Cotyledons facilitate the adaptation of early-maturing soybean varieties to high-latitude long-day environments

Xin Xu\textsuperscript{1,2} | Lixin Zhang\textsuperscript{1} | Xiaoning Cao\textsuperscript{1} | Lifeng Liu\textsuperscript{1} | Bingjun Jiang\textsuperscript{1} | Chunlei Zhang\textsuperscript{1} | Hongchang Jia\textsuperscript{1} | Xiangguang Lyu\textsuperscript{1} | Yumei Su\textsuperscript{1} | Yupeng Cai\textsuperscript{1} | Luping Liu\textsuperscript{1} | Shengrui Zhang\textsuperscript{1} | Fulu Chen\textsuperscript{1} | Cunxiang Wu\textsuperscript{1} | Bin Liu\textsuperscript{1} | Wensheng Hou\textsuperscript{1} | Shi Sun\textsuperscript{1} | Jinsheng Lai\textsuperscript{2} | Tianfu Han\textsuperscript{1}

\textsuperscript{1}Ministry of Agriculture and Rural Affairs Key Laboratory of Soybean Biology (Beijing), Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China
\textsuperscript{2}State Key Laboratory of Agrobiotechnology, Department of Plant Genetics and Breeding, China Agricultural University, Beijing, China

Correspondence
Tianfu Han, Ministry of Agriculture and Rural Affairs Key Laboratory of Soybean Biology (Beijing), Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing 100081, China.
Email: hantianfu@caas.cn

Funding information
the CAAS Agricultural Science and Technology Innovation Project; the China Agriculture Research System, Grant/Award Number: CARS-04; the National Key R&D Program of China, Grant/Award Number: 2017YFD0101400

Abstract
Soybean (\textit{Glycine max}), a typical short-day plant (SDP) domesticated in temperate regions, has expanded to high latitudes where daylengths are long from soybean emergence to bloom, but rapidly decrease from seed filling to maturity. Cotyledons are well known as the major storage organs in seeds, but it is unclear whether developing cotyledons store flowering substances at filling stage in SD for upcoming seedlings, or instead respond to photoperiod for floral induction after emergence of matured seeds in long-day (LD). Here, we report that cotyledons accelerate flowering of early-maturing varieties not resulting from stored floral stimuli but by perceiving photoperiod after emergence. We found that light signal is indispensable to activate cotyledons for floral induction, and flowering promoting gene \textit{GmFT2a} is required for cotyledon-dependent floral induction via upregulation of floral identity gene \textit{GmAP1}. Interestingly, cotyledons are competent to support the entire life cycle of a cotyledon-only plant to produce seeds, underlying a new photoperiod study system in soybean and other dicots. Taken together, these results demonstrate a substantial role for cotyledons in flowering process, whereby we propose a ‘cotyledon-based self-reliance’ model highlighting floral induction from emergence as a key ecological adaptation for rapid flowering of SDPs grown in LD environments at high latitudes.

KEYWORDS
\textit{GmFT2a}, high-latitude adaptation

1 | INTRODUCTION

Soybean [\textit{Glycine max} (L.) Merr.] is a typical short-day plant (SDP) domesticated in the temperate regions (Hymowitz & Newell, 1981; Hyten et al., 2006). However, the high-latitude regions with long-day (LD) environments in the northern hemisphere, such as the regions of northeast China, the Korean peninsula, north Japan, the Far East of Russia, the Northern Midwest of the United States and the Canadian prairie, are among the major areas of soybean production (Sinegovskii, Yuan, Sinegovskaya, & Han, 2018; Wilcox, 2004). Indeed, the production of soybean at high-latitude regions relies on the use of early-maturing and photoperiod-insensitive varieties which belong to the
early ‘maturity groups’ (MGs) resulting from natural evolution, artificial domestication and variety improvement (Hartwig, 1970; Jia et al., 2014).

Numerous studies have focused on the genetic and molecular basis of high-latitude adaptation of soybean. To date, the E1 (Xia et al., 2012), E2 (GmGlIA) (Watanabe et al., 2011), E3 (GmPHYA3) (Watanabe et al., 2009) and E4 (GmPHYA2) (Liu et al., 2008) have been shown as the key regulatory loci which influence the latitude adaptation and are thought to explain about 60% of the molecular basis of soybean maturity diversity (Liu, Song, et al., 2020; Tsubokura et al., 2014). Additionally, PSEUDO-RESPONSE REGULATOR (PRR) homologs of Tof11 and Tof12 (GmPRR3b or GmPRR37) (Li et al., 2020; Lu et al., 2020; Wang et al., 2020) and flowering promoting FLOWERING LOCUS T (FT) homologs of GmFT2a/2b/5a (Chen, Cai, et al., 2020; Kong et al., 2010, 2014; Nan et al., 2014; Sun et al., 2011; Zhao et al., 2016) also control soybean flowering and maturity. Importantly, the early-maturing varieties are usually characterized with the recessive genotypes of e1, e2, e3, e4 and prr because the expression of downstream GmFT2a/5a are elevated when the E and PRR genes are mutated (Jiang et al., 2014; Li et al., 2020; Liu, Song, et al., 2020; Lu et al., 2020; Tsubokura et al., 2014; Wang et al., 2020).

Leaf is the major organ that perceives light signals and produces flowering stimuli (Knott, 1934; Thomas & Vince-Prue, 1997). The first fully expanded leaf has initiated the photoperiodic response until plant senescence, although the sensitivity is distinct in leaves with different ages and node positions (Han et al., 2006; Thomas & Vince-Prue, 1997; Wu et al., 2006). Molecular genetics studies have revealed that FT is a long-distance signal that is transmitted from leaves to the shoot apex meristem via the phloem and interacts with FLOWERING LOCUS D (FD) to activate APETALA1 (AP1) expression and induce flowering (Abe et al., 2005; Corbesier et al., 2007; Liljegren, Gustafson-Brown, Pinyopich, Ditta, & Yanofsky, 1999; Mandel, Gustafson-Brown, Savidge, & Yanofsky, 1992; Wigge et al., 2005). This FT/FD-AP1 module is also conserved in soybean (Chen, Nan, et al., 2020; Chi, Huang, Liu, Yang, & Yu, 2011; Nan et al., 2014; Takeshima et al., 2019).

