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Surface Plasmon Resonance Spectroscopy (SPR) Interaction Studies of the Circadian-Controlled Tomato LHCa4*1 (CAB 11) Protein with Its Promoter

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ABSTRACT

Feedback regulation is an important biochemical mechanism which is also able to direct the circadian timing at the transcriptional level. Independent investigations highlighted a conserved ca. 10 nucleotide motif present in many circadian regulated Lhc genes. Two of such nucleotide motifs exist within 119 nucleotides of the Lhca4*1 promoter from tomato. This promoter fragment was used as a bait in a yeast one hybrid screen and interestingly a clone encoding with sequence identity to the LHCa4*1 protein was isolated as an interaction partner. The LHCa4*1 protein was heterologous expressed and binding to the 119 bp promoter fragment was demonstrated by surface plasmon resonance spectroscopy (SPR, Biacore). This result allows to postulate an autoregulatory feedback loop involved in expression of the Lhca4*1 gene.

Key Words: Light harvesting complex protein (LHCa4*1); Circadian regulation; Autoregulatory feedback loop; Yeast one hybrid screen; Surface plasmon resonance spectroscopy (SPR); Tomato.

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INTRODUCTION

In plants the light harvesting complex (Lhc) genes were the first for which circadian mRNA oscillations were demonstrated (Kloppstech, 1985; Piechulla and Gruissem, 1987) as well as circadian regulation of LHC protein synthesis (Riesselmann and Piechulla, 1992). Recently, a microarray-based analysis indicated that ca. 450 genes in *Arabidopsis thaliana* exhibit this characteristic mRNA accumulation pattern (Harmer et al., 2000). The oscillations vary in respect to the amplitude and the time points when peaks or troughs occur, e.g., photosynthesis-related genes accumulate to highest levels around noon (middle of subjective day), while genes encoding enzymes related to secondary metabolism peak at the end of night (before subjective dawn). This coordinated gene expression demands for a mechanism that has the ability to synchronize transcriptional and translational activities, biosynthetic pathways, metabolic activities, etc., a function that can be realized by the circadian clock.

Nuclear run-off experiments and transcriptional gene fusion experiments established that circadian regulation occurs at the transcriptional level, where DNA binding proteins (transcription factors) interact with the promoter regions of specific genes. It can be anticipated that coordinately expressed genes are regulated by the same set of *cis-* and *trans-*acting elements, e.g., genes that are transcribed during the day share a promoter motif that binds a specific transcription factor and genes that are transcribed around dusk posses different promoter motifs and interact with other clock-regulated transcription factors.

Three independent experimental approaches revealed DNA motifs that are implicated to be involved in circadian control of transcription in plants, (i) to understand the phytochrome signal transduction chain of the A. thaliana Lhcb1*3 gene a consensus promoter sequence .AAAAATCT' and a myb-related transcription factor CCA1 were identified (Wang et al., 1997); (ii) a promoter sequence analysis combined with promoter deletion studies of four tomato Lhc genes in transgenic plants revealed the motif $,CAA(N)_{3-4}ATC^{\circ}$ (Piechulla et al., 1998); and (iii) the survey of ca. 450 oscillating A. thaliana genes identified 46 times a nine nucleotide motif AAAATATCT' in 31 cycling genes peaking at the end of the subjective day and therefore it was called the evening element (EE) (Harmer et al., 2002). Two facts are interesting to note: (i) the identified motifs have an ,ATC' sequence in common and (ii) while for the first and second case the motifs were found in promoters of day specific genes (Lhc mRNAs accumulate around noon), the latter motif was found in promoters of genes where mRNAs peak around dusk. In a detailed analysis it was recently shown that nucleotides surrounding the ,ATC' consensus sequence determine the evening and morning specificity, a ,T^c 5' of ,ATC^c is responsible for evening expression while the ,A' 5' of ,ATC' determines the morning specific expression. Furthermore, other nucleotides in the context are also necessary for full circadian activity (Michael and McClung, 2002).

