Short Communication

Increase in vascular pattern complexity caused by mutations in *LHY* and *CCA1* in *Arabidopsis thaliana* under continuous light

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Abstract Circadian rhythms in *Arabidopsis thaliana* (*Arabidopsis*) are controlled by clock components such as LATE ELONGATED HYPOCOTYL (*LHY*) and CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*). Plants with mutations in both *LHY* and *CCA1* (*lhy;cca1*) show a wavy leaf phenotype under continuous light (LL). The circadian clock regulates both the biosynthesis and signaling of plant hormones, including auxin. Auxin plays a key role in vascular pattern formation in leaves. For example, plants with mutations in either *VASCULAR NETWORK DEFECTIVE 3* (*VAN3*) or *FORKED 1* (*FKD1*) exhibit reduced complexity in their leaf vascular patterns. However, the molecular mechanism underlying the decrease in flatness of *lhy;cca1* leaves under LL has not been elucidated. To address this question, the leaf vascular patterns of *lhy;cca1* were compared with those of wild-type, *van3*, and *fkd1* plants under LL. As reported previously, the numbers of areoles and branch points in *van3* and *fkd1* plants grown for 14 days under LL were much lower than those of wild-type plants. In contrast, the numbers of free ends, areoles, and branch points increased in *lhy;cca1*. This is the first demonstration of *Arabidopsis* mutants with increased vascular pattern complexity. Our results suggest that the circadian clock plays a key role in controlling the vascular pattern of leaves.

Key words: Auxin, *CCA1*, circadian clock, *LHY*, vascular patterning.

Plant growth and metabolism are coordinated with the time of day by rhythmic gene expression with an approximately 24-h period (McClung 2006; Niinuma et al. 2007). Flowering time, leaf movement, organ elongation, and stomatal opening are regulated by the circadian clock (de Montaigu et al. 2010; Doherty and Kay 2010; Nozue et al. 2007; Niinuma et al. 2008). LATE ELONGATED HYPOCOTYL (*LHY*) and CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*) are key components of the circadian clock in *Arabidopsis thaliana* (*Arabidopsis*). *LHY* and *CCA1* encode similar transcription factors with a single MYB repeat (Schaffer et al. 1998; Wang and Tobin 1998). A gain-of-function in either gene causes the downregulation of other genes and aberrant rhythmic phenomena (Green and Tobin 1999; Mizoguchi et al. 2002; Schaffer et al. 1998; Wang and Tobin 1998). A double loss-of-function of *LHY* and *CCA1* (*lhy;cca1*) results in severe effects on the circadian rhythm and other output phenomena (Alabadi et al. 2002; Fujiwara et al. 2008; Mizoguchi et al. 2002; Mizoguchi et al. 2005).

lhy;cca1 plants show photoperiod-insensitive early flowering under long-day (LD; i.e., 16 h of light/8 h of dark) and short-day (SD; i.e., 8 h of light/16 h of dark) conditions, and arrhythmic expression of both circadian clock-related and output genes under continuous light (LL; Fujiwara et al. 2008; Mizoguchi et al. 2002; Mizoguchi et al. 2005).

The circadian clock coordinates plant growth with respect to time of day by producing rhythms of gene expression with a roughly 24-h period. One major factor in plant growth is the hormone auxin. Plants exhibit varying levels of sensitivity to auxin at different times of day, and the circadian clock regulates plant sensitivity to auxin by controlling both the level of transcription and stem growth. An intimate connection between the circadian clock and auxin signaling has been demonstrated by showing the rhythmic expression of components from nearly every step in the auxin signaling pathway (i.e., synthesis to response; Covington and Harmer 2007; Rawat et al. 2009). Plant development

Abbreviations: *Arabidopsis*, *Arabidopsis thaliana*; *CCA1*, CIRCADIAN CLOCK ASSOCIATED 1; *FKD1*, FORKED 1; *LHY*, LATE ELONGATED HYPOCOTYL; LL, continuous light; *VAN3*, VASCULAR NETWORK DEFECTIVE 3.

