RESEARCH PAPER

Light controls phospholipase A$_{2\alpha}$ and $\beta$ gene expression in Citrus sinensis

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Abstract

The low-molecular weight secretory phospholipase A$_{2\alpha}$ (CssPLA$_{2\alpha}$) and $\beta$ (CsPLA$_{2\beta}$) cloned in this study exhibited diurnal rhythmicity in leaf tissue of Citrus sinensis. Only CssPLA$_{2\alpha}$ displayed distinct diurnal patterns in fruit tissues. CssPLA$_{2\alpha}$ and CsPLA$_{2\beta}$ diurnal expression exhibited periods of approximately 24 h; CssPLA$_{2\alpha}$ amplitude averaged 990-fold in the leaf blades from field-grown trees, whereas CsPLA$_{2\beta}$ amplitude averaged 6.4-fold. Diurnal oscillation of CssPLA$_{2\alpha}$ and CsPLA$_{2\beta}$ gene expression in the growth chamber experiments was markedly dampened 24 h after transfer to continuous light or dark conditions. CssPLA$_{2\alpha}$ and CsPLA$_{2\beta}$ expressions were redundantly mediated by blue, green, red and red/far-red light, but blue light was a major factor affecting CssPLA$_{2\alpha}$ and CsPLA$_{2\beta}$ expression. Total and low molecular weight CsPLA$_2$ enzyme activity closely followed diurnal changes in CssPLA$_{2\alpha}$ transcript expression in leaf blades of seedlings treated with low intensity blue light (24 $\mu$mol m$^{-2}$ s$^{-1}$). Compared with CssPLA$_{2\alpha}$ basal expression, CsPLA$_{2\beta}$ expression was at least 10-fold higher. Diurnal fluctuation and light regulation of PLA$_2$ gene expression and enzyme activity in citrus leaf and fruit tissues suggests that accompanying diurnal changes in lipophilic second messengers participate in the regulation of physiological processes associated with phospholipase A$_2$ action.

Key words: Citrus sinensis, diurnal, light regulation, phospholipase A.

Introduction

Phospholipases are diverse enzymes that cleave phospholipids into fatty acids and other lipophilic substances. Three major forms, known as phospholipases A, C, and D, have been characterized in plants based upon the positional specificity of catalysis on phospholipids (Wang, 2001). Phospholipase A$_2$ (PLA$_2$) hydrolyses the 2-acyl ester linkage of 1,2-diacyl-sn-3-phosphoglycerides to free fatty acids and 1-acyl-2-lysophospholipids (Six and Dennis, 2000; Wang, 2001). Within four major biological subfamilies of PLA$_2$ identified in animals, only low molecular weight secretory PLA$_2$ (sPLA$_2$) and intracellular Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$), but not cytosolic Ca$^{2+}$-dependent PLA$_2$ (cPLA$_2$) or PAF acetyl hydrolase/oxidized lipid Lp PLA$_2$ have been identified in plants (Wang, 2001; Burke and Dennis, 2008). The action of PLA$_2$ has been implicated in various cellular processes including lipid signalling and metabolism (Chapman, 1998; May et al., 1998; Munnik et al., 1998), wounding responses (Narváez-Vásquez et al., 1999), plant–pathogen interactions, defence signalling (Munnik et al., 1998), fruit abscission (Alferez et al., 2005), auxin-regulated responses (Andre and Scherer, 1991; Ryu and Palta, 1999), and stomatal movement (Seo et al., 2008). Such diverse physiological actions begin with receptor(s) that receive the released lipid signals, such as lysosphatidylcholine, lysosphatidylethanolamine, and fatty acids, and initiate a cascade of events leading to a physiological response (Wang et al., 2002; Meijer and Munnik, 2003; Wang, 2004). Signals required to activate PLA$_2$ have not been understood clearly.

