A Heteromeric RNA-Binding Protein Is Involved in Maintaining Acrophase and Period of the Circadian Clock[1][W]

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The RNA-binding protein CHLAMY1 from the green alga *Chlamydomonas reinhardtii* consists of two subunits. One (named C1) contains three lysine homology motifs and the other (named C3) has three RNA recognition motifs. CHLAMY1 binds specifically to uridine-guanine-repeat sequences and its circadian-binding activity is controlled at the posttranslational level, presumably by time-dependent formation of protein complexes consisting of C1 and C3 or C1 alone. Here we have characterized the role of the two subunits within the circadian system by measurements of a circadian rhythm of phototaxis in strains where C1 or C3 are either up- or down-regulated. Further, we have measured the rhythm of nitrite reductase activity in strains with reduced levels of C1 or C3. In case of changes in the C3 level (both increases and decreases), the acrophase of the phototaxis rhythm and of the nitrite reductase rhythm (C3 decrease) was shifted by several hours from subjective day (maximum in wild-type cells) back towards the night. In contrast, both silencing and overexpression of C1 resulted in disturbed circadian rhythms and arrhythmicity. Interestingly, the expression of C1 is interconnected with that of C3. Our data suggest that CHLAMY1 is involved in the control of the phase angle and period of the circadian clock in *C. reinhardtii*.

Circadian rhythms are biological rhythms that persist with a period of about 24 h under constant conditions of light and temperature. They have been described in many organisms where they regulate a wide variety of physiological and cellular processes. While the physiological properties of circadian rhythms are well conserved among different organisms including bacteria (Kondo et al., 1993), only some of the molecular components are conserved across evolution. But, in all model systems studied so far a similar mechanism consisting of positive and negative feedback loops was found to be a key feature of the endogenous pacemaker (Harmer et al., 2001; Reppert and Weaver, 2002; Dunlap and Loros, 2004; Johnson, 2004). Thus, transcription factors and their protein-protein interactions, as well as temporal phosphorylation events, have been found to play important regulatory roles within the circadian oscillator.

In the green alga *Chlamydomonas reinhardtii* several processes are under control of the circadian clock such as phototaxis, chemotaxis, stickiness to glass surfaces, UV sensitivity, and the cell cycle (for review, see Mittag et al., 2005). Phototaxis, also known as photoaccumulation, was already demonstrated more than 30 years ago by Bruce (1970). As biflagellate unicell, *C. reinhardtii* moves to regions of brighter light during day phase, allowing the cells to optimize their light absorption for photosynthesis. Automated computerized measurement devices have been developed (Mergenhagen, 1984; Johnson et al., 1991; Kondo et al., 1991) to study this circadian rhythm under free-running conditions over several days.

Based on the availability of the entire genome sequence of *C. reinhardtii* (Grossman et al., 2003; Grossman, 2005), homology searches have been undertaken to check if known clock genes of other model organisms are conserved in this green alga. Thereby, it was found that two cryptochrome (CRY)-like proteins are present in *C. reinhardtii* (Mittag et al., 2005). One of them is more closely related to higher plants, while the other is closer to animal CRY. Even though both still have to be functionally characterized, the occurrence of both in *C. reinhardtii* is of high interest, since mammalian CRY is part of the endogenous oscillator while plant CRY acts as photoreceptor. Thus, it has been hypothesized that *C. reinhardtii* might have an amalgam clock (Breton and Kay, 2006).

In addition, kinases and phosphatases that are involved in posttranslational modifications of key components of the oscillatory system in other eukaryotic model systems are well conserved in *C. reinhardtii* (Mittag et al., 2005). First approaches have been undertaken to characterize one of these proteins functionally. Silencing of casein kinase1 (CK1) by RNAi caused a period shortening for several days and finally arrhythmicity of circadian phototaxis (Schmidt et al., 2006). Thus, at

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least one of the well-conserved kinases also plays an important role in the circadian system of *C. reinhardtii*. However, the target proteins of these kinases such as frequency (FRQ) in *Neurospora crassa* or period in *Drosophila melanogaster* have not been found through homology searches (Mittag et al., 2005). Thus, it was concluded that *C. reinhardtii* may also have its own clock proteins.

Based on functional homology with the circadian controlled translational regulator (CCTR) from the dinoflagellate *Gonyaulax polyedra*, an RNA-binding protein named CHLAMY1 has been identified in *C. reinhardtii* (Mittag et al., 1994; Mittag, 1996). The CCTR binds to a uridine-guanine (UG)-repeat region situated in the mRNA of the luciferin-binding protein (LBP) in a circadian manner (Mittag et al., 1994). LBP was indeed, to our knowledge, the first example where it was shown that its circadian expression is regulated at the translational level (Morse et al., 1989). While LBP reaches a peak in the night phase, its mRNA is constantly present. The CCTR was considered to inhibit translation of *lbp* mRNA during day phase when it is bound to it. CHLAMY1 also recognizes specifically $UG_{\geq 7}$-repeat sequences that are present in the 3′ untranslated regions (UTRs) of several mRNAs of *C. reinhardtii* and its binding activity is controlled by the circadian clock (Mittag, 1996; Waltenberger et al., 2001). The proteins encoded by these mRNAs include members of nitrogen and CO_2 metabolism such as chloroplastic Gln synthetase2, nitrite reductase (NII), or one of the small subunits of Rubisco. Further investigations showed that CHLAMY1 represents a novel type of heteromeric RNA-binding protein that consists of two subunits comprising three RNA recognition motifs (RRMs; C3 subunit) and three Lys homology motifs (KH; C1 subunit), respectively (Zhao et al., 2004). The *c1* cDNA encodes a protein of 51,706 D, and the *c3* cDNA encodes a protein of 44,979 D. While the C3 subunit migrates according to its molecular mass in SDS-PAGE, the C1 subunit migrates at a higher apparent mass of approximately 60 kD (Zhao et al., 2004). This was observed not only in *C. reinhardtii* extracts, but also in the recombinant protein overexpressed in *Escherichia coli*. Interaction of the C1 and C3 subunits was confirmed by immunoprecipitation assays as well as by supershift mobility assays along with a labeled UG-repeat transcript and anti-C1 or anti-C3 antibodies (Zhao et al., 2004). Both C1 and C3 bear domains that may be involved in their protein-protein interaction. C1 has a WW domain at its C terminus that is known to communicate protein-protein interactions (Sudol and Hunter, 2000). C3 that is also conserved in other organisms including mammals belongs to the CELF family of RNA-binding proteins. The spacer region between the second and third RRM domains has been postulated to be involved in protein-protein interactions (Ladd et al., 2001).