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is caused by cell elongation promoted by auxin. This growth promotion does not occur unless auxin is at the optimal concentration. Auxin moves to avoid light, and it moves only toward the basal portion of plants. These two characteristics of auxin (i.e., light avoidance and polar movement) are used to explain plant tropism and vascular patterning (Hasenstein and Evans 1988). The polar flow of auxin is under the control of auxin efflux carriers called localization of PIN-FORMED (PIN). These carriers mediate the import and export of auxin through cells in an apical–basal direction (Jacobs and Gilbert 1983; Palme and Galweiler 1999).

The leaf venation pattern consists of a complex vascular network. Although vascular patterning is a major theme in plant research, the mechanism has not been fully elucidated. However, previous studies have indicated that auxin plays an important role in vein pattern formation (Scarpella et al. 2006). Complex vein patterns are formed by the dynamic alteration of PIN localization during the differentiation of veins. Plants with mutations in *VASCULAR NETWORK* (*VAN*) genes show aberrant vein patterning, and *VAN3* and *VAN7/GNOM* are key factors in vesicular transport (Koizumi et al. 2000; Koizumi et al. 2005). Vesicular transport has been proposed to be closely related to PIN localization (Koizumi et al. 2005; Naramoto et al. 2009; Scarpella et al. 2006). *fkdl* was isolated as an abnormal vein pattern mutant (Steynen et al. 2003). *FKD1* was shown to be the same protein as *VAN3* binding protein (*VAB*) and has thus been proposed to be related to vascular pattern formation via the auxin-dependent regulation of PIN localization (Hou et al. 2010; Naramoto et al. 2009).

The circadian clock is intimately tied with the auxin signaling pathway (Covington and Harmer 2007; Rawat et al. 2009). Auxin plays important roles in vein pattern formation (Mattson et al. 1999; Scarpella et al. 2006). Thus, the circadian clock is expected to function in leaf vascular pattern formation through auxin. *VAM3* protein is required for proper localization of PIN1 in leaf cells. There was a report on correlation between a decrease of vascular network complexity and a decrease in flatness of leaves in *vam3* mutant plants (Shirakawa et al. 2009). Therefore, we thought that a change (increase or decrease) of vascular pattern complexity might be responsible for the decrease in flatness of *lhy;cca1* leaves under LL.

To investigate vascular patterning, pigments were removed from the leaves of seedlings grown on Murashige and Skoog (MS) medium for 7, 10, and 14 days under LL by soaking overnight in 100% ethanol. Images of the leaves were captured with a microscope (Leica EZ 4D) and inspected. The vascular patterns in *van3-2* and *fkdl* were simpler than those of wild-type plants, as reported previously (Hou et al. 2010; Koizumi et al. 2000; Naramoto et al. 2009; Figure 1A, C, and D).

Figure 1. Vascular pattern of the first leaves of wild-type, *lhy;cca1*, *fkdl*, and *van3* plants grown under continuous light (LL). Images of the vascular pattern in cleared first leaves from wild-type (WT) Ler (A), *lhy-12;cca1-101* (*lhy;cca1*; B), *van3-2* (*van3*; C; Naramoto et al. 2009), and *fkdl* (D; Steynen and Schultz 2003) were captured under a microscope with a digital camera (Leica EZ 4D). Seedlings grown at 24°C under LL for 14 days on MS medium were soaked overnight in 100% ethanol. Next, the ethanol was removed and the leaves were inspected. AR, BP, and FE indicate areoles (any area of the leaf blade completely bounded by veins), branch points (two or more veins meeting), and free ends (free-ending veins), respectively. Morphological characterization was performed at least twice with independent samples with similar results. Scale bars = 1 mm.

In contrast, the vascular pattern of *lhy;cca1* was more complicated than that of wild-type plants (Figure 1A and B).

