



# A flowering time locus dependent on *E2* in soybean

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Received: 7 December 2020 / Accepted: 12 April 2021 / Published online: 25 May 2021  
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**Abstract** Soybean [*Glycine max* (L.) Merrill] is very sensitive to changes in photoperiod as a typical short-day plant. Photoperiodic flowering influences soybean latitudinal adaptability and yield to a considerable degree. Identifying new quantitative trait loci (QTLs) controlling flowering time is a powerful initial approach for elucidating the mechanisms underlying flowering time and adaptation to different latitudes in soybean. In this study, we developed a Recombinant Inbred Lines (RILs) population and recorded flowering time under natural long-day conditions. We also constructed a high-density genetic map by genotyping-by-sequencing and used it for QTL mapping. In total, we detected twelve QTLs, four of which are stable and named by *qRI-2*, *qRI-4*, *qRI-6.1*, and *qRI-10*, respectively. Among these four

QTLs, *qRI-4* and *qRI-6.1* are novel. QTL mapping in two sub-populations classified by the genotype of the maturity locus *E2*, genetic interaction evaluation between *E2* and *qRI-2*, and qRT-PCR indicated that *E2* has an epistatic effect on *qRI-2*, and that causal gene of *qRI-2* acts upstream of *E2*. We presumed the most likely candidate genes according to the resequencing data and briefly analyzed the geographic distributions of these genes. These findings will be beneficial for our understanding of the mechanisms underlying photoperiodic flowering in soybean, contribute to further investigate of *E2*, and provide genetic resources for molecular breeding of soybean.

**Keywords** Soybean · Flowering time · Quantitative trait loci · Genotyping-by-sequencing · *E2*

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## Introduction

Soybean [*Glycine max* (L.) Merrill] is particularly important to world agriculture as the main economical oil and protein crop, providing more than 25% of the world's source of protein for food and animal feed (Lu et al. 2020). Flowering time is one of the most important factors affecting soybean yield (Zhang et al. 2004), and it is controlled by several major gene loci and quantitative trait loci (QTLs). To date, twelve major genetic loci and genes associated with flowering time have been identified: *E1* and *E2* (Bernard 1971; Xia et al. 2012; Watanabe et al. 2011), *E3* (Buzzell 1971; Watanabe et al. 2009), *E4* (Buzzell and Voldeng 1980; Saindon et al. 1989a, b; Liu et al. 2008), *E5* (McBlain and Bernard 1987), *E6* (Bonato and Vello 1999; Li et al. 2017; Fang et al. 2020), *E7* (Cober and Voldeng 2001), *E8* (Cober et al. 2010), *E9* (Kong et al. 2014; Zhao et al. 2016), *E10* (Samanfar et al. 2017), *E11* (Wang et al. 2019), and *J* (Ray et al. 1995; Lu et al. 2017). Among these, *E1* is a legume-specific transcriptional factor that contributes the most to flowering time and maturity and delays flowering by suppressing the expression of *FLOWERING LOCUS T (FT)* orthologs in soybean (Xia et al. 2012). *E2* is an ortholog of *Arabidopsis GIGANTEA*, which plays conserved roles in flowering in many crops (Hecht et al. 2007; Itoh et al. 2010; Izawa et al. 2011). *E2* regulates flowering time in soybean by controlling the expression of *GmFT2a* but not *GmFT5a* (Watanabe et al. 2011). The effect of *E2* on delaying flowering has been stably detected in different environments (Watanabe et al. 2011; Xu et al. 2013). The diversification of *E2* haplotypes contributes to the adaptation of soybean to different latitudes by affecting flowering time. Compared with soybean varieties from the north, the *E2* allele appears more frequently in southern soybean varieties (Wang et al. 2016). However, the mechanism underlying how *E2* regulates flowering time in soybean is not clearly understood. *E3* and *E4* are reported as the orthologs of *Arabidopsis Phytochrome A (phyA)* and regulate flowering under different red-to-far-red (R:FR) quantum ratios (Cober et al. 1996; Liu et al. 2008; Watanabe et al. 2009; Xu et al. 2013). Under long-day conditions, either *E3* or *E4* could induce the expression of *E1* (Xia et al. 2012; Xu et al. 2013). *E5* has been considered as an unexpected outcrossing with pollen having an *E2*-dl allele and no unique *E5* gene exists (Dissanayaka et al. 2016). The *E7* locus is located on Chr06 and is closely linked to *E1* (Cober and Voldeng

2001), while the *E8* locus is located on Chr04 near *E1L* gene (Cober et al. 2010). Both *E7E7* and *E8E8* delay flowering or maturity under low R:FR light quality or natural daylight conditions (Cober and Voldeng 2001; Cober et al. 2010). The candidate genes of *E9* and *E10* are *GmFT2a* and *GmFT4*, respectively, both of which are the members of *GmFT* family (Kong et al. 2014; Zhao et al. 2016; Samanfar et al. 2017). *E11* is a recently identified locus, which is located on Chr07. *E11E11* could promote flowering under long-day conditions (Wang et al. 2019). *E6* and *J* are identified as the promotion of flowering time under short-day conditions (Lu et al. 2017; Li et al. 2017). A recent study indicated that *E6*, an ortholog of *Arabidopsis EARLY FLOWERING 3*, is a novel allele of *J* (Fang et al. 2020). Among them, various combinations of *E1* to *E4* greatly contribute to the adaptation and yield of soybean (Tasma et al. 2001; Liu et al. 2011; Harada et al. 2011; Watanabe et al. 2012; Xu et al. 2013; Jia et al. 2014; Jiang et al. 2014; Li et al. 2017). Meanwhile, the variation contribution rate of these genes was as much as 62–66% (Liu et al. 2008; Tsubokura et al. 2014). In addition, *Tof11* and *Tof12* have been recently reported to sequentially contribute to soybean adaptation to higher latitudes (Lu et al. 2020). They undergone stepwise selection and loss-of-function alleles of them were enriched in landraces, particularly in the northeast region of China (Lu et al. 2020).

