The APRR1/TOC1 Quintet Implicated in Circadian Rhythms of Arabidopsis thaliana: II. Characterization with CCA1-Overexpressing Plants

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We previously identified a novel class of proteins, named pseudo-response regulators (APRRs) in Arabidopsis thaliana, each of which (APRR1, APRR3, APRR5, APRR7, and APRR9) has an intriguing structural design containing an N-terminal pseudo receiver domain and a C-terminal CONSTANS motif. Among them, APRR1 is identical to TOC1, previously proposed to be a candidate component of the Arabidopsis circadian clock. Intriguingly, expressions of the APRR1/TOC1 family of genes are under control of coordinate circadian rhythms at the level of transcription, in the manner that each APRR-transcript starts accumulating sequentially after dawn with 2 to 3 h intervals in the order: APRR9→APRR7→APRR5→APRR3→APRR1/TOC1. Here we examined this circadian-related event, “circadian waves of the APRR1/TOC1 quintet”, by employing CCA1-overexpression (CCA1-ox) transgenic plants, based on the fact that CCA1 is a well-characterized and the most plausible oscillator component. It was found that aberrant overexpression of the CCA1 gene severely perturbed free-running and sequential rhythms of the APRR1/TOC1 family of genes. In the accompanying paper, it was shown that overexpression of APRR1 also results in a marked alteration of the CCA1 circadian rhythm, and vice versa. Taken together, it was suggested that there are intimate and mutual links between these two types of circadian-associated components (APRRs and CCA1).

Key words: Arabidopsis — Circadian rhythm — Clock components — Transgenic plants.

In Arabidopsis thaliana, biological and genetic studies have begun to shed light on the molecular bases of biological clocks that regulate many biological rhythms in this higher plant (for reviews see Anderson and Kay 1996, Thomas and Vince-Prue 1997, Kreps and Kay 1997, Koornneef et al. 1998, Pichulka 1999, Staiger and Heintzen 1999, Murtas and Millar 2000, Samach and Coupland 2000, Barak et al. 2000, Carre 2001). In general, such a biological clock consists of input pathways, central oscillators, and output pathways (Dunlap 1999). With regard to the Arabidopsis central oscillator(s), several genes were recently proposed to encode potential clock-associated components, although none of them has yet been firmly established as a bona fide clock component (for a review see Barak et al. 2000). Among them, TIMING OF CAB 1 (TOC1) is intriguing, because a semi-dominant (toc1-1) mutant interferes with a wide range of clock-controlled output processes (Somers et al. 1998), TOC1 was thus proposed to function close to, or as part of, the oscillator itself.

We have been extensively characterizing a novel family of proteins, termed Arabidopsis pseudo response regulators (designated as APRRs), with special reference to circadian rhythms (Makino et al. 2000, Matsushika et al. 2000, Makino et al. 2001, Makino et al. 2002), based on the fact that one of them (APRR1) is identical to TOC1 (Matsushika et al. 2000, Strayer et al. 2000). More importantly, each transcript of APRRs starts accumulating after dawn rhythmically and sequentially at approximately 2 h intervals in the order: APRR9→APRR7→APRR5→APRR3→APRR1/TOC1 (this event was previously termed “circadian waves of the APRR1/TOC1 quintet”) (Matsushika et al. 2000). In an accompanying paper (Makino et al. 2002), we showed that overexpression of APRR1 (TOC1) in plants results in abolishment of circadian rhythms of a number of circadian-associated genes, including CCA1 and LHY (for references, see below). It was also assumed that not only APRR1/TOC1 itself, but also other APRR1/TOC1 family of proteins might be implicated as part of the Arabidopsis circadian clock.

