The Mechanism of Cell Cycle Arrest Front Progression Explained by a KLUH/CYP78A5-dependent Mobile Growth Factor in Developing Leaves of Arabidopsis thaliana

Toshiya Kazama¹, Yasunori Ichihashi²,³, ⁵, Satoshi Murata⁴,⁶ and Hirokazu Tsukaya²,³, ∗

¹Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashi-hiroshima, 739-8526 Japan
²Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo, 113-0033 Japan
³National Institute for Basic Biology, National Institutes of Natural Sciences, Okazaki, Aichi, 444-8585, Japan
⁴Department of Computational Intelligence and Systems Science, Interdisciplinary Graduate School of Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama, 226-8502 Japan
⁵Present address: Department of Bioengineering and Robotics, Tohoku University, 6-6, Aobayama, Sendai, 980-8579 Japan
⁶Present address: Section of Plant Biology, University of California, Davis, CA 95616, USA

∗Corresponding author: E-mail, tsukaya@biol.s.u-tokyo.ac.jp; Fax, +81-3-5841-4047

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The size and shape of leaves are influenced by the progression of the cell cycle arrest front (AF). However, the AF progression with leaf growth has not been characterized quantitatively. Moreover, the mechanism linking AF progression and genetic factors is not fully understood. Recently, it was proposed that a KLUH/CYP78A5 (KLU)-dependent signal acts as a mobile growth factor (MGF) for cell proliferation and controls the lateral organ size of Arabidopsis. This study examines this hypothesis under the assumption that the gradient field dynamics of the KLU-dependent MGF provide the mechanism of AF progression using molecular markers and computer simulations. First, we measured the exact AF position with leaf growth using the pCYCB1;1::CYCB1;1::GUS expression pattern, which visualizes mitotic cells. As a result, we found that the AF stayed at an almost constant distance from the leaf blade base (stage 1) and then progressed towards the base and disappeared relatively quickly (stage 2), which previously had not been identified. Secondly, we showed that KLU may generate a concentration gradient of MGF in leaves, if KLU really controls cell division via the biosynthesis of MGF, by comparing the expression patterns of pKLU::GUS and pCYCB1;1::CYCB1;1::GUS. Finally, we built a simulation model using a diffusion equation with a decay term, in which the rate of MGF production estimated from the KLU expression level was included in the boundary condition. Our simulation model successfully reproduced both stages 1 and 2 of the AF, suggesting that the proposed mechanism does explain the AF progression under some restricted conditions.

**Keywords:** Arabidopsis thaliana • Cell cycle arrest front • KLUH/CYP78A5 • Leaf development • Mobile growth factor • Simulation model.

**Abbreviations:** AF, arrest front; DAS, days after sowing; GUS, β-glucuronidase; KLU, KLUH/CYP78A5; MGF, mobile growth factor; 4MU, 4-methyl-umbelliferone; RAM, root apical meristem; SAM, shoot apical meristem.

**Introduction**

The control of the size of multicellular organisms is one of the fundamental questions in developmental biology. Plant leaves show determinant growth, indicating that the number and size of cells are important determinants of the final leaf size. Additionally, cell proliferation and cell expansion within leaves are coordinated at a multicellular level (Tsukaya 2002, Beemster et al. 2003, Tsukaya 2003, Tsukaya 2005, Horiguchi et al. 2006, Tsukaya 2006, Tsukaya 2008). The leaf primordia show a gradient of cell proliferation along the proximal–distal axis, and cell expansion occurs from the tip to the base of leaf primordia (Donnelly et al. 1999, Ferjani et al. 2007). Concerning the coordination between cell proliferation and cell expansion, the boundary where the switch from proliferation to expansion occurs within leaf primordia is important for regulating leaf size. This boundary is known as the cell cycle arrest front (AF) (Donnelly et al. 1999, Nath et al. 2003, White 2006).

