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Review

The tonoplast functioning as the master switch for circadian regulation of crassulacean acid metabolism

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Abstract. From the initial discovery of free-running endogenous circadian oscillations of Crassulacean acid metabolism (CAM) under constant conditions in the light and in air, it has been disputed whether the underlying oscillator is enzymic or biophysical. The hypothesis of a biophysical hysteresis switch or beat oscillator started from osmotic considerations of malate accumulation and remobilisation, indicating a tonoplast tension/relaxation mechanism. It then advanced to application of non-linear dynamics theory for the analysis of rhythmic and arrhythmic time series of CO_2 exchange under the regime of external control parameters, mainly temperature, and the implementation of models for computer simulations of CAM rhythms. This provided strong evidence for the tonoplast functioning as a master switch for circadian regulation of CAM. Conversely, the hypothesis of an enzymic beat oscillator strongly developed on the experimental basis of phosphorylation/dephosphorylation of phospho*enol*pyruvate carboxylase (PEPC) regulating the enzyme activity, and hence CO_2 fixation and malate synthesis via this enzyme. It was much supported by the discovery that PEPC-kinase gene-transcription was under circadian control. However, biochemical and molecular analysis, as well as model simulation, strongly suggests that this is a secondary and not the primary oscillator. The synchronisation/desynchronisation of leaf patches has revealed spatio-temporal characteristics of circadian rhythmicity that may open new ways for understanding biological clocks.

Key words: Circadian rhythm – Crassulacean acid metabolism – Minimal model (CAM rhythm) – Oscillator – Non-linear dynamics (CAM model) – Patchiness (leaf photosynthesis)

Introduction: free running endogenous rhythmicity of CAM posing the problem of equilibrium thermodynamics versus non-linear dynamics

Crassulacean acid metabolism (CAM) is a specific mechanism of inorganic carbon acquisition for photosynthesis. Carbon dioxide is fixed via phospho*enol*pyruvate carboxylase (PEPC) in darkness. The resulting malic acid is accumulated to high concentrations of up to several hundred millimolar and stored in the cell sap vacuole (Lüttge 1987; Winter and Smith 1996). The ecological advantage of this nocturnal CO_2 fixation is, on the one hand, gaining and concentrating carbon intracellularly when inorganic carbon availability is low and competition is high, i.e. for submerged freshwater CAM plants, or, on the other hand, reducing transpiratory water loss due to the low evaporative demand at night-time, i.e. for terrestrial CAM plants under limited water supply (Lüttge 1987; Winter and Smith 1996). During the subsequent light period malic acid is remobilised again and decarboxylated, so that the internally regenerated CO_2 can be re-fixed via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and assimilated in the Calvin cycle (Lüttge 1987; Winter and Smith 1996).

Malic acid accumulation in the vacuole is driven by the H^+-pumping V-ATPase at the tonoplast with malate^{2-} passively following the electrochemical proton gradient, $\Delta\bar{\mu}_{H^+}$, set up by the pump (Lüttge et al. 1981). Equilibrium thermodynamics, however, provide no reason why a net malic acid influx should switch to a net malic acid efflux unless equilibrium is disturbed. This could be a consequence of changing external parameters, e.g. a switch from darkness to light, altered temperature etc., with manifold consequences for cytological and metabolic machineries, leading to a

Abbreviations: CAM = crassulacean acid metabolism; PEPC = phospho*enol*pyruvate carboxylase

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destruction and rearrangement of equilibrium. Hence, in a natural night/day cycle the switching from net acid accumulation to net mobilisation does not at all pose a new fundamental problem beyond the standard question of metabolic regulation during dark-light-transitions of photosynthesising cells. Thus, the discovery that CAM is also free running endogenously under constant environmental conditions, and that this occurs not only in continuous darkness in an initially CO_2-free atmosphere (Warren and Wilkins 1961) but also under more natural conditions in continuous light and in air allowing photosynthesis (Lüttge and Ball 1978; Buchanan-Bollig et al. 1984), initially appeared most unfortunate. Simple explanations arrived at by changing external parameters were no longer valid – instabilities must be generated internally. Non-linear dynamics in general, came into play, together with a harsh demand to find the endogenous oscillator. How this apparently unfortunate event turned into good fortune as a result of its challenge to reveal new basic properties of CAM and the biological clock of a plant is reported in this review.

Early notions: membrane tension of the tonoplast functioning as hysteresis switch

Oscillations between two discrete different states require a beat-oscillator, or hysteresis-switch, which elicits jumps back and forth between the two states (''discrete'' hysteresis-switch) or somewhat gradually shifts between the two states (''dynamic'' hysteresis-switch). Whether the hysteresis-switch might be a biochemical or a biophysical mechanism was an early consideration. The original proposal of an enzymic oscillator (Morel and Queiroz 1974; Brulfert et al. 1975) remained somewhat vague until the advent of molecular biology (see below), while experiment-based reasoning led to a distinct hypothesis for a biophysical oscillator at the level of the tonoplast membrane.