Cotyledon is an ‘embryo leaf’ that perceives daylength to influence plant development (Hanley & May, 2006; Ogawa & King, 1990; Paton, 1971; Yoo, Hong, Jung, & Ahn, 2013; Zhang, Zhao, & Lamb, 2011). However, the role of cotyledon in regional adaptation remains largely unknown. In our previous grafting experiments (Cao, 2013), the grafted donor cotyledons (attaching with root and hypocotyl) of early-maturing varieties from high-latitude regions could cause a flowering response in receptor shoots of late-maturing varieties, while varieties from low- and middle-latitude regions as donors did not showed the same function. Here, we try to determine the role of cotyledons in daylength adaptation of soybean particularly for the early-maturing varieties to fit the LD environment in the higher latitudes.

The daylength changes greatly during the life cycle of soybean plants at higher latitudes, where it is getting long from emergence to bloom (mid-May to mid-July), but becoming short rapidly during seed filling and maturing stages (early-August to late-September) (Figure S1). Since the soybean cotyledon acts as storage organ in the developing seeds at filling stage, and the flowering stimuli are relatively stable and transmissible, we propose that cotyledon may facilitate the adaptation of soybean to high-latitude regions in accordance with one or both of the following two hypotheses: (a) maternal plants produce flowering stimuli under SD and store the substances in cotyledons (seeds) for flowering of next generation in the subsequent year or (b) the cotyledons produce flowering stimuli after emergence and accelerate flowering at the seedling stage under LD condition.

In this study, we investigated the function of cotyledons and the molecular mechanism of cotyledon-based floral induction to understand the role of cotyledons in the adaptation of early-maturing soybean varieties to high-latitude regions. We proposed a cotyledon-based self-reliance model summarizing existing molecular features of LD adaptation in soybean and incorporating the observed flowering induction effect of cotyledons. Furthermore, we established a cotyledon-based research system for the study of photoperiodism in soybean and other dicots.

2 | MATERIALS AND METHODS

2.1 | Grafting methods

For studying the photoperiod responses of soybean cotyledons, we constructed three types of grafted plants (RC-grafting, R-grafting and C-grafting) at the cotyledon stage and named them in accordance to the major organs used as stock (Figure 1a,b). In RC-grafted plants, the stock genotypes provided the root (R), the hypocotyl and the cotyledons (C), while the scion genotypes provided the apex (Figure 1b,c; Cao, Sun, Wu, Han, & Yang, 2013). In R-grafted plants, the stock genotypes provided the root (R) and the hypocotyl, while the scion genotypes provided the cotyledons and the apex (Figure 1b,c). In RC- and R-grafted plants, the seedlings used as stock were grown to 4 days after emergence (DAE) and the seedlings used as scions were grown to 7 DAE. In C-grafted plants, the stock 1, which provided the root and the hypocotyl, was grafted with scion 1, which provided the cotyledons (C) attaching with a ~1-cm-long hypocotyl; we used a scion 1/stock 1 combination as the stock to graft with the scion 2, which provided the apex (Figure 1b,c). In C-grafted plants, the seedlings used as stock 1 and the scion 1 were grown to 4 DAE, while the seedlings used as scion 2 were grown to 7 DAE. The stocks and scions were grafted by a splicing method and fixed with parafilm (Figure 1d).

2.2 | Plant materials and growth conditions

To clarify the soybean ecotypes (maturity group, MG) which showed flowering induction effect from cotyledons, we selected 21 early-maturing (MG 00 to MG I) soybean varieties (Table S1) from Northeast China (NE), and 25 medium-maturing (MG II to MG IV) varieties (Table S1) from Huang-Huai-Hai River Valleys (HHH) and a late-
maturing variety Zigongdongdou (ZGDD, MG VIII; Table S2) from south China. The early- and medium-maturing varieties were used as stocks, and ZGDD was used as the common scion, respectively. We prepared RC-grafted plants on May 15, 18 and 21 in 2011, respectively, and placed the grafted plants under natural daylength (ND) in Beijing (N39°57′, E116°19′), China. The dates of emergence (VE) and flowering time (R1) were recorded as described by Fehr, Caviness, Burmood, and Pennington (1971).

For making clear the function of cotyledons, hypocotyl and root in floral induction, we used the early-maturing variety Heihe27 (HH27, MG 0; Table S2) and ZGDD as the either stocks or scions to establish RC, C and R-grafted plants and placed them in growth chambers at 26°C under LD (14 hr:10 hr, light:dark) condition. To test the impact of scion on the flowering of grafted plants, the RC-grafted plants in which late-maturing varieties of Nandou17 (MG VII), Jupiter (MG IX) and I.C.-192 (MG X) (Table S2) were used as the scions and HH27 as the common stock were constructed and the plants were grown in chambers at 26°C under continuous dark condition and then transferred to dark, SD (10 hr:14 hr, light:dark) and LD (14 hr:10 hr, light:dark) treatments.

For GmFT2a, SOC1-like and GmMFT expression analysis in the cotyledons, HH27 and ZGDD plants were grown in chambers at 26°C under LD (14 hr:10 hr, light:dark) condition. To analyse the expression of GmFT2a and E1 in cotyledons under different photoperiods, HH27 and ZGDD plants were grown in chambers at 26°C under continuous dark condition and then transferred to dark, SD (10 hr:14 hr, light:dark) and LD (14 hr:10 hr, light:dark) conditions, respectively.

