


doi 10.18699/vjgb-26-06

Soybean locus qDTF-7 as an example of genetic heterogeneity associated with flowering and maturity time

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Abstract. Genome-wide association studies (GWAS) have become a standard approach for identifying quantitative trait loci associated with diverse phenotypic traits. Further investigation of the locus – specifically, the search for the causal gene and mutation – may present various challenges. One of the challenges is genetic heterogeneity (or locus heterogeneity), when alleles from different closely located genes can influence the same trait. Recently, using GWAS, we found the qDTF-7 locus on soybean chromosome 3, which is associated with flowering time under Novosibirsk conditions. Initially, we identified *GmTOE1*, an ortholog of *TOE1* (*TARGET OF EAT1*), a known flowering-time regulator in Arabidopsis, as the most likely candidate gene for this locus. Four major haplotypes were identified in *GmTOE1*, which are associated with soybean flowering and maturity and are likely to provide soybean adaptation to northern latitudes. However, this gene showed only a very weak association with soybean flowering in the Novosibirsk region compared to the Oryol region, suggesting the presence of another gene within the locus that influences flowering time. We therefore re-analyzed genes in the qDTF-7 locus and identified *GmRVE8c*, an Arabidopsis *RVE8* (*REVEILLE8*) ortholog, located ~21 kb upstream of *GmTOE1*; *RVE8* is a circadian clock component involved in plant development. After studying the natural variation of the *GmRVE8c* genes, we found four major haplotypes that arose due to three nonsynonymous substitutions and one 19-bp deletion leading to a frameshift. To identify three haplotypes, *GmRVE8c*^{hap1, 3, 4}, which are predominant in improved soybean cultivars, we developed DNA markers. Using these markers, we genotyped 129 soybean accessions, the developmental time of which had been studied in the Novosibirsk and Oryol regions. Using our data and data from SoyOmics, we found the *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4} haplotypes to be associated with late flowering and maturity in soybean. The early-maturing haplotype *GmRVE8c*^{hap1} is predominant in cultivars from northern regions and is likely associated with the adaptation of soybean to high latitudes. The *GmRVE8c*^{hap4} haplotype is in complete linkage with the early-maturing allele *GmTOE1*^C, whereas the *GmRVE8c*^{hap3} haplotype shows strong linkage with the late maturing allele *GmTOE1*^T. Furthermore, the ANOVA results indicate an interaction between *GmRVE8c* and *E1*, the major regulator of flowering in soybean. This interaction is manifested as a stronger effect of the *GmRVE8c*^{hap3, 4} haplotypes on flowering and maturity in the genetic background of the *e1-as* allele compared with *E1*. Together, these findings define a complex and intriguing locus, which may serve as a possible example of a genetically heterogeneous locus.

Key words: soybean; flowering; *TOE1*; *RVE8*; genetic heterogeneity

For citation: Perfil'ev R.N., Shmatova M.I., Shcherban A.B., Salina E.A. Soybean locus qDTF-7 as an example of genetic heterogeneity associated with flowering and maturity time. *Vavilovskii Zhurnal Genetiki i Seleksii = Vavilov J Genet Breed.* 2026;30(1):43-52. doi 10.18699/vjgb-26-06

Funding. This research was funded by the Russian Science Foundation (RSF project No. 21-76-30003-П). The design of markers and their verification was carried out within the framework of a budget project FWNR-2025-0033.

Локус сои qDTF-7 как пример генетической гетерогенности, ассоциированной с продолжительностью цветения и созревания

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Аннотация. В настоящее время полногеномный анализ ассоциаций (GWAS) стал стандартным подходом для идентификации локусов количественных признаков, ассоциированных с различными фенотипическими признаками. При последующем изучении локуса, т. е. поиске гена и мутации, которые влияют на признак, можно столкнуться с разными проблемами. Одна из проблем – генетическая (или локусная) гетерогенность, ситуация, когда аллели из разных близко расположенных генов в одном локусе влияют на один признак. Ранее с помощью

GWAS на хромосоме 3 сои мы обнаружили локус qDTF-7, ассоциированный с продолжительностью цветения в условиях Новосибирска. Первоначально для данного локуса в качестве наиболее вероятного гена-кандидата мы определили *GmTOE1*, ортолог *TOE1* (*TARGET OF EAT 1*), известного регулятора цветения у арабидопсиса. У *GmTOE1* были обнаружены четыре основных гаплотипа, которые ассоциированы с цветением и созреванием сои и, вероятно, связаны с адаптацией сои к северным широтам. Однако этот ген демонстрировал очень слабую ассоциацию с цветением сои в Новосибирской области по сравнению с Орловской, что указывало на наличие в составе локуса другого гена, который может влиять на цветение сои. Повторно проанализировав гены в составе локуса qDTF-7, мы примерно на 21 т.п.н. выше гена *GmTOE1* обнаружили *GmRVE8c*, ортолог гена *RVE8* (*REVEILLE 8*), который вовлечен в процессы развития у арабидопсиса в качестве компонента циркадных ритмов. Изучив естественное нуклеотидное разнообразие *GmRVE8c*, мы выявили четыре основных гаплотипа, которые возникли из-за трех однонуклеотидных замен и одной делеции длиной 19 п.н., приводящей к сдвигу рамки считывания. Для определения трех гаплотипов *GmRVE8c^{hap1,3,4}*, преобладающих в современных сортах сои, мы разработали ДНК-маркеры. С помощью этих маркеров мы генотипировали 129 сортообразцов сои, для которых ранее изучили продолжительность развития в условиях Новосибирской и Орловской областей. В результате с помощью наших сведений и данных из SoyOmics мы обнаружили ассоциацию гаплотипов *GmRVE8c^{hap3}* и *GmRVE8c^{hap4}* с поздним цветением и созреванием сои. Раннеспелый гаплотип *GmRVE8c^{hap1}* преобладает в сортообразцах из северных регионов и, вероятно, связан с адаптацией сои к северным широтам. Гаплотип *GmRVE8c^{hap4}* полностью сцеплен с раннеспелым аллелем *GmTOE1c*, а гаплотип *GmRVE8c^{hap3}* сильно сцеплен с позднеспелым аллелем *GmTOE1T*. Кроме того, результат ANOVA показывает наличие взаимодействия *GmRVE8c* с главным регулятором цветения сои – геном *E1*. Данное взаимодействие выражается более сильным эффектом гаплотипов *GmRVE8c^{hap3,4}* на цветение и созревание на генетическом фоне аллеля *e1-as* в сравнении с *E1*. Все это формирует достаточно комплексный и интересный локус, который может выступать как возможный пример генетически гетерогенного локуса.