Although much work has been done to elucidate the control of flowering time in soybean, the mechanism has yet to be fleshed out. In the present work, a recombinant inbred lines (RILs) population called S4W was developed to identify new QTLs related to flowering time under long-day conditions. A total of twelve QTLs were detected, four of which are stable. Moreover, the roles of *qRI-2* and *qRI-6.1* in regulating flowering depend on *E2* and that the causal gene of *qRI-2* acts upstream of *E2*. Additionally, we presumed the most likely candidate genes according to resequencing data and briefly analyzed the geographic distributions of the candidate genes.

## Materials and methods

### Plant materials and growth conditions

For QTL mapping, an  $F_{6:8}$  RIL population named S4W was developed via a single-seed descent method (Fehr 1987). The population ( $n=116$ )

was generated from a cross between Suinong4 (*e1<sup>as</sup>e2e3E4*) and Williams 82 (*e1<sup>as</sup>E2E3E4*). The RIL population and parents were grown under natural long-day conditions in 2018 and 2019 at the Experimental Station of the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, China (45°75'N, 126°63'E). The seeds were sown with a row length of 2 m, row spacing of 60 cm, and distance between rows of approximately 10 cm. Approximately 20 seeds were sown per line. Standard cultivation practices were applied equally to each trial to control insects and weeds (Lu et al. 2017). The day of emergence was defined as the day when 50% of plants in a line reached emergence. The day when 50% of plants in a row began to flower was recorded as R1 (Fehr et al. 1971). The accessions in the 424-panel obtained from Lu et al. (2020) were used for analysis in this study. The 424-accession panel was grown under natural day-length conditions in Zhengzhou (34°44'N, 113°42'E) and Hefei, China (31°51'N, 117°15'E) in 2018, and in Guangzhou (23°16'N, 113°23'E) (short-day conditions), Wuhan (30°52'N, 114°31'E), Zhengzhou, and Harbin, China (45°75'N, 126°63'E) (long-day conditions) in 2019 and used to evaluate flowering time (Lu et al. 2020). Plants used for qRT-PCR were grown under long-day conditions (16 h light/8 h dark) in a plant growth cabinet.

#### DNA extraction

Young and fully developed trifoliate leaves were well-collected, frozen in liquid nitrogen, and transferred to a -80 °C freezer. Total genomic DNA was extracted from the samples with Genomic DNA kit (CWBIO, Beijing, China). The integrity and quality of the extracted DNA were tested by 1% agarose gel electrophoresis. The DNA concentrations in each sample were analyzed with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States) and NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States).

#### Genotyping by high-throughput sequencing

To guarantee the whole genome sequencing, about 1.5 µg DNA samples were analyzed. The sequencing database used in this study was generated which described by Cheng et al. (2015). The Illumina

HiSeq2500 platform (Illumina, Inc., San Diego, CA, USA) was used to sequence both parental libraries and there were 150 bp paired-end reads with insert sizes of about 300 bp generated. Genotyping-by-sequencing (GBS) technology was used to genotype S4W population. Low-coverage sequencing was used to identify the genotypes of single-nucleotide polymorphisms (SNPs) in the RIL population based on the reference polymorphic loci of the parents (Huang et al. 2009; Davey et al. 2013). Genomic DNA was incubated at 37 °C with restriction endonuclease *MseI* (New England Biolabs, NEB, Ipswich, MA, USA), T4 DNA ligase (NEB), ATP (NEB), and *MseI* Yadapter N containing barcode. The reaction was terminated by changing the temperature to 65 °C at the proper time. The enzymes *MseI*+*NlaIII* were then added to digest the products at 37 °C. Following digestion, the products were purified using Agencourt AMPure (Beckman Coulter, Brea, CA, USA) and amplified by PCR technology. The PCR system included diluted restriction-ligation DNA samples, dNTPs, Phusion-Master Mix (NEB) universal primer, and index primer. Also, the PCR products were purified as digested products by the same method and were separated by 2% agarose gel electrophoresis. Among all the separated strips, fragments ranging from 375 to 400 bp were isolated and extracted from the gels with a Gel Extraction kit (Qiagen). The purified fragment products were diluted prior to sequencing. Then, according to the manufacturer's recommendations, the paired-end sequencing which end was 150 bp in length was performed on the Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA).

#### Sequence data grouping and SNP identification

In order to have a reliable data without artificial bias, a series of quality control (QC) procedures using in-house C programs were used for raw data (raw reads). The QC standards included removing data at following situations: (1) under the Phred quality <5 condition while reads with >50% bases; (2) recognizing at least 10% unidentified nucleotides; (3) reads with *MseI* and/or *NlaIII* remnants; and (4) reads with > 10 nt aligned to the adapter while allowing mismatch was 10% and low-quality data obtained under situation above would be remove at the first round of distinguish attempt. Aligning the clean reads from each

sample against the reference genome was performed using Burrows-Wheeler Aligner (setting: mem-t4-M-R) (BWA v0.7.10) (Li and Durbin 2009), then SAMtools software (v1.7.6) (Li et al. 2009) was used to convert the alignment files to BAM files. GATK (v3.8.0) was used to perform for all samples' variant calling (Wang et al. 2010). Markers with > 30% missing genotype data, markers with segregation distortion ( $P < 0.01$ ), or markers containing abnormal bases were filtered out in map construction.

### Map construction

All SNPs were subjected to Chi-square tests to detect all segregation distortion. On the progress of SNP mapping, Python script was used to divide the markers with the same genotype as SNP marker. According to physical position, all markers were distributed into 20 linkage groups (or chromosomes). In this study, 3285 high-quality markers were detected in S4W population. The genetic distances of the markers were analyzed using QTL IciMapping (ICIM) software (Meng et al. 2015).

