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Circadian Rhythms of Leaf and Stomatal Movements in Gymnosperm Species

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Abstract

It is generally accepted that various physiological, morphological and gene expression phenomena are under the control of a circadian clock, and that this time keeping mechanism is universally present. Although such endogenously regulated phenomena have first been documented in plants more than 250 years ago and much work has been accumulated particularly in the past 70 years, it was not obvious from the literature whether such time keeping mechanisms exist in gymnosperms.

Two prominent parameters were investigated in several gymnosperm species which have been demonstrated to be under the control of a circadian clock in many plants: (i) leaf movement and (ii) stomata movement. In young plants of *Pinus sylvestris*, *Picea abies*, *Taxus baccata*, *Araucaria angustifolia*, *Araucaria heterophylla* and *Ginkgo biloba* leaf oscillations could be recorded for about 5 days. However, compared to angiosperm plants, the amplitude was small. The period length under free running conditions (constant temperature and continuous light) was characteristic for the species.

Stomatal movement was observed in *Ginkgo biloba* leaves by electron microscopy. Stomata were open at noon and closed at midnight under normal day/night conditions (LD) as well as under constant light conditions (LL), indicating that stomatal aperture is under circadian control in the gymnosperm *Ginkgo biloba*. Online recordings of stomata conductance however, exhibited diurnal but not circadian oscillations of net CO_2-exchange in *G. biloba* leaves.

Our results show that a circadian clock controls leaf and stomatal movements in gymnosperm species indicating that endogenous time keeping mechanisms are present.

Keywords: circadian rhythm, leaf movement, stomata movement, stomata conductance, gas exchange, gymnosperms, *Pinus*, *Picea*, *Taxus*, *Araucaria*, *Ginkgo*.

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Introduction

Many physiological, morphological and gene expression processes need to be tightly coordinated during the growth of plants. This occurs with the help of an endogenous timing system universally present in animals, plants and even in prokaryotic organisms. The timing system that coordinates processes at the daily basis is called a circadian clock. In fact, the first process mentioned in the literature demonstrating circadian rhythmicity was the movement of the leaves of *Mimosa pudica* (De Mairan, 1729). About 200 years later this original observation was reinvestigated and extended by Pfeffer, Bünning and others and a new era of plant chronobiology was initiated. Particularly Bünning pointed out the importance of biological clocks in plants. In 350 publications he contributed much information to the field, developed methods and designed experiments which helped to understand the basic rules of the circadian mechanism (summarized in: Plesse, 1996; Bünning, 1977). Although he has investigated many different plant species apparently no gymnosperm was among them. Since the circadian clock is generally thought to be universal we started to investigate different gymnosperm species in order to find out whether they also possess a circadian system.

For several years we have studied the gene expression of the light harvesting complex genes (*Lhc*) of tomato. It was found that all 19 genes are under the control of the circadian clock (Kellmann et al., 1993). Later approximately 50 other genes of higher plants were shown to display diurnal or nocturnal expression patterns, indicating that this time keeping system controls and coordinates the expression of many genes in higher plants (Piechulla et al., 2001). In gymnosperm species, however, the situation turned out to be more complicated. RNA accumulation patterns were not rhythmic in the following species: *Pinus sylvestris, Picea abies, Abies alba, Taxus baccata, Ephedra campylopoda, Welwitschia mirabilis* (Oberschmidt et al., 1995; Brinker et al., 2000). This result seemed to indicate that in these species a circadian control of gene expression was lacking. However, it was found that the half life time of the *Lhc* mRNA in *Pinus sylvestris* is very long and apparently responsible for concealing possible clock controlled gene expression (Brinker et al., 2000). Therefore it cannot be excluded that a circadian clock functions in gymnosperms. The photoperiodic response on budset of *Picea abies* supports the notion of the existence of a time keeping system in such species (Clapham et al., 1998).

Since only circumstantial evidence supports the existence of a circadian clock in gymnosperm species, it was necessary to investigate other physiological and morphological observable phenomena such as leaf movement, stomata movement and gas exchange.

Results and Discussion

Rhythms of leaf movement

Quite a number of plants are able to position their leaves in such a way that photosynthetic reactions are optimized. In this way light can be received and harvested independent of environmental circumstances such as clouds and shading by other leaves. It is apparently advantageous to anticipate sunrise by using a circadian clock and not just react to the daily light. Such a clock controls the diurnal leaf movement of many plants. It remained uninvestigated whether gymosperms perform such leaf movements. Here we present the results of leaf movement measurements obtained with several gymnosperm plant species. The angiosperm species *Lycopersicon esculentum* and *Nicotiana tabacum* were used as control plants.