The AF can be divided into the primary and secondary AF (White 2006). In leaf development, cell division initially occurs throughout the leaf primordium. Then, the primary AF, which is the AF of the general proliferation of cells, appears and progresses with basiplastic polarity. Following the primary AF, the secondary AF, which is the AF of stomatal and vascular precursors, appears and progresses. Class II TCP genes, CIN in Antirrhinum (Nath et al. 2003) or TCP genes (TCP2, 3, 4, 10
and 24) in Arabidopsis (Palatnik et al. 2003, Palatnik et al. 2007, Schommer et al. 2008), influence the shape of the primary AF and its progression speed. A loss-of-function mutation of CIN results in uneven growth of the leaf surface, because the longer growth of the marginal regions compared with the medial regions causes a concave shape and delays the AF in mutants. Recently, TCP4 was shown to induce miR396 and repress GROWTH REGULATING FACTOR (AtGRF) activity, and the repression of AtGRF by miR396 was suggested to be a component of the AF (Rodriguez et al. 2010). In contrast, the progression of the secondary AF is affected by the PEAPOD (PPD) gene (White 2006). The loss-of-function mutant of PPD develops large, dome-shaped leaves, because of the additional growth caused by the delayed secondary AF.

Recently, molecular genetic studies have identified several genes involved in cell proliferation during leaf development. For example, genes encoding the AP2-type transcription factor AINTEGUMENTA (ANT) (Mizukami et al. 2000, Horiguchi et al. 2009), a nuclear factor of unknown function, ARGOS (Hu et al. 2003), the transcription co-activator ANGUSTIFOLIA3 (AN3) and one of its interaction partners AtGRFS (Horiguchi et al. 2005), the C2H2 zinc finger protein JAGGED (JAG) (Ohno et al. 2004), a subunit of the Mediator complex STRUWWELPETER (SWP) (Autran et al. 2002) and KLUH/CYP78A5 (KLU) (Anastasiou et al. 2007) are constituent factors positively controlling cell proliferation in leaves. In contrast, the short polypeptide ROTUNDIFOLIA4 (ROT4) (Narita et al. 2004), the RING-finger protein BIG BROTHER (BB) (Disch et al. 2006), two putative ubiquitin receptors DA and DA-RELATED (DAR) (Li et al. 2008), several members of class II TCP genes that are targeted by microRNA (miRNA) JAW (Palatnik et al. 2003) and the basic helix–loop–helix transcription factor SPATULA (SPT) (Ichihashi et al. 2010) are negative regulators of cell proliferation. Although the AF-related molecular networks are beginning to be revealed (Hay et al. 2004, Krizek 2009, Rodriguez et al. 2010), the mechanism linking these genes and AF progression is not well understood.

Among these cell proliferation regulators, KLU is postulated to regulate a mobile growth factor (MGF). Anastasiou et al. (2007) proposed a model by which the MGF promoted cell proliferation and determined organ size. Specifically, the MGF is generated at the KLU expression domain, and then diffuses throughout the organ, homogenously. As the organ grows, the MGF concentration decreases because the KLU expression domain is restricted compared with the entire organ. Finally, when the MGF concentration falls below some critical threshold, the cells stop dividing and differentiate into post-mitotic cells. However, this function of KLU has not yet been characterized or examined in detail in leaves.

The morphogen gradient model is one of the important traditional ideas for examining pattern formation in cell differentiation in multicellular organisms (Wolpert 1969, Wolpert 1996). This model can represent varieties of patterns that form in nature (Gilbert 2000). Here, we examined the idea that the AF progression in leaves is controlled by KLU-dependent MGF (Fig. 1). Although it has been suggested that the distribution of MGF is largely homogenous throughout a petal, resulting in an obscure gradient in proliferation (Anastasiou et al. 2007), we postulated that KLU has the potential to produce a gradient of MGF in a leaf primordium and controls AF progression in leaves.

First, we measured the exact AF position from the blade base based on the pCYCB1;1::CYCB1;1::GUS expression pattern, using an image processing method, to determine the detailed behavior of the AF. Then, we showed that KLU generated an MGF gradient field in leaves by comparing the spatiotemporal patterns of KLU expression and a cell proliferation distribution in leaves using pKLU::GUS and pCYCB1;1::CYCB1;1::GUS. Thirdly, to investigate the mechanism by which the MGF gradient controls AF progression, we constructed a simulation model of AF progression, described using a diffusion equation with a decay term. We found that within a certain parameter range, our model could reproduce the observed AF progression pattern.