In contrast to malic acid influx into the vacuole, malic acid efflux is a passive process (Lüttge and Smith 1984). Efflux from leaf slices submerged in an aqueous medium appeared to depend on cell turgor. It was reversibly inhibited by osmotica in the external medium (Lüttge and Ball 1974; Lüttge et al. 1975) where the comparison of non-permeating (mannitol) and permeating (ethylene glycol) osmotica allowed it to be ascertained that reduced turgor and not the water potential of the cells caused the effect (Lüttge et al. 1977). In vivo, strong correlations between oscillations of vacuolar malate levels, cell sap osmotic pressure and turgor pressure, including direct measurements with an intracellular pressure probe, showed that nocturnal malic acid accumulation was associated with osmotic uptake of water and increased turgor pressure at the end of the dark period (Lüttge and Ball 1977; Steudle et al. 1980; Lüttge and Nobel 1984; Smith and Lüttge 1985; Lüttge 1986, 1987). This work supported the hypothesis of CAM functioning as a ''tension/relaxation rhythm'' (Lüttge et al. 1975), where a high tonoplast tension at high turgor marks the maximum malate filling state of the vacuole and net efflux starts at a critical threshold of turgor pressure or membrane tension, i.e. the tonoplast functions as a discrete hysteresis switch. The localisation of a beat-oscillator at the tonoplast was, at that time, also supported by Wilkins (1984).

Temperature regimes: non-linear dynamics of tonoplast functions

When the endogenous rhythm of net CO_2-exchange, J_{CO2}, of the CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie is run under continuous illumination at constant temperature, then raising the constant temperature level above a critical threshold within only a fraction of a degree Celsius induces a dramatic shift from regular rhythmicity to arrhythmic behaviour (Lüttge and Beck 1992). This has been shown to be a reversible effect. Similarly, lowering the temperature below a critical lower threshold results again in a reversible loss of rhythmicity. Reversibility of these transitions indicates the existence of several dynamically separated states of the same system. The critical threshold temperature depends on growth temperature of the plants. By homeoviscous adaptation, *K. daigremontiana* plants growing under higher temperature regimes produce more rigid or less-permeable tonoplast membranes than plants growing under lower temperature regimes (Kluge et al. 1991; Kliemchen et al. 1993; Schomburg and Kluge 1994). The critical upper temperature threshold for switching from endogenous rhythmicity to arrhythmicity is increased when plants are grown under higher temperature regimes (Grams et al. 1995).

A thought-experiment would suggest that when rhythmicity is lost at too high a temperature this should occur when the vacuole is in an ''empty'' state with respect to malate because increased temperature increases tonoplast permeability and malate efflux (Friemert et al. 1988; Kliemchen et al. 1993) while, conversely, at too low a temperature this should happen with a ''full vacuole'' as tonoplast permeability is decreased (Kliemchen et al. 1993). In consequence, one would then expect that when the rhythm is re-initiated by lowering the temperature again from too high, the phase starting the rhythm would be the increased CO_2 uptake, i.e. the filling of the empty vacuole by malate synthesis. Conversely, when the rhythm is re-initiated by increasing the temperature again from too low the rhythm should recommence with the phase of decreasing CO_2 uptake, i.e. emptying the full vacuole by malate remobilisation. This has been shown exactly by measurements of J_{CO2} (Wilkins 1962; Grams et al. 1997; Fig. 1). Moreover, measurements of PEPC activity, as well as measurements of carbon-isotope ratios detected on line in samples taken from the air-stream passing the gas-exchange chamber that allow the relative activities of PEPC and Rubisco to be followed due to their different $^{13}CO_2$ discrimination, show that the re-initiated rhythm-

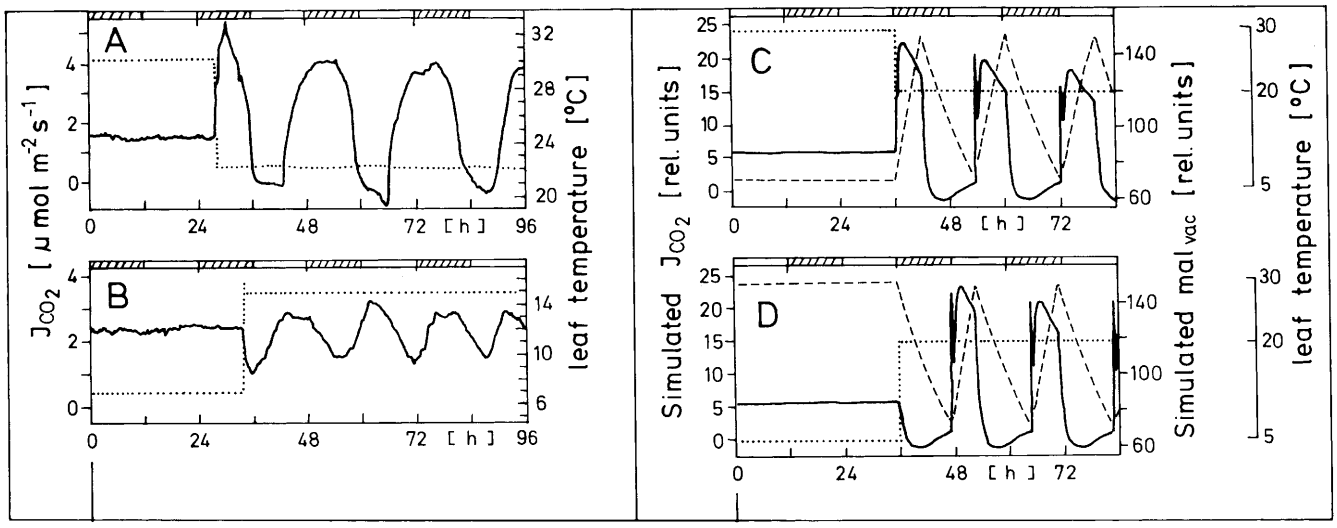

Fig. 1A,B. Time series of net CO_2 exchange, J_{CO2} (*solid lines*), generated in two leaves of *K. daigremontiana* under continuous light, where white and dark bars above the graphs indicate subjective light and dark periods, respectively. Temperature first was too high (**A**) and too low (**B**), respectively, for regular rhythmicity, but rhythmicity was re-initiated by lowering (**A**) or increasing (**B**) leaf temperature (*dotted lines*) into the appropriate temperature range. **C, D** The corresponding computer simulation for (**A**) and (**B**), respectively, where vacuolar malate levels, mal_{vac} (*dotted lines*), were also simulated. Simulations used the CAM model of Fig. 2A. (After Grams et al. 1997)

