**RESEARCH ARTICLES**

**Ln Is a Key Regulator of Leaflet Shape and Number of Seeds per Pod in Soybean**

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Narrow leaflet soybean (*Glycine max*) varieties tend to have more seeds per pod than broad leaflet varieties. Narrow leaflet in soybean is conferred by a single recessive gene, *ln*. Here, we show that the transition from broad (*Ln*) to narrow leaflet (*ln*) is associated with an amino acid substitution in the EAR motif encoded by a gene (designated Gm-JAGGED1) homologous to *Arabidopsis JAGGED* (*JAG*) that regulates lateral organ development and the variant exerts a pleiotropic effect on fruit patterning. The genomic region that regulates both the traits was mapped to a 12.6-kb region containing only one gene, Gm-JAG1. Introducing the Gm-JAG1 allele into a loss-of-function *Arabidopsis jagged* mutant partially restored the wild-type *JAG* phenotypes, including leaf shape, flower opening, and fruit shape, but the Gm-jag1 (*ln*) and EAR-deleted Gm-JAG1 alleles in the jagged mutant did not result in an apparent phenotypic change. These observations indicate that despite some degree of functional change of Gm-JAG1 due to the divergence from *Arabidopsis JAG*, Gm-JAG1 complemented the functions of *JAG* in *Arabidopsis thaliana*. However, the Gm-JAG1 homoeolog, Gm-JAG2, appears to be sub- or neofunctionalized, as revealed by the differential expression of the two genes in multiple plant tissues, a complementation test, and an allelic analysis at both loci.

**INTRODUCTION**

Leaves and flowers develop continuously at the flanks of the shoot apical meristem in flowering plants. A single mutation often causes pleiotropic phenotypes during leaf and flower development (Tsuchiya, 2006), suggesting that a common regulatory circuit is involved in the production of leaves and flowers. For example, *Leafy/UNIFOLIATA* regulates both leaf and flower morphogenesis in pea (*Pisum sativum*; Hofer et al., 1997). Conversely, combinations of floral homeotic mutations result in the conversion of floral organs to leaf-like structures (Bowman et al., 1993). The pea mutant *crispa* (*cri*), which is defective in *PHANTASTICA* (*PHAN*), has a reduced leaflet width-to-length ratio and exhibits pleiotropic effects, including longer internodes, reduced peduncle length, and lower seed production per pod (Tattersall et al., 2005). However, the molecular genetic basis of the relationship between leaf/flower development and pods or fruits, which are derived from the flower and associated tissues, is poorly understood.

Seed yield is determined by the number of seeds per unit area and seed weight. During soybean (*Glycine max*) production, the number of seeds per unit area is a product of the number of plants per unit area, the number of pods per plant, and the number of seeds per pod (NSPP) (Pedersen and Lauer, 2004). The NSPP is primarily determined by the number of ovules per placenta as well as the frequency of embryonic abortions. Soybean ovaries contain from one to four ovules, indicating that the NSPP is determined at the early stage of flower development (Carlson and Lersten, 2004). A quantitative trait loci (QTL) analysis using three recombinant inbred populations derived from reciprocal crosses of three cultivars demonstrated that the average NSPP in soybean is determined genetically by multiple significant QTL that account for ~50% of the heritable variation (Tischner et al., 2003). These QTL were linked to the *Ms1*, *Ms6*, or *St5* genes for male and female sterility and *Lf1* and *In* for leaflet number and leaflet shape, respectively, although further molecular genetic analyses have not been conducted. Among the genes associated with NSPP, the *ln* gene has been more frequently studied, as the *In* locus named after narrow leaflet has long been suggested to be tightly linked to the NSPP trait or to exert a major pleiotropic effect on the NSPP trait (Takahashi, 1934; Domingo, 1945; Bernard and Weiss, 1973). However, the genetic relationship between the two traits has not been easy to resolve partly due to additional minor modifying gene(s) in the *ln* genetic background (Weiss, 1970; Jeong et al., 2011).

Previous agronomic studies on the *ln* locus using isogenic lines (Mandl and Buss, 1981), diverse cultivars (You et al., 1995), and broad (*Ln/Ln*), heterozygous (*Ln/Ln*), and narrow (*ln/ln*) leaflet types (Dinkins et al., 2002) have repeatedly found that broad and narrow leaflet soybean genotypes have similar seed yields, but narrow leaflet plants consistently produce smaller seeds than those of broad leaflet plants. The fact that the narrow leaflet plants tend to produce a greater number of seeds has garnered a great deal of attention, particularly in the development of soybean cultivars.
We performed map-based cloning of the proving yield, one of the most complex plant traits. In this study, yield components may provide molecular genetic tools for improving yield, one of the most complex plant traits. Consequently, our results should facilitate development of new soybean cultivars with high yield potential.

