agronomy

MDPI

Article

Mechanism of Early Flowering in a Landrace Naked Barley *eam8.l* Mutant

Chengzhi Mao [1,2,3,4], Jinqing Xu [1,2,3], Handong Wang [1,2,3,5], Tongrui Chen [1,2,3,4], Haiyan Bian [1,2,3], En You [1,2,4], Yuhu Shen [1,2,3,4,6] and Lei Wang [1,5,*]

1 Laboratory for Research and Utilization of Qinghai Tibetan Plateau Germplasm Resources, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
2 Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
3 Qinghai Provincial Key Laboratory of Crop Molecular Breeding, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
4 University of Chinese Academy of Sciences, Beijing 100049, China
5 State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining 810016, China
6 Innovation Academy for Seed Design, Chinese Academy of Sciences, Xining 810008, China
* Correspondence: wanglei@nwipb.cas.cn

Abstract: The *EARLY MATURITY 8* (*EAM8*) gene of barley is homologous to the *EARLY FLOWERING 3* (*ELF3*) gene in Arabidopsis, as loss-of-function mutations in this circadian clock gene promote rapid flowering. A previous study demonstrated that the early flowering phenotype of a hulless barley, Lalu, was due to allele *eam8.l* carrying an alternative splicing mutation in intron 3 that led to intron retention. In the present study, we verified that *eam8.l* encoded a truncated protein. Although *EAM8* was expressed at a higher level in Lalu than in other barley lines with a longer growth period, it did not negatively regulate flowering time. This result further proved that the *eam8.l* protein was nonfunctional in regulating flowering in barley. The early flowering phenotype of Lalu plants was strongly dependent on the biosynthesis of gibberellin (GA). The *eam8.l* mutation stopped the suppression of GA biosynthesis, and Lalu accumulated excessive GA, especially in leaves. This was achieved through the upregulated expression of genes in the GA pathway, including *GA20ox2*, *LFY1*, *SOC1*, *PAP2*, and *FPF3*. The mutation of the *EAM8* gene also abolished the inhibition of *FLOWERING LOCUS T-like* (*FT1*) gene expression at night. During the night, expression levels of the *FT1* gene were higher than those during the day in Lalu. However, the GA-dependent pathway and *FT1* gene mechanism are two independent pathways that promote flowering in Lalu. Alleles of *EAM8*, therefore, demonstrated an important breeding value in barley, which is probably effective in many other day-length-sensitive crop plants as well; thus, they could be used to tune adaptation in different geographic regions and climatic conditions, a critical issue in times of global warming.

Keywords: naked barley; early flowering; *EARLY MATURITY 8*; gibberellin; *FLOWERING LOCUS T-like*

Citation: Mao, C.; Xu, J.; Wang, H.; Chen, T.; Bian, H.; You, E.; Shen, Y.; Wang, L. Mechanism of Early Flowering in a Landrace Naked Barley *eam8.l* Mutant. *Agronomy* **2022**, *12*, 2137. https://doi.org/10.3390/agronomy12092137

Academic Editor: Andras Cseh

Received: 10 August 2022
Accepted: 5 September 2022
Published: 8 September 2022

1. Introduction

Plant flowering is a complex regulatory process of development that is influenced by a number of internal and external factors. Under the influence of internal and external factors, plants initiate related signal transduction processes and promote the control of plant gene expression, thereby determining plant flowering. Based on an in-depth study of the model plant *Arabidopsis thaliana*, at least four types of pathways that control plant flowering have been discovered, including photoperiod, vernalization, autonomic promotion, and various hormonal pathways in response to specific exogenous signals, which synergistically control the flowering process of plants [1,2]. These pathways construct a very complicated and large regulatory network involving a large number of genes. For example, *FLOWERING LOCUS T* (*FT*) encodes a protein with a phosphatidylethanolamine-binding

protein domain, and the FT protein interacts with the leucine zipper transcription factor *FLOWERING LOCUS D*, activating the floral meristem gene *APETALA 1* and enhancing the expression of *LEAFY* (*LFY*), thereby promoting flowering [3]. During crop domestication, *FLOWERING LOCUS T-like* genes (*FT1* in barley) were a key determinant of the flowering response. The reduced expression of *FT1* always leads to delayed flowering. *EARLY FLOWERING 3* (*ELF3*) is usually regarded as a zeitnehmer (time taker) and an essential component of the plant circadian clock. It plays a crucial role as a negative regulator of flowering time in *A. thaliana* [4]. Mutants that lose the function of this gene have attenuated photoperiod sensitivity and flower much earlier under both short-day (SD) and long-day (LD) conditions [5]. The survival of such mutations, occupying broad geographical ranges, indicates that early flowering is advantageous for crops in any environment where the growing season is short [5–7]. Breeders have identified the pattern in crops by reducing photoperiod sensitivity to assist with migration to high-latitude regions where SDs impede floral induction [5,7,8].

In barley, the *EARLY MATURITY 8* (*EAM8*) gene, also known as *Mat-a* (Praematur*um-a*), is responsible for the generation of circadian rhythms and the regulation of photoperiodic flowering. It is located on the long arm of barley chromosome 1H and is homologous to the *ELF3* gene in Arabidopsis [5]. The loss of *EAM8* gene function can induce photoperiod insensitivity in barley, increasing the expression of the *FT1* gene and promoting rapid heading and flowering of barley (*Hordeum vulgare*) under LD and SD conditions. Zakhrabekova et al. found that among 87 induced eam8 mutants, 6 carried a splice-site mutation [5]. Among them, there were only two spontaneous mutations in *EAM8*: *eam8.k* (found in cvs. Kinai 5 and Kagoshima Gold) and *eam8.w* (cv. Early Russian) [5]. The heading of *eam8_ea8.k* and *eam8_ert.016* with a Bowman or an Igri genetic background occurred earlier than that of the parental cultivars under both SD (25 days earlier) and LD (~2 weeks earlier) conditions [9]. Studies revealed that the early flowering phenotype in *mat.a-8* mutants was explained by increased production of the hormone gibberellin (GA), and that GA played an important role in the transition from vegetative to reproductive growth in barley, cooperating with the *FT1* gene [9,10]. A third spontaneous *EAM8* mutation (*eam8.l*) was identified in a six-rowed hulless barley landrace Lalu adapted to the Qinghai–Tibetan Plateau [11]. The *eam8.l* line flowered approximately 21 days and 15 days ahead of Diqing1, a normal six-rowed hulless cultivated barley line, under LD and SD conditions, respectively. *eam8.l* carries an A/G alternative splicing mutation at position 3257 in intron 3, which results in intron retention and a putative truncated protein [11].

