Loss of Function of the E1-Like-b Gene Associates With Early Flowering Under Long-Day Conditions in Soybean

Jianghui Zhu¹, Ryoma Takeshima², Kohei Harigai¹, Meilan Xu¹,³, Fanjiang Kong³,⁴, Baohui Liu⁴*, Akira Kanazawa¹, Tetsuya Yamada¹ and Jun Abe¹* 

¹ Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, ² Institute of Crop Science, National Agriculture and Food Research Organization, Tsukuba, Japan, ³ Key Laboratory of Soybean Molecular Design Breeding, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, China, ⁴ School of Life Sciences, Guangzhou University, Guangzhou, China

Keywords: soybean, Glycine max, flowering, E1Lb, photoperiodism, adaptation

INTRODUCTION

Photoperiod response of flowering determines the adaptation of crops to a wide range of latitudes with different daylengths during growing seasons. Its regulatory mechanisms vary with plant species, and may rely on both evolutionally conserved and species-specific gene systems. In Arabidopsis, a long-day (LD) plant, CONSTANS (CO) plays a key role in regulation of photoperiodic
However, unlike in *Arabidopsis*, *Hd1* activates *Hd3a* expression under inductive LD conditions, which in turn activates *FLOWERING CO* 

**Coupland, 2012; Song et al., 2013).** Similarly, in rice, a short-day (SD) plant, a *CO* ortholog, *Heading date 1* (*Hd1*) (Yano et al., 2000), regulates the *FT* orthologs *Heading date 3a* (*Hd3a*) and *Rice FT-like 1* (*RFT1*) (Kojima et al., 2002; Tamaki et al., 2007).

Moreover, *Arabidopsis* *Hd1* is controlled by a complex of *Hd1* with the monocot-specific CCT domain protein Grain HD1 with the monocot-specific CCT domain protein Grain* (Izawa et al., 2002). This functional switch, which is absent in *Arabidopsis*, is controlled by a complex of *Hd1* and the monocot-specific CCT domain protein Grain HD1 with the monocot-specific CCT domain protein Grain* (Izawa et al., 2002). This functional switch, which is absent in *Arabidopsis*, is controlled by a complex of *Hd1* and the monocot-specific CCT domain protein Grain HD1 with the monocot-specific CCT domain protein Grain (Izawa et al., 2002). This functional switch, which is absent in *Arabidopsis*, is controlled by a complex of *Hd1* and the monocot-specific CCT domain protein Grain HD1 with the monocot-specific CCT domain protein Grain (Izawa et al., 2002). This functional switch, which is absent in *Arabidopsis*, is controlled by a complex of *Hd1* and the monocot-specific CCT domain protein Grain HD1 with the monocot-specific CCT domain protein Grain (Izawa et al., 2002).

In rice (Cao et al., 2015), Ghd7 represses the expression of the B-type response regulator *Early heading date 1* (*Ehd1*) (Doi et al., 2004), an activator of *Hd3a* and *RFT1* expression, by binding to its cis-regulatory region (Nemoto et al., 2016).

**Soybean (Glycine max) has multiple CO orthologs** (Fan et al., 2014; Wu et al., 2014), of which two pairs of homoeologs, *CO-like (COL) 1a/COL1b* and *COL2a/COL2b*, fully complement the function of *CO* in *Arabidopsis* (Wu et al., 2014). *COL1a* overexpression in soybean causes late flowering, and artificial *COL1b* mutants flower significantly earlier than the wild type, indicating that both *COL1a* and *COL1b* function as floral suppressors under LD conditions, as in rice (Cao et al., 2015). However, unlike in the case of *Hd1*, the overexpression of *COL1a* does not promote flowering under inductive SD conditions, although it up-regulates major soybean *FT* genes by virus-induced gene silencing (VIGS) in a cultivar (Fan et al., 2012; Xu et al., 2015). Down-regulation of the *COL1a* ortholog, *Samanfar et al., 2017*, four maturity genes, *E1*-like-a (*E1Lb*), *E1Lb*, and *E3* or *E4* or *E4*, and (group 3) a combination of *e1-as* (hypomorphic allele), *e3*, and *E4*. Because *E4* inhibits flowering under ILD conditions (Saindon et al., 1989; Cober et al., 1996; Abe et al., 2003; Liu and Abe, 2010), the group 3 cultivars have novel genes that abolish or reduce ILD sensitivity. One such gene is an early-flowering allele at *qDTP-I*, a QTL for days to flowering in linkage group J, which encodes FT5a; early flowering is caused by its increased transcriptional activity or mRNA stability associated with an insertion in the promoter and/or deletions in the 3′ UTR (Takeshima et al., 2016). Here, we describe a novel loss-of-function allele at the *E1Lb* locus, which is most likely involved in the gain of photoperiod insensitivity in group 3 soybean cultivars. Our data suggest that *E1Lb* inhibits flowering under LD conditions, independently of *E1*, and play major roles in the control of flowering in soybean.