To elucidate the molecular mechanism of cotyledons’ role in floral induction, we cloned the GmFT2a CDS into pTF101.1-GFP vector (Paz, Martinez, Kalvig, Fonger, & Wang, 2006) and generated transgenic soybean lines overexpressing 35S:GmFT2a in the background of medium-maturing variety Jack (MG III; Table S2). The primers used for vector construction and the identification of transgenic lines are listed in Table S3. RC-grafted plants made with transgenic lines of GmFT2a OE (Jack) (in Jack background), GmFT2a OE (ZGDD) (in ZGDD background; Sun et al., 2011) and E1 RNAi (in ZGDD background; Liu, Gao, et al., 2020) plants, and CRISPR/Cas9-derived gmft2a (in Jack background; Cai et al., 2018) and gmft5a (in Jack background; Cai et al., 2020) mutant plants as stocks and Jack as the common scion were grown in chambers at 26°C under LD (14 hr:10 hr, light:dark) condition.
To evaluate the roles of cotyledons in the development of soybean plants in high-latitude region, we grew HH27 and another early-maturing variety of Jinyuan55 (MG 0; Table S2) in Heihe (NS0‘22’, E127‘53’), China, in the spring of 2020. The seeds were sown in May 15, and 0, 1 or 2 cotyledons were dark-treated/removed, respectively after emergence. In dark treatment, the cotyledons were wrapped with tin-foil to completely to exclude light penetration until cotyle- don’s abscission.

For observing the life cycle of cotyledon-only soybean plant, we used HH27 and ZGDD plants as materials and removed all the organs above the cotyledonary node at VC (unifoliolate leaf edges are not touching) (Fehr et al., 1971). Subsequently, the newly produced leaves or stems at the cotyledonary node were removed soon when they began to emerge. The cotyledon-only plants were grown in chambers at 26°C under SD (10 hr:14 hr, light:dark) or LD (14 hr:10 hr, light: dark) condition and the flowering time (R1) and maturity (R7) were recorded (Fehr et al., 1971).

2.3 E1/E2/E3/E4 genotype identification

The genomic DNA of HH27, I.C.-192, Jack, Jinyuan55, Jupiter, Nandou17 and ZGDD plants were extracted using the CTAB method (Solarbio, Beijing, China) from fresh leaves. These varieties were gen- otyped with the Amplified fragment length polymorphism (Tsukakura et al., 2014) and Kompetitive allele-specific PCR (Liu, Song, et al., 2020) markers, as previously described.

2.4 Transcriptome analysis and gene function annotation

Soybean cv HH27 and ZGDD plants were grown in a chamber at 26°C under LD (14 hr:10 hr, light:dark) condition. Their cotyledons were sampled at 4 hr after dawn 4 DAE. Each sample consisted of material collected from three individual plants. Two biological replicates were analysed. mRNA extracts from these samples were sequenced with the Hiseq 4000 platform (Illumina, San Diego, CA) fol- lowing the manufacturer’s protocols. The analysis of the data was done as described in our previous study (Liu et al., 2018).

2.5 RNA extraction and gene expression analysis

mRNA was extracted using a TransZol Up kit (TransGen Biotech, Bei- jing, China) and cDNA was synthesized with Superscript II reverse transcriptase (TransGen Biotech). We performed qPCR using an ABI7500 Thermocycler (Applied Biosystems, Foster City, CA) with the KAPA SYBR DNA Polymerase (KAPA Biosystems, Cape Town, South Africa). We measured three biological replicates per sample. The qPCR data were analysed by the 2−ΔΔCT method with GmActin as the internal reference gene. The primer sequences used in the qPCR experiment are listed in Table S3.

2.6 Phenotyping and statistical analysis

We recorded the number of days from VE to V1 (unifoliolate leaves fully expanded), R1 (i.e., beginning bloom: one open flower at any node on the main stem) or R7 (one pod on the main stem has reached mature pod colour) (Fehr et al., 1971), and the types of terminal racemes (STR, short terminal raceme; RTR, reversed terminal raceme; VT, vegetative terminal) on the shoot apex of ZGDD or I.C.-192 plants (Figure S2) (Han, Gai, Wang, & Zhou, 1998; Washburn & Thomas, 2000; Wu et al., 2006). STR is a short inflorescence that fea- tured with opened or unopened flowers, while RTR is an under- developed inflorescence which featured with reversed flowers (RF), or RF accompanied with a few opened flowers (Figure S2). In our system, STR and RTR both indicated reproductive features. The data were analysed using Excel and R-packages and are presented as the mean ± SD. Student’s t tests were used to assess the significance of the observed differences.

3 RESULTS

3.1 Cotyledons of early-maturing varieties as donor promote the flowering of late-maturing varieties as receptor in grafted plants

In an attempt to examine the varietal variations of cotyledons’ roles in soybean development, we conducted the RC-grafting experiments (Figure 1) with 21 early-maturing varieties from Northeast China (NE) and 25 medium-maturing varieties from the Huang-Huai-Hai River Valleys (HHH) as stocks, and a late-maturing variety Zigongdongdou (ZGDD) as the common scion. Interestingly, we observed that the grafted plants with early-maturing varieties as stocks all underwent reproductive growth (including RTR or STR) (Figure S2) under ND (Beijing) condition, while the majority (88.1%) of grafted plants with medium-maturing varieties as stocks and grafted ZGDD/ZGDD (scion/stock) plants failed to reach the reproductive stage (Table S1), suggesting that the stocks of early-maturing varieties, not medium-maturing varieties, have ability to induce the flowering of scion in grafted plants. Furthermore, we used the early-maturing vari- ety Heihe27 (HH27) and ZGDD as either stocks or scions to construct the RC-grafting plants. The results showed that 44.8% of grafted ZGDD/HH27 plants exhibited reproductive growth under LD condi- tion, whereas the grafted ZGDD/ZGDD plants displayed continuous vegetative growth throughout the experiment (Table 1). No significant differences of flowering time were observed between the grafted plants in which HH27 and ZGDD were used as stocks, respectively, and HH27 as the common scion (27.4 ± 1.9 and 28.4 ± 2.3 days after grafting (DAG), respectively) (Table 1).

Besides cotyledons, the stock also contains root and hypocotyl in RC-grafting. To determine the independent roles of cotyledons, we subsequently conducted two additional grafting. R-grafting (the stock provides the root and the hypocotyl) and C-grafting (the scion 1 pro- vides the cotyledons) to shed light on their respective function. We
found that 41.9% of C-grafted ZGDD/(HH27/ZGDD) [scion 2/(scion 1/stock 1)] plants exhibited reproductive growth under LD condition, whereas C-grafted ZGDD/(ZGDD/ZGDD), R-grafted ZGDD/HH27 (scion/stock) and ZGDD/ZGDD plants all displayed vegetative growth (Table 1). This indicates that only the cotyledons have flowering induction effects, while the root and the hypocotyl do not.

