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Article Addendum

Are there multiple circadian clocks in plants?

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We have reported that Arabidopsis might have genetically distinct circadian oscillators in multiple cell-types.[1] Rhythms of *CHLOROPHYLL A/B BINDING PROTEIN2* (*CAB2*) promoter activity are 2.5 h longer in *phytochromeB* mutants in constant red light and in *cryptocrome1 cry2* double mutant (*hy4*-1 *fha*-1) in constant blue light than the wild-type.[2] However, we found that cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) oscillations were undetectable in these mutants in the same light conditions.[1] Furthermore, mutants of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) have short period rhythms of leaf movement but have arrhythmic $[Ca^{2+}]_{cyt}$ oscillations. More important, the *timing of cab1*-1 (*toc1*-1) mutant has short period rhythms of *CAB2* promoter activity (~21 h) but, surprisingly, has a wild-type period for circadian $[Ca^{2+}]_{cyt}$ oscillations (~24 h). In contrast, *toc1*-2, a *TOC1* loss-of-function mutant, has a short period of both *CAB2* and $[Ca^{2+}]_{cyt}$ rhythms (~21 h). Here we discuss the difference between the phenotypes of *toc1*-1 and *toc1*-2 and how rhythms of *CAB2* promoter activity and circadian $[Ca^{2+}]_{cyt}$ oscillations might be regulated differently.

The plant circadian clock controls a multitude of physiological processes such as photosynthesis, organ and stomatal movements and transition to reproductive growth. A plant clock that is correctly matched to the rhythms in the environment brings about a photosynthetic advantage that results in more chlorophyll, more carbon assimilation and faster growth.[3] One of the first circadian clock mutants to be described in plants was the short period *timing of cab1*-1 (*toc1*-1), which was identified using the rhythms of luciferase under a *CHLOROPHYLL A/B BINDING PROTEIN2* (*CAB2*) promoter as a marker for circadian period.[4]

Circadian rhythms of both *CAB2* promoter activity and cytosolic-free Ca^{2+} ($[Ca^{2+}]_{cyt}$) oscillations depend on the function of a *TOC1*, *CIRCADIAN CLOCK ASSOCIATED1* and *LATE ELONGATED HYPOCOTYL* (*TOC1/CCA1/LHY*) negative feedback loop.[5] In tobacco seedlings, *CAB2:luciferase* (*CAB2:luc*) rhythms and circadian $[Ca^{2+}]_{cyt}$ oscillations can be uncoupled in undifferentiated calli.[6] In Arabidopsis, we reported that *toc1*-1 has different periods of rhythms of *CAB2* promoter activity (~21 h) and circadian $[Ca^{2+}]_{cyt}$ oscillations (~24 h). The mutant allele *toc1*-1 has a base pair change that leads to a full protein that has an amino acid change from Ala to Val in the CCT domain (CONSTANS, CONSTANS-LIKE and TOC1).[7] On the other hand, the mutant *toc1*-2 has short period of both rhythms of CAB2 promoter activity and circadian $[Ca^{2+}]_{cyt}$ oscillations (~21 h).[1,7] This allele has a base pair change that results in changes to preferential mRNA splicing, resulting in a truncated protein with only 59 residues.[7] Thus, the mutated CCT domain in *toc1*-1 might lead to the uncoupling of rhythms of *CAB2* promoter activity and circadian $[Ca^{2+}]_{cyt}$ oscillations while the absence of TOC1 in *toc1*-2 causes the shortening of the period of both rhythms. Indeed, *zeitlupe*-1 (*ztl*-1) mutants, that have higher levels of TOC1, have long periods of both rhythms of *CAB2* promoter activity and circadian $[Ca^{2+}]_{cyt}$ oscillations.[1] The biochemical function of the CCT domain is unknown but it is predicted to play an important role in protein-protein interactions[8] and nuclear localization.[9]

One model to explain the period difference of *CAB2:luc* expression and circadian $[Ca^{2+}]_{cyt}$ oscillation is that the *toc1*-1 mutation has uncoupled two oscillators in the same cell. Uncoupled oscillators are a predicted outcome of certain mutations in the recently described three-loop mathematical model.[10-11] However, both rhythms of *TOC1* and *CCA1/LHY* expression, which would be in uncoupled oscillators accordingly to the model, are described as short-period in *toc1*-1.[5] Thus, we have favored the model in which *CAB2:luc* expression and circadian $[Ca^{2+}]_{cyt}$ oscillation are reporting cell-types with different oscillators that are affected differently by *toc1*-1.

