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Update section

Mini review

'Circadian clock' directs the expression of plant genes

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Abbreviations: Lhc, genes encoding proteins of the light-harvesting complex of PSI/PSII (ref. 16; old nomenclature is given in { }); *rbcS* and *rbcL*, genes encoding the small and large subunit of Rubisco, respectively; *elip*, gene encoding early light-induced protein; *psbA*, quinone-binding protein; *psaA*, gene encoding P700 protein of PSI; chl, chlorophyll

Introduction

Plants as well as animals can tell the time without the aid of wrist watches [53]. This fact is due to the universal phenomenon of endogenous clocks. Depending on the natural environmental periodicities organisms display a range of biological rhythms. A rhythm is defined as a self-sustaining repeating fluctuation pattern. The time required to complete one cycle is called period. According to the free-running periods (e.g. periods in constant conditions) different biological rhythms are classified: ultradian, circadian, infradian, circannual, etc. The entrainment by the naturally occurring 24 h light/dark cycle reveals *circadian oscillations*. All eukaryotic organisms appear to have evolved circadian rhythms, for example in enzyme activity, body temperature, locomotion, mitotic index, fragrance formation, conidia formation, O_2 evolution, CO_2 exchange, ion uptake, leaf movement, bioluminiscence and photosynthetic activity [reviewed in 7, 19, 53]. Plants, exhibiting rhythmic 'sleep' movements, stomatal opening and closure, stem elongations and so on, have been historically important to study circadian rhythms [53]. Recently, attention was drawn to a new parameter exhibiting diurnal and circadian rhythms in plants: oscillations of messenger RNA accumulations (Fig. 1A).

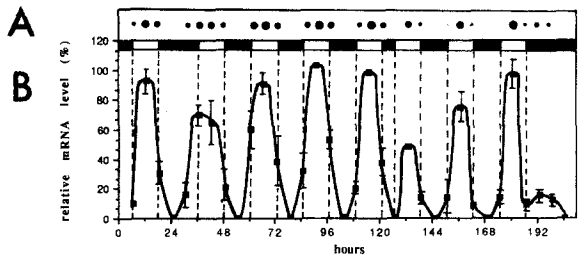

Fig. 1. Diurnal *Lhc* transcript oscillations in young tomato plants. Dark/light regime was presented by filled and open bars, respectively. The dark periods were either from 18:00–07:00 (normal day) or 24:00–13:00 (after the phase shift). A. Detection of mRNAs via dot blot hybridization. B. Quantitative determination of relative *Lhc* mRNA levels. (See [41] for further details).

This paper summarizes (1) the genes which show diurnal or circadian oscillations at the transcript level in plants, (2) the features of these rhythms, and (3) experimental approaches that can elucidate possible control steps or the mechanism(s) involved. The latter may provide indications that ultimately may lead to the understanding of the 'hands of the clock' or the 'oscillator(s)'.

Steady-state mRNA oscillations

Accurate development and growth of all living organisms require a precise temporal and spatial

control of gene expression. In plants, spatial expression (e.g. tissue and organ specificity) and developmentally controlled expression have been intensively investigated in the past and components involved in regulatory mechanisms seem to be unraveled. In contrast, interest on temporal controlled gene expression was recently documented when oscillations of transcript levels were demonstrated to follow an endogenous rhythm (e.g. 'biological clock').

