SHORT PROMOTER REGIONS ARE SUFFICIENT TO MEDIATE CIRCADIAN EXPRESSION OF TOMATO LHC GENES IN TRANSGENIC TOBACCO

Birgit Piechulla, Nicole Merforth, Daniel Dreesmann, Ulrike Schäfer
Institut für Biochemie der Pflanze, Untere Karspüle 2, 37073 Göttingen, Germany

1. Introduction

To capture sun light and transfer the energy to the photosynthetic reaction centers I and II plants developed complex light harvesting systems (LHC) localized in the thylakoid membranes and surrounding the photosynthetic reaction centers with an inner and outer antenna (1). The antenna system of higher plants is constituted of ten structurally and functionally homologous protein types. Each LHC protein type is at least represented by one gene on the nuclear genome. In tomato (*Lycopersicon esculentum*) up to now nineteen Lhc genes have been identified and characterized. The regulation of the expression of these genes has intensively been investigated in the past and it turned out that several exogenous signals (e.g. light, temperature) as well as endogenous signals (e.g. developmental program, organ and tissue specificity, circadian clock) influence the expression. As a consequence of the action of the circadian clock the Lhc mRNA levels oscillate throughout a normal light/dark day as well as under continuous darkness (2). Oscillations with a period length of approximately 24 hours were determined for all nineteen Lhc genes of tomato (3). Since the circadian expression patterns are so similar one would expect a similar or even an identical regulatory mechanism. Surprisingly the promoter sequences upstream of the transcription start sites of these genes are in some cases nearly identical but for the majority of the Lhc genes they are very diverse (4). Up to now no sequence motif has been detected that is present in all upstream sequences which may confer the circadian mRNA accumulation.

In order to investigate possible regulatory elements of the circadian clock we choose three Lhc genes (Lhcb 1*1, cab 1A; Lhcb 1*2, cab 1B; Lhca3, cab 8) and started to investigate the promoters. Deleted 5' upstream promoters plus coding regions were transferred into tobacco by *Agrobacterium*-mediated transformation. The tomato Lhc mRNA levels were and are analysed in transgenic tobacco plants.

2. Procedure

2.1 Tomato Lhc genes and construction of 5' deletions

Lhcb 1*1 (cab 1A) (5), Lhcb 1*2 (cab 1B) (5), Lhca 3 (cab 8) (6). The 5' upstream region of the Lha 3 gene was determined using the didesoxy-chain termination method (7). Deletions were prepared according to the protocol of the "double-stranded nested deletion kit" (Pharmacia-LKB, Freiburg, Germany). Plasmid DNA containing the genomic DNA fragments (cab 1A: app. 1.4 kb partial digest EcoRI; cab 1B: 2 kb EcoRI/EcoRI; cab 8: 4.7 kb, Sal I/EcoRI) were digested with restriction enzymes, Exonuclease III removed nucleotides from the blunt or 5' overhang ends and S1 nuclease eliminated ss DNA. After ligation *E. coli* XL1-blue was transformed (7) and the mutated plasmid was sequenced to determine the exact position of the deletions. The inserted DNA was isolated and cloned into BIN 19 using the same restriction sites (8). These plasmids were transferred via transformation into *Agrobacterium tumefaciens* C5801, containing the helper plasmid pGV2260 (9).

2.2 Plant transformation

Leaf discs of *Nicotiana tabacum* (L cv. Samsun NN) were incubated with *A. tumefaciens*, calli were regenerated. Approximately after ten weeks plants were transferred to soil and grown at 21°C, 70% humidity.

2.3 RNA analysis

Leaves of transgenic tobacco plants were harvested at indicated time points, immediately frozen in liquid nitrogen and stored at -40 °C. RNA was extracted (10) and absolute transcript levels (fmol/mgRNA) were determined by the primer extension technique described by Kellmann et al. (3).

3. Results and Discussion

The promoter sequence of the Lhca 3 gene (cab8) (6; accession No. X15258) was determined (12). Genomic clones of Lhcb 1*1 (cab 1A), Lhcb 1*2 (cab 1B) and Lhca 3 were used to delete the 5' upstream regions (Fig. 1). Transgenic tobacco plants were generated. For the reason which is known as 'position effect' we screened approximately 40 plants of each construct. Leaves of similar age were harvested at noon and the mRNA levels of the three Lhc genes were determined by northern blot analysis. Statistical analyses (Lilliefors'-test; 12, 13) were performed to select plants for further experiments which

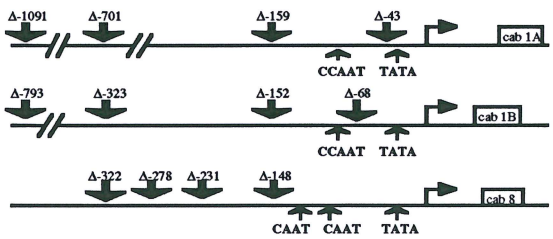

Figure 1: 5' upstream deletions of three Lhc genes from tomato. Thick arrows: position of deletion.

allowed a comparison of individual lines of transformants. An untransformed tobacco plant was used to determine the expression level of the internal tobacco Lhc gene. As an example the hybridization signal (cpm) of each Lhca 3 construct with standard error and median deviation is presented in Fig 2. The elimination of 5' upstream promoter sequences results in a continuous reduction of Lhca 3 mRNA accumulation. Low levels of the tomato Lhc transcripts are detectable in the Δ-43 Lhcb 1*1, Δ-68 Lhcb 1*2 and Δ-148 Lhca 3 deletions.

