Circadian control of isoprene emissions from oil palm (*Elaeis guineensis*)

Running title: Circadian control of isoprene emissions from oil palm

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Summary

The emission of isoprene from the biosphere to the atmosphere has a profound effect on the Earth's atmospheric system. Until now, it has been assumed that the primary short-term controls on isoprene emission are photosynthetically active radiation and temperature. Here we show for the first time that isoprene emissions from a tropical tree (oil palm, *Elaeis guineensis*) are under strong circadian control and that the circadian clock is potentially able to gate light induced isoprene emissions. These rhythms are robustly temperature compensated with isoprene emissions still under circadian control at 38 ºC. This is well beyond the acknowledged temperature range of all previously described circadian phenomena in plants. Furthermore, rhythmic expression of *LHY/CCA1,* a genetic component of the central clock in *Arabidopsis thaliana*, is still maintained at these elevated temperatures in oil palm. Maintenance of the CCA1/LHY-TOC1 molecular oscillator at these temperatures in oil palm allows for the possibility that this system is involved in the control of isoprene emission rhythms. This study contradicts the accepted theory that isoprene emissions are primarily light induced.

Keywords: Isoprene, oil palm, diurnal variability, circadian clock, circadian gating.

Abbreviations: *IE* (isoprene emission rate); $IE_{1000/30}$ (isoprene emission rate at PAR of 1000 µmol m^{-2} s^{-1} and 30 ºC leaf temperature); LL (constant light).

Introduction

Emissions of the volatile organic compound isoprene (2-methyl-1,3-butadiene; C_5H_8) from the biosphere affect the oxidative capacity of the atmosphere (Shallcross and Monks, 2000) and hence have the potential to modify regional ozone pollution (Pierce *et al.*, 1998), lead to secondary aerosol formation (Claeys *et al.*, 2004) and indirectly control the lifetime of the greenhouse gas methane (Poisson *et al.*, 2000). Isoprene is synthesized in the chloroplast, mainly from recently assimilated carbon (Karl *et al.*, 2002), and although synthesis is loosely coupled to photosynthetic processes through substrate availability and requirements for adenosine triphosphate, its emission may become uncoupled from photosynthesis, particularly at elevated temperatures (Harley *et al.*, 1999). On time scales of minutes to hours, the primary controls that are known to affect isoprene emission rate (*IE*) are photosynthetically active radiation (PAR) and leaf temperature (Harley *et al.*, 1999), which regulate substrate availability and enzyme kinetics (Sharkey *et al.*, 2001). Isoprene is emitted through stomata but changes in stomatal aperture have negligible effects on its emission due to its high volatility and low solubility in water (Niinemets and Reichstein, 2003a). In the longer term, carbon dioxide concentration (Possell *et al.*, 2004; Possell *et al.*, 2005), leaf phenology (Kuzma and Fall, 1993), leaf nitrogen content (Harley *et al.*, 1994; Rosenstiel *et al.*, 2004), and environmental stresses such as soil water potential (Pegoraro *et al.*, 2004) and herbivory (Funk *et al.*, 1999) also affect the magnitude of *IE*.

To compile emission inventories for this important compound, branch and leaf enclosure techniques are commonly employed to determine the isoprene emission rate for individual plant species at 1000 µmol m^{-2} s^{-1} (PAR) and 30 ℃ leaf temperature. Here we define this parameter as $IE_{1000/30}$. An estimate of *IE* at ambient PAR and

temperature throughout the day for each species is obtained by scaling $IE_{1000/30}$ using light and temperature correction factors (Guenther *et al.*, 1995). Species composition and density are then used to estimate a regional average emission flux. However, there is high variability in $IE_{1000/30}$ measurements with considerable day to day, seasonal and inter- and intra-species variability (e.g. http://www.acd.ucar.edu:8080/voc/vocIndex.jsp; http://www.es.lancs.ac.uk/cnhgroup/iso-emissions.pdf.). Significant diurnal variability in the magnitude of $IE_{1000/30}$ for several tree species has also been reported. Isoprene emission rates from cottonwood and oak, standardized to $IE_{1000/30}$, were observed to be significantly higher in the afternoon than in the morning (Funk *et al.*, 2003). Yet in the same study no variability in $IE_{1000/30}$ for eucalyptus was observed. In other studies, $IE_{1000/30}$ for oak has been shown to increase over a day by as much as 50 %; this increase could be predicted using changes in weather conditions over the previous 24 hours (Sharkey *et al.*, 1999; Geron *et al.*, 2000). However, some of the variability in $IE_{1000/30}$ cannot be explained by present understanding of the effects of short-term environmental and biological controls on isoprene synthesis. As well as changes in $IE_{1000/30}$ over the course of a day, significant changes in the concentration of the isoprene precursor, dimethylallyl diphosphate (DMAPP), have been observed (Geron *et al.*, 2000; Wolfertz *et al.*, 2003; Rosenstiel *et al.*, 2004) but without a link being made to possible circadian control.