### Results

#### Spatiotemporal pattern of AF progression

No reported study of the AF has quantitatively characterized AF progression in accordance with leaf growth (Donnelly et al. 1999, Nath et al. 2003, White 2006). To understand further the AF progression mechanism, it is necessary to characterize the

![Fig. 1 Schematic of the AF progression mechanism explained by the KLU-dependent MGF.](image-url)
AF progression quantitatively. For example, to know the dynamics of AF progression, such as the timing of its appearance and disappearance, the exact distance of the AF from the leaf blade base at each developmental stage should be measured. For this observation, we define the AF as the position where proliferating cells obviously exist along the proximal–distal axis of the leaf blade, which corresponds to the primary AF.

To determine the timing of the appearance and disappearance of the AF, we observed changes in the distribution of mitotic cells during leaf development using a pCYCB1;1::CYCB1;1::GUS transgenic line (Ler) (Tamaki et al. 2009); this can be used to visualize cells at the G2/M phase of the cell cycle, based on the staining of β-glucuronidase (GUS)-positive spots. Mitotic cells were observed throughout the leaf primordium 3 and 4 days after sowing (DAS; Fig. 2A, B). At 5 DAS, the distribution of mitotic cells in the leaf primordium showed a gradient, as described previously (Donnelly et al. 1999). This gradient occupied three-quarters of the leaf blade (Fig. 2C). At 6 and 7 DAS, a region of proliferation was observed one-third and one-sixth of the length, respectively, of the leaf blade at the leaf primordium (Fig. 2D, E). At the leaf primordium at 8 DAS, no mitotic cell was observed in the leaf blade (Fig. 2F). Consequently, the AF appears 4–5 DAS and disappears 7–8 DAS.

To determine the exact AF position, we used an image-processing method, applied to GUS staining spots using pCYCB1;1::CYCB1;1::GUS (details are given in the Materials and Methods and Fig. 3). The estimated average AF positions and leaf lengths during leaf development are shown in Fig. 4. The AF remained within 100 µm of the leaf blade base, suggesting that the AF stays at a constant distance from the leaf blade base during leaf development.

In summary, these results showed that there were two distinct stages in AF progression: stage 1 (4–7 DAS) and stage 2 (7–8 DAS). During stage 1, the AF appeared 4–5 DAS and maintained the same position until 7 DAS. During stage 2, the AF moved quickly towards the leaf base and then disappeared.

Comparison of the spatiotemporal expression patterns of pKLU::GUS and pCYCB1;1::CYCB1;1::GUS

Previously, KLU has been shown to promote the growth of leaves, floral organs, ovules and seeds, and to prolong the plastochron (Anastasiou et al. 2007, Wang et al. 2008, Adamski et al. 2009). KLU is a positive cell proliferation regulator in petals and leaves, although the KLU expression pattern is inconsistent with the proliferative region in petals. This suggests that KLU generates an MGF (Anastasiou et al. 2007). However, the expression pattern of KLU in leaves has not been reported in detail (Zondlo et al. 1999, Anastasiou et al. 2007). Thus, it is still
not known whether KLU also generates an MGF in leaves. To examine this possibility, we compared the spatiotemporal patterns of KLU expression and cell proliferation distribution in leaves using pKLU::GUS and pCYCB1;1::CYCB1;1::GUS.

At 3–4 DAS, the mitotic cells were distributed uniformly (Fig. 2A, B) and pKLU::GUS was expressed throughout the leaf primordium, with especially strong expression in the basal region (Fig. 2G, H). At 5–6 DAS, a strong pKLU::GUS expression pattern was observed at the base and both sides of the primordium, but there was no signal in the central area (Fig. 2I, J). In contrast, during the same stage, mitotic cells were observed in the central area of the leaf primordium (Fig. 2C, D). At 7 DAS, no pKLU::GUS signal was detected in the leaf blade (Fig. 2K), although mitotic cells were distributed at the base (Fig. 2E). Neither pKLU::GUS nor pCYCB1;1::CYCB1;1::GUS was found in the leaf blade at 8 DAS (Fig. 2F, L). These observations showed that there was a difference between pKLU expression and the mitotic cell distribution after 5 DAS in the leaf primordium. Additionally, KLU is expressed at the leaf blade base during early development. These results suggest that KLU generated an MGF gradient at the leaf blade base and this appeared to affect AF positioning.