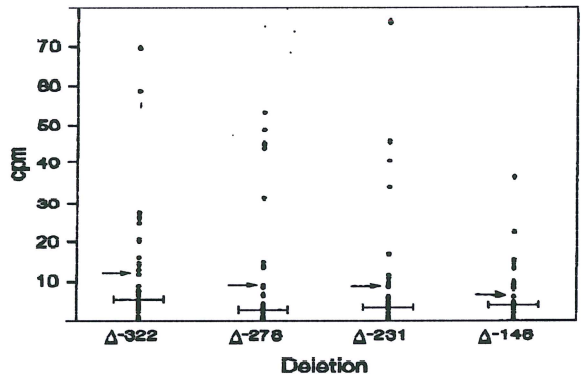

Figure 2: Variation of mRNA accumulation levels of four populations of the promoter deletions of the tomato Lhca 3 (cab 8) gene in transgenic tobacco (arrow: standard error, bar: median deviation).

After determining the levels of the different deletion mutants we were interested to know which of these constructs express the tomato Lhc mRNA in a circadian manner. For this reason plants were grown under light/dark and one day in continuous darkness. Leaves were harvested at appropriate time points and the mRNA levels were analysed with the primer extension technique. Four examples are presented in Fig 3. Each deletion mutant expresses a circadian Lhc mRNA oscillation pattern with a period length of approximately 24 hours in light/dark and continuous dark conditions indicating that the circadian clock mediating regulatory components have not been removed. Apparently a 5' promoter length of approximately 300 nucleotides is sufficient for circadian Lhc gene expression.

The data obtained for the tomato Lhc genes are in agreement with the results published for the wheat cab 1 (Lhcb1; 14) and the Arabidopsis cab 2 (Lhcb1; 15) genes. A deletion Δ-211 of the wheat cab 1 gene and a Δ-111 to -33 construct of the Arabidopsis cab 2 gene exhibit circadian transcript accumulation in transgenic tobacco plants. Comparison of the nucleotide sequences of all four examples investigated so far does not exhibit significant sequence similarities or conservation of sequence motifs. Apparently no specific cis-element is present to confer 'circadian clock' dependent transcription. A sequence motif which was correlated with the circadian expression of Lhc and nia genes is the 'GATA'- and/or its adjacent sequence 'ACTT'-motif (16). Neither one or both sequence motifs are present in the promoter region of all four genes compared. However, it should be pointed out that the complementary sequence 'CTAT' is present in the tomato cab 8 and wheat cab 1

Figure 3: Steady-state mRNA levels of tomato deletion constructs in transgenic tobacco. White and black bars refer to light and dark phases.

promoter. It is possible that a specific 'clock factor' binds to this cis-element, regardless of the orientation.

To decipher the machinery of the 'circadian clock' we continue to analyse the deletions constructs of the three tomato Lhc genes and with 'site directed mutagenesis' we are hopefully able to define 'clock'-regulatory elements.

4. Addendum

We like to thank Dr. U. Sonnewald for many discussions and the plant transformations and the DFG for a grant to B.P. (Pi 153/8-2)

References

1 Jansson S. (1994) Biochem. Biophys. Acta 1184, 1-19
2 Piechulla B. (1993) Plant Mol. Biol. 22,533-542
3 Kellmann JW., Merforth N., Wiese M., Pichersky E., Piechulla B. (1993) Mol. Gen. Genet. 237, 439-448
4 Piechulla B., Kellmann JW., Pichersky E., Schwartz E., Förster HH. (1991) Mol. Gen. Genet. 230, 413-422
5 Pichersky E., Bernatzky R., Tanksley SD., Breidenbach RB., Kausch AP., Cashmore AR. (1985) Gene 40, 247-258
6 Pichersky E., Brock T., Nguyen D., Hoffman N., Piechulla B., Tanksley S., Green B. (1989) Mol. Gen. Genet. 227, 277-284
7 Sambrook J., Frisch E., Maniatis T (1989) Cold Spring Harbor Laboratory Press
8 Bevan M. (1984) Nuc. Acid Res. 12, 8711-8721
9 Deblaere R., Bytebier B., De Greve H., Debroeck F., Schell J, van Montagu M., Leemans L. (1985) Nuc. Acid Res. 13, 4777-4788
10 Logemann J., Schell J., Willmitzer L. (1987) Anal. Biochem. 163, 16-20
11 Schäfer U. (1993) Diploma thesis, University of Göttingen
12 Nap JP., Keizer P., Jansen R. (1993) Plant Mol. Biol Rep. 11, 156-164
13 Sprent P. (1993) Chapman and Hall, Suffolk
14 Fejes E., Pay A., Kanevsky I., Szell M., Adam E., Kay S., Nagy F. (1990) Plant Mol. Biol. 15, 921-932
15 Anderson SL., Teakle GR., Martino-Catt SJ., Kay SA. (1994) Plant J. 6, 457-470
16 Borello U., Ceccarelli E., Giuliano G. (1993) Plant J. 4, 611-61