A wide range of fundamental processes in plant biology are optimized and coordinated each day due to regulation by an endogenous circadian clock. Examples include leaf movements, hypocotyl elongation, gene transcript and protein abundance rhythms and the photoperiodic control of flowering time (McClung, 2001). A number of the genes that constitute the molecular components of the positive-negative

autoregulatory feedback loops that comprise the plant circadian clock have been identified in *Arabidopsis thaliana*. *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) and the pseudo-response regulator/CONSTANS-domain protein *TIMING OF CAB EXPRESSION1* (*TOC1*) form at least one of the interlocking loops at the core of the plant clock (Alabadi *et al.*, 2001). *CCA1* and *LHY* transcript abundance is under circadian control and peaks at dawn (Schaffer *et al.*, 1998; Wang and Tobin, 1998). Orthologous clock genes have been characterised in plant species as diverse as rice (Izawa *et al.*, 2003), common ice plant (*Mesembryanthemum crystallinum*) (Boxall *et al.*, 2005), beans (*Phaseolus vulgaris*) (Kaldis *et al.*, 2003) and chestnut (*Castanea sativa*) (Ramos *et al.*, 2005) suggesting that the genetic components of the clock are broadly conserved in higher plants. In *Arabidopsis*, all known clock-controlled processes have been shown to be ultimately responding to the central *CCA1/LHY-TOC1* oscillator including gas exchange, leaf movements, gene expression rhythms and flowering time (Dodd *et al.*, 2005). The CCA1 and LHY proteins belong to the Myb family of transcription factors (Schaffer *et al.*, 1998; Wang and Tobin, 1998) and thus act at the transcriptional level to control gene expression.

Here we report that isoprene emission from the fronds of oil palm trees are under the control of a circadian clock. Circadian rhythms of isoprene emission persist for several days at constant light and temperature, are robust and temperature compensated between 25 ºC and 38 ºC and are gated in a circadian fashion. Using an oil palm *CCA1/LHY* orthologue as a marker, we establish that the molecular clock also operates in oil palm trees up to 38 ºC and thus that this clock is potentially capable of coordinating isoprene emission rhythms.

Results

Isoprene emission in oil palm is under circadian control

As noted above, diurnal variability in *IE* standardised to $IE_{1000/30}$ has been observed previously (Sharkey *et al.*, 1999; Geron *et al.*, 2000; Funk *et al.*, 2003). We have confirmed this using field measurements of $IE_{1000/30}$ from oil palm growing on a plantation in Malaysia, which showed a strong diurnal trend (Fig 1a). This trend was also observed in laboratory grown plants under controlled conditions (Figure 1b). To determine whether this short-term variability in $IE_{1000/30}$ is caused by circadian regulation, we monitored $IE_{1000/30}$ from a frondlet under constant illumination (LL) for 70 h at 1000 μmol m^{-2} s^{-1} and constant 30 ^{o}C leaf temperature. If isoprene synthesis is under circadian control, we would expect to observe free running rhythms in $IE_{1000/30}$ over a period of several days. The frondlet was illuminated from dark at 06:00 (normal 'lights on'). $IE_{1000/30}$ increased for 4 h, reaching a maximum (IE_{max}) of ~40 nmol C m^{-2} s^{-1}, before declining over the rest of the day (Figure 2a). $IE_{1000/30}$ fell during subjective night (i.e. when the frondlet is not normally illuminated) to approximately 10% of IE_{max} before rising again the following subjective morning. Over the following 48 h, oscillations in $IE_{1000/30}$ continued but with a dampening of IE_{max}. The period of oscillation in this free running cycle was 23.1 h. The persistence of rhythms in the absence of a dark-light cycle or other exogenous signals clearly indicates that isoprene synthesis is regulated by an internal biological clock.

To investigate the effect of different leaf temperatures on these free running rhythms in *IE*, we repeated the experiment at leaf temperatures of 25 ºC ($IE_{1000/25}$) and 38 ºC ($IE_{1000/38}$) (Figure 2b and 2c respectively). At these temperatures, isoprene

emissions oscillated over several days with the magnitude and amplitude of the oscillations dependent on leaf temperature and correlated positively with these parameters. The increase in absolute emission rates with increasing temperature is consistent with present understanding of the effects of temperature on isoprene synthase activity (Sharkey *et al.*, 2001). As well as *IE* increasing with temperature, Figures 2a-c show that the oscillations in *IE* become more robust at elevated temperatures when the central components of the plant circadian clock are believed to become arrhythmic. The period of the *IE* rhythm was 25.5 h at 25 ˚C and 21.4 h at 38 ˚C, revealing that the rhythm shows significant temperature compensation. The robust circadian rhythm in *IE* at all three temperatures is not reflected in assimilation rates (A); no circadian rhythm in A was observed (Figure 2d-f). This suggests a total uncoupling of *IE* from photosynthesis. Rhythmicity was observed in stomatal conductance (g_s) (Fig 2d-f) but the period length differed to that of *IE* such that the two parameters were not in phase with one another (see Table 1). These results confirm previous findings which have shown that *IE* is independent of g_s (Fall, 1999; Niinemets and Reichstein, 2003a; 2003b).

Table 1. Length of period in *IE* and g_s at varying temperatures

Leaf temperature (ºC)	*IE* period (h)	g_s period (h)
25	25.5	21.2
30	23.1	26.7
38	21.4	22.1

Figure 1. Variability in isoprene *IE* at 1000 µmol m^{-2} s^{-1} and 30 °C leaf temperature (*IE*$_{1000/30}$). (a) Field measurements of *IE*$_{1000/30}$ from oil palm taken in an oil palm plantation in Sabah, Malaysia. PAR and temperature were held constant at 1000 µmol m^{-2} s^{-1} and 30 °C from 10:00 to 16:00 hours. (b) *IE*$_{1000/30}$ measured in the laboratory. PAR and temperature were held constant at 1000 µmol m^{-2} s^{-1} and 30 °C from 10:00 to 18:00 hours.

