RESEARCH PAPER

CsPLDα1 and CsPLDγ1 are differentially induced during leaf and fruit abscission and diurnally regulated in Citrus sinensis

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Received 5 June 2008; Revised 26 July 2008; Accepted 4 August 2008

Abstract

Understanding leaf and fruit abscission is essential in order to develop strategies for controlling the process in fruit crops. Mechanisms involved in signalling leaf and fruit abscission upon induction by abscission agents were investigated in Citrus sinensis cv. ‘Valencia’. Previous studies have suggested a role for phospholipid signalling; hence, two phospholipase D cDNA sequences, CsPLDα1 and CsPLDγ1, were isolated and their role was examined. CsPLDα1 expression was reduced in leaves but unaltered in fruit peel tissue treated with an ethylene-releasing compound (ethephon), or a fruit-specific abscission agent, 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP). By contrast, CsPLDγ1 expression was up-regulated within 6 h (leaves) and 24 h (fruit peel) after treatment with ethephon or CMNP, respectively. CsPLDα1 expression was diurnally regulated in leaf blade but not fruit peel. CsPLDγ1 exhibited strong diurnal oscillation in expression in leaves and fruit peel with peak expression around midday. While diurnal fluctuation in CsPLDα1 expression appeared to be light-entrained in leaves, CsPLDγ1 expression was regulated by light and the circadian clock. The diurnal expression of both genes was modulated by ethylene-signalling. The ethylene-induced leaf abscission and the ethephon- and CMNP-induced decrease in fruit detachment force were enhanced by application during rising diurnal expression of CsPLDγ1. The results indicate differential regulation of CsPLDα1 and CsPLDγ1 in leaves and fruit, and suggest possible roles for PLD-dependent signalling in regulating abscission responses in citrus.

Key words: Abscission, circadian, Citrus sinensis, diurnal, ethephon, phospholipase D.

Introduction

Abscission is a tightly regulated process that ultimately results in organ detachment from the parent plant. Abscission occurs primarily at developmentally predetermined and anatomically distinct regions termed abscission zones (AZ). Knowledge of mechanisms involved in leaf and fruit abscission is essential to develop strategies to control them and to improve harvesting practices or unwanted crop loss in fruit crops such as citrus. Ethylene plays a primary role in signalling and accelerating abscission responses (Jackson and Osborne, 1970; Bleecker and Patterson, 1997). Increased ethylene biosynthesis through over-expression of aminocyclopropene-1-carboxylate (ACC) synthase, an enzyme involved in ethylene biosynthesis, leads to premature flower abscission, while a block in ethylene perception in the never ripe (nr) mutant, delays petal abscission in tomato (Lanahan et al., 1994; Wilkinson et al., 1995). Interplay between ethylene and other plant hormones also play a critical role in regulating the progression of organ abscission. A balance of ethylene and auxin levels at the AZ is considered a key factor determining cell separation (Sexton and Roberts, 1982; Brown, 1997; Patterson, 2001; Taylor and Whitelaw, 2001). While ethylene promotes cell separation, auxin inhibits it and also reduces sensitivity of the AZ to ethylene. In addition, factors such as wounding and stress regulate organ abscission (Taylor and...
In citrus, ethylene-releasing compounds such as ethephon (2-chloroethane-phosphonic acid) or chemical wounding agents such as CMNP (5-chloro-3-methyl-4-nitro-1H-pyrazole), reduce mature fruit detachment force (Goren, 1993; Burns, 2002; Yuan and Burns, 2004). In addition, ethephon application results in extensive leaf abscission. Blocking ethylene perception through the application of 1-MCP (1-methylcyclopropene) prevents ethephon-induced leaf abscission, although it has a minimal effect on mature citrus fruit detachment force (Pozo et al., 2004). The application of two putative heterotrimeric guanine nucleotide–binding protein (G-protein) receptor agonists, clonidine and guanfacine, mitigates ethephon-induced leaf abscission in citrus (Burns et al., 2003; Yuan et al., 2005). These agonists reduce ethephon-induced expression of ACS1 (ACC synthase) and ACO (ACC oxidase) and markedly decrease ethylene production in citrus leaves (Yuan et al., 2005). Hence, a role for heterotrimeric G-protein signalling in mediating ethylene-regulated abscission responses seems plausible.