icity sets in with PEPC activation after lowering the temperature again from too high and with PEPC deactivation after increasing the temperature from too low (Grams et al. 1997). Evidently, temperature modulates tonoplast fluidity and hence permeability determines the maximum filling state of the vacuole. Therefore, the direction of temperature changes sets the phase of the rhythm via malate loading or unloading of the vacuole with its effects on PEPC. The experimental observation has been excellently mimicked by computer simulations using a minimal model of CAM. In this model the entire complex biochemical network of CAM was reduced to six metabolite pools coupled by flows of substrates and feedback loops and governed by the external control parameters light and temperature, as indicated in Fig. 2A. The essential point is that this model contained a discrete hysteresis switch jumping between two states, i.e. net influx and net efflux, respectively, of malate in the vacuole.

Using a simplified lipid membrane model and testing a parameter space with three dimensions, namely the order parameter $\langle S \rangle$ of the membrane (i.e. fluidity/rigidity), mean available area for a lipid molecule (f) and temperature (T), it could be shown that a membrane can indeed function as a hysteresis switch (Neff et al. 1998; Fig. 3). For a range of temperatures, T, as shown in Fig. 3 at a given f, three solutions are obtained for the thermodynamics of average $\langle S \rangle$, two of which are stable, i.e. at high and at low $\langle S \rangle$, and one is instable, i.e. at medium $\langle S \rangle$. Thus, for a given parameter region of f and T the system jumps between the two stable states of $\langle S \rangle$, i.e. low and high permeability, respectively. This means that it operates as a beat oscillator. The energy required for these jumps calculated for this thermodynamic model is ca. 5×10^{-11} J. This is two orders of magnitude less than the energy actually afforded by the plants when the vacuoles of cells of *K. daigremontiana* swell by ca. 4.6% in volume with an increase in tonoplast surface of ca. 3.0% due to osmotic uptake of water following malate accumulation (Lüttge 1986). This reassuring estimation of feasibility provided confidence for reducing the minimal model of CAM still further, strongly emphasising tonoplast membrane control (Blasius et al. 1999; Fig. 2B). This new minimal model also gave an excellent simulation of actual experimental observations (Fig. 4).

Genes and transcription: metabolic oscillators

Rhythm genes have been identified in several organisms (Dunlap 1993, 1999; Takahashi and Kornhauser 1993; Somers 1999), e.g. *Drosophila* as well as *Neurospora* (McClung et al. 1989) and *Arabidopsis* (Somers et al. 1998; Millar 1999; Park et al. 1999). Since the same genes may affect different rhythms in an organism they must be very close to a basic master oscillator. Evidently, however, a gene per se is not an oscillator. Its regulation is itself the result of the operation of a very complex network system with signal transduction chains, transcription factors, etc. Although a wealth of transcription and translation products, and in several cases also the corresponding genes, are now known to be under circadian control, in no case has it been actually possible to deduce the discrete structure of an oscillator. Revealing metabolic oscillators appears to remain a matter of system analysis just like revealing biophysical oscillators as shown above.

Anderson and Wilkins (1989) have supported an enzymic mechanism for a beat oscillator in CAM. Indeed, understanding the diel and circadian regulation of PEPC has much advanced the concept of a metabolic