To eliminate the possibility of cross-talk signalling between neighbouring cells outside the gas exchange cuvette, which were at a different temperature to the measured cells inside the cuvette, whole frond measurements of IE were made using excised fronds, with the whole frond kept at a constant temperature. Isoprene emissions from these fronds were measured at leaf temperatures of 25 and 38 ºC at light intensity of 120 µmol m^{-2} s^{-1} ($IE_{120/25}$ and $IE_{120/38}$; Figures 2g and h respectively). At 25 ºC (Figure 2g), whole frond isoprene $IE_{120/25}$ oscillated with a similar period to that seen in the leaf cuvette (Figure 2b). At 38 ºC (Figure 2h), the periodicity in $IE_{120/38}$ was seen for 48 h, after which wilting of the excised frond occurred and IE became arrhythmic. From these whole frond measurements at both 25 ºC and 38 ºC we conclude that no cross-talk signalling occurred between neighbouring cells during the cuvette experiments.
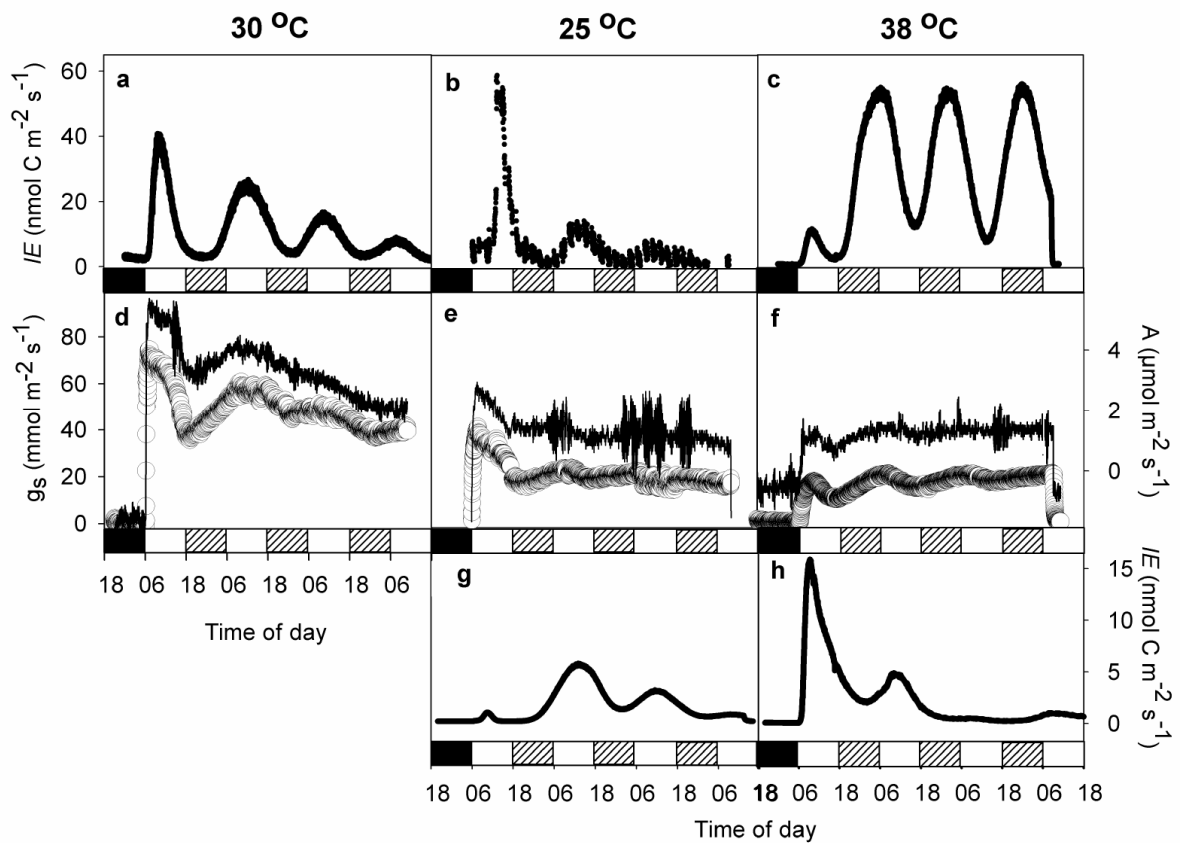
Figure 2. Leaf level free running isoprene emission rates (*IE*) (a-c) and assimilation rates (A) (top trace) and stomatal conductance (g$_s$) (bottom trace) (d-f) from oil palm measured in LL (constant light; 1000 µmol m^{-2} s^{-1}) for 70 h at 30 ℃ (a + d), 25 ℃ (b + e) and 38 ℃ (c + f) leaf temperature. Full frond *IE* measured in LL (120 µmol m^{-2} s^{-1}) at 25 ℃ and 38 ℃ are also shown in (g) and (h) respectively. Note: in panel b, *IE* values have been multiplied by 10.