**MATERIALS AND METHODS**

**Plant Materials and Segregation Analysis**

The indeterminate Far-Eastern Russian soybean cultivars Zeika (ZE), Yubileinaya (YU), and Sonata were crossed with the Canadian indeterminate cultivar Harosoy (L58-266; HA); ZE and YU were also crossed with a Harosoy near-isogenic line (NIL) for *e3* (PI547716; H-e3). The three Russian cultivars have the same genotype as H-e3 at five maturity loci, *E1*, *E2*, *E3*, *E4*, and *E9* (e1-as/e2/e3/E4/E9), but unlike H-e3 they flower without any marked delay under ILD conditions in comparison with natural daylength (ND) conditions (maximum daylength, 15.2 h) in Sapporo, Japan (43°07′N, 141°35′E) (Xu et al., 2013). The ILD condition was set at an experimental farm of Hokkaido University by extending the ND to 20 h by supplemental lighting from 2:00 to 7:00 and from 18:00 to 22:00 with incandescent lamps with a red-to-far-red (R:FR) quantum ratio of 0.72 (Abe et al., 2003). Seeds of *F*2 populations and parents were sown in paper pots (Paperpots No. 2, Nippon Beet Sugar Manufacturing Co., Tokyo, Japan) on 28 May 2013 for the crosses with HA and 26 May 2014 for crosses with H-e3. The pots were put under the
ILD condition, and 12 days later seedlings were transplanted into soil. The progeny test was carried out for 48 F₂ plants randomly selected from the H-e₃ × ZE cross and recombinant plants used in fine mapping. Seeds of these plants were sown in paper pots on late May in 2015 to 2017 (25 May, 2015; 28 May, 2016; and 26 May, 2017). After 12 days under the ILD condition, 15 seedlings per plant were transplanted into the same field. The number of days from sowing to the first flower opening (R1) (Fehr et al., 1971) of each plant was recorded.

**Association Test, Linkage Map Construction, and Fine Mapping**

A total of 16 F₂ plants from the H-e₃ × ZE cross were used to test the association of ILD sensitivity with simple sequence repeat (SSR) marker genotypes. They were selected based on the segregation pattern in their progeny, and included 8 plants fixed for ILD insensitivity and 8 plants fixed for ILD sensitivity. SSR markers were chosen from those located in genomic regions that harbored the soybean orthologs of Arabidopsis flowering genes (Song et al., 2004; Watanabe et al., 2012). The SSR markers significantly associated with ILD sensitivity were genotyped for a total of 306 F₂ plants from the H-e₃ × ZE and H-e₃ × YU crosses to confirm the detected association. Plants recombinant in the targeted region were subjected to fine mapping; the genotypes for the target gene were estimated based on the segregation of flowering under the ILD condition in the progeny and were compared with the graphical genotypes constructed by using additional 11 BARCSOY SSR markers (Song et al., 2010) (Supplementary Table S1).

**Development of NILs**

Four sets of NILs, each including one NIL for ILD insensitivity and another one for sensitivity, were developed from heterozygous inbred F₅ plants derived from different F₂ plants (#4 and #21) from the H-e₃ × ZE cross and those (#11 and #20) from the HA × ZE cross. The former two sets of NILs had the recessive e₃ allele, whereas the latter two had the dominant E₃ allele. These lines, together with parents and an ILD-insensitive NIL of HA for e₃ and e₄ (P1546043; H-e₃e₄), were cultivated in a growth chamber (25°C, 20-h daylength) with an average photon flux of 120 µmol m⁻² s⁻¹ and an R:FR ratio of 2.2 at 1 m below light sources, or in the field under the ILD condition (sowing date, May 26, 2018), as described above. For comparison, indeterminate NILs for alleles, e₁-nl and e₁-as, at E₁ (NIL-E₁; e₂/e₃/E₄/E₉), which were developed from a heterozygous inbred F₅ plant derived from a cross between the Japanese determinate cultivar Toyomusume (e₁-nl/e₂/E₃/E₄/e₉) and HA, were included in the evaluation of flowering under the ILD condition.

**DNA Extraction and SSR Marker Analysis**

Total DNA was extracted from trifoliate leaves of each of 150 H-e₃ × ZE and 156 H-e₃ × YU F₂ plants as described by Doyle and Doyle (1990), and from each of 492 seeds from two F₂ plants from the H-e₃ × ZE cross, as described by Xia et al. (2012). Each PCR mixture for SSR marker analysis contained 30 ng of total genomic DNA as a template, 0.2 µl of each primer (10 µM), 0.8 µl of dNTPs (2.5 mM), 0.1 µl of Taq DNA polymerase (Ampliqon), and 1 µl of 10× ammonium buffer (Ampliqon) in a total volume of 10 µl; amplification conditions were 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were separated by electrophoresis in 10.5% (w/v) polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

**Expression Analysis**

A new fully expanded leaflet was sampled from each of four plants per parent and NIL at Zeitgeber time 3 in two different growing stages, the 2nd and 3rd leaf stages. The sampled leaves were bulked, immediately frozen in liquid N₂, and stored at −80°C. Total RNA was isolated from frozen tissues with TRIzol Reagent (Thermo Fisher Scientific). DNase I (Takara) was used to remove genomic DNA. The complementary DNAs (cDNAs) were synthesized from 1 µg of total RNA by using the M-MLV reverse transcriptase system (Invitrogen) with an oligo (dT) 20 primer in a volume of 20 µL. Transcript levels of E₁L₁, E₁L₂, E₁L₅, FT2a, and FT5α were determined by quantitative real-time PCR. The PCR mixture (20 µL) contained 0.1 µL of the cDNA synthesis reaction mixture, 5 µL of 1.2 µM primer premix, and 10 µL SYBR Premix Ex Taq II (Takara). A CFX96 Real-Time System (Bio-Rad) was used. The PCR cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 59°C for 30 s, 72°C for 20 s, and 78°C for 2 s. Fluorescence was quantified before and after the incubation at 78°C to monitor the formation of primer dimers. The mRNA for β-tubulin was used for normalization. A reaction mixture without reverse transcriptase was also used as a control to confirm the absence of genomic DNA contamination. Amplification of a single DNA fragment was confirmed by melting curve analysis and gel electrophoresis of the PCR products. Averages and standard errors of relative expression levels were calculated from PCR results for three independently synthesized cDNAs. Primer sequences used in expression analyses are listed in Supplementary Table S1.