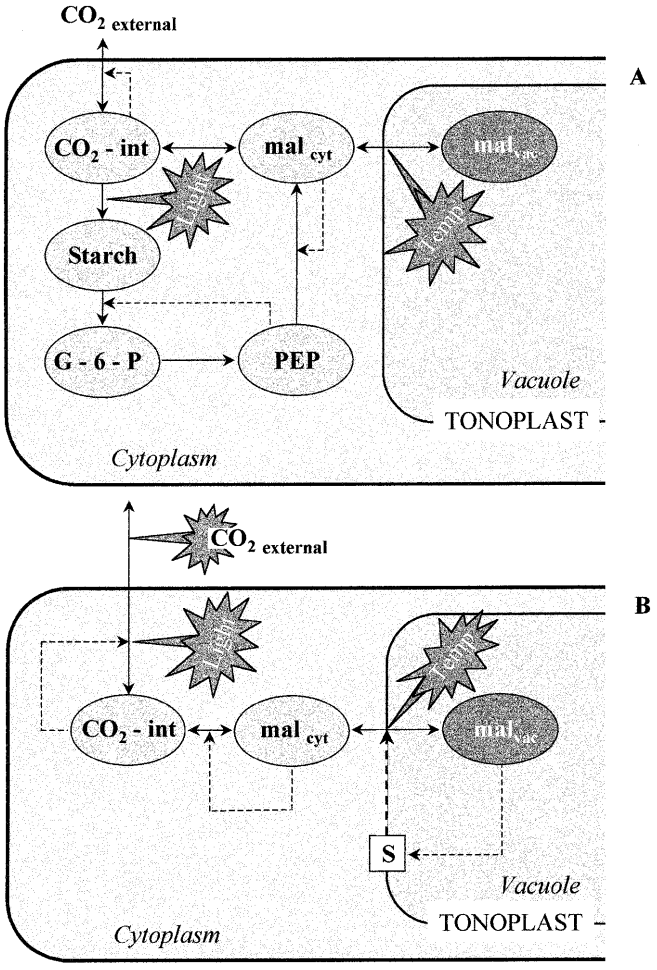

Fig. 2A,B. Heuristic minimal models of CAM derived from experimental observation of the relevant metabolic pools as independent degrees of freedom in the non-linear rate equations. The model in **A** has six pools of metabolites, namely internal CO_2 (*CO_2-int*), starch, glucose-6-phosphate (*G-6-P*), phospho*enol*pyruvate (*PEP*) and cytoplasmic and vacuolar malate (mal_{cyt}, mal_{vac}). Feedback loops (*dotted lines*) are the known feedback inhibitions of glycolysis by PEP and of PEPC by malate as well as effects of CO_2-int on CO_2 uptake from the outside. External control parameters are temperature and light. In the model in **B** metabolite pools are reduced to only three, namely internal CO_2, cytoplasmic and vacuolar malate, the feedback loops (*dotted lines*) of CO_2-int on CO_2 uptake and mal_{cyt} on PEP-carboxylase are retained, and another important feedback loop is now vacuolar malate acting on vacuolar net influx and efflux of malate via the state of order $\langle S \rangle$ of the tonoplast. The metabolite pools left out in **B** are strongly tied to the remaining degrees of freedom and so do not establish their own independent dynamics. External control parameters are temperature, light and CO_2. Pools are connected by fluxes (*solid lines*). This is formulated by appropriate sets of non-linear coupled differential equations allowing computer implementation and simulations. [After Lüttge and Beck 1992 (**A**); Blasius et al. 1999 (**B**)]

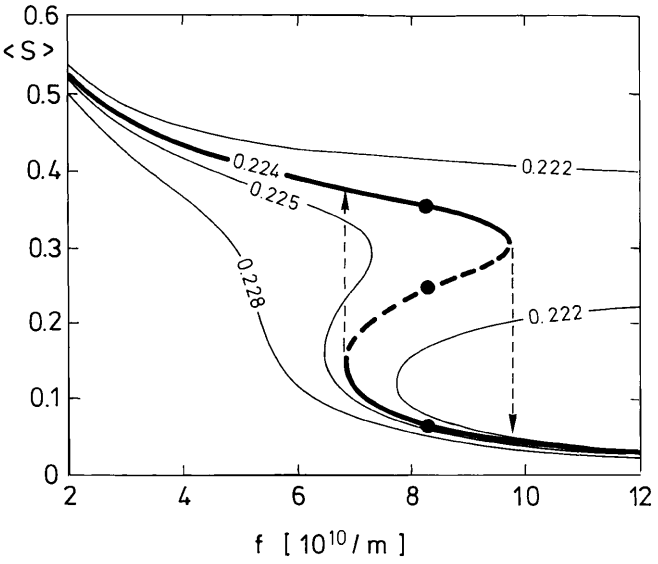

Fig. 3. Cuts across the parameter space of a thermodynamic lipid membrane model with the state of order of the membrane (*y*-axis, $\langle S \rangle$) and the mean area available per lipid molecule (*x*-axis, *f*). The *z*-axis (temperature) of the parameter space is not drawn, and temperature effects are represented by four curves of selected fixed temperatures in the two-dimensional cut of the three-dimensional parameter space. The curve for the computed effective temperature 0.224 has two stable branches (*bold solid lines*) and an instable branch (*bold dashed line*, i.e. three solutions for $\langle S \rangle$ with a given *f* (*circles*) two of which are stable and one of which is instable. In the manner of a hysteresis switch the state of order $\langle S \rangle$ can jump between the two stable branches (*thin dashed lines with arrows*). (After Neff et al. 1998.)

oscillator of CAM, as an alternative or in addition to a biophysical oscillator. Substrate affinity, as well as pH and malate sensitivity, i.e. feedback inhibition by malic acid formed in the cytoplasm at the site of PEPC, are regulated by phosphorylation/dephosphorylation by a PEPC-kinase and a phosphatase. The active night-form of PEPC is phosphorylated, whereas the less active or inactive day-form is dephosphorylated (Nimmo et al. 1987; Kusumi et al. 1994; Carter et al. 1995a, b, 1996). Thus, it is a distinct possibility that phosphorylation/dephosphorylation of PEPC constitutes a hysteresis switch. Another key element of CAM, where the transcription is under rhythmic control with period lengths of 12, 24 and 48 h, i.e. the circadian frequency and its harmonic overtone and undertone, is the membrane-integral H^+-translocating subunit *c* of the H^+-pumping V-ATPase in the tonoplast (Rockel et al. 1997).

The gene of PEPC-kinase from *K. fedtschenkoi* has been cloned (Hartwell et al. 1999). Phospho*enol*pyruvate carboxylase-kinase is under circadian control (Carter et al. 1991; Hartwell et al. 1996). Notwithstanding the hard work and attempts to show on this basis, that molecular PEPC-regulation represents the circadian CAM oscillator (Nimmo 2000), it turned out that PEPC-kinase activity cannot be regarded as an essential driving force of the endogenous CAM rhythm (Carter et al. 1995b). Detailed metabolic and molecular analyses now show clearly that metabolic control overrides circadian regulation of PEPC-kinase mRNA (Borland et al. 1999; Nimmo 2000). The details of how this metabolic control works are not yet clear. Most likely, parameters such as cytoplasmic pH, which we know changes by about 0.3 pH units in diel CAM oscillations of *K. daigremontiana* (J. Hafke, Darmstadt, personal communication), and malate levels are involved, because … "the circadian control of kinase mRNA and activity