The oil palm molecular clock maintains function at high temperatures

The robust circadian rhythm of isoprene emissions at 38 ˚C (Figs. 2c and 2h) indicate that a circadian clock must be operating in oil palm at a significantly higher temperature than any previously recorded plant circadian rhythm. For example, the circadian rhythm of CO_2 fixation in the tropical CAM species *Kalanchoë daigremontiana* becomes arrhythmic above 32 ˚C, even in plants that were pre-adapted to a daytime temperature of 34 ˚C for one month prior to analysis (Grams *et al.*, 1995). It was therefore important to examine the operation of the molecular clock in oil palm at 38 ˚C to determine whether the molecular oscillator was operating and, therefore, capable of controlling the observed *IE* rhythm. We identified an oil palm expressed sequence tag (EST), which we called *EgCCA1/LHY*, which is an orthologue of the *Arabidopsis* central clock components *CCA1* and *LHY* (see Materials and Methods). We collected frondlet samples every six hours from excised oil palm fronds maintained in LL at both 25 ˚C and 38 ˚C using a whole-frond chamber. We isolated RNA from each frondlet sample and used a real time-polymerase chain reaction (RT-PCR) technique to examine changes in the steady-state transcript abundance of *EgCCA1/LHY* (Fig. 3). This analysis revealed robust rhythms in *EgCCA1/LHY* transcript abundance at both 25 ˚C and 38 ˚C, supporting the hypothesis that *IE* could be controlled by the *CCA1/LHY-TOC1* central molecular clock. It is noteworthy that the period of the *EgCCA1/LHY* rhythm at 38 ˚C is significantly shorter than the rhythm at 25 ˚C (Fig. 3); this correlates well with the shortening of the period of the *IE* rhythm at 38 ˚C relative to 25 ˚C (Figs 2a-c).
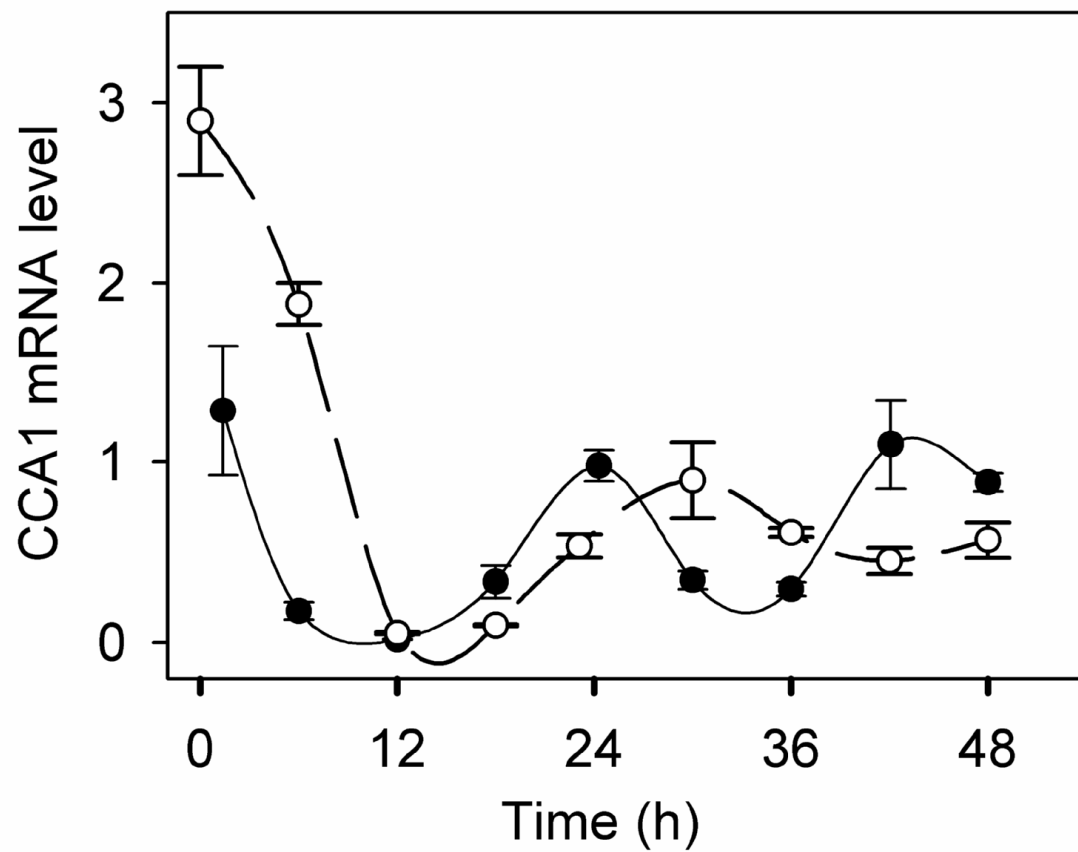
Figure 3. Steady state changes in mRNA transcript abundance of *EgCCA1/LHY* at leaf temperatures of 25 ℃ (open circles) and 38 ℃ (closed circles) under constant illumination.

Isoprene emissions are circadian-gated

Circadian clock outputs are often gated such that they can only be activated at a specific phase in the 24 h cycle. For example, the circadian rhythm of nocturnal CO_2 fixation in the Crassulacean acid metabolism species *Kalanchoë fedtschenkoi* is gated in response to temperature (Hartwell *et al.*, 2002). When the temperature was reduced from the non-permissive 30 ˚C to the permissive 15 ˚C during the subjective light period, no CO_2 fixation was observed in this species, but when the temperature was reduced to 15 ˚C during the subjective dark period, CO_2 fixation was initiated. *IE* has previously been assumed to respond directly to light, with zero emission in the dark, i.e. emissions will only occur if the frondlet is illuminated. However, if the induction of isoprene is gated by the circadian clock, we would predict that emissions should only occur when a frondlet is illuminated during the subjective light period. Illumination of a frondlet at the beginning of the subjective dark period should not give rise to isoprene emissions until the subsequent subjective light period.