### Classification of S4W population based on the genotypes of *E2* and *qR1-2*

The interval of *qR1-10*, with a high logarithm of odds (LOD) score and percentage of phenotypic variance explained by the QTL (PVE), is consistent with *E2* locus, which indicates that *E2* has an extremely strong effect on flowering in S4W population. To exclude the effect of *E2* on other QTLs, S4W population was divided into two sub-populations using allele-specific DNA marker of *E2*. One group included 58 individuals with *E2E2*, and the other included 51 individuals with *e2e2*. IciMapping software was used to detect QTLs.

To examine the genetic relationships between *qR1-2* and *E2*, S4W population was classified into four groups based on the homozygous allelic combinations of *E2* and *qR1-2*. SNP markers (2:15078759 and 2:12259445) based on the genetic map with the highest LOD scores of *qR1-2* and the functional marker of *E2* were used.

### QTL analysis using high-density genetic map

The IciMapping (ICIM) method with QTL IciMapping software was used to detect QTLs for flowering

time, with default software parameters (probability in stepwise regression = 0.001, step = 1.0 cM). The criteria and the IciMapping protocol used in this study were described by Li et al. (2007). The LOD threshold of declaring significant QTLs was calculated using a permutation test (PT) at a significance level of  $P < 0.05$ ,  $n = 1000$ .

### RNA extraction and reverse transcription

Fresh leaves were sampled from plants at ZT8 at 20 days after emergence (20 DAE), frozen in liquid nitrogen, and stored at  $-80\text{ }^{\circ}\text{C}$  freezer. Total RNA was extracted from the samples using an Ultrapure RNA Kit (CW BIO). The integrity and quality of RNA were tested by 0.8% agarose gel electrophoresis. NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States) was used to analyze the RNA concentrations in each sample. Reverse transcription was performed using Super Script First-strand cDNA Synthesis System (Takara, China) from 500 ng RNA.

### Quantitative PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using Roche LightCycle480 system (Roche, Germany) and SYBR Green II Master Mix Kit (Takara, Japan). cDNA was diluted tenfold prior to qRT-PCR. The PCR cycling conditions were  $95\text{ }^{\circ}\text{C}$  for 30 s followed by 40 cycles of  $95\text{ }^{\circ}\text{C}$  for 10 s and  $60\text{ }^{\circ}\text{C}$  for 60 s. The relative expression levels of *E2* were calculated based on the expression level of *Tubulin* (*Glyma.05G157300*). The primers used for real time PCR were obtained from Li et al. (2013). Three biological replicates were used in the assays, and the results were presented as mean  $\pm$  s.e.m. ( $n = 8$  plants).

## Results

### Phenotypic analysis

In order to detect new QTLs related to flowering time, we constructed a RIL population of 116 individuals developed from the crosses between Suinong4

and Williams 82. Suinong4 is one of the major soybean varieties with the high yield that is planted in MG0 (maturity group 0) in Heilongjiang Province, China. Williams 82 is a soybean variety from Northern America and that is planted in MG III. Suinong4 flowered approximately 10 days earlier than Williams 82 in 2018 and 2019 (Table 1 and Supplementary Fig. 1). The days to flowering ranged from 27 to 53 in  $F_{6:7}$  population in 2018 and from 31 to 57 in  $F_{7:8}$  population in 2019 in Harbin (Table 1). A transgressive segregation of flowering time was observed in S4W population indicating that there may exist at least two loci which associated with flowering time.

#### Analysis of sequencing data and construction of a genetic linkage map

In this study, total bases of 15,797,212,800 and 13,223,878,500 were identified in the two parents, with a Q30 ratio and GC content of 35.87% and 35.84%, respectively (Supplementary Table 1). The average sequencing depth of Suinong4 was 13.18 $\times$ , and Williams 82 was 11.20 $\times$ . A total of 3285 SNP markers fell within 20 linkage groups (LGs) were used to construct a high-density genetic map. The genetic length was 2720.53 cM with an average marker interval distance of 0.828 cM (Supplementary Fig. 2).

#### QTL mapping for flowering time in S4W population

Using the data of flowering time and the genetic map, the QTL identification was performed using ICIM method. Loci located in the similar regions in 2 years were considered as consistent QTLs. A LOD value of 2.5 was used as the threshold to acquire QTLs contributed to the flowering time trait.

We primarily detected seven QTLs related to flowering time in S4W population (Table 2 and Fig. 1). Among these QTLs, *qRI-2*, *qRI-4*, and *qRI-10* were stably detected in 2018 and 2019. With the highest LOD scores, *qRI-10* was located between the interval of 39,966,171–45,852,401 on chromosome 10 which was consistent with the position of *E2* and accounted for approximately 23–43% of the observed phenotypic variation (PV). *qRI-2* was detected on chromosome 2 with a physical position of 12,296,249–15,179,120 and explained 4.31–5.68% of the observed PV. Another QTL, *qRI-4* with a LOD score of 3.94–4.51, could explain 4.77–7.39% of the observed PV and was located on chromosome 4. *qRI-2* and *qRI-4* showed positive additive effects. This suggested that the effects of these two QTLs on delayed flowering were derived from Suinong4. *qRI-19* was another QTLs with the high LOD score. Located in the interval of 47,664,574–47,774,155 on chromosome 19, the position of *qRI-19* covered *E3*

**Table 1** Statistical analysis of the parents and S4W population

Trait	Environment	Parents				S4W population							
		Females		Males		Flowering time		Flowering time					
		SN4	W82	Range	<i>P</i> -value	Min	Max	Range	Mean	CV (%)	Skewness	Kurtosis	
R1	Harbin,2018	31	42	11	1.14E–04	27	53	26	36.95	20.30	0.45	–0.99	
R1	Harbin,2019	34	44	10	3.66E–04	31	57	26	43.58	16.82	0.20	–1.38	
R1	Average R1	32	42	10	8.81E–08	29	55	26	40.21	17.53	0.41	–1.11	

R1: flowering time trait (days from emergence to first open flower appeared)