In 1985, Kloppstech reported for the first time that mRNA levels of nuclear genes in pea (*Lhc*, *rbcS*, *elip*) follow a diurnal and circadian expression pattern [24]. Other investigators came across this phenomenon and described the periodic fluctuations of mRNAs in other plant species (summarized in Table 1). Impressive oscillations of steady state or synthesis of mRNAs were consistently presented for the *Lhc* genes of monocotyledonous and dicotyledonous plants. Increasing *Lhc* mRNA levels were detected either a few hours prior to or right after the transition from darkness to light, maximum levels were generally reached around noon (about 5 hours after the D/L transition), and decreasing levels were detected in the afternoon and night. Although in higher plants the LHC (light-harvesting complex) proteins are encoded by several genes combined in the *Lhc* gene family [17], most of the investigations deal with the expression pattern of the type I LHC proteins of photosystem II. Three *Lhc* genes (encoding type I LHC II proteins) were investigated in *Arabidopsis thaliana* [32] and five *Lhc* genes (encoding four type I LHC II proteins and one type II LHCI protein) were investigated in petunia [52]. The most comprehensive analysis of individual *Lhc* gene expression was undertaken in tomato, where nineteen genes (types I, II, III, CP29, CP24 of LHC II proteins and types I–IV of LHC I proteins [23, 43] and *Lhcb* 1 {*cab1*}, *Lhca* 1 {*cab6*} [16, 59]) were demonstrated to be under the control of a circadian rhythm (Fig. 2). It appears that the period lengths and time points of minimum and maximum of all individual *Lhc* transcript accumulation patterns are very similar under LD and constant conditions while the amplitudes of the mRNA oscillation patterns are significantly different [23]. These concerted transcript level oscillations could be the result of either one mechanism (e.g. one oscillator) or, alternatively, different mechanisms (e.g. many oscillators) reveal the same mRNA accumulation patterns. Such mechanisms must be or are able to drive a concerted expression of many *Lhc* genes although they reside on different chromosomes of the tomato genome. A differential expression modus is present in petunia, where the mRNA of one out of five genes exhibits a different circadian accumulation pattern [52], and in *A. thaliana*, where mRNAs of two genes accumulate with circadian periodicity, while a third (*Lhcb* 1 * 3 {*cab1*}) is only weakly, if at all, regulated by a circadian clock [6, 32]. A differential expression was also documented for three catalase genes in maize; only *cat3* mRNA accumulation exhibits a circadian pattern and the steady-state mRNA fluctuations are not entirely due to shifts in transcription rates [46]. The transcript synthesis of distinct *Lhc* genes has been investigated in less detail. Up to now the majority of the data support that diurnal and circadian mRNA oscillations are due to changes in transcript initiation and/or transcript synthesis (*Lhcb* 1 {*cab1*} *and Lhca* 1 {*cab6*} of tomato [16, 59]; *Lhcb* 1 * 2 {*cab1B*}, 2 * 1 {*cab4*}, 5 * 1 {*cab9*} and *Lhca* 2 * 1 {*cab7*} of tomato (H. Meyer and B. Piechulla, unpublished results); *Lhcb* 1 of maize [56]; *Lhcb* 1 * 3 {*cab1*} and 1 * 1 {*cab2*} of *A. thaliana* [32]). However, RNA stability may additionally play an important role (LI818 gene of *Chlamydomanas eugametos* [14]).

While the diurnal and circadian *Lhca/b* mRNA accumulation pattern is well documented in angiosperms, only few investigations deal with this aspect in plants of other sections or genera of the plant kingdom. The lack of information does not presently legitimate a general conclusion. Due to a different light-responsive system in gymnosperms [60], diurnal *Lhc* mRNA oscillations are minor in *Pseudotsuga menziesii* (Douglas fir) and no circadian expression in continuous illumination or darkness was detectable [2]. While in the fern *Ceratopteris richardii* and the algae *Dunaliella tertiolecta* a diurnal mRNA accumulation pattern

Table 1. Plant genes express diurnal or circadian mRNA accumulation patterns.