We investigated this by keeping two leaves in the dark at the end of subjective day; one for 24 h, the other for 36 h. The frondlet kept in the dark for 24 h was illuminated with 1000 µmol m^{-2} s^{-1} at 18:00, at the onset of subjective dark, at a constant leaf temperature of 30 ˚C. Following illumination, $IE_{1000/30}$ was essentially zero until the start of the next subjective day, at which time it increased significantly (Figure 4: black line). This demonstrates an uncoupling of *IE* from the light stimulus, and is in contrast to all previous assumptions about the light dependency of *IE*. Continued illumination of the frondlet, for a further 24 h, resulted in diurnal oscillations in $IE_{1000/30}$ with a period ~24 h. During the second subjective night, $IE_{1000/30}$ did not fall to zero but decreased to approximately 20% of the preceding IE_{max} before increasing during the

following subjective day. The frondlet that was kept in the dark for 36 h (Figure 4: open circles) was illuminated at the start of subjective day. $IE_{1000/30}$ was zero for the first hour after the frondlet was illuminated and then increased significantly, reaching IE_{max} at 09:40 before starting to decline. This circadian gating of isoprene emission is similar to the gating described for the acute induction of the chlorophyll A/B binding protein (CAB) by light in *Arabidopsis thaliana* (Millar and Kay, 1996). Figure 4 also shows that the time of day that IE_{max} was achieved for the frondlet illuminated at 18:00 (black line) was delayed by 9 h when compared to the frondlet illuminated at 06:00 (open circles). This suggests there was a shift in phase of the circadian rhythm due to the frondlet being illuminated at the beginning of a subjective dark period, i.e. 12 h out of phase with the prior light entrainment of the frondlet. These results demonstrate that the isoprene emissions are circadian-gated.
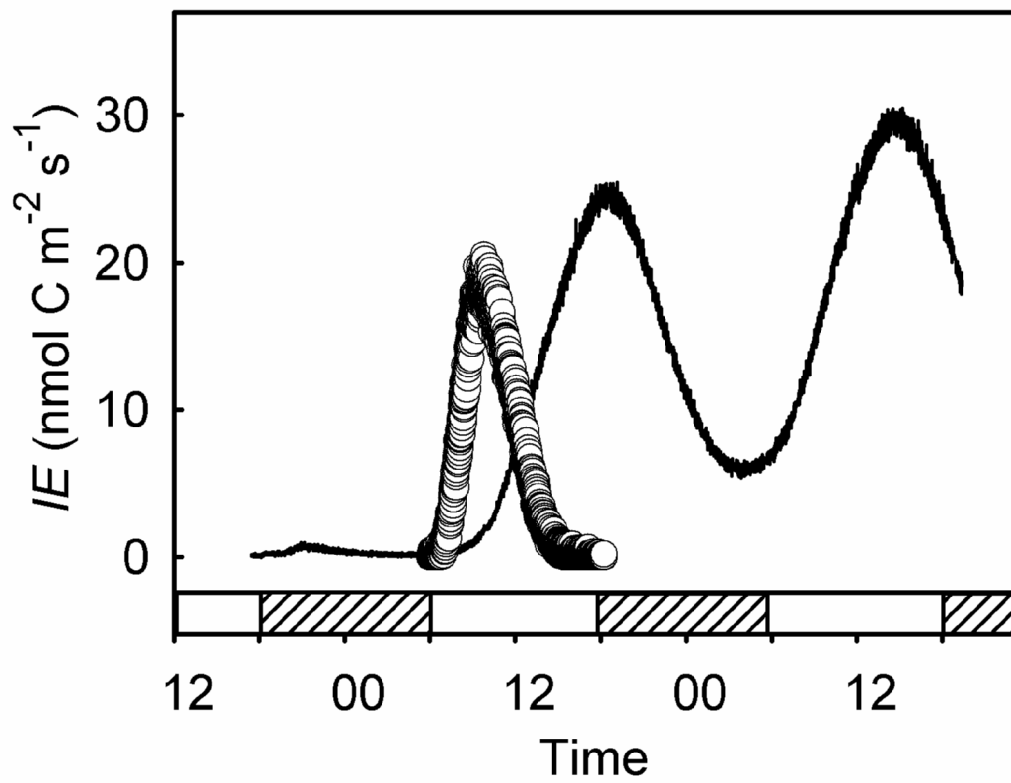
Figure 4. Circadian gating of isoprene emission rates from oil palm at 1000 µmol m^{-2} s^{-1} and 30 $^{\circ}$C. Note that when a frondlet was kept in the dark for 24 h and illuminated at the start of subjective day, initiation of *IE* was delayed by 12 h (black line). When a frondlet kept in the dark for 36 h was illuminated at the start of subjective day (open circles) no delay in the onset of isoprene emission was observed. Comparison of emission rates between the two leaves shows that illuminating the frondlet at the start of subjective night resulted in a shift of phase of ~9 h.

