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Ecotype-Dependent Genetic Regulation of Bolting Time in the *Arabidopsis* Mutants with Increased Number of Leaves

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Leaves are the major biomass-producing organs in herbaceous plants and mainly develop during vegetative stage by activities of shoot apical meristem. There is a strong correlation between leaf number and bolting, a characteristic phenotype during the transition to reproductive phase in *Arabidopsis thaliana*. In order to study interactions between leaf number and bolting, we isolated a Landsberg *erecta*-derived mutant named *multifolia1* (*mfo1*) that produces increased number of leaves and bolts at the same time as the wild type. Through positional cloning and allelism test, *mfo1* was found to be an allele of a previously reported mutant, *altered meristem program1-1* (*amp1-1*) that is defective in a glutamate carboxypeptidase and bolts earlier than its wild type, Columbia ecotype, with the increased number of leaves. The bolting time differences between *mfo1* and *amp1*, despite the same phenotype of many leaves, suggest the existence of genetic factor(s) differently function in each ecotype in the presence of *mfo1*/*amp1* mutation.

Keywords: Biomass, leaf number, bolting, plastochron, *multifolia*, *altered meristem program*

Plant biomass is a major resource of cellulose-based biofuel production [21]. Plant structure and morphology affects the plant biomass and yield [24]. In herbaceous plants, leaves are the main organ that determines the plant biomass. Plant structures are elaborated by the activities of shoot apical meristems (SAM) and root apical meristems (RAM). Particularly, the leaf development is regulated by the activities of shoot apical meristem. Leaf numbers are determined by spacing (phyllotaxy) and timing (plastochron) patterns of leaf initiation at the flanks of the shoot apical meristem. Each plant species has its unique spacing and timing patterns to develop its typical morphology. While phyllotaxy defines the geometrical arrangement of leave along the stem, plastochron indicates the time interval between initiations of two sequential leaves. Molecular mechanisms of phyllotaxy are recently explained in detail with accumulation and distribution of auxin at SAM [17, 18]. Cytokinin signaling is also implicated in this leaf spacing pattern regulation [4].

Several mutants with altered plastochron (*i.e.*, altered leaf number) have been isolated; rice *plastochron1* (*pla1*) and *plastochron2* (*pla2*), maize *terminal ear1* (*te1*), and *Arabidopsis altered meristem program1* (*amp1*), *phytochrome b* (*phyb*), *serrate* (*se*) and *argonaut1-27* (*ago1-27*) [2, 9, 14–16, 22, 23]. *phyb*, *se* and *ago1-27* mutants show longer plastochron than the wild type while the others display shortened plastochron. Studies on these mutants revealed that plastochron is nonautonomously regulated by the activities of the genes expressed in leaf primordia or the SAM periphery [9, 14, 23]. These genes include the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, miR156, a cytochrome P450 gene (*CYP78A5*), and the *AMP1* gene in *Arabidopsis*. The *SPL* genes are the targets of miR156 and suppression of the *SPL9* gene expression by overexpressing miR156 shortens plastochron length, resulting in increased leaf numbers in *Arabidopsis* [23]. *AMP1* encodes a putative glutamate carboxypeptidase and mutations in *AMP1* caused the high accumulation of *CYP78A5* transcript at the shoot apical region, which appears to be correlated with the big SAM size of *amp1* mutants [5]. The miR156-SPL pathway and the *AMP1*/*CYP78A5*-mediated pathway appear acting independently in plastochron regulation [23].

At vegetative stage, *Arabidopsis* grows rosette leaves that are the results from repetitive leaf production from leaf primordia at the flanks of the shoot apical meristem without node elongation. Turning into reproductive stage, *Arabidopsis* SAMs develop into floral buds and dramatic node elongation takes place to give rise to an inflorescence, or bolting stem [10]. This bolting process is tightly regulated by environmental stimuli and developmental programs. For example, short day conditions (*i.e.*, 8 h light/16 h dark) favor vegetative growth (*i.e.*, rosette leaf production) and

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long day conditions (*i.e.,* 16 h light/8 h dark) promote reproductive growth (*i.e.,* bolting and flowering) in *Arabidopsis*. It is well-known that rosette leaf numbers are strongly correlated with the bolting and flowering program in *Arabidopsis* [11]. Despite this strong correlation, mechanisms of the correlation between leaf number and bolting time are not well-understood.