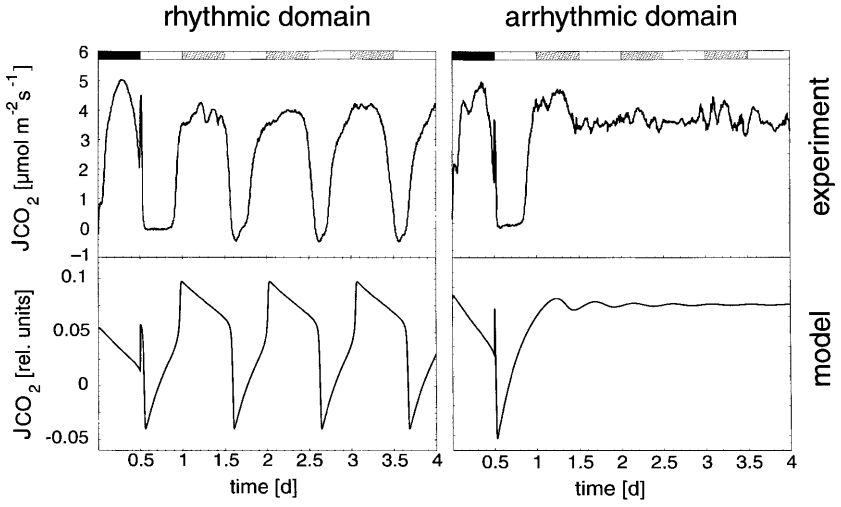

Fig. 4. Simulations of net CO_2 exchange with the CAM model of Fig. 2B in comparison to experimentally measured curves in the rhythmic and arrhythmic domains. The *dark bar* indicates the last normal dark period, the *hatched bars* give the times of subjective dark periods in continuous light (ca. 120 µmol photons m^{-2} s^{-1}, $\lambda = 400$–700 nm); temperatures were 25 and 28 °C, respectively, for the rhythmic and arrhythmic domains

can be influenced by metabolic status, specifically by treatments that affect the content or compartmentation of malate …" (Borland et al. 1999). This means, however, that we are back to the tonoplast functioning as the master switch of circadian regulation of CAM. Thus, PEPC-kinase oscillations are an epiphenomenon, which nature has possibly selected for stabilising a complex reaction network. Indeed, in attempts at model simulations a PEPC beat-oscillator alone failed to function. In addition to the tonoplast beat oscillator it stabilised the simulations against perturbations (Blasius 1997). Conversely, the tonoplast beat-oscillator alone always functions well (see above).

Temperature regimes: harmonies, disharmonies and noise

Time-series of J_{CO2} in the arrhythmic domains (Fig. 1 and especially Fig. 4) look irregular. Fast Fourier Transform (FFT) analysis shows, however, that they are by no means stochastic. They are structured with a distinct spectrum of characteristic frequencies. This is most wonderfully revealed in entrainment experiments with symmetric rectangular temperature jumps (Fig. 5). When the period length of the external temperature rhythm of a higher and a lower temperature of similar duration is close enough to the circadian period, there is entrainment, i.e. the exposed rhythm drags J_{CO2} along with it. When the period of the exposed rhythm is too short, the endogenous J_{CO2} rhythm comes through. When the period of the exposed rhythms is too long, J_{CO2} shows arrhythmicity. In any of these cases, all the frequencies revealed by FFT are related to the circadian ground frequency by fractions of 1/1, 1/2, 1/3, 1/4, 1/5 and 2/1, 3/1, i.e. they correspond to the ground frequency (1/1) and its harmonic overtones (1/2 to 1/5) and undertones (2/1, 3/1) and represent a musical harmony in *K. daigremontiana* (Lüttge et al. 1996).

In a vacuolar malate-content/tonoplast order $\langle S \rangle$ diagram the temperature relations shown above (Fig. 1) are simulated with the minimal model of Fig. 2B as follows (Beck et al. 2000, Fig. 6). At a low relative simulation temperature (0.2240, in relative units obtained by scaling the model, in Fig. 6) the system directly moves into a fixed point with a full vacuole (high value of vacuolar malate content), i.e. rhythmicity is lost. At a high temperature (0.2265) an opposite fixed point is reached with an empty vacuole, and again rhythmicity is lost. At a medium temperature (0.2245) the hysteresis switch keeps the system cycling rhythmically, the curve presents a so-called limit cycle. Most interesting is a somewhat higher temperature (0.2254) where the system "tries" to cycle but gradually spirals into a fixed point and rhythmicity again is lost (Fig. 6A). If now a certain degree of stochastic ("white") noise is imposed on the system the behaviour at the temperatures of 0.2240 and 0.2265 (fixed points with full and empty vacuole, respectively) and 0.2245 (limit cycle) is unchanged, the trajectories only becoming more squiggly. However, at the temperature 0.2254 the system now oscillates. When it reaches the fixed point, stochastical noise-kicks throw it out again and it has to return to the fixed point via nearby limit cycles, i.e. along an extended trajectory in the tonoplast order/malate level phase space. Thus, the system at temperature 0.2254 has now entered a disturbed rhythm (Fig. 6B). It can be easily shown that the intensity of noise must be in a defined range (Fig. 7). Noise that is too low or noise that is too high cannot generate the quasi-rhythmicity. In general, with increasing noise intensity the signal-to-noise ratio rapidly increases, but after a peak with further increasing noise it gradually declines again. Such phenomena, well known from nonlinear dynamics in theoretical physics, are called stochastic coherence and stochastic resonance (Wiesenfeld and Moss 1995; Russel et al. 1999). They are becoming increasingly important in biology and medicine (Pikovsky and Kurths 1997; Gammaitoni et al. 1998). For