Diurnal variability in *IE* at low light intensities

To determine the effect of different light intensities on the observed diurnal variability in *IE*, we measured *IE* at PAR of 100, 200 and 300 µmol m^{-2} s^{-1} on successive days at a constant leaf temperature (30 ºC). For each light treatment *IE$_{max}$* occurred 2-3 hours after illumination (Figure 5). Following *IE$_{max}$* there was a steady decline in *IE* during the afternoon. This is in contrast to the square wave function previously assumed, in which *IE* rapidly stabilises at a given value, irrespective of the time of day, as long as PAR and temperature remain constant. However, the magnitude of *IE$_{max}$* was PAR dependent, which is consistent with previous reports (Guenther *et al.*, 1993; Lerdau and Gray, 2003).
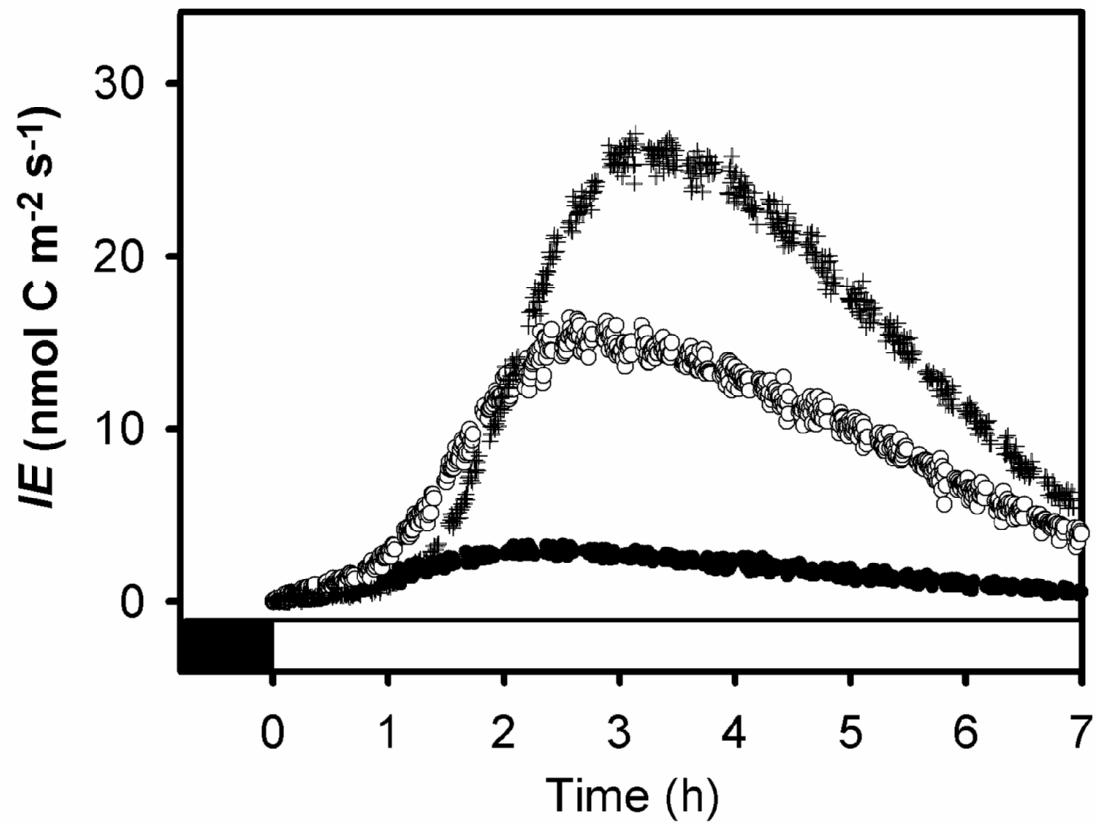
Figure 5. Diurnal variability in isoprene emission rates (*IE*) at a constant leaf temperature of 30 ˚C and PAR of 100 (closed circles), 200 (open circles) and 300 (crosses) µmol m^{-2} s^{-1} respectively.

Discussion

Despite considerable efforts to elucidate the physiological role of isoprene, its biological function still remains unclear. Several hypotheses have been proposed to explain its production by plants, including: a role as a mechanism to alleviate oxidative stress (Loreto *et al.*, 2001; Affek and Yakir, 2002); a protectant of photosynthetic apparatus against thermal damage (Sharkey and Yeh, 2001); or as a mechanism to protect against excessive light (Penuelas and Munne-Bosch, 2005). Therefore, a circadian mechanism that allows plants to adjust to the diurnal variability of these different stresses and more efficiently protect the photosynthetic apparatus in high light and temperature environments would help to increase productivity and would be of evolutionary and ecological benefit. Circadian clocks represent an evolutionary adaptation allowing plants to optimise their biochemical and physiological activities in anticipation of the varying demands of the day/night cycle and to react to environmental signals (Millar and Kay, 1996). From an evolutionary perspective, this ability to synchronise with both external and internal environments may be critical to the plant's survival (Dodd *et al.*, 2005).

The circadian rhythm in *IE* can be measured across a broad range of light and temperature conditions, with the magnitude and amplitude dependent on these two parameters. Furthermore, by studying the transcriptional regulation of the central clock-component *CCA1/LHY*, we have determined that the oil palm molecular clock maintains rhythmicity at a higher temperature (38 ˚C) than any previously recorded plant circadian rhythm. This suggests that a tropical species such as oil palm, which has evolved under temperatures that commonly reach a daytime maximum of 38 ˚C or more, has a clock that is pre-adapted to function at elevated temperatures. In contrast, circadian rhythms in

many other plants, including species native to hot climates, have been reported to become arrhythmic at temperatures in the range 30-32 ˚C (Grams *et al.*, 1995). Importantly, the period of the *EgCCA1/LHY* rhythm is shorter at 38 ˚C than it is at 25 ˚C and this correlates well with the shortening of the *IE* period at 38 ˚C. Together, these observations provide circumstantial evidence for a *CCA1/LHY-TOC1*-type molecular oscillator underlying isoprene emission rhythms in oil palm trees.