In the present study, the early flowering mechanism of Lalu plants (*eam8.l*) was further investigated. The objectives of this study were as follows: (1) to verify the existence of the truncated protein; (2) to show the expression pattern of *eam8.l*; (3) to confirm whether the early flowering phenotype of Lalu was caused by GA synthesis regulation; and (4) to show how GA synthesis regulates early flowering. Here, the hypothesis was verified that the precocious phenotype was dependent on an increase in GA synthesis. In *eam8.l*, the results showed a significant increase in the expression of genes related to GA synthesis, especially *GA20oxidase 2* (*GA20ox2*). Moreover, *FT1* also played an important role in promoting Lalu flowering, but its expression was not affected by GA. Thus, GA and *FT1* acted via two independent pathways to promote the early flowering of Lalu plants. This study provides a theoretical basis for future barley precocity breeding under SD conditions.

2. Materials and Methods

2.1. Plant Lines

The plant lines comprised landrace barley Lalu (*eam8.l* mutant, with a G to A mutation at the 3257 bp site of *EAM8* leading to early flowering), the cultivar Diqing1 (wild type, flowering later than Lalu), the cultivar Bonus (wild type, a general reference line of barley, kindly provided by the Qinghai Academy of Agricultural Sciences), and Tibetan wild barley 86587 (wild type, different to the 3257 bp site of *EAM8*; the remaining sequences of the gene were the same as Lalu; flowering later than Lalu). All plants were grown in gallon

pots (3L) filled with general nutritional soil in standard growth rooms at 25 °C with an air humidity of 53% and all plants were under the same SD photoperiod (8 h light/16 h dark).

Germination assays, chlorophyll extraction and measurements, GA and PAC (paclobutrazol, used to inhibit GA synthesis) treatments, apex dissection, developmental flowering time measurements, and GA extraction and quantification were performed as described by Boden et al. [10].

2.2. Western Blotting

The antibodies were prepared by QiWei YiCheng Tech Co., Ltd. (Beijing, China). The polypeptide was synthesized according to the partial exon 1 amino acid sequence of the *EAM8* gene: MRRAGGGGGGSGEDKVMGPLFPRLHVNDTTLKGGGPRAPPRNKMALYEQFSVPSQRFAANTAPAAHRPAASFAAVSSASAGQIGGIDRPLF. After compounding of the polypeptide, a rabbit anti-EAM8 immune serum was prepared as the primary antibody, and anti-rabbit IgG H+L (HRP) was used as the second antibody.

The protein of plant lines was extracted using a total protein extraction kit (Solarbio, Beijing, China). After polyacrylamide gel electrophoresis, the gel area with the targeted band was selected for transmembrane use. Then, the following procedures were performed: blocking, primary antibody incubation, and secondary antibody incubation. Finally, the membrane containing the protein compound was exposed in a dark room.

2.3. RNA Extraction and Expression Analysis

RNA was extracted from the barley lines as follows: (1) The sampling time for the lines used for spatial expression was 22:00. (2) The sampling time for the lines (leaves and apices) treated with GA, PAC, PAC+GA, and the control was also 22:00 at the four-leaf stage. (3) Leaves from Lalu under PAC-treatment (1 μM) conditions and leaves from Lalu, Diqing1, 86587, and Bonus without any treatment were harvested at defined intervals until the four-leaf stage. Each leaf was the youngest tissue that emerged from the fourth leaf at the four-leaf stage. Each apex RNA sample contained eight pooled apices and was harvested from the main stem. All barley lines used in the experiment contained three biological replicates. Total RNA extraction kits (TaKaRa MiniBEST Plant RNA Extraction Kit), reverse transcription kits (PrimeScript™ RT Reagent Kit with gDNA Eraser), and kits for real-time PCR (RT-qPCR) (TB Green™ Premix Ex Taq™ II) were from TaKaRa Bio Inc. Details of the nucleotide primer sequences used in this study are shown in Supplementary Table S1. The internal reference gene used in the quantitative experiment was α-tubulin.

2.4. Statistical Analysis

Student's *t*-test of the significance of the differences between treatment groups was performed with GraphPad Prism software using a 95% confidence level ($p < 0.05$). The results are shown in the figures as mean ± SE.

3. Results

3.1. eam8.l Encodes a Truncated Protein

Previous studies have demonstrated that the G/A mutation at 3257 bp in the intron of *eam8.l* in Lalu results in a translation frame shift and leads to truncated proteins [11]. A bioinformatics analysis predicted that the theoretical molecular weight of the *eam8.l* protein in Lalu would be 46.40 kDa, whereas that of the EAM8 protein in Dingqing1 would be 84.32 kDa (Figure 1A). To verify this conjecture, Western blotting was performed to determine the total proteins of new leaves of Lalu and Diqing1 at the four-leaf stage (Figure 1B). The results showed that the molecular weight of the EAM8 protein in Lalu was apparently lower than that of the corresponding protein in Diqing1, indicating that the protein encoded by *eam8.l* in Lalu was indeed a truncated incomplete sequence (Figure 1). This deficiency causes a change in protein function.