Light was shown to mediate stomatal movement (Suh et al., 1998; Seo et al., 2008), and sPLA$_2$ was shown to play a role in regulating stomatal opening via action on the
proton pump (Palmgren et al., 1988; Lee et al., 1994; Seo et al., 2008). Evidence suggests that PLA2-derived lipid products and downstream physiological processes are diurnally regulated by a biological clock that responds to 24 h rhythms. Transcript accumulation of lipoxygenase was found to be under circadian control in maize (Nemchenko et al., 2006), and linoleic acid content fluctuated diurnally in sunflower seeds (Pleite et al., 2008). Diurnal oscillations can be controlled by internal elements and exogenous environmental signals, such as temperature, humidity, and light/dark (LD) conditions (McClung, 2006). Several photoreceptors, such as phytochromes and crytochromes, provide light information to the biological clock (Devlin and Kay, 2000; Salomé et al., 2002) leading to a wide range of physiological responses. The plant growth hormone auxin is influenced by circadian rhythms in tobacco leaves (Nováková et al., 2005). Notably, many auxin response genes, response factors, and efflux carriers were found to be diurnally regulated (Harmer et al., 2000; Covington and Harmer, 2007; Lau et al., 2008). Auxin-mediated hypocotyl elongation was found to be regulated by PLA2β (Lee et al., 2003). PLA2-derived lysophospholipids regulate auxin-related physiological processes (Scherer, 2002). Such evidence suggests a link between PLA2 involved auxin-mediated responses and the biological clock. Thus, PLA2-mediated signalling and metabolites may mediate many physiological processes that follow diurnal cycles controlled by an oscillator. However, no direct evidence has connected PLA2 gene expression, enzyme activity, and diurnal rhythms. Here, it is reported that two PLA2s isolated from Citrus sinensis, CssPLA2α and CssPLA2β, were modulated diurnally by exogenous stimuli. It is demonstrated that select wavelengths of lights, especially blue light, control these gene expressions and total PLA2 activity.

Materials and methods

Plant material and growth conditions

To examine the circadian rhythm in CssPLA2α and CssPLA2β gene expression in citrus, 17-year-old Citrus sinensis cv. ‘Valencia’ citrus trees on ‘Swingle’ rootstock were used. Field samples of leaf blades (LB), leaf abscission zones (LAZ), fruit flavedo (FF), and fruit abscission zones (FAZ) were collected as described by Malladi and Burns (2008). Four biological replicates of each tissue were harvested from random canopy locations of four trees at 4 h intervals over 48 h from 19 June to 21 June in 2007. To test diurnal changes under controlled conditions, leaf blades were collected from light-entrained Citrus sinensis cv. ‘Valencia’ potted citrus trees as described by Malladi and Burns (2008). Briefly, 7-year-old trees were entrained to test conditions by transferring to growth rooms set at 25/19 °C in light/dark (LD) for 12:12 h photoperiod period for 7 d prior to sampling. The plants were then transferred into either constant light (LL), constant dark (DD) or kept in LD. Metal halide lamps (1000 W Clear BT37, Philips, Somerset, NJ) with a fluence rate of 245 μmol m−2 s−1 were used as the light source in the growth chambers. Temperature was maintained at 24 °C during the experiments. Mature leaf blades were collected at 4 h or 8 h intervals for 48 h. At least four biological replications from eight trees were collected for each time period.

To determine the effects of different spectra of light on CssPLA2α and CssPLA2β gene expression, and CssPLA2 enzyme activity, 1-year-old seedlings were transferred to light boxes (see below) set to LD photoperiods with various light spectra and intensity for at least 1 week prior to sampling. Leaf blades were harvested at 4 h intervals over 60 h. Temperature and humidity in all light boxes ranged from 23.5 °C to 24.5 °C and 60–65% RH during the sampling period. At least four biological replications from 16 seedlings were collected for each time period.

Light boxes and light resources

Light boxes were constructed with blue, green, red, and red/far-red spectra LED lights as described by Folta et al. (2005). The light boxes containing blue, green, and red lights were supplied with 36 Luxdrive 7007 Endor Star LED light modules whereas the red/far-red lights were supplied by Snap-Lite 1 (Quantum Devices, Barneveld, WI). Light emission range and fluence rate were measured using a spectroradiometer (SpectraWiz PS-100R, Apogee, Roseville, CA). Blue, green, red, and red/far-red LED lights emitted light from 410–540, 470–620, 580–670, and 600–780 nm, respectively, with emission peaks of blue, green, and red light of 456, 530, and 630 nm, respectively. Two emission peaks of 655 nm and 725 nm were measured with red/far-red light. Light intensity (μmol m−2 s−1) in each box was adjusted to the appropriate illuminating rate as indicated below for experiments.