**Sequencing and Marker Analysis of E₁Lb**

The coding sequences of the three gene models, Glyma.04G143300, Glyma.04G143400 and Glyma.04G143500, were analyzed for H-e₃ and ZE. The coding sequences were amplified from the cDNAs by using primers listed in Supplementary Table S1. The amplified fragments were cloned into a pGEM-T Easy vector (Promega) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRISM 3100 Avant Genetic Analyzer (both from Applied Biosystems, Japan) according to the manufacturer’s instructions. A derived cleaved amplified polymorphic sequence (dCAPS) marker targeting a single-base deletion observed in ZE was developed to discriminate the functional E₁L₅ allele of H-e₃ from the loss-of-function e₁Lb allele of ZE. The 275-bp DNA fragment amplified from ZE by PCR with the forward primer 5'-GTGTAAACACTCAAGTCCT-3' and the reverse primer 5'-CGTCTTCTTGACTTCTCAACG-3' was digested with HpyCH4IV (New England Biolabs Japan) into two fragments, 254 bp and 21 bp, but the 276-bp fragment amplified from H-e₃ was resistant to HpyCH4IV digestion. The PCR products
were treated with HpyCH4IV for 1 h and then separated by electrophoresis in 2.5% NuSieve 3:1 gel (Lonza), stained with ethidium bromide, and visualized under UV light.

**Survey of the Dysfunctional Allele in ILD-Insensitive Accessions**

A total of 62 ILD-insensitive accessions including the three Russian cultivars were surveyed for the E1Lb genotype using the allele-specific DNA marker. They included 9 accessions from northern Japan, 26 from north-eastern China, 16 from Far-Eastern Russia, 8 from Ukraine, and 3 from Poland (Supplementary Table S2). The maturity genotypes at E1 to E4 of 50 accessions were determined previously by Xu et al. (2013), and those of the remaining 12 accessions were assayed according to Xu et al. (2013) and Tsubokura et al. (2014).

**RESULTS**

**Segregation of Flowering Time in F2 and F3 Populations**

The three Russian cultivars are photoperiod insensitive (Xu et al., 2013). They flowered 45–47 days after sowing (DAS) under the ND condition of Sapporo, whereas H-e3 and HA flowered approximately 5 and 10 days later, respectively. Under the ILD condition, the three cultivars and H-e3 flowered 2–4 days and around 20 days later than under ND, respectively, whereas HA continued vegetative growth and did not develop any flower buds until the end of light supplementation (10 August, 76 DAS).

Flowering time under the ILD condition in F2 populations of the H-e3 × ZE and H-e3 × YU crosses varied continuously from that of ILD-insensitive parents (45 DAS for ZE and 46 DAS for YU) to the end of light supplementation; 10 out of 150 and 12 out of 156 plants had no flower buds in the H-e3 × ZE and H-e3 × YU F2 populations, respectively (Figure 1). In both populations, the distribution of flowering time tended to be bi-modal; plants which flowered at 56 DAS and later or remained vegetative segregated more than those which flowered earlier. We randomly selected 48 H-e3 × ZE F2 plants and tested their progeny for flowering time segregation under the ILD condition. Based on the segregation pattern, the 48 F2 plants could be classified into three groups: (1) plants fixed for ILD insensitivity (all F3 plants tested flowered as ZE did; e/e); (2) those segregating for flowering time (E/e) and (3) those fixed for ILD sensitivity (all F3 plants tested showed delayed or no flowering; E/E) (Figure 1A). The number of plants was 8 in e/e, 23 in E/e, and 17 in E/E, in consistence with a monogenic 1:2:1 ratio (χ2 = 3.81, df = 2, p = 0.18), suggesting the involvement of a single recessive gene for ILD insensitivity. Based on the results of the progeny test, we classified 306 F2 plants into early-flowering ILD-insensitive plants, which flowered before 56 DAS, and late- or non-flowering ILD-sensitive plants (Figure 1). The segregation ratios of the two classes fit the expected 3:1 ratio (χ2 = 0.33, df = 1, p = 0.56 for H-e3 × ZE, χ2 = 3.28, df = 1, p = 0.07 for H-e3 × YU), confirming that ILD insensitivity is controlled mainly by a single recessive gene.

We also examined the segregation of flowering time under the ILD condition for the crosses between HA and the three Russian cultivars. Because HA had the E3 allele and the three cultivars had the e3 allele, we predicted that, in addition to the gene for ILD insensitivity segregated in the crosses with H-e3, the E3 locus would also segregate in the F2 populations. In the three crosses, however, ILD-insensitive plants segregated at frequencies of 21.1–33.9%; the remaining plants remained vegetative until the end of light supplementation (Table 1). These segregation frequencies were thus inconsistent with those of a two-gene model, but were close to those expected from monogenic inheritance, as in the crosses with H-e3 (Table 1).

**FIGURE 1** Segregation of flowering time in F2 populations of crosses between a Harosoy NIL for e3 (H-e3) and the incandescent-long daylength (ILD)-insensitive cultivars Zeika (ZE) and Yubileinaya (YU) under far red light–enriched ILD conditions. (A) H-e3 × ZE; (B) H-e3 × YU. In a cross between H-e3 and ZE, 48 F2 plants were selected for the progeny test; ILD-sensitivity genotypes were estimated based on the segregation in the progeny. Pink bars, homozygotes for ILD insensitivity (E/E); yellow–green bars, heterozygotes (E/e); light-blue bars, homozygotes for ILD sensitivity (e/e). Arrows indicate mean values of flowering time in parents. Dotted vertical lines indicate the threshold for classification of F2 plants into early-flowering ILD-insensitive and late- or non-flowering ILD-sensitive. nf, no flower buds by the end of light supplementation. DAS, days after sowing.
### TABLE 1 | Segregation of ILD-insensitivity in F2 of crosses of an ILD-sensitive cultivar Harosoy with ILD-insensitive Russian cultivars.