Surprisingly, the amounts of both subunits were found to be rather constant over the circadian cycle, indicating that posttranslational events control the circadian-binding activity of CHLAMY1. At the same time, it was found that a ≥670 kD protein complex consisting of the C1 subunit occurs only during subjective day, while an approximately 158 kD complex consisting of C1 and C3 is present both during day and night phase (Zhao et al., 2004). Thus, circadian-binding activity of CHLAMY1 appears to be controlled at the posttranslational level by time-dependent formation of different protein complexes involving C1 and C3. Thereby, it cannot be ruled out that the ≥670 kD protein complex may consist not only of C1, but may have some novel interaction partners.

Here we have characterized the role of CHLAMY1 within the circadian system by independent up- or down-regulation of its two subunits and measurements of the phototaxis rhythms in the transgenic strains. Further, we measured the rhythms of NII activities in the silenced strains. We can show that changes in the level of the C1 or the C3 subunit result in severe disturbances of the circadian clock either affecting its acrophase or causing arrhythmicity. Further, changes in the expression of the C1 subunit (slight overexpression or silencing below a critical level) resulted in large changes in the level of the C3 subunit, suggesting that C3 regulation is strongly dependent on C1. In contrast, C3 up- and down-regulation influence the C1 level only slightly (C3 up-regulation) or not significantly (C3 down-regulation).

RESULTS

Overexpression and Silencing of the C3 Subunit of CHLAMY1 Phase Shift Circadian Phototaxis by Several Hours

To obtain functional information about the role of the C3 subunit in the circadian system of *C. reinhardtii*, it was either silenced or overexpressed, and circadian phototaxis of the modified strains was measured. For overexpression of C3, the major part of its open reading frame (ORF) was put under the control of the strong truncated hsp70A/rbcS2 tandem promoter (Fig. 1A; for details, see ''Materials and Methods'' and Supplemental Protocol S1). Transformed strains growing under selection of paromomycin were used for further analysis. Cells were grown to a cell density of about 1 to 5 $\times$ 10^6 cells/mL and crude extracts were prepared. For comparison, a crude extract from nontransformed wild-type cells was used. C3 overexpression was analyzed in westerns with anti-C3 antibodies (Fig. 1B). Different amounts of proteins from wild type (90, 60, and 30 μg per lane) were separated on SDS-PAGE and quantitatively compared to proteins from transformed strains (30 μg per lane) after immunoblotting with the anti-C3 antibody. Equal loading was checked by Ponceau staining. Two strains where C3 was overexpressed at least 3-fold were used for further analysis ($C3\text{-}ox_{90}$ and $C3\text{-}ox_{100}$).

In all studied model organisms the oscillatory system is driven by positive and negative feedback loops.

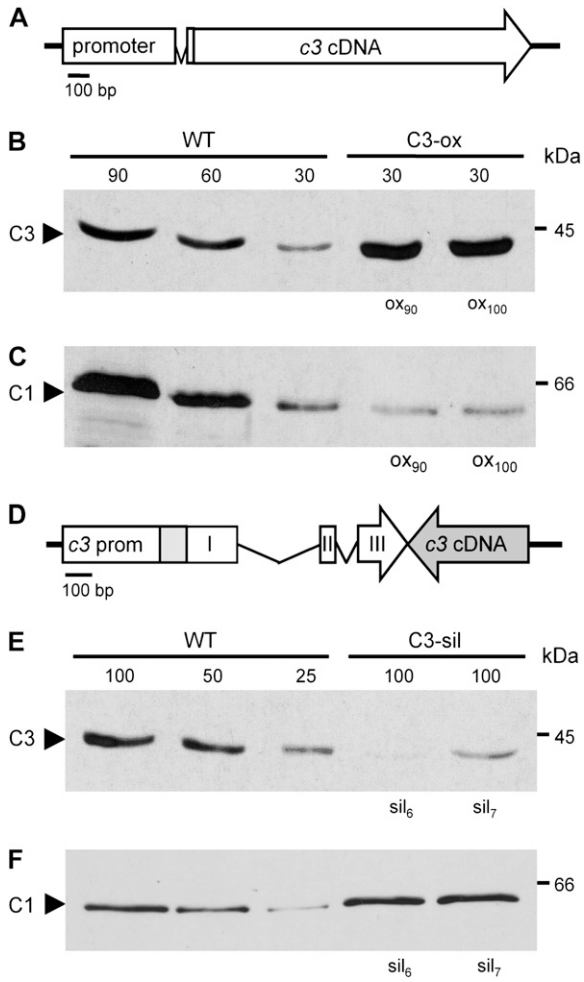

Figure 1. Overexpression and silencing of C3 and the effects on C1 expression. A, The overexpression construct used for C3 is shown. Promoter indicates the truncated *hsp70A/rbcS2* tandem promoter along with the first intron of *rbcS2* that bears an enhancer (see "Materials and Methods"). The tagged *c3* cDNA bears the major part of the *c3* ORF (the first 17 amino acids are missing) and the entire 3′ UTR (see Supplemental Protocol S1). B and C, Different amounts of proteins from a crude extract (90, 60, and 30 μg per lanes) of wild-type (WT) cells were separated on SDS-PAGE and used for western analysis with anti-C3 (B) or anti-C1 (C) antibodies along with proteins of crude extracts (30 μg per lane) from two different C3-overexpressing strains (C3-ox$_{90}$ and C3-ox$_{100}$). The positions of C3 and C1 are indicated by arrowheads. D, The RNAi construct used for silencing of C3 is shown. The numbers represent the involved exons. Introns are indicated by lines. The light-gray area represents the predicted 5′ UTR, and *c3* prom the potential promoter region of *c3* (see Supplemental Protocol S2). E and F, Different amounts of proteins from a crude extract (100, 50, and 25 μg per lanes) of wild-type (WT) cells were separated on SDS-PAGE and used for western analysis with the anti-C3 (E) and anti-C1 (F) antibodies along with proteins of crude extracts (100 μg per lane) from two different C3-silenced strains (C3-sil$_6$ and C3-sil$_7$). The positions of C3 and C1 are indicated by arrowheads.