**Simulation of AF progression**

As described before, we found that the behavior of AF showed the two distinct stages: 1 and 2. This behavior of AF could not be explained intuitively by only the characteristic of diffuseness in KLU-dependent MGF. In this study, therefore, we examined how the behavior of AF was explained in the case where the KLU-dependent MGF might be a managing factor for AF positioning. We estimate the condition of MGF characteristics, the diffusion coefficient and the decay coefficient, by computer simulation analysis.

**Temporal expression of KLU in leaf primordia.** The gradient field dynamics depend on the chemical characteristics of the MGF (i.e. the diffusion, decay and production rates). Because the rate of MGF production should be reflected in the level of KLU expression, we first estimated the level of KLU expression per leaf during leaf development. The estimation consisted of two steps: (i) quantification of pKLU::GUS activity by fluorometry; and (ii) estimation of KLU expression using a mathematical model. With this method, we were able to evaluate the KLU expression level per leaf during the developmental stages.

First, we measured the KLU expression using promoter::GUS, because it is difficult to dissect only the KLU expression regions using quantitative real-time PCR. We quantified the pKLU::GUS activity using fluorescence analysis. The plot of the measured pKLU::GUS activity was bell-shaped, with a peak at 6 DAS (Fig. 5).

We then estimated the level of KLU expression from the pKLU::GUS activity, because GUS protein is stable in living mesophyll protoplasts, with a half-life of ~50h (Jefferson et al. 1987). We estimated the KLU expression level using the observed GUS activity with the following procedure. First, by assuming that the rate of GUS protein production was proportional to the KLU expression level and that the decay of GUS protein depended only on its destruction, via its half-life, we obtained the following relationship between GUS protein and the KLU expression level (Goodwin 1965):

\[
\frac{dG}{dt} = r_A L_{KLU} - \lambda G \quad (1)
\]

where \(G\) is the concentration of GUS protein, \(L_{KLU}\) is the level of KLU expression, \(\lambda\) is the decay coefficient of GUS protein, \(r_A\) is the production coefficient and \(T_{hl}\) is the half-life of GUS protein. Then, we assumed that the GUS activity was proportional to the concentration of GUS protein:

\[
G = r_A A \quad (3)
\]

where \(A\) is the GUS activity and \(r_A\) is the proportionality coefficient. Substituting Equation (3) into Equation (1) gives the following equation for \(L_{KLU}\):

\[
L_{KLU}(t) = \frac{r_A}{\lambda} \left( \frac{dA(t)}{dt} + \lambda A(t) \right) \quad (4)
\]
The second assumption is that MGF is produced at the leaf base, diffuses through the leaf blade and decays in each position. The concentration of MGF $c = c(x,t)$ is a function of time $t$ and position $x$. The dynamics of MGF are described by the following equation:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} - Kc$$  \hspace{1cm} (7)

where $D$ is the diffusion coefficient and $K$ is the decay coefficient. The first term represents the diffusion of the signal and the second term represents the decay of the signal. From our observation of the $pKLU::GUS$ expression patterns, $KLU$ is expressed strongly at the leaf base (Fig. 2G–L). Thus, we assumed that the MGF was produced only at the leaf base, and that this was directly proportional to the level of $KLU$ expression, $l_{KLU}$. Consequently, the boundary condition at the leaf base is given by

$$-D \frac{\partial c}{\partial x} = r_l l_{KLU}(t) \quad \text{at} \ x = 0$$  \hspace{1cm} (8)

Moreover, we imposed a no-flux condition at the leaf tip, thus

$$\frac{\partial c}{\partial x} = 0 \quad \text{at} \ x = l(t)$$  \hspace{1cm} (9)