In order to study interplay between leaf number and bolting, we mutagenized Landsberg *erecta* (L*er*) ecotype seeds of *Arabidopsis thaliana* with ethyl methane sulfonate (EMS) to produce the M1 generation. M1 generation seedlings were grown under the conditions previously reported [13, 19]. Seedlings of the following M2 generation were screened and a mutant with increased number of leaves was isolated and named *multifolia 1* (*mfo1*) (Figs. 1A and 1C). We further characterized the bolting timing of *mfo1* grown under long day conditions (16 h light/8 h dark) at 22°C. *mfo1* bolting time was almost the same (15.71±0.29 days after germination (DAG)) as the wild type (15.00±0.00 days DAG) (Table 1). A known mutant with many leaves, *amp1-1* is known to bolt earlier than the wild type [2]. Therefore, we concluded that *mfo1* is a different mutant from *amp1-1* in that bolting time in *mfo1* is not affected by disrupted plastochron.

To examine if *mfo1* is dominant or recessive and if *mfo1* is caused by a single mutation, *mfo1* was crossed to L*er* and F1 generation was obtained. Tested all 27 F1 seedlings showed the wild type phenotype indicating that the *mfo1* mutation is recessive. The following F2 seedlings were segregated for the wild type and mutant phenotype at an ~ 3:1 ratio (131:42). This result suggests that *mfo1* is a single gene-defected mutation.

To identify the gene responsible for the *mfo1* phenotypes, we took positional cloning approach. The

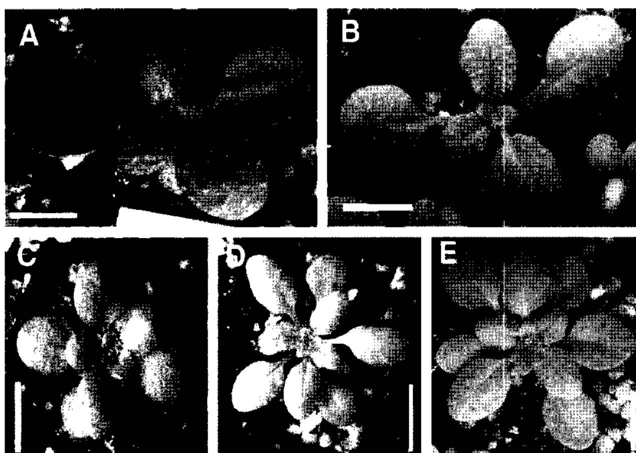

Fig. 1. Morphological phenotypes of mfo1 and related plants.
A. *Ler*, wild type for *mfo1*; **B**. Col, wild type for *amp1-1*; **C**. *mfo1*; **D**. *amp1-1*; **E**. F1 seedling from *amp1-1* x *mfo1*. All seedlings were 16-day old grown under long day conditions (16 h light/8 h dark) at 22°C. Bars=0.5 cm

Table 1. Comparisons of leaf numbers at bolting and days to bolting in each genotype.

Genotype	Leaf number	Bolting (DAG)
L*er*	5.70±0.15	15.00±0.00
mfo1 (L*er*)	12.28±0.78	15.71±0.29
pt1 (L*er*)	9.90±0.38	15.00±0.00
Col	9.00±0.19	20.00±0.00
amp1-1 (Col)	11.13±0.28	16.25±0.41
Salk_087303 (Col)	13.17±0.65	17.43±0.20
amp1-1 x *mfo1* F1	13.00±0.37	11.78±0.22

Plants were grown under long day conditions (16 h light/8 h dark) at 22°C and leaf numbers were counted at bolting. The experiment was repeated three times and all gave similar results. Numbers are averages with standard errors (n≥7). Mutant background ecotypes are shown in parentheses. DAG, days after germination.

mapping population was generated by crossing *mfo1* to Columbia ecotype and allowing F1 plants to be self-crossed. Seedlings with many leaves from the resulting F2 population were selected and their genomic DNAs were isolated as previously described [8].