RESULTS

Physical Map

Our fine genetic map was used to delimit the genomic region that regulates both leaflet shape and NSPP traits between microsatellite markers Ln_at004 and Ln_atre04 on soybean chromosome 20 (Jeong et al., 2011), a region that has a sequence length of 66 kb in the soybean genome (Schmutz et al., 2010), corresponding to 0.7 centimorgans (Figure 1A). To further dissect the 66-kb genomic region, 4219 F2 seeds derived from 162 F2 plants heterozygous for both Ln_at004 and Ln_atre04 were grown and screened for recombination between Ln_at004 and Ln_atre04. As a result, 17 recombinants with the genotype homozygous at one marker and heterozygous at the other were selected. The recombinants and their progeny were grown for a phenotypic evaluation of leaflet shape and NSPP traits. By further genotyping the recombinant plants using markers Ln_43k, Ln_44k, Ln_attre, Ln_AH, Ln_54k, and Ln_57k (see Supplemental Table 1 online), the In locus was localized to the interval between Ln_44k and Ln_57k (Figure 1B). Three markers (Ln_attre, Ln_AH, and Ln_54k) cosegregated with Ln. One recombination event between Ln_44k and Ln and one recombination event between Ln_57k and Ln were detected. Thus, the mutation responsible for the derivation of both narrow leaflet shape and high NSPP value in soybean was delimited to a 12.6-kb region between Ln_44k and Ln_57k (Figure 1C).

Only one gene (Glyma20g25000_1) has been predicted in this 12.6-kb region of the Glyma1.0 soybean gene annotation database (accessible at Phytozone v5.0, www.phytozone.net, accessed August 2012) (Figures 1C and 1D). When we conducted de novo gene annotation with AUGUSTUS (Stanke et al., 2006) for this sequence and queried Next-Gen Sequence Databases for small RNAs (http://mpss.udel.edu/scoy.shtml, accessed August 2012), no additional gene or small RNA could be predicted. The gene was predicted to be homologous to Arabidopsis thaliana JAGGED (JAG), which was reported to be involved in the development of lateral organs, including leaves and flowers (Dinneny et al., 2004; Ohno et al., 2004), and its coding region was physically located between 34,688,514 and 34,690,379 bp on the Gm20 pseudomolecule of soybean chromosome 20 (http://www.phytozone.net). A BLAST search against the soybean genome sequence revealed that Glyma20g25000_1 has a close paralog gene, Glyma10g42020_1, on the Gm10 of soybean chromosome 10. Glyma20g25000_1 and Glyma10g42020_1 were named Gm-JAG1 and Gm-JAG2, respectively.

BLAST searches of the 12.6-kb region against the GenBank EST database hit more than 10 ESTs, including GD767462 and GD866768, corresponding to the 5’ untranslated and 3’ untranslated regions of the annotated gene, respectively. However, alignment of those ESTs did not reveal a full-length open reading frame (ORF) and suggested different coding sequences from the annotated Glyma20g25000_1 and Glyma10g42020_1. Thus, the ORF of Gm-JAG1 was determined by cloning and then by sequencing RT-PCR products from RNA isolated from the shoot tips of ‘Sowon’ (Ln) and ‘V94-5152’ (Ln) using the primer pairs designed from the exon regions flanking the included intron (see Supplemental Table 2 online). Most of the RT-PCR products contained sequences of both Gm-JAG1 and Gm-JAG2, except the product of a cJAG-m-3 primer pair. Thus, the ORF sequence of Gm-JAG2 was also completely determined using an additional cJAG-mh-3 primer pair. A comparison of the multiple RT-PCR product sequences revealed that although rare, Gm-JAG1 contains an alternative splicing site of 6 bp (two amino acids) on the 5’ side of the third exon (Figure 1D). Thus, Gm-JAG1 encodes 256 (predominant) or 258 amino acid proteins. Gm-JAG1 and Gm-JAG2 showed 90.7% identity at the amino acid level of their predicted coding regions (see Supplemental Figure 1 online).

Four conserved domains were readily identified in the predicted Gm-JAG1 and Gm-JAG2 sequences when compared with Arabidopsis JAG and its paralog NUBBIN (NUB), tomato (Solanum lycopersicum) JAG, and maize (Zea mays) JAG: an EAR motif, a putative nuclear localization signal sequence, a single C2H2-type zinc finger motif, and a Pro-rich motif (Figure 1E; see Supplemental Figure 1 online). The putative nuclear localization signal sequence and Pro-rich motif are poorly conserved in NUB and Zm-JAG. Interestingly, our BLAST searches indicated that no soybean homolog of the NUB gene is present in the current soybean genome sequence assembly (Schmutz et al., 2010). A 1-bp substitution or change from Asp (Lr) to His (ln), which is the only difference between the ORF sequences of the Gm-JAG1 genes in V94-5152 (Ln) and Sowon (ln) plants, occurred at the EAR motif. As the conserved EAR motif contained a nonsynonymous mutation, it could be hypothesized that the mutation might be responsible for the phenotypic differences between the Ln and ln plants. Outside of these described domains, amino acid sequence conservation between Gm-JAG1 and At-JAG/Si-JAG was low, indicating possible functional dissimilarities (see Supplemental Figure 1 online).

A comparison of the 12.6-kb sequences between the mapping parents revealed eight polymorphisms between two single nucleotide substitutions for markers Ln_44k and Ln_57k (Figures 1C and 1D). These included five polymorphisms in the putative promoter region within 1.5 kb upstream of the 5’ untranslated exon, one in the 5’ untranslated exon, one in the second exon, and one (Ln_54k site) 3’ downstream of the stop codon.