Ключевые слова: соя; цветение; *TOE1*; *RVE8*; генетическая гетерогенность

Introduction

Advances in high-throughput plant phenotyping and genotyping methods, as well as the development of mathematical models that enable the identification of links between phenotype and genotype, have led to the discovery of numerous quantitative trait loci in plants (Zhang et al., 2022). The flowering and maturity time of soybean is no exception, and genes controlling these traits have been identified using GWAS (Genome-Wide Association Studies), for example: *Tof5* (Dong et al., 2022), *Tof8* (Li H. et al., 2023a), *Tof13* (Li H. et al., 2023b), *Tof16* (Dong et al., 2021), *qFT13-3* (Li Y.F. et al., 2024), *GmAP1d* (Guo et al., 2024). Thus, the *Tof16* locus was found to encode an ortholog of the *Arabidopsis CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*) gene, which is a key component of the circadian clock and a transcription factor containing a DNA-binding MYB domain (Dong et al., 2021). Recessive *tof16* alleles that partially or completely lose the original function of the encoded protein delay flowering and maturity time in soybean, which is more favorable for cultivars grown in southern latitudes (Dong et al., 2021).

A total of 54 CCA1-Like proteins have been identified in the soybean genome (Bian et al., 2017), four of which belong to the same phylogenetic clade and show the highest similarity to the *Arabidopsis* genes *RVE4* (*REVEILLE 4*) and *RVE8* (Bian et al., 2017; Shan et al., 2021; Bao et al., 2024). Loss of *RVE8* function in *Arabidopsis* reduces flowering time under both short-day (SD) and long-day (LD) conditions, whereas overexpression delays flowering (Rawat et al., 2011). In addition to reduced flowering time, the *rve4 6 8* and *rve3 4 5 6 8* *Arabidopsis* mutants exhibit accelerated development and increased cell size due to disrupted expression of *PIF4* (*PHYTOCHROME INTERACTING FACTOR 4*) and *PIF5*, as well as reduced proteasomes activity (Gray et al., 2017; Scandola et al., 2022). The functional role of *RVE* genes in flowering

and maturity time in soybean is still poorly understood (Shan et al., 2021), although only recently it was shown that overexpression of *GmMYB133* (the gene most closely related to *Arabidopsis RVE5*) and *GmRVE8a* in *Arabidopsis* leads to delayed flowering (Shan et al., 2021; Bao et al., 2024). Interestingly, overexpression of *GmPIF4b* in soybean accelerates not only flowering but also subsequent developmental stages (Arya et al., 2021). In soybean, a *GmMYB133-PRR5-PIF4* module has also recently been proposed, as *GmMYB133* in *Arabidopsis* can bind to the *PRR5* (*PSEUDO-RESPONSE REGULATOR 5*) promoter, and *PRR5* can, in turn, bind to *PIF4* (Shan et al., 2021).

At present, genes and mutations affecting traits have been identified for only a relatively small number of quantitative trait loci in plants. Several challenges interfere with fine-mapping, that is, the identification of the gene and mutation within a locus that influence the expression of a trait (Clauw et al., 2024). One of the challenges is allelic heterogeneity, a situation in which different mutations in a single gene lead to the same or similar phenotype (Sasaki et al., 2021; Clauw et al., 2024; Liu H.-J. et al., 2024). One of the classic examples in plants is the multiple alleles of the *FRI* (*FRIGIDA*) gene in *Arabidopsis*, which have a confirmed effect on the expression of *FLC* (*FLOWERING LOCUS C*) but are not detected by GWAS unless these alleles are included as covariates in the statistical model (Atwell et al., 2010). Another less-studied challenge is genetic heterogeneity (or locus heterogeneity) – a situation in which alleles from different closely located genes at the same locus influence the same trait (Sasaki et al., 2021; Clauw et al., 2024; Liu H.-J. et al., 2024). Significantly fewer examples of genetic heterogeneity are currently known. Recently, E. Sasaki et al. (2021) characterized such a locus in *Arabidopsis*, showing that the association of the *AOP2/AOP3* (*ALKENYL HYDROXALKYL PRODUCING*) genes with

flowering time can, in fact, be explained by two neighboring genes, *NDX1* (*NODULIN HOMEODOMAIN GENES 1*) and *GAI* (*GIBBERELIC ACID REQUIRING 1*). A similar locus was also found in soybean, where pod shattering resistance was shown to arise from mutations in two nearby genes, *Sh1* and *Pdh1* (Li S. et al., 2024).

In this article, we discuss a possible example of a genetically heterogeneous locus in soybean. By reanalyzing genes within the soybean flowering-associated *qDTF-7* locus, we identified a new candidate gene, *GmRVE8c*, located ~21 kb upstream of another strong candidate gene, *GmTOE1*. Mutations in *GmRVE8c* were found to form distinct haplotypes associated with soybean flowering and maturity. Furthermore, the effects of *GmRVE8c* haplotypes on flowering and maturity time depend on the allelic state of *E1*, the main regulator of flowering in soybean. Altogether, this forms a complex and intriguing locus, and our work may serve as a theoretical example of the natural mutations underlying associations identified through GWAS.

Materials and methods

Plant materials and phenotypes. As plant material, we used 129 soybean accessions (63 cultivars and 66 breeding lines), which were described and used in our previous works (Perfil'ev et al., 2023, 2024). For these cultivars, the duration from emergence to flowering (DTF, days from emergence to flowering) and to maturity (DTM, days from emergence to maturity) was previously studied in Novosibirsk and Oryol in 2021 and 2022. The details of this field experiment were described in a previous work (Perfil'ev et al., 2023). We used BLUP (Best Linear Unbiased Prediction) values as phenotypes for these 129 accessions, which were calculated separately for each region, as described previously (Perfil'ev et al., 2024).