Gene	Plant species	Steady state				Run-on			Steady state			Devel. stage	Protein, activity	Ref.
		LD[1]	LD[2]	DD	LL	LD	DD	LL	R	FR	R/FR			
bbp[3]	*L. esculentum*		−		+		+	+	+ −			s		16
cat3	*Z. mays*		+	+	+				+	+	+	m/s		46
chs	*A. sativa*		+	−	+ −							m/s	+	38
cpnA, B	*A. thaliana*	+		+	+							m		44 (ns)
elip	*P. sativum*	+			+							m		24, 36
gs-2	*L. esculentum*		+									m		
	N. tabacum	−										m		5
hsp	*P. sativum*	+			+							m		36
lha/b	*A. thaliana*	(3 type I LHCII genes)												
		+		+		+						m		32
	G. max	+										m		31
	H. vulgare	+										m		31, 35
	L. esculentum	(type I, II, III, CP29, CP24 of LHCII, type I–IV of LHCI)												
		+		+	+							m		23, 43
		+										m	+	49
		+		+		+	+	+				s		16
						+			+	+	+	s		59
	N. tabacum	+		−	+	+		−				m/s		37
						+			+	+	+	s		59
	P. coccineus	+										m		31
	P. hybrida	(4 type I LHCII genes, 1 type II LHCI gene)												
			+	+								m		52
	P. sativum	+		+	+							m	+	1, 24, 36
			+		+							m		51
	P. vulgaris		+	+	+				+	+	+	m		54, 55
		+										m		31
	S. alba	+										m		31
	S. cereale		+	+	+				+			s		12
	T. aestivum	+		+	+				+	+	+	m/s		34
		+												35
	V. faba	+										m		31
	Z. mays					+	+					m/s		56
	Chlamydomonas		+		+			−	'new *lha/b*' (L818 clone)					
	C. eugametos	+		−					LHC II (pMH32 clone)					14
	C. richardii		+									sporophyt		Hücking *et al.*
	D. tertiolecta	+		+										27
	P. menziesii	+		+	+							s		2
nir	*L. esculentum*		+									m		4
nr	*L. esculentum*		+									m		4, 15
	N. tabacum	+		+	+							m	+	10, 15
	A. thaliana	+		+	+							m	+	44 (ns)
oee	*L. esculentum*		+		+		+	+	+			s		16
pepC	*S. vulgare*			+								m		57
rca	*A. thaliana*	+		+	+							m		44 (ns)
	L. esculentum	+		+		+							+	29

[1] Examined on one day; [2] Examined on two or more days; [3] Gene encoding a biotin-binding protein. ns, data not shown; s, seedling; m, mature plants.