Thereby, variations in protein level of any clock component can affect the normal expression of other components. For example, in *N. crassa*, a knockout of FRQ leads to decreased levels of white collar-1 (WC-1; Lee et al., 2000). Also, coregulation was found in some multiprotein complexes of *C. reinhardtii*, such as components of the photosystems (Wostrikoff et al., 2004; Göhre et al., 2006). Therefore, we were interested to understand if up- or down-regulation of any of the two subunits of CHLAMY1 could cause an effect on the normal expression of the other subunit. Using the C3-overexpressing strains, we examined if the C1 level was affected in parallel. In comparison to wild type, the level of C1 was slightly decreased in the C3-ox strains (Fig. 1C), indicating that there may be a weak coregulation.

For silencing of C3, an RNAi construct was created (Fig. 1D; for details, see "Materials and Methods" and Supplemental Protocol S2). C3 silencing was checked by western analysis with the anti-C3 antibodies (Fig. 1E). Different amounts of proteins from wild type (100, 50, and 25 μg per lane) were separated on SDS-PAGE and quantitatively compared to proteins from transformed strains (100 μg per lane) after immunoblotting with the anti-C3 antibody. Again, equal loading was checked by Ponceau staining. In the two strains C3-sil$_6$ and C3-sil$_7$, the C3 level was reduced to about 25% (C3-sil$_7$) or clearly below 25% (C3-sil$_6$; Fig. 1E) in comparison to the wild-type protein level. In this case, no significant coregulation with regard to the level of C1 was observed in the C3-silenced strains (Fig. 1F). Thus, the C1 levels in C3-sil$_6$ and C3-sil$_7$ were very close to that of the wild type.

The strains where C3 is up- or down-regulated were used for checking automated circadian phototaxis. As a control, the wild-type strain SAG 73.72 was used. Its period ranges between 24.3 and 24.7 h as determined in several independent experiments. A representative circadian phototaxis rhythm of the wild-type strain (period of 24.7 h) is shown (Fig. 2A). Phototaxis reaches its maximum during the middle of the subjective day and a minimum at the end of the subjective night. Both strains where C3 was overexpressed showed a period that is slightly increased in comparison to wild type (25.2 and 25.5 h, respectively; Fig. 2, B and C). With both C3-ox strains significant, phase angle (acrophase) shifts could be observed in comparison to wild type. This can be best visualized when the phototaxis data are amplified and compared to wild type, which has been done for the C3-ox$_{100}$ strain (Fig. 2D). Thus, the maximum of circadian phototaxis was shifted by a few hours to the very early subjective day and no longer occurred during the middle of the subjective day.

Two C3-silenced strains (C3-sil$_6$ and C3-sil$_7$) showed a period of 24.3 and 24.2 h, respectively (Fig. 2, E and F), that is either in the range found for wild-type cells or very close to it. Again, acrophase shifts could be observed that were even more pronounced as with the C3-ox strains. This is again best highlighted by amplification of the phototaxis data as done with one of the two strains and comparison to wild type (Fig. 2G). Thus, the maximum of phototaxis was shifted by about 6 h to the very end of the subjective night and the minimum to the middle of the subjective day.

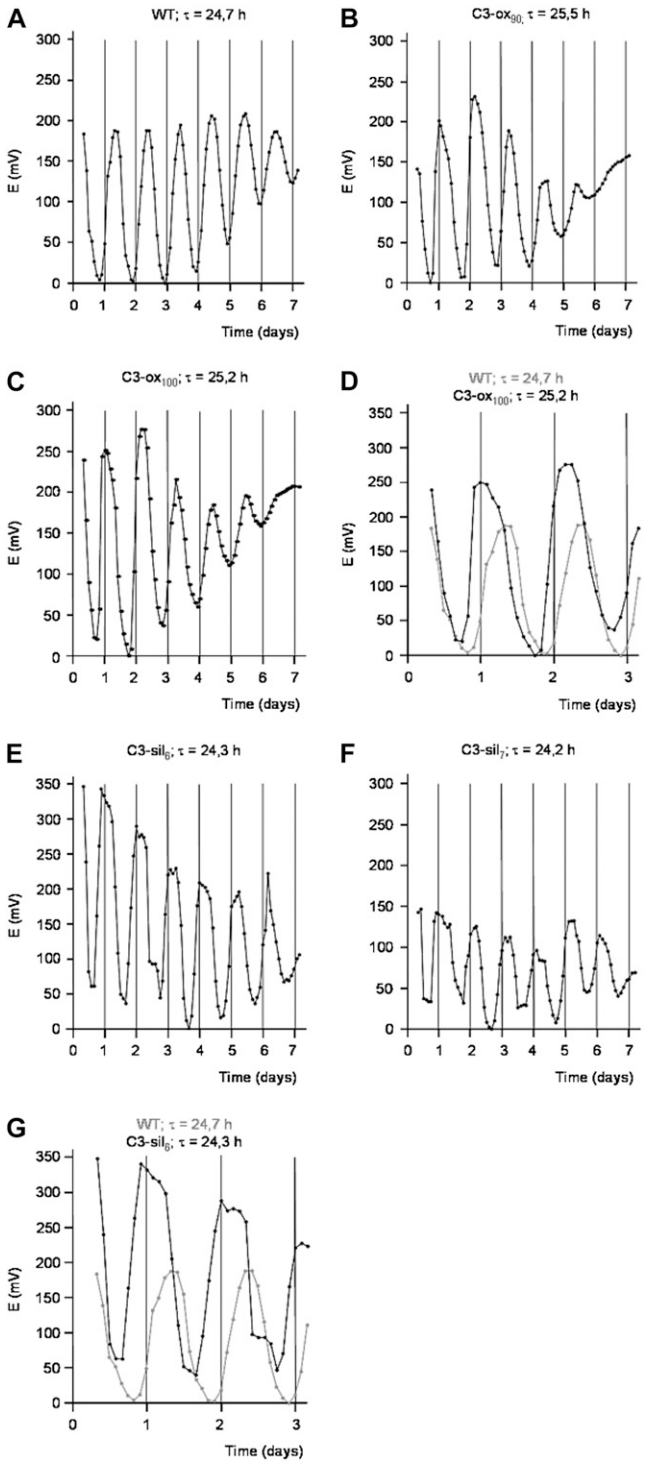

Figure 2. The peak time of circadian phototaxis of cells maintained in constant darkness is significantly shifted toward the night phase in C3-ox and C3-sil strains as compared to the wild-type strain SAG 73.72. Ordinate, Cell density documented as extinction E in mV; abscissa, time in days. The free-running period of the strains is indicated. Phototaxis curves of wild-type (A), C3-ox strains (C3-ox_{90} [B] and C3-ox_{100} [C]) and C3-sil strains (C3-sil_6 [E] and C3-sil_7 [F]) are shown. Amplification and comparison of phototaxis curves of C3-ox_{100} and WT (D) and of C3-sil_6 and WT (G) over the first 3 d under constant conditions are also shown.