The In Phenotype and Its Expression

Differences in leaflet shape and pod type between Ln and In soybean plants have been repeatedly assessed in different soybean
Control of Fruit Patterning by Ln

Figure 1. Molecular Cloning of ln.

*ln = GAT (Asp) to CAT (His)
genetic backgrounds (Takahashi, 1934; Domingo, 1945; Weiss, 1970; Bernard and Weiss, 1973; Mandl and Buss, 1981; You et al., 1995; Dinkins et al., 2002; Jeong et al., 2011). These studies have consistently shown that Ln plants tend to have broader leaflet shapes (Figures 2A and 2B) and lower NSPP than In plants (Figures 2C and 2D). As the mutations of the Arabidopsis JAG gene, the soybean homolog of which is presumed to encode Ln by the physical mapping described above, were reported to display defects in floral morphology including narrow floral organs (Dinneny et al., 2004; Ohno et al., 2004), we examined morphology of flowers. The flowers of In plants (Figure 2F) appear smaller than those of Ln plants (Figure 2E) likely because floral organs, including sepals and petals of In flowers, are smaller or narrower than those of Ln flowers. When we measured width of banner petals (Figure 2H) and length of carpels (Figure 2I), those of In plants were found to be significantly narrower and shorter than those of Ln plants (t test, P < 0.01). An extra petal or carpel occasionally appears in the In-containing parent Sowon (Figure 2G). However, these abnormal flowers likely are not determined by In, as they appear in both Ln- and In-recombinant plants.

To investigate whether promoter region mutations are responsible for the soybean leaflet shape and NSPP, JAG1 transcript levels in different soybean lateral organs of V94-5152 (Ln) and Sowon (In) plants were examined using RT-PCR. The patterns of RNA accumulation in both Ln and In plants were quite similar to each other. The JAG1 transcript was detected during vegetative and reproductive development in the shoot apex (meristem) and in open flowers (Figure 2J), as observed for At-JAG in different lateral organs of Arabidopsis (Ohno et al., 2004). In soybean, a low level of RNA transcript was detected in young In pods, but not in those of Ln at a higher cycle number. Validation of these results with quantitative RT-PCR (qRT-PCR) analysis suggested that JAG1 is most strongly expressed in the meristem and flowers of both Ln and In plants and is greatly reduced or not detectable in other organs (Figure 2K). Despite the lack of expression studies for this gene at the tissue level conducted previously in Arabidopsis studies (Dinneny et al., 2004; Ohno et al., 2004), these results indicate that the mutations in the promoter region of Gm-JAG1 did not affect overall mRNA expression patterns of the gene. Furthermore, as we observed only a single nucleotide substitution in the coding region of the Gm-JAG1 gene, which led to a single amino acid change, our subsequent analyses focused on determining whether this was the causal mutation of the phenotypic differences between the Ln and In genotypes.

Complementation of the Arabidopsis jag-3 Mutant with Gm-JAG1

To validate the function of Gm-JAG1 for broad leaflet and low NSPP (versus Gm-jag1 for narrow leaflet and high NSPP), we introduced the V94-5152 GmJAG1 allele driven by the Arabidopsis JAG promoter (PATJAG:gGmJAG1) into an Arabidopsis jag loss-of-function mutant (jag-3). Since Sowon is not readily transformed using current technology, complementation of the In phenotype with the Ln Gm-JAG1 allele was not performed. The jag-3 mutant expresses a defective truncated JAG protein by disruption of the 3′-splice acceptor sequence (Ohno et al., 2004). The presence of the soybean Gm-JAG1 allele in the transgenic lines was detected by PCR analysis and sequencing of PCR fragments and then further verified by RT-PCR (Figure 3A). The transgene (Figures 3B and 3H) showed substantial rescue of the jag-3 phenotypes in 34 of 107 primary transformants. Notably, the transformation of Gm-JAG1 converted narrow leaves and floral organs of the Arabidopsis jag-3 mutant into relatively broad leaves and floral organs, nearly the same characteristics as wild-type Arabidopsis Landsberg erecta (Ler) (Figures 3B to 3D and 3F to 3H). The serrated leaves (fifth leaf) in the PATJAG:gGmJAG1 transgenics appeared slightly later than those in jag-3 (fourth leaf) but appeared slightly earlier than in the wild type (seventh leaf) (Figures 3F to 3H). However, extra petals and rosette leaves relative to numbers in the wild type as previously reported for jag-3 (Ohno et al., 2004) were occasionally observed in the PATJAG:gGmJAG1 transgenics (Figures 3B to 3D and 3F to 3H), indicating that the transformation of Gm-JAG1 did not rescue these abnormalities in a notable manner. These results suggest that, despite substantial divergence in amino acid sequence, Gm-JAG and Arabidopsis JAG show a high degree of functional homology. The question remained of whether the one nonsynonymous nucleotide substitution (Gm-jag1 allele) detected at the EAR motif region of the Gm-JAG1 locus in the cultivated soybean had no or diminished functions relative to the Gm-JAG1 allele for leaflet shape and NSPP. To address this question, we introduced the Sowon GmJag1 allele driven by the Arabidopsis JAG promoter (PATJAG:gGmJag1) into the jag-3 mutants and obtained 63 transgenic (GmJag1) lines. Expression of the GmJag1 allele was confirmed by RT-PCR (Figure 3A). All PATJAG:gGmJag1 transgenics...
showed phenotypes identical to that of the Arabidopsis jag-3 mutant (Figures 3C, 3E, 3G, and 3I). These results substantiated that the mutation at the EAR motif is sufficient for the conversion of Gm-JAG1 to Gm-jag1.