SN4: abbreviation of Suinong4

W82: abbreviation of Williams 82

Min: the minimum value of the population

Max: the maximum value of the population

Range: Max–Min

Mean: the average data

CV (%): coefficient of variance of percentage type

A Student's *t*-test was used to generate the *P* values

**Table 2** Details of the QTLs detected by IciMapping 4.0 in S4W population

QTL name	Year	Chr	Interval (cM)	Physical position	Max LOD	PVE (%)	ADD
<i>qRI-2</i>	2018	2	73.78–77.04	14,993,906–15,179,120	2.86	4.31	1.57
<i>qRI-4</i>		4	54.04–56.73	4,990,596–5,106,785	4.51	7.39	2.10
<i>qRI-6</i>		6	8.60–9.22	1,502,327–1,636,070	3.35	5.15	–1.71
<i>qRI-10</i>		10	151.50–153.21	44,738,813–44,769,837	19.93	43.26	–5.06
<i>qRI-20</i>		20	23.84–24.61	34,450,004–34,521,943	3.58	5.48	–1.78
<i>qRI-2</i>	2019	2	63.98–65.20	12,296,249–12,356,831	4.65	5.68	1.55
<i>qRI-4</i>		4	34.04–35.58	3,405,370–3,493,695	3.94	4.77	1.43
<i>qRI-10</i>		10	116.03–166.03	39,966,171–45,852,401	14.22	23.74	–3.21
<i>qRI-11</i>		11	45.50–6.30	8,264,639–8,264,964	4.02	4.91	–1.44
<i>qRI-19</i>		19	83.18–84.52	47,664,574–47,774,155	6.89	9.33	–1.98

Max LOD: maximum logarithm-of-odds (LOD) scores

PVE (%): percentage of phenotypic variance explained by the QTL

ADD: the additive effects contributed by QTLs. Additive effects with a positive value (+) indicate that the allele from Suinong4 enhances the phenotype; additive effects with a negative value (–) indicate that the allele from Williams 82 enhances the phenotype

locus, which encodes a homolog of the *Arabidopsis* photoreceptor phyA (Watanabe et al. 2009). *qRI-19* could explain 9.33% of the observed PV in 2019. Based on analysis of additive effects, the effect of *qRI-19* on extended flowering time was derived from Williams 82. In addition to these QTLs, *qRI-6*, *qRI-11*, and *qRI-20* which were located on chromosome 6, 11, and 20, respectively, were also detected in a single year. They could explain 5.15%, 4.91%, and 5.48% of the observed PV, respectively.

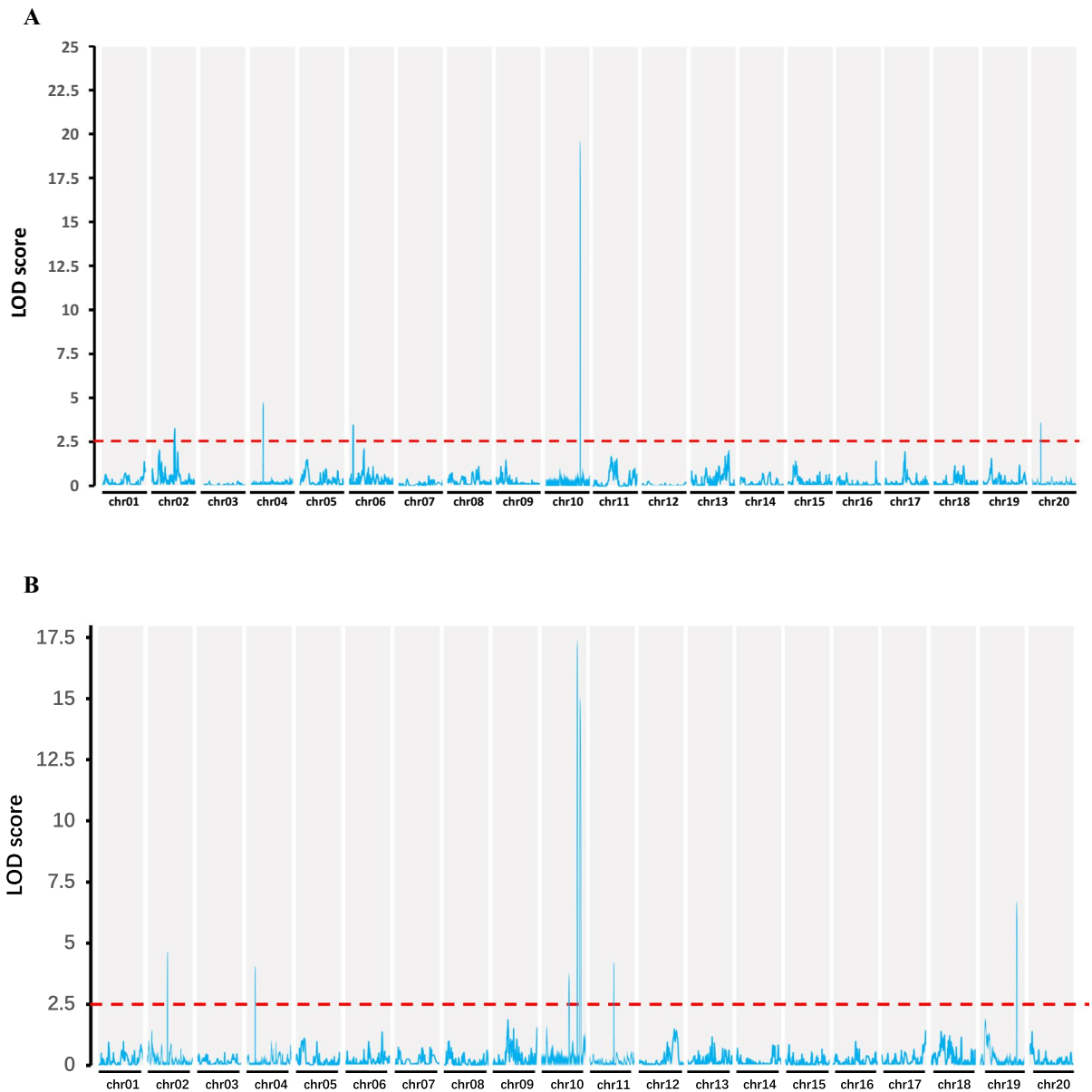
#### QTL mapping in two sub-populations classified by the genotype E2