To understand the complete transcriptional regulation DNA binding proteins interacting with promoter sequences have to be isolated and characterized. Our analysis of the promoters of the 19 tomato Lhc genes (Kellmann et al., 1993) revealed the ,CAA (N)₃₋₄ ATC' motif which is present in 81% of all published Lhc promoters (Piechulla et al., 1998). We therefore used this motif in a yeast one hybrid interaction screening as a bait to isolate potential binding proteins. The previous characterization (Kellmann et al., 1999) and the presence of two respective motifs in the Lhca4*1 (formerly called cab 11) promoter were good reasons to choose this promoter for promoter interaction studies.

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MATERIALS AND METHODS

Preparation of an Activation Domain cDNA Library

Lycopersicon esculentum Mill. var. VNFT LA 1221 was grown for 4 weeks in a 12h dark/light regime. Leaves were harvested 15 min prior to the dark/light or light/dark transition, RNA was extracted and purified using guanidinium thio cyanate (GTC) and CsCl cusions (Kellmann et al., 1990; Sambrook and Russell, 2001). Poly A^+ RNA was enriched using oligo-dT₃₀-Oligotex (Qiagen, Hilden, Germany) and cDNA synthesis and ligation was performed with the HybriZAP-2.1 Two Hybrid cDNA Synthesis Kit (Stratagene, Heidelberg, Germany). The ligated phage cDNA was packaged into particles using the Gigapack III Gold Packaging Extract (Strategene, Heidelberg, Germany). After amplification of phages in *E. coli* (XL1 Blue, MRF') phage suspension was used for a second infection together with a helper plasmid (ExAssist TM; Strategene, Heidelberg, Germany) to obtain a phagemid DNA library (pAD-GAL4-2.1) by in vivo excision process in *E. coli*. Cells were lysed and phagemids were obtained from the supernatant.

Yeast One Hybrid Screen

Following the instruction manual for the One-Hybrid System (Clontech, Heidelberg, Germany) both reporter genes (histidin His and beta-Galactosidase LacZ) were fused with the 119 bp promoter fragment of Lhca4*1 gene from tomato (form -119 to +3; Kellmann et al., 1999). Prior to the transformation (40% PEG/100 mM lithium acetate in TE buffer pH 7.5) into yeast cells the constructs pHisi and pLacZi were linearized with XhoI and Ncol, respectively, to allow integration into the yeast genome. Consecutive transformed yeast cells were incubated on minus L-histidin, minus L-uracil single dropout medium (Ausubel et al., 2001) at 30°C for 4–6 days. After yeast transformation with the pHisiconstruct colonies were tested on 15-60 mM 3-AT (Aminotriazol) medium, to select transformants that did grow on lower or up to 15 mM 3AT-concentration. Only such transformants were useful for transformations with pLacZi and further screening experiments. Positive clones were than tested for β -galactosidase expression using the filter lift assay. Such reporter yeast cells were then transformed with 200 µg DNA of the tomato AD-cDNA-library DANN (pAD-Gal4-2.1), yeast cells were regenerated and incubated on minus Leu, minus His, minus Ura plus 3-AT for 4-7 days at 30°C. 3-AT (60 mM) was added to repress leaky transcription of the pHisi construct. Positive clones were transferred to fresh plates and the β -galactosidase filter assay was performed. pAD-Gal4-2.1 plasmids were isolated from positive yeast cells (Bartels and Fields, 1995), amplified in E. coli and analyzed on agarose gels and transferred again into the reporter yeast cells to verify a second time the interaction with the 119 bp promoter of the tomato Lhca4*1 gene. Only clones which showed growth after 4-6 days and were positive in the beta-galactosidase filter assay were considered positive and were sequenced. PCR for sequencing were performed with the SequiTherm EXCEL II Long-Read DNA Sequencing kit (Biozym, Hameln, Gemany) and analyzed on the LiCor 4200 (MWG, Ebersberg, Germany). Obtained sequences were aligned in a BLAST SEARCH to gain information about structural homologies and possible functions of the cloned sequences.

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Heterologous Expression in E. coli

A cDNA clone containing the full-length sequence (including the leader sequence) of the Lhca4*1 gene from *L. esculentum* was a gift from Dr. Ralf Werner (University of Hamburg). The complete gene was cloned into the expression vector pQE31 plasmid (Qiagen, Hilden, Germany) via BamHI and SalI and transformed into *E. coli* (M15). Cells were grown and Lhca4*1 expression was induced with IPTG (1 mM final concentration) for 4–5h.