Recent progress in G-protein signalling research in plants suggests their involvement in mediating physiological responses to plant growth regulators (Ashikari et al., 1999; Ueguchi-Tanaka et al., 2000; Romanov et al., 2002; Coursol et al., 2003). Transduction of signals perceived by the G-protein signalling pathway is achieved in part through interaction with phospholipases, enzymes that catalyse the hydrolysis of membrane phospholipids (Assmann, 2002; Wang, 2005). D-type phospholipases (PLDs) can bind to and interact with G\textalpha, a heterotrimeric G-protein subunit, through a motif analogous to the ‘DRY’ motif in animal G-protein coupled receptors (GPCRs) (Zhao and Wang, 2004). Activation of heterotrimeric G-proteins through the release of the G\textalpha subunit removes inhibition of PLDs (Lein and Saalbach, 2001). Through direct or indirect enzymatic action of PLDs, a variety of lipid signalling molecules such as phosphatic acid (PA) are produced. PLD activity and associated signalling molecules are regulated by various types of stress, wounding, and ABA and ethylene, plant growth regulators often associated with senescence and/or abscission (Lee et al., 1998; Ritchie and Gilroy, 1998; Frank et al., 2000; Taylor and Whitelaw, 2001; Wang, 2002; Welti et al., 2002; Hong et al., 2008).

Several classes of plant PLDs have been identified (Pappan et al., 1997a, b; Qin et al., 1997; Qin and Wang, 2002; Wang, 2004). The Arabidopsis PLD family has 12 PLD-encoding genes classified into six types: PLD\alpha, PLD\beta, PLD\gamma, PLD\delta, PLD\epsilon, and PLD\zeta (Wang, 2005). PLD\alpha is involved in mediating hyperosmotic stress responses, and ABA- and ethylene-dependent senescence of detached leaves in Arabidopsis (Fan et al., 1997; Hong et al., 2008). PLD\beta regulates active oxygen species production and polyphenol oxidase activity in tomato (Laxalt et al., 2001; Bargmann et al., 2006). PLD\gamma may regulate freezing tolerance as its over-expression enhances freezing tolerance (Li et al., 2004). By contrast, PLD\delta negatively regulates freezing tolerance in Arabidopsis as PLD\delta anti-sense plants exhibit increased survival at low temperature (Welti et al., 2002). PLD\epsilon is involved in mediating auxin responses in Arabidopsis (Li and Xue, 2007). These data indicate diverse functions for PLDs in plant growth and development, and also suggest specific roles for individual isoforms. As with Arabidopsis, citrus probably has multiple PLD genes that participate in a variety of growth- and development-related processes. However, little is known about the citrus PLD family.

In previous studies, expression of a PLD was found to be up-regulated in Arabidopsis following application of CMNP (Alferez et al., 2007). CMNP also induces phospholipase A2 and lipoygenase activities in citrus fruit flavedo (peel), suggesting modulation of lipid-signalling during abscission and a role for fruit flavedo in the response (Alferez et al., 2005). In addition, expression of several genes involved in phospholipid metabolism and signalling was altered in various citrus tissues treated with abscission-inducing agents (J Burns et al., unpublished results). Hence, it is hypothesized that PLDs and phospholipid signalling played a role in mediating leaf and mature fruit abscission responses in Citrus sinensis cv. ‘Valencia’ sweet orange. In this study, the isolation and characterization of two abscission agent-regulated PLDs are reported. Evidence is presented for diurnal, light- and ethylene signalling-dependent regulation of PLD expression. A relationship is suggested between PLD-dependent signalling and the regulation of AZ sensitivity.

**Materials and methods**

*Leaf abscission in whole trees*

Seventeen-year-old Citrus sinensis cv. ‘Valencia’ citrus trees on ‘Swingle’ rootstock located at the Citrus Research and Education Center, Lake Alfred, FL, USA, were used for field abscission experiments. Leaf abscission was studied using the ethylene-releasing agent, ethephon (Ethrel®). Ethephon concentrations were selected based on previous experiments and forecasted temperatures at application, as high temperatures are known to increase efficacy (Yuan and Burns, 2004). Canopy sections on 10 trees were tagged and randomly assigned to water (control) or ethephon treatments (n=5). Water (control) and ethephon (600 mg l⁻¹) were applied to “run-off” at 10.00 h using a backpack sprayer. Temperature at the time of application was 26 °C. A branch was tagged in each section and leaf number was counted daily for 6 d, and then at 10, 14, and 20 d after application. Leaf samples were collected from water- and ethephon-treated trees for analysis of PLD gene expression at 0, 3, 6, 24, and 48 h after treatment. To determine the effect of time of ethephon application on abscission response, four separate trees were sprayed either with water or ethephon (400 mg l⁻¹) at 09.00 h and 13.00 h and leaf number on tagged branches was counted at
various times up to 10 d after application. Temperature at the time of ethephon application was 30 °C (09.00 h) and 35°C (13.00 h).