It is possible that TOC1 could interact with a family of cell-type specific proteins. The interaction of TOC1 with each member of the family could be affected differently by the mutation in the CCT domain (Fig. 1). Two-hybrid assays have shown that TOC1 interacts with PIF proteins (PHYTOCHROME INTERACTING FACTOR3 and PIF4) and related PIL proteins (PIF3-LIKE PROTEIN 1, PIL2, PIL5 and PIL6).[8] In fact, TOC1 interaction with both PIF3 and PIL1 is stronger when the N-terminus receiver domain is taken out and the CCT domain is left intact.[8] Thus, it is possible that TOC1 and different PIF/PIL proteins interact to regulate the central oscillator.

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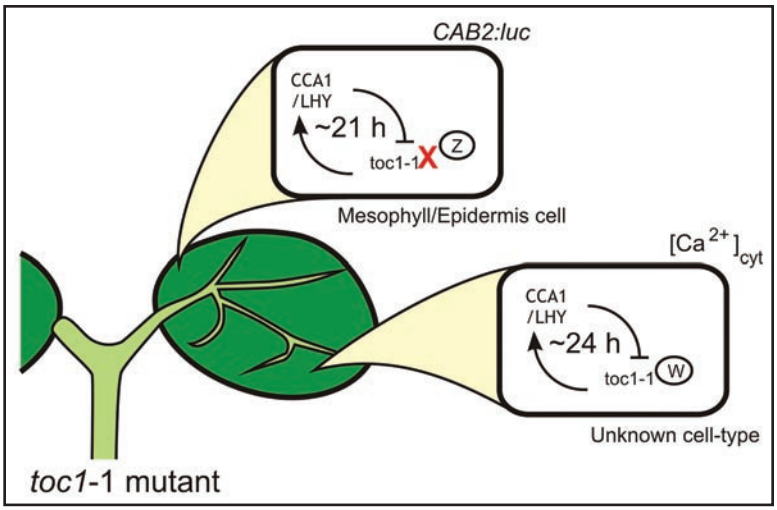

Figure 1. Models of how the toc1-1 mutation might differently affect cell-type specific circadian oscillators. The single mutant *toc1*-1 have 21 h rhythms of *CAB2* promoter activity and 24 h-rhythms of $[Ca^{2+}]_{cyt}$ oscillations. The *toc1*-1 mutation is a single amino acid change in the CCT domain. The CCT domain is involved in protein-protein interaction and/or nuclear localization. We have proposed that circadian oscillators with different periods are present in different cell-types. The luminescence generated by *CAB2* promoter-drived luciferase (from the *CAB2:luc*) is probably originated in the epidermis and mesophyll cells. In this model, we propose that the mutation on the CCT domain impairs the mutated TOC1 interaction with the hypothetical protein Z in these cells-types. In contrast, in other cell-types, the mutated TOC1 still interacts with other hypothetical proteins (W), despite the mutation in the CCT domain. In those cell-types, the circadian oscillator could still run with a 24 h period for $[Ca^{2+}]_{cyt}$ rhythms (from the *35S:AEQ* construct). One possible identity for Z and W are the members of the PHYTOCHROME INTERACTING FACTOR (PIF) related PIF3-LIKE (PIL) family.