| Cross combination | Number of plants | $\chi^2$ value for 1:3 | P-value |
|-------------------|------------------|------------------------|--------|
| ILD-insensitive   | ILD-sensitive    | Total                  |        |
| Harosoy × Zeika   | 19               | 37                     | 56     | 3.57  | 0.059 |
| Harosoy × Yubileinaya | 28             | 105                    | 133    | 1.66  | 0.198 |
| Harosoy × Sonata  | 19               | 54                     | 73     | 0.06  | 0.803 |

#### FIGURE 2 | Simple sequence repeat (SSR) marker analyses in F2 plants from a Harosoy isoline for e3 (H-e3) × Zeika (ZE) cross. (A) Gel electrophoresis for the analysis of Satt190 and Sat_085. Eight plants homozygous for the ILD-insensitive allele (e/e) and 8 plants homozygous for the ILD-sensitive allele (E/E) were selected on the basis of the results of the progeny test. M1, φX174/HaeIII digest; M2, 100 bp DNA ladder. (B,C) Association between Sat_085 and flowering time in H-e3 × ZE (B) and H-e3 × YU (C). F2 plants were classified based on the genotype at Sat_085. Pink bars, homozygotes for the allele from ILD-insensitive parents (I/I); yellow-green bars, heterozygotes (I/S); light-blue bars, homozygotes for the allele from ILD-sensitive H-e3 (S/S). Arrows indicate mean values of flowering time in parents. DAS, days after sowing.

### Association Test, Linkage Map Construction, and Fine Mapping

To determine the genomic position of the gene for ILD insensitivity from ZE, we tested the association between ILD sensitivity and SSR marker genotypes. Based on the results of the progeny test, we selected 16 F2 plants from the H-e3 × ZE cross, 8 homozygous for ILD insensitivity (e/e), and 8 homozygous for ILD sensitivity (E/E). Among the SSR markers tested, Satt190 and Sat_085 in linkage group C1 (chromosome 4; Chr04) showed genotypic variation in complete accordance with the ILD sensitivity (Figure 2A). Then we determined the genotypes of the two markers in the whole F2 plants of H-e3 × ZE and H-e3 × YU populations (Figures 2B,C). The two markers were tightly linked to each other with a recombination value of 2.1, and were closely associated with ILD sensitivity. All of the plants homozygous for the allele from ILD-insensitive parents at Sat_085 (I/I) flowered before 56 DAS (H-e3 × ZE) or 52 DAS (H-e3 × YU), whereas those homozygous for the allele from ILD-sensitive H-e3 (S/S) flowered at ≥60 DAS or did not flower in both crosses. Heterozygous plants (I/S) mostly flowered at ≥58 DAS (H-e3 × ZE) or ≥54 DAS (H-e3 × YU), which partly overlapped with the flowering date ranges of the S/S plants; only a few plants flowered as early as the I/I plants. These results strongly suggested that a gene for ILD insensitivity is located near the two SSR markers.

Satt190 and Sat_085 are located 17.3 Mb from each other in the pericentromeric region of Chr04 (Schmutz et al., 2010) (Phytozome v12.1/Glycine max Wm82.a2.v1). To delimit the genomic region of the gene for ILD insensitivity more precisely, we selected plants with recombination between the two markers (7 from 306 F2 plants from the H-e3 × ZE and H-e3 × YU crosses and 3 from 492 F3 plants from the H-e3 × ZE cross) and constructed their graphical genotypes with 11 SSR markers. A comparison of the graphical genotypes with the genotype of ILD insensitivity estimated by the progeny test revealed that the gene for ILD insensitivity was located between SSR markers M5 (BARC-18g-0889) and M6 (BARC-18g-0895) (Figure 3). The physical distance between the two markers is 17.3 Mb, as reported previously (Schmutz et al., 2010). This result is consistent with the results of the association test and linkage map construction, supporting the hypothesis that the gene for ILD insensitivity is located near the two SSR markers.
markers was 842 kb, and the delimited region contained only 6 annotated genes (Phytozome v12.1/Glycine max Wm82.a2.v1) (Figure 3 and Table 2). RNA-sequencing Atlas in Phytozome v12.1/Glycine max Wm82.a2.v1 indicates that Glyma.04G143000, Glyma.04G143100 and Glyma.04G143200 are expressed only in flower or root tissues, whereas Glyma.04G143300, Glyma.04G143400, and Glyma.04G143500 are expressed in leaves (Severin et al., 2010). Because ZE exhibited significantly higher expressions for \(\text{FT2a} \) and \(\text{FT5a} \) in leaves in the 2nd and 3rd trifoliate leaf stages than \(H^{-e3} \) under R-enriched LD condition (Supplementary Figure S1), we focused on the three genes expressed in leaves as a possible candidate of the gene for ILD insensitivity that upregulates the two \(\text{FT} \) genes.

### Sequence Analysis

Sequence analysis revealed that ZE and \(H^{-e3} \) possessed identical sequences for Glyma.04G143400 and Glyma.04G143500, whereas one of cytosines at the 162th nucleotide to 164th nucleotide from the adenine of the start codon was deleted in the Glyma.04G143300 from ZE; this deletion generated a premature stop codon, and the Glyma.04G143300 from ZE was predicted to encode a truncated protein of 61 amino acids (Figure 4). Glyma.04G143300 is \(\text{E1Lb} \) of floral repressor \(\text{E1} \) (Xia et al., 2012). Because the down-regulation of \(\text{E1La} \) and \(\text{E1Lb} \) expressions by VIGS promotes flowering under non-inductive conditions such as LD and night break (Xu et al., 2015), we considered the loss-of-function allele of \(\text{E1Lb} \) (designated \(\text{e1lb} \) hereafter) as the most probable causal factor for the ILD-insensitivity.