**Fruit abscission in whole trees**

*Citrus sinensis* cv. ‘Valencia’ sweet orange trees were used for fruit abscission experiments. Ethephon (600 mg l⁻¹), CMNP (250 mg l⁻¹) and water were applied at 09.00 h to canopy sections until runoff (*n* = 3). Ethephon and CMNP concentrations were selected based on previous experiments on fruit abscission. The temperature at the time of application in this study was 29 °C. Fruit detachment force (FDF) was measured using a digital force gauge (Force One, Wagner Instruments, Greenwich, CT, USA) at 0, 2, 4, and 7 d after application. Fruit were sampled from treated canopies at various intervals up to 96 h after application for gene expression analysis. Fruit peel (flavedo) was removed from the equatorial region of sampled fruit, frozen in liquid N2, and snap-frozen in liquid N2. Laminar abscission zones (LAZs) expanded leaves was excised from the mid-section of the leaf blade for leaf tissue, approximately 4 cm of leaf blade from fully expanded leaves was excised from the mid-section of the leaf blade and snap-frozen in liquid N2. Tissues were ground in liquid N2 and RNA from all tissues was extracted using the guanidine isothiocyanate method. RNA was precipitated using a salt solution (1.4 M sodium chloride and 0.8 M sodium citrate) in ethanol. The resulting RNA was pelleted, washed in 70% ethanol, dried, and dissolved in diethyl-pyrocarbonate (DEPC) until further analysis. Ethephon application was 30 °C (09.00 h) and 35°C (12/12 h; light/dark) for at least 7 d prior to sampling. Halogen lamps were used as light source (230–260 μmol m⁻² s⁻¹). Mature leaves were sampled at 4 h or 8 h intervals for 48 h. RNA was extracted, and gene expression analysis was performed (*n* = 4). Diurnal oscillation in PLD gene expression in fruit flavedo was studied using citrus trees in the field. Fruit flavedo was collected from citrus fruit harvested from the exterior region of the canopy at 4 h or 8 h intervals, RNA was extracted and gene expression analysis was performed (*n* = 4).

To determine the effect of continuous light and continuous dark exposure on PLD gene expression, potted citrus trees were entained in the growth room under the above described light/dark conditions (LD) for 1 week and subsequently transferred to either constant light (LL) or constant dark (DD). A constant temperature of 24 °C was maintained during entrainment and during continuous light and dark treatments. Leaves were sampled at 4 h or 6 h intervals and gene expression analysis was performed (*n* = 4).

**Gene expression analysis**

An Applied Biosystems (Foster City, CA, USA) 7500 Fast-Real-Time PCR system was utilized for quantitative real-time RT-PCR analyses. Analysis was performed on 1 μl of diluted cDNA in a final reaction volume of 20 μl using the SYBR™ Green PCR Master Mix (Applied Biosystems). PCR conditions were 50 °C 2 min; 95 °C 10 min followed by 40 cycles of 95 °C 15 s; 60 °C 1 min. Melting curve analysis was performed to confirm target-specific amplification. Primer concentration was optimized and primer validation was performed to enable relative gene expression analysis using the ΔΔCt method. Citrus glyceraldehyde-3-phosphate-dehydrogenase (CsGAPDH) was used as the calibrator. A citrus actin sequence was also used for confirmation in some experiments with similar results. Data were normalized to a control replicate (time = 0 h). At least three biological replicates were utilized in every experiment and all analyses were performed in duplicate. Primers used for real-time analyses are listed in the Supplementary data (Table S2) that can be found at JXB online.

**Gene expression in 1-MCP on diurnal PLD gene expression**

‘Valencia’ field trees from the above-mentioned field block were treated with water (control) or 1-MCP (5 mM SmartFresh®) according to Pozo et al., 2004 to inhibit ethylene perception (*n* = 4). Applications were made at 10.00 h on a clear and calm day. Leaf samples were collected from treated trees at 4 h intervals for 48 h and PLD gene expression analysis was performed. 1-MCP applications were followed by ethephon (400 mg l⁻¹) application on selected branches. 1-MCP decreased the extent of ethephon-induced leaf abscission on these branches, indicating that it reduced ethylene perception.