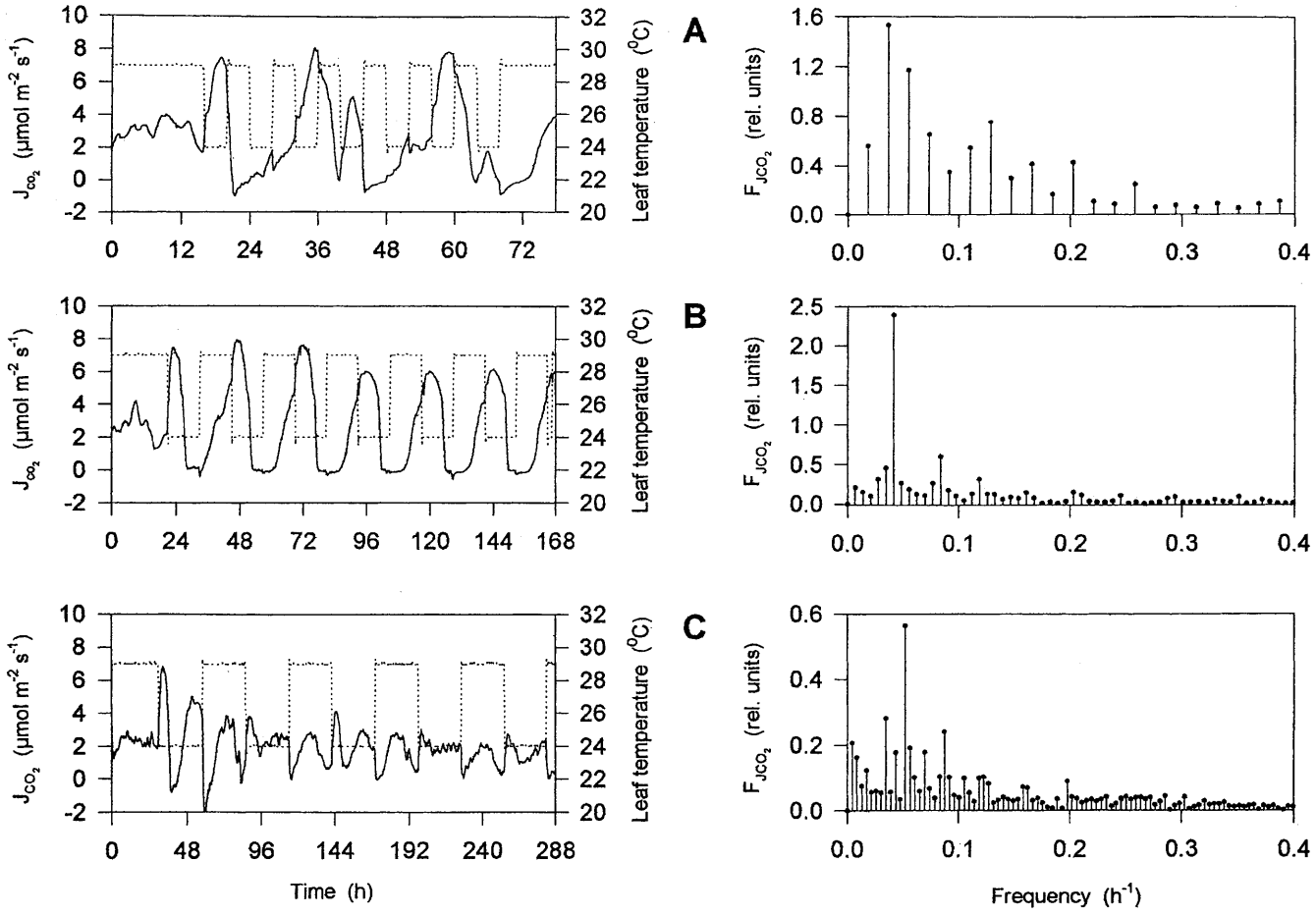

Fig. 5A–C. Time series of CO_2 gas exchange (J_{CO2}: *left*) and corresponding power spectra obtained by Fourier transformation analysis (*right*). **A** Short imposed temperature rhythm (8 h), where the gas-exchange curve shows rhythmicity and the dominating frequency of the power spectrum corresponds to the period length of the endogenous rhythm. **B** Medium imposed temperature rhythm (24 h) where the gas-exchange curve also shows rhythmicity and the dominating frequency corresponds to the period length of the imposed temperature rhythm indicating entrainment. **C** Long imposed temperature rhythm (56 h), where the gas-exchange curve looks irregular and the power spectrum is complex. *Left panels*: *solid lines* J_{CO2}; *dotted lines* leaf temperatures. *Right panels*: *vertical lines* frequency distribution in the power spectra. The Fourier amplitudes of net CO_2 exchange have been normalised so that comparisons can be made between the three experiments shown; however, the different scales should be noted. (From Lüttge et al. 1996; with permission)

plants, we discuss them for the first time in relation to the CAM oscillator. What does this tell us?

The theoretical prediction that noise may affect the CAM oscillator has led to experiments questioning if a strong signal is needed to elicit J_{CO2} rhythmicity out of arrhythmicity in constant light. Indeed, if out of a regime with a too high temperature, in contrast to Fig. 1, temperature is lowered very slowly, rhythmicity is not generated, although under similar conditions a fast temperature change of the same magnitude readily elicits a J_{CO2} rhythm (Rascher et al. 1998). By allowing a certain number of cells or leaf patches to behave independently rather than treating the whole leaf as a single entity in the model, this behaviour is simulated very accurately, so that without knowing, the eye cannot distinguish between measured and simulated time series of J_{CO2}. Hence, the leaf may consist of a number of independent (or weakly coupled), although structurally and cytologically identical oscillators, needing strong signals for phase synchronisation.