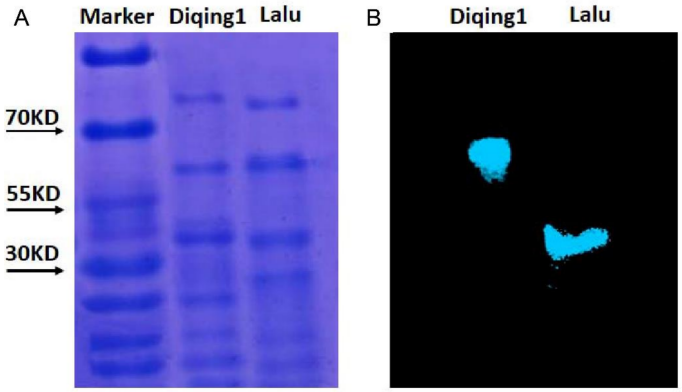

Figure 1. Comparison of EAM8 protein abundance in leaves of Lalu and Diqing1. (**A**) Total proteins in leaves of Lalu and Diqing1. (**B**) Western blotting of the EAM8 protein of Lalu and Diqing1.

3.2. Spatial Expression of the EAM8 Gene at Different Developmental Stages

Owing to the mutation of gene *EAM8*, the flowering time of Lalu is significantly earlier than that of other lines. To further investigate the mechanism of the earlier flowering phenotype, the expression levels of the *EAM8* gene were tested in different organs of Lalu, Diqing1, 86587, and Bonus at different stages. Under SD conditions, none of the lines other than Lalu could be headed. Therefore, the expression test was carried out at three periods: the four-leaf stage, tillering stage, and jointing stage.

At the four-leaf stage, the expression of the *EAM8* gene in the Lalu apex was significantly higher than that in Diqing1 and 86587 (Figure 2A), but in leaves and roots, there was no significant difference among Lalu, Diqing1, and 86587 in the expression level of *EAM8* at this stage (Figure 2B,D). At the jointing stage, the expression levels of the *EAM8* gene in the apex and leaves of Lalu were significantly higher than those in Diqing1 and 86587 (Figure 2E,F), whereas the difference in expression in stems and roots was not significant (Figure 2G,H). In the tillering stage, the transcript levels for *EAM8* were significantly higher in Lalu than in Diqing1 and 86587 except for in the root; especially in the apex, the difference was extremely significant (Figure 2I–L).

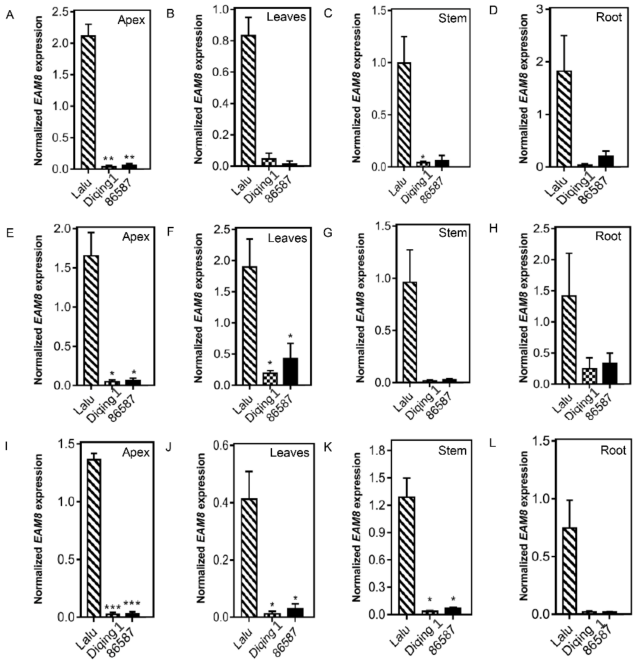

Figure 2. Spatial expression of the *EAM8* gene at different stages in Lalu, Dingqing1, and 86587. (**A**–**D**) Four-leaf stage. (**E**–**H**) Tillering stage. (**I**–**L**) Jointing stage. Data are the mean ± SE of three biological replicates (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

From an organ perspective, in the apex the expression of *EAM8* in Lalu was significantly higher compared with that in Diqing1 and 86587 at all three stages (Figure 2A,E,I). In contrast, no significant difference was detected among the three lines in the three stages in the roots (Figure 2D,H,L). Given that *EAM8* is regarded as a negative regulator of flowering time [11], the higher its expression, the later the flowering should be. In the present study, although the transcript levels in various organs during the growth process were higher in Lalu compared with those in other barley lines, Lalu flowered much earlier. This result further indicated that the *EAM8* protein in Lalu was nonfunctional and promoted flowering.

3.3. The Early Flowering Phenotype in Lalu Depends on Increasing GA Production

To further analyze the mechanism of the early flowering of Lalu caused by *eam8.l*, four experimental lines (Lalu, Diqing1, 86857, and Bonus) were planted under SD conditions to compare the flowering phenotype. At early vegetative growth, it was observed that Lalu was superior to Diqing1, 86587, and Bonus; for instance, it had longer coleoptiles and leaf length, but a lighter leaf color than the other three lines. Qualitatively measuring these phenotypes showed that Lalu had advantages over Diqing1, 86587, and Bonus in terms of germination rate, leaf growth, and coleoptile development (Figure 3A–C), whereas the chlorophyll content of Lalu was significantly lower than that of the other three lines (Figure 3D).