Initial mapping with SSLP markers suggested that the *mfo1* gene is linked to nga112 (5'-CTCTCCACCTCCTCCA GTACC-3' and 5'-TAATCACGTGTATGCAGCTGC-3') located on the bottom of chromosome 3 (Fig. 2A). We developed new SSLP markers using Columbia (Col)/L*er* insertion/ deletion polymorphism information at the *Arabidopsis* Information Resource (http://www.arabidopsis.org) around the linked SSLP marker, nga112 [6]. With the new SSLP markers, T32N15-61K (5'-GTGCACGACGGATCTGATT-3' and 5'-CGGAGTAGGCTTTGTGGAAT-3'), CIW4 (5'-GT TCATTAAACTTGCGTGTGT-3' and 5'-TACGGTCAGAT TGAGTGATTC-3'), and F28P10-46K (5'-TTAGAGGAC ATTCCGGTTCG-3' and 5'-ACGATCCAAGTGGAACCA AC-3'), we were able to narrow down the *MFO1* locus between F28P10-46K and nga112 with a tight linkage to F28P10-46K (Fig. 2A). While surveying candidate genes around the region, we found that the *AMP1* gene (At3g54720, a putative glutamate carboxypeptidase) is located in this region. Surprisingly, despite the different phenotype (e.g., bolting time difference), *mfo1* contained a mutated *AMP1* gene with a nucleotide change (G to A) at 758th nucleotide from the start codon, ATG (Fig. 2B). This sequence change causes a change of tryptophan at 253rd amino acid to stop codon in the MFO1/AMP1 protein. To confirm the cloning, we carried out allelism test by examining the phenotypes of F1 seedlings from a cross of *mfo1* to *amp1-1* obtained from the *Arabidopsis* Biological Resource Center (ABRC). The F1 seedlings also showed increased number of leaves (Fig. 1E and Table 1), suggesting that *mfo1* is an allele of *amp1-1* and confirming that we cloned the correct gene responsible for the *mfo1* mutant phenotype.

Despite the same phenotype of "many leaves" between *mfo1* and *amp1-1*, the phenotype of bolting time was