Nucleotide extraction and gene expression analysis

For CssPLA2 promoter analysis and gene structure characterization, total DNA was extracted from leaf blades of Citrus sinensis cv. ‘Valencia’ using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). For gene cloning and expression analysis using quantitative RT-PCR, LB, LAZ, FF, and FAZ were frozen in liquid N2, and stored at −80 °C as described by Malladi and Burns (2008). Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA), and DNA contamination was removed using DNase treatment (Qiagen). Total RNA (1 μg) was reverse transcribed using ‘Superscript III’ reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA was diluted 1.5-fold and stored at −20 °C until quantitative RT-PCR analysis. Gene expression analysis was performed using quantitative RT-PCR. Citrus glyceraldehyde-3-phosphate-dehydrogenase (CgAPDH) was used as the constitutively expressed internal calibrator. Optimal primer concentration used in quantitative RT-PCR analysis for CssPLA2α (PLA2a1: 5'-GTGGGCGTCGCTGATCACTGGAT-3'; PLA2a2: 5'-CAAGCATCAATACATCAACA-3') was 0.25 μM, and was 0.125 μM for CssPLA2β (PLA2b1: 5'-TCGCCATTTCTCGGGAATGT-3'; PLA2b2: 5'-CATGTCACCTACCCCAACCAATG-3'). Gene expression analysis was performed on each biological replicate in duplicate and results averaged for each replicate. The lowest expression value was used to compare all others within the experiment. To compare expression levels between CssPLA2α and CssPLA2β, relative expressions were compared within an experiment to the lowest expression sample of CssPLA2α.

Gene cloning and promoter and amino acid analysis

Genes and promoters were obtained from total RNA and genomic DNA extracted from leaf blades in Citrus sinensis cv. ‘Valencia’. Gene specific primers designed based on the partial sequences of PL2α (5'-TCTGATATGAGAATACACTCGTGTC-3') and β (5'-GCCACGAAAGTGTATAGAGGTC-3') obtained from citrus HarvEst database (http://harvest.ucr.edu/) were used for CssPLA2α and CssPLA2β cloning. 5'-RACE (Rapid Amplification of cDNA End, Invitrogen), and 3'-RACE were performed for specific gene amplification via Polymerase Chain Reaction (PCR) using Taq DNA polymerase (Qiagen). Inverse PCR was performed to obtain CssPLA2α and CssPLA2β promoter sequences by restriction enzyme digestion of genomic DNA, self-ligation, followed by PCR amplification using Elongase enzyme mix (Invitrogen).
with inverse primers. The amplified DNA fragments were cloned into pGEM-T easy vector (Promega). Transformation of Escherichia coli JM109 cells (Promega) was performed, and the resulting plasmid DNA was purified using a Wizard DNA purification kit (Promega). Sequencing was performed at the Interdisciplinary Center for Biotechnology Research, University of Florida (Gainesville, FL). Nucleotide sequences of CssPLA₂ (accession no. GU075396), CsPLA₂β (accession no. GU075398), and 1000 bp promoter regions of CssPLA₂ (accession no. GU075397) from Citrus sinensis cv. ‘Valencia’ were used for this study. Alignment of amino acid sequences were performed using ClustalW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide prediction was performed using SIG-Pred: Signal Peptide Prediction web server (http://bmbpcu36.leds.ac.uk/prot_analysis/Sig.html).

CssPLA₂ enzyme extraction and activity analysis

Citrus leaves (1 g) were ground in liquid N₂, dissolved in 2.5 ml of cold extraction buffer (1 mM EDTA, 100 mM TRIS-HCl, 2% (w/v) PVPP, 0.15 M sorbitol, pH 7) with 1 mM PMSF added, and the cold extraction buffer (1 mM EDTA, 100 mM TRIS-HCl, 2% (w/v) sorbitol, pH 7) with 1 mM PMSF added. Supernatant proteins were desalted using a PD-10 column (GE Healthcare, Buckinghamshire, UK) equilibrated and eluted with column buffer (10 mM HEPES, 1 mM MgCl₂, pH 7). Total proteins in the eluate were precipitated overnight at 4 °C using ammonium sulphate to saturation, centrifuged 14 000 rpm at 4 °C, washed with acetone, and resuspended in extraction buffer. Concentrated proteins were quantified and stored at –20 °C for the total PLA₂ enzyme activity assay. Total PLA₂ activity assay (Cayman Chemical Co, Ann Arbor, MI, USA) was performed as described (Alferez et al., 2005). sPLA₂ was fractionated from total protein samples using centrifugal filter (Microcon, Bedford, MA, USA) with a molecular weight cut-off of 30 kDa, and enzyme activity measured.