### TABLE 2 | Genes annotated in an 842-kb genomic region in chromosome 4 delimited by fine-mapping.

| No. | Gene                  | Annotation (Phytozome V12.1/Glycine max Wm82.a2.v1) | Expressed tissues |
|-----|-----------------------|-----------------------------------------------------|-------------------|
| (1) | Glyma.04G143000       | Diacylglycerol kinase 7                              | Flower            |
| (2) | Glyma.04G143100       | RNA-binding (PRR/RBD/RNP motifs) family protein     | Root              |
| (3) | Glyma.04G143200       | Pectin lyase-like superfamily protein                | Flower            |
| (4) | Glyma.04G143300       | AP2/3-like transcriptional factor family protein, \(\text{E1Lb} \) | Leaf              |
| (5) | Glyma.04G143400       | Cytidine/deoxyxycytidylate deaminase family protein | Leaf, root        |
| (6) | Glyma.04G143500       | Mitochondrial substrate carrier family protein       | Flower, leaf      |

Data on expressed tissues are referred from Glycine max Wm82.a2.v1. (Severin et al., 2010).
We developed a dCAPS marker to discriminate e1lb from E1Lb (Figure 5). The PCR-amplified fragment of 275 bp from ZE produced a shorter fragment of 254 bp when digested with HpyCH4IV, whereas that from H-e3 (276 bp) was not digested. The digestion of the PCR products from YU and Sonata (Russian cultivar) produced 254-bp fragments, indicating that these two cultivars had the same deletion as ZE (Figure 5B). Therefore, the segregation of ILD-insensitive plants in the crosses of these cultivars with HA and H-e3 were most likely caused by e1lb.

Comparison of Flowering Time and Gene Expression Among NILs

We evaluated the allelic effects of E1Lb and e1lb on flowering under the R-enriched LD condition (daylength, 20 h) in four sets of NILs, each for E1Lb and e1lb, developed from different F2 plants from the H-e3 × ZE cross (#4 and #21) and the HA × ZE cross (#11 and #20). In the two sets of the e3/E4 NILs, each NIL for e1lb flowered at the same or almost the same time (#4, 31.7 DAS; #21, 30.3 DAS) as ZE (30.3 DAS); this was on average 6.7–7.6 days earlier than the respective NILs for E1Lb, which flowered at almost the same time as H-e3 (Figure 6A). Flowering times of the E3/E4 NILs were around 20 days or more later than those of the e3/E4 NILs. e1lb also promoted flowering in the E3/E4 background: each NIL for e1lb flowered around 10 days earlier than the respective NIL for E1Lb and HA. This flowering-promoting effect of e1lb versus E1Lb under the R-enriched LD condition was smaller than that of e4 vs. E4 and that of e3 vs. E3, because H-e3e4 and H-e3 flowered, on average, 13 and 25 days earlier than H-e3 and HA (E3E4), respectively.

We also evaluated the effect of e1lb vs. E1Lb on flowering under the FR-enriched ILD condition (Figure 6B). e1lb induced flowering at 58 DAS (#4) or 49 DAS (NILs #21) in the e3/E4 genetic background and at 56 DAS (#11 and #20) in the E3/E4 genetic background. All these NILs produced pods of up to 3 cm in length at the end of light supplementation, similar to those of ZE and H-e3e4. In contrast, the e3/E4 NILs for E1Lb and H-e3 flowered around 20 days later, and E3/E4 NILs for E1Lb and HA continued vegetative growth and did not produce any flower buds until the end of light supplementation.
Therefore, $e1lb$ was sufficient to induce flowering under the ILD condition, irrespective of the $E3$ genotype (Figure 6B). Interestingly, a similar flowering-promoting effect was observed in the NIL-$E1$ for a loss-of-function allele $e1-nl$ ($e1$); it initiated flowering under the ILD condition, as the $E3/E4$ NILs for $e1lb$, whereas the NIL for $e1-as$ ($E1$) did not (Figure 6B).

We tested the expression levels of $E1$, two $E1L$ genes, and two $FT$ orthologs in two different growing stages (the 2nd and 3rd leaf stages) in the $E3/E4$ NILs grown under the R-enriched LD condition (Figure 7). The expression levels of $E1$ and $E1La$ were similar between the NILs for $E1lb$ and $e1lb$ at both stages in NILs #4 or at the 3rd stage in NILs #21; both $E1$ and $E1La$ were significantly up-regulated in the 2nd leaf stage in NILs (#21) for $e1lb$ relative to those for $E1Lb$. On the other hand, the expression of $E1lb$ was significantly down-regulated in the NILs for $e1lb$ at both stages (#4) or at the 3rd leaf stage (#21). In contrast, the expression of both $FT2a$ and $FT5a$ was up-regulated at both stages in the NILs for $e1lb$ relative to those for $E1lb$ in both NIL sets. The similar effect of $e1lb$ vs. $E1lb$ on the expression of $FT2a$ and $FT5a$ was also observed at the 3rd leaf stage in both sets of $E3/E4$ NILs (#11 and #20; Figure 8). As observed in the $E3/E4$ NILs for $e1lb$, the expression levels of $FT2a$ and $FT5a$ were significantly upregulated in the $E3/E4$ NIL for $e1lb$.