All available observations for BBD (Beginning bloom date) and MD (Pod maturity date) were downloaded from SoyOmics (<https://ngdc.cnpc.ac.cn/soyomics/index>) (Liu Y. et al., 2023). BLUP values for these phenotypes were calculated using the “lmer” function from the lme4 package (Bates et al., 2015), with genotype, location, and year included as random effects in the model. The resulting BLUP values were used for further analyses.

Characterization of the *qDTF-7* locus and natural variation in the *GmRVE8c* gene. The list of genes located within the *qDTF-7* locus was obtained using SoyBase (<https://www.soybase.org/>).

Data on haplotypes of the *GmRVE8c/SoyZH13_03G159500* gene within the coding sequence were obtained using the “Hap-

Snap” module from SoyOmics. The settings in “HapSnap” were kept at default, except for the “Variation type” option, which excluded “Synonymous” and “Unclassed” variation.

The ModiDB database was used to search for the main protein domains of *GmRVE8c/Glyma.03G177300* (<https://mobidb.org/>) (Piovesan et al., 2025).

Co-localization of *qDTF-7* with previously identified QTLs was examined using the “BreedingTips” module from the SoyOmics website.

DNA extraction, development of DNA markers, genotyping. Genomic DNA was extracted from 3–4-day-old seedlings grown in Petri dishes using a CTAB buffer (Rogers, Bendich, 1985).

To develop DNA markers for the *GmRVE8c/Glyma.03G177300* gene, the nucleotide sequence obtained from the Ensembl Plants database was used (<https://plants.ensembl.org/index.html>). UGENE v48.0 software was used for primer design (Okonechnikov et al., 2012) (<http://ugene.net/ru/>). The nucleotide sequences of the primers are provided in the Table.

PCR was performed using BioMaster HS-Taq PCR-Color (2×) (BiolabMix, Novosibirsk, Russia). The mixture with a total volume of 25 µl contained 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 0.1 % Tween 20, 2 mM MgCl₂, 0.25 mM of each primer, 50–100 ng of genomic DNA, and 1 U Taq DNA polymerase. The primers used are presented in the Table. PCR conditions: 95 °C, 5 min; (95 °C, 15 s; 55 °C, 10 s; 72 °C, 20 s) for 34 cycles; 72 °C, 1 min.

Digestion of PCR products using the restriction enzyme Mhl I (SibEnzyme, Novosibirsk, Russia) was carried out in a reaction mixture with a total volume of 20 µL, which contained: 8 µl of PCR product; 2 µl 10x buffer recommended by the manufacturer; 1 U enzyme; 10 µl ddH₂O. Restriction and amplification products were separated on a 2–2.5 % agarose gel with ethidium bromide. Electrophoresis results were photographed using Gel Doc™ XR+ (BioRad, USA). The “Step100+50” DNA ladder (BiolabMix, Novosibirsk, Russia) was used as a molecular size marker.

Genotypes of the *E1* and *GmTOE1* genes for 129 soybean accessions were previously obtained and published (Perfil'ev et al., 2023, 2024). For improved cultivars from the SoyOmics database, genotypes for the mutations soy8699425 for the *E1/SoyZH13_06G195900* gene and soy4989324 for the *GmTOE1/SoyZH13_03G159700* gene were obtained using the “HapSnap” module, and for the mutation soy8699425, they were additionally recoded as C → *e1-as*, G → *E1* allele.

DNA markers used to determine the main haplotypes of *GmRVE8c* (*Glyma.03G177300/SoyZH13_03G159500*)

| Gene | 5'–3' direction | Primer sequences | Haplotypes | Expected amplification/restriction products, bp | Restriction enzyme |
|----------------|-----------------|---------------------------|------------|---|--------------------|
| <i>GmRVE8c</i> | F | AGATAGGATATGATACCATATATTG | hap1–3 | 173 | – |
| | R | CGGACCAGCTCTCCCTTGAC | hap4 | 154 | |
| <i>GmRVE8c</i> | F | AAGAACGGGGCAGTAGCACACG | hap2, 3 | 251 | Mhl I |
| | R | TTCATCCCATGTGACATACCCA | hap1, 4 | 221+30 | |

Statistical analysis. The association of *GmRVE8c* haplotypes with flowering and maturity time was analyzed using the basic function “aov” in R version 4.4.2 (<https://www.r-project.org/>). Multiple haplotype comparisons were performed using the “TukeyHSD” function. Boxplots and heatmaps illustrating the results of multiple comparisons were generated using the R packages “ggplot2” (Wickham, 2016) and “ggcorplot” (Kassambara, 2023).

Results

Revisiting the qDTF-7 locus identified a new candidate gene, *GmRVE8c*

Previously, using the FarmCPU multilocus model on soybean chromosome 3, we identified a QTN (Gm03_40959110_A_G) that was associated with flowering time in Novosibirsk conditions (Perfil'ev et al., 2024). This QTN was located within a 553 kb LD block, which was designated as the qDTF-7 locus (QTL days from emergence to flowering 7) (Perfil'ev et al., 2024). This locus contains 71 genes (Table S1 in Supplementary Materials)¹, and we initially identified *Glyma.03G177500* (hereafter referred to as *GmTOE1*) as the most likely candidate gene (Perfil'ev et al., 2024). However, this gene exhibited a much weaker association with soybean flowering in Novosibirsk compared to Oryol (Perfil'ev et al., 2024). Therefore, we decided to revisit the genes within this locus more closely and, as a result, found *Glyma.03G177300*. This gene encodes one of four soybean homologs that are most closely related to the *Arabidopsis* genes *RVE8* (*REVEILLE 8*) and *RVE4* (Bao et al., 2024). In this article, the *Glyma.03G177300* gene will be referred to as *GmRVE8c*.