Purification under native conditions: proteins were isolated from 100 mL cell culture pellets by adding 5 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazol, pH 8.0) and 5 mg lysozyme, 30–60 min incubated at 4°C, six times 20 sec of ultra sonification, centrifugated 30 min 10,000 × g at 10°C. The supernatant was combined with 1 mL Ni-NTA-agarose and shaked for 2h on ice. The agarose was applied to a column and washed twice with wash solution (lysis buffer + 20 mM imidazol). Bound proteins were eluted with elution buffer (lysis buffer + 250 mM imidazol).

Purification under denaturing conditions: The lysis buffer contained 100 mM NaH₂PO₄, 10 mM Tris–HCl, and 8 M urea, pH 8.0.; the washing solution (lysis buffer, pH 6.3); the elution buffer (lysis buffer, pH 5.9 or 4.5). Beside the different buffers the procedure was the same as under native conditions. Native and denatured proteins were applied to SDS-PAGE and tested with polyclonal antibodies against LHCb protein in Western Blots (Sambrook and Russell, 2001) and used for SPR measurements.

Preparation of Promoter-Streptavidin Chips

Two promoter fragments of the Lhca4*1 gene were obtained (p119: from -119 to -1; p40: from -119 to -80) by PCR amplification using biotinylated primers (for the 3'B-p119 promoter fragment: sense: 3'GGA ATT CGC ACA ACT CAA TCC AAC CGT-5'; antisense: biotin-5'AAC TTT GTA TGT TTT GTA T3'; for the 5'B-p40 fragment: sense: biotin-5'GCA CAA CTC AAT CCA ACC GT4', antisense: 5'CTA AGA TTT TTT GGA ATC CA-3'). Biotin labeled PCR products were immobilized to the streptavidin sensor chips (Sensor Chip SA, BIAcore): the chip was transferred into the BIAcore 3000 instrument and was prepared by washing three times with $20 \,\mu$ L 1M NaCl, 50 mM NaOH (flow rate: $20 \,\mu$ L min⁻¹, 25° C). Then 0.3 μ g biotin-promoter DNA in $60 \,\mu$ L 0.5 M NaCl (flow rate $2 \,\mu$ L min⁻¹) were applied. The immobilized p119 fragment revealed an increase of >2000 resonance units (RU) and such chips were stable for several weeks in H₂O at 4°C.

Protein-DNA Interaction Studies (Surface Plasmon Resonance Spectroscopy)

The streptavidin chips immobilized with the promoter DNA were applied to the BIAcore 3000 and washed with buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA) (flow rate $20 \,\mu L \,min^{-1}$, 30° C). $30 \,\mu L$ (20–50 ng) of the purified protein samples were applied in the same buffer with a flow rate of 5–10 $\mu L \,min^{-1}$. The sample was also applied to an unloaded flow cell of the streptavidin chip, to allow automatical substraction of resonance differences of the unloaded cell from the values obtained from the

loaded flow cell. Association and dissociation of proteins were automatically recorded. With 10 min postinjection the dissociation reached a new base line which was higher than the base line at the beginning of the experiment, the resonance difference accounts for bound protein and is expressed in RU.

RESULTS

Screening for Promoter Interacting Proteins/DNA Binding Proteins

The 119 bp of the Lhca4*1 promoter were used as a bait to screen an activation domain (AD) cDNA library from tomato leaves in a yeast one hybrid interaction assay. The promoter was ligated to the his3 and the lacZ reporter genes and yeast cells were transformed with 200 μ g of the tomato AD cDNA library. Addition of 3-aminotriazol (3-AT), a competitive inhibitor of the histidin synthesis, was used to increase the stringency of the assay conditions. Twenty clones appeared within 6 days under 60 mM 3-AT on a histidin minus medium. These clones were retransformed and again tested on 30 and 60 mM 3-AT and a filter lift galactosidase assay was performed. Positive clones contained inserts between 150 and 2900 bp which were sequenced. The BLAST SEARCH revealed four interesting clones with sequence similarities to: (i) the *L. esculentum* Lhca4*1 gene; (ii) the *A. thaliana* transaldolase gene; (iii) the *Gossypium hirsutum* photosystem II 5 kD-precursor protein; and (iv) the *L. esculentum* phytoene synthase I precursor protein.