Results

Cloning and sequence analysis

Gene specific primers designed based on the EST sequences of PLA₂ and β from the citrus HarvEST database were used for CssPLA₂ and CsPLA₂β cloning. 5’- and 3’-RACE was performed to obtain the full-length sequences. Full-length CSSPLA₂ and CsPLA₂β cDNA sequences encoded 17.1 and 31.6 kDa proteins of 156 and 279 amino acids, respectively, and shared 31% identity (Fig. 1). CSSPLA₂ and CsPLA₂β contained key phospholipase catalytic sites of a Ca²⁺-binding loop, the active site motif with a conserved his/asp dyad (HD), and 12 conserved Cys residues that form six disulphide bonds for conformational integrity. A eukaryotic signal peptide sequence was present in the N-terminus of CSSPLA₂ but not CsPLA₂β. The KxEL endoplasmic reticulum (ER) retention sequence in the 3’-terminus is only present in CsPLA₂β. Eukaryotic signal peptide cleavage site present only in CSSPLA₂ is underlined. ‘.’, identical amino acids; ‘;’, conserved substitutions; ‘,’ semi-conserved substitutions; ‘–’, sequence gaps; ‘|’, conserved Cys residues. Ca²⁺-binding loop, PLA₂ catalytic motifs, and endoplasmic reticulum (ER) retention sequence are enclosed in boxes.

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**Fig. 1.** Amino acid sequence alignment of CSSPLA₂ and CsPLA₂β. The KxEL endoplasmic reticulum (ER) retention sequence in the 3’-terminus is only present in CsPLA₂β. Eukaryotic signal peptide cleavage site present only in CSSPLA₂ is underlined. ‘.’, identical amino acids; ‘;’, conserved substitutions; ‘,’ semi-conserved substitutions; ‘–’, sequence gaps; ‘|’, conserved Cys residues. Ca²⁺-binding loop, PLA₂ catalytic motifs, and endoplasmic reticulum (ER) retention sequence are enclosed in boxes.

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**Tissue-specific and diurnal expression of CSSPLA₂ and CsPLA₂β**

CSSPLA₂ transcript accumulation displayed a distinct diurnal rhythm in leaf blades (LB), leaf abscission zones (LAZ), fruit flavedo (FF), and fruit abscission zones (FAZ) in field samples (Fig. 3). Minimum and maximum CSSPLA₂ transcript accumulation in all tissues occurred at approximately 10.00 h and 18.00 h, respectively. The order of maximum amplitude of CSSPLA₂ relative expression was LB>LAZ>FAZ>FF (Fig. 3A). At its maximum, relative expression in LB was 1337-fold and 643-fold higher than its minimum over two diurnal cycles. Accumulation of CsPLA₂β RNA oscillated in LB and much less in LAZ. CsPLA₂β transcript accumulation maxima and minima occurred at 18.00 h and 10.00 h, respectively, however, the decline in expression during the dark period was gradual compared with CSSPLA₂. Relative CsPLA₂β expression in promoter regulatory elements. Core regulatory TATA box (TATAAA) and CAAT/CCAAT elements, light regulatory elements, and some of clock regulated elements, such as G-box and CCA1 binding sites, were found in CSSPLA₂ and CsPLA₂β promoter sequences (Fig. 2; Table 1). Clock-regulated elements associated with blue light and evening element were present in CSSPLA₂ but absent in CsPLA₂β, whereas a phytochrome A element was present in CsPLA₂β but not CSSPLA₂. The abundance of light/clock and evening regulatory elements in the promoter region of CSSPLA₂ suggests CSSPLA₂ may function as a light-activated gene diurnally governed with an evening-specific expression pattern. CsPLA₂β may be regulated by light but less responsive.
LB was 7.7-fold and 5.0-fold higher at its maxima than its minima over two diurnal cycles. Closer inspection of LAZ, FF, and FAZ expression revealed the order of maximum rhythmic amplitude of \( \text{CsPLA}_2^b \) was LB > LAZ, but no oscillations occurred in FAZ or FF (Fig. 3B). The basal expression of \( \text{CsPLA}_2^a \) was over 50-fold higher in LB compared to LB expression.