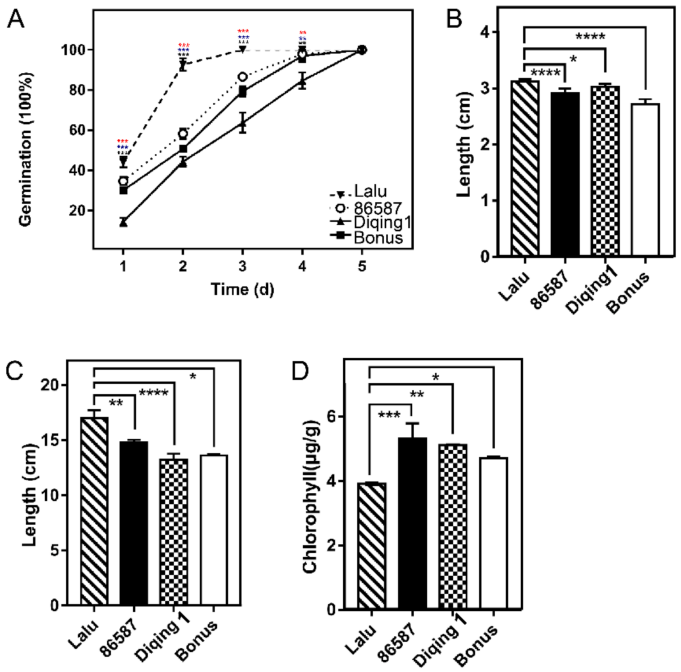

Figure 3. Phenotypes of Lalu, 86587, Diqing1 and Bonus plants at germination stage. (**A**) Quantification of germination rate. (**B**) Coleoptile length of Lalu, Diqing1, 86587, and Bonus. (**C**) Leaf length of Lalu, Diqing1, 86587, and Bonus. (**D**) Chlorophyll concentrations of Lalu, Diqing1, 86587, and Bonus. Data are the mean ± SE of three biological replicates (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Red asterisk indicates the difference between Lalu and 86587; blue asterisk indicates the difference between Lalu and Bonus; black asterisk indicates the difference between Lalu and Diqing1).

The trend of these phenotypic changes was similar to the response to increasing GA [12–14]. GA can promote plant development in many ways; thus, it was suspected that the GA-dependent pathway might also be involved in the early flowering pathway of Lalu plants. To this end, we analyzed the flowering phenotypes of Lalu, Diqing1, 86587, and Bonus under the GA3 and GA biosynthesis inhibitor PAC. Anatomic photographs of the developmental inflorescence of Lalu, Diqing1, 86587, and Bonus were taken under treatment and control conditions. PAC strongly inhibited the development of the inflorescence

of the four accessions, and Diqing1, 86587, and Bonus did not flower during the course of the experiment (Figure 4A). In addition, the higher the PAC concentration, the stronger the inhibition (Figure S1A). This inhibition was significantly relieved by the application of GA3 (Figure 4A; Figure S1A). Additionally, increasing GA content markedly promoted flowering in Lalu but did not lead to the complete flowering of Diqing1, 86587, and Bonus (Figure S1). Therefore, GA-dependent pathways have a major impact on the early flowering of Lalu.

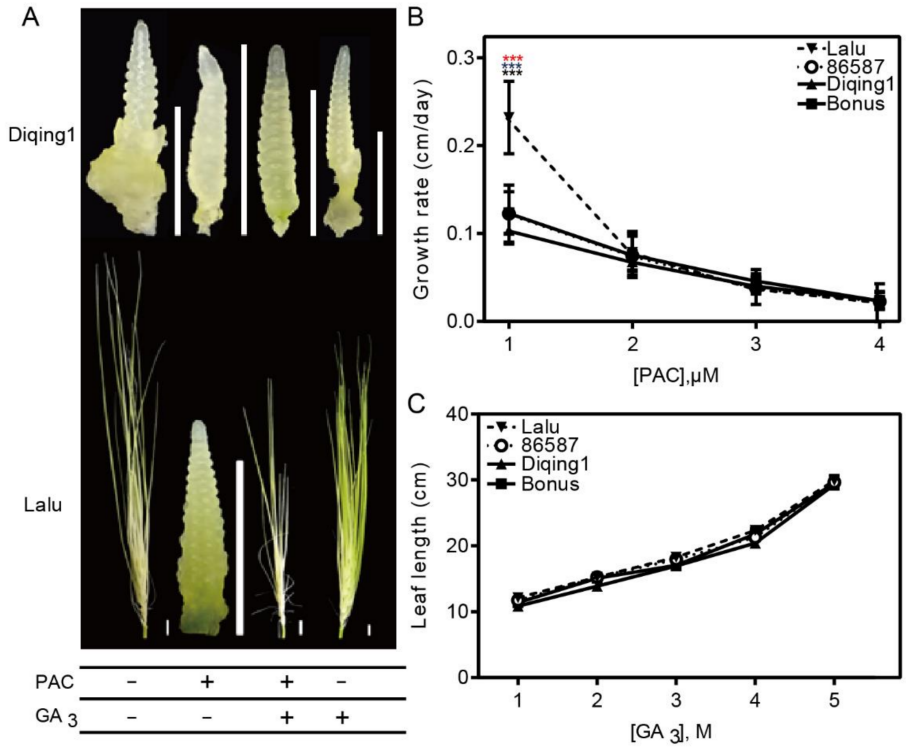

Figure 4. Effect of GA and PCA application on phenotypes of Lalu, 86587, Diqing1 and Bonus. (**A**) Inflorescence development of Diqing1 and Lalu plants treated with PAC (1 μM), PAC (1 μM) and GA3 (10-8 M), or GA3 (10-8 M). The images of inflorescences were taken on the day when the spike of the Lalu control plant emerged from the boot. Bars for immature florescences, 1 mm, and for mature spikes, 1 cm. (**B**) Growth rates of Lalu, 86587, Diqing1, and Bonus plants under GA treatment (10-2 M). (**C**) Leaf length of Lalu, 86587, Diqing1, and Bonus plants under PAC treatment (1 μM). Data are the mean ± SE of three biological replicates (*** $p < 0.001$. Red asterisk indicates the difference between Lalu and 86587; blue asterisk indicates the difference between Lalu and Bonus; black asterisk indicates the difference between Lalu and Diqing1).

Four lines were treated with the GA biosynthesis inhibitor PAC at different concentrations to verify whether Lalu synthesizes higher levels of GA or is more sensitive to GA. The leaf growth rates of all four lines slowed down and reached an equal level as the concentration of PAC increased (Figure 4B). Significantly, the inhibition of Lalu was more remarkable. As Lalu was more sensitive to the increase in PAC concentration, the assumption that Lalu had a constitutive GA response was disproved. Four lines were treated with different concentration gradients of GA3 to verify whether Lalu displayed a similar trend in GA sensitivity. Diqing1, 86587, and Bonus plants showed similar responses to exogenous GA, and their leaves grew as fast as those of Lalu plants under all GA3 concentrations. Lalu was not as sensitive to exogenous GA as the other lines (Figure 4C). Taken together, these results indicate that the growth of Lalu plants is highly dependent on endogenous GA biosynthesis.