Since both up- and down-regulation of the C3 subunit significantly shift the acrophase of the circadian clock, it can be postulated that the C3 subunit represents a component of the circadian clock in *C. reinhardtii* that is involved in phase maintenance, as will be discussed later.

Up-Regulation of the C1 Subunit of CHLAMY1 Causes Coregulation of the C3 Subunit and Arrhythmic Phototaxis

For overexpression of C1, the same strategy was used as for C3 (Fig. 3A; Supplemental Protocol S1). An overexpression of C1 of about 2-fold was observed in the transgenic strains (C1-ox_{18}, C1-ox_{93}; Fig. 3B). Also in this case, it was determined if the other subunit C3 was influenced in its expression level. Clearly, the level of C3 was up-regulated in the C1-ox_{18} and C1-ox_{93} strains (Fig. 3C) with an even higher amplitude (3- to 4-fold) as observed for C1. Thus, in the C1-ox strains the entire C1 and C3 comprising CHLAMY1 complex should be overexpressed. Analysis of phototaxis with the two C1-ox strains revealed that their circadian clock is severely disturbed when examined over a 7 d time period under constant conditions of darkness (Fig. 4). C1-ox_{18} showed a normal rhythmic behavior over the first 2 d in constant conditions as could be seen in several measurements, but then always became altogether arrhythmic (Fig. 4A). C1-ox_{93} had a tendency to arrhythmicity from the very beginning

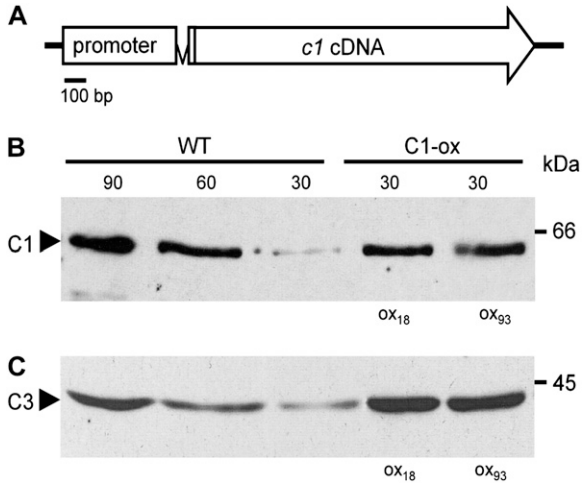

Figure 3. Overexpression of C1 and coregulation of C3. A, The overexpression construct used for C1 is shown. Promoter indicates the truncated *hsp70A/rbcS2* tandem promoter along with the first intron of *rbcS2* that bears an enhancer (see "Materials and Methods"). The tagged *c1* cDNA bears the major part of the *c1* ORF (the first 23 amino acids are missing) and the entire 3′ UTR (see Supplemental Protocol S1). B and C, Different amounts of proteins from a crude extract (90, 60, and 30 μg per lanes) of wild-type (WT) cells were separated on SDS-PAGE and used for western analysis with anti-C1 (B) and anti-C3 (C) antibodies along with proteins of crude extracts (30 μg per lane) from two different C1-overexpressing strains (C1-ox_{18} and C3-ox_{93}). The positions of C1 and C3 are indicated by arrowheads.

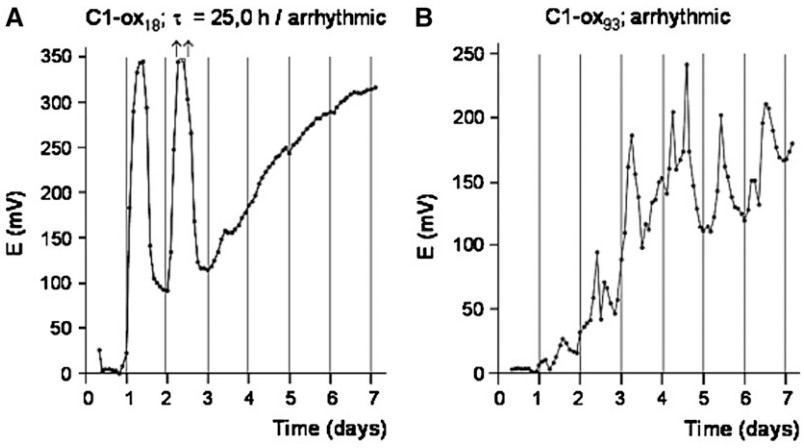

Figure 4. Overexpression of C1 causes arrhythmic behavior. A, Circadian phototaxis of C1-ox_{18} was measured using the automated phototaxis-measuring unit developed by Mergenhagen (1984). Ordinate, Cell density documented as extinction E in mV; abscissa, time in days. White circles over layered by an arrow indicate that the scale for mV measurement was exceeded. The free-running period of 25 h that is observed during the first 2 d is indicated, then the cells became completely arrhythmic. B, Phototaxis of C1-ox_{93} under constant conditions showing disturbances in the circadian clock.

under constant conditions including double peaks during day and night phase (Fig. 4B) or showing rather complete arrhythmicity in some measurements (data not shown).

These data show that a wild-type-like level of the C1 subunit is essential for an intact circadian clock and that its overexpression of about 2-fold can already cause arrhythmicity for the endogenous clock of *C. reinhardtii*.

Down-Regulation of the C1 Subunit of CHLAMY1 Can Cause Arrhythmicity

For silencing of C1, the same RNAi strategy as for C3 was applied (Fig. 5A; Supplemental Protocol S2). Silencing down to a level of about 25% to 30% was observed in some transgenic strains (e.g. C1-sil_{35}), but some strains also showed only silencing down to 40% to 70% (e.g. C1-sil_{72}; Fig. 5B). Again, we examined in the C1-sil strains if the level of C3 was changed in parallel. In strains where C1 was silenced to 25% to 30%, a strong coregulation of the C3 level was observed (e.g. C1-sil_{35}; Fig. 5C), whereby C3 was even more silenced than C1 (below 25%). In contrast, a less pronounced or no coregulation of the C3 subunit was found in the transgenic strains that had shown a smaller degree of C1 silencing (e.g. C1-sil_{72}; Fig. 5C). These data indicate that strong coregulation of C3 in case of C1 silencing depends on a critical low level of C1 that has to be reached in the cell.