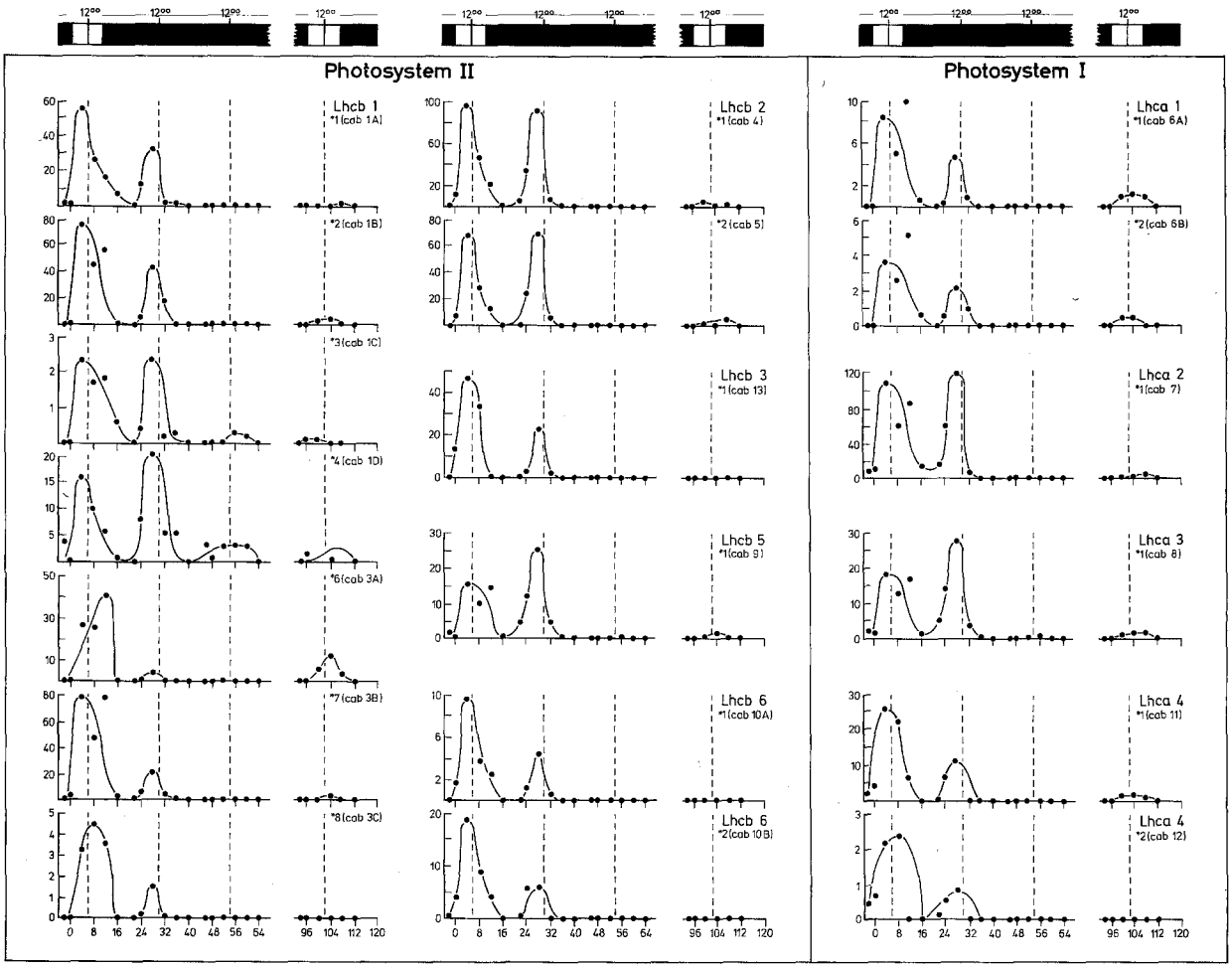

Fig. 2. Circadian rhythms of nineteen *Lhc* genes of *Lycopersicon esculentum*. Steady-state mRNA levels of individual *Lhc* genes were calculated based on the primer extension analysis. (See [23] for further details).

was detected, none was measured in the moss *Conicephalum conicum* ([27], Hücking and Piechulla, unpublished results). More complex is the situation in *C. eugametos*; one gene encoding the major PSII LHC protein (pMH32) expresses a diurnal mRNA accumulation pattern but mRNA levels do not oscillate under constant darkness, while the mRNA of another gene encoding a LHC-related protein (LI818) oscillates under LD and DD conditions [14]. Furthermore, the LI818 gene transcription rates are not affected by the clock, indicating a post-transcriptionally controlled circadian oscillation.

In contrast to the accumulation of consistent data about diurnal and circadian expression patterns of *Lhc* genes in plants, different results have been published for other photosynthesis-specific genes, such as *oee*, *rbcS*, *rbcL*, *psb* and *psa* genes. Circadian oscillations are less pronounced for the gene encoding the oxygen-evolving enzyme (*oee*) in tomato [16]. Controversial are the results presented for the expression of *rbcS* genes: no diurnal or circadian oscillations of steady-state mRNA levels were observed in wheat [34], tomato [16, 29, 31], tobacco [11, 37] and petunia [52], while Kloppstech [24], Spiller *et al.* [51], Ernst *et al.* [12] and Pilgrim *et al.* [44] come to the conclusion that the *rbcS* genes in *Pisum sativum*, *Secale cereale* and *A. thaliana*, respectively, are under the control of a 'circadian clock'. How-

ever, run-off experiments with *rbcS* genes of tomato revealed diurnal and circadian variations indicating a regulating step of a 'circadian clock' at the level of transcript synthesis [16]. The inconsistent or varying results may be due to species-specific differences, but it seems likely that in some cases an inadequate experimental design is the reason. It is worth pointing out that repeatedly occurring rhythmic fluctuations with a constant period (ca. 24 h) in LD and/or under constant conditions is a critical parameter for the decision whether a true diurnal and/or circadian oscillation pattern exists or not. Therefore it is necessary to examine consecutive days in LD or DD or LL. We ourselves misinterpreted the diurnal fluctuations of *rbcS* mRNA levels in tomato [39, 40], since investigations over several days in LD showed irregular fluctuations but not rhythmic diurnal oscillations of the steady-state *rbcS* mRNA levels [31]. Environmental variations such as shading of leaves [45], variations of metabolit concentrations [50] or other parameters may be the reason(s) for the irregular diurnal mRNA level fluctuations of *rbcS* or other genes.

Conflicting data have accumulated about the diurnal expression of *rbcL*, *psbA*, *psbB*, *psaA* and *psaD* [16, 37, 39, 40, 51] which, in my opinion, are most likely due to the fact that in many cases only one day in LD, LL or DD was examined. It is presently not definite, but more indications suggest that plastid-encoded genes are not under the control of a circadian rhythm.