In *Arabidopsis*, the *RVE8* gene is a known component of the circadian clock and also functions to delay flowering (Rawat et al., 2011). Overexpression of one of the soybean *RVE8* orthologs (*GmRVE8a*) in *Arabidopsis* leads to delayed flowering (Bao et al., 2024). Collectively, these findings suggest that *GmRVE8c* is a candidate gene for regulating flowering and maturity in soybean.

Association of *GmRVE8c* haplotypes with flowering and maturity of soybean

Using the SoyOmics database, we studied natural variation in the coding region of the *GmRVE8c* gene. As a result, we found four haplotypes, which arose due to one deletion causing a frameshift and three nonsynonymous substitutions (Table S2). This deletion is predicted to cause a frameshift after four amino acids, likely leading to a complete loss of the gene's original function. All nonsynonymous substitutions found are located outside the predicted MYB DNA-binding domains of the protein (Fig. S1). It is difficult to assume whether these substitutions have consequences for the structure and function of the encoded protein.

We studied the association of three common haplotypes with BLUP_BBD and BLUP_MD in improved soybean cultivars. In cultivated varieties, according to the results of the association analysis for BLUP_BBD, cultivars carrying

GmRVE8c^{hap3} flower significantly later than those carrying *GmRVE8c*^{hap1} and *GmRVE8c*^{hap4} (Fig. 1a). According to the results of the association analysis for BLUP_MD, cultivars carrying *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4} mature significantly later than those carrying *GmRVE8c*^{hap1} (Fig. 1a).

To identify these three haplotypes, we developed DNA markers for the soy32494699 and soy4988787 mutations (see the Table, Fig. S2) and used them to genotype 129 soybean accessions from our collection, in which the qDTF-7 locus was initially identified. As a result, *GmRVE8c*^{hap1} was found in 109 accessions, while the remaining 12 and 8 carried haplotypes *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4}, respectively (Table S3). In addition, *GmRVE8c* shows a significant association with BLUP_DTF and BLUP_MD under the conditions of the Novosibirsk and Oryol regions, and accessions carrying the *GmRVE8c*^{hap4} haplotype flower and mature significantly later (Fig. 1b, c).

In cultivars Graciya and VNIIS-1, we previously identified the *GmTOE1*^T allele (Perfil'ev et al., 2024); however, genotyping for *GmRVE8c* revealed the *GmRVE8c*^{hap4} haplotype in these cultivars, which was unexpected, since *GmRVE8c*^{hap4} is linked to the *GmTOE1*^C allele. It should be noted that the *GmRVE8c* gene is physically located very close to *GmTOE1*, making the occurrence of crossover genotypes *GmRVE8c*^{hap4} *GmTOE1*^T extremely unlikely. For example, no such genotype was found in the SoyOmics database. Therefore, we re-extracted DNA from these cultivars and genotyped them using a previously developed DNA marker for the *GmTOE1*^C allele (Perfil'ev et al., 2024), confirming the presence of the *GmTOE1*^C allele in these cultivars (Table S3).

The *GmRVE8c* gene exhibits an epistatic interaction with the *E1* gene and is also located near another flowering regulator, *GmTOE1*

In soybean, *GmCCA1s* (*CIRCADIAN CLOCK ASSOCIATED 1*) genes promote flowering by repressing the expression of the major flowering repressor, *E1* (Dong et al., 2021). *RVE* genes belong to a family of transcription factors with CCA1-like MYB domains, so we suggested that the function of *GmRVE8c* in flowering regulation might also depend on the *E1* gene.

The distance between *GmRVE8c* and *GmTOE1* is approximately 21 kb, and *GmTOE1* is nearly the immediate downstream gene following *GmRVE8c* in the 5'–3' direction (Table S1). *GmTOE1* harbors natural variation that is associated with flowering and maturity time in soybeans (Perfil'ev et al., 2024). Previously, we identified four main haplotypes, including two early-maturing and two late-maturing haplotypes (hereafter referred to as the *GmTOE1*^C and *GmTOE1*^T alleles, respectively), which differ from each other by the nucleotide substitution T316C, resulting in the replacement of serine with proline at the 106th amino acid (Perfil'ev et al., 2024).

We examined the association of *GmRVE8c* haplotypes, taking into account the *E1* and *GmTOE1* genetic backgrounds, using data from SoyOmics and from our experiment (Fig. S3–S6). The ANOVA results show a significant interaction between *GmRVE8c* and *E1*, based on data from SoyOmics (Tables S4, S5). This indirectly suggests the presence of an

¹ Supplementary Tables S1–S6 and Figures S1–S6 are available at: <https://vavilovj-icg.ru/download/pict-2026-30/appx3.zip>