At present, it is unknown at which level(s) circadian regulation is applied to isoprene emission. Investigation of available promoter regions (1000-2000 bp) from poplar isoprene synthase genes (GenBank accession numbers AM084344 and AY341431 and sequences in the *Populus trichocarpa* genome, http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) reveals the presence of multiple copies of circadian-associated *cis* elements. These include: the binding site for CCA1 (AAa/cAATCT), which is conserved in light-harvesting complex (*Lhc*) gene promoters (Wang and Tobin, 1998) and is found in a number of circadian-regulated gene promoters (reviewed in (McClung, 2000; Nozue and Maloof, 2006); the core conserved sequence (CANNNNATC) of a region required for circadian regulation of the tomato *Lhc* genes (Piechulla *et al.*, 1998); and the 'evening element' (AAATATCT), which was identified by *in silico* analyses of circadian-regulated gene promoters (Harmer *et al.*, 2000). These elements are all closely related and may in fact represent the same conserved sequence. Their presence in the isoprene synthase gene promoters suggests that expression of isoprene synthase genes may be controlled in a circadian fashion at the transcriptional level. (Arimura *et al.*, 2004) showed significant variability in isoprene synthase transcript levels between day and night in poplar leaves; this is consistent with the possibility that transcript abundance is under circadian control. Post-transcriptional

regulation of circadian rhythms is also known to occur at several levels (reviewed in (Somers, 1999; Nozue and Maloof, 2006).

Circadian regulation may also be imposed on genes upstream in the MEP pathway for isoprene synthesis, resulting in circadian fluctuation of *IE.* It has been suggested that isoprene emissions may act as a biological "safety valve" for excess chloroplastic energy and carbon (Rosenstiel *et al.*, 2004). Competition for phosphoenylpyruvate (PEP) between cytosolic and chloroplastic processes may result in changes in isoprene emission by preferentially diverting PEP away from the chloroplast (and hence isoprene synthesis) via the anapleurotic pathway for nitrate assimilation. It has long been known that nitrate assimilation is itself under circadian control at the level of nitrate reductase. As a result, when nitrate levels begin to increase during the afternoon to a late evening/early morning maximum (Yang and Midmore, 2005), isoprene emissions may decrease due to increased cytosolic competition for PEP. It is also known that several other components of the PEP metabolic pathway are under circadian control at levels of transcript abundance and protein activity (Hartwell *et al.*, 1999; Streatfield *et al.*, 1999; Sullivan *et al.*, 2004; Hartwell, 2005), though circadian regulation of all of these components does not occur in all species. Clearly, the circadian control of PEP metabolism and chloroplast-cytosol partitioning, and its relation to the circadian rhythms of isoprene emissions reported here, merit further detailed investigation in a species such as oil palm. We are currently investigating this and other molecular levels at which circadian control may be imposed on *IE*.

Several attempts have been made to scale up isoprene emissions to produce regional and global scale emission inventories (Guenther *et al.*, 1995; Simpson *et al.*, 1999; Stewart *et al.*, 2003). There are substantial uncertainties in these estimates already, and our discovery of strong circadian control of isoprene emissions is an additional complication that now must be taken into consideration when modelling

isoprene fluxes from the biosphere to the atmosphere. While it is not known how widespread circadian regulation of isoprene emissions is, it is possible that other plant species as well as oil palm, especially in the tropics, exhibit this characteristic.

Experimental procedures

Field measurements of $IE_{1000/30}$

Isoprene emission rates ($IE_{1000/30}$) were measured from three frondlets of the same frond of a 5 year old oil palm tree growing in an oil palm plantation (Sabah, Malaysia, February 2004). Samples were collected using a leaf cuvette system (LCpro; ADC Biosciences, Hoddesdon, UK) which controlled PAR and leaf temperature (1000 μmol m^{-2} s^{-1}; 30 °C). Inflow air (350 ml min^{-1}) was filtered through charcoal to remove ozone and other ambient trace gases. Gas samples from the cuvette were taken into Perkin Elmer stainless steel sample tubes (89 mm x 6 mm O.D) at a rate of 100 ml min^{-1} for 15 min. The sample tubes contained 100 mg Carbotrap to trap isoprene. After sampling, tubes were sealed with ¼" Swagelock caps and PTFE ferrules. Previous work has shown that samples can be stored for up to 8 weeks at 4 °C without significant loss of compound, provided samples are collected from an ozone-free cuvette system (Owen *et al.*, 1997). Isoprene analysis was carried out by thermal desorption/capillary gas chromatography with mass selective detection (Possell *et al.*, 2005).

Plant material and growth conditions

Three oil palm (*Elaies guineenensis* J. Gaertn.) trees were grown from seed for two years in Levingtons M3 compost (Fisons PLC, Ipswich, UK) in 10 litre containers under 12 h light (250 umol m^{-2} s^{-1})/12 h dark with the tree illuminated between the

hours of 06:00 and 18:00 at ambient temperatures of 30 ºC day and 20 ºC night. These conditions reflect typical under-storey conditions in a tropical plantation.

Laboratory measurements of isoprene, CO_2 assimilation and stomatal conductance

Isoprene emission rates were measured using two systems. First we used a controlled environment broadleaf cuvette (PP-Systems, Hitchin, U.K.), modified for isoprene analysis, which allowed real time measurement of isoprene emission rates with simultaneous measurement of plant physiology (carbon assimilation rate and stomatal conductance) from a 2.5 cm^2 section of frondlet held at precise conditions of T and PAR. Secondly a custom built chamber for whole-frond measurements, containing ~1200 cm^2 of frond was used. The latter system allowed real time measurements of isoprene emission rates but not of physiology.