This interaction could be impaired by the Ala to Val change in the *toc1*-1 mutation, leading to the period shortening. However, lines misexpressing *PIF3*, *PIL1* and *PIL6* showed no changes in their circadian rhythms.[12-16]

One possible explanation for the absence of alterations in the period of circadian rhythms in lines misexpressing PIF/PIL is that they only have roles in certain cell-types. As an example, PIL6 and PIF3 are involved with flowering time and hypocotyl growth in red light[12-15] while PIL1 and PIL2 are involved with hypocotyl elongation in shade-avoidance responses.[16] Both hypocotyl growth and flowering time require cell-type specific regulation: vascular bundle cells in the case of the flowering time[17] and the cells in the shoot in the case of the hypocotyl elongation.[16] If TOC1 interaction with certain PIF/PIL is indeed cell-type specific, the mutated CCT domain found in the *toc1*-1 mutant could affect the clock in different ways, depending on the type of PIF/PIL protein expressed in each cell-type. Therefore, a question that arises is: which cell-types are sensitive to the *toc1*-1 mutation?

There is evidence that *CAB2* and *CATALASE3* (*CAT3*) are regulated by two oscillators that respond differently to temperature signals.[18] These genes might be regulated by two distinct circadian oscillators within the same tissues or a single cell.[18] Interestingly, the spatial patterns of expression of *CAB2* and *CATALASE3* overlap in the mesophyll of the cotyledons.[18] Furthermore, rhythms of *CAB2* and *CHALCONE SYNTHASE* (*CHS*) promoter activity have different periods and they are equally affected by *toc1*-1 mutation.[19] Whereas *CAB2* is mainly expressed in the mesophyll cells, *CHS* is mainly expressed in epidermis and root cells.[19] However, rhythms of AEQUORIN luminescence, which reports $[Ca^{2+}]_{cyt}$ oscillation, were insensitive to *toc1*-1 mutation and appear to come from the whole cotyledon.[20] One cell-type which is found in the whole cotyledon but is distinct from either mesophyll or epidermis cells is the vascular tissue and associated cells.

Another approach to determine which cell-types are insensitive to *toc1*-1 mutation is to compare the *toc1*-1 and *toc1*-2 phenotypes. The period of circadian $[Ca^{2+}]_{cyt}$ oscillations is not the only phenotype that is different in *toc1*-1 and *toc1*-2 mutants. Rhythms in *CAB2* promoter activity in constant red light are short period in *toc1*-1 but arrhythmic in *toc1*-2.[21,22] *COLD*, *CIRCADIAN RHYTHM* AND *RNA BINDING 2/GLYCINE-RICH RNA BINDING PROTEIN 7* (*CCR2/GRP7*) is also arrhythmic in *toc1*-2 but short period in *toc1*-1 in constant darkness.[7,22] When the length of the hypocotyl was measured for both *toc1*-1 and *toc1*-2 plants exposed to various intensities of red light, only *toc1*-2 had a clear reduction in sensitivity to red light. Therefore, *toc1*-2 has long hypocotyl when maintained in constant red light while hypocotyl length in *toc1*-1 is nearly identical to that in the wild-type.[22] These differences may allow us to separate which cell-types are sensitive to the *toc1*-1 mutation and which not.

Hypocotyl growth is regulated by a large number of factors such as light, gravity, auxin, cytokinins, ethylene, gibberellins and brassinosteroids.[23] There is also a correlation between the size of the hypocotyl in red light and defects in the circadian signaling network.[24,25] The fact that *toc1*-1 has different hypocotyl sizes from *toc1*-2 suggests that circadian $[Ca^{2+}]_{cyt}$ oscillations could be involved in the light-dependent control of hypocotyl growth. Circadian $[Ca^{2+}]_{cyt}$ oscillations might encode temporal information to control cell expansion and hypocotyl growth. [26-28] *toc1*-1 have short-period rhythms of hypocotyl elongation, which indicates that the cells in the hypocotyl have a 21 h oscillator.[29] However, *toc1*-1 might also have a wild-type hypocotyl length in continuous red light because cells which generate the signal to regulate hypocotyl growth might have 24 h oscillators.

The *toc1*-1 mutation was the first to be directly associated with the plant circadian clock, revitalizing the field of study.[4] Now, by either uncoupling two feedback loops or by distinct TOC1 protein-protein interaction in different cell-types, *toc1*-1 has shown new properties of the circadian clock that may deepen our understanding of this system.

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