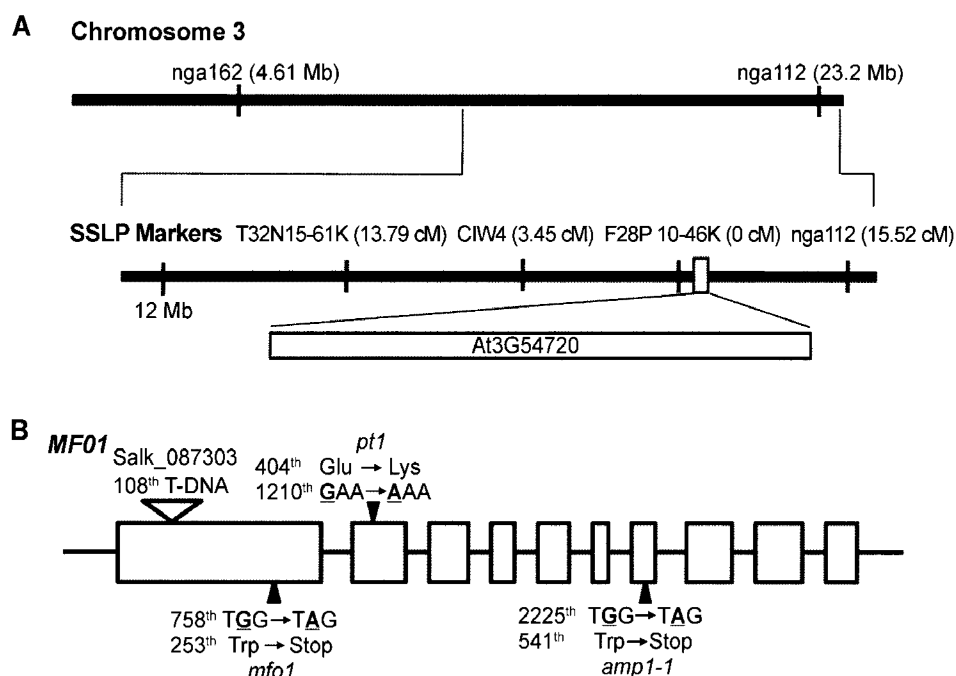

Fig. 2. Molecular cloning of MFO1.
MFO1 locus is located on the bottom of chromosome 3 closely linked to a SSLP markers, F28P10-46K. Twenty nine F2 seedlings were used for the mapping. cM, centimorgan to indicate the distance between the maker and the MFO1 gene. Structure of the MFO1 gene and mutations in mfo1 and its alleles. Exons and introns are shown in boxes and lines, respectively. Arrowheads indicate the position of mutations in mfo1 and other alleles. Solid arrowheads represent point mutations and a hollow arrowhead marks T-DNA insertion position in Salk_087303. Gene and protein sequences can be found at The Arabidopsis Information Resource (http://www.arabidopsis.org) with the ID number of At3G54720.

different between *mfo1* and *amp1-1* when compared with respective wild type control; *mfo1* bolts at the same time as its background, L*er* ecotype and *amp1-1* bolts earlier than its background, Col ecotype (Table 1 and Fig. 3). We assumed that this is because of mutant background differences between *mfo1* (L*er* background) and *amp1-1* (Col background). Thus, other *mfo1* alleles were obtained from the ABRC; *primordia timing 1* (*pt1*) and Salk_087303, whose background is L*er* and Col, respectively. We evaluated the leaf numbers and days to bolting in all the alleles along with the wild

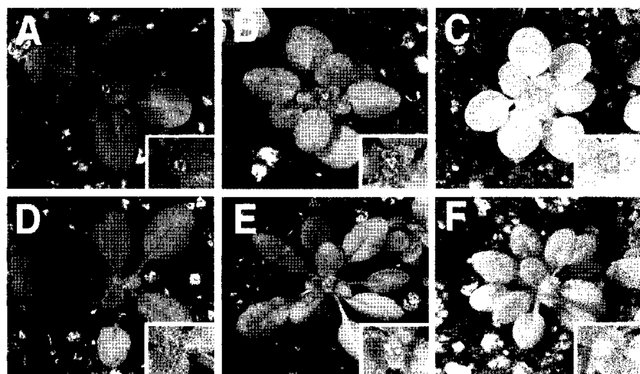

Fig. 3. Leaf number and bolting time differences in each genotype.
A. *Ler*; **B**. *mfo1*; **C**. *pt1*; **D**. Col; **E**. *amp1-1*; **F**. Salk_087303. Plants were grown under long day conditions (16 h light/8 h dark) at 22°C. Insets are to show the floral bud formation, the characteristic event of bolting initiation.