In order to uncover the specific flowering influence of scion in the above RC-grafted plants, we selected three additional late-maturing varieties as scions, specifically Nandou17, Jupiter and I.C.-192, with the early-maturing variety of HH27 as the common stock. The accelerated flowering was also observed in all three grafted plants including Nandou17/HH27 (55.6 ± 15.1 DAG), Jupiter/HH27 (43.1 ± 4.9 DAG) and I.C.-192/HH27 (55.5 ± 2.6 DAG) compared with the late-flowering in the grafted plants of Nandou17/Nandou17 (86.9 ± 3.6 DAG), Jupiter/Jupiter (102.8 ± 7.4 DAG) and I.C.-192/I.C.-192 (not to flower), under LD condition (Table S4).

### 3.3 | Differential expression of flowering-time genes in the cotyledons of early-maturing and late-maturing soybean varieties

To identify the relevant molecular mechanisms behind cotyledon-induced flowering, we examined differentially expressed genes (DEGs) by performing transcriptome sequencing (RNA-seq) of HH27 and ZGDD cotyledons grown under LD condition. We found a total of 4890 DEGs between the cotyledons of HH27 and ZGDD plants. Data analyses revealed that 21 DEGs show homology with known flowering-time associated genes (Figure 2d). Furthermore, the majority of the uncovered DEGs are associated with the photoperiodic pathway. The first set of genes including GmLHY1b/2a/2b, GmPHYA3 (E3), Tof12 (GmPRR3b or GmPRR37), GmGLa (E2), GmGlb, GmCOL9, Glyma10g042800 (PIF1-like) and Glyma06g095700 (SVP-like1) were downregulated in HH27 cotyledons. In contrast, the another set of genes like GmCRY1b, Glyma16g027200 (SPA1), GmMFT, GmFT2a, Glyma03g019300 (SOC1-like), Glyma14g019400 (AGL5), GmmGR156b, Glyma02g282100 (PIF4-like), Glyma01g023500 (SVP-like2), Glyma08g137500 (TPPL) and Glyma06g184200 (TPS1-like) were upregulated in HH27 cotyledons (Figure 2d). We confirmed the top three upregulated DEGs in HH27 cotyledons by qPCR analysis, although the estimated fold changes in gene expression differed from that calculated by RNA-seq (Figure 2e). Collectively, these findings indicate that the cotyledons promote flowering mainly through the photoperiodic flowering pathway.

### 3.4 | Cotyledons respond to photoperiod by perceiving light signals immediately after emergence

To address the question of whether cotyledons store flowering stimuli at filling stage or produce flowering substances at seedling stage, we obtained seeds from HH27 plants grown in SD and LD. There were no significant differences in flowering time between the progenies of SD- and LD-treated plants (26.7 ± 1.9 and 26.3 ± 1.8 DAE, respectively) under LD condition (Figure 2a). Importantly, the RC-grafted ZGDD/HH27 plants maintained vegetative growth all the time when the cotyledons were dark-treated, while exhibited reproductive growth when treated with SD and LD photoperiods (Figure 2b). These results suggested that the cotyledons produce floral stimuli only after perceiving light signals at seedling stage. In addition, we found 17.1% of reproductive plants with RTR in grafted ZGDD/ZGDD plants, when the cotyledons were exposed to SD (Figure 2c), indicating that the cotyledons of the late-maturing variety ZGDD impact flowering under the inductive SD photoperiod.
FLOWERING TIME LOCUS T (FT) has been previously confirmed as the major component of ‘florigen’, the floral signal molecule that promotes early flowering in Arabidopsis (Corbesier et al., 2007). In soybean, FT homologs functioned diversely in flowering pathway and GmFT2a/GmFT5a promoted flowering and maturity (Cai et al., 2018, 2020; Kong et al., 2010; Nan et al., 2014; Sun et al., 2011). In the current study, we found a significant upregulation of GmFT2a in HH27 cotyledons (Figure 2d,e). To gain further insights into the photoperiodic response...
of the cotyledons, we analysed the expression profiles of GmFT2a in cotyledons of HH27 and ZGDD plants under various photoperiodic conditions, including dark. We found that the expression of GmFT2a was dramatically increased in the cotyledons of HH27 plants compared with ZGDD under SD and LD conditions (Figure 3a), and peaked at 30 hr after exposure to LD following dark condition (Figure 3c). However, GmFT2a was hardly detectable under dark condition (Figure 3a) which was consistent with the observation that cotyledons have no flowering effect in dark, indicating that light signal is necessary to activate the expression of GmFT2a. In addition, we also detected the expression pattern of E1, a key flowering repressor that is induced by LD and suppresses the expression of GmFT2a (Xia et al., 2012), and found that E1 showed a higher expression in LD compared with SD which was negatively correlated with GmFT2a (Figure 3a,b). However, E1 was slightly upregulated in the first day of LD treatment and then exhibited a stable expression in HH27 plants compared with an increasing expression pattern in ZGDD plants (Figure 3b).