Unexpected and interestingly, one of the clones showed 100% sequence identity to the C-terminal sequence of the LHCa4*1 protein (Fig. 1). This protein fragment was 109 aminoacids long and comprises part of the second (aminoacid 143–152) and the complete third membrane spanning helix (aminoacid 197–225), a hydrophilic region of 17 aminoacids (aminoacid 179–196) was located between these helices, and an amphiphatic alphahelix was found in the C-terminal end of the protein. Particularly the hydrophobic and amphiphatic regions are presumably responsible for the interaction of the protein fragment with DNA. To investigate further the possible binding between the LHCa4*1 protein with its promoter sequence, in vitro experiments such as SPR spectroscopy were performed.

Heterologous Expression of Proteins

The yeast one hybrid screen demonstrated that the C-terminal fragment of the tomato LHCa4*1 protein interacted with the 119 bp promoter fragment of the same gene. To test this binding by SPR it was necessary to enrich or isolate the protein. The complete cDNA sequence of the Lhca4*1 gene was cloned into the expression vector pQE30 (including a $6 \times$ his tag), transformed into *E. coli* and after induction with IPTG the protein was isolated under native or denatured conditions (Fig. 2). In this *E. coli* extracts a protein band of approximately 28 kD was enriched [Fig. 2(A), lanes 3 and 4] compared to extracts prepared from *E. coli* carrying the vector without insert [Fig. 2(A), lanes 1 and 2]. Western blot analysis with specific polyclonal antibodies against tomato LHCb proteins identified the 28 kDa protein and an additional band of approximately 60 kDa [Fig. (2B)]. Further detailed analysis (not shown) indicated that this protein tends to form aggregates, preferentially dimers and trimers, oftenly composing inclusion bodies in *E. coli*. Further purification via



T2 109 aa Gal4-AD 3'UTR ...Poly(A) LHCa4*1 (tomato) 251 aa 3'UTR ...Poly(A) м С ASRTR GSSGKLNREVSFRPST 0 Α IFRP L т 41 51 64 s S N S F к Е AKK GO T. P G т. s Р DYL D GSL Ρ G D LG 81 84 84 118 D NL I QAEL L G G GMLLPE v SIGI 121 133 152 LN VPKWYDAGKSEYFASSSTLF FHYVE IRRWQDI 161 179 196 KN VNQDPIFKNYSLPPNKC G YPGGI FNPLNFAPTEEA 201 225 ΚE K E L A N G R L A M L A F L G F I V Q H N V T G K G P F D N L L Q H L S D P 241 WHNTIIQTLSN*

Figure 1. Sequence and domains of the LHCa4*1 protein from tomato. The activation domain (AD) cDNA clone T2 isolated via the yeast one hybrid screen shares 109 amino acids with the C-terminal region of the tomato LHCa4*1 protein (100% identity). The amino acid sequence of the LHCa4*1 protein is presented. Underlined sequence represents clone T2, aminoacid 1–51: transit peptide; 64–84: hydrophilic region; 87–118: 1st membrane spanning α -helix; 133–152: 2nd membrane spanning α -helix; 179–196 hydrophilic region; 197–225: 3rd membrane spanning α -helix.

Ni-NTA affinity chromatography revealed enriched denatured and native LHCa4*1 protein fractions [Fig. 2(C), (D), respectively], containing also the putative 60 kD dimer.

Surface Plasmon Resonance Spectroscopy Studies

Binding of purified LHCa4*1 protein with its promoter was studied via SPR. Two Lhca4*1 promoter regions (p119 and p40) were synthesized, labeled with biotin and bound to streptavidin chips (Fig. 3). The two different promoter fragments were orientated in two ways to allow different expositions of the two motifs to the LHCa4*1 proteins. The purified protein solutions containing the native or denatured enriched LHCa4*1 proteins were injected to the BICcore 3000 to allow binding to the DNA promoter regions. Different binding buffers were tested with the p119 promoter fragment and *E. coli* extracts [as in Fig. 2(A)], showing that additon of 0 or 1 mM EDTA and 80 mM NaCl revealed good binding. An increase of 500–600 RU above empty vector extracts was obtained (Fig. 4). The association and dissociation curves of the binding kinetics are presented in Fig. 5. The denatured LHCa4*1 protein preparation applied to the p40 chip revealed 300 RU [Fig. 5(A)] and the native LHCa4*1 interacting with the longer promoter fragment (p119) revealed 500 RU after 10 min of dissociation [Fig. 5(C)]. In each case the empty vector *E. coli* extract did not bind and no change in resonance units could be measured [Fig. 5(B),





