Early flowering phenotype of the Arabidopsis altered meristem program1 mutant is dependent on the FLOWERING LOCUS T-mediated pathway

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Abstract Controlling the flowering time is crucial for propagating plant species and crop production. ALTERED MERISTEM PROGRAM1 (AMP1) in Arabidopsis thaliana encodes a putative carboxypeptidase, and an AMP1 mutant (amp1) was found to cause highly pleiotropic phenotypes including a short plastochron, an enlarged shoot apical meristem, and reduced apical dominance. Although amp1 also shows an early flowering phenotype, its mechanism has not been investigated in detail. The most important floral integrator or florigen gene, FLOWERING LOCUS T (FT), has a close relative, TWIN SISTER OF FT (TSF). In this report, we generated a new allele of tsf using a genome-editing technique and produced ft tsf double and amp1 ft tsf triple mutants. The flowering time of amp1 ft tsf was equally as late as ft tsf under long-day conditions. In addition, the expression level of FT in amp1 was 2.4-fold higher than that in wild-type, even five days after germination under long-day conditions. These results suggest that the elevated expression level of FT is responsible for the early flowering phenotype of amp1. Furthermore, expression of FLOWERING LOCUS C (FLC), a negative regulator of FT expression, is severely repressed in amp1, raising the possibility that low expression levels of FLC contributes to upregulation of FT expression and the early flowering phenotype of amp1.

Key words: AMP1, Arabidopsis, CRISPR/Cas9, flowering, FT.

A pleiotropic gene often has an essential role in the development and homeostasis of an organism. One such gene in Arabidopsis is ALTERED MERISTEM PROGRAM1 (AMP1), which encodes a putative carboxypeptidase that localizes to the endoplasmic reticulum (Helliwell et al. 2001; Li et al. 2013). A double mutant of AMP1 and its close relative LAMP1 show a highly pleiotropic phenotype that includes abnormal embryogenesis, an occasional increase in the number of cotyledons, loss of apical hook formation, enlarged shoot apical meristem (SAM), a short plastochron, early flowering, small organ size, sterility, early leaf senescence, reduced apical dominance, and occasional fasciation (Chaudhury et al. 1993; Griffiths et al. 2011; Nobusawa et al. 2021). Furthermore, the amp1 mutant shows modulated abscisic acid sensitivity (Shi et al. 2013). The double mutant of plant-specific cytochrome P450 family members, CYP78A5 and CYP78A7, showed very similar pleiotropic phenotypes, and genetic interaction between AMP1/LAMP1 and CYP78A5/CYP78A7 has been demonstrated in some traits (Nobusawa et al. 2021).

The biochemical function of AMP1 has not been elucidated. Li et al. (2013) reported that AMP1 is involved in miRNA-mediated translational repression, whereas other studies have shown that AMP1 may act as a carboxypeptidase to synthesize a novel bioactive compound partly because AMP1 acts in a non-cell-autonomous manner (Kong et al. 2015; Nobusawa et al. 2021).

Several phenotypes observed in an amp1 lamp1 mutant are related to cell proliferation/cell activity and SAM function, suggesting a fundamental function of AMP1/LAMP1 in plant development (Huang et al. 2015). Meanwhile, the early flowering phenotype of the amp1 mutant has not been investigated in detail. Controlling the flowering time in response to field conditions is very important for producing offspring and maintaining species, and fine and complex systems...
Regulate the flowering time. The flowering time is also important in crop production, and various varieties, in a wide range of flowering times, have been bred for most crops to facilitate adaptation to local environments. The flowering of the long-day plant Arabidopsis thaliana is regulated by not only the photoperiod pathway but also age, ambient temperature, gibberellin, vernalization, and autonomous pathways (Teotia and Tang 2015). The florigen FLOWERING LOCUS T (FT) protein is synthesized in leaf phloem during the evening under long-day conditions and moves to shoot apices to induce flower development. FT is a central floral regulator involved in most flowering pathways. Thus, we first examined FT expression to investigate the mechanism of early flowering in the amp1 mutant.