Finally, the AF is assumed to be the position where the concentration of MGF equals a critical value at which cells stop dividing. The MGF concentration distribution is expressed by a gradient field, where the concentration of the signal is maximal at the base and falls to an almost constant value toward the tip, because in our observations, the AF appeared between 4 and 5 DAS. The AF is defined as the position where the MGF concentration exceeds the threshold, while cells stop dividing at the position where the MGF concentration is below the threshold. In the simulation, we chose the concentration at the tip at 4 DAS to be the threshold value $c_{th}$, i.e., $c_{th} = c(l(t_b), t_{th})$ where $t_{th} \approx 4$ DAS, because in our observations, the AF appeared between 4 and 5 DAS. The AF is defined as the position where $c = c_{th}$ holds. If the concentration of MGF at the blade tip exceeds the threshold, the AF position is at the blade tip. Equation (7) was solved using an explicit scheme (Kytht et al. 1997). The simulation was initiated at $t = 3$ DAS, at which the initial concentration of MGF is $c = 0$ at every $x$. The leaf field expansion is represented by extending the $x$-axis with additional mesh points, where the concentration of MGF $c = 0$, to satisfy Equation (6) at time $t$. The program was written in C language. The constant values used in the simulations are listed in the Supplementary table.
whether our model could represent the observed AF progression pattern, we carried out simulations for various values of each parameter. To evaluate whether the simulated AF behavior had the characteristics of the observed AF behavior, we defined the numerical expression of stages 1 and 2. For stage 1, we used the standard deviation (SD) of the AF positions 5, 6 and 7 DAS. The SD of the observed AF was 14 µm. If the SD in a simulation was ≤14 µm, we defined the AF progression to have the characteristics of stage 1. For stage 2, we use the AF position at 8 DAS. The AF position 8 DAS was 0 µm because the AF disappeared at 8 DAS (Figs. 2, 3). If this condition is satisfied, the simulated AF progression has the characteristics of stage 2.

Fig. 6 shows a diagram of the AF behavior in the D–K phase plane. Generally, the AF patterns tend to consist only of stage 1 at low D, and have stage 2 at high D. The AF patterns with both stages appear for a limited range of D and K. (Fig. 7) shows examples of the AF behaviors at different D (K = 0.12 h⁻¹). In all cases, the AF increased gradually until some time and then decreased abruptly. Our model corresponds best with the observations at D = 120 µm² h⁻¹, suggesting that the MGF is a diffusible molecule with specific characteristics. Additionally, the SD of the AF position at 5–7 DAS obtained in the simulation was about 13 µm, which represents both stages 1 and 2 of the AF behavior well.

Discussion

Restricted cell proliferation region in the leaf blade

In plants, new cells are produced actively in tissues called meristems, such as the shoot apical meristem (SAM) and root apical meristem (RAM). Although stem cells divide continuously and provide cells to new organs in the meristems, the size of the meristem and the number of constituent cells are kept constant (Miwa et al. 2009). In contrast, in Arabidopsis leaves, the region showing active cell division is thought to change dynamically, rather than remaining static like the SAM or RAM. The conventional understanding of AF progression is that the AF progresses consistently toward the blade base during leaf development. In this study, we conducted a detailed temporal analysis of the AF positioning with leaf growth. Our findings on AF behavior alter the conventional understanding, namely the AF does not continue to progress but remains at an almost constant position during a certain time period (Fig. 4). This suggests that there is a meristematic region in the blade base, which maintains a constant size, like the SAM or RAM, and undergoes cell proliferation activity during a certain time period.

Mechanism of AF progression

Although many biological and mathematical studies have examined pattern formation in multicellular organisms (Meinhardt 1982, Gilbert 2000, Murray 2002, Berleth et al. 2007), there is no previously reported model of the AF progression in leaves. In this study, we showed that the AF progression can be explained by KLU-dependent MGF gradient dynamics using our model. We evaluated the model using both experimental observations (Fig. 4) and computer simulation (Figs. 6, 7).

