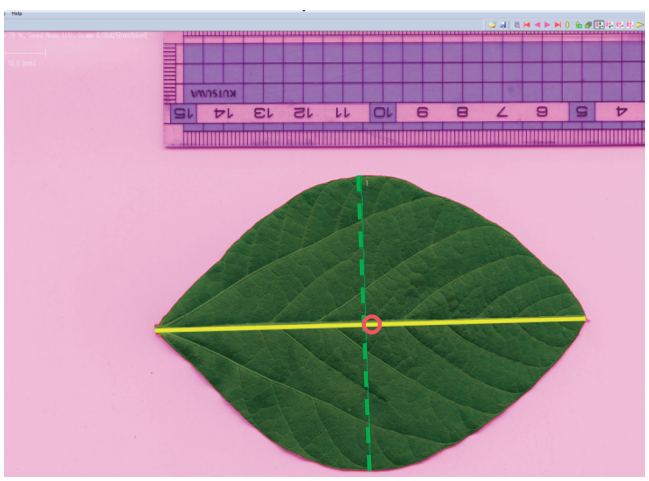

Fig. 3. Picture of an 'Enrei' leaflet analyzed using the SmartLeaf software. SmartLeaf accurately recognized the leaf and background indicated by pink region (light grey region in black and white; green region recognized in the SmartLeaf software). It also measured the length indicated by yellow line (solid line in black and white); width indicated by green line (broken line in black and white); their ratio; the centroid indicated by circle with a hole in the middle; circularity; perimeter; size; and the L*a*b* colorimetric values, which was the additional function to SmartLeaf.

(**Table 4a**, **4b**). The NSP, SSW, NP, and SWP of 541m was not significantly different from those of 541w and 541h (**Table 4b**). All the four NILs showed significantly higher

Table 4. Traits related to seed yield components

a) Experiment conducted in Tsukuba

Plant materials	Genotype	NSP	SSW [mg]	NP	SWP [g]
EN	*Ln/Ln*	2.12	342	69.7	50.5
685w	*Ln/Ln*	2.20^b	305^a	70.5	47.1
685h	*Ln/L10F*	2.24^b	295^a	69.5	46.0
685m	*L10F/L10F*	2.43^a	284^b	65.3	45.1
SC	*Ln/Ln*	2.16^b	357	87.6^b	67.9^b
SC_*ln*	*ln/ln*	2.32^a	361	96.0^a	80.8^a
FK	*Ln/Ln*	2.07^b	342^a	96.7	68.9^b
FK_*ln*	*ln/ln*	2.41^a	333^b	99.7	80.2^a
CL	*Ln/Ln*	2.84^b	208^a	111.7^a	65.9^a
CL_*ln*	*ln/ln*	3.22^a	185^b	81.9^b	49.4^b
HR	*Ln/Ln*	2.76^b	179^a	104.2^a	51.3^a
HR_*ln*	*ln/ln*	2.98^a	160^b	91.4^b	43.6^b

b) Experiment conducted in Tsukubamirai

Plant materials	Genotype	NSP	SSW [mg]	NP	SWP [g]
EN	*Ln/Ln*	2.25	292	46.7	30.6
685w	*Ln/Ln*	2.23^b	291	37.7	24.2
685h	*Ln/L10F*	2.22^b	263	33.8	20.5
685m	*L10F/L10F*	2.68^a	266	29.1	20.7
541w	*Ln/Ln*	2.23	250	26.2	14.4
541h	*Ln/M1null*	2.11	230	24.4	11.5
541m	*M1null/M1null*	2.19	240	22.6	12.0

^{a,b} Different letters indicate significant difference at the 5% level between respective NILs.

NSP values in the *ln* genotype than in *Ln*. Like 685m, CL_*ln* and HR_*ln* showed smaller SSW than that of the respective recurrent parents. In contrast, SC_*ln* and FK_*ln* showed almost the same SSW as that of the recurrent parents. With regard to NP and SWP, the differences between *Ln* and *ln* varied depending on the genetic background, that is, CL_*ln* and HR_*ln* had significantly lower NP and SWP than those of CL and HR, whereas SC_*ln* and FK_*ln* had higher values than those of SC and FK.

Discussion

Assessing genetically pleiotropic effects of a single locus is difficult, because the locus cannot be separated from the effects of closely linked genes. Mutation is an attractive choice to verify the pleiotropic gene function. The present study first showed that two lines ('EnT-0541' and 'EnT-0685') of the mutant lines screened from the high-density soybean mutant library (Tsuda *et al.* 2015) having independent missense and null mutations exhibited narrow leaflet phenotype. Therefore, this mutant library was confirmed to be useful to determine gene functions not only for seed components (Yano *et al.* 2017) but also for plant morphology.