**Light regulates oscillation of LB \( \text{CssPLA}_2^a \) and \( \text{CsPLA}_2^b \) expression**

\( \text{CssPLA}_2^a \) and \( \text{CsPLA}_2^b \) expression in LB from potted citrus plants held in light/dark (LD) and constant temperature in the growth room demonstrated diurnal oscillation (Fig. 4). Average maximum amplitude was similar to that from field samples. Transfer of plants from LD to constant dark (DD) resulted in the cessation of \( \text{CssPLA}_2^a \) expression oscillation within 24 h, whereas transfer of plants from LD to constant light (LL) markedly dampened expression. The period of \( \text{CsPLA}_2^b \) expression shifted when transferred from LD to LL or DD conditions. Thus, light was a major factor regulating \( \text{CssPLA}_2^a \) and \( \text{CsPLA}_2^b \) expression.

\( \text{CssPLA}_2^a \) and \( \text{CsPLA}_2^b \) gene expression in seedling LB oscillated in blue and green light (Fig. 5). The amplitude of \( \text{CsPLA}_2^a \) was much higher than \( \text{CsPLA}_2^b \) under all light spectra examined. Rhythmicity of \( \text{CsPLA}_2^b \)
gene expression in blue and green light was erratic, but oscillations were more pronounced with blue light treatment. *CssPLA2α* expression was lowest 1 h before the end of dark period through 3 h after the light period was initiated. Expression increased 7–11 h after the light treatment commenced and peaked 11 h after illumination.

Table 1. Promoter analysis of 1 kb sequences upstream of translational start codon (ATG) of *CssPLA2α* and *CsPLA2β*. Cis-regulatory elements, sequence and references are listed.

| Cis-regulatory element | Sequencea | No. of elements | Reference                  |
|------------------------|-----------|-----------------|----------------------------|
|                        |           | *CssPLA2α* | *CsPLA2β* |
| Core regulatory elements |           |       |                   |
| TATA box               | TATAAA    | 2    | 1                 | Breathnach and Chambon, 1981 |
| CAAT                   | CAAT      | 13   | 5                 | Terzaghi and Cashmore, 1995 |
| CCAAT                  | CCAAT     | 2    | 1                 | Terzaghi and Cashmore, 1995 |
| Light-regulatory element |         |       |                   |
| WC-1/WC-2 binding element | GATA    | 4    | 2                 | Giuliano et al., 1988     |
| GT-1                   | GRWAAG    | 4    | 3                 | Terzaghi and Cashmore, 1995; Zhou 1999 |
| I-box                  | GATAAG    | 1    | 1                 | Chatterjee et al., 2006   |
| Shade response element | TAATTA    | 1    | 1                 | Devlin et al., 2003       |
| Clock regulated element |         |       |                   |
| G-box                  | CACG      | 3    | 1                 | Hudson and Quail, 2003    |
| CCA1 binding element   | ATCT      | 7    | 5                 | Wang and Tobin, 1998      |
| CCA1ATLHCB1            | AA N1.2ATCT | 1   | 0                 | Chatterjee et al., 2006   |
| CIACADIANLELHC         | CAA N1.2ATC | 1  | 1                 | Chatterjee et al., 2006   |
| Clock and blue light-regulated element | ACTN1.2CCAA | 2 | 0                | Folta and Kaufman, 1999   |
| Evening element        | AATATHT   | 4    | 0                 | Harmer et al., 2000; Xu and Johnson, 2001 |
| Evening element        | TTAATATCT | 1    | 0                 | Harmer et al., 2000; Xu and Johnson, 2001 |
| Phytochrome A-induced element |       |       |                   |
| SORLIP1                | GCCAC     | 0    | 1                 | Hudson and Quail, 2003    |
| SORLIP2                | GGGCC     | 0    | 0                 |                           |
| SORLIP3                | CTCAAGTGA | 0    | 0                 |                           |
| SORLIP4                | GTATGATGG | 0    | 0                 |                           |
| SORLIP5                | GAGTGAG   | 0    | 0                 |                           |

a R=A/G; W=A/T; M=A/C, H=A/C/T, N=A/C/T/G.