A challenging task was the analysis of the strong C1-silenced strains since the levels of C1 and of C3 reverted within a few weeks back to wild-type level. After transformation and selection on paramomycin plates, colonies were always grown up and checked by western analysis for their degree of silencing. If silencing of C1 was occurring, the same colony was grown up and checked again by western analysis to verify the result. In most but not all cases, silencing was still maintained within this time range. But in case of a further repeat 1 or 2 weeks later, reversion back to wild-type level was already completed in most strains analyzed, including C1-sil_{35}. As will be discussed later, such a quick reversion of RNAi strains is unusual and also did not occur with the C3-silenced strains that were prepared by the same strategy. Phototaxis measurements with several strains, where C1 was silenced below 40% and C3 was strongly cosilenced, were done directly after the second verification of C1 silencing and in all cases they showed wild-type-like behavior of phototaxis. For example, phototaxis of C1-sil_{35} is shown (Fig. 6A). However, the wild-type-like behavior could be due to the fact that the C1 level had already been reverted to wild type since every analysis of the silencing level of C1 after the phototaxis assay revealed complete reversion of the C1 level back to wild type in these strains (data not shown).

Only in the case of strains where silencing was not as pronounced, reduced levels of C1 could still be found by western analysis after the phototaxis assay showing that the cells were still silenced in C1 during the assay. Notably, these transgenic lines showed arrhythmic behavior from the very beginning under constant darkness (C1-sil_{72}; Fig. 6B) or after 3 d under constant conditions (data not shown). These data, in combination with the data from the C1-ox strains, suggest that C1 is an essential component of the circadian clock in *C. reinhardtii*.

The Circadian Rhythm of NII Activity Is Also Affected in Strains Where the C1 or C3 Levels Are Reduced

Automated measurement of the phototaxis rhythm over 7 d is well suited to obtain comprehensive information about the effects on phase and period in the transgenic strains. However, it was also of interest to find out if another circadian output rhythm was disturbed in the same way. Therefore, we have manually measured the rhythm of NII activity in strains where either the C3 or C1 levels are reduced in comparison to wild type. NII represents one of the key enzymes of nitrogen metabolism. Its activity was shown before to be diurnally regulated with a maximum during the middle of the light period (Pajuelo et al., 1995). *Nii* mRNA bears an UG repeat in its 3′ UTR that is recognized by CHLAMY1 (Waltenberger et al., 2001). Thus,

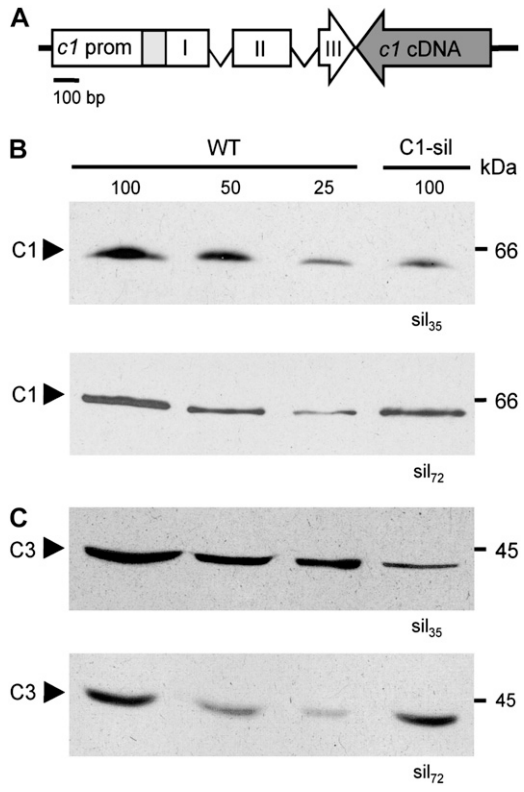

Figure 5. Silencing of C1 below a critical level causes strong coregulation of C3. A, The RNAi construct used for silencing of C1 is shown. The numbers represent the involved exons. Introns are indicated by lines. The light-gray area represents the 5′ UTR, and *c1* prom the potential promoter region of *c1* (Supplemental Protocol S2). B and C, Different amounts of proteins from a crude extract (100, 50, and 25 μg per lanes) of wild-type (WT) cells were separated on SDS-PAGE and used for western analysis with the anti-C1 (B) and anti-C3 (C) antibodies along with proteins of crude extracts (100 μg per lane) from different C1-silenced strains (C1-sil$_{35}$ and C1-sil$_{72}$). The positions of C1 and C3 are indicated by arrowheads.

it can be seen if CHLAMY1 may regulate one of its direct output targets in the same way as phototaxis. For this purpose, we have released cells that were grown under a light-dark (LD) cycle to constant dim light (LL) and measured NII activity starting from the second day during subjective night (LL 38) until the end of the next subjective day (LL 58) in a 4 h interval. In wild-type cells, NII showed a circadian rhythm in its activity, however, with a rather modest amplitude. Maximal activity occurred during early day phase (Fig. 7A). In the C3-sil$_6$ strain, the acrophase of the peak of NII activity was shifted toward the night phase (Fig. 7B). Thus, maximal NII activity appeared at the end of the subjective night and decreased already during early subjective day. Thereby, the amplitude was enhanced in comparison to wild type.

For analyzing NII activity in a C1-silenced strain, we chose a most recent characterized strain, which is not very strongly silenced to avoid potential reversion during the experiments, as mentioned above. In C1-sil$_{81}$ (Fig. 7D) the C1 level was reduced to about 53%. Immediately after the western analysis, the strain was used for the measurements of NII activity and it was verified after the experiment that the C1 level was still reduced. In this case, arrhythmic NII activity was observed (Fig. 7C). Thus, reduced levels of C3 or C1 cause the same effects (shifts in acrophase or arrhythmicity) on the rhythm of NII activity as on the phototaxis rhythm.