Leaf physiological responses for the cuvette system were obtained using a CIRAS-1 differential CO_2/H_2O infrared gas analyser (PP-Systems, Hitchin, U.K.) to control humidity, CO_2 concentration, leaf temperature, PAR and air flow rate through the cuvette. Water vapour concentration and CO_2 concentration were measured with a precision of 0.7 mbar and 0.2 µl l^{-1} respectively. PAR was measured using a filtered silicon detector with a response range of 400-700 nm with a controllable flux precision of ±10 µmol m^{-2} s^{-1}. Inlet CO_2 concentration was maintained at 360 µl l^{-1}. Leaf temperature was measured with an infrared radiation sensor. The air flow rate through the cuvette was 350 ml min^{-1}, giving a residence time of ~1 second.

For whole frond branch measurements, excised palm fronds were cut under water. The cut end of the frond was kept underwater and the whole frond placed in the chamber. Illumination was from cold fluorescent lighting tubes (120 µmol m^{-2} s^{-1}). Leaf temperature was controlled using thermocouples connected to a heat mat via a

temperature controller (PXZ 4; Fuji Electric (UK Ltd), London), giving a frond surface

temperature of 25 ºC or 38 ºC (± 0.3 ºC). Air temperature, relative humidity and PAR

were measured simultaneously (RHT2nl and QS2 sensors; Delta-T Devices Ltd.,

Cambridge, UK). All environmental parameters were recorded every 5 minutes using a

DL2e data logger (Delta-T Devices Ltd., Cambridge, UK). The air flow rate through the

chamber was 3 L min^{-1}, giving a residence time of ~10 min. The rate at which air was

introduced into both cuvette and frond chamber was mass flow controlled and scrubbed

of isoprene using activated charcoal.

Air from both leaf and frond chambers was introduced at a rate of 100 ml min^{-1} into a proton transfer reaction mass spectrometer (PTR-MS) (Ionicon GmbH,

Innsbruck, Austria) for isoprene analysis. The PTR-MS was calibrated by dilution of a

700 ppb isoprene gas standard (Linde UK Ltd. Aberdeen, Scotland) and operated with

an *E/N* ratio of 125 Td (Hayward *et al.*, 2002). Air samples from the cuvette were also

collected on 6 mm OD stainless steel sample tubes containing absorbent resin

(Carbotrap; Supelco, Bellefonte, PA) for isoprene identification using gas

chromatography–mass spectrometry (Turbomatrix Gold; Perkin Elmer, Boston. USA.).

The analytical procedure followed that of Possell *et al.* (2005).

Identification of an oil palm *CCA1/LHY* orthologue

We identified an oil palm *CCA1/LHY* EST in the GenBank EST database using

the TBLASTN search algorithm. The translated *EgCCA1/LHY* EST (GenBank

accession number CN600590) aligns with the *Arabidopsis AtLHY* protein from amino

acid residue 235 to residue 350 and shares 35.4 % identity with *AtLHY* across this

region. Phylogenetic tree analysis confirmed that this EST was a good orthologue of

AtCCA1/LHY and was most closely related to *AtLHY* (data not shown). We also

identified an oil palm EST that is an orthologue of the *Arabidopsis* polyubiquitin 10

gene (*UBQ10*), which we used as a loading control in our real-time PCR expression

analysis. This gene is routinely used in *Arabidopsis* as a loading control for real-time

PCR analysis (Gould *et al.*, 2006). The *EgUBQ10* EST corresponds to GenBank

accession number CN600235. Gene specific primers for real-time PCR of

EgCCA1/LHY and *EgUBQ10* were designed as follows: *EgCCA1/LHYF* 5'-

ACCCTTTACCCAATTCCACA-3', *EgCCA1/LHYR* 5'-

CAGGCTTGGATAAGGGTTCA-3', *EgUBQ10F* 5'-

GACAACGTCAAGGCCAAGAT-3' and *EgUBQ10R* 5'-

CTCGAGGGTGATCGTCTTTC-3'.

RNA isolation

Frondlet samples were collected and frozen in liquid nitrogen every six hours

from an excised oil palm frond maintained in constant light (120 µmol m^{-2} s^{-1}) and at a

constant temperature of either 25 ºC or 38 ºC in the branch chamber. Frozen frondlet

samples were ground in liquid nitrogen using a mortar and pestle, and total RNA was

isolated using an acetyl-trimethyl ammonium bromide-based RNA extraction procedure

(Hartwell *et al.*, 1996). The quantity and purity of the RNA were determined

spectrophotometrically as described (Sambrook, 1989).

Reverse transcription and real-time PCR

Total RNA samples (1µg) were reverse-transcribed using random hexamers and

the QuantiTect^{TM} Reverse Transcription kit with integrated genomic gDNA Wipeout

buffer (Qiagen, Crawley, UK) according to the manufacturer's instructions.

EgCCA1/LHY transcript abundance was measured in each sample relative to *EgUBQ10* using real-time PCR in a Rotor-Gene 3000^{TM} (Corbett Research Ltd, Cambridge UK). Each reaction contained 2 µl of cDNA and 6 µl of QuantiTect^{TM} SYBR Green PCR mix (Qiagen, Crawley, UK) together with the gene specific primers for *EgCCA1/LHY* or *EgUBQ10*. The efficiency value of amplification for each set of primers was determined by measuring the abundance of transcripts from a cDNA dilution series. Efficiency values were computed for each primer set using REST (Pfaffl *et al.*, 2002). Each RNA sample was assayed in triplicate. The *EgCCA1/LHY* transcript abundance levels were normalised to *EgUBQ10* using Q-gene software (http://www.biotechniques.com/softlib/qgene.html) (Muller *et al.*, 2002).

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