**DISCUSSION**

The soybean maturity loci, $E1$ to $E4$, are major flowering loci that determine the ability of cultivars to adapt to different latitudes. Diverse allelic combinations at the $E1$, $E3$, and $E4$ loci, each of which has multiple loss-of-function alleles (Tsukimura et al., 2013, 2014; Xu et al., 2013; Jiang et al., 2014; reviewed by Cao et al., 2017), have resulted in cultivars with various sensitivities to photoperiod. Photoperiod insensitivity is an adaptive trait for cultivars at high latitudes; such cultivars are classified into three genotypic groups according to the allelic
functions at each of the three loci (Xu et al., 2013). Among the ILD accessions tested, the predominant group has the loss-of-function alleles of the phyA genes E3 and E4 (e3/e4), followed by a group which has the loss-of-function of the E1 repressor for FT2a and FT5a in combination with a dominant E3 or E4 allele. Cultivars of the third group have a novel genetic mechanism that abolishes or reduces sensitivity to daylength, because they have the same genotype (e1-as/e3/E4) as an HA NIL for e3, which is sensitive to FR-enriched ILD conditions (Saindon et al., 1989; Cober et al., 1996; Abe et al., 2003; Liu and Abe, 2010; Xu et al., 2013). Takeshima et al. (2016) carried out QTL analysis of ILD insensitivity by a testcross of a Chinese cultivar of group 3 with the HA NIL for e3 and demonstrated that an early-flowering allele at qDTF-J, which encodes the FT5a protein, up-regulates FT5a expression by cis-activation in the presence of E4 to induce flowering under ILD conditions.

In the present study, we detected a novel loss-of-function allele that resulted from a frameshift mutation at the E1Lb locus in Far-Eastern Russian group 3 photoperiod-insensitive cultivars. E1Lb and its tandemly linked homolog, E1La, have high sequence similarity to E1, suggesting their functional similarity, although a certain degree of subfunctionalization is suggested by the presence of a number of amino acid substitutions and indels between the E1 and E1L genes (Xia et al., 2012). Down-regulation by VIGS revealed that, similar to E1, E1L genes inhibit flowering under LD and night-break conditions (Xu et al., 2015), but the function of each homolog has remained undetermined. Comparison of NILs for E1Lb and e1lb in this study suggests that e1lb promotes flowering under both R-enriched and FR-enriched LD conditions. In particular, the effect of e1lb vs. E1Lb in the FR-enriched LD condition was similar to that of e4 vs. E4, irrespective of the E3 genotype, suggesting that e1lb completely cancels the inhibitory effect of FR-enriched LD on flowering.
modulated by $E_4$. These flowering-promoting effects are most likely due to the up-regulation of $FT_{2a}$ and $FT_{5a}$; their expression levels were not associated with the expression levels of $E_1$ and $E_{1La}$. One likely explanation for this observation is that the total expression level and/or activity of $E_1$, $E_{1La}$ and $E_{1lb}$ may be important for the repression of $FT_{2a}$ and $FT_{5a}$ expression. The induction of flowering by $e_{1lb}$ in the $E_3/E_4$ genetic background under ILD conditions is in good accordance with monogenic segregation observed in the crosses of HA with Russian ILD-insensitive cultivars. $e_{1lb}$ also promoted flowering under R-enriched LD conditions, but its effect was small and it could not cancel flowering inhibition by $E_3$ as efficiently as $e_3$ did. The function of $E_{1Lb}$ may therefore depend on light quality of LD. Interestingly, $e_{1-nl}$ (loss-of-function allele at the $E_1$ locus) could also cancel the inhibitory effect of FR-enriched LD conditions on flowering, as efficiently as $e_4$ could. Because the effects of
e1lb under the e1-as background were similar to those of e1-nl under the E1lb background (Figure 6B). E1 and E1lb may inhibit flowering under LD conditions, independently of each other. It may be tempting in a further study to develop double recessive lines for the loss-of-function alleles at the E1 and E1lb loci not only to elucidate the interaction between the two genes and the function of another E1 homolog, E1La, but also to explore the regulatory mechanisms of these E1 family genes by E3 and E4 under different light conditions. In addition, a further study is also needed to determine why the loss-of-function allele at E1lb can singly upregulate the FT2a and FT5a expression under LD condition, even though the remaining E1 family genes are expressed normally.

E1, E2, and E3 have large effects on flowering in a wide range of latitudes, whereas the allelic effect of E4 is rather limited to high latitudes (Yamada et al., 2012; Tsubokura et al., 2013, 2014; Xu et al., 2013; Lu et al., 2015). Among these four soybean genes, E1 has the most marked effect on time to flowering (McBlain et al., 1987; Upadhyay et al., 1994; Tsubokura et al., 2014). The polymorphism of E1 (or its flanking genomic region) largely accounts for the variation in flowering time and related agronomic traits in segregating populations of different genetic backgrounds (Yamanaka et al., 2000; Wang et al., 2004; Zhang et al., 2004; Funatsuki et al., 2005; Liu et al., 2007; Zhai et al., 2015). In contrast to the E1 gene, only a few reports have demonstrated the presence of major genes or QTLs for flowering and maturing associated with the genomic region of Chr04 harboring E1La and E1lb (Cober et al., 2010; Cheng et al., 2011; Watanabe et al., 2017; Kong et al., 2018). Cober et al. (2010) determined that the E8 gene, which was identified in a photoperiod-insensitive genetic background, is located in a genomic region harboring two E1 genes 10, 640 kb apart from each other (Xu et al., 2015), suggesting either of E1La and E1lb as a candidate for E8. The QTLs for flowering and maturity were also detected in the positions of Chr04 similar to that of E8 (Cheng et al., 2011; Watanabe et al., 2017; Kong et al., 2018). It would be interesting to determine whether the E8 gene is E1La or E1lb and to identify the responsible genes for these QTLs. Genotyping with an allele-specific DNA marker in this study revealed that e1lb is a rare and region-specific allele even in early maturing photoperiod-insensitive cultivars, suggesting that e1lb has neither largely contributed to the diversity of flowering behaviors nor been used widely in soybean breeding. The e1lb allele may therefore be useful as a new resource to broaden the genetic variability of soybean cultivars for flowering under LD conditions at high latitudes.