Figure 2. Heterologous expression of LHCa4*1. (A) SDS–PAGE of *E. coli* extracts after IPTG induction carrying the empty pQE 30 vector (lanes 1 and 2) or harboring the pQE vector with the full length Lhca4*1 gene from tomato (lanes 3 and 4). Arrows indicate the 28.8 kD (monomer) and 60 kD (dimer) proteins which are expressed after induction. (B) Western blot with polyclonal antibodies against LHCb proteins. Arrows indicate the 28.8 kD and 60 kD proteins which are expressed after induction (lanes 3–5 respectively). In lane 6 a leaf extract from *A. thaliana* was separated. (C) SDS-PAGE of crude extract (cr.ex.) and denatured isolated, Ni-NTA-purified LHCa4*1 protein (LHC). Arrows indicate the 28.8 kD (monomer) and 60 kD (dimer) proteins. (D) SDS-PAGE of crude extract (cr.ex.) and native isolated, Ni-NTA-purified LHCa4*1 protein (LHC). Arrows indicate the 28.8 kD (monomer) and 60 kD (dimer) proteins.

(D)]. Together, these in vitro experiments indicate an interaction between the Lhca4*1 promoter sequence and the LHCa4*1 protein and support the in vivo interactions determined in the yeast one hybrid screen.

DISCUSSION

The results obtained by the in vivo yeast one hybrid screen and the in vitro SPR spectroscopy suggest an additional function of the LHC protein beside the well-known importance in light harvesting. The results presented indicate an interaction of the protein

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Figure 3. Lhca4*1 promoter streptavidin chips. (A) Doublestranded DNA fragments complementary to the Lhca4*1 promoter from tomato were amplified by PCR using biotinylated primers (p40: from -119 to -80, from p119: from -119 to -1) and bound to streptavidin chips in 3' or 5' orientation in respect to the chip surface. (B) Sequence of the Lhca4*1 promoter region from -1 to -119. The two 10 nucleotide motifs are underlined.

or protein fragment with its promoter. This is an unexpected result since mature LHC proteins posses three membrane-spanning helices and are therefore thought to be exclusively integral proteins of the thylakoid membranes (Green and Pichersky, 1994). However, hydrophilic amino acid regions are found before the first and between the second and third membrane-spanning regions and C-terminal of the third trans-membrane



Figure 4. Optimization of buffer conditions for the SPR experiments. Application of heterologous expressed LHCa4*1 protein to the p119 chip using HEPES buffer (50 mM, pH 7.5) with different NaCl and EDTA concentrations. Extracts from *E. coli* pQE-LHCa4*1 (gray column), extracts with the empty pQE vector (white column).

helix is a region predicted to be an amphiphatic alpha-helix, which is exposed at the interphase between the polar and nonpolar milieus (Green et al., 1991). The T2 clone initially isolated from the yeast one hybrid screen contained only part of the second and the third membrane-spanning region but significant stretches of hydrophilic and



Figure 5. Surface plasmon resonance spectroscopy interaction experiments. (A) Presentation of the association and dissociation kinetic between the denatured isolated and Ni-NTA purified LHCa4*1 protein and the 5'B-p40 streptavidin chip. LHCa4*1 protein was applied to the p40 chip and dissociation was performed with HEPES buffer (10 mM, pH 7.4, 1 mM EDTA, 150 mM NaCl). After 10 min resonance difference was determined. (B) Presentation of the association and dissociation kinetic between denatured isolated protein from *E. coli* with empty pQE vector and the 5'B-p40 streptavidin chip. (C) Presentation of the association and dissociation kinetic between the native isolated and Ni-NTA purified LHCa4*1 protein and the 3'B-p119 streptavidin chip. LHCa4*1 protein was applied to the p119 chip and dissociation was performed with HEPES buffer (20 mM, pH 7.5, 40 mM NaCl). After 10 min resonance difference was determined. (D) Presentation of the association and dissociation and dissociation with the p119 streptavidin chip. EMCa4*1 protein from *E. coli* with empty pQE vector and the 3'B-p119 streptavidin chip.