It has been suggested that the KLU-dependent MGF diffuses homogenously throughout petals, and KLU does not affect the
spatial dynamics of cell proliferation (Anastasiou et al. 2007). In this study, we focused on leaves and found that KLU was expressed at the leaf base during development. The expression pattern in leaves was different from that in petals, where KLU is expressed in the periphery. This suggests that the MGF generates a concentration gradient in leaves, which is maximal at the base and falls to an almost constant value toward the tip.

Computer simulations are especially important in studies of multicellular pattern formation during organ development, because spatiotemporal processes, such as cell differentiation, can be difficult to grasp intuitively (Berleth et al. 2007). In this study, we constructed a simple simulation model to describe the mechanism of AF behavior under the assumption that KLU might be the managing factor of AF positioning. We found that our model reproduced both stages 1 and 2 of AF positioning, suggesting that the MGF gradient is important for coordinating cell proliferation and expansion in leaves. Moreover, we estimate the condition of MGF chemical characteristics where its gradient field explains the behavior of AF by the computer simulation analysis. Diffusion coefficients have been published for some small molecules in plant tissues (Goldsmith 1966, Richter et al. 1983, Canny 1990, Canny et al. 1994, Bayliss et al. 1996, Kramer et al. 2007), and that of the MGF deduced from our simulation model is classified as the small permeability class in the above-reported range. Alternatively, the characteristics of MGF suggest that some physical and/or biochemical regulation(s) may operate in the signal transduction from MGF to AF behavior. Further biochemical analyses of KLU and MGF are needed to verify our proposed model. Our present study supplies an important basis for this.

Materials and Methods

Preparation of plant material

Seeds were sown on rock wool and the plants were grown on rock wool at 23°C under continuous illumination (Ichihashi et al. 2010). We used pCYCB1;1::CYCB1;1::GUS (Tamaki et al. 2009) and pKLU::GUS transgenic lines (Anastasiou et al. 2007).

Histological observation

Whole leaves were observed using a stereoscopic microscope (MZ16 F) and a Nomarski differential interference contrast microscope (DM4500 B), both from Leica Microsystems (Tokyo, Japan). The leaves were fixed in a formalin–acetic acid–alcohol (FAA) solution, and cleared using a chloral hydrate solution, as described previously (Tsuge et al. 1996). The samples were then photographed under the microscope.

GUS staining

The GUS activity patterns in leaf primordia were determined as described previously (Donnelly et al. 1999).

Quantification of GUS activity

We quantified the pKLU::GUS activity using fluorescence analysis. GUS-specific activity was detected using the procedure described by Jefferson et al. (1987). Based on the observations of pKLU::GUS in leaf primordia (Fig. 2), the first set leaves including the SAM from 3–6 DAS and the first set leaves from 7–8 DAS were used. The numbers of leaves sampled from 3–8 DAS plants was 600, 360, 360, 300 and 160, respectively. The fluorogenic product [4-methyl-umbelliferone (4MU)] was estimated using an Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) using excitation at 365 nm and emission at 455 nm. To obtain detailed differences among the DAS, GUS specific activity data were converted into GUS activity; the GUS specific activity data were multiplied by the total mass of protein in each extract, and then divided by the number of leaves used to obtain each extract.

Measurement of AF position

The process consisted of the following steps. (i) An image of a leaf capturing the promoter::GUS expression pattern was prepared (Fig. 3A). (ii) A binary version of the image was created to distinguish the GUS staining (Fig. 3B). The stained value of each pixel in the image was assigned a value of 1 if the pixel was highlighted and a value of 0 otherwise. (iii) The average stained value of the pixels across the width of the leaf was calculated for each distance from the base of the leaf blade. The value was standardized using the maximum average value and plotted on a graph (e.g. Fig. 3C). (iv) The position of the AF was defined as the position where the average stained value was 1/2 on scanning the graph from the blade tip (Fig. 3C). This approach is simple, but more objective than detection by the naked eye. We used GIMP (The GIMP Development Team, available at http://www.gimp.org/) for image processing, and Microsoft Visual Studio.NET (Microsoft, WA, USA) for calculations. The programs were written in C language.

Supplementary data

Supplementary data are available at PCP online.

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