Next, three indices of vascular patterning, free ends, areoles, and branch points, were analyzed (Figure 1, Supplemental Table 1, and Table 1). For each of these indices, the number in 14-day-old *lhy;cca1* was greater than that in the wild type (Table 1, Figure 2, and Supplemental Figure 1). This tendency was not observed in seedlings grown for 7 or 10 days (Table 1 and Figures 3 and 4). In contrast, as reported previously (Hou et al. 2010; Koizumi et al. 2000; Naramoto et al. 2009), the numbers of areoles and branch points in *van3-2* and *fkdl* plants grown for 7, 10, and 14 days were much smaller compared to those of the wild type (Table 1 and Figures 2–4). The numbers of free ends in *van3-2* and *fkdl* plants grown for 7 or 10 days were greater than that in the wild type (Table 1 and Figures 3 and 4).

Our results indicate that the *lhy;cca1* vascular pattern is more complex than that of the wild type. The greater

Table 1. Vascular pattern characters of the first leaves of wild-type (WT), *lhy;cca1*, *fkdl*, and *van3* plants.

	Free ends/size (mm ²)	Areoles/size (mm ²)	Branch points/ size (mm ²)
7 days			
WT (30)	3.7±2.0	9.8±0.4	17.6±0.6
<i>lhy;cca1</i> (25)	5.1±0.3*	9.3±0.4	17.2±0.6
<i>fkdl</i> (0)	ND	ND	ND
<i>van3</i> (30)	5.8±0.2*	2.6±0.2*	8.5±0.3*
10 days			
WT (30)	1.2±0.1	2.6±0.1	4.7±0.2
<i>lhy;cca1</i> (30)	1.6±0.1*	2.7	5.1±0.2
<i>fkdl</i> (30)	3.3±0.1*	1.1±0.1*	4.6±0.2
<i>van3</i> (30)	2.3±0.2*	0.5*	2.9±0.2*
14 days			
WT (30)	1.2	1.9±0.1	3.9±0.1
<i>lhy;cca1</i> (30)	1.5±0.1*	2.6±0.2*	5.3±0.3*
<i>fkdl</i> (30)	1.4±0.1*	0.5*	2.0±0.1*
<i>van3</i> (30)	1.2±0.1*	0.3*	1.6±0.1*

The numbers in parentheses represent the number of organs scored. ND, not determined. Asterisks indicate statistically significant differences between the WT and mutant plants ($p < 0.05$). Plants were grown for 7, 10, and 14 days on MS medium in a controlled environment at 24°C under continuous light (LL).

number of free ends, areoles, and branch points created a more complex network of vascular bundles. Several mutations have been reported that simplify the vascular pattern in Arabidopsis leaves (Hou et al. 2010; Koizumi et al. 2000; Naramoto et al. 2009). In contrast, no mutant has been described that has a more complicated vascular pattern than that of the wild type. Although *aux1-7* leaves have a larger number of free ends, areoles, and branch points, they are larger in size than wild-type leaves (Steynen and Schultz 2003). Therefore, these data do not necessarily mean that *aux1-7* leaves have a more complicated vascular pattern than those of the wild type. Therefore, this is the first demonstration of an Arabidopsis mutant with a more complex vascular pattern than that of the wild type.

lhy;cca1 plants have severe defects in maintaining circadian rhythms under LL (Fujiwara et al. 2008; Mizoguchi et al. 2002). The numbers of free ends, areoles, and branch points in *lhy;cca1* were significantly greater than those of the wild type under LL (Table 1, Figure 2, and Supplemental Figure 1), which suggests that the circadian clock has an important function in vascular pattern formation. However, testing how the vascular patterns in *lhy;cca1* plants grown under different light and dark conditions develop should be performed before drawing a conclusion. Although the phases of clock-controlled genes were altered in *lhy;cca1*, the expression of these genes showed diurnal rhythms under light and dark conditions (Fujiwara et al. 2008). The *lhy;cca1* plants did not develop wavy leaves under LD and SD conditions. If the arrhythmic phenotype of *lhy;cca1* is associated with the increase in complexity of the vascular pattern, *lhy;cca1* grown under different light and dark conditions would not show an aberrant vascular