3.4. The GA-Dependent Pathway and FT1 Gene Represent Two Independent Pathways That Promote Flowering in Lalu

The *FT1* gene is located at the junction of various flowering pathways in plants and has a key role in the regulation of plant flowering. Under appropriate light conditions, the *FT1* gene is exposed to vascular-bundle-specific zinc finger proteins in the photoperiod pathway. The *FT1* protein synthesized in plant leaves is transferred to the shoot apex meristem to regulate downstream gene expression and promote flowering [10]. Therefore, we presumed that the PAC treatment would affect the expression of the *FT1* gene in Lalu plants. The *FT1* transcript levels from leaves of PAC-treated and control Lalu plants at the four-leaf stage were compared. No transcripts of *FT1* were detected in Diqing1 or 86587 leaves under PAC-treatment and control conditions. *FT1* gene expression was not affected significantly by the PAC treatment in Lalu (Figure 5). Combined with the previous results, this demonstrated that flowering could be delayed by reducing GA biosynthesis in Lalu plants, but this reduction did not affect the expression of *FT1*.

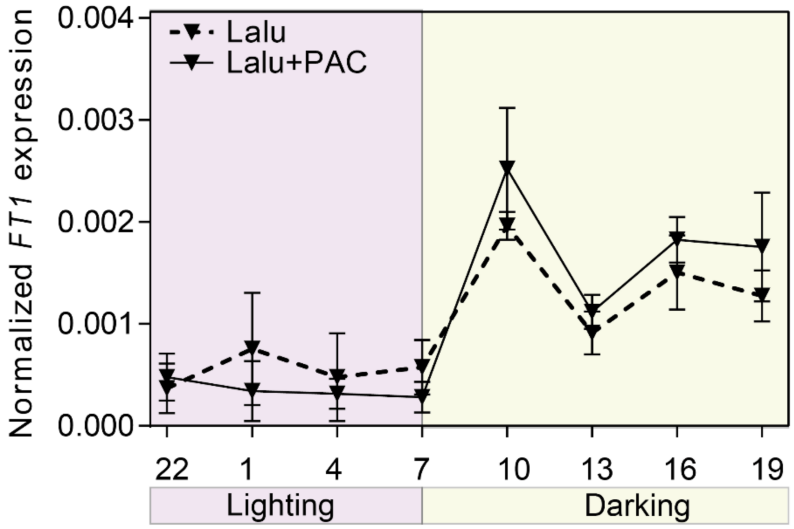

Figure 5. *FT1* gene expression throughout the 24 h period in Lalu under control and PAC-treated (PAC; 1 μM) conditions. Data are the mean ± SE of three biological replicates. The lines for *FT1* gene analysis were harvested from plants at the four-leaf stage.

To further explore the mechanism of early flowering in Lalu plants, we measured the expression of flowering-related genes downstream of the GA pathway, including *LEAFY* (*LFY1*), *SUPPRESSOR OF CONSTANS 1* (*SOC1*), *FLORAL PROMOTING FACTOR 3* (*FPF3*), and *PANICLE PHYTOMER 2* (*PAP2*). Likewise, the expression of *MADS8* and *MADS14*, which are downstream genes of *FT1*, was also measured. The transcription of these genes was compared in the developing spikes of Lalu, Diqing1, and 86587. The expression levels of the *LFY1*, *SOC1*, *FPF3* and *PAP2* genes in Lalu were significantly higher than those in Diqing1 and 86587 (Figure S2A). Furthermore, we compared the expression of these genes in developing spikes from control Lalu plants and plants that had been treated with PAC and PAC/GA to determine whether their expression was GA dependent. When PAC was applied to Lalu plants, the expression levels of these four genes in the GA pathway decreased significantly, and the suppressive effect was eliminated by the application of GA (Figure S2A). The expression levels of *MADS8* and *MADS14* genes in Lalu plants were also significantly higher than those in Diqing1 and 86587, but there was no significant change resulting from the treatment with PAC or PAC/GA (Figure S2B). These results indicate that GA promotes the early flowering phenotype in Lalu plants by increasing the expression of genes including *LFY1*, *SOC1*, *FPF3*, and *PAP2*.

3.5. Mutation of the EAM8 Gene (eam8.l) Suppresses GA Synthesis in Lalu

The abovementioned experimental results demonstrated that Lalu plants exhibited a strong response to GA biosynthesis inhibitors (Figure 4C). It was presumed that the mutation of *EAM8* made Lalu synthesize more GA than Diqing1 and 86587. First, the amount of GA molecules in leaves was measured, including bioactive GA1, precursor molecules GA19 and GA20, and the inactivate product GA8, as direct evidence to support this assumption. It was found that the four small GA molecules in Lalu plants were all higher than those in Bonus, 86587 and Diqing1 plants (Figure S3, Table S1). Moreover, the GA1, GA19, and GA8 contents in Lalu plants were significantly higher than those in Diqing1 plants (Figure S3, Table S1). The results indicated that the leaves of Lalu contain excessive GA production. Previous studies have shown that the production of GA19 and GA20 needs to be catalyzed by a *GA20ox* gene, GA20 is used to form GA1, and GA8 is produced after catabolism of GA1 [15,16]. Therefore, the expression of *GA20ox* genes is very important in the GA synthesis process and has been measured. The expression pattern over the day–night cycle of the *GA20ox2* gene in leaves showed that the expression of the *GA20ox2* gene in Lalu plants was significantly higher than that of Diqing1 and 86587 during the whole day, and this relatively high expression trend was much more evident at night (Figure 6A). We also found that the expression levels of genes *GA20ox1*, *GA20ox3*, and *GA20ox4* in the leaves of Lalu plants were higher than those in Diqing1 and 86587 during the night at ZT 10 h (Figure 6B). In contrast to the *GA20ox2* gene, the *EAM8* gene showed significantly higher expression during the daytime than at night (Figure 6A,C). Therefore, although the expression of the *EAM8* gene decreased, the production of GA showed an increasing trend. Moreover, when GA3 (10^{-4} M) was applied to either Lalu, Diqing1, or 86587, the expression levels of the *GA20ox2* and *GA20ox3* genes were significantly lower than that in the control (Figure S4A). Taken together, these results demonstrate that the trend of GA synthesis gene expression was positively correlated with GA abundance.