**Results**

**Isolation of phospholipase D genes from citrus**

Full-length citrus *PLDα* and *PLDγ* sequences, designated *CsPLDα1* and *CsPLDγ1*, were isolated and characterized. The predicted amino acid sequence of *CsPLDα1* has 86% and 81% identity with PLDα from castor bean and *Arabidopsis*, respectively. *CsPLDα1* possesses an N-terminal C2 domain thought to be involved in calcium

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**RNA isolation and RT-PCR**

For leaf tissue, approximately 4 cm of leaf blade from fully expanded leaves was excised from the mid-section of the leaf blade and snap-frozen in liquid N2. Laminar abscission zones (LAZs) were removed by excising approximately 5 mm of tissue proximal and distal to the abscission zone plane. Fruit flavedo was removed using a kitchen-type potato peeler and snap-frozen. Tissues were ground in liquid N2 and RNA from all tissues was extracted using the guanidine isothiocyanate method. RNA was precipitated using a salt solution (1.4 M sodium chloride and 0.8 M sodium citrate) and isopropanol, washed in 70% ethanol, and precipitated overnight in ethanol. The resulting RNA was pelleted, washed in 70% ethanol, dried, and dissolved in diethyl-pyrocarbonate (DEPC) treated water. Total RNA (0.5 μg) was treated with DNase I (Promega) to remove genomic DNA contamination and reverse transcribed using ‘Superscript III’ reverse transcriptase (Invitrogen) to remove genomic DNA contamination and reverse transcribed using ‘Superscript III’ reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The cDNA was diluted 5-fold and stored at –20 °C until further analysis.

**Isolation of full-length CsPLDα1 and CsPLDγ1**

Phospholipase genes were isolated from fruit flavedo cDNA using degenerate primers, RT-PCR, and 3′ and 5′ rapid amplification of cDNA ends (RACE). At each step, the amplified fragments were cloned into pGEM-T-Easy and sequenced at the University of Florida Core Sequencing Facility. Degenerate primers P1 and P2, designed based on amino acid sequence similarity among several plant PLDs, amplified a 537 base pair fragment with similarity to plant *PLDα* from citrus flavedo cDNA. Another degenerate primer (P3) similarly designed and used with a gene-specific primer (P4) amplified a fragment from the 5′ region of citrus *PLDα*. The 5′ RACE system (Invitrogen, Carlsbad, CA, USA) was used along with a gene specific primer (P5) to isolate the 5′ region of *PLDα*. A 3′ RACE strategy was used with primers P6 and P7 to amplify the 3′ region. Similarly, degenerate primers (P8 and P9) were used to amplify a 1047 bp fragment with similarity to *PLDγ*. The 5′ region was amplified using 5′ RACE with P10 as a gene specific primer. Extension of the 3′ region was achieved with a degenerate primer (P11) and a gene specific primer (P12). Finally, 3′ RACE was performed with P13 and P7. Primer sequences used for PLD gene isolation are presented in the Supplementary data (Table S1) that can be found at JXB online.
and phospholipid binding and two HKD domains essential for catalytic activity. A motif containing ‘ERF’ residues followed by a hydrophobic chain (VYIVV), and analogous to the ‘DRY’ motif of GPCRs was identified within the catalytic region. The ERF motif, a modified form of the DRY motif, has been implicated in direct interactions of PLD with the heterotrimeric protein Gα subunit (Zhao and Wang, 2004). CsPLDγ1 shares 71% identity with Arabidopsis PLDγ1 and has a calcium-binding C2 and two catalytic HKD domains. An ‘ERF’ motif was also identified within its catalytic region along with a modified hydrophobic region immediately downstream of this site. CsPLDα1 and CsPLDγ1 sequences were deposited in GenBank and assigned accession numbers, EU340031 and EU340032, respectively.

**Ethephon induces CsPLDγ1 and decreases CsPLDα1 expression in citrus leaves and LAZ**

Ethephon application to whole citrus tree canopy sections induced rapid defoliation. Leaf abscission began 24 h after application (Fig. 1A). The rate of abscission increased from 24 h to day 3 and resulted in >70% leaf drop by day 5 after treatment. Ethephon induced a significantly greater leaf drop when compared with controls, beginning from day 1 to the end of the experiment. In comparison to the untreated control, ethephon decreased CsPLDα1 expression in the leaf blade and LAZ by 30% and 36%, respectively, 6 h after application (Fig. 1B, D). Reduction in expression was also observed at 24 h and 48 h after treatment. In contrast, CsPLDγ1 expression was rapidly induced (Fig. 1C, E). CsPLDγ1 expression in leaf blade tissue increased by 3-fold within 3 h and >5-fold within 6 h of ethephon application (Fig. 1C). Increased expression was also observed 24 h and 48 h after application. A 4-fold increase in CsPLDγ1 expression occurred in the LAZ 6 h after application but no significant difference was noted at 48 h after application (Fig. 1E). These data indicate a rapid induction of CsPLDγ1 and suppression of CsPLDα1 expression in the leaf blade and LAZ by ethephon, and that trends in expression were similar in leaf blade and LAZ.