Fig. 3. Diurnal oscillation in *CssPLA2α* (upper graph) and *CsPLA2β* (lower graph) gene expression under field conditions. (A) Oscillation of gene expression in leaf blades (LB, closed circles), leaf abscission zones (LAZ, open circles), fruit flavedo (FF, closed triangles), and fruit abscission zones (FAZ, open triangles) during natural daylight (white area) and darkness (grey area) over a 48 h period. (B) Enlargement of LAZ, FF, and FAZ gene expression showing diurnal pattern. Actual time of days is indicated on the x-axis. Vertical bars represent standard error of mean (n=4).
Expressions of both genes in blue light were greater at 24 l mol m⁻² s⁻¹ intensity compared with 3 l mol m⁻² s⁻¹. CSSPLA₂ gene expression in seedling LB also oscillated in red, red/far-red, and the combination of blue, green, and red light held in LD (see Supplementary Fig. S1 at JXB online). The amplitudes of CSSPLA₂ expression were 18-fold and 8-fold lower in red and red/far-red compared to blue light at 24 l mol m⁻² s⁻¹ intensity, respectively. Blue light was as effective as the combination light treatment in inducing maximum CSSPLA₂ gene expression. Rhythmicity of CsPLA₂ gene expression in red/far-red light was also erratic and the amplitude was much lower than blue light. Increasing green, red or red/far-red light intensity from 3 to 24 l mol m⁻² s⁻¹ had minor effects on expression of both genes (see Supplementary Fig. S1 at JXB online).

PLA₂ enzyme activities

CSSPLA₂ relative gene expression in LB of seedlings treated with blue light under LD conditions and total CsPLA₂ enzyme activity showed similar rhythmicity, with the peak occurring 11 h after the light period commenced (Fig. 6A, B). Like CSSPLA₂ expression, total PLA₂ activity remained low and constant under DD conditions. The peak activity of the low-molecular-weight PLA₂ fraction corresponded with the maximum amplitude of CSSPLA₂ expression.

**Discussion**

Phospholipase A₂ are enzymes that hydrolyse phospholipids to free fatty acids and 1-acyl-2-lysophospholipids; such hydrolysis products have downstream signalling functions (Schaffer et al., 2001; Wang, 2004). Thus, PLAs represent an important control point for phospholipid signalling. This study demonstrated diurnal fluctuation of two PLA₂ transcripts, PLA₂ enzyme activity, and their regulation by light. These data suggest that increased production of PLA₂-derived signals during the light period may have significant influence over physiological processes influenced by PLA₂ action.

Sequence analysis of CSSPLA₂ indicated that CSSPLA₂ encoded a low molecular-weight secretory Ca²⁺-dependent lipase. The presence of an N-terminal eukaryotic secretory signal peptide in CSSPLA₂ suggests that the mature peptide functions as a secretory protein, whereas the C-terminal ER retention-like signal KxEL in CsPLA₂ indicates the mature peptide may be localized to ER (Pagny et al., 1999; Seo et al., 2008). The higher molecular weight of CsPLA₂, the presence of the Ca²⁺-binding loop, and its predicted target in the ER indicates it may represent a cPLA₂ or related sequence.

The presence of diurnal and circadian promoter elements in 5'-upstream sequences of CSSPLA₂ and CsPLA₂ suggests their regulatory control over oscillating expressions. A tight resetting pattern at ‘dusk’ was evident with CSSPLA₂ expression in all tissues examined but more prominent in leaf tissue; however, CsPLA₂ diurnal expression was leaf
failure of oscillation in an important external factor controlling diurnal expression and McClung, 2002) may regulate its expression. Light was applied at 24/12 h blue/dark cycle, or 24 h constant dark. Blue light was symbol expression, and (B) CsPLA2 total (open symbols) and low molecular weight (sPLA2) (closed triangles) enzyme activity under mean (n = 4). Vertical bars represent standard error of background, respectively.

Fig. 6. (A) Diurnal CssPLA2x (open symbols) and CsPLA2β (closed symbols) expression, and (B) CsPLA2 total (open symbols) and low molecular weight (sPLA2) (closed triangles) enzyme activity under light harvesting chlorophyll binding (Lhcb) gene family strongly respond to increasing blue light fluence by increasing transcription rate (Folta and Kaufman, 1999; Harmer et al., 2000). The CCA1 binding element implicated in circadian blue light responses found in the promoter region of Lhcb (Wang and Tobin, 1998) is present in CssPLA2x and CsPLA2β. Thus, CCA1 may function as a core oscillator and connect blue-light and clock signals with diurnal control of CsPLA2 gene expression.