AUTHOR CONTRIBUTIONS

JZ, BL, and JA conducted the experiments. JZ, RT, and KH conducted genetic analyses, fine-mapping, and sequencing analyses. JZ and MX developed allele-specific DNA markers and analyzed the variation of genotypes in soybean accessions. JZ, MX, and TY conducted the expression analyses. JZ and JA drafted the manuscript with edits from RT, KH, MX, FK, BL, TY, and AK. All authors read and approved the final manuscript.

FUNDING

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (17K07579) to JA, by the National Natural Science Foundation of China (Grant No. 31501330) to MX, the Program of the General Program of the National Natural Science Foundation of China (Grant No. 31771815) to BL, and by the Natural Key R&D Program of China (2017YFE01111000 and 2016YFD0100400) to FK.

ACKNOWLEDGMENTS

The authors are grateful to Drs. AY Ala (All Russian Research Institute of Soybean, Russia) and ER Cober (Agriculture and Agri-Food Canada, Canada) for providing us seeds of soybean cultivars and experimental lines.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01867/full#supplementary-material

REFERENCES

Abe, J., Xu, D., Miyano, A., Komatsu, K., Kanazawa, A., and Shimamoto, Y. (2003). Photoperiod-insensitive Japanese soybean landraces differ at two maturity loci. Crop Sci. 43, 1300–1304. doi: 10.2135/cropsci2003.1300

Andrés, F., and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. Nat. Rev. Genet. 13, 627–639. doi: 10.1038/nrg3291

Buzzell, R. I. (1971). Inheritance of a soybean flowering response to fluorescent-daylength conditions. Can. J. Genet. Cytol. 13, 703–707. doi: 10.1139/g71-1100

Buzzell, R. I., and Voldeng, H. D. (1980). Inheritance of insensitivity to long daylength. Soyb. Genet. NewsL. 7, 26–29. doi: 10.1093/oraesp/117

Cao, D., Li, Y., Lu, S., Wang, J., Nan, H., Li, X., et al. (2015). GmCOLLa and GmCOLLb function as flowering repressors in soybean under long-day conditions. Plant Cell Physiol. 56, 2409–2422. doi: 10.1093/pcp/pcv152

Cao, D., Takeshima, R., Zhao, C., Liu, B., Abe, J., and Kong, F. (2017). Molecular mechanisms of flowering under long days and stem growth habit in soybean. J. Exp. Bot. 68, 1873–1884. doi: 10.1093/jxb/erw394

Cheng, L., Wang, Y., Zhang, C., Wu, C., Xu, J., Zhu, H., et al. (2011). Genetic analysis and QTL detection of reproductive period and post-flowering photoperiod responses in soybean. Theor. Appl. Genet. 123, 421–429. doi: 10.1007/s00122-011-1594-8

Cober, E. R., Molnar, S. J., Charette, M., and Voldeng, H. D. (2010). A new locus for early maturity in soybean. Crop Sci. 50, 524–527. doi: 10.2135/cropsci2009.04.0174

Cober, E. R., Tanner, J. M., and Voldeng, H. D. (1996). Genetic control of photoperiod response in early-maturing, near-isogenic soybean lines. Crop Sci. 36, 601–605. doi: 10.2135/cropsci1996.0011183X003600030013x

Doi, K., Iizawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., et al. (2004). Ehd1, a B-type response regulator in rice, confers short-day promotion of
flowering and controls FT-like gene expression independently of Hd1. Genes Dev. 18, 926–936. doi: 10.1101/gad.1189604

Doyle, J. J., and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus 12, 13–15.

Fan, C., Hu, R., Zhang, X., Wang, X., Zhang, W., Zhang, Q., et al. (2014). Conserved CC-FT regulons contribute to the photoperiod flowering control in soybean. BMC Plant Biol. 14:9. doi: 10.1186/1471-2229-14-9

Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. (1971). Stage of development descriptions for soybeans, Glycine max (L.) merrill. Crop Sci. 11, 929–931. doi: 10.2135/cropsci1971.0011183X0001100006051x

Funatsuhi, K., Kawaguchi, K., Matsuoka, S., Sato, Y., and Ishimoto, M. (2005). Mapping of QTL associated with chilling tolerance during reproductive growth in soybean. Theor. Appl. Genet. 111, 851–861. doi: 10.1007/s00122-005-0007-2

Izawa, T., Okawa, T., Sugiyama, N., Tanisaka, T., Yano, M., and Shimamoto, K. (2002). Phytocrome mediates the external light signal to repress FT orthologs in photoperiodic flowering of rice. Genes Dev. 16, 2006–2020. doi: 10.1101/gad.999202

Jia, H., Jiang, B., Wu, C., Lu, W., Hou, W., Sun, S., et al. (2014). Maturity group classification and maturity locus genotype of early-maturing soybean varieties from high-latitude cold regions. PLoS One 9:e94139. doi: 10.1371/journal.pone.0094139

Jiang, B., Nan, H., Gao, Y., Tang, L., Yue, Y., Lu, S., et al. (2014). Allelic combinations of soybean maturity loci E1, E2, E3 and E4 result in diversity of maturity and adaptation to different latitudes. PLoS One 9:e106042. doi: 10.1371/journal.pone.0106042

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T., et al. (2002). Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of HD1 under short-day conditions. Plant Cell Physiol. 43, 1096–1105. doi: 10.1093/pcp/pcf156

Kong, F., Liu, B., Xia, Z., Sato, S., Kim, B. M., Watanabe, S., et al. (2010). Two maps for photoperiod insensitivity of a Japanese soybean landrace Sakamotowase. J. Hered. 101, 251–256. doi: 10.1093/jhered/esf009