The Pinoopsida *Araucaria angustifolia*, *Araucaria heterophylla*, *Picea abies*, *Pinus sylvestris*, and *Taxus baccata* show circadian leaf movements for two to six days under continuous light conditions (LL) (Figs. 1A, B, C, D, E). Furthermore, the sole survivor of the Ginkgopsida *Ginkgo biloba* exhibits also a circadian rhythm of leaf movement under LL (Fig. 1F). The period length (τ) varies between 17 hours and 32 hours and is characteristic for each plant species. For comparison the angiosperms tomato and tobacco were also recorded (Figs. 1G, H).

In general, the oscillations of the gymnosperm species have a much smaller amplitude and are therefore more difficult to detect. A very robust rhythmic leaf movement with a relatively high amplitude was detected in young *A. angustifolia* plants. This rhythm disappeared after five days, probably due to cessation of leaf growth. It is known that in quite a number of plants the leaf movements are the result of differential growth of the upper and lower leaf parts (Darwin & Darwin, 1880). They are therefore detectable only in young leaves.

Although it is difficult to detect circadian leaf movements in gymnosperm species because of the small amplitude and because the oscillations are restricted to a defined and short period of growth, we succeeded in demonstrating circadian control. This supports the hypothesis that a circadian clock exists also in gymnosperms.

Stomatal movement

Plants control their gas exchange (CO_2 uptake and O_2 evolution) via opening and closing of stomata in the epidermis of the leaves. In some plants this movement is controlled by a circadian clock (summarized in: Bünning, 1977; Sweeney, 1987) and is therefore independent of diurnal light/dark and temperature changes. To our knowledge no analyses on stomatal movements in gymnosperm species have been reported. We therefore investigated the motion of stomata in leaves of *Ginkgo biloba* and used tobacco leaves as a control (Figs. 2A, B). Under normal day/night conditions (LD) stomata were closed during the night and were open during the day. Similar opening and closing was observed under constant conditions when illumination (LL) continued during the night. This result shows that stomata opening and closing in *G. biloba* leaves is under circadian control. The stomata of tobacco leaves are closed at midnight and opened at noon under LD and LL conditions (Fig. 2B). Circadian rhythmicity of stomatal resistance was previously demonstrated for tobacco by Macdowall (1964). The results indicate a circadian control of stomatal movement in *G. biloba* leaves, although only few data points are available. More data are necessary for confirmation.