DISCUSSION

Strategies Used to Silence and Overexpress the C1 and C3 Subunits, Respectively

In this study, we have analyzed the role of the two subunits of CHLAMY1 within the circadian system by either silencing them via RNAi or overexpressing them under the control of a strong promoter. For RNAi, the method that was developed by Fuhrmann et al. (2001) for silencing of chlamyopsin was chosen that involved usage of the endogenous promoter of the gene. Thus, the potential promoter regions of the c1 and c3 genes together with the first exons and introns were fused to an inverted cDNA part so that a double-stranded RNA will be formed in the cell that triggers silencing. Such an approach had already been successfully applied for silencing of CK1 in our lab (Schmidt et al., 2006). Maximal silencing down to about 25% (with C1) or even below 25% (with C3) of wild-type level was observed, which is in the range of other RNAi silencing approaches conducted in *C. reinhardtii* (for review, see Schroda, 2006). While silencing of CK1 and C3 was relatively stable for a few months when the cells were kept on paromomycin plates, and only little reversion was observed during this time frame, strong silencing of C1 was not stable over time and could only be maintained for a few weeks. Reversion of RNAi strains back to wild-type level over a few months has been observed with RNAi and antisense constructs of *C. reinhardtii* (Schroda, 2006). However, the relatively quick reversion of C1 seems to be a particular case of C1 especially when it is silenced below a critical level of about 40% and may be triggered by a specific mechanism within the cell.

For overexpression, the strong truncated hsp70A/rbcS2 tandem promoter together with the first rbcS2 intron that bears an enhancer (Lumbreras et al., 1998; Sizova et al., 2001) was used. The truncated rbcS2 promoter was shown to be independent of light (Fuhrmann et al., 2004). The truncated tandem promoter was checked recently with regard to a potential circadian expression and found to mediate rather constant expression over a circadian cycle (S. Kiaulehn, O. Voytsekh, M. Fuhrmann, and M. Mittag, unpublished data). While C3 could be overexpressed more than 3-fold using this promoter, C1 overexpression ranged about 2-fold at most. Also, in the case of C1 overexpression, reversion back to wild type was

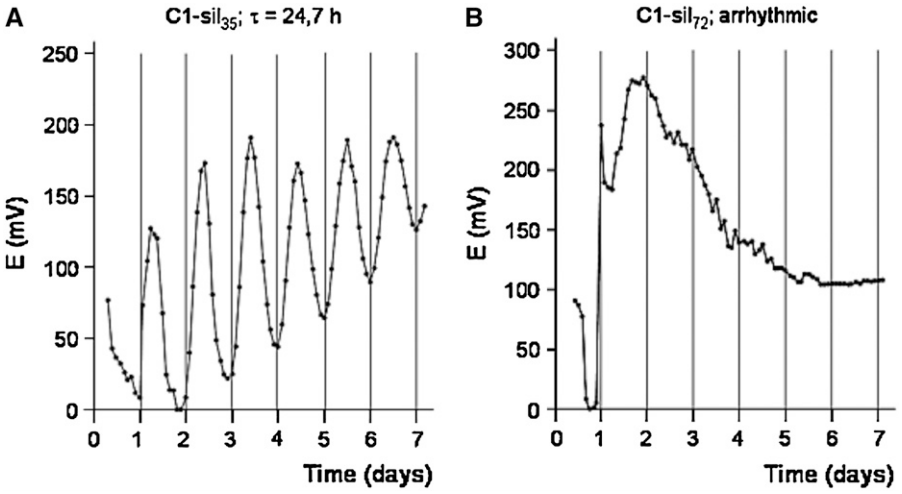

Figure 6. Silencing of C1 can cause arrhythmic behavior. A, Circadian phototaxis of C1-sil$_{35}$ was measured using the automated phototaxis-measuring unit developed by Mergenhagen (1984). Ordinate, Cell density documented as extinction E in mV; abscissa, time in days. The free-running period of 24.7 h is indicated. It should be noted that the C1 as well as the C3 levels were fully reverted back to wild-type level when C1-sil$_{35}$ cells were checked after the phototaxis experiment. B, Phototaxis of C1-sil$_{72}$ under constant conditions showing complete arrhythmicity. In this case, silencing was still observed after the phototaxis experiment.

observed, however, not in such a short time range as was found with its silencing. These data give some indications that high up- or down-regulation of C1 might have some toxic effects for the cell.

Changes in the Level of C3 Phase Shift the Peak of Circadian Phototaxis and of NII Activity

The major purpose of the up- and down-regulation of the C1 and C3 subunits was to study the function of the heteromeric RNA-binding protein within the circadian system. For this purpose, measurement of circadian phototaxis is well suited as an indicator for potential disturbances within the circadian clock since it can be automatically measured under constant conditions over a 7 d time range. Thus, sound conclusions can be drawn about effects on the phase and period of the circadian clock.

To examine the influence of up- and down-regulation of the C3 subunit, the phototaxis rhythm was measured in the transgenic C3-ox and C3-sil strains. Increasing amounts of the C3 level caused a slight period lengthening (about 0.5 h) in comparison to wild type. Decreases in the C3 amounts did not change the period in a significant way. However, both overexpression and silencing of C3 shifted the acrophase of the rhythm significantly. Thus, the maximum of circadian phototaxis, which occurs during the middle of the day phase in wild type, appeared at either the end of the night phase in C3-sil strains or the very beginning of the day phase in C3-ox strains. Thereby, the acrophase was shifted up to 6 h towards the night and this shift remained stable over the 7 d of measurement (Fig. 2). The shift in acrophase toward the night was also observed when another output rhythm, namely NII activity, was measured in C3-sil$_6$. While the rhythm of NII activity has its maximum during early day phase in wild-type cells, maximal activity of NII in the C3-sil strain occurred during late night phase. These data suggest that the C3 subunit is involved in maintaining the correct phase angle of output rhythms such as phototaxis or NII activity.

Physiologically, phase shifts can be caused, for example, by pulses of light to which the cells are exposed at specific times of a circadian cycle as was first demonstrated by Hastings and Sweeney (1958) with a marine dinoflagellate. In *C. reinhardtii* action spectra have been determined for shifting the phase of its circadian phototaxis rhythm by light (Johnson et al., 1991; Kondo et al., 1991). These experiments suggested the involvement of blue- and red-light photoreceptors.

A disturbance in the acrophase of a circadian rhythm caused by up- or down-regulation of specific proteins had been already shown, for example, in case of the PAS/LOV protein VIVID (VVD) of *N. crassa* (Heintzen et al., 2001; Elvin et al., 2006). In those experiments, knockout of vvd resulted in a phase shift of about 4 h with regard to the conidiation rhythm in wild-type cells. Also, it was shown that temporal overexpression of the cyanobacterial clock gene kaiC can reset the phase of the rhythm (Ishiura et al., 1998). In *Arabidopsis thaliana*, phase-specific circadian regulation of transcription was recently found (Harmer and Kay, 2005). Further, it was shown in *N. crassa* that WC-1 possesses a promoter element that can modulate phase (Káldi et al., 2006). Thus, molecular components that are involved in phase adjustment are under investigation. The finding that an RNA-binding protein such as C3 can be involved in phase determination is, however, novel.