Further investigations demonstrated that not only transcript levels of photosynthetic genes express diurnal mRNA patterns, but also genes encoding enzymes such as nitrate reductase (*nr* [11, 15]), catalase 3 (*cat* [46]), phosphoenolpyruvate carboxylase (*pepC* [57]), chalcone synthase (*chs* [38]), nitrite reductase (*nir* [4]), glutamine synthetase (*gs* [5]), RuBPase activase (*rca* [29, 44]), A and B chaperonin subunits (*cpn* [44]), two heat-inducible proteins [36] and a biotin-binding protein [16]. Free-running oscillations under constant conditions with a period length of ca. 24 h were observed for nitrate reductase [11], catalase 3 [46], heat-induced proteins [36], RuBPase activase [29, 44], the chaperonin subunits [44] and the biotin-binding protein [16]. These are a few known examples of genes which are under the control of a circadian oscillator (Table 1). At present it is not known how many genes of a plant genome display a diurnal or circadian expression pattern. Cremer *et al.* [9, 10] estimated that about 10% of the mRNAs in *Sinapis alba* are affected by complex diurnal rhythmical changes. They analysed *in vitro* translation products on two-dimensional gels and identified 19 different diurnal rhythmic patterns. Many are characterized by increasing levels after the dark-to-light transition and decreasing levels during the dark period. The opposite patterns with decreasing levels after the dark/light transition were also detectable, which may present the mRNA accumulation patterns for the NADPH-protochlorophyllide oxidoreductase or phytochrome [9].

Features of circadian mRNA oscillations of plant genes

Circadian rhythms are the observable result of complex mechanisms. The underlying oscillatory processes are not comprehended and many investigations have failed to detect or define the oscillator(s) in any investigated system. Models for generating circadian rhythms have been proposed by several groups (for example [21, 28]), but none is completely identified. Also, it is presently not known whether one or several oscillators per organism or cell exist. As a result of these uncertainties only phenomenological descriptions of the oscillations (e.g. of steady-state transcript levels or transcript synthesis) are possible. Two important features that describe circadian rhythms are free-running rhythm and temperature compensation. However, at least to my knowledge, no detailed investigations have been performed into the latter aspect with respect to transcript oscillations in plants.

Under constant conditions, circadian rhythms free-run with a periodicity slightly different from 24 h. This phenomenon is due to the entrainment of the rhythm by the daily light-and-dark cycles, and affects the accumulation of certain transcripts

in plants. For several genes, such as biotin-binding protein, *cpnA/B*, *elip*, *Lhc*, *nr*, *oee* and *rca*, circadian oscillations were detectable either in constant illumination (LL) or darkness (DD) (Table 1). The time points of minima and maxima of the oscillations vary between the different genes; for example, the steady-state mRNA levels of *Lhc* genes reach their maxima about 5 h after the subjective transition from darkness to light [31], the steady-state mRNA levels of the nitrate reductase peak 4–8 hours after the subjective light/dark transition and decrease during the subjective light phase [11], *cat3* mRNA levels peak by the end of the light phase but before the subjective light/dark transition [46], and *elip* mRNAs peak at the end of the subjective night phase [24].

To characterize oscillations, including the oscillator(s), factors or control steps of the complex 'circadian clock' machinery, two experimental procedures were classically followed in chronobiology: either the influence of various substances (antibiotics, enzyme inhibitors, organic and inorganic compounds) was tested or the variation of environmental conditions was investigated [58]. To my knowledge, the first approach was not applied to characterize steady-state mRNA oscillations in plants and only a few experiments were performed altering the entraining light/dark regimes. For example, a delay of the light period (phase shift) results in a shift of the time point of the maximum of *Lhc* mRNA level by the respective delay time, and an adaptation to the new environmental conditions occurs within three days (Fig. 1; [41]). Further, an entrainment of 6hL/6hD reveals two *Lhc* mRNA accumulation maxima within 24 h, although the heights of the amplitudes are different [41]. From another series of experiments it was concluded that the transition from light to darkness is the measure for synchronizing the phase of the periodic cycle [26, 48]. So far, phase shifts have only been detected in mature plants. In contrast, the induction of circadian *Lhc* mRNA accumulation by monochromatic light was demonstrated in etiolated seedlings of several plant species [32, 34, 54, 59] and a whole complement of diurnal *Lhc* gene expression was demonstrated in tomato (J.W. Kellmann and B. Piechulla, unpublished results). In *Phaseolus vulgaris* a second red light pulse, applied 36 h after initiation of the rhythm, induces a phase shift which is prevented by far-red light immediately after the second red light pulse [55]. Furthermore, the mRNA of the non-photosynthetic gene *cat3* accumulates in maize seedlings after treatment with red and far-red light [46]. The red/far-red reversibility of the amount of transcript indicates strongly that the photoreceptor phytochrome is involved in the expression of respective genes possibly via a phytochrome-controlled circadian oscillator [28, 59]. In addition, phytochrome, particularly the destruction of the active form of the phytochrome complex is thought to be responsible for the characteristic damping of the *Lhc* mRNA oscillations in DD [20]. Although light was demonstrated to substantially influence the expression of *Lhc* genes the time point of the day when light is applied is also very important. Four hours of white light given at different time points during the day resulted in significantly different accumulation levels of *Lhc* mRNAs [31]. From these experiments it was concluded that either a 'negative regulator' maintains a different extend of transcription (possibly because of different concentrations or different DNA-protein or protein-protein interactions), or a 'positive regulator' has to be synthesized or recovered in order to support transcription during the day.