**CsPLDγ1 expression in citrus fruit flavedo increases during abscission-agent induced fruit abscission**

The abscission agents ethephon and CMNP significantly decreased mature fruit detachment force within 4 d after application (Fig. 2A). Ethephon decreased fruit detachment force to 59% and 80% of the control at 4 d and 7 d after application, respectively. CMNP application was more effective in loosening mature fruit. FDF was reduced to 42% that of the control by 4 d after application and did not change thereafter.

Ethephon and CMNP did not alter CsPLDα1 expression in fruit flavedo (Fig. 2B). Ethephon application transiently increased CsPLDγ1 expression by 60% at 24 h after application, but the increase was not statistically significant (Fig. 2C; Fisher’s LSD 0.05). Ethephon did not have any effect on CsPLDγ1 expression at the later stages (48 h and 96 h). By contrast, CMNP application increased CsPLDγ1 expression by almost 2-fold within 24 h after application. CMNP application also resulted in a sustained increase in CsPLDγ1 expression by ~2.5-fold at 48 h and at 96 h after application. The above data indicate differential regulation of PLD gene expression by abscission agents in the fruit flavedo.

**Diurnal oscillation of PLD gene expression in citrus leaves and fruit**

During our analysis of PLD gene expression in the leaf blade, an increase in expression within control samples was noted at 6 h after application (sampling time: 16.00 h; Fig. 1B, C). Hence, diurnal oscillation in PLD gene expression was investigated. CsPLDα1 and CsPLDγ1 exhibited diurnal oscillation in gene expression within the leaf blade (Fig. 3A). CsPLDα1 and CsPLDγ1 expression were low early in the light period (09.00 h), and increased as light duration increased, reaching a maximum around 17.00 h, and then declined during the dark period. The maximum/minimum change in CsPLDα1 expression was about 1.7-fold. CsPLDγ1 exhibited a similar phase but greater amplitude in the diurnal rhythm of expression with maximum/minimum change of almost 5-fold. These data indicate that PLD gene expression is diurnally regulated in citrus leaves.

In contrast to the expression pattern in the leaf blade, expression of CsPLDα1 did not oscillate diurnally in the fruit flavedo (Fig. 3B). However, CsPLDγ1 expression oscillated with a diurnal pattern. CsPLDγ1 expression was lowest during the early part of the day (09.00 h), increased with the progression of the day, and reached peak expression during midday (13.00 h and 17.00 h). Peak expression of CsPLDγ1 was 3-fold higher than that early in the day.

**PLD gene expression in citrus leaves is regulated by light and the circadian clock**

The role of temperature, light, and the circadian clock in entraining PLD diurnal gene expression was studied in citrus leaves. Entrainment of potted citrus plants at constant temperature and LD-entrained plants to continuous light and the circadian clock PLD gene expression in citrus leaves is regulated by light and the circadian clock. The role of temperature, light, and the circadian clock in entraining PLD diurnal gene expression was studied in citrus leaves. Entrainment of potted citrus plants at constant temperature (24 °C) and 12/12 h light/dark (LD) for 1 week did not abolish diurnal rhythms in CsPLDα1 or CsPLDγ1 expression (Fig. 4A, B; the initial two time-of-day samples), but the magnitude of CsPLDγ1 expression increased (compare with Fig. 3A). The transfer of constant temperature and LD-entrained plants to continuous light (LL) resulted in the loss of oscillation in CsPLDα1 expression (Fig. 4A). By contrast, oscillation of CsPLDγ1 expression persisted after transfer to LL. However, peak
CsPLD\(\gamma 1\) expression was lower and a phase change occurred, resulting in earlier peak expression at 14.00 h followed by a decline in expression.

Transfer of constant temperature- and LD-entrained plants to continuous dark (DD) resulted in a loss of oscillation in CsPLD\(\alpha 1\) expression (Fig. 4B). CsPLD\(\gamma 1\) expression in leaves exhibited diurnal oscillation on day 1 after transfer to DD (2–2.5-fold increase during midday), but remained at basal levels during prolonged exposure to DD. These data suggest that light strongly influences CsPLD\(\alpha 1\) expression, and that CsPLD\(\gamma 1\) expression is regulated by light as well as the circadian clock.