3.4 | Cotyledons induce flowering by expressing the floral promoting gene GmFT2a

To address whether GmFT2a acts as a flowering stimulus in the cotyledons, we conducted RC-grafting experiments in which the soybean variety Jack, overexpression transgenic lines of GmFT2a OE (Jack) and GmFT2a OE (ZGDD), and CRISPR/Cas9-derived gmft2a mutants in which an adenine nucleotide was inserted at position 153 in the first exon of GmFT2a in Jack background (Cai et al., 2018) served as stocks, and ZGDD as the common scion. The reproductive features were assessed for each grafted plant under LD condition. We found that 61.9% of the grafted ZGDD/GmFT2a OE (Jack) plants underwent reproductive growth, which represents a higher proportion than observed in the grafted ZGDD/Jack plants (19.5%) (Figure 4a,b). Similar early flowering was observed in RC-grafted ZGDD/GmFT2a OE (ZGDD) plants, corresponding to a total of 37.5% reproductive plants, compared with the grafted ZGDD/ZGDD plants, which failed to

---

**Figure 3**: The expression patterns of GmFT2a and E1 in soybean cotyledons. (a,b) The expression profiles of GmFT2a (a) and E1 (b) in soybean cotyledons. The Heihe27 (HH27) and Zigongdongdou (ZGDD) seeds were sown under continuous dark condition and then the seedlings (2 day-old after emergence) were transferred to dark, SD (10 hr:14 hr, light: dark) and LD (14 hr:10 hr, light:dark) conditions, respectively. Samples were collected 4 hr after dawn. (c) Expression dynamic of GmFT2a in soybean cotyledons in the first two days in LD. The samples were collected every 2 hr starting at dawn (ZT 0). All data are represented as mean ± SD of three biological replicates [Colour figure can be viewed at wileyonlinelibrary.com]
flower (Figure S3). Moreover, all the grafted ZGDD/gmft2a plants maintained vegetative growth during the 90-day experiment (Figure 4a,b). Taken together, these results suggest that GmFT2a may play a central role in the floral induction of cotyledons.

We measured the expression levels of GmFT2a in the cotyledons of stocks and the downstream floral identity gene GmAP1a in the apex of scions, under LD condition. Corresponding to flowering phenotypes, transgenic plants overexpressing GmFT2a in stocks showed higher expression of GmAP1a in scions compared with the WT (Jack) as stocks (Figure 4c,d). However, despite the loss-of-function of GmFT2a in the stock cotyledons of grafted ZGDD/gmft2a plants, we observed a slight reduction of GmAP1a in the scion apex (Figure 4d). This suggests that GmAP1a is not the only downstream target gene of GmFT2a and may not necessarily result in floral induction. In addition, we detected a very high expression levels of GmFT2a in the stock cotyledons of grafted ZGDD/GmFT2a OE (Jack) plants compared with grafted ZGDD/Jack and ZGDD/gmft2a plants (Figure 4c), while no difference of GmFT2a expression in the scion apex was observed (Figure 4e), suggesting that GmFT2a mRNA may not act as a mobile signal in the grafted plants.

Most soybean varieties grown in higher latitudes have loss-of-function alleles of E1 (Jiang et al., 2014; Liu, Song, et al., 2020; Tsubokura et al., 2014). To determine whether E1 mediates GmFT2a expression in cotyledons as it does in the true leaves, we conducted RC-grafting experiments in which E1 RNAi lines (ZGDD background) were used as stocks and ZGDD as the scion. The results showed that 36.7% of the grafted ZGDD/E1 RNAi plants carried on reproductive growth under LD condition, while the grafted ZGDD/ZGDD plants displayed vegetative growth (Figure 4f). However, the expression levels of GmFT2a and GmFT5a were all significantly increased in the cotyledons of E1 RNAI lines with low levels of E1 transcripts compared with ZGDD plants (Figure 4g). Furthermore, we used CRISPR/Cas9-derived gmft5a mutants in which two thymine nucleotides (TT) were inserted at position 66 in the first exon of GmFT5a in Jack background (Cai et al., 2020) as stocks and ZGDD as the scion. The results showed that 19.8% of the grafted ZGDD/gmft5a plants underwent reproductive growth (Figure 4f), which suggests that GmFT5a may have a minor contribution to the cotyledon-induced flowering.

3.5 | Cotyledons accelerate the flowering of early-maturing soybean varieties in the high-latitude regions

To confirm the contribution of cotyledons to flowering of early-maturing soybean varieties in their original high-latitude region, we dark-treated and removed 0, 1 or 2 cotyledons of early-maturing HH27 and Jinyuan55 plants and investigated the flowering time under ND (Heihe, Heilongjiang province) condition. The HH27 and Jinyuan55 plants with 2 dark-treated cotyledons all exhibited significantly delayed flowering than plants with 0 dark-treated cotyledon (intact plant) (Figure 5a,c). However, plants with 1 dark-treated cotyledon displayed no difference in flowering compared with intact plants (Figure 5a,c), suggesting that one cotyledon is enough to produce floral stimuli to induce flowering of the early-maturing varieties. Moreover, the delayed flowering was also detected in HH27 and Jinyuan55 plants with 2 cotyledon removal and HH27 plants with 1 cotyledon removal (Figure 5b,d), it is a case that the absence of nutrients and photoperiodic signals both contribute to this result.

3.6 | Cotyledons have ability to complete the life cycle of cotyledon-only plants

To investigate whether the cotyledons are sufficient to support a plant through an entire life cycle, we generated ‘cotyledon-only’ HH27 and ZGDD plants and monitored their flowering time and maturity under SD and LD conditions. When the cotyledons were the only above-ground vegetative organs, the HH27 and ZGDD cotyledon-only plants produced normal flowers and bloomed only 3.9 and 4.0 days later than intact HH27 and ZGDD plants under SD condition (Figure 6a,b) and matured later by 28.8 and 35.6 days, respectively (Figure 6c,d). Under LD condition, the cotyledon-only and intact HH27 plants flowered at 28.4 ± 2.9 and 26.7 ± 1.5 DAE and matured at 93.1 ± 9.3 and 76.2 ± 2.1 DAE, respectively (Figure S4). In contrast, ZGDD cotyledon-only plants under LD kept vegetative growth throughout the entire 120-day experiment (Figure S4), suggesting that the cotyledon-only plants responded to photoperiod as intact plants. The seeds of HH27 and ZGDD cotyledon-only plants were smaller than intact plants, while their germination rate showed no difference (Figures 6e,f and S4), indicating that cotyledon-only plants are able to carry a successful reproduction. Moreover, the longevity of cotyledon in the cotyledon-plants of HH27 and ZGDD was 106.8 ± 5.7 and 107.1 ± 7.6 DAE in SD and 96.9 ± 5.1 and 140.4 ± 6.3 DAE in LD, respectively (Figures 6g and S4). The results of these SD and LD experiments indicate that cotyledons are enough to produce the signals and nutrients for soybean flowering and maturation. The prolonged cotyledon longevity and small size of cotyledon-only-plant make it possible to establish a novel research system for studying photoperiodic responses in plants.