As mentioned above, we detected a major QTL called *qRI-10*. The position of *qRI-10* coincides with *E2*, a major soybean maturity locus (Watanabe et al. 2011). The additive effect of *qRI-10* is negative, suggesting that its effect on delayed flowering is attributed to Williams 82. This corresponds with the genotypes of *E2* in the parents: the genotype of Suinong4 is *e2e2*, while that of Williams 82 is *E2E2*. Based on these results, *E2* is probably the candidate gene of *qRI-10*. Previous studies showed that the maturity locus *E2* is a *GIGANTEA* ortholog with a significant effect on flowering time in soybean (Watanabe et al. 2011). The high LOD scores and PVE of *qRI-10* indicate that *qRI-10* has an extremely strong impact on flowering time which might cover the effects of some other QTLs in S4W population. Thus, we divided S4W population into two sub

populations: 58 individuals with *E2E2* called *E2-S4W* and 51 individuals with *e2e2* called *e2-S4W*. Interestingly, a described QTL named *qRI-2* was detected in *E2-S4W*, while it was not detected in *e2-S4W*, in both 2018 and 2019 (Table 3, Supplementary Table 2, Supplementary Fig. 3, and Supplementary Fig. 4). The interval position of *qRI-2* was 15,078,759–15,263,588 on chromosome 2. The LOD scores and PVE of *qRI-2* were higher in *E2-S4W* than in *S4W* in both 2018 and 2019. Another QTL, *qRI-6.1*, was initially detected in *E2-S4W* in 2018 and 2019 (Table 3 and Supplementary Fig. 3). Like *qRI-2*, *qRI-6.1* was not detected in *e2-S4W* (Supplementary Table 2 and Supplementary Fig. 4). Considering the low LOD scores of *qRI-6.1*, the genetic effect of *qRI-6.1* was not explored further. With the physical interval of 8,542,345–13,420,505, *qRI-6.1* accounted for 15.97–17.38% of the observed PV. These results suggest that the effects of *qRI-2* and *qRI-6.1* might rely on the presence of *E2*. Several other QTLs were detected in only 1 year: *qRI-7* and *qRI-18* were detected in *E2-S4W* (Table 3); *qRI-13* and *qRI-19.1* were detected in *e2-S4W* (Supplementary Table 2). These QTLs accounted for 15.66%, 9.30%, 36.66%, and 23.30% of the observed PV, respectively.

#### Genetic effects between *E2* and *qRI-2*

As an ortholog of *Arabidopsis GI (GIGANTEA)*, *E2* plays major roles in adaptation and delays flowering under long-day conditions in soybean (Watanabe et al.



**Fig. 1** Quantitative trait locus (QTL) mapping in S4W population by IciMapping 4.0. **a** QTLs of flowering time in 2018. **b** QTLs of flowering time in 2019. The dashed lines indicate the LOD score of 2.5

2011; Xu et al. 2015; Wang et al. 2016). The expression of *E2* is suppressed in the *e3e4* background (Cao et al. 2015). QTL mapping indicated that *qRI-2* regulates flowering time based on *E2*. To further explore the genetic effect between *qRI-2* and *E2*, we classified S4W population into four groups based on different homozygous allelic combinations at *E2* and *qRI-2* using the functional marker of *E2* and SNP markers with highest LOD scores of *qRI-2* (2:15,078,759 was

used in 2018 and 2:12,259,445 was used in 2019). Individuals with the same genotype of *qRI-2* as SN4 were labeled as *qRI-2*-SN4, while individuals with the same genotype of *qRI-2* as W82 were labeled as *qRI-2*-W82. We then examined the flowering time in the four groups. In the *E2E2* background, *qRI-2*-SN4 lines exhibited significantly later flowering than *qRI-2*-W82 lines in both years (Fig. 2a and b). However, *qRI-2*-SN4 lines and *qRI-2*-W82 lines showed no

**Table 3** Details of the QTLs detected by IciMapping 4.0 in *E2-S4W*

QTL name	Year	Chr	Interval (cM)	Physical position	Max LOD	PVE (%)	ADD
<i>qRI-2</i>	2018	2	75.00–77.24	15,078,759–15,263,588	4.381	29.8297	2.4049
<i>qRI-6.1</i>		6	31.71–33.88	11,367,510–12,394,322	2.5861	17.3789	–1.871
<i>qRI-2</i>	2019	2	75.00–77.24	15,078,759–15,263,588	6.2791	20.6417	2.168
<i>qRI-6.1</i>		6	27.21–41.01	8,542,345–13,420,505	3.8162	15.9654	–1.8913
<i>qRI-7</i>		7	71.56–76.55	39,974,697–46,510,240	2.6832	15.6593	1.8912
<i>qRI-18</i>		18	0–0.38	670,036–670,042	3.3552	9.3043	–1.4418

Max LOD: maximum logarithm-of-odds (LOD) scores

PVE (%): percentage of phenotypic variance explained by the QTL

ADD: the additive effects contributed by QTLs. Additive effects with a positive value (+) indicate that the allele from Suinong4 enhances the phenotype; additive effects with a negative value (–) indicate that the allele from Williams 82 enhances the phenotype

difference in flowering time in the *e2e2* background (Fig. 2a and b). These results indicate that *qRI-2* depends on *E2* for its activity in regulating flowering and that *E2* has an epistatic effect on *qRI-2*. An epistatic effect usually implies the relationship on upstream and downstream. Therefore, the expression levels of *E2* in the four groups were also measured. Eight individuals were randomly selected from each of the four groups, and samples were taken at ZT8 on 20 DAE and used for qRT-PCR (Li et al. 2013). Not unexpectedly, *E2* had higher expression levels in *qRI-2-SN4* than in *qRI-2-W82* in both the *E2* and *e2* backgrounds (Fig. 2c and d). These results suggest that the causal gene in the *qRI-2-SN4* segment promotes the transcription of *E2* and that the causal gene of *qRI-2* acts upstream of *E2*.