Diurnal oscillation in PLD gene expression in citrus leaves is partially dependent on ethylene signalling

As in Arabidopsis seedlings (Thain et al., 2004), ethylene emission from citrus leaves occurs in a diurnal pattern with peak emission during the day (A Malladi et al., unpublished data). It is hypothesized that diurnal changes in ethylene levels and signalling may modulate diurnal
PLD expression. The ethylene perception inhibitor 1-MCP was utilized to reduce ethylene perception in citrus leaves. Application of 1-MCP did not prevent oscillation in $\text{CsPLD}a_1$ and $\text{CsPLD}c_1$ gene expression (Fig. 5A and B, respectively); however, the amplitude of oscillation decreased. These data indicate that ethylene perception and signalling modulate the magnitude of diurnal oscillation in $\text{CsPLD}a_1$ and $\text{CsPLD}c_1$ expression in citrus leaves. Diurnal oscillation in $\text{CsPLD}a_1$ expression (Fig. 5A) was higher than in Fig. 3A, possibly as this experiment was performed in the field.

**Sensitivity to abscission agent-induced leaf and fruit abscission increases during midday**

The relationship between diurnal oscillation in PLD gene expression and leaf and fruit abscission sensitivity was investigated by the application of abscission agents during minimal (early-day) and rising (midday) $\text{CsPLD}c_1$ expression. Early-day application of ethephon (09.00 h) induced 47% leaf abscission by day 10, with the majority of leaf drop occurring by day 4 (40%; Fig. 6A). Midday (13.00 h) application increased total leaf abscission to almost 60% by day 10, with the majority of leaf drop...
occurring by day 3 (48%). These data indicate greater sensitivity to ethephon when applied during rising CsPLD1 expression.

The application of ethephon at 09.00 h decreased FDF to 50% of the control while the midday (13.00 h) application decreased it to 42% of the control (Fig. 6B). Application of CMNP during midday (13.00 h) had a greater effect on reducing FDF. While early-day (09.00 h) application of CMNP decreased FDF to 42% of the control, midday application of CMNP decreased FDF to less than 9% of the control. The above data indicate that sensitivity to abscission-agent induced leaf and fruit abscission increased during midday.

Discussion

Initial signals during ethylene-induced leaf abscission are thought to be generated within the leaf blade, while the trigger for citrus mature fruit abscission is generated within the fruit flavedo (Beyer, 1975; Alferez et al., 2005). Hence, our analysis of citrus PLD expression was largely focused in the above tissues. Expression of CsPLD1 was rapidly induced in ethephon-treated citrus leaf blades while that of CsPLDγ1 decreased, indicating an important role for PLDs during the early responses to induced leaf abscission. The G-protein-related signalling pathway was implicated in modulating ethephon-induced leaf abscission in citrus as the application of G-protein receptor agonists blocked ethephon-induced leaf abscission (Yuan et al., 2005). Ethylene may promote leaf abscission by modulating interactions within the heterotrimeric G-protein complex (Gα, Gβ, and Gγ), thereby affecting G-protein signalling. Rapid and differential

Fig. 4. Light dependence of CsPLDα1 and CsPLDγ1 expression in citrus leaves. Potted ‘Valencia’ citrus trees were entrained under 12/12 h: light/dark cycles (LD) and constant temperature (24 °C) conditions for 1 week. Trees were then transferred to either (A) continuous light (LL) or (B) continuous dark (DD), and CsPLDα1 and CsPLDγ1 expression in the leaf blade was analysed by quantitative RT-PCR at the indicated times. Vertical lines through markers depict SE mean (n=4). The absence of SE lines indicates a marker larger than the SE. Short white bars under the graph depict the end of the entrainment period. Long white and black bars depict the length of LL (A) and DD (B) treatment, respectively.

Fig. 5. Effect of 1-MCP on diurnal fluctuation in PLD expression in citrus leaves. 1-MCP (5 mM) was applied to mature field-grown citrus trees at 10.00 h and its effect on diurnal CsPLDα1 (A) and CsPLDγ1 (B) expression in the leaf blade was measured using quantitative RT-PCR at the indicated times. Vertical lines through the markers depict the SE mean (n=4). Absence of SE lines indicates a marker larger than the SE.
changes in CsPLDα1 and CsPLDγ1 expression by ethephon may facilitate this process directly through interactions with the Gα subunit via the putative ‘DRY’ motif or indirectly through effects on Gβ and Gγ subunits of the heterotrimeric G-protein complex.

Differential regulation of PLD expression was associated with ethephon-induced, early leaf abscission responses. Diversity in products and/or physiological outcomes generated by different PLD isoforms may account for the seemingly opposing functions of CsPLDα1 and CsPLDγ1. Opposing roles for different PLDs have previously been reported in Arabidopsis (Wang et al., 2002; Welti et al., 2002; Li et al., 2004; Wang, 2005).