Other well-known potential clock components are the gene products of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCHOTYL (LHY) genes, both of which encode homologous Myb-related transcription factors (Wang et al. 1997, Wang and Tobin 1998, Schaffer et al. 1998). It has been demonstrated that overexpression of CCA1 in plants stops overt rhythmicity, including the expression of certain circadian-controlled genes, such as CAB2 encoding a light-harvesting chlorophyll-a/b-binding protein (Wang and Tobin 1998). It is thus crucial to see if rhythmic expressions (circadian waves) of the APRR1/TOC1 family of genes are also affected in such CCA1-overexpressing plants (CCA1-ox).

In this short communication, we address this particular issue.

Homozygous plants carrying a 35S::CCA1 transgene were constructed and established previously (Wang and Tobin 1998),

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wild-type plants, the \textit{CCA1}-transcript oscillates rhythmically
with a peak at dawn, as expected (Wang and Tobin 1998). In
contrast, a high and constitutive level of the \textit{CCA1}-transcript
was detected for all RNA samples of CCA1-ox plants (from leaves), irrespective of the timing of preparation (Fig. 1A).

To examine whether or not such constitutive expression of
\textit{CCA1} affects the circadian-regulated expressions of the
\textit{APRR1}/\textit{TOC1} family of genes, first of all, we examined light-
dependent expression of \textit{APRR9} in CCA1-ox plants (Fig. 1B, C). The reason for this is twofold. Among the five members
of \textit{APRRs}, only the expression of \textit{APRR9} is light inducible
(Matsushika et al. 2000). Furthermore, the expression of \textit{CCA1}
is also rapidly induced by light (Wang and Tobin 1998). Plants
(Col. and CCA1-ox) were grown in the dark for 6 d, and then the
resulting etiolated seedlings were exposed to white light.
Total RNA samples were prepared at short intervals to examine
both the \textit{CCA1}- and \textit{APRR9}-transcripts by Northern hybridi-
zation analyses. In wild-type plants (Fig. 1B), the results con-
irmed the previous notions that both the expression of \textit{CCA1}
and \textit{APRR9} are induced very rapidly by light in a similar man-
nner to each other. For CCA1-ox plants (Fig. 1C), here it was
found that \textit{APRR9} was normally induced by light, while \textit{CCA1}
was constitutively expressed independently of light. This
result suggests that, in the light signal input pathways, the
induction of \textit{APRR9} occurs independently of \textit{CCA1}. In other
words, it is unlikely that \textit{CCA1} acts upstream of \textit{APRR9} in the
light signal input pathways.

We then examined possible effect of CCA1-ox on the
\textit{APRR1}/\textit{TOC1} circadian waves by extensive Northern hybridi-
zation analyses (Fig. 2, 3). Plants (Col and CCA1-ox) were
grown for 20 d under conditions of 12 h light/12 h dark. In one
line of experiments the photoperiod conditions were main-
tained (LD conditions), whereas in the other line of experi-
ments plants were transferred to continuous light (LL condi-
tions). RNA samples from these plants (leaves) were prepared
at appropriate intervals, followed by Northern hybridization
analyses. The specific probes used were those for \textit{UBQ10}
(loading reference), \textit{CCA1}, \textit{APRR1}, \textit{APRR3}, \textit{APRR5},
\textit{APRR7}, and \textit{APRR9} (Makino et al. 2002). Some representative
raw data are shown in Fig. 2. In the case of \textit{APRR3} (Fig. 2A),
when plants were grown under LD conditions, rhythmic
expression of \textit{APRR3} was seen with a peak at evening in
CCA1-ox plants. This profile is essentially the same as that
observed for \textit{APRR3} in wild-type plants grown under the same
conditions (Matsushika et al. 2000). However, when CCA1-ox
plants were transferred to LL conditions, such robust rhythmic-
ity of \textit{APRR3} was rapidly dampened (Fig. 2A). It has already
been reported that the free-running oscillation of \textit{APRR3} is
maintained in wild-type plants even under such LL conditions
(Matsushika et al. 2000). It was thus found that overexpression
of \textit{CCA1} in plants stops overt rhythmicity of \textit{APRR3}. Essen-
tially the same phenomena were seen for \textit{APRR7}. It peaked
after dawn under LD conditions, but the rhythmicity of \textit{APRR7}
was dampened under LL conditions (Fig. 2B).
The above results of Northern hybridization, together with those carried out with probes each specific for CCA1, CAB2, APRR1, APRR5, and APRR9, were quantitatively characterized by using UBQ10 as an internal reference (Fig. 3). These results provided us with the following insights. In wild-type plants, the CCA1-transcript accumulated rhythmically with a peak at dawn, whereas a higher and constitutive level of the CCA1-transcript was detected for CCA1-ox plants, irrespective of whether under LD or LL conditions (these confirmative data are not shown, see Wang and Tobin 1998). The expression of CAB2 was also examined in this experiment with CCA1-ox plants, as a critical reference (Wang and Tobin 1998). It was confirmed that the rhythmic expression of the well-known circadian-controlled CAB2 gene was no longer observed in CCA1-ox plants under LL conditions (Fig. 3A). These events are fully consistent with those reported previously (Wang and Tobin 1998), supporting the view that aberrant overexpression of CCA1 severely perturbs the free-running and sequential rhythms of the APRR1/TOC1 family of genes. It is worth mentioning that a significant level of each APRR-transcript was still seen in CCA1-ox plants grown even under LL conditions, although its rhythmicity disappeared, as emphasized above. These profiles are similar to that observed for CAB2 in CCA1-ox plants (see Fig. 3A).