Outlook: the clock, a spatio-temporal phenomenon

This last conclusion led to the need for experimentation to show that there is patchiness which changes in time and in space over a CAM leaf and also in its degree as defined by units of homogeneity or inhomogeneity. The choice was to use photographic analysis of chlorophyll fluorescence, i.e. a non-intrusive method of depicting photosynthetic activity over an entire leaf of *K. daigremontiana*. From chlorophyll fluorescence recorded by a camera sensitive in the appropriate red wavelength region of the light spectrum, efficiency of photosystem II (PSII) is computed as $(F'_m - F)/F'_m$, i.e. effective quantum yield, where F is basic fluorescence and F'_m is light-saturated fluorescence of a light-adapted leaf (Schreiber and Bilger 1993). This allows the patchiness of photosynthethic activity on leaves to be analysed (Osmond et al. 1998, 1999). Efficiency of PSII is related to availability of the photosynthetic substrate CO_2, i.e. it responds to vacuolar net influx/efflux, where malic acid

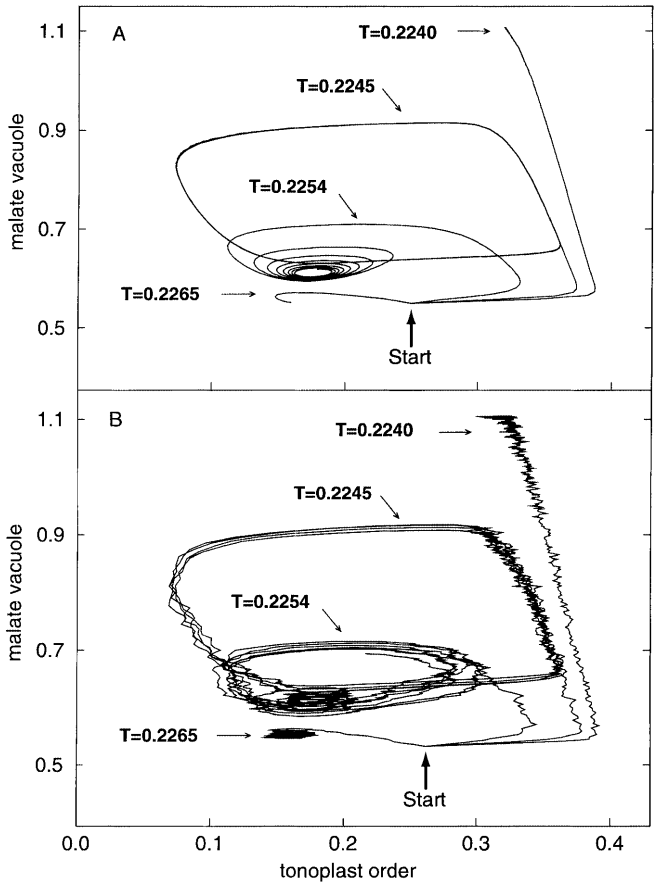

Fig. 6A,B. Trajectories in the parameter plane of vacuolar malate levels (y-axis) and the state of order of the tonoplast membrane (x-axis) all starting at the same point. Simulation using the minimal model of Fig. 2B. **A** Trajectories without noise. For the scaled relative temperature (0.2240) the trajectory runs into the upper fixed point characterised by a full vacuole. At high temperatures the trajectory reaches the lower fixed point characterised by an empty vacuole on a spiral path (0.2254) or on a direct path (0.2265). At intermediate temperatures, here exemplified for a temperature of 0.2245, limit cycle oscillations occur. **B** The same trajectories shown in **A** but adding a noise component to the equation describing them. This did not affect the trajectories at temperatures 0.2240, 0.2245 and 0.2265 in principle. A fundamental difference, however, is obtained with the trajectory at temperature 0.2254 which, in **A,** spiralled into the fixed point without noise and now is thrown by noise-kicks stochastically into nearby limit cycles from where it returns to the fixed point after a full cycle, so that now almost regular oscillations are obtained at this temperature. (By courtesy of F. Beck, Darmstadt)