Although JAG promotes the formation of the valve margin that facilitates the detachment of the valves from the replum during development of Arabidopsis fruit, which is also referred to as siliques (Dinneny et al., 2005), the role of JAG in siliques growth and seed set has been poorly characterized. To substantiate common functional homology between Arabidopsis JAG and Gm-JAG1 in terms of the fruit development, it was necessary to examine changes in siliques morphology, seeding pattern in the siliques, length of siliques, and the number of seeds per siliques in the Arabidopsis wild-type, jag-3, Gm-JAG transgenics, and Gm-jag transgenics. The siliques in wild-type plants were longer and thicker than those in the jag-3 plant (Figures 3J and 3K). However, seeds were more densely packed in the siliques in jag-3 than those in the wild type (Figures 3N and 3O). The Gm-JAG1 transgenic lines (Figures 3L and 3P) showed substantial rescue of the jag-3 siliques phenotypes. However, the siliques of the Gm-jag1 transgenic lines were similar in morphology to those of jag-3 (Figures 3M and 3Q). Siliques length was measured for each genotype, and seeds from one side of the septum after removing a valve in each siliques were counted for each genotype. Siliques

Figure 2. The ln Phenotype and Its Expression Pattern in Plant Tissues.

(A) and (B) Trifoliolate leaves of V94-5152 (Ln) and Sowon (ln). Bar = 50 mm.
(C) and (D) Pod types occurring in Ln (C) and ln (D) allele plants. Predominant two-seeded pods are indicated by an asterisk. Bar = 10 mm.
(E) and (F) Ln whole flower (E) and ln whole flower (F) and their detached banner petals, wing petals, keel petals, and carpels. Bar = 5 mm.
(G) Abnormal flowers having two banner petals, three keel petals, and two carpels, respectively. Bar = 5 mm.
(H) and (I) Distributions of banner petal width (H) and carpel length (I) in Ln and ln plants (n = 21). Perpendicular lines span from maximum to minimum of data, boxes span the interquartile range, and the horizontal lines indicate the median.
(J) RT-PCR analysis of Gm-JAG1 in different tissues of ln and Ln allele plants. Numbers refer to PCR cycles. RNA was extracted from tissues of field-grown plants. Amplification of actin 11 was used as a control.
(K) Quantitative RT-PCR analysis of Gm-JAG1 expression levels in different tissues of ln and Ln allele plants. Two biological replicates and three technical replicates were performed. Values were normalized to the expression of Actin 11 and are expressed relative to the level (set to 1.0) in the meristems of the Ln plants. Error bars indicate SE.
length and number of seeds in siliques were significantly longer and higher, respectively, in the wild type than those in jag-3 in the Fisher’s Protected LSD test (see Supplemental Figure 2 online). The number of seeds per unit silique length was significantly lower in the wild type than in jag-3. Silique length and number of seeds in the Gm-JAG1 transgenic lines were significantly shorter and lower than in the wild type but significantly longer and higher than those in jag-3. Collectively, our results suggest that introducing Gm-JAG1 into the jag-3 mutant partially rescued the wild-type silique phenotypes by restoring silique length and seed distribution.

Functional Divergence between Soybean JAG1 and Arabidopsis JAG

Our genetic and physical maps and complementation tests indicated that the single nucleotide substitution at the EAR motif...
of Gm-JAG1 was likely the causal mutation responsible for the phenotypic difference between Ln and ln soybean plants. However, more mutations were observed at the Gm-JAG1 promoter region and the sequence conservation between the Arabidopsis JAG and soybean JAG1 proteins was low outside of the conserved motifs, making it crucial to determine the extent to which the two proteins and their promoters are functionally similar. To address this question, we introduced several different gene constructs into the Arabidopsis jag-3 mutant. First, we compared the effect of Gm-JAG1 and Gm-jag1 misexpression in the jag-3 genetic background with the published effects of Arabidopsis JAG overexpression (Dinneny et al., 2004; Ohno et al., 2004) (Figure 4; see Supplemental Figure 3 online). The 35S:GmJAG1 transgenics displayed phenotypes highly reminiscent of PAtJAG:gGmJAG1 transgenic plants but did not display the aberrant phenotypes, including fusion of rosette leaves and stipule outgrowth from the lateral margins at the base of the leaf, observed in JAG overexpressing Arabidopsis. The 35S:Gmjag1 transgenics showed phenotypes nearly identical to those of the Arabidopsis jag-3 mutant. These results demonstrate that although Arabidopsis JAG and soybean JAG1 maintain common essential functions observed in the initial lateral organ development, the substantial divergence in amino acid sequence might have led to diminished function of G. max JAG1 in ectopic tissues. Second, to investigate whether the promoter regions that had more variation than coding regions in the comparison of Gm-JAG1 and Gm-jag1 sequences maintained any functional conservation between soybean and Arabidopsis, V94-5152 Gm-JAG1 (PGmJAG1:gGmJAG1) and Sowon Gm-jag1 (PGmjag1: gGmjag1) driven by their own promoter, respectively, were introduced into the Arabidopsis jag-3 mutant. We obtained 20 PGmJAG1: gGmJAG1 and 57 PGmjag1: gGmjag1 transgenics. RT-PCR did not detect expression of Gm-JAG1 and Gm-jag1 mRNA (Figure 4A), and all transgenics showed phenotypes identical to that of the Arabidopsis jag-3 mutant (see Supplemental Figure 3 online). The results indicate that the promoter region of Gm-JAG1 was no longer functional in Arabidopsis due to substantial divergence. Third, an EAR-deleted version of Gm-JAG1 (PAtJAG: gGmJAG1ΔEAR) was transformed into the Arabidopsis jag-3 mutant. We obtained 49 PAtJAG:gGmJAG1ΔEAR transgenic lines. All