(continued)







Figure 5. Continued.

amphipathic amino acids increase the chances that this fragment is more soluble than the mature protein. Therefore it is possible that the LHCa4*1 fragment can bind to DNA. Furthermore, helix-loop-helix structures are also found in other DNA binding proteins (e.g., CCA1/LHY) and GIGANTEA has six putative transmembrane spanning helices, but so far it was not shown to be a membrane integral protein. Reasons which additionally support this result are: (i) out of all the other LHC proteins it was particularly the LHCa4*1 that was isolated via a binding screen with viroid nucleic acids (Ralf Werner, personal communication); (ii) out of all the other LHC proteins including the highly expressed LHCb1*2 (220 fmol mg⁻¹ RNA; Kellmann et al., 1993), the minor expressed LHCa4*1 protein (40 fmol mg⁻¹ total RNA) was the one which interacted with its promoter sequence; and (iii) interaction sustained very stringent yeast one hybrid screening conditions and repeated rescreenings. It has to be stressed that the screen revealed the low expressed LHCa4*1 and not the prominant expressed LHCb1*2 protein, despite the fact that the latter appeared quite often as a false positive in yeast two hybrid screens (Hengen, 1997; Serebriiskii et al., 2000).

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The yeast one hybrid screen resulted in a 109 amino acid C-terminal LHCa4*1 fragment interacting with the 119 bp Lhca4*1 promoter region. Above are reasons mentioned why this fragment might act as a soluble rather than an integral membrane protein but so far it has not been investigated whether the respective precursor or mature protein interacts with its promoter in a yeast interaction assay. However, the in vitro SPR experiments with the complete LHCa4*1 precursor protein support such binding. The results presented here show that the interaction between the LHCa4*1 protein with its promoter is stronger with the 119 bp promoter fragment and the native protein than the denatured protein with the shorter 40 bp promoter fragment, while no or very low resonance units were obtained with proteins of the E. coli, which carries the empty pQE vector. These results indicate the specificity of the binding and suggest that in addition to the two DNA motifs which make up the majority of the 40 bp sequence tested in the SPR spectroscopy other sequences of the promoter support the binding and increase the binding affinity. That contextual nucleotides are needed beside the importance of the T and A nucleotides adjacent to the ATC' sequences in the morning and evening specific elements was recently also suggested by Michael and McClung (2002).

Precursor LHC proteins are usually synthesised on cytoplasmatic ribosomes and in successive stages transported through the cytosol into the chloroplasts where the leader sequence is removed for correct integration into the thylakoid membranes (Robinson and Klösgen, 1994). It cannot be excluded that some of these precursor proteins migrate to the nucleus to bind to respective promoter regions to fulfill autoregulatory feedback regulation. However, the hypothesized presence of LHCa4*1 protein in the nucleus needs to be demonstrated, for example, with GFP fusion protein constructs or in situ immuno histological stainings. To decide whether the observed autoregulatory feedback loop is a special case or whether the LHCa4*1 protein can interact with the promoters of the other members of the Lhc gene family, or vice versa, other LHC proteins posses interaction potential with Lhc promoters, needs to be investigated as well. Such experiments can clarify whether an autoregulatory feedback mechanism is a general theme in the expression of LHC genes.