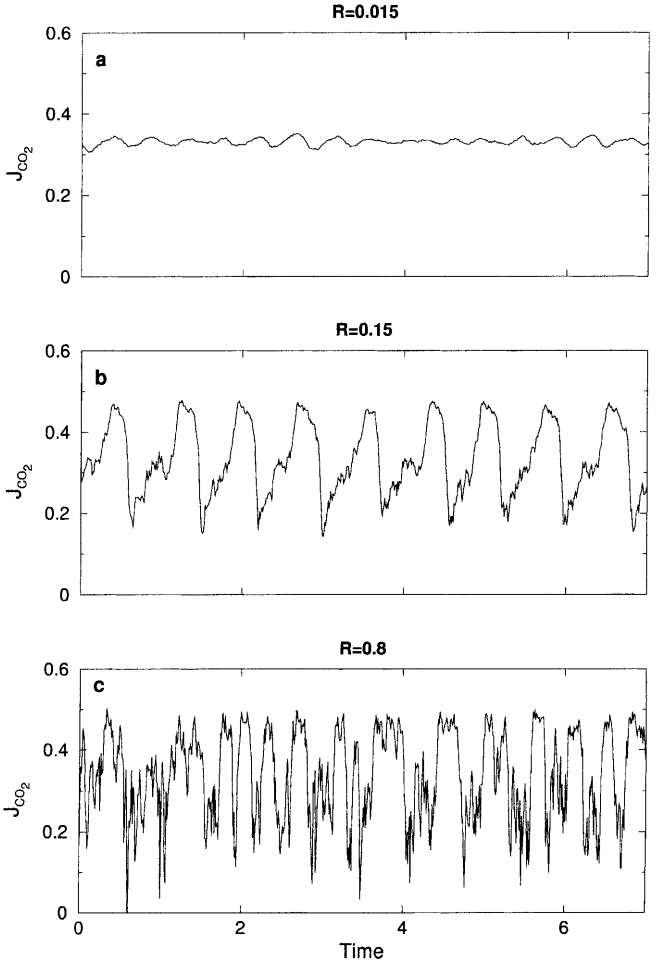

Fig. 7A–C. Net CO_2-exchange (J_{CO2}) simulated with the minimal model of Fig. 2 at various levels of white noise (R) increasing from top to bottom. (By courtesy of F. Beck, Darmstadt)

synthesis and net accumulation lead to low internal CO_2 concentration, and malic acid remobilisation and decarboxylation lead to high CO_2 concentration with low and high PSII efficiency, respectively. When cells or flecks in a leaf are not synchronised, this may build up patchiness in as much as CO_2 diffusion within a *Kalanchoë* leaf is constrained (Maxwell et al. 1997).

These studies show in fact, that there is patchiness during the endogenous CAM rhythm in leaves of *K. daigremontiana* of $(F'_m - F)/F'_m$ in continuous light (Fig. 8). This patchiness is variable in space and also in time, both for location of patches on the leaf and for intensity, viz. degree of homogeneity/inhomogeneity. We have obtained a wealth of time series over many days at 20-min intervals for the normal day/night rhythm as well as under constant conditions with the rhythmic and arrhythmic behaviour, respectively, of CAM leaves. A detailed mathematical analysis of these pictures is underway. Nevertheless, this already shows that we are arriving at a totally new perception of rhythmicity.

It is a long-standing experience in developmental biology that spatial morphological patterns develop in time, and much has been learned about how this is organised by cascades and hierarchies of genes (Mayer et al. 1991; Gering 1992; Pyke 1994; Theißen and Saedler 1997). The studies reviewed above show that rhythmic time series may evolve in space. Considerations of both development and biological clocks merge in the unique spatio-temporal conception. This new view of endogenous oscillators may pave the way for an understanding of the physiological mechanisms and molecular structures of biological clocks.

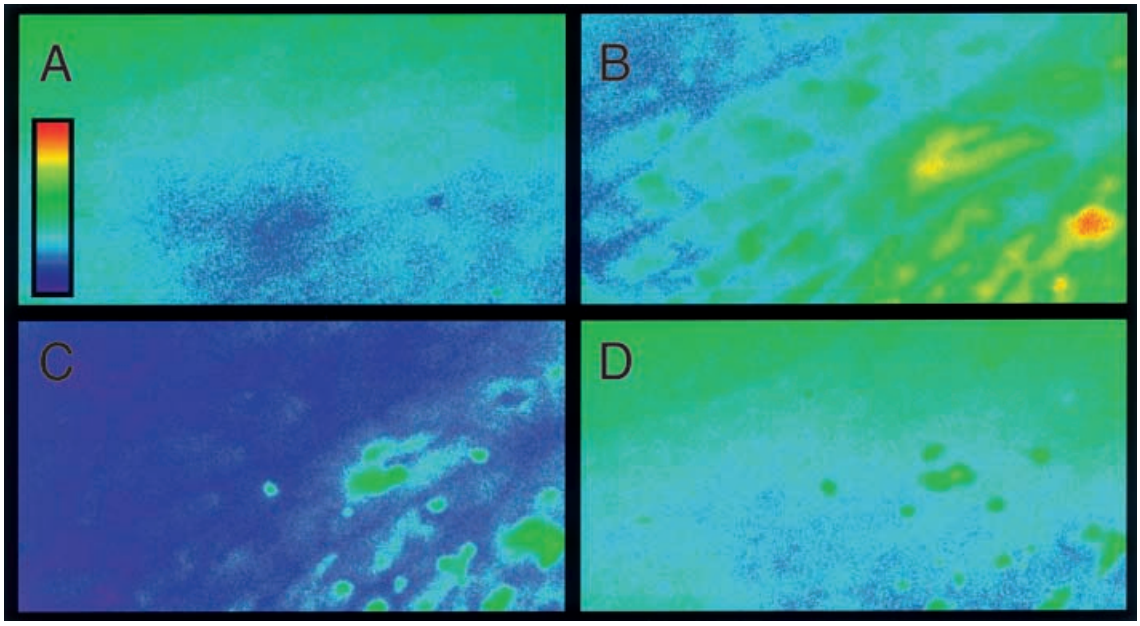

Fig. 8A–D. Chlorophyll fluorescence of a given part of one leaf of *K. daigremontiana* kept under continuous light (ca. 195 µmol photons m^{-2} s^{-1}, $\lambda = 400$–700 nm) and at a constant temperature of 21 °C as obtained by photographs with the chlorophyll-fluorescence camera. The four frames (**A–D**) are 7 h apart in time and represent a leaf area of ca. 4×8 cm (32 cm^2). The colour scale given in **A** indicates decreasing effective quantum yield of PSII, i.e. $(F'_m - F)/F'_m$ from top to bottom. This means red corresponds to the highest and blue to the lowest $(F'_m - F)/F'_m$. (By courtesy of U. Rascher, Darmstadt)

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