Figure 4. Functional Analysis of 35S:GmJAG1, 35S:Gmjag1, PGmJAG1:gGmJAG1, PGmjag1:gGmjag1, PAtJAG:gGmJAG1ΔEAR, and PAtJAG: gGmJAG2 Gene Constructs in the Arabidopsis jag-3 Mutant.

(A) Confirmation of expression of the introduced gene constructs in the transgenic Arabidopsis jag-3 lines by RT-PCR analysis. RNA was extracted from inflorescence tissues of 35S:GmJAG1, 35S:Gmjag1, PAtJAG:gGmJAG1, PAtJAG:gGmjag1, and PAtJAG: gGmJAG2 transgenic plants, wild-type Ler, and the jag-3 mutant.

(B) Confirmation of expression of the introduced gene constructs in the transgenic Arabidopsis jag-3 lines by RT-PCR analysis. RNA was extracted from inflorescence tissues of PAtJAG:gGmJAG2 transgenic plants, wild-type Ler, and the jag-3 mutant.

(C) to (H) Fruit morphology. Fully elongated fruits of Ler (C), jag-3 (D), 35S:GmJAG1 (E), 35S:Gmjag1 (F), PAtJAG:gGmJAG1ΔEAR (G), and PAtJAG: gGmJAG2 (H). Bar = 1 mm.
Evolutionary Diversification of JAG in Soybean

The BLAST search indicated that Gm-JAG1 and its homoeolog Gm-JAG2 are located on large duplicated blocks between soybean chromosomes 20 and 10, respectively, in the soybean genome, which were presumably generated by a soybean lineage–specific paleoalotetraploidy event dated to ~13 million years ago (Schmutz et al., 2010). Therefore, these two genes are a homoeologous pair. The presence of only two copies of the Gm-JAG homolog was substantiated by DNA gel blot analysis (see Supplemental Figure 4 online). The sequences of the Gm-JAG2 promoter and coding region, determined from Sowon and V94-5152, were identical, indicating that Gm-JAG2 is not responsible for the phenotypic difference between Sowon and V94-5152. However, as amino acid sequences of Gm-JAG1 and Gm-JAG2 are identical in the three conserved domains except the Pro-rich domain out of the four conserved domains, Gm-JAG2 may be functional. To address this question, we introduced Gm-JAG2 driven by the Arabidopsis JAG promoter (PAtJAG:gGmJAG2) into the Arabidopsis jag-3 mutant. Surprisingly, 13 of 35 PATJAG:gGmJAG2 transgenics showed phenotypes nearly identical to PATJAG:gGmJAG1 transgenic plants relative to the wild-type Arabidopsis (Figure 4H; see Supplemental Figure 3 online).

The expression patterns of Gm-JAG1 and Gm-JAG2 were compared to better understand their functional diversity. RT-PCR and qRT-PCR were used to profile Gm-JAG2 expression in different lateral organs. Consistent with the expression patterns of Arabidopsis JAG, Gm-JAG1 was mainly expressed in the meristem and flowers (Figure 2J). By contrast, the Gm-JAG2 transcript was strongly expressed in broader types of tissues than was Gm-JAG1 (Figure 5). At similar levels to that of the shoot apex and open flowers, the Gm-JAG2 transcript was expressed in young pod tissues of both In and Ln plants, and it was markedly detected in young leaf tissues of Ln plants but greatly reduced in those of In plants (Figure 5B). These results indicate that Gm-JAG2 may be sub- or neofunctionalized.

Allelic variation of Gm-JAG1 in the wild and cultivated soybean populations was evaluated in the 59 accessions containing a broad leaflet type and in 12 accessions containing a narrow leaflet type. The genotypes scored by the Ln_AH marker generated from the 1-bp substitution mutation at the EAR correlated perfectly with the genotypes scored by leaflet types (see Supplemental Table 3 online). We observed no additional allelic variation presumed to affect the amino acid sequence encoded by Gm-JAG1 from a comparison of sequences of Gm-JAG1 determined from 14 accessions selected from the above 71 accessions (see Supplemental Figures 5 and 6 online).