Besides light, temperature is undoubtedly another important environmental factor in regulating circadian rhythmicity. Experiments performed with tomato, pea and barley clearly demonstrated that alternating high and low temperatures (simulation of day and night temperatures) and cyclic heat-shock treatments can function as entraining signal(s) to synchronize *Lhc*, *rbcS* and *elip* mRNA oscillations [3, 25, 42, 48]. Furthermore, it was demonstrated that low temperature suspended the timing of the rhythm and upon rewarming, the circadian control was reestablished but was displaced from the actual time of day by the length of the chilling exposure [29].

Based on the results mentioned above it can be

concluded that alternating L/D as well as low/high temperature cycles synchronize and entrain the expression and/or accumulation of transcripts in plants, although the latter is probably a weaker 'Zeitgeber'. The adaptation process, including the transfer of information about the changes in environmental conditions to the oscillator(s) and the manifestation of the 'new' rhythm for the expression of particular genes, can be reached in a fairly short period of time in plants. Apparently at least two aspects are important for the establishment of a 'biological clock', firstly: the expression of genes is under the control of a circadian rhythm (to anticipate upcoming conditions and being ready prior to the anticipated situation; preparedness) and secondly, the adaptation of circadian rhythms to environmental conditions/entraining regimes (to support flexibility and survival through temporarily optimized photosynthesis).

Physiological relevance of circadian mRNA oscillations

The finding of circadian mRNA oscillations requires an answer for the physiological relevance or the necessity of such oscillations. Reasoning will be presented for three different cases: (1) genes involved in photosynthesis (*Lhc*, *elip*, *oee*); (2) nitrate reductase (*nr*) and (3) catalase (*cat3*).

1. The prerequisite of true circadian rhythms is the free-running rhythm most likely to anticipate recurrent situations during the development and life of the plant. Photosynthesis is such a repeatedly occurring process. It is likely that due to the natural half-life times of the proteins or the external (stress) factors such as high irradiance the thylakoid membrane loses its optimal configuration and/or composition. It was calculated that less than 1% of LHC proteins is degraded during a day [49]. To compensate this loss LHC proteins are synthesized on a daily basis, controlled by a circadian clock to anticipate upcoming situations [29, 49]. This concept is also true for other thylakoid membrane proteins, such as Elip, Fe-S-Rieske protein and Q_B-binding protein, and the mRNA of the oxygen-evolving enzyme (*oee*) in *P. sativum*, *Spirodela oligorrhiza* and *L. esculentum*, respectively [1, 16, 30]. In accordance with the daily new synthesis of the LHC proteins and the circadian *Lhc* mRNA oscillations are the diurnal variations of the chl content chlorophyll *a*/*b* ratio, chlorophyll *a* fluorescence yield and the relative amount of LHC complexes in wheat seedlings [8]. Together, these data support the notion that for optimal diurnal reconstitution of the components of the photosynthetic machinery a regulation by a 'circadian clock', and therefore primarily independent of environmental signals, is optimal for plant development. Superimposed signals, such as light (quality and quantity), and length of light and dark phases, are necessary for the adaptation to the actual environmental situation.