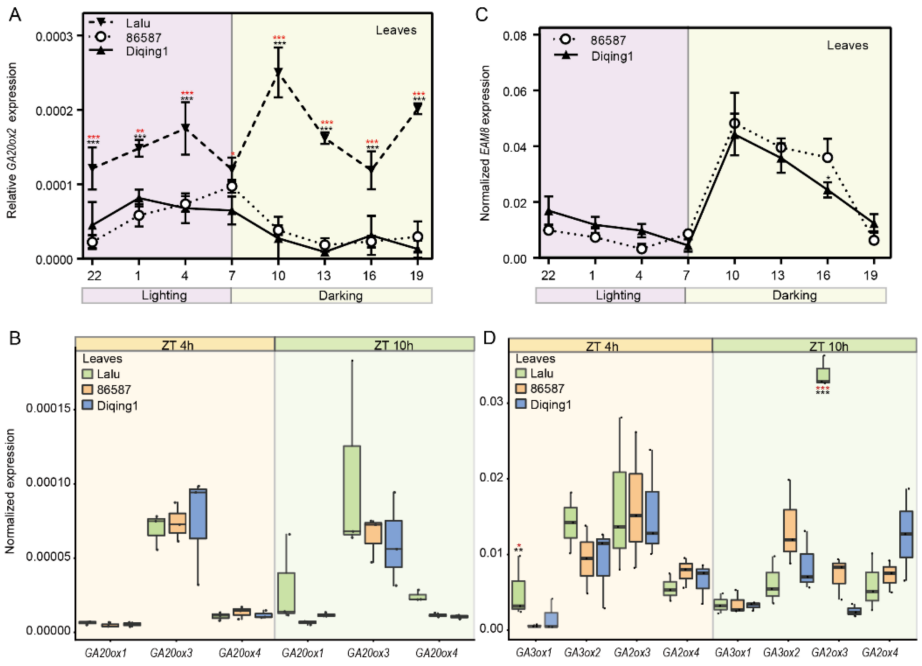

Figure 6. Expression of GA biosynthesis-related genes in Lalu, 86587, and Diqing1 plants. (**A**) Expression of *GA20ox2* in Lalu leaves during the diurnal cycle. (**B**) Quantification of transcript levels of *GA20ox1*, *GA20ox3*, and *GA20ox4* during t during the day (ZT 4 h) and night (ZT 10 h). Data are the mean ± SE of three biological replicates. (**C**) Expression of *EAM8* in 86587 and Diqing1 leaves during the diurnal cycle. Data are the mean ± SE of three biological replicates. (**D**) Quantification of transcript levels of *GA3ox1*, *GA3ox2*, *GA2ox3*, and *GA2ox4* in leaves of 86587, Diqing1, and Lalu plants during the day (ZT 4 h) and night (ZT 10 h). Data are the mean ± SEM of

three biological replicates. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; red asterisk indicates the difference between Lalu and 86587; black asterisk indicates the difference between Lalu and Diqing1).

We also analyzed the expression levels of *GA20ox* in the apex of Lalu, Diqing1, and 86587. *GA20ox1* was not expressed in the apex, and the expression levels of *GA20ox3* and *GA20ox4* did not differ significantly between the treatment and control groups (Figure S4B). However, under PAC-treated conditions, the expression level of the *GA20ox2* gene in the apex of Lalu plants was significantly higher than that in the Lalu control group, and the expression decreased when GA3 was applied (Figure S4C). Taken together, these results indicate that GA does not accumulate excessively in the apex of Lalu plants with the *eam8.l* mutation.

The transcripts of other GA synthesis-related genes, including *GA3ox1*, *GA3ox2*, *GA2ox3*, and *GA2ox4*, could be detected in leaves. The quantitative RT-qPCR showed that during the daytime, *GA3ox1* transcripts were significantly higher in Lalu than in Diqing1 and 86587, although the transcript level was very weak compared with *GA3ox2* and *GA2ox3*. During the nighttime, the *GA2ox3* transcript level of Lalu was much higher than that of the other two lines, and there was no significant difference in *GA3ox1*, *GA3ox2*, or *GA2ox4* transcript levels among the three lines (Figure 6D). The *GA2ox3* transcription pattern was consistent with the feedback mechanism of the GA biosynthesis pathway. In the apex of Lalu, Diqing1, and 86587, we only detected the *GA3ox2* and *GA2ox3* genes. The expression level of the *GA3ox2* gene in Diqing1 and 86587 plants was significantly higher than that in Lalu plants. The differences in the expression levels of the *GA2ox3* gene between Lalu and the other two lines (Diqing1 and 86587) were not significant (Figure S4D). Furthermore, the expression levels of these genes in Lalu plants responded to PAC and PAC/GA treatment to different degrees. These results further indicated that the increase in GA production occurred in the leaves of Lalu plants, but not in apices.

4. Discussion

Plants that flower rapidly can be grown in marginal environments where the growing season is restricted by the climate. Photoperiod insensitivity is also beneficial in regions with a long growing season, as it extends the length of the vegetative phase, with positive effects on reproductive potential [5,17].

The *EAM8* gene in barley is a homolog of *ELF3* that is involved in regulating the sensitivity of the barley photoperiod. Loss of function of the *EAM8* gene causes photoperiod insensitivity and precocity in barley under both LD and SD conditions [5,9,10]. Mutations in *EAM8* have aroused the interest of barley breeders. Lalu is a six-rowed hulless landrace barley with an early flowering phenotype that is natively grown on the Tibetan Plateau. Lalu was demonstrated to be a spontaneous recessive *EAM8* mutant (*eam8.l*) by members of our laboratory, and the mutation was shown to lead to intron retention and a putative truncated protein [11]. In the present study, both a bioinformatics analysis and Western blotting verified that the *EAM8* protein in Lalu was indeed a truncated protein. *EAM8*, a negative regulator of flowering, was expressed at higher levels in Lalu than in other barley lines that flowered later than Lalu. These results provided the evidence that the *eam8.l* protein was nonfunctional. The loss of function was responsible for the early flowering of Lalu.