DISCUSSION

We isolated the classical soybean locus (In) that shapes narrow leaflets and increases seed number in soybean by positional cloning. Ln encodes Gm-JAG1, a homolog of an Arabidopsis transcription factor that acts to promote lateral outgrowth in aerial organs (Dinneny et al., 2004; Ohno et al., 2004). The gene expression analysis indicated that the transition from dominant Ln (V94-5152 cultivar) to recessive In (Sowon cultivar) phenotypes in soybean was associated with a single nucleotide substitution at a region encoding the Gm-JAG1 EAR motif, which led to a single amino acid change. When Gm-jag1 was introduced into the Arabidopsis jag-3 mutant, it restored the wild-type phenotypes of the flowers, leaves, and silique shape to some degree. When Gm-jag1 was introduced into the Arabidopsis jag-3 mutant, it did not restore the wild-type phenotypes, indicating that the single nucleotide mutation is equivalent to a loss-of-function mutation. Collectively, our results show that phenotypic variations for narrow leaflet and high NSPP are predominantly the result of the pleiotropic effects of the In gene and thus provided an answer to a long debated hypothesis of whether leaflet shape and NSPP are regulated by the In gene alone or by the tightly linked loci including In.

The molecular basis of the relationship between floral organs and leaves has been actively studied ever since the formation of the ABC model (Coen and Meyerowitz, 1991). While there are distinct morphological differences in flower, leaf, and silique (fruit)
shapes between the wild-type Arabidopsis and jag mutants and between the wild-type tomato and lyrate mutant, Ln (Gm-JAG1) and ln (Gm-jag1) soybean plants showed less distinct morphological differences in leaf, pod, and flower shapes. This may have been due to the extent of the mutation or differences in the organization of development in soybean, Arabidopsis, or tomato or due to the existence of a neo- or subfunctionalized homoeologous gene, JAG2. Similar observations have been reported by functional studies of PHAN. phan mutations have similar phenotypes in tomato (Kim et al., 2003) and tobacco (Nicotiana tabacum) (McHale and Koning, 2004), but in the homologous maize rs2 and Arabidopsis as1 mutants, the leaves develop with essentially normal polarity (Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). Unlike the cri mutant, another PHAN-defective pea mutant, which shows decreased seed production per pod in the mutant compared with the wild type (Tattersall et al., 2005), the ln mutant led to an increase in the NSPP.

The only difference between Sowon (ln) and V94-5152 (Ln) at the coding region of Gm-JAG1 was a single nucleotide substitution at the EAR motif region. The results from the expression patterns, allele diversity analysis, and transformations of the Gm-jag1 and EAR-deleted Gm-JAG1 genes into the Arabidopsis jag-3 mutant supported that the mutation was solely responsible for the phenotypic difference between Ln and ln. The EAR motif is found in broad gene families and is conserved across diverse plant species (Kagale et al., 2010). The EAR motif is composed of LxLxL, DLNxxP, or an overlap of the two (Kagale et al., 2010). The Gm-JAG1 EAR motif is composed of LDLNNLP. Gm-jag1 contains LHLLNLNP, in which the second D, Asp, which has an acidic side chain, is replaced by H, His, which has a basic side chain. Arabidopsis JAG and tomato JAG contain LDLNLNP, supporting that the JAG EAR motif is conserved among different plant species. The EAR motif contains alternating hydrophilic and hydrophobic amino acids, and the amphiphilic Asp is necessary for its repressive function (Ohta et al., 2001; Hiratsu et al., 2003). When the EAR motif is fused to transcription factors, the transcription factors become repressors, and transcription factors that activate other transcription factors become transrepressors (Ohta et al., 2001). Thus, our finding that the single mutation at the EAR motif leads to loss of JAG activity indicates that JAG functions as a repressor, thereby providing further insight into efforts to find the position of JAG in genetic regulatory networks that coordinate lateral organ development. Many genes upstream or downstream of JAG have been reported recently. JAG expression is negatively regulated by the BLADE-ON-PETIOLE genes in lateral organs, particularly in the Arabidopsis cryptic bract (Hepworth et al., 2005; Norberg et al., 2005). JAG activity is negatively regulated by REPLUMLESS (Dinneny et al., 2005), which promotes replum development in Arabidopsis fruit (Roeder et al., 2003). JAG, together with ASYMMETRIC LEAVES1 (AS1) and AS2, represses the expression of the boundary-specifying genes CUP-SHAPED COTYLEDONS1 and 2 and PETAL LOSS, which maintains the low cell proliferation rate near the organ primordia for the development of the sepal and petal (Xu et al., 2008). JAG negatively regulates KNOTTED1 LIKE HOMEBOX expression in developing tomato leaves and positively regulates expression of the auxin-responsive genes PIN-FORMED1, AUXIN/INDOLE-3-ACETIC ACID 9 (IAA9), and IAA4, which are important for leaflet initiation and for lamina outgrowth in tomato (David-Schwartz et al., 2009). As our results support the function of JAG as a repressor that uses the EAR motif, it will be interesting to further elucidate what genes/signals upstream or downstream of JAG in the genetic regulatory networks interact directly with JAG at the molecular level.