**FIGURE 4** Expression of GmFT2a in stock cotyledon influences the flowering time of scion in grafted plants. (a) The phenotypes of RC-grafted plants under LD (14 hr:10 hr, light:dark) condition. Close-up views of RTR (reversed terminal raceme), STR (short terminal raceme) and VT (vegetative terminal) framed by the boxes (upper panel). Scale bar, 5 cm. The photos were taken at blooming stage. ZGDD, Zigongdongou. (b,f) The reproductive growth of RC-grafted plants in LD. Data are represented as mean ± SD of two replicates. Fifteen grafted plants were analysed for each replicate. (c–e) The expression patterns of GmFT2a in the cotyledons of stocks (c) and GmAP1a (d) and GmFT2a (e) in the apex of scions of RC-grafted plants. The samples were collected at 4 hr after dawn 11 days post grafting in LD. Data are represented as mean ± SD of five biological replicates. (g) The expression pattern of E1, GmFT2a and GmFT5a in the cotyledons of E1 RNAi (ZGDD) lines. The samples were collected 4 hr after dawn 4 days after emergence in LD. Data are represented as mean ± SD of three biological replicates. Statistical significance was determined by applying a Student’s t test (*p < .05; **p < .01) [Colour figure can be viewed at wileyonlinelibrary.com]
DISCUSSION

4.1 The effects of cotyledons on floral induction in soybean plants

The cotyledon is a storage organ that provides nutrients for seed germination and an ‘embryo leaf’ that can photosynthesize carbohydrates that are essential for seedling development (Cao, Sun, Zhang, et al., 2013; Saito, Chang, Walling, & Thomson, 1990; Zhang et al., 2011). In this study, we demonstrated that the cotyledons perceive and respond to light signals immediately after emergence, which enables soybean seedlings to produce flowering stimulus at a very early stage and mediate reproductive development. These findings support our previously proposed hypothesis that photoperiodic response exists from emergence to maturity in soybean (Han et al., 1998, 2006; Wu et al., 2006). Moreover, the light signal is essential to induce GmFT2a expression and activate cotyledons for floral induction. Furthermore, we found that the cotyledons accelerated the flowering of early-maturing varieties in their original high-latitude region, confirming the cotyledons’ important contribution for the soybean adaptation in the LD environment. This is not consistent with the observations in Arabidopsis (Yoo et al., 2013), suggesting that the cotyledons may experience functional diversity in different species. Our work identified the major ecological/evolutionary implications for cotyledons and indicated that the floral induction from emergence may act as a key ecological adaptation for rapid flowering of SDP grown in LD condition.

4.2 A putative model for the role of cotyledons in the adaptation of soybean to high-latitude regions

Soybean has been domesticated from wild soybean in temperate regions of China (Hymowitz & Newell, 1981; Hyten et al., 2006). As human farming moved to higher latitudes, soybean cultivation expanded northwards and flourished. In these regions, the growing season is shorter and features a LD environment before flowering. Therefore, early flowering and maturity is crucial for soybean plants to complete their life cycle. In this study, we provide evidence that the floral induction of cotyledons during the seedling stage may play an important role in controlling flowering of soybean. Here, we propose a ‘cotyledon-based self-reliance’ model for soybean adaptation to high-latitude regions (Figure 7). In this model, mutations of E1/E2/E3/E4 release the inhibition on downstream floral promoting genes, endowing soybean plants with a floral competent state in the absence of a juvenile stage. Once the germinated seed emerges, photoreceptors receive light signals to upregulate the expression of GmFT2a in a photoperiod-dependent pathway, which then activates GmAP1 expression in the stem apex that induces flowering. Cotyledons, as the first emerging ‘leaf’ organs, perceive light and regulate the expression of GmFT2a as flowering stimulus, which consequently

![FIGURE 5](https://www.wileyonlinelibrary.com)
The flowering time and maturity of cotyledon-only plants under SD condition. (a,c) The flowering (a) and maturity (c) phenotypes of cotyledon-only plants that were grown under short-day (SD, 10 hr:14 hr, light:dark) condition. HH27, Heihe27. ZGDD, Zigongdongdou. Scale bar: 2 cm. The photos were taken at the blooming (a) and maturing (c) stages. (b,d) The flowering time (b) and maturity (d) of cotyledon-only plants in SD. (e) The seed weight of cotyledon-only plants in SD. All data are represented as mean ± SD (n = 15). (f) The germination rate of seed produced by cotyledon-only plants in SD. Data are represented as mean ± SD of two replicates. Fifteen individual plants were analysed for each replicate. (g) Longevity of cotyledons in cotyledon-only plants in SD. Data are represented as mean ± SD (n = 15). Statistical significance was determined by applying a Student's t test (**p < .01) [Colour figure can be viewed at wileyonlinelibrary.com]
4.3 | A cotyledon-based research system for studying the mechanism of photoperiodic response in plants

Soybean has a pair of large cotyledons, which are rich in stored high-energy nutrition (protein and oil) and can also produce photosynthates after emergence (Cao, Sun, Zhang, et al., 2013). The results of this study showed that soybean cotyledons possess a complete photoperiodic flowering pathway, and that cotyledon-only plants are able to complete the whole life cycle, ensuring that it can become a model system for studying photoperiodic response due to the following special characteristics: (a) the cotyledon-only plant is very small sized that can be grown in a limited space, which allows for conducting a large number of experiments and precisely controlling light and temperature factors, for example. (b) The cotyledon is the major above-ground vegetative organ in the cotyledon-only plant and has the potential to exist from emergence to new seed filling or even nearly to maturation, which allows for the implementation of long-term and consistent tracking experiments. (c) Studies can be targeted at one or a pair of cotyledons in avoiding the influence of signals and substances transmitting between leaves with different ages. (d) The cotyledon is a fully developed organ in seedling plants and rapidly converts to a competent state of photoperiodic perception after emergence. This feature is helpful to study the mechanism in the early stage of photoperiodic responses, such as light signal perception, daylength discrimination and gene regulation. (e) The cotyledon-only plant could live for a long time even under low-light or dark condition to prevent the influence of photosynthesis to photoperiod-dependent development. (f) As an integrator in the flowering pathway, GmFT2a expression can potentially serve as a ‘indicator’ to follow the effects of internal and external signals on flowering in soybean plants. Clearly, our new cotyledon-based system is likely to facilitate studies of photoperiodic response in soybean and other dicots.