#### Candidate gene prediction

QTLs with similar positions and the same additive effect in different years were identified as the same QTL. To analyze candidate genes of the QTLs, we combined the different intervals and chose the longest distance as the candidate interval. Since we detected four stable QTLs (*qRI-2*, *qRI-4*, *qRI-6.1*, and *qRI-10*) and *qRI-19* with high LOD scores and PVE, we primarily predicted the candidate genes of these QTLs.

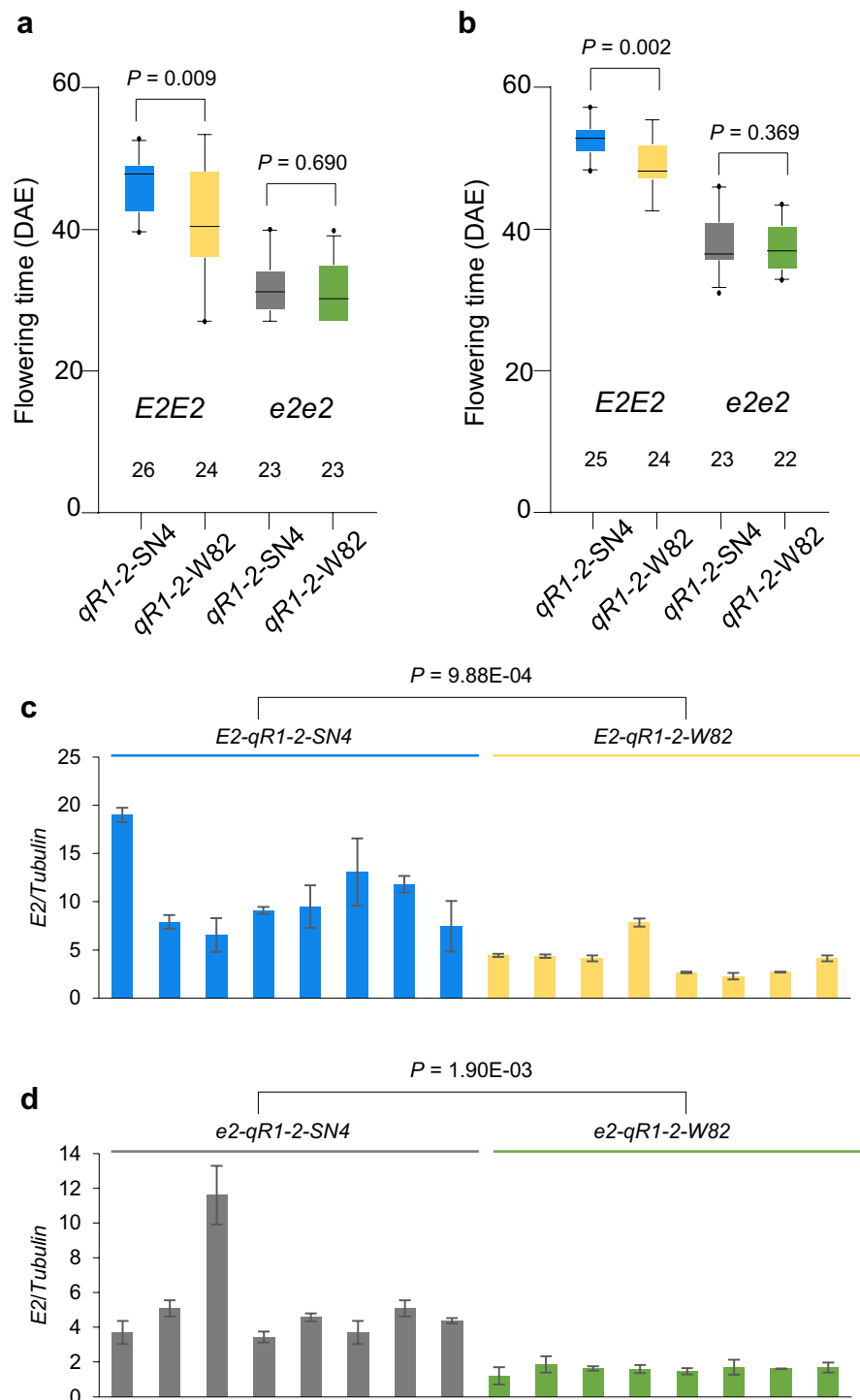
As mentioned above, the interval of *qRI-10* includes the region containing *E2* (Watanabe et al. 2011). Since there were different alleles of *E2* between parents, we presumed that *E2* is the candidate gene of *qRI-10*. In the position interval of *qRI-19*, the locus of *E3*, a maturity locus in soybean, was

found (Watanabe et al. 2009). As the parents possess different alleles of *E3*, *E3* is likely the candidate gene of *qRI-19*.