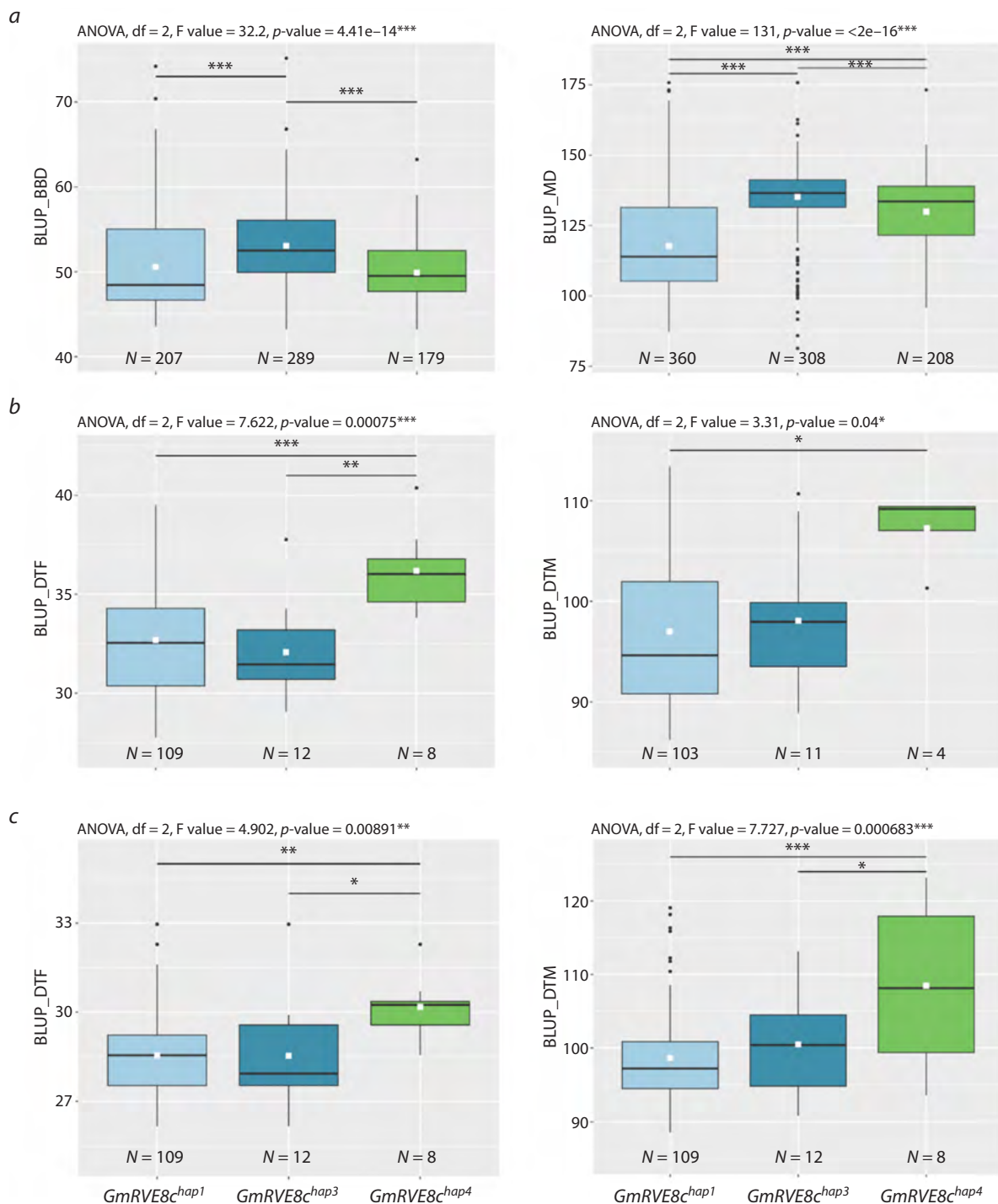


Fig. 1. Association of haplotypes *GmRVE8c* with flowering and maturity time.

a – association of haplotypes *GmRVE8c* with BLUP_BBD and BLUP_MD; *b* – association of haplotypes *GmRVE8c* with BLUP_DTF and BLUP_DTM in Novosibirsk; *c* – association of haplotypes *GmRVE8c* with BLUP_DTF and BLUP_DTM in Oryol. White squares on the boxplots indicate the mean value. Asterisks indicate significant differences between the compared genotypes: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

epistatic interaction between *GmRVE8c* and *E1*. Analysis of the established associations using data from SoyOmics showed that, on the *e1-as* allele background, the *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4} haplotypes have a stronger effect on flowering and maturity time (Fig. S3a, b). In our collection, plants carrying the *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4} haplotypes also

carry the *e1-as* or *e1-nl* alleles, and plants with *GmRVE8c*^{hap4} exhibit later flowering and maturity compared to other genotypes (Fig. S3c, d).

According to BLUP_BBD data, plants with *GmRVE8c*^{hap1} *GmTOE1*^T and *GmRVE8c*^{hap3} *GmTOE1*^T flower significantly later than those with *GmRVE8c*^{hap4} *GmTOE1*^C and

GmRVE8c^{hap1} GmTOE1^C (Fig. S4a). A similar pattern is observed for BLUP_MD, with the exception of the *GmRVE8c^{hap4} GmTOE1^C* genotype, which matures later than *GmRVE8c^{hap1} GmTOE1^C* (Fig. S4a). According to data from our collection, plants with *GmRVE8c^{hap4} GmTOE1^C* flower and mature significantly later than those with *GmRVE8c^{hap1} GmTOE1^C* in both regions (Fig. S4b–c). According to BLUP_MD and BLUP_BBD, plants with the *GmRVE8c^{hap1} GmTOE1^C* genotype flower and mature significantly earlier than those with *GmRVE8c^{hap1} GmTOE1^T* (Fig. S4a). According to the data from our collection, plants with the *GmRVE8c^{hap1} GmTOE1^C* genotype flower significantly earlier than those with *GmRVE8c^{hap1} GmTOE1^T*, but only under the conditions of Oryol (Fig. S4b). The *GmTOE1^C* allele is associated with early flowering and maturity, as we described previously, and *GmRVE8c^{hap4}* appears to partially restore the early-maturing phenotype of *GmTOE1^C* plants, making them late-maturing (Fig. S4a).

When considering all three genes simultaneously, we interpreted the results by comparing genotypes that differ from each other at only one gene. According to BLUP_BBD and BLUP_MD, plants with the *GmRVE8c^{hap1} e1-as GmTOE1^C* genotype flower and mature later than those with *GmRVE8c^{hap4} e1-as GmTOE1^C* (Fig. S5). According to BLUP_BBD, there are no significant differences between genotypes *GmRVE8c^{hap1} E1 GmTOE1^C* and *GmRVE8c^{hap1} E1 GmTOE1^T*, or between *GmRVE8c^{hap1} e1-as GmTOE1^C* and *GmRVE8c^{hap1} e1-as GmTOE1^T* (Fig. S5a). According to BLUP_MD, of the two genotypes, only *GmRVE8c^{hap1} E1 GmTOE1^C* and *GmRVE8c^{hap1} E1 GmTOE1^T* differ significantly from each other (Fig. S5b). In our collection, plants with the *GmRVE8c^{hap1} e1-nl GmTOE1^C* genotype flower significantly earlier than those with *GmRVE8c^{hap1} e1-nl GmTOE1^T* under Oryol conditions (Fig. S6c). In addition, genotypes with *GmRVE8c^{hap4} e1-as GmTOE1^C* flower and mature later than the other genotypes (Fig. S6). Overall (although not completely) this repeats the result of the two-gene analysis from the previous paragraph.