The present study aimed to determine how *eam8.l* regulates the early flowering of Lalu. Previous studies have shown that GA, a phytohormone, is not the only factor regulating flower formation, but works in concert with other factors under the conditions of an induced photoperiod [18–20]. Our results showed that the *eam8.l* gene can regulate the expression of GA biosynthesis-related genes in Lalu plants, especially *GA20ox2*, which is also confirmed as a factor in the early flowering phenotype of Lalu plants. Moreover, the role of the *EAM8* gene in Lalu plants was consistent with the increased expression of *GA20ox2* in the circadian clock mutant of *A. thaliana* [21]. In Lalu, high levels of *GA20ox2* expression and GA accumulation were found mainly in leaves rather than apices; however,

genes associated with flowering in the GA pathway, including *LFY1*, *SOC1*, *FPF3*, and *PAP2*, were highly expressed in apices. Therefore, the GA high-content signal may be transmitted from leaves to apices, followed by the expression of downstream genes in the GA pathway.

In barley, *FT1* is the main *FT-like* gene involved in the transition from vegetative to reproductive development and is highly expressed under LD photoperiods relative to SDs [9,22]. The circadian cycle expression patterns of the *FT1* and *GA20ox2* genes showed that the mutation of the *EAM8* gene makes early flowering inseparable from these two genes in Lalu plants. In Lalu plants, the expression of *GA20ox2* was significantly higher than that of Diqing1 and 86587 in the diurnal expression pattern, which was consistent with *ELF3* being a key inhibitor in the evening loop of the circadian cycle [23,24]. Interestingly, the development of Lalu was obviously inhibited by PAC, but was not sensitive to exogenous GA3. Moreover, the *FT1* expression level in Lalu was higher than that in Diqing1 and 86587; however, it was not affected by the PAC treatment. Therefore, increasing GA production and *FT1* expression are two independent means of accelerating the flowering process in Lalu (Figure S5). This pattern is consistent with the early flowering mechanism of the *elf3* mutant *mat.a-8* [10]. The mechanism was further confirmed by the relationship between GA and *FT1* found in *Lolium*, but not in *Arabidopsis* [25–27].

5. Conclusions

In conclusion, *eam8.l* indeed encoded a truncated protein. The loss of function relieved the suppression of GA synthesis by *EAM8*, and more GA was produced in Lalu than in wild types. The early flowering phenotype in Lalu was responsible for the excessive production of GA and the high expression of the *FT1* gene. These two pathways were independent of each other in promoting the flowering process in Lalu. This study clearly elucidates the early flowering mechanism in Lalu and could provide help for barley breeding programs. Lalu could be used as a parental line by breeders to modify flowering time and create new cultivars adapted to broader geographical ranges.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092137/s1, Figure S1: Inflorescence development of Lalu, 86587, Diqing1, and Bonus plants; Figure S2: Expression analysis of flowering-related genes in the developing apex; Figure S3: Quantification of GA1, GA19, GA20, and GA8 levels in leaves of Lalu plants relative to Bonus, 86587, and Diqing1; Figure S4: GA biosynthesis-related genes expression in Lalu, 86587, and Diqing1 plants under control, GA-treated, and PAC-treated conditions; Figure S5: Model of *EAM8* regulation of flowering in barley. Table S1: Measurements of GA1, GA19, GA20, and GA8 levels from Lalu, Bonus, 86587, and Diqing1; Table S2: Oligonucleotide sequences used in qRT-PCR assays.