The SmartLeaf software revealed the leaflet characteristics of four pairs of *ln* NILs (**Table 3**); the leaflet of *ln* NILs was the longer and narrower, and thus the L/W ratio was notably higher than that in the original *Ln* varieties. This

suggests the importance of the L/W ratio in determining the narrow leaflet shape as previously reported (Chen and Nelson 2004, Dinkins *et al.* 2002, Jeong *et al.* 2011, Sawada 1992). The image analysis software revealed identical features of the two *Gm-jag1* mutant lines, which showed higher L/W ratio than that of the original cultivar 'Enrei' and sib lines having a wild *Gm-JAG1* allele. Interestingly, in this study, image analysis indicated no apparent changes in the perimeter of mutant leaflets despite the decrease in leaf area. The leaf morphology is controlled genetically in either the length or width (Tsukaya 2005). In this case, the perimeter consequentially changes. Although *Gm-JAG1* coincidentally controls the length and width in the soybean leaflet, no apparent changes were noted in the perimeter. This observation might contribute to the elucidation of the function of *JAGGED*-like gene in cell division and/or cell expansion. SmartLeaf can allow the use of new evaluation methods for leaf morphology, which is known to vary across environments, and leaf positions, because of its high-throughput image processing.

Jeong *et al.* (2012) introduced the soybean *Gm-JAG1* gene into *Arabidopsis jag* mutant line and showed that soybean *Gm-JAG1* complemented the leaf shape and silique length in the *Arabidopsis* mutant. However, the function of *Gm-JAG1* in soybean was not determined. In this study, we found that *Gm-JAG1* is responsible for the function of *Ln* in soybean based on the narrow leaflet shape of the two different *jag* soybean mutant lines, 'EnT-0541' (M1null) and 'EnT-0685' (L10F; **Fig. 2**). Considering that the other seven mutants did not form narrow leaflets (**Fig. 1**), mutations on the EAR motif (Ohta *et al.* 2001) in Gm-JAG1 (**Supplemental Fig. 2**) were thought to affect the leaflet shape. Deduced amino acid substitutions occurred in the conserved EAR motif in both the 685m mutant line (L10F) and soybean *ln* NILs (D9H; Jeong *et al.* 2012). In addition, a mutation in the 541m mutant line (M1null) possibly resulted in the loss-of-function allele of *Gm-JAG1*. These mutations likely weaken the transcriptional property of *Gm-JAG1* and change cell shape and/or proliferation rate, thereby leading to the formation of narrow leaflets in soybean. The *jag* mutant lines of *Arabidopsis* and tomato have irregular cell size and develop malformed leaves (David-Schwartz *et al.* 2009, Ohno *et al.* 2004); further, a mutant in rice showed abnormal flowers, especially stamens (Xiao *et al.* 2009). However, the narrow leaflet shape is retained in the elite cultivars of soybean. The reason for this difference can be attributed to the existence of a paralog in soybean, *Gm-JAG2* (*Gm10g42020* in Phytozome), which, like *Gm-JAG1*, is also expressed in young leaves, meristems, flowers, and young pods (Jeong *et al.* 2012). Determining the cause of such mild phenotype in soybean mutants requires the elucidation of the function of *Gm-JAG2*.

The NSP of the mutant line 685m with narrow leaflets significantly increased like that in the four *ln* NILs that were grown under the same conditions, indicating that soybean *Ln* gene pleiotropically controls NSP as well as leaflet shape (**Table 4**). This is consistent with the findings of Takahashi (1934) who showed that narrow leaflet phenotype is closely associated with NSP. The pleiotropy is conceivable in part because the leaf shape and silique length were also affected in the *Arabidopsis jag* mutant (Jeong *et al.* 2012). In contrast, no significant differences were found in the NSP, SSW, NP, and SWP among 541w, 541h, and 541m. Since the parental (M_2') line ('EnT-0541') was estimated to contain more than 15,000 base changes in the genome (Tsuda *et al.* 2015; no data for 'EnT-0685'), such a high density of mutations in these lines might affect other traits such as SWP that was almost the half of that in EN (**Table 4b**). The gynoecium form (Ohno *et al.* 2004) and fruit length (Jeong *et al.* 2012) in *Arabidopsis* are known to be controlled by the *JAG* gene. However, the molecular genetic basis of the relationship between leaf and pod/fruit development, which are derived from flowers and their associated tissues, and EAR-motif of the *JAG* gene are poorly understood. The findings that a close relationship exists between the EAR motif and leaflet shape or NSP might allow the elucidation of the genetic network controlled by the *JAG* gene and the improvement of seed productivity in soybean and other crops.

Mutations in the EAR motif (**Supplemental Fig. 2**) certainly increased the NSP, whereas the SSW of the mutant line 685m and the two NILs, CL_*ln* and HR_*ln*, was significantly smaller than that of 685w or the recurrent parents of the NILs (**Table 4a**, **4b**). Generally, a negative correlation exists between the total number of seeds and seed size per plant in various plant species. Our results might suggest that competition of resource allocation among seeds occurs inside a pod. Mandl and Buss (1981) proposed that the narrow leaflet trait offers neither yield advantage nor disadvantage compared with the broad leaflet trait. Similarly, in this study, 685m did not show considerable changes in SWP, because the increase in NSP led to a concomitant decrease in SSW. Therefore, we suggest that *ln* alone could not improve the seed yield. However, the SWP of SC_*ln* and FK_*ln* was higher than that of their recurrent parents, suggesting that *ln* might improve seed yield under some particular genetic background(s) in soybean. Further studies are warranted to identify the genetic factors associated with these phenotypes and determine the synergistic effect of *ln* on NSP in such a genetic background.

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