Kurasch, A. K., Hahn, V., Leiser, W. L., Vollmann, J., Schori, A., Betrix, C. A., et al. (2017). Identification of mega-environments in Europe and effect of allelic variation at maturity E loci on adaptation of European soybean. Plant Cell Environ. 40, 765–778. doi: 10.1111/pce.12896

Langewisch, T., Zhang, H., Vincent, R., Joshi, T., Xu, D., and Bilyeu, K. (2014). CONSTANS in rice, functions as an Hd1a repressor through interaction with moncot-specific CCT-domain protein Ghd7. Plant J. 86, 221–233. doi: 10.1111/tpj.13168

Mclain B. A., Hesketh J. D., and Bernard R. L. (1987). Genetic effects on reproductive phenotype in soybean isolines differing in maturity genes. Can. J. Plant Sci. 67, 105–115. doi: 10.4141/cips87-012

Nemoto, Y., Nonouye, Y., Yano, M., and Izawa, T. (2016). Hd1a, CONSTANS ortholog in rice, functions as an Hd1 repressor through interaction with monocot-specific CCT-domain protein Ghd7. Plant J. 86, 221–233. doi: 10.1111/tpj.13168

Song, Q., Jia, G., Zhu, Y., Grant, D., Nelson, R. T., Hwang, E. Y., et al. (2010). Abundance of SSR motifs and development of candidate polymorphic SSR markers (BARSCOYSSR_1.0) in soybean. Crop Sci. 50, 1950–1960. doi: 10.2135/cropsci1006.0060

Song, Q., Marek, L. F., Shoemaker, R. C., Lark, K. G., Consibido, V. C., Delanany, X., et al. (2004). A new integrated genetic linkage map of the soybean. Theor. Appl. Genet. 109, 122–128. doi: 10.1007/s00122-004-1602-3

Song, Y., Ito, S., and Imaizumi, T. (2013). Flowering time regulation: photoperiod- and temperature-sensing in leaves. Trends Plant Sci. 18, 575–583. doi: 10.1016/j.tplants.2013.05.003

Takeshima, R., Hayashi, T., Zhu, J., Zhao, C., Xu, M., Yamaguchi, N., et al. (2016). A soybean quantitative trait locus that promotes flowering under long days is identified as FT5a, a FLOWERING LOCUS T ortholog. J. Exp. Bot. 67, 5247–5258. doi: 10.1093/jxb/erw283

Takami, S., Matsu, S., Song, W., Hany, Y., Kokoi, S., and Shimamoto, K. (2007). Hd3a protein is a mobile flowering signal in rice. Science 316, 1033–1036. doi: 10.1126/science.1141753

Tubuskaya, Y., Matsumori, H., Xu, M., Liu, B., Nakashima, H., Anai, T., et al. (2013). Genetic variation in soybean at the maturity locus Ed is involved in adaptation to long days at high latitudes. Agronomy 3, 117–134. doi: 10.3390/agronomy3010117

Tubuskaya, Y., Watanabe, S., Xia, Z., Kanamori, H., Yamagata, H., Kaga, A., et al. (2014). Natural variation in the genes responsible for maturity loci E1, E2, E3 and E4 in soybean. Ann. Bot. 111, 429–441. doi: 10.1093/annbot/mnu259

Upadhyay, A. P., Ellis, R. H., Summerfield, R. J., Roberts, E. H., and Qi, A. (1994). Characterization of photothermal flowering responses in maturity isolines of soyabean [Glycine max (L.) Merrill] cv. Clarkar. Ann. Bot. 74, 87–96. doi: 10.1093/aob/74/1.87

Wang, D., Graef, G. L., Procopiuk, A. M., and Diers, B. W. (2004). Identification of photoperiod responses of flowering in soybean. Plant Cell Physiol. 45, 545–553. doi: 10.1093/pcp/pci112

Watanabe, S., Harada, K., and Abe, J. (2012). Genetic and molecular bases of photoperiod flowering in soybean. Breed. Sci. 61, 531–543. doi: 10.1270/jsbbs.61.531

Watanabe, S., Hideshima, R., Xia, Z., Tubuskaya, Y., Sato, S., Nakamoto, Y., et al. (2009). Map-based cloning of the gene associated with the soybean maturity locus E3. Genetics 182, 1251–1262. doi: 10.1534/genetics.108.098772

Watanabe, S., Tsukamoto, C., Oshita, T., Yamada, T., Anai, T., and Kaga, A. (2017). Identification of quantitative trait loci for flowering time by a combination of restriction site-associated DNA sequencing and bulked segregant analysis in soybean. Breed. Sci. 67, 267–285. doi: 10.1270/jsbbs.17013

Watanabe, S., Xia, Z., Hideshima, R., Tubuskaya, Y., Sato, S., Yamanaka, N., et al. (2011). A map-based cloning strategy employing a residual heterozygous line reveals that the GIGANTEA gene is involved in soybean maturity and flowering. Genetics 188, 395–407. doi: 10.1534/genetics.110.125062
Zhu et al.  
E1Lb Represses Flowering Under LD

Wu, F., Price, B. W., Haider, W., Seufferheld, G., Nelson, R., and Hanzawa, Y. (2014). Functional and evolutionary characterization of the CONSTANS gene family in short–day photoperiodic flowering in soybean. PLoS One 9:e85754. doi: 10.1371/journal.pone.0085754

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Xia, Z., Watanabe, S., Yamada, T., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. Proc. Natl. Acad. Sci. U.S.A. 109, E2155–E2164. doi: 10.1073/pnas.1117982109

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Zhu, Takeshima, Harigai, Xu, Kong, Liu, Kanazawa, Yamada and Abe. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.