types. As previously reported by Chaudhury *et al.* (1993), *amp1-1* made more leaves upon bolting and took fewer days for bolting than the wild type (Col); 11.13±0.28 vs. 9.0±0.19 leaves and 16.25±0.41 vs. 20.00±0.00 days (Table 1, Figs. 3D and 3E). Similarly, Salk_087303, whose background is Col, also required more leaves and fewer days for bolting than the wild type (Col). Salk_087303 produced 13.17±0.65 leaves and took 17.43±0.20 days for bolting (Table 1, Figs. 3D and 3F). However, two L*er* -derived mutants, *mfo1* and *pt1* bolted almost at the same number of days (15.71±0.29 in *mfo1* and 15.0±0.00 in *pt1*) as L*er* (15.00±0.00 days) with increased number of leaves upon bolting (12.28±0.78 in *mfo1* and 9.90±0.38 in *pt1* vs. 5.70±0.15 in L*er*) (Table 1, Figs. 3A, 3B, and 3C). The significance of leaf number and bolting time differences between the wild types and the mutants were confirmed by statistical analysis. First, the leaf number differences between the wild types and the mutants were substantially significant (student's *t*-test, $P<0.005$). In addition, bolting time was statistically the same between L*er* and *mfo1* or *pt1* (student's *t*-test, $P>0.005$), but was significantly different between Col and *amp1-1* or Salk_087303 (student's *t*-test, $P<0.005$).

These results above suggest that there is/are genetic factor(s) that make(s) this difference between the Col- and the L*er* -derived mutants. The genetic factor(s) was/were capable of shortening number of days to bolting in response to shortened plastochron in Col, but not in L*er*.

ampl-1 x *mfo1*-derived F1 seedlings displayed 13.00±0.37 leaves and took 11.78±0.22 days for bolting. The F1 seedling phenotypes of increased leaf number at bolting and decreased number of days to bolting are similar to those in Col-background *ampl-1*. Thus, the genetic factor(s) in Col could be dominant to that/those in L*er*.

There are some examples of genetic factors differently acting in Col and L*er*, particularly among the floral transition genes. *FLOWERING LOCUS C* (*FLC*), the central player for the transition represses the expression of genes for the floral transition and its own expression is promoted by *FRIGIDA* (*FRI*) [1]. Natural variations in *FRI* and *FLC* contribute to flowering time differences in *Arabidopsis* ecotypes [3, 7, 12, 20]. Indeed, there are natural variations of *FRI* and *FLC* between Col and L*er* [7]. Bolting in *Arabidopsis* is characteristic phenotype during the transition from the vegetative growth to the reproductive growth for flowering. Therefore, *FRI* and *FLC* could be good candidates of the genetic factor(s) that determine(s) the bolting time in response to plastochron changes. It should be noted that the bolting time difference is not likely due to the nature of the mutations. Both *mfo1* and *ampl-1* has a point mutation (G to A) and result in early termination (540-amino acid polypeptide and 252-amino acid polypeptide from the original 705-amino acid polypeptide, respectively) (Fig. 2B). *pt1* also has a point mutation causing a amino acid change at 404th position (Glutamic acid to Lysine) and Salk_087303 has T-DNA insertion at 1st exon, most likely a null mutant (Fig. 2B). Thus, correlation does not seem to exist between the nature of the mutations and the bolting time differences.

In this study, we identified the *mfo1* mutant that produces more leaves than the wild type and bolts at the same time as the wild type. The *MFO1* gene was cloned through positional cloning approach and was found to encode a putative glutamate carboxypeptidase, the previously reported *AMP1* gene. Interestingly, mutant alleles of the gene in the Col ecotype bolted earlier than Col, while L*er*-background alleles bolted at the same time as L*er*. These results suggest the existence of genetic factor(s) differentiated in Col and L*er*. This genetic variation might cause the alterations in the sensitivity of "leaf number counting clock" to the *mfo1/amp1* mutations in each ecotype. This genetic factor(s), thus, could play important roles in linking between plastochron and bolting. The current view of regulation of plastochron supports the existence signal from leaf primordia to SAM where major regulations of bolting take place [9, 14, 23]. Our study and future identification of such genetic factor(s) will help understand how plastochron-regulated leaf number is recognized by the SAM and how the regulation is integrated into the developmental programs in the SAM. In addition, as leaves are the major parts determining total biomass in herbaceous plants, understanding of regulation of leaf numbers and bolting will be helpful in manipulating plant biomass for biofuel production.

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