ACKNOWLEDGMENTS
We gratefully acknowledge Prof. Haiyang Wang for his valuable suggestions and comments, Ms Weiwei Yao for her assistance with soybean transformation, Dr Li Chen in generating CRISPR/Cas9-derived gmft2a and gmft5a mutants and Dr John H. Snyder for improving the language of the manuscript. This research was supported by grants from the National Key R&D Program of China (2017YFD0101400), the China Agriculture Research System (CARS-04) and the CAAS Agricultural Science and Technology Innovation Project.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
Tianfu Han planned and designed the study. Xin Xu, Lixin Zhang, Xiangguang Lyu, Yumei Su and Bin Liu performed the experiments. Xiaoning Cao, Chunlei Zhang, Hongchang Jia and Cunxiang Wu conducted fieldwork. Luping Liu performed the genotype analysis. Lifeng Liu, Yupeng Cai and Wensheng Hou generated the RNAi lines and CRISPR/Cas9-derived mutants. Bingjun Jiang and Shengrui Zhang analysed the data. Shi Sun collected the soybean varieties. Xin Xu, Tianfu Han, Jinsheng Lai and Fulu Chen wrote the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Xin Xu https://orcid.org/0000-0002-8571-5034
Bingjun Jiang https://orcid.org/0000-0002-8172-4646
Wensheng Hou https://orcid.org/0000-0002-6342-4308

REFERENCES
Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., ... Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science, 309(5737), 1052–1056.
Cai, Y., Chen, L., Liu, X., Guo, C., Sun, S., Wu, C., ... Hou, W. (2018). CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. Plant Biotechnology Journal, 16(1), 176–185.

Cai, Y., Wang, L., Chen, L., Wu, T., Liu, L., Sun, S., ... Hou, W. (2020). Mutagenesis of GmFT2a and GmFT5a mediated by CRISPR/Cas9 contributes for expanding the regional adaptability of soybean. Plant Biotechnology Journal, 18(1), 298–309.

Cao, X. N. (2013). Studies on the evolution of root traits of soybean varieties from different decades by using grafting technique. (doctoral dissertation). Yaan, China: Sichuan Agricultural University.

Cao, X. N., Sun, S., Wu, C. X., Han, T. F., & Yang, W. Y. (2013). GmFT2a in soybean AP1 homologs controls flowering time and plant height. Journal of Integrative Plant Biology, 62(12), 1868–1879. https://doi.org/10.1111/jipb.12988

Chen, L., Cai, Y., Qu, M., Wang, L., Sun, H., Jiang, B., ... Hou, W. (2020). Soybean adaption to high-latitude regions is associated with natural variations of GmFT2b, an ortholog of FLOWERING LOCUS T. Plant Cell Environment, 43, 934–944.

Cao, X. N., Han, K., Kong, L., Yue, L., Yang, H., Zhao, Q., ... Dong, L. (2020). Soybean AP1 homologs control flowering time and plant height. Journal of Integrative Plant Biology, 62(12), 1868–1879. https://doi.org/10.1111/jipb.12988

Chi, Y., Huang, F., Liu, H., Yang, S., & Yu, D. (2011). An APETALA1-like gene of soybean regulates flowering time and specifies floral organs. Journal of Plant Physiology, 168(18), 2251–2259.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., ... Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science, 316(5827), 1030–1033.

Fehr, W. R., Cavinness, C. E., Burmood, D. T., & Pennington, J. S. (1971). Stage of development descriptions for soybeans. GmFT2a (L.) Merr. Crop Science, 116(2), 929–931.

Han, T., Gai, J., Wang, J., & Zhou, D. (1998). Discovery of flowering reversion in soybean plants. Acta Agronomica Sinica, 24(2), 168–171.

Han, T., Wu, C., Tong, Z., Mentreddy, R. S., Tan, K., & Gai, J. (2006). Post-flowering photoperiod regulates vegetative growth and reproductive development of soybean. Environmental and Experimental Botany, 55(1-2), 120–129.

Hanley, M. E., & May, O. C. (2006). Cotyledon damage at the seedling stage affects growth and flowering potential in mature plants. New Phytologist, 169(2), 243–250.

Hartwig, E. E. (1970). Growth and reproductive characteristics of soybeans [Glycine max (L.) Merr.] grown under short-day conditions. Tropical Science, 12, 47–53.

Hymowitz, T., & Newell, C. A. (1981). Taxonomy of the genus Glycine, domestication and uses of soybeans. Economic Botany, 35(3), 272–288.

Hyten, D. L., Song, Q., Zhu, Y., Choi, I. Y., Nelson, R. L., Costa, J. M., ... Cregan, P. B. (2006). Impacts of genetic bottlenecks on soybean genome diversity. Proceedings of the National Academy of Sciences, USA, 103(45), 16666–16671.

Jia, H., Jiang, B., Wu, C., Lu, W., Hou, W., Sun, S., ... Han, T. (2014). Maturity group classification and maturity locus genotyping of early-maturing soybean varieties from high-latitude cold regions. PLoS One, 9(4), e94139.

Jiang, B., Nan, H., Gao, Y., Tang, L., Yue, Y., Lu, S., ... Liu, B. (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(8), e106042.

Knott, J. E. (1934). Effect of a localized photoperiod on spinach. Proceedings of American Society for Horticultural Science, 31, 152–154.

Kong, F., Liu, B., Xia, Z., Sato, S., Kim, B. M., Watanabe, S., ... Abe, J. (2010). Two coordinately regulated homologs of FLOWERING LOCUS T are involved in the control of photoperiodic flowering in soybean. Plant Physiology, 154, 1220–1231.