The interval of *qRI-2* contained 57 predicted genes with nonsynonymous/frameshift deletion/non-frameshift deletion/stopgain/frameshift insertion/nonframeshift insertion based on the resequencing data (Supplementary Table 3). Among these genes, we notice that *Glyma.02G139200* is an ortholog of *Arabidopsis PHYTOCHROME AND FLOWERING TIME 1 (PFT1)*. As reported, PFT1 acts as a co-activator of TCPs and FBHs to active the transcription of *CONSTANS*, thereby regulating flowering (Liu et al. 2017). The short tandem repeat (STR) of *PFT1* is essential for its role in regulating flowering (Rival et al. 2014). We used PCR approach to confirm the mutations of *Glyma.02G139200* between Suinong4 and Williams 82. An indel of an 84-bp STR was found between the two parents (Supplementary Fig. 5a). Furthermore, using InterPro (<https://www.ebi.ac.uk/interpro/search/sequence/>), we predicted the protein domain of *Glyma.02G139200* (Supplementary Fig. 6b). The 84-bp STR was located in the predicted domain of *Glyma.02G139200*. Subsequently, we examined the variations of *Glyma.02G139200* associated with flowering time in the 424-accession soybean panel at five field sites located in different latitudes in China (Lu et al. 2020). *Glyma.02G139200* contains fifteen haplotypes in the 424-accession panel (Supplementary Fig. 6a). Unfortunately, the allele (H11) of *Glyma.02G139200* from SN4 is a rare allele, thus it was not involved in the statistical analysis. We therefore selected two haplotypes (H14 and H15) covering a large group of individuals and analyzed



**Fig. 2** Genetic interaction evaluation and qRT-PCR analysis of *E2* and *qR1-2*. **a** Flowering time of different combinations of *qR1-2* and *E2* in 2018. **b** Flowering time of different combinations of *qR1-2* and *E2* in 2019. **c** Expression levels of *E2* in *E2-qR1-2-SN4* and *E2-qR1-2-W82*. **d** Expression levels of *E2* in *e2-qR1-2-SN4* and *e2-qR1-2-W82*. All data were given as mean  $\pm$  s.e.m. ( $n=8$  plants). Plants were grown under LD (16L/8D) and sampled at ZT8, 20 DAE. *qR1-2-SN4*, individuals have the same genotype of *qR1-2* as SN4; *qR1-2-W82*, individuals have the same genotype of *qR1-2* as W82; DAE, days after emergence. A Student's *t*-test was used to generate the *P* values



their association with flowering time. In all locations, accessions harboring H14 showed significantly later flowering than those harboring H15, suggesting that *Glyma.02G139200* might influence flowering in

soybean (Supplementary Fig. 7). We further examined the geographic distributions of H14 and H15 of *Glyma.02G139200* in subsets of Chinese landraces and cultivars (Supplementary Fig. 6c). The proportion

of H14 was very low in Huanghuai and the northern region of China. Whereas, a higher proportion of H14 was detected in the southern region of China. These results imply that the later flowering allele of *Glyma.02G139200* is already being used in southern region of China. Accordingly, *Glyma.02G139200* might be the candidate of *qR1-2*.

The region of *qR1-4* harbored 76 predicted genes with nonsynonymous/frameshift deletion/nonframeshift deletion/stopgain/frameshift insertion/stoploss according to the resequencing data (Supplementary Table 4). Among these genes, *Glyma.04G042700* is an ortholog of *Arabidopsis MRG*. In *Arabidopsis*, MRG2 physically interacts with CONSTANS to promote flowering by activating the expression of *FT* (Bu et al. 2014). According to the resequencing data and PCR sequencing results, a nonsynonymous mutation in codon 346 existed between Suinong4 and Williams 82 which was not located in the domain of *Glyma.04G042700* predicted by InterPro (Supplementary Table 4, Supplementary Fig. 5b, and Supplementary Fig. 8b). In the soybean 424-panel, four haplotypes of *Glyma.04G042700* were detected. The allele of *Glyma.04G042700* from Suinong4 belongs to haplotype 3 (H3), while the allele from Williams 82 belongs to haplotype 4 (H4) (Supplementary Fig. 8a). In all locations, accessions harboring H3 showed significantly later flowering than accessions harboring H4 (Supplementary Fig. 9). These results indicate that the mutation in codon 346 of *Glyma.04G042700* could alter flowering time in soybean. Consequently, we presume that the candidate gene of *qR1-4* might be *Glyma.04G042700*. The geographic distributions of H3 and H4 of *Glyma.04G042700* were also examined in the Chinese accessions (Supplementary Fig. 8c). A relatively high proportion of H4 was detected in the northern region of China, whereas this haplotype was hardly existed in the southern region of China. Considering that accessions with H4 showed earlier flowering than those with H3 and that the proportion of H4 was higher in the north of China, we presume that H4 has already been used during the breeding process in northern China.

*qR1-6.1* was first detected in E2-S4W. The interval of *qR1-6.1* contained 50 predicted genes with nonsynonymous/frameshift deletion/stopgain/nonframeshift insertion according to the resequencing data (Supplementary Table 5). Twenty-four of these genes were

functionally annotated and categorized into three classes: biological processes, cellular components, and molecular function using WEGO2.0 (<http://wego.genomics.org.cn/>), respectively (Supplementary Table 6 and Supplementary Fig. 10). No genes that were obviously associated with regulation of flowering were found, thus the genes mentioned above are considered as the candidate genes of *qR1-6.1*.

## Discussion

In summary, we developed a RIL population from the crosses between Suinong4 and Williams 82. With the combination of high-density map (constructed by GBS approach) and phenotypic data of flowering time, twelve QTLs were identified in total. Among these QTLs, four of them (*qR1-2*, *qR1-4*, *qR1-6.1*, and *qR1-10*) are stable which were detected in 2018 and 2019. Others (*qR1-6*, *qR1-7*, *qR1-11*, *qR1-13*, *qR1-18*, *qR1-19*, *qR1-19.1*, and *qR1-20*) were detected in only 1 year. Additionally, *qR1-2* and *qR1-10* were reported previously (Bernard 1971; Pooprompan et al. 2006; Khan et al. 2008). Whereas no QTL related to flowering time has been reported before for the position of *qR1-4* or *qR1-6.1* according to the SoyBase (<https://www.soybase.org/search/index.php?searchterm=First+flower&search=true>).