C3 does not have a PAS/LOV domain such as VVD or WC-1 that could perceive light information. We therefore hypothesize that the C3 subunit functions as a transducing component that can receive light information from a circadian photoreceptor and then alter the phase angle of circadian output rhythms.

The Duo of C1 and C3 Is Essential for an Intact Circadian Clock in *C. reinhardtii*

Previous experiments have demonstrated that CHLAMY1 consists of the C1 and C3 subunits and that their interaction is necessary to bind to their RNA targets (Zhao et al., 2004). Interestingly, overexpression

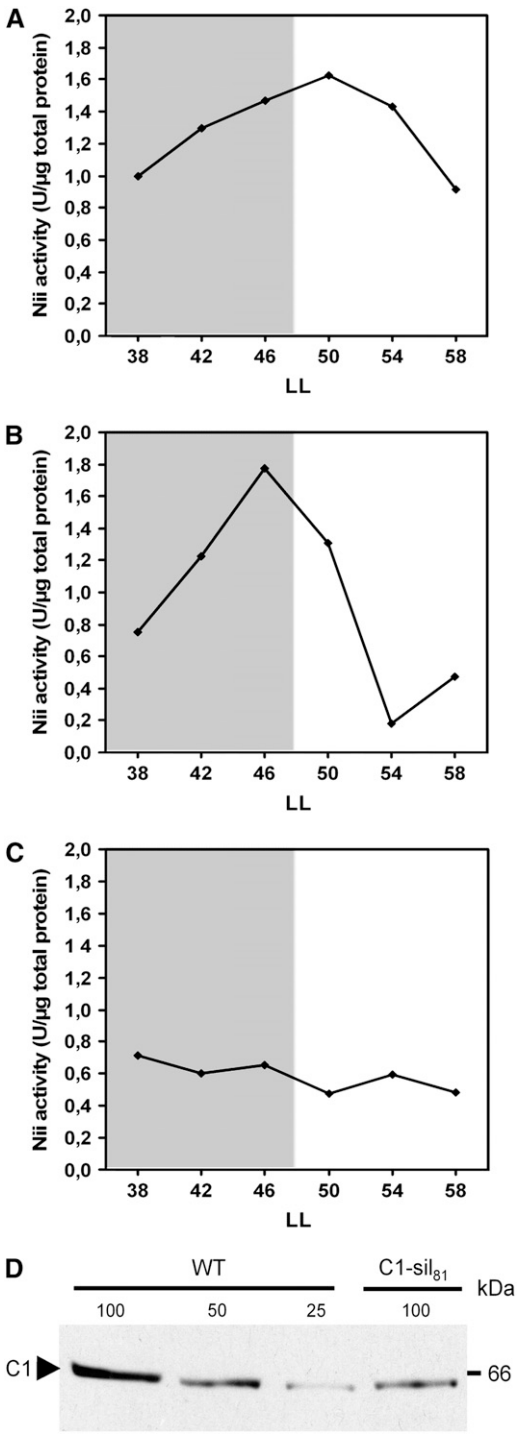

Figure 7. NII activities over the circadian cycle in wild-type cells compared to cells where C3 or C1 are silenced. Cells were grown under a LD (12:12) cycle and then transferred to constant conditions of dim light (LL 0). After 38 h, cells were harvested every 4 h at the indicated times. Crude extracts were prepared and enzyme activities were determined as described in "Materials and Methods." Subjective night, gray background; subjective day, white background. A, Wild-type cells SAG 73.72. B, Strain C3-sil$_6$. C, Strain C1-sil$_{81}$. Repetition of the NII measurements in the different strains resulted in similar results as shown. D, Different amounts of proteins from a crude extract (100, 50, and 25 μg per lanes) of wild-type (WT) cells were separated on SDS-PAGE and used for western analysis with the anti-C1 antibodies along with proteins of a crude extract (100 μg per lane) from the C1-sil$_{81}$ strain.

of C1 caused a parallel increase in C3. Thus, the level of the CHLAMY1 complex as a whole should be up-regulated in the C1-ox strains. In these strains the circadian clock, as judged by measuring the phototaxis rhythm, is severely disturbed and arrhythmicity occurs. While C1-ox$_{18}$ still shows circadian behavior for the first 2 d under constant conditions before it becomes arrhythmic, C1-ox$_{93}$ has disturbances in its circadian rhythm from the very beginning. This is especially impressive when one considers that the level of C1 is altered very little (increase of only about 2-fold) in the two C1-ox strains. In the case of C1-ox$_{18}$, where a wild-type-like circadian behavior was still visible during the first 2 d under constant conditions, there was no phase shift observed over this time range even though C3 was strongly co-up-regulated. Thus, one has to assume that the influence of increased C3 on acrophase shifting can be counteracted by the increased level of C1.

In the case of C1 silencing, arrhythmic behavior of phototaxis could be found in strains where C1 was only slightly silenced (e.g. C1-sil$_{72}$). In such strains silencing of C1 was still observed after the phototaxis assay was finished when analyzed by westerns. C1-sil strains that had a more pronounced silencing below 40% (e.g. C1-sil$_{35}$) were not stable over time. Reversion of C1 and in parallel of C3 back to its wild-type level occurred within a relatively short period of time and in each case examined the reversion was already completed when cells were analyzed after the phototaxis experiment. Thus, the wild-type-like circadian rhythm of C1-sil$_{35}$, for example, cannot be interpreted in an unambiguous way. We assume that the cells were already reverted before the phototaxis measurement and thus a wild-type-like behavior occurred. It would be rather surprising if slight overexpression (about 2-fold) as well as slight silencing of C1 (e.g. down to about 70%) can cause arrhythmicity, while stronger silencing of C1 would not. However, we cannot rule out this possibility for sure.

Also in the case of C1 silencing, we checked the rhythm of NII activity as a second circadian output process using C1-sil$_{81}$. In this strain the C1 level was reduced to about 53% and the reduced level could be still confirmed after the NII assay. Clearly, arrhythmicity of NII activity was observed. These data show that changes in the C1 level can cause arrhythmicity with regard to both analyzed output rhythms and corroborate its important role in the circadian clock of *C. reinhardtii*.