Author Contributions: L.W. and Y.S. conceived and designed the research study. C.M. performed the experiments and analyzed the data in the research study. J.X., H.W., T.C., H.B. and E.Y. assisted in the experiments and figure editing. C.M. and L.W. wrote the manuscript. Y.S. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Second Tibetan Plateau Scientific Expedition and Research (STEP) program (Grant No. 2019QZKK0303), the National Natural Science Foundation of China (General Program 32171958), the Qinghai Province Natural Science Foundation (2020-ZJ-908), the Construction Project for Innovation Platform of Qinghai province (2022-ZJ-Y04 and 2022-ZJ-Y01), and the Open Project of State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University (2022-KF-06).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Ruijun Duan (Qinghai University) and the National Duplicate Genebank for Crops of China for providing the seeds of Bonus and wild barley 86587.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Simpson, G.G. The autonomous pathway: Epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Curr. Opin. Plant Biol.* **2004**, *7*, 570–574. [CrossRef] [PubMed]
2. Michaels, S.D.; Himelblau, E.; Kim, S.Y.; Schomburg, F.M.; Amasino, R.M. Integration of Flowering Signals in Winter-Annual Arabidopsis. *Plant Physiol.* **2005**, *137*, 149–156. [CrossRef] [PubMed]
3. Kardailsky, I.; Shukla, V.K.; Ahn, J.H.; Dagenais, N.; Christensen, S.K.; Nguyen, J.T.; Chory, J.; Harrison, M.J.; Weigel, D. Activation Tagging of the Floral Inducer *FT*. *Science* **1999**, *286*, 1962–1965. [CrossRef] [PubMed]
4. Zhao, H.; Xu, D.; Tian, T.; Kong, F.; Lin, K.; Gan, S.; Zhang, H.; Li, G. Molecular and functional dissection of EARLY-FLOWERING 3 (ELF3) and ELF4 in *Arabidopsis*. *Plant Sci.* **2021**, *303*, 110786. [CrossRef] [PubMed]
5. Zakhrabekova, S.; Gough, S.P.; Braumann, I.; Müller, A.H.; Lundqvist, J.; Ahmann, K.; Dockter, C.; Matyszczak, I.; Kurowska, M.; Druka, A. Induced mutations in circadian clock regulator *Mat-a* facilitated short-season adaptation and range extension in cultivated barley. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4326–4331. [CrossRef] [PubMed]
6. Lundqvist, U. *Eighty Years of Scandinavian Barley Mutation Genetics and Breeding*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2009; pp. 39–43.
7. Faure, S.; Turner, A.S.; Gruszka, D.; Christodoulou, V.; Davis, S.J.; von Korff, M.; Laurie, D.A. Mutation at the circadian clock gene 13 *EARLY MATURITY 8* adapts domesticated barley (*Hordeum vulgare*) to short growing seasons. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 8328–8333. [CrossRef] [PubMed]
8. Pugsley, A.T. The impact of plant physiology on Australian wheat breeding. *Euphytica* **1983**, *32*, 743–748. [CrossRef]
9. Beales, J.; Turner, A.; Griffiths, S.; Snape, J.W.; Laurie, D.A. A Pseudo-Response Regulator is misexpressed in the photoperiod insensitive Ppd-D1a mutant of wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* **2007**, *115*, 721–733. [CrossRef]
10. Boden, S.A.; Weiss, D.; Ross, J.J.; Davies, N.W.; Trevaskis, B.; Chandler, P.M.; Swain, S.M. *EARLY FLOWERING3* Regulates Flowering in Spring Barley by Mediating Gibberellin Production and *FLOWERING LOCUS T* Expression. *Plant Cell* **2014**, *26*, 1557–1569. [CrossRef]
11. Xia, T.; Zhang, L.; Xu, J.; Wang, L.; Liu, B.; Hao, M.; Chang, X.; Zhang, T.; Li, S.; Zhang, H. The alternative splicing of EAM8 contributes to early flowering and short-season adaptation in a landrace barley from the Qinghai-Tibetan Plateau. *Theor. Appl. Genet.* **2017**, *130*, 757–766. [CrossRef]
12. Wolf, F.T.; Haber, A.H.; Wolf, A.H.H.F.T. Chlorophyll Content of Gibberellin-Treated Wheat Seedlings. *Nature* **1960**, *186*, 217–218. [CrossRef] [PubMed]
13. Huang, S.; Raman, A.S.; Ream, J.E.; Fujiwara, H.; Cerny, R.E.; Brown, S.M. Overexpression of 20-Oxidase Confers a Gibberellin-Overproduction Phenotype in *Arabidopsis*. *Plant Physiol.* **1998**, *118*, 773–781. [CrossRef] [PubMed]
14. Hauvermale, A.L.; Ariizumi, T.; Steber, C.M. Gibberellin Signaling: A Theme and Variations on DELLA Repression. *Plant Physiol.* **2012**, *160*, 83–92. [CrossRef] [PubMed]
15. Davies, L.J.; Rappaport, L. Metabolism of Tritiated Gibberellins in d-5 Dward Maize. *Plant Physiol.* **1975**, *56*, 60–66. [CrossRef] [PubMed]
16. Xu, Y.L.; Li, L.; Wu, K.; Peeters, A.J.; Gage, A.D.; Zeevaart, A.J. The GA5 locus of Arabidopsis thaliana encodes a multifunctional gibberellin 20-oxidase: Molecular cloning and functional expression. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6640–6644. [CrossRef] [PubMed]
17. Turner, A.; Beales, J.; Faure, S.; Dunford, R.P.; Laurie, D.A. The Pseudo-Response Regulator *Ppd-H1* Provides Adaptation to Photoperiod in Barley. *Science* **2005**, *310*, 1031–1034. [CrossRef] [PubMed]
18. Lang, A. The Effect of Gibberellin upon Flower Formation. *Proc. Natl. Acad. Sci. USA* **1957**, *43*, 709–717. [CrossRef]
19. Chailakhyan, M.K. Hormonale faktoren des pflanzenblühens. *Biol. Zent. Bl.* **1958**, *77*, 641–662.
20. Bernier, G.; Havelange, A.; Houssa, C.; Petitjean, A.; Lejeune, P. Physiological Signals That Induce Flowering. *Plant Cell* **1993**, *5*, 1147–1155. [CrossRef]
21. Blázquez, M.A.; Trénor, M.; Weigel, D. Independent Control of Gibberellin Biosynthesis and Flowering Time by the Circadian Clock in *Arabidopsis*. *Plant Physiol.* **2002**, *130*, 1770–1775. [CrossRef]
22. Yan, L.; Loukoianov, A.; Tranquilli, G.; Helguera, M.; Fahima, T.; Dubcovsky, J. Positional cloning of the wheat vernalization gene *VRN1*. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6263–6268. [CrossRef] [PubMed]
23. Dixon, L.E.; Knox, K.; Kozma-Bognar, L.; Southern, M.M.; Pokhilko, A.; Millar, A.J. Temporal Repression of Core Circadian Genes Is Mediated through EARLY FLOWERING 3 in *Arabidopsis*. *Curr. Biol.* **2011**, *21*, 120–125. [CrossRef] [PubMed]
24. Fowler, S.; Lee, K.; Onouchi, H.; Samach, A.; Richardson, K.; Morris, B.; Coupland, G.; Putterill, J. GIGANTEA: A circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *EMBO J.* **1999**, *18*, 4679–4688. [CrossRef] [PubMed]
25. King, R.W.; Moritz, T.; Evans, L.T.; Martin, J.; Andersen, C.H.; Blundell, C.; Kardailsky, I.; Chandler, P.M. Regulation of Flowering in the Long-Day Grass *Lolium temulentum* by Gibberellins and the *FLOWERING LOCUS T* Gene. *Plant Physiol.* **2006**, *141*, 498–507. [CrossRef]

26. Hisamatsu, T.; King, R.W. The nature of floral signals in *Arabidopsis*. II. Roles for *FLOWERING LOCUS T* (FT) and gibberellin. *J. Exp. Bot.* **2008**, *59*, 3821–3829. [CrossRef]
27. Porri, A.; Torti, S.; Romera-Branchat, M.; Coupland, G. Spatially distinct regulatory roles for gibberellins in the promotion of flowering of *Arabidopsis* under long photoperiods. *Development* **2012**, *139*, 2198–2209. [CrossRef]