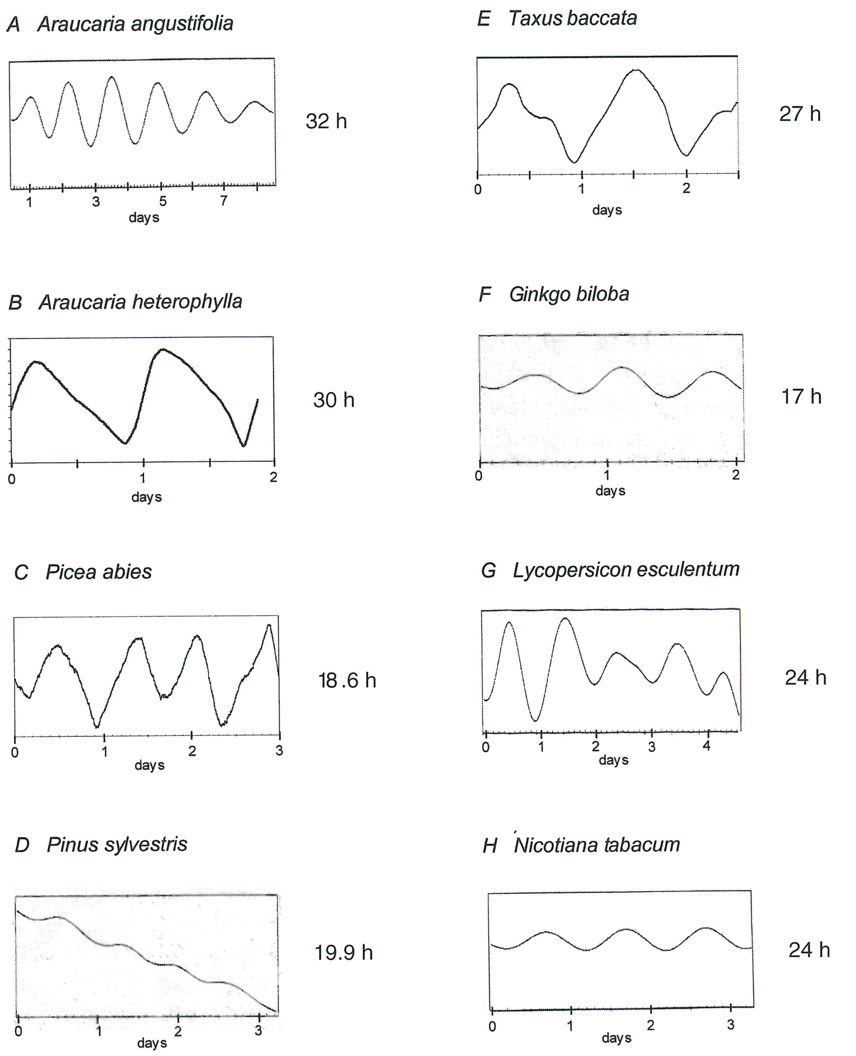

Figure 1. Circadian leaf movement in different gymnosperm plant species. Oscillations of the movement of leaves were measured under continuous light conditions in different gymnosperm species. (A) *Araucaria angustifolia*, 9 weeks old; (B) *Araucaria heterophylla*, 15 year old plant, young leaves were used for measurements; (C) *Picea abies*, 20 weeks old; (D) *Pinus sylvestris*, 13 weeks old; (E) *Taxus baccata*, 4 years old, young leaves were observed; (F) *Ginkgo biloba*, 6 months old; and the angiosperm species (G) *Lycopersicon esculentum*, cherry line, VFNT LA1221, 12 weeks old and (H) *Nicotiana tabacum*, 8 weeks old. Leaf movements were detected by using a video camera connected to an Atari computer and an imaging analysis software (Schuster & Engelmann, 1990). Leaves were marked with a small styrofoam bead or with white ink. The movement was recorded under continuous light ($75\,\mu E\,m^{-2}s^{-1}$) and the data were digitally filtered (Oxaldifi program by Schuster) to obtain the periods. The mean period length (τ) is presented for each species at the right side of the diagrams.

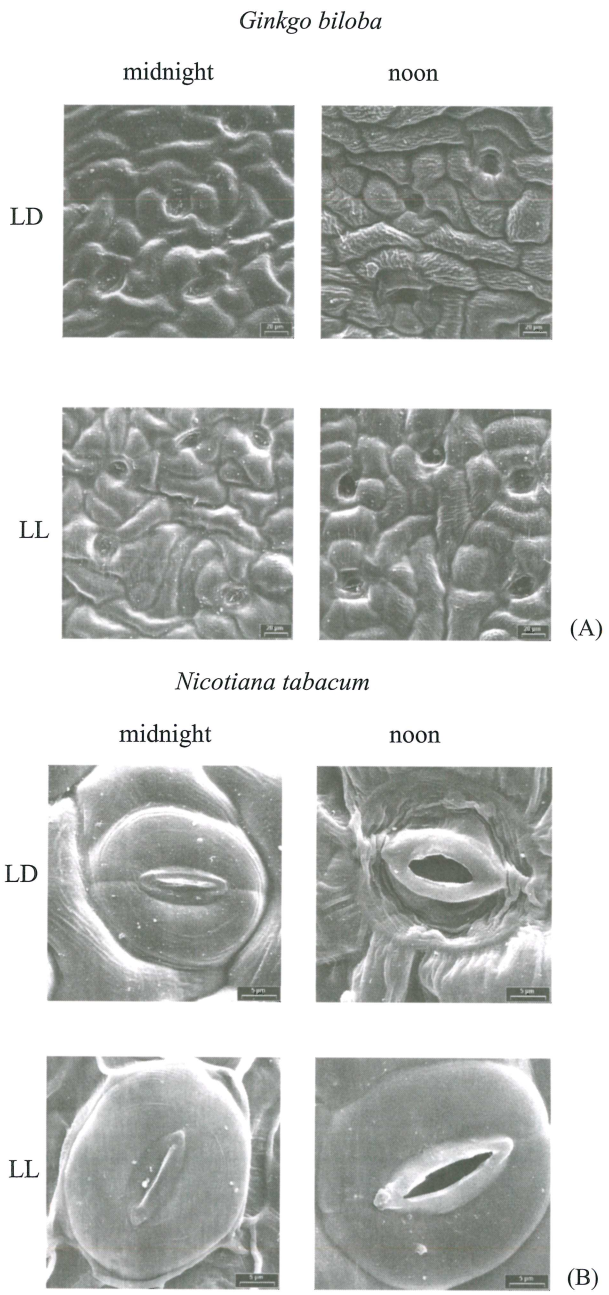

Figure 2. Electron micrographs of stomata of *Ginkgo biloba* (A) and *Nicotiana tabacum* (B). Pieces of leaves from both plant species were harvested at indicated time points and immediately fixed in glutaraldehyde (4% in 0.1 M sodium phosphat buffer) for 48 h. Samples were air dried in the critical point dryer K850. The surface of the leaves was sputtered with gold and analysed in a SEM.