Our transformation and expression studies indicate that both Gm-JAG1 and Gm-JAG2, which are located on homoeologous chromosomes, are functional, as indicated by the partial restoration of the wild-type phenotypes in the Arabidopsis jag-3 mutant. Dinneny et al. (2006) showed that the functional differences between JAG and its paralog NUB in Arabidopsis are caused by slightly different tissue expression patterns. Our expression analysis indicated that Gm-JAG1 and Gm-JAG2 are expressed in a different range of tissues, suggesting sub- or neofunctionalization of Gm-JAG2. Therefore, the results from the previous Arabidopsis JAG paralogs are somewhat reminiscent of how a defective mutation in one of two highly similar soybean genes, Gm-JAG1 and Gm-JAG2, causes phenotypic difference between mutated and wild-type soybeans.

In this study, we demonstrated that the recently completed whole-genome sequence of soybean could serve as bridging information to translate the information gained from a model species into gene discovery and functional characterization in a crop species, soybean. We then further analyzed silique phenotypes in order to gain insight into yield, which has been generally regarded as a complex trait. Direct evidence of pleiotropy for the leaflet shape and NSPP by the cloning of the ln gene has fundamental implications for yield improvement, at least for sprout specialty soybeans, by individually manipulating the component traits using molecular-assisted selection and providing insight into locating the position of JAG as a repressor in genetic regulatory networks.

METHODS

Plant Materials

A BC2F2 population was developed from four self-fertilized BC1F1 hybrids made between an elite narrow leaf soybean cultivar for sprouts (the female parent, Sowon) and a broad leaflet soybean cultivar (the male parent, V94-5152) (hereafter, referred to as the SV population). A total of 309 F2 individuals were previously used to establish linkage relationships among molecular markers, the NSPP, and the narrow leaflet shape trait presumed to be determined by the ln gene (Jeong et al., 2011). To further dissect the 66-kb genomic region detected in the previous study (Jeong et al., 2011), 4219 F3 seedlings derived from 162 F2 plants heterozygous for both Ln_at004 and Ln_atre04 were grown and screened for recombinants between Ln_at004 and Ln_atre04. Plants with the homozygous genotype at one marker and heterozygous genotype at the other locus were selected, and a total of 17 individuals were grown for phenotypic evaluation.

The genetic correlation between markers and leaf shape was assessed in a collection of 71 presumably diverse soybean cultivars or wild accesses listed in Supplemental Table 3 online. Among them, 14 variants were further analyzed by sequencing: Sowon, Pungsannamul, Myeonggymul, IT182932, IT178535, IT191201, IT184014, and PI549049 collected or developed in Korea, V94-5152 and Williams 82 developed in the US, and four wild soybean accessions PI378691 (collected in the Japan), PI407290 (China), PI423991 (Russia), and PI518282 (Taiwan).
DNA Isolation and Marker Analysis

Young trifoliolate leaf tissues from soybean accessions were collected. Soybean genomic DNA was isolated as described previously by Saghai Marof et al. (1984). For quick preparation from the F23 plants of the SV population, soybean genomic DNA was isolated using a FastDNA kit in accordance with the manufacturer’s protocols (MP Biomedicals) from a single young leaflet. The extracted DNA was dissolved in 200 μL water, and then 2 μL of the solution was used in a 20-μL PCR to amplify DNA fragments for marker genotyping. Microsatellite and single nucleotide polymorphism markers were analyzed as previously described (Cregan et al., 1999; Jeong and Saghai-Marof, 2004).

PCR of Genomic DNA and Sequencing

Genomic DNA isolation, PCR primer design, PCR amplification, PCR fragment purification, and sequencing of PCR fragments were conducted as described previously (Jeong and Saghai-Marof, 2004). Primers used for PCR amplification of Gm-JAG1 and Gm-JAG2 are listed in Supplemental Table 2 online. In brief, the PCR mixture contained 20 ng of total genomic DNA, 1 × PCR buffer (10 mM Tris-HCl and 50 mM KCl, pH 8.3), 2.5 mM MgCl2, 100 nM of each forward/reverse primer, 0.16 mM of each deoxy-nucleotide triphosphate, and 0.25 units of Taq polymerase in a total volume of 20 μL. Standard PCR was conducted as follows: a denaturation step at 94°C for 5 min, 34 cycles at 94°C for 30 s, 43 to 58°C for 30 s, 72°C for 30 s, and an extension step at 72°C for 5 min followed by a 4°C soak. Alignment of the nucleotide and amino acid sequences was performed using ClustalW implemented in BioEdit (Hall, 1999).

Vector Construct for Transformation

For the PAtJAG:gGmJAG1 vector, the JAG promoter was amplified first from the Arabidopsis thaliana Ler ecotype using primers pA-JAG and Xhol-pA-JAG listed in Supplemental Table 2 online. The promoter was then cloned into the pENTR/D-TOPO entry vector (Invitrogen) following the manufacturer’s protocol. The genomic fragments of Gm-JAG1 (Ln) were amplified from soybean cultivar V94-5152 using primers Xhol-soyJAG-1 and soyJAG. The DNA fragment was then cloned into the pENTR/D-TOPO entry vector. The genomic Gm-JAG1 in V94-5152 was subcloned into the PAtJAG entry vector using Ascl and Xhol sites. The PAtJAG:gGmJAG1 cassette was transferred to the destination vector pHGWL7 using the attL × attR reaction. For the PAtJAG:gGmJAG2 vector, the genomic fragment of Gm-JAG2, the Gm-JAG1 homoeolog, was amplified from V94-5152 using primers Xho1-hm-soyJAG and hm-soyJAG. The genomic fragment was then cloned into the pENTR/D-TOPO entry vector. Then, GmJAG2 was subcloned into the pPAtJAG entry vector using Ascl and Xhol sites. The Gateway cassette of PAtJAG:gGmJAG2 was transferred into pHGWL7 by the attL × attR reaction.