Notwithstanding the numerical increase in expression, ethephon application did not significantly alter CsPLDα1 and CsPLDγ1 expression in fruit flavedo. Hence, a PLD-independent mechanism may regulate ethephon-induced reduction in FDF. These data indicate that diverse mechanisms regulate ethephon-induced citrus leaf and fruit abscission responses. Such mechanisms may include differential ethylene sensitivity between organs. The ethylene perception inhibitor, 1-MCP, modulates ethephon-induced leaf abscission responses but not FDF responses (Pozo et al., 2004). Also, G-protein agonists modulate ethephon-induced leaf abscission responses but have little effect on fruit abscission (Burns et al., 2003).

CMNP is a fruit-specific abscission agent that elicits unique physiological reactions when compared with ethephon or ethylene (Li et al., 2008; Alferez et al., 2005). Although mechanisms through which CMNP specifically induces citrus mature fruit abscission remain unclear, chemical wounding, transient alteration in membrane permeability, and reduction in ATP levels are important factors contributing to the acceleration of abscission. The application of CMNP rapidly increased and sustained CsPLDγ1 expression in citrus fruit flavedo and this was correlated with higher and consistent reduction in FDF. Rapid elevation of PA levels occurs in several plants upon wounding and this may be involved in wound-induced and phospholipid-based signal transduction mechanisms (Lee et al., 1997). A similar, PA-dependent wound-induced signalling mechanism may operate in triggering abscission responses to CMNP. An increase in PA levels facilitated by CsPLDγ1 may trigger downstream signalling mechanisms leading to fruit abscission. In addition, modification of membrane phospholipid composition by CsPLDγ1 activity and PA may further contribute to the acceleration of fruit abscission responses. Mechanical wounding of flavedo induces fruit abscission in citrus (Kostenyuk and Burns, 2004). It may be that CsPLDγ1 is similarly involved in signalling mechanical wounding-induced fruit abscission responses. Together, the above data suggest that rapid and sustained increase in CsPLDγ1 expression may constitute a key mechanism that mediates abscission responses to diverse abscission-agents in different organs of citrus. CsPLDγ1 may have dual functions in abscission signalling: to facilitate ethylene and G-protein interaction-dependent modulation of leaf abscission, and to mediate wound-induced fruit abscission responses.

Additional functions for phospholipid-signalling in facilitating abscission cannot be excluded. PA has been shown to interact directly with and inhibit the activity of CTR1 (constitutive triple response 1) a negative regulator of ethylene signalling (Testerink et al., 2007). Hence, PA
generated by CsPLDγ1 activity may directly mediate ethylene-induced leaf abscission responses through the inhibition of CTR1 and the activation of ethylene signalling. In addition, PA may also modify auxin transport, another important regulator of ethylene-induced abscission (Patterson, 2001; Taylor and Whitelaw, 2001), through interaction with RCN1 (roots curl in NPA 1), a regulator of auxin transport (Testerink et al., 2004; Muday et al., 2006). Also, the Arabidopsis PLD isoform, PLDζ2, regulates auxin responses through the modification of auxin transport (Li and Xue, 2007). A decrease in auxin transport from the leaf blade or fruit flavedo to the AZ, facilitated by an increased expression of CsPLDγ1, may alter the critical auxin-ethylene balance at the AZ and promote abscission. The above possible mechanisms involved in phospholipid signalling-mediated abscission responses warrant further investigation.

Analysis of PLD expression in citrus leaves revealed diurnal oscillation in transcript abundance in this gene family. Expression of CsPLDα1 and CsPLDγ1 was highest at midday but declined later in the night, reaching the lowest levels early in the day. CsPLDα1 expression was not diurnally regulated in fruit flavedo; however, CsPLDγ1 expression followed a similar diurnal pattern to that seen in leaves, but with lower amplitude. Diurnal regulation of PLD gene expression in leaves may have implications for several physiological functions including diurnal regulation of stomatal opening and closure as PLDα plays a role in this process (Mishra et al., 2006; Hong et al., 2008).

Factors that contributed to diurnal oscillation in PLD gene expression in citrus leaves were investigated. While diurnal CsPLDα1 expression was regulated by light, oscillation of CsPLDγ1 expression was under light as well as circadian control. These data indicate that light- and circadian clock-dependent mechanisms modulate PLD expression in citrus leaves. Interestingly, studies in etiolated oat seedlings indicate that PLD activity is red-light dependent (Park et al., 1996; Kabachevskaya et al., 2007). Furthermore, application of 1-MCP reduced the magnitude of diurnal oscillation in CsPLDα1 and CsPLDγ1 gene expression. Hence, diurnal oscillation in PLD gene expression is, in part, dependent upon ethylene perception. Phospholipid-signal generation may therefore be regulated by light, the circadian clock, and ethylene-signalling and such diurnal regulation of phospholipid signals may mediate physiological responses including abscission.