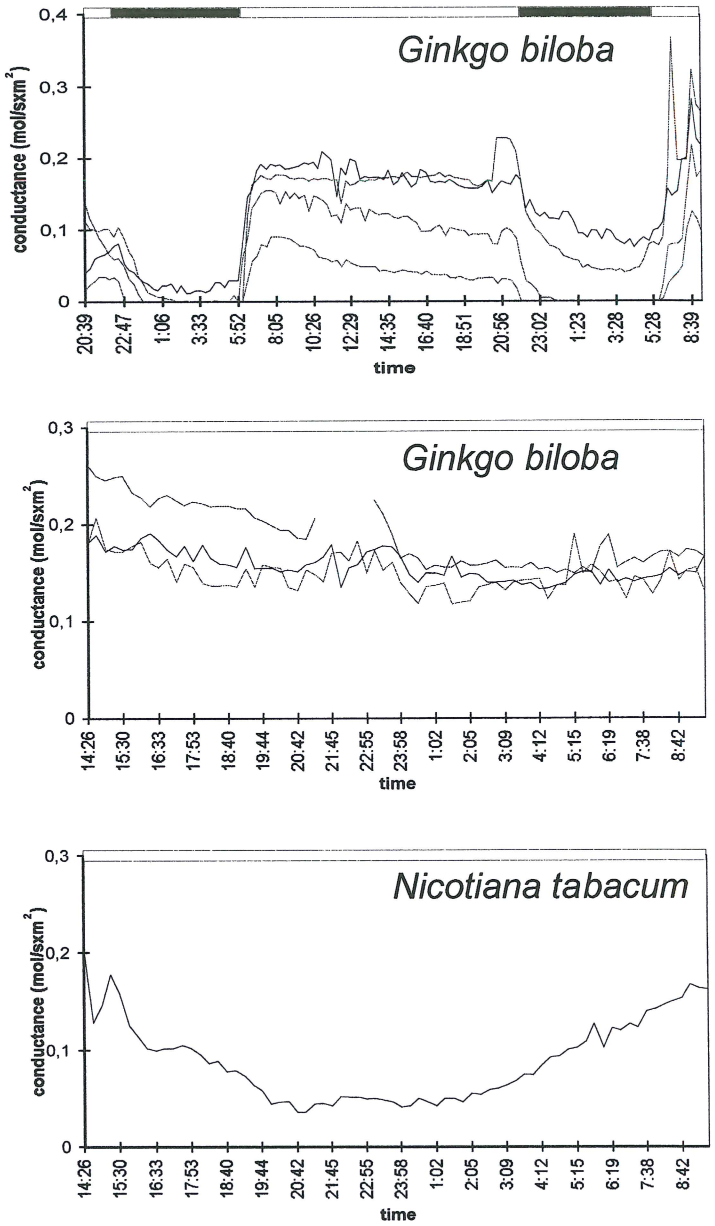

Figure 3. Gas exchange measurements in *Ginkgo biloba* and *Nicotiana tabacum* leaves. Online recording of conductance (gas exchange) of attached leaves under day/night conditions (LD) (upper panel) and under continuous illumination (LL, 140 µmol m^{-2} s^{-1}, temperature 30–32°C) (middle and lower panel) are plotted. Open and dark bars on top of the diagrams indicate day and night, respectively. Conductance measurements with *Ginkgo biloba* leaves are shown in the upper and middle panel, with tobacco leaves in lower panel.

Gas exchange measurements

To confirm the circadian control of stomatal aperture in *Ginkgo biloba* leaves we performed online gas exchange measurements with *G. biloba* and tobacco leaves. Leaves of one year old *G. biloba* plants were enclosed in a gas exchange chamber and the conductance of stomata was determined by measuring CO_2 uptake (Fig. 3). Under LD conditions diurnal changes were detectable in *G. biloba* (Fig. 3, upper panel). However, under constant conditions (LL) no rhythmic changes of conductance were detected (Fig. 3, middle panel). In comparison, under free running conditions a decrease of conductance was observed during the subjective night in tobacco leaves (Fig. 3, lower panel). These data seem to indicate that gas exchange under our recording conditions is not under circadian control in *G. biloba* while it is in the angiosperm *tobbacco*. This result contradicts the observation of stomatal movements in *G. biloba* leaves (Fig. 2A). A possible explanation is that although stomatal movement occurs under continuous illumination, the conductance changes are too small to be detected. Alternatively, no changes in conductance are recorded because photosynthetic reactions do not occur under the light and temperature conditions applied to the *G. biloba* leaves.

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