There are also significant differences in BLUP_MD and BLUP_BBD between genotypes *GmRVE8c^{hap1} E1 GmTOE1^C* and *GmRVE8c^{hap4} E1 GmTOE1^C*, with plants carrying *GmRVE8c^{hap4}* maturing later but flowering slightly earlier (Fig. S5). In the background of the *e1-as* allele, the *GmRVE8c^{hap4}* haplotype shows a stronger effect on BLUP_BBD (Fig. S5a). For the *GmRVE8c^{hap3}* haplotype, a similar pattern is observed, with no significant differences in BLUP_BBD and BLUP_MD between the *GmRVE8c^{hap3} E1 GmTOE1^T* and *GmRVE8c^{hap1} E1 GmTOE1^T* genotypes (Fig. S5). However, a significant difference in BLUP_MD is observed between the *GmRVE8c^{hap3} e1-as GmTOE1^T* and *GmRVE8c^{hap1} e1-as GmTOE1^T* genotypes (Fig. S5b). In our collection, plants carrying the *GmRVE8c^{hap4}* haplotype, even in the presence of the null *e1-nl* allele, show late flowering and maturity (Fig. S5).

Distribution of *GmRVE8c* haplotypes in soybean accessions of different origin

For the *GmRVE8c* gene, we examined haplotype frequencies in soybean cultivars from China across three major soybean

growing regions (China I, China II, and China III) and one mixed region (China IV–VI), as well as in our collection, which was divided into three main groups: A – from West Siberia (Novosibirsk and Omsk regions); B – from other Russian regions; and C – from other countries (Fig. 2).

The *GmRVE8c^{hap1}* haplotype predominates in cultivars from northern China, whereas *GmRVE8c^{hap3}* predominates in the other China regions. In our collection, accessions from Western Siberia predominantly carry the *GmRVE8c^{hap1}* haplotype, whereas the late-maturing *GmRVE8c^{hap4}* haplotype is present only in accessions from other regions of Russia and from other countries (Fig. 2b). This demonstrates the role of *GmRVE8c* haplotypes in the adaptation of soybean to different cultivation regions.

Discussion

The *GmRVE8c* gene is a new candidate gene for regulating flowering and maturity in soybeans

We identified the *Glyma.03G177300/GmRVE8c* gene as a new candidate gene for the previously found qDTF-7 locus associated with soybean flowering (Perfil'ev et al., 2024). *GmRVE8c* was found to have four major haplotypes resulting from three single nucleotide substitutions and a 19-bp deletion (Table S2). Among the mutations identified in *GmRVE8c*, the soy32494699 substitution (a 19-bp deletion) in the *GmRVE8c^{hap4}* haplotype may lead to a complete loss of the encoded protein's original function due to a frameshift occurring after the fourth amino acid (Table S2). The remaining three *GmRVE8c^{hap1–3}* haplotypes arose due to three nonsynonymous substitutions (Table S2). Although all three substitutions are located outside the predicted DNA-binding domains (Fig. S1), they may still affect the 3D structure of the protein or its interactions with other proteins (Fig. S1). Notably, two of the identified mutations are located within the predicted LIP (Linear Interacting Peptides) regions of the protein (Fig. S1). A complete loss of *GmRVE8c* function appears to result in delayed soybean development, as plants carrying the *GmRVE8c^{hap4}* haplotype exhibit late flowering and maturity (Fig. 1). It is also possible that the *GmRVE8c^{hap3}* haplotype has also lost the original function of the protein, since, according to SoyOmics data, samples carrying this haplotype also exhibit late flowering and maturity (Fig. 1a).

The frequency of *GmRVE8c* haplotypes shifts from wild to cultivated soybean; compared with wild soybean, the *GmRVE8c^{hap2}* haplotype is almost absent in landrace and modern cultivars (Table S2). Moreover, the *GmRVE8c^{hap4}* haplotype is not detected in the wild soybean population and likely arose and was selected after soybean domestication (Table S2). The distribution of *GmRVE8c* haplotypes across different cultivation regions shows that the early-maturing haplotype *GmRVE8c^{hap1}* predominates in cultivars from northern China, whereas *GmRVE8c^{hap3}* predominates in other regions (Fig. 2a); similarly, in our collection, almost all cultivars from Western Siberia carry the *GmRVE8c^{hap1}* haplotype (Fig. 2b). This highlights the role of *GmRVE8c* haplotypes in the adaptation of soybean to different cultivation regions. Due to its association