Figure 2. Average for three vascular pattern indices/sizes of the first leaves of seedlings grown for 14 days. (A) Average number of free ends/sizes. (B) Average number of areoles/sizes. (C) Average number of branch points/sizes. Plants of wild-type (WT) *Ler*, *lhy-12;cca1-101* (*lhy;cca1*), *van3-2* (*van3*), and *fkdl* were grown for 14 days on MS medium in a controlled environment at 24°C under continuous light (LL). The numbers of free ends, areoles, and branch points were divided by each size value. The data are presented as the mean ± S.E. ($n = 30$). Morphological characterization was performed at least twice with independent samples with similar results. Asterisks represent statistical significance compared to values for the wild type (Student's *t*-test, $p < 0.05$).

pattern. This possibility could be confirmed through investigations of the vascular pattern in other arrhythmic mutants. PSEUDO RESPONSE REGULATOR (PRR)9, PRR7, and PRR5 also play key roles in the function of the circadian clock in Arabidopsis (Nakamichi et al. 2005). Plants with mutations in PRR9, PRR7, and PRR5 (*prp9;prp7;prp5*) grown under LL showed similar characteristics (e.g., late flowering, semidwarf, and wavy/dark green leaf phenotypes) to those of *lhy;cca1* (Niinuma et al. 2008). If the circadian clock plays a key role in regulating vascular pattern formation, the vascular pattern of *prp9;prp7;prp5* grown under LL may show similarities to that of *lhy;cca1*. In addition, we have not yet understood why the *lhy;cca1* showed a clear difference on 14 days after sowing. Investigation of the *lhy;cca1* and wild-type together with other clock mutant

Figure 3. Average values for three vascular pattern indices/sizes of the first leaves of seedlings grown for 10 days. (A) Average number of free ends/sizes. (B) Average number of areoles/sizes. (C) Average number of branch points/sizes. Plants of wild-type (WT) Ler, *lhy-12;cca1-101* (*lhy;cca1*), *van3-2* (*van3*), and *fkd1* were grown for 10 days on MS medium in a controlled environment at 24°C under continuous light (LL). The numbers of free ends, areoles, and branch points were divided by each size value. The data are presented as the mean $\pm$ S.E. ($n=30$). Morphological characterization was performed at least twice with independent samples with similar results. Asterisks represent statistical significance compared to values for the wild type (Student's *t*-test, $p<0.05$).

plants grown for longer periods (e.g., 3, 4 and 5 weeks) under LL would be helpful to understand a molecular mechanism underlying the vascular pattern complexity.

Auxin is related to vascular pattern formation (Scarpella et al. 2006), and the circadian clock regulates auxin biosynthesis and signaling (Covington and Harmer 2007). Our results indicate that the vascular pattern in *lhy;cca1* is more complex than that in wild-type plants. This suggests a role for auxin in the link between the circadian clock and vascular pattern formation. Changes of distribution or concentration of auxin in leaves might be responsible for the phenotype of *lhy;cca1*. This possibility should be tested in the near future. Nevertheless, other plant hormones may act as intermediates between the circadian clock and vascular pattern formation. For example, brassinosteroids are

Figure 4. Average values for three vascular pattern indices/sizes of the first leaves of seedlings grown for 7 days. (A) Average number of free ends/sizes. (B) Average number of areoles/sizes. (C) Average number of branch points/sizes. Plants of wild-type (WT) Ler, *lhy-12;cca1-101* (*lhy;cca1*), and *van3-2* (*van3*) were grown for 7 days on MS medium in a controlled environment at 24°C under continuous light (LL). The numbers of free ends, areoles, and branch points were divided by each size value. The data are presented as the mean $\pm$ S.E. (WT and *van3*, $n=30$; *lhy;cca1*, $n=25$). Morphological characterization was performed at least twice with independent samples with similar results. Asterisks represent statistical significance compared to values for the wild type (Student's *t*-test, $p<0.05$).

plant hormones that are essential regulators of plant growth and development (Clouse and Sasse 1998), and brassinosteroids conceivably may be involved in the connection between vascular patterning and the clock components LHY and CCA1. The relationship between vascular patterning and hormones should be investigated using mutants that are defective in the biosynthesis and signaling of various hormones, thus helping to elucidate which hormone mediates between the circadian clock and vascular pattern formation.

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