Clarification of plant biological clocks is a paradigm of the current Arabidopsis molecular biology. In this respect, the rhythmic waves of the APRR1/TOC1 family of genes are intriguing. In a accompanying paper (Makino et al. 2002), we examined APRR1-ox plants to gain insight into the molecular mechanism underlying the circadian rhythms in Arabidopsis. In this study, we further employed CCA1-ox plants to gain deeper insight into possible interplays of these different types of candidate oscillator components, namely, APRR1/TOC1 and CCA1. We showed that not only constitutive expression of CCA1, but also that of APRR1 results in abolishment of the free-running robust oscillation of CAB2, which is a hallmark of the plant circadian rhythms. More importantly, the results of this study, together with those of the accompanying paper, showed that these two types of potential oscillator components (APRR1/...
TOC1 and CCA1) appear to mutually interact with each other, in such a manner that constitutive expression of CCA1 in plants abolishes the free-running rhythmic expression of APRR1/TOC1 (and other APRRs), and vice versa. Such a reciprocal regulation between APRR1/TOC1 and CCA1 was consistent with the view that was reported recently by Alabadi et al. (2001). However, it may be noted that the robust free-running rhythm of CCA1 was dampened in APRR1-ox, resulting in a longer interval, whereas the free-running rhythm of APRR1 in CCA1-ox was abolished, and a certain level of non-oscillated expression was seen (see Fig. 3B). In any event, our results further supported the view that no matter whether they are bona fide oscillator components or not, these components (and other APRRs also) most likely play concerted roles in the mechanism underlying the circadian rhythms. The mode of presumed interplay between CCA1 and APRRs is not yet clear. In this respect, Alabadi et al. (2001) proposed that the CCA1 protein negatively regulates the expression of APRR1/TOC1 through its direct binding to the APRR1/TOC1 promoter. In this connection, we showed that APRR9 was normally induced by light in CCA1-ox plants (Fig. 1), but severely repressed in APRR1-ox plants (Makino et al. 2002). It was also found that APRR3 and APRR7 are not severely repressed in CCA1-ox plants (Fig. 2, 3), although the free-running rhythms of these genes are abolished (or dampened) under LL conditions. This suggests that CCA1 acts on other APRRs through a manner distinct from that observed between CCA1 and APRR1/TOC1. No matter the interactions of other APRRs and CCA1 are direct or indirect, it would be of interest to clarify the underlying mechanism, by which these potential clock components interact mutually.

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References


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