The concept of feedback regulation which has been demonstrated to be a perfect system for regulating enzymes of biosynthetic pathways as well as controlling networks of biological signaling pathways (Bhalla and Iyengar, 1999) is also a useful control mechanism at the transcriptional level, and very important for circadian regulation. Several recent reviews demonstrate that this concept is lived in Drosophila, in Neurospora, in mammals, in cyanobacteria as well as in plants (summarized in reviews: Dunlap, 1999; Iwasaki and Dunlap, 2000; Young and Kay, 2001). The importance of autoregulatory feedback loops for central oscillators of a circadian system were already claimed and discussed in 1994 (Aronson et al., 1994; Sassone-Corsi, 1994). Although the obtained results support the presence of an autoregulatory feedback loop for LHCa4*1 gene expression, at this point we do not consider LHC proteins as a central part of the oscillator but rather believe that it is an output unit of the circadian system of plants (Fig. 6; green arrow shown in web version). Other proteins such as APRR1-9/TOC1 or CCA1/LHY are discussed to be essential parts of the plants central circadian system. Evidence is obtained by overexpression studies examining the effects on the transgene, the original gene(s), other clock-controlled genes as well as on rhythms of output phenomena (e.g., leaf movement). Additional information is taken from loss-of-function mutants and genetic crosses between rhythmic or arrhythmic plants. Furthermore, screening for new





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nelix DNA binding domain; motif C, DNA binding domain, originally from CO; F-Box, protein recruitment for ubiquitination; KELCH repeats, likely to Genes/proteins: APRR1-9, Arabidopsis pseudo response regulator gene 1-9 (homologous to timing of cab expression TOC1); CCA1, circadian clock associated (homologous to LHY late elongated hypocotyl); CCR2, cold circadian rhythm (homologous to glycine rich protein GRP7); CHS, chalcone production of anthocyanin pigment; PIF 3, phytochrome interacting factor; PIL1, pif like protein (homologous to PFL1), PHY A-E: phytochrome; Regulatory circadian network in plants. A complex network of the circadian system was constructed primarily based on the information 2001. The new feedback loop of LHCa4 is shown in dark green. Solid black lines with arrowheads at both ends indicate interaction; blue lines with one synthase; CK 2, casein kinase (homologous to CKB3 and Hd6 in rice); CO, constans; COL 1/2, constans-like; CRY1, 2, cryptochrome; ELF3, early light Jowering; FHY1/3, protein of phytochrome signal transduction chain; FKF 1/2, flavin binding, kelch repeat, F-box protein (homologous to ZTL, and ADO 3); GI, gigantea; LHCa4, light harvesting complex protein (formerly cab 11); LKP, lov kelch protein (homologous to FKL1 and ADO2); PAP1, xSF1/HFR1/REP1, proteins of the phytochrome signal transduction chain; WNK1, protein kinase of WNK family: ZGT, clock and light regulated presented in the recent publications by Alabadi et al., 2001; Covington et al., 2001; Frankhauser and Staiger, 2002; Jarillo et al., 2001; Makino et al., 2002; McClung, 2001; Murakami-Kojima et al., 2002; Nelson et al., 2000; Park et al., 1999; Somers, 2001; Xu and Johnson, 2001; Yanowsky and Kay, arrowhead indicate positive direct or indirect regulation/influence; blue lines with bar at the end indicate negative direct or indirect regulation/influence; wave symbol: circadian accumulation of mRNA or protein; P: phosphorilation; ZT: Zeitgeber time. Abbreviations: Protein domains: β-HLH, helix-loopbe important for protein-target interaction; PAS, protein-protein interaction domain and chromophore binding, originally found in PER, ARNT, and SIM. zhong guang tiaokong); ZTL, zeitlupe (homologous to LKP1 and ADO1) Figure 6.

SPR Interaction Studies



components of the circadian clock in plants in combination with in vitro interaction studies as well as studies of flowering mechanisms and photoperiodic responses contributed a lot to allowed a construction of a circadian network. In Fig. 6 it was tried to include and interlock all genes and proteins known or suggested to be related to the circadian control process in plants. This scheme of the network demonstrates feedback as well as autoregulatory feedback loops based on protein-protein as well as protein-DNA interactions. Interactions or bindings are presented by solid lines with arrowheads. If positive (blue) and negative (red) regulatory effects were directly or indirectly determinated or suggested, this interaction is illustrated by broken lines. We are aware that the compartmentalization adds another cue to the regulatory network, this aspect however has been neglected from our scheme, because only limited information for most of the components is presently available. It becomes apparent that some proteins are yet not interlooked in this network since the interaction or regulation is not fully understood, and further investigations are necessary to fill the information gaps. It is also very likely that components of the circadian network are still not known. Nevertheless, a picture emerges that convincingly demonstrates that a complex network of feedback and feed-forward reactions is responsible for sustaining the timing process in plants (and other organisms).

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