Arabidopsis Transformation

The gene constructs were introduced into Agrobacterium tumefaciens strain GV3101. Transgenic plants were generated by vacuum infiltration of Arabidopsis Ler mutant jag-3 recipient plants kindly provided by E. Meyerowitz using the Agrobacterium strain GV3101 (Bechtold and Pelletier, 1998), and plants were selected using resistance against the antibiotic hygromycin.

DNA Gel Blot Analysis

Eight micrograms of the genomic DNA from both V94-5152 and Sowon were digested overnight with restriction enzymes, fractionated on 1% agarose gel, alkaline transferred onto Hybond N+ nylon membranes (Amersham Pharmacia), and probed using biotin-labeled Gm-JAG1 gene probes amplified using SoyJAG-2and SoyJAG-2 listed in Supplemental Table 2 online. DNA probe preparation was conducted using the Nick Translation System (Invitrogen) in accordance with the manufacturer’s instructions. The biotin-labeled DNA was then detected via chemiluminescence using streptavidin alkaline phosphatase and CDP-Star (Amersham Pharmacia). Hybridization, membrane washing, and detection were all conducted in accordance with the manufacturer’s instructions. The size of the DNA band was compared with a 1-kb DNA ladder (Bioneer).

RNA Isolation and Expression Analysis

For analysis of the expression of Gm-JAG1 and Gm-JAG2, total RNA was isolated from expanded leaves, young leaves, meristems, flowers, and young pods collected from three individuals. For the examination of Gm-JAG1 and its variants and Gm-JAG2 in the transgenic Arabidopsis jag-3 lines, total RNA was isolated from Arabidopsis inflorescences. The tissues were frozen immediately in liquid nitrogen and then ground with a mortar and pestle. RNA was extracted using the RNaseasy plant mini kit (Qiagen). Before cDNA synthesis, all RNA samples were treated using RNase-free RNAse (Promega). For RT-PCR, cDNA was synthesized by one-step RT-PCR (Qiagen) using gene-specific primers as designed by the manufacturer. The PCR products were generated using the following primer sets: cJAG-sp for Gm-JAG1 and cJAG-hsp for Gm-JAG2. The soybean Actin 11 gene was used as an internal control for RT-PCR analysis in soybean (Jian et al., 2008), and the Arabidopsis UBQ10 gene was used as an internal control in Arabidopsis (Czechowski et al., 2005). The PCR products were separated by agarose gel electrophoresis on a 1.5% gel with ethidium bromide staining. For qRT-PCR, first-strand cDNA was synthesized from 1 μg total RNA in a 20-μL reaction mixture using the AccuPower RocketScript RT PreMix kit (Bioneer). All qRT-PCR reactions were performed in an ABI
The following materials are available in the online version of this article.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Gm-JAG1 (JX119212 to JX119213 and JX119216 to JX119226), Gm-JAG2 (JX119214 to JX119215), At-JAG (AY465924), NUB (NM_101210), LYRATE (EU490064), and Zm-JAG (NM_001153894).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Alignment of Amino Acid Sequences of Soybean JAG1 and Its Homoeolog JAG2, At-JAG, and NUBBIN from *Arabidopsis thaliana*, LYRATE from Tomato, and Zm-JAG from Maize.

**Supplemental Figure 2.** Silique Phenotypes Observed in *Arabidopsis* Wild-Type Landsberg erecta, jag-3 Mutant, Three jag-3 Mutant Lines Transformed with Transgene PAtJAG:gGmJAG1, and Three jag-3 Mutant Lines Transformed with Transgene PAJAG:gGmJAG1.

**Supplemental Figure 3.** Comparison of Phenotypes of 3SS:GmJAG1, 3SS:Gnmjag1, PGMJAG1:gGmJAG1, PGMnjag1:gGmnjag1, PAJAG: gGmJAG1:3EAR, and PAJAG:gGmJAG2 Gene Constructs in the *Arabidopsis* jag-3 Mutant.

**Supplemental Figure 4.** Copy Number Analysis of Gm-JAG1 and Gm-JAG2.

**Supplemental Figure 5.** Variation in Leaflet Shape among Cultivated and Wild Soybean Accessions.

**Supplemental Figure 6.** Polymorphic Sites Identified from Comparison of Gm-JAG1-Containing Sequences from 14 Soybean Lines.

**Supplemental Table 1.** Attributes of Single Nucleotide Polymorphism Markers for Fine Mapping.

**Supplemental Table 2.** Primers for Sequencing, RT-PCR, and Probe Generation Used in This Study.

**Supplemental Table 3.** Leaflet Shapes of Cultivated and Wild Soybean Accessions and Their Genotype at Marker Ln-AH Locus.

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**AUTHOR CONTRIBUTIONS**

N.J., S.J.S., and M.K. performed the experiments. S.L., J.-K.M., and H.S. K. contributed the experimental soybean accessions and population and designed the research. S.-C.J. designed the research and wrote the article.

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