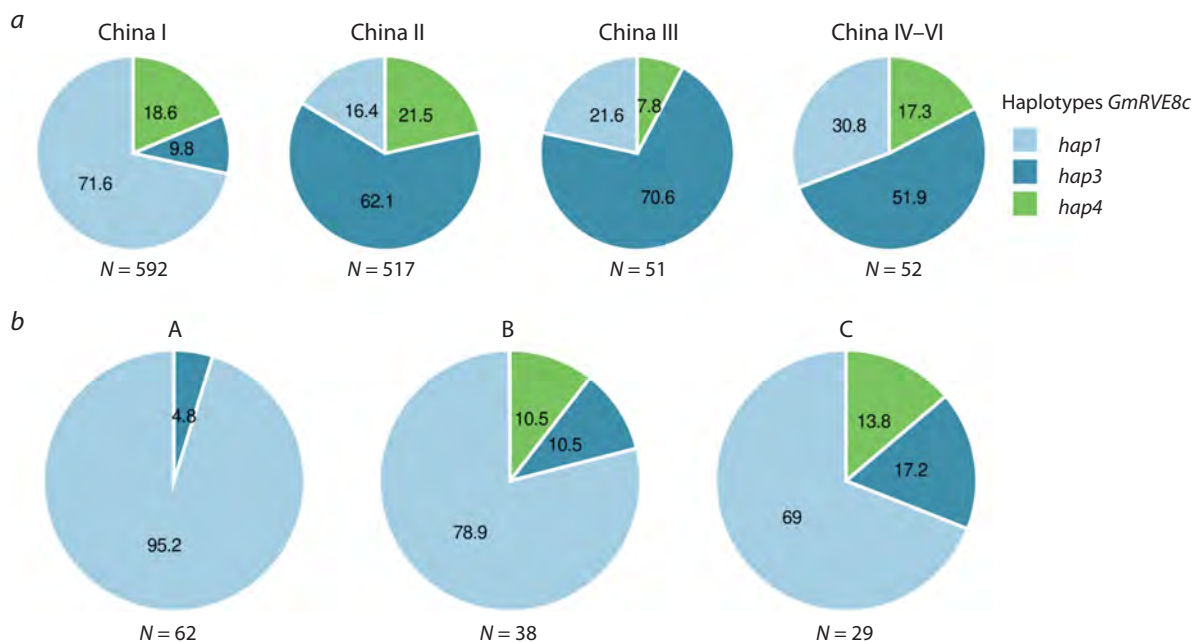


Fig. 2. Distribution of *GmRVE8c* haplotypes: *a* – in improved soybean cultivars from China (China I designates northern provinces of China, and China II, III, IV–VI designate southern provinces); *b* – distribution of *GmRVE8c* haplotypes in three groups of soybean accessions: A – from West Siberia (Novosibirsk and Omsk regions); B – from other Russian regions; and C – from other countries.

Numbers on pie charts indicate percentages; *N* denotes the number of accessions.

with late flowering and maturity, the *GmRVE8c*^{hap4} haplotype is unfavorable for northern latitudes but, conversely, advantageous for southern latitudes. We believe that *GmRVE8c*^{hap4} could be valuable in breeding soybean cultivars adapted to southern regions.

The *GmRVE8c* gene shows epistatic interaction with the *E1* gene

The *RVE* gene family encodes transcription factors with CCA1-like MYB domains and is closely related to the *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*) and *LHY* (*LATE ELONGATED HYPOCOTYL*) genes (Rawat et al., 2009). In soybean, the *CCA1/LHY* genes are known regulators of flowering, and under SD conditions, *GmLHYs* directly bind to the *E1* promoter and repress its expression, thereby accelerating flowering, whereas loss of *GmLHYs* function delays flowering (Dong et al., 2021). Based on this, we hypothesized that the role of *GmRVE8c* in flowering regulation may also depend on the *E1* gene. ANOVA results based on SoyOmics data show a significant interaction between *E1* and *GmRVE8c*, suggesting that *GmRVE8c* may also act as a repressor of the *E1* gene (Tables S3, S4). Association analysis of these data shows that the *GmRVE8c*^{hap3} and *GmRVE8c*^{hap4} haplotypes more strongly delay flowering and maturity in the background of the semi-functional *e1-as* allele (Fig. S4a, b). This is an unexpected situation, because if we assume that *GmRVE8c* can suppress the expression of the *E1* gene, then in the presence of the weakly functional *e1-as* allele, loss of *GmRVE8c* function should not lead to a phenotypic change, or at least the effect

should be weaker. Similarly, the effect on the flowering time of the loci/genes *Tof16/LHYa*, *Tof8/FKF1b*, and *J/ELF3* under the background of the *e1-as* allele completely disappeared or was reduced (Lu S. et al., 2017; Dong et al., 2021; Li H. et al., 2023a). Phenotypic analysis in our collection shows that the *GmRVE8c*^{hap4} haplotype affects flowering and maturity even in the presence of the *e1-nl* allele, which has a deletion of the entire *E1* gene (Fig. S3c, d). It is likely that, under our conditions, *GmRVE8c* may be involved in the transcriptional regulation of the entire *E1* gene family (*E1*, *E1La*, *E1Lb*), or that *GmRVE8c* controls flowering time through an *E1*-independent mechanism.

In *Arabidopsis*, proteins LNK1 (NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1) and LNK2 recruit RVE4 and RVE8 to activate transcription of *TOC1* (*TIMING OF CAB EXPRESSION 1*) and *PRR5* (*PSEUDO-RESPONSE REGULATOR 5*), and loss of function of *LNK1–4* results in delayed flowering under LD conditions (Xie et al., 2014; De Leone et al., 2018). Knockout of the four *GmLNK2s* orthologs in soybean accelerates flowering under LD conditions due to reduced expression of the *E1* gene (Li Z. et al., 2021). A characteristic feature of *LNK* genes is the absence of their own DNA-binding domains, for which they recruit *CCA1*, *LHY*, *RVE4* and *RVE8*, and switch them from repressors to activators of transcription (Xie et al., 2014). Further studies will clarify whether the function of *GmRVE8c* in flowering control depends on the *E1* gene family and whether *GmLNK2s* proteins recruit *GmRVE8s* proteins to regulate *E1* gene transcription.

The qDTF-7 locus as a possible example of a heterogeneous locus in soybean

Locus or genetic heterogeneity is a situation in which alleles from different closely located genes at the same locus influence the same trait. For the qDTF-7 locus, we previously proposed a strong candidate gene, *GmTOE1* (Perfil'ev et al., 2024), which has been shown to play a role in the regulation of flowering in soybean (Wang T. et al., 2016). In this study, we identified a new candidate gene, *GmRVE8c*, located ~21 kb upstream of *GmTOE1*, which may also be involved in the regulation of flowering and maturity in soybean. Using the data available to us, we attempted to analyze how mutations in the *GmRVE8c* and *GmTOE1* genes correspond to each other. In the genetic background of the early-flowering/maturing *GmTOE1^C* allele, the late-maturing *GmRVE8c^{hap4}* haplotype arose. We did not find any *GmRVE8c^{hap4} GmTOE1^T* genotypes in either the SoyOmics database or our collection (Fig. S4). Based on SoyOmics phenotypic data, the effect of the *GmRVE8c^{hap4}* haplotype appears to partially restore the effect of the early-maturing *GmTOE1^C* allele on flowering and maturity time (Fig. S4a, S5a, b).