*E1* to *E4* play important roles in soybean at high latitudes (Liu et al. 2008; Watanabe et al. 2009; Xu et al. 2013; Lin et al. 2021). Under long-day conditions, *E3* and *E4* promote the expression of *E1* and *E2* to control flowering time (Kong et al. 2010; Watanabe et al. 2011). The effect of *E3* is enhanced under a high R:FR ratio under long-day conditions (Watanabe et al. 2009). In the current study, *E3* was identified as the candidate gene of *qR1-19*. However, *qR1-19* was only detected in 2019, perhaps since, both parents possess *e1<sup>as</sup>*, the effects of *E3* might be weakened (Upadhyay et al. 1994; Watanabe et al. 2004; Yamanaka et al. 2001). In addition, weather conditions in 2018 and 2019 were widely different (Supplementary Table 7). There were more cloudy and rainy days in 2018 than in 2019, leading to lower R/FR ratios, suggesting that *E3* presumably had less of an effect in 2018 (Supplementary Table 7) (Mao et al. 2017). Furthermore, the average high temperature and the difference between the high and low temperatures were lower in 2019 than in 2018 (Supplementary Table 7). The difference

in temperature conditions might have influenced the effect of *E3*. Therefore, the phenotypes are influenced not only by genotypes but also by the environments.

Based on the results described above, *E2* (*qRI-10*) had a crucial effect on flowering time in S4W population. *E2* participates in the regulation of circadian clock and flowering, its effect on flowering time is relatively stable under different light conditions (Watanabe et al. 2011; Lee et al. 2016). Combination of different *E2* alleles with other *E* loci partially determines the maturity groups of soybean cultivars (Li and Lam 2020). Furthermore, selection of *E2* haplotypes has contributed to the changes in flowering time during the adaptation of soybean to different latitudes (Watanabe et al. 2011; Wang et al. 2016). Nonetheless, the actual molecular mechanism of *E2* in regulating flowering has not been fully clarified. Another stable QTL, *qRI-2*, completely lost its effect on flowering time in the *e2e2* background, suggesting that *qRI-2* is dependent on *E2* function (Fig. 2). In addition, *Glyma.02G139200*, an ortholog of *Arabidopsis PFT1*, might be the candidate gene of *qRI-2*. *PFT1* regulates the signal intensity downstream of *phyA* and *phyB* in *Arabidopsis* (Cerdan and Chory 2003). *GI* acts as a positive regulator of very-low-fluence response (VLFR) which is mediated by *phyA* (Huq et al. 2000; Kim et al. 2007; Oliverio et al. 2007; Sawa et al. 2007; Song et al. 2014). Thus, *GI* and *PFT1* might function in the same pathway to regulate flowering. Similarly, we presume that *E2* and *qRI-2* might participate in the same pathway to regulate flowering in soybean. The analysis of genetic interaction evaluation of *qRI-2* and *E2* and the results of qRT-PCR suggest that *E2* has an epistatic effect on *qRI-2* and that the causal gene *qRI-2* acts upstream of *E2* (Fig. 2). Molecular cloning of *qRI-2* will further facilitate the understanding of regulatory relationships between *qRI-2* and *E2* and the mechanisms controlling flowering of them. Meanwhile, *qRI-2* could be utilized in molecular breeding to improve soybean varieties that possess *E2* by fine-tuning flowering time.

A panel of 424 soybean accessions was used to analyze the associations between candidate genes and flowering time in this study. We attempted to analyze the genetic relationship between *E2* and *Glyma.02G139200* in 424-accession panel. However, after classification, H14 contained so few individuals in the *E2* or *e2* background that

statistical analysis cannot be carried on. In addition, the different haplotypes of *Glyma.02G139200* and *Glyma.04G042700* lead to changes in flowering time at five sites, suggesting that the two genes may play important roles in controlling flowering time in regions ranging from high latitudes (Harbin: 45°75'N, 126°63'E) to low latitudes (Guangzhou: 23°16'N, 113°23'E). For *Glyma.02G139200*, H14, which leads to delayed flowering time compared to H15, was present at relatively low frequency in wild soybean ( $n=3$ ), this might be a mutant allele of *Glyma.02G139200* (Supplementary Fig. 6). Considering that soybean varieties in low latitudes have a relatively high frequency of *E2* allele, mutants of *Glyma.02G139200* might also have potential in breeding varieties with LJ trait (long-juvenile trait: extending the vegetative phase and improving yield under short-day conditions), which is consistent with the analysis of geographic distributions of *Glyma.02G139200* in our study (Supplementary Fig. 6c) (Wang et al. 2016). For *Glyma.04G042700*, analysis of the geographic distributions of H3 and H4 implied that H4 of *Glyma.04G042700* which leads to early flowering has been used for breeding in the north of China (Supplementary Fig. 8c).

Identifying QTLs related to flowering time is an important initial step in investigating the molecular mechanism underlying flowering time, even latitudinal adaptivity. In the present study, we detected several QTLs associated with flowering time, including *qRI-2*, whose role in regulating flowering is dependent on *E2*. These findings are conducive to understand the flowering regulatory network in soybean

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11032-021-01224-1>.

and provide genetic resources for molecular breeding.

**Acknowledgements** We would like to acknowledge Mrs. Yafeng Liu for phenotyping and managing the filed.

**Author contribution** CF designed the experiments. TS, YW, SL, LW, KK, LK, QC, and LD carried out the experiments. TS, BL, FK, SL, and CF analyzed the data. TS, FK, SL, and CF wrote the paper.

**Funding** This work was supported by Natural Key R&D Program of China (2017YFE0111000); EUCLEG Horizon 2020

of European Union (727312); National Natural Science Foundation of China (31930083, 31725021); the Major Program of Guangdong Basic and Applied Research (2019B030302006); the Open Project Foundation of National Key Laboratory for Crop Genetics and Germplasm Enhancement (ZW201901); Heilongjiang Academy of Agricultural Sciences Fund for Distinguished Young Scholars (2020JCQN005); and Heilongjiang Provincial Science and Technology Major Project (2019ZX16B01).

**Data availability** Not applicable.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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