The amp1-1 (CS8324) and ft-10 (CS9869) mutants were obtained from the Arabidopsis Biological Resource Center. Seeds were sown on Jiffy-7 peat pellets and kept for three days in the dark at 4°C. Plants were grown under long-day conditions (16 h light/8 h dark, 100 μmol m⁻² s⁻¹) at 22°C. Total RNA was extracted from whole aerial-part tissues using the TRI reagent (Molecular Research Center). After DNase I (TaKaRa) treatment at 37°C for 30 min, first-strand cDNA was synthesized from 500 ng total RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO). qPCR was performed using a KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and a Rotor-Gene Q 2PLEX thermocycler (Qiagen). The primers used for RT-qPCR analyses are listed in Supplementary Table S1.

Figure 1A shows the growth of the null mutant of AMP1 (amp1-1) under long-day conditions. amp1-1 produces small leaves with apparent short plastochrons. The wild-type (Col-0) species bolted about 20 days after germination (DAG), whereas amp1-1 bolted about 11 DAG, confirming the very early flowering phenotype of the amp1 mutant (Figure 1A).

Next, we examined the change in FT expression from early germination to bolting in wild-type and amp1-1 using RT-qPCR (Figure 1B). FT expression was measured at zeitgeber time (ZT) 15. The FT expression level was high even from 5 DAG and later in wild-type because long-day conditions induced FT expression. The expression level of FT in the amp1-1 mutant was 2.4-fold higher on average than that observed in wild-type, and this high expression level was retained until bolting. Overexpression of FT substantially promotes bolting even under long-day conditions (Kardailsky et al. 1999; Kobayashi et al. 1999), suggesting that the early flowering phenotype of amp1-1 is caused by the higher expression of FT than that found in wild-type from early development.

We examined the genetic interactions between AMP1 and FT to determine the contribution of FT to the early flowering phenotype in the amp1 mutant. FT has a close paralog, TWIN SISTER OF FT (TSF), in A.
Although the loss of function mutant of \textit{TSF} (tsf-1) does not show a substantial delay in flowering, the \textit{ft-10} tsf-1 double mutant showed further delayed flowering than \textit{ft-10} under long-day conditions (Yamaguchi et al. 2005). Overexpression of \textit{TSF} dramatically promoted flowering in a similar manner to that observed for overexpression of \textit{FT}, indicating that these proteins have functional redundancy. The mutant allele of \textit{TSF} used in previous reports is tsf-1, which carries a T-DNA insertion in the second intron (Figure 2A). Although tsf-1 has been suggested to be an almost null allele because the \textit{TSF} transcript was not detected in tsf-1 (Yamaguchi et al. 2005), we decided to use a more assured null allele for the analysis. Thus, we generated a null mutation of \textit{TSF} using the CRISPR/Cas9 system for a definitive study of the contribution of the \textit{FT} pathway.

To generate the tsf mutant, \textit{A. thaliana} plants were transformed with \textit{Agrobacterium tumefaciens} strain EHA-105 harboring the pK11.1R vector (Tsutsui and Higashiyama 2017) with a gRNA sequence (GGT CAC TTA TGG CCA TAG AG) targeting \textit{TSF} via the floral dip method. We obtained tsf-2, a null tsf mutation with a single nucleotide insertion in the first exon that causes a frameshift (Figure 2A), and established the \textit{ft-10} tsf-2 double mutant. We then measured the bolting time of this line under long-day conditions (Figure 2B). Bolting time is indicated by the number of days to bolting and not by the leaf number because the amp1 mutant has a short plastochron. Although wild-type bolted at a median of about 22 DAG, the \textit{ft-10} tsf-2 mutant bolted 47 DAG, confirming the lengthy delay in bolting by the \textit{ft tsf} mutant.

Double RNAi lines of \textit{Heading date3a} (Hd3a) and \textit{RICE FLOWERING LOCUS T1} (RFT1), which are coorthologs of \textit{FT} in rice, were shown to not flower even 300 days after sowing, suggesting that the \textit{FT} pathway is essential for flowering in rice (Komiya et al. 2008). Our results using the \textit{ft-10} tsf-2 mutant confirm previous observations that there is an \textit{FT}-independent flowering pathway, although the \textit{FT} pathway is critical in \textit{Arabidopsis}.

The \textit{amp1-1} ft-10 tsf-2 triple mutant bolted at a median of 47 DAG, which was not statistically different from that of the \textit{ft-10} tsf-2 mutant, suggesting that the early flowering phenotype of the \textit{amp1} mutant is dependent on the \textit{FT} pathway (Figure 2B). However, in contrast to \textit{FT}, the expression level of \textit{TSF} was not statistically different at 5 and 8 DAG between wild-type and the \textit{amp1} mutant, suggesting that the contribution of \textit{TSF} to the early flowering phenotype of the \textit{amp1} mutant may be limited.