A similar pattern is observed in our collection; however, the *GmRVE8c^{hap4}* haplotype shows a much stronger effect on flowering and maturity time than either *GmTOE1^C* or *GmTOE1^T* (Fig. S4b, c, S6a, b). *GmTOE1* alleles have a minor effect on flowering and maturity under our conditions; for example, genotype *GmRVE8c^{hap1} GmTOE1^C* differs significantly from *GmRVE8c^{hap1} GmTOE1^C* only for BLUP_DTF in Oryol conditions (Fig. S4b, c, S6a, b). Interestingly, the *GmRVE8c^{hap3}* haplotype predominates in the background of the *GmTOE1^T* allele, and, for example, in the SoyOmics database and in our collection, we found only two accessions with the *GmRVE8c^{hap3} GmTOE1^C* genotype (Fig. S4). Therefore, it is difficult to determine whether the delayed flowering and maturity are caused by *GmRVE8c^{hap3}* or by *GmTOE1^T*, although SoyOmics data suggest that both genes influence these traits (Fig. S4a, S5). However, phenotypic analysis in our collection did not reveal any significant effect of *GmRVE8c^{hap3}* on flowering and maturity time (Fig. S4b, c, S6a, b).

It should be mentioned that the mutations detected in the *GmRVE8c* and *GmTOE1* genes require functional validation to confirm whether they actually affect the phenotype. If this proves true, the qDTF-7 locus could serve as an interesting example of how natural mutations shape phenotypic diversity in plants, particularly in soybean.

The influence of the *GmRVE8c* gene on other soybean traits

The early-maturing *GmTOE1^C* allele likely contributes to soybean adaptation to northern latitudes by reducing flowering time (Perfil'ev et al., 2024). In contrast, the *GmRVE8c^{hap4}* haplotype is unfavorable for northern latitudes due to its association with late flowering and maturity, whereas in our collection, all accessions from Western Siberia carry the early-maturing *GmRVE8c^{hap1}* haplotype (Fig. 2). As discussed above, *GmRVE8c^{hap4}* appears to restore the early-maturing phenotype of *GmTOE1^C*, raising the question of why the *GmRVE8c^{hap4}* mutation arose in the presence of the early-maturing *GmTOE1^C* allele. A possible explanation for this is the pleiotropic effect

of *GmRVE8c* on other soybean traits. Interestingly, several loci associated with soybean drought tolerance have been identified on chromosome 3 using bi-parental QTL mapping (Table S6). The genes *GmRVE8c* and *GmTOE1* are also positioned within these loci. However, it should be noted that these loci also contain the known drought tolerance regulator *GmSALT3* (Shi et al., 2018; Guan et al., 2021).

Overexpression of *GmRVE8a* in *Arabidopsis* enhances drought and salt tolerance, and *GmRVE8a* can interact with the GmNAC019 protein (Bao et al., 2024), which is involved in the ABA-mediated drought response (Hoang et al., 2019). *GmLHYs* genes are also involved in the ABA-mediated drought response in soybean, and knockout of all four *GmLHYs* genes enhances drought tolerance (Wang K. et al., 2021). Recently, it has been shown that the soybean circadian clock component *GmPRR3b* directly suppresses the expression of *GmABF3* (*ABSCISIC ACID-RESPONSIVE ELEMENT-BINDING FACTOR 3*), the overexpression of which enhances drought tolerance in soybean (Li C. et al., 2024). We hypothesize that loss of *GmRVE8c* function may also enhance drought tolerance in soybean. Interestingly, in *Arabidopsis*, the *TOE1* and *TOE2* genes have been shown to play key roles in drought escape, and loss of *TOE1/2* function accelerates flowering but reduces drought tolerance (Chen et al., 2025). For the early-maturing *GmTOE1^C* allele, we assume at least a partial loss of function of the encoded protein (Perfil'ev et al., 2024). It is possible that the *GmRVE8c^{hap4}* haplotype arose to compensate for drought sensitivity in plants carrying the *GmTOE1^C* allele. In our view, this is an intriguing hypothesis that warrants further exploration.

According to Table S6, the *GmRVE8c* gene colocalizes with loci associated with plant height and weight, oil and isoflavone content, seed thickness, and resistance to soybean cyst nematode. Theoretically, *GmRVE8c* may also influence some of these traits, such as plant height, and weight, potentially through regulation of the *PRR5-PIF4* module, as has been proposed for *GmMYB133* (Shan et al., 2021). *GmMYB133* has also been shown to be a positive regulator of isoflavone biosynthesis in soybean (Bian et al., 2018). In *Arabidopsis*, *rve 4 6 8* mutants exhibit altered metabolism of carbohydrates, fatty acids, and lipids, as well as reduced proteasome activity, which leads to an increase in cell size (Scandola et al., 2022). Various circadian clock genes have been studied and shown to contribute to plant defense against pathogens (Lu H. et al., 2017). In particular, the role of *GmCCA1-1* in soybean response to infection by the nematode *Heterodera glycines* has recently been demonstrated (Niraula et al., 2022). All of this makes *GmRVE8c* an interesting gene for further study, not only in the context of soybean flowering and maturity but also for other agriculturally important traits.

Conclusion

In this study, we identified a new candidate gene, *GmRVE8c*, and two of its haplotypes, *GmRVE8c^{hap3}* and *GmRVE8c^{hap4}*, which are associated with late flowering and maturity in soybean. In addition, to distinguish these haplotypes from each other, we developed DNA markers that can be used for marker-assisted selection in soybean. The *GmRVE8c* gene is located

in close proximity to another soybean flowering regulator, *GmTOE1*, which also has mutations associated with flowering and maturity time. *GmTOE1* and *GmRVE8c* are likely to form a genetically heterogeneous locus, i. e. a locus in which alleles of different nearby genes influence the same trait. Also, the influence of the *GmRVE8c^{hap3}* and *GmRVE8c^{hap4}* haplotypes on the flowering and maturity time shows partial dependence on the allelic state of the *E1* gene. Altogether, these findings make *qDTF-7* a highly complex and intriguing locus, and we plan to further validate the mutations in *GmRVE8c* and *GmTOE1* using hybrid soybean populations.

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Conflict of interest. The authors declare no conflict of interest.

Received August 14, 2025. Revised November 25, 2025. Accepted December 23, 2025.