2. As argued for the photosynthetic proteins, the functional relevance of diurnal/circadian mRNA oscillations of the nitrate reductase is found in the diurnal need of the enzyme, since nitrate accumulates during the night [47] and is reduced to ammonia or assimilated in glutamine during the day. The absence of glutamine or ammonia during the night (or experimentally applied nitrogen starvation) may lead to a depression and accumulation of *nr* mRNA during the night, reaching highest levels shortly after the dark-to-light transition. This mRNA accumulation pattern is accompanied by the circadian oscillation of the enzyme content and the activity, both parameters reaching their maximum levels early during the day [11, 15]. The regulation of the *nr* gene expression is very complicated and interferes at the transcriptional and post-transcriptional level. Based on the complexity of the regulation process it is presently difficult to explain why a control step mediated by a 'circadian clock' is needed both at the level of mRNA and protein and enzyme activity.

3. Degradation of H_2O_2 in maize is accomplished by three catalases. In maize the expression of the *cat1* gene is 'constitutive' and the CAT-1 isozyme may be present in the peroxisomes of all maize

tissues at low levels while the CAT-2 protein is thought to be associated with the degradation of H_2O_2 generated during the oxidation of photosynthetically produced glycollate in the shoot [46]. It appears that the *cat3* mRNA accumulates to highest levels to the end of the light and beginning of the dark phase in the mesophyll of green maize leaves. In analogy to the interpretation for the *Lhc* genes it is assumed that the CAT-3 isozyme accumulates for specific metabolic processes (although as yet undefined) that occur late in the light or early in the dark phase.

Analysing molecular mechanisms of the 'circadian clock'

Consequently, after finding that plant genes are under the control of a 'circadian clock', the regulatory mechanisms functioning at the level of gene transcription were started to be investigated. The present knowledge of regulation of gene expression includes the interaction of *trans*-factors (DNA- and protein-binding proteins) and *cis* elements (5′-upstream sequences of genes). Fejes *et al.* [13] analysed 5′ deletion mutants of the wheat *Lhcb* 1 * 1 {*cab1*} gene in transgenic tobacco plants and defined a 268 bp sequence mediating the 'circadian clock' controlled transcription. Similarly, Millar and Kay [32] reported that 249 bp or 319 bp upstream of the *Lhcb* 1 * 3 {*cab1*} and *Lhcb* 1 * 1 {*cab2*} genes, respectively, of *A. thaliana* are sufficient to direct circadian accumulation of a reporter mRNA. Neither the 'clock-responsive element' of wheat, other uniform regions nor any DNA-motive were found to be present upstream of sixteen *Lhc* genes of tomato (approximately −400 bp upstream of the translational start point) [43]. It cannot be excluded that a universal 'clock-responsive element' is present further upstream of the *Lhc* genes, but it is also likely that different combinations of *cis*- and *trans*-elements or other yet not defined regulatory elements reveal the similar circadian accumulation patterns of the individual *Lhc* transcripts (Fig. 2). It should also be kept in mind that a possible role of mRNA degradation as a function of the 'circadian clock' has not been investigated at all. From the deletion mutant analysis can be concluded that the monocot 5′-upstream region can direct 'circadian clock'-controlled gene expression in dicot plants, indicating that the mechanisms (*cis*- and *trans*-element interactions) at least in higher plants are exchangeable or universal.

An interesting experimental approach to investigate and elucidate the mechanism(s) of the 'circadian clock' was established by S. Kay and coworkers [32, 33]. They constructed chimeric genes including 5′-upstream regions of *A. thaliana* *Lhcb* 1 * 3 {*cab1*} and 1 * 1 {*cab2*} genes fused either to the bacterial chloramphenicol acetyltransferase gene or to the firefly luciferase gene. The *A. thaliana* 319 bp 5′-upstream sequence of *Lhcb* 1 * 1 {*cab2*} can direct the expression of the reporter genes and the respective mRNAs or luciferase activity accumulates in LD, LL and DD with free-running periods of ca. 24 h. The luciferase gene containing tobacco seedlings gloom during the day and this novel phenotype can be imaged and recorded in a quantitative fashion with a video camera. Using this approach it is expected to gain some more insight into the mechanisms of the 'circadian clock' in the future.

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