Kong, F., Nan, H., Cao, D., Li, Y., Wu, F., Wang, J., ... Liu, B. (2014). A new dominant gene E9 conditions early flowering and maturity in soybean. Crop Science, 54(6), 2529–2535.

Li, C., Li, Y. H., Li, Y., Lu, H., Hong, H., Tian, Y., ... Qiu, L. J. (2020). A domestication-associated gene GmPRR3b regulates the circadian clock and flowering time in soybean. Molecular Plant, 13(5), 745–759.

Liljegren, S. J., Gustafson-Brown, C., Pinyopich, A., Ditta, G. S., & Yanofsky, M. F. (1999). Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. Plant Cell, 11(6), 1007–1018.

Liu, B., Kanazawa, A., Matsumura, H., Takahashi, R., Harada, K., & Abe, J. (2008). Genetic redundancy in soybean photoreponses associated with duplication of the phytochrome A gene. Genetics, 180(2), 995–1007.

Liu, L., Gao, L., Zhang, L., Cai, Y., Song, W., Chen, L., ... Han, T. (2020). Co-silencing E1 and its homologs in an extremely late-maturing soybean cultivar confers super-early maturity and adaptation to high-latitude short-season regions. Journal of Integrative Agriculture. https://doi.org/10.1016/S2095-3119(20)63391-3

Liu, L., Song, W., Wang, L., Sun, X., Qi, Y., Wu, T., ... Han, T. (2020). Allele combinations of maturity genes E1–E4 affect adaptation of soybean to diverse geographic regions and farming systems in China. PLoS One, 15(7), e0225397.

Liu, W., Jiang, B., Ma, L., Zhang, S., Zhai, H., Xu, X., ... Han, T. (2018). Functional diversification of Flowering Locus T homologs in soybean: GmFT1a and GmFT2a/5a have opposite roles in controlling flowering and maturation. New Phytologist, 217(3), 1335–1345.

Lu, S., Dong, L., Fang, C., Liu, S., Cheng, Q., Kong, L., ... Kong, F. J. (2020). Stepwise selection on homeologous PRR genes controlling flowering and maturity during soybean domestication. Natural Genetics, 52, 428–436.

Mandel, M. A., Gustafson-Brown, C., Savidge, B., & Yanofsky, M. F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature, 360(6401), 273–277.

Nan, H., Cao, D., Zhang, D., Li, Y., Lu, S., Tang, L., ... Kong, F. (2014). GmFT2a and GmFT5a redundantly and differentially regulate flowering through interaction with and upregulation of the E3 bZIP transcription factor GmFTD19 in soybean. PLoS One, 9(5), e97669.

Ogawa, Y., & King, R. W. (1990). The inhibition of flowering by non-induced cotyledons of Pharbitis nil. Plant and Cell Physiology, 31(1), 129–135.

Paton, D. M. (1971). Photoperiodic induction of flowering in the late pea cultivar greenfeast: The role of exposed cotyledons and leaves. Australian Journal of Biological Sciences, 24(3), 609–618.

Paz, M. M., Martinez, J. C., Kalvig, A. B., Fonger, T. M., & Wang, K. (2006). Improved cotyledony node method using an alternative explant derived from mature seed for efficient Agrobacterium-mediated soybean transformation. Plant Cell Reports, 25(3), 206–213.

Saito, G. Y., Chang, Y. C., Walling, L. L., & Thomson, W. W. (1990). Chloroplast development and nuclear gene expression in cotyledons of soybean seedlings. New Phytologist, 114(4), 547–554.

Sinegovskii, M., Yuan, S., Sinegovskaya, V., & Han, T. (2018). Current status of the soybean industry and research in the Russia Federation. Soybean Science, 37(1), 1–7.

Sun, H., Jia, Z., Cao, D., Jiang, B., Wu, C., Hou, W., ... Han, T. (2011). GmFT2a, a soybean homolog of FLOWERING LOCUS T, is involved in flowering transition and maintenance. PLoS One, 6(12), e29238.

Takeshima, R., Nan, H., Harigai, K., Dong, L., Zhu, J., Lu, S., ... Abe, J. (2019). Functional divergence between soybean FLOWERING LOCUS T orthologues FT2a and FT5a in post-flowering stem growth. Journal of Experimental Botany, 70(15), 3941–3953.
Xia, Z., Watanabe, S., Yamada, T., Tsubokura, Y., Nakashima, H., Zhai, H., ... Harada, K. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus $E1$ that regulates photoperiodic flowering. *Proceedings of the National Academy of Sciences, USA*, 109(32), 2155–2164.

Yoo, S. J., Hong, S. M., Jung, H. S., & Ahn, J. H. (2013). The cotyledons produce sufficient FT protein to induce flowering: evidence from cotyledon micrografting in Arabidopsis. *Plant Cell Physiology*, 54(1), 119–128.

Zhang, S., Zhao, C., & Lamb, E. G. (2011). Cotyledon damage affects seed number through final plant size in the annual grassland species *Medicago lupulina*. *Annals of Botany*, 107(3), 437–442.

Zhao, C., Takeshima, R., Zhu, J., Xu, M., Sato, M., Watanabe, S., ... Abe, J. (2016). A recessive allele for delayed flowering at the soybean maturity locus $E9$ is a leaky allele of FT2a, a FLOWERING LOCUS T ortholog. *BMC Plant Biology*, 16(1), 20.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

---

**How to cite this article**: Xu, X., Zhang, L., Cao, X., Liu, L., Jiang, B., Zhang, C., Jia, H., Lyu, X., Su, Y., Cai, Y., Liu, L., Zhang, S., Chen, F., Wu, C., Liu, B., Hou, W., Sun, S., Lai, J., & Han, T. (2021). Cotyledons facilitate the adaptation of early-maturing soybean varieties to high-latitude long-day environments. *Plant, Cell & Environment*, 1–14. [https://doi.org/10.1111/pce.14120](https://doi.org/10.1111/pce.14120)