As much as 11–13.4% of the Arabidopsis transcriptome is diurnally regulated with gene changes more than 2-fold (Schaffer et al., 2001; Bläsing et al., 2005). The amplitude of most diurnally regulated genes treated with low intensity light (130 μmol m⁻² s⁻¹) was less than 2-fold. Only 2.4% reached amplitudes of 4 or more and only 0.001% were grouped in the lipid metabolism functional category (Bläsing et al., 2005). The 990-fold and 300-fold amplitude change in CssPLA2x gene expression under high light intensity in the field and low intensity blue-light-LD cycle, respectively, suggests important functions for CssPLA2x in citrus and perhaps other plants. However, the remarkable changes in expression resulted in only a 2-fold increase in total PLA2 enzyme activity. The striking reduction in efficiency of output implies post-transcriptional regulation, loss of mRNA stability, or suppression of translation (Lidder et al., 2005).

Diurnal fluctuation in CsPLA2 gene expression and resulting enzyme activity may allow plants to respond to daily changes in environment. Abscission, stomatal conductance, and hypocotyl elongation respond to light and diurnal cues (Decoteau and Craker, 1983; Somers et al., 1998; Chatterjee et al., 2006; Xu et al., 2007; Malladi and Burns, 2008). It was previously demonstrated that inhibiting sPLA2 enzyme activity markedly reduced lipid hydroperoxides and efficacy of a citrus abscission agent (Alferez et al., 2005), suggesting that production of lipid-derived signals was associated with the acceleration of abscission. Phospholipase D gene expression diurnally fluctuated in citrus leaf and fruit tissues, with peak amplitude occurring at the end of the light period (Malladi and Burns, 2008). Natural fruit attachment force fluctuated diurnally, with maximum loosening occurring mid-day (Pozo et al., 2007). Similarly, the response to abscission agents was greater when applied at midday, even when temperatures were held constant (Pozo et al., 2007; Malladi and Burns, 2008).

sPLA2 action was shown to play a role in regulating stomatal opening during the light hours in C₄ plants. Inhibition of sPLA2 inhibited light-induced stomatal opening (Suh et al., 1998). Application of lysophospholipids and free fatty acids activated the stomatal proton pump, enhanced blue light-induced stomatal opening, and reversed the effect of PLA2 gene silencing (Palmgren et al., 1988; Lee et al., 1994; Seo et al., 2008). sPLA2 activity is enhanced by auxin application, and PLA2-derived lysophospholipids can regulate auxin-related physiological processes (Scherer, 2002). Diurnal hypocotyl elongation and leaf expansion that occurred with blue light treatment (Cashmore, 1997) appeared to be regulated via auxin signalling (Lee et al.,
2003; Wilmoth et al., 2005). Cell elongation and auxin content diurnally fluctuated (Harmer et al., 2000; Nováková et al., 2005), and peak auxin content occurred near the end of the light cycle. Our work demonstrated that low intensity blue light increased total CsPLA₂ enzyme activity and activity associated with the low molecular weight fraction, presumably sPLA₂, and activity oscillations corresponded with gene expression in citrus seedling leaves. Taken together, these results indicate the potential for production of diurnally oscillating lipid signals that may regulate organ loosening, stomatal conductance, cell-elongation and perhaps other light-regulated diurnal physiological processes.

Although blue light appears to play key roles in these processes, red and red/far-red light interact with blue light (Folta and Maruhnich, 2007), and in our study promoted diurnal expression, albeit at lower amplitude. Red and red/far-red light regulates K⁺ accumulation and stomatal opening in guard cells ( Göring et al., 1984; Talbott et al., 2003; Doi and Shimazaki, 2008), auxin-regulated cell and hypocotyl elongation (Goto and Suzuki, 1980; Reed et al., 1993; Takase et al., 2003), and influences abscission (Craker et al., 1987). CssPLA₂α and CssPLA₂β redundantly oscillated with low amplitude under low intensity red/far-red light (see Supplementary Fig. S1 at JXB online), implying such light interactions participate in PLA₂ regulation. Low-intensity green light also promoted CssPLA₂α and CssPLA₂β diurnal oscillation. Green light reversed blue-induced stomatal opening (Frechilla et al., 2000; Talbott et al., 2006), but accelerated stem elongation (Folta and Maruhnich, 2007). How green, red, and red/far-red light interact with blue light and contribute to the regulation of CssPLA₂α and CssPLA₂β gene expression and downstream biological activities are unclear.

Supplementary data are available at JXB online.

Supplementary Fig. S1. Diurnal expression of CssPLA₂α (A) and CssPLA₂β (B) under 12 h different light/12 h dark cycles.

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