Coregulation of the C1 and C3 Subunits

In all model organisms studied so far the mechanism of circadian oscillation is triggered by positive

and negative feedback loops (Harmer et al., 2001; Reppert and Weaver, 2002; Dunlap and Loros, 2004; Johnson, 2004) whereby transcription factors are involved. Here, we show that changes in a heteromeric duo of RNA-binding proteins, the KH-domain bearing C1, and the RRM-spanning C3 proteins, which can interact with each other, are able to modulate acrophase, alter slightly period, and result in arrhythmicity. Thus, key features of a circadian clock are affected in strains where C1 and/or C3 levels have been altered. It is of interest that the two subunits are interconnected in their expression level. An increase in C1 causes up-regulation of C3 and a decrease in C1 below a critical point results in strong down-regulation of C3. In both cases, the expression of C3 is altered with an even higher amplitude than C1 in comparison to wild type. In contrast, changes in the C3 level have little (C3 overexpression) or no significant (C3 silencing) effect on C1 expression. Therefore, C1 seems to be the core unit in the CHLAMY1 complex. A slight increase in C1 triggers an increase of C3, as observed with both C1-ox strains. In case of C1 silencing, there seems to be a critical low level of C1 that has to be reached to cause strong cosilencing of C3. Thus, the well-silenced C1-sil strains (e.g. C1-sil$_{35}$) showed strong coregulation of C3, while the less-silenced C1 strains (e.g. C1-sil$_{72}$) showed only weak coregulation.

Currently, we do not know by which mechanism this coregulation occurs, but this matter will be of great interest in future studies. In the case of the assembly governed regulation of the complex biogenesis of subunits of PSI and PSII, it was found that translation is involved in this process and that the 5′ UTRs communicate this regulation (Wostrikoff et al., 2004; Minai et al., 2006). If a comparable mechanism occurs with C1 and C3, there exists the possibility that C1 itself as an RNA-binding protein might regulate the translation of C3, for example. However, C1 might also activate some yet unknown factor that influences the expression of C3.

MATERIALS AND METHODS

Cell Culture

Chlamydomonas reinhardtii cells (wild-type strain SAG 73.72) were grown in Tris-acetate phosphate (TAP) medium (Harris, 1989) under a 12-h light/12-h dark cycle (LD 12:12) with a light intensity of 71 μE m^{-2} s^{-1} (1 E = 1 mol of photons) at 24°C. Cells were grown under LD cycle unless otherwise indicated. The beginning of the light period is defined as time zero (LD 0) and the beginning of the dark period is LD 12.

Preparation of Plasmid Constructs for Overexpressing of C1 and C3

For overexpression of C1 or C3, major parts of their cDNAs were put under control of the strong truncated *hsp70A/rbcS2*-tandem promoter of pRbcBRL that is fused with a two-amino acid spacer to the first intron of rbcS2 that bears an enhancer (Lumbreras et al., 1998; Sizova et al., 2001; Fuhrmann et al., 2004). The detailed cloning procedures for the overexpressing vectors can be found in Supplemental Protocol S1.

Preparation of Plasmid Constructs for Silencing of C1 and C3 via RNAi

Silencing of C1 and C3 by RNAi was done according to Fuhrmann et al. (2001). The detailed cloning procedures for the RNAi vectors can be found in Supplemental Protocol S2.

Transformation of *C. reinhardtii* with the RNAi and Overexpressing Vectors

C. reinhardtii wild-type strain SAG 73.72 was grown in TAP medium under a LD 12:12 cycle with a light intensity of 71 μmol·m^{-2}·s^{-1} at 24°C up to a cell density of 2 to 5 $\times$ 10^6 cells/mL for transformation. Twenty micrograms of DNA of each plasmid DNA that was linearized with *Sca*I were used for transformation according to Kindle (1990) with the following modification. Vortexing of the cells with glass beads was carried out for 15 s. Cells were then transferred in liquid TAP medium in light overnight according to Davies et al. (1992) and plated with 0.5% cover agar on TAP-paromomycin plates (selection medium) following the protocol of Sizova et al. (2001).

Preparation of Crude Extracts of *C. reinhardtii* and Western-Blot Analysis

Cells were grown to a cell density of 1 to 5 $\times$ 10^6 cells per mL, harvested by centrifugation at LD6, and stored at −80°C after being frozen in liquid nitrogen. Aqueous extracts were prepared according to Zhao et al. (2004). All extracts were prepared in the presence of Complete Proteinase Inhibitor Cocktail (Roche-Applied-Science) according to the user's manual.

Western-blot analysis was done as described in Zhao et al. (2004) with chemiluminescence detection.

Automated Measurement of Circadian Phototaxis (Photoaccumulation) with Wild-Type, RNAi, and Overexpressing Strains

Phototaxis measurements were done with a custom-made machine, developed and described by Mergenhagen (1984). Briefly, a light beam that passed through the culture in a cuvette (30 mL flat Falcon tube) was measured by a photocell, such that the amount of light transmitted depended on the number of cells in the light path. The accumulation of the cells in the light path was determined by the cell density (documented as extinction in mV) with the minimum value (few or no cells in the light path) being arbitrarily set at zero and was measured every 2 h for a period of 20 min. The recording system was installed in a temperature-controlled dark room of 22.5°C.

Assay for NII Activity

C. reinhardtii cells (wild-type strain SAG 73.72) were grown in TAP medium with NO_3^- as nitrogen source under a LD 12:12 cycle and then put under constant conditions of dim light (LL; 15 μE m^{-2} s^{-1}) before further use. The beginning of the dim light period is defined as LL 0. Hours under which cells have been kept under dim light are given.

For the NII assay, cell pellets were washed with 50 mM Tris/5 mM EDTA, pH 8, two times before their storage at −80°C. For extracts, cells were resuspended in a buffer of 50 mM Tris/5 mM EDTA pH 8/14 mM dithiothreitol, and lysed by vortexing (highest speed) with glass beads (diameter: 0.25–0.30 mm) for 5 $\times$ 1 min. After each min of vortexing, they were placed on ice for 2 min. Cell debris was removed as described in Zhao et al. (2004) and the resulting crude extract was immediately further used. NII activity was determined by a dithionite assay as described by Vega et al. (1980) along with 15 μg of total protein from the crude extract. The reaction was carried out for 20 min at 40°C. The assay involved sodium dithionite as reductant and methyl viologen as electron carrier. Enzymatic activity was followed by measuring colorimetrically (540 nm) the rate of disappearance of nitrite. One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of nitrite per minute.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AAR90343 (C1) and AAR90344 (C3).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Protocol S1. Plasmid constructs for overexpressing C1 and C3.

Supplemental Protocol S2. Plasmid constructs for silencing of C1 and C3.

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