Thus, these observations suggest that elevated \textit{FT} expression during the early stage of growth contributes to the early flowering phenotype of the \textit{amp1} mutant. \textit{FT} is a central floral regulator and various flowering pathways regulate its expression. To investigate a flowering pathway that elevates \textit{FT} expression in the \textit{amp1} mutant, we examined the expression of several genes that regulate \textit{FT} expression.

\textit{CONSTANS} (CO) is the key transcriptional activator for \textit{FT} induction in promoting long-day-mediated flowering (Samach et al. 2000; Song et al. 2012). Under long-day conditions, CO protein induces \textit{FT} expression through coincidence of upregulation of mRNA level and protein stabilization by the light signal (Suárez-López et al. 2001; Valverde et al. 2004). The \textit{CO} expression level was high throughout the experiment for wild-type under long-day conditions. Although higher expression of \textit{CO} was observed 8 and 12 DAG in the \textit{amp1} mutant in comparison with wild-type but not 5 DAG, expression of \textit{FT} was higher even 5 DAG in the \textit{amp1} mutant, suggesting that elevated \textit{CO} expression is not the primary

\begin{figure}[h]
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\caption{The early-flowering phenotype of the \textit{Arabidopsis} \textit{amp1} mutant is dependent on the \textit{FT/TSF} pathway. (A) Schematic diagram of \textit{TSF} and mutation sites. (B) Days to bolting under long-day conditions, \textit{n}=48. Data were analyzed by one-way ANOVA using the Tukey test (\textit{p}<0.05).}
\end{figure}
cause of elevated FT expression in the amp1 mutant (Figure 3).

FLOWERING LOCUS C (FLC) is a transcriptional repressor for FT expression. FLC is involved in the vernalization and autonomous pathways (Blázquez et al. 2001). Interestingly, the expression level of FLC was very low from 5 DAG to bolting in the amp1 mutant, raising the possibility that this low expression level of FLC contribute to the upregulation of FT expression in the amp1 mutant.

We examined the expression of other regulators of FT expression; however, no good candidate that upregulates FT expression in the amp1 mutant was identified. For example, expression levels of the transcriptional repressors of FT, SHORT VEGETATIVE PHASE (SVP) (Hartmann et al. 2000), SCHLAFLMUTZE (SMZ) (Mathieu et al. 2009), TEMPRANILLO 1 (TEM1) (Matías-Hernández et al. 2014), and EARLY FLOWERING3 (ELF3) (Nieto et al. 2015), in the amp1 mutant were similar to or higher than those in wild-type, thus inconsistent with the elevated FT expression in the amp1 mutant (Supplementary Figure S1).

We examined the expression of another important floral regulator gene, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1). The SOC1 expression level was higher in amp1-1 than in wild-type from 5 DAG to bolting (Figure 3). The elevated expression level of SOC1 may contribute to the early flowering of amp1-1. However, SOC1 expression is reportedly enhanced by FT. Taking into consideration that genetic analysis suggested that the early flowering phenotype of amp1 is dependent on FT (Figure 2), it is more likely that SOC1 contributes to the early flowering phenotype in amp1 via upregulation by FT.

In this study, it is revealed that AMP1 negatively regulates flowering via the regulation of FT expression. Furthermore, our study suggests that FLC is a possible component regulating FT expression via AMP1. FT is associated with a number of flower-promoting pathways, whereas FLC is involved in the autonomous and vernalization pathways. Therefore, AMP1 probably functions to prevent the misactivation of such floral induction pathways. Although only long-day conditions were used for floral induction experiments in this study, examining this process under different conditions should help to reveal the precise contribution of AMP1 to floral induction. FLC expression is regulated in an epigenetic manner (He 2009). Thus, the involvement of AMP1 in extremely pleiotropic phenomena raises the possibility that AMP1 regulates expression of FLC and other genes downstream of AMP1 through an epigenetic regulator(s).

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Author contribution
T.N., H.Y., and M.K. conceived and designed the research; T.N. and H.Y. performed the experiments; and T.N. and M.K. wrote the manuscript.

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Conflict of interest
None declared.

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