Response to abscission agent-induced abscission appears to be modulated depending upon the time of day (Pozo et al., 2007). Preliminary studies suggest that changes in leaf abscission response during different times of the day persist under constant temperature conditions (A Malladi et al., unpublished results). Fruit held under constant temperature probably respond in a similar manner, but this remains to be tested. These data indicate diurnal regulation of abscission in citrus. Peak sensitivity of abscission during the day corresponded with rising diurnal CsPLDγ1 expression in citrus leaf blade and flavedo. Assuming CsPLDγ1 enzyme activity closely follows diurnal changes in CsPLDγ1 gene expression, changes in PA and related lipid product levels in the leaf blade and flavedo during midday may more effectively trigger and propagate signalling mechanisms involved in facilitating abscission responses. Such mechanisms may also be diurnally regulated. Analysis of diurnal changes in PLD activity and/or PA levels should provide novel insights into their roles in mediating changes in abscission sensitivity.

**Supplementary data**

The following supplementary data for this article are available at JXB online.

**Table S1.** List of primers used for cloning CsPLDα1 and CsPLDγ1 from *Citrus sinensis* cv. ‘Valencia’.

**Table S2.** List of primers used for quantitative RT-PCR analysis (5′–3′).

**Acknowledgement**

The authors wish to thank Dr Luis Pozo for help with field experiments.

**References**

Alferez F, Singh S, Umbach AL, Hockema B, Burns JK. 2005. Citrus abscission and Arabidopsis plant decline in response to 5-chloro-3-methyl-4-nitro-1H-pyrazole are mediated by lipid signaling. *Plant, Cell and Environment* 28, 1436–1449.

Alferez F, Zhong GY, Burns JK. 2007. A citrus abscission agent induces anoxia- and senescence-related gene expression in *Arabidopsis*. *Journal of Experimental Botany* 58, 2451–2462.

Ashikari M, Wu J, Yano M, Sasaki T, Yoshimura A. 1999. Rice gibberellin-insensitive dwarf mutant gene Dwarf 1 encodes the α-subunit of GTP-binding protein. *Proceedings of the National Academy of Sciences, USA* 96, 10284–10289.

Assmann SM. 2002. Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *The Plant Cell* 14, S355–S373.

Bargmann BOR, Laxalt AM, Riet BT, Schouten E, van Leeuwen W, Dekker HL, de Koster CD, Haring MA, Munnik T. 2006. LePLDβ1 activation and relocation in suspension-cultured tomato cells treated with xylanase. *The Plant Journal* 45, 358–368.

Beyer E. 1975. Abscission: the initial effect of ethylene is in the leaf blade. *Plant Physiology* 55, 322–327.

Bleecker AB, Patterson SE. 1997. Last exit, senescence, abscission, and meristem arrest in *Arabidopsis*. *The Plant Cell* 9, 1169–1179.

Brown KM. 1997. Ethylene and abscission. *Physiologia Plantarum* 100, 567–576.

Burns JK. 2002. Using molecular biology tools to identify abscission materials for citrus. *HortScience* 37, 459–464.
Burns JK, Pozo LV, Yuan R, Hockema B. 2003. Guanfacine and clonidine reduce defoliation and phytotoxicity associated with abscission agents. *Journal of the American Society for Horticultural Science* 128, 42–47.

Coursol S, Fan LM, Le Stunff H, Spiegel S, Gilroy S, Assmann SM. 2003. Sphingolipid signalling in Arabidopsis guard cells involves heterotrimERIC G proteins. *Nature* 423, 651–654.

Fan L, Zheng SQ, Wang XM. 1997. Antisense suppression of phospholipase Dα retards abscissic acid- and ethylene-promoted senescence of postharvest Arabidopsis leaves. *The Plant Cell* 9, 2183–2196.

Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D. 2000. Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. *The Plant Cell* 12, 111–123.

Goren R. 1993. Anatomical, physiological and hormonal aspects of abscission in citrus. *Horticultural Reviews* 15, 145–182.

Hong Y, Pan X, Welti R, Wang X. 2008. Phospholipase D δ3 is involved in the hyposmotic response in *Arabidopsis*. *The Plant Cell* 20, 803–816.

Jackson MB, Osbourn D. 1970. Ethylene, natural regulator of leaf abscission. *Nature* 225, 1019–1022.

Kabachevskaya AM, Liakhnovich GV, Kisel MA, Volotovsky ID. 2007. Red/far-red light modulates phospholipase D activity in oat seedlings. Relation of enzyme photosensitivity to photosynthesis. *Journal of Plant Physiology* 164, 108–110.

Kostenyuk IA, Burns JK. 2004. Mechanical wounding and abscission in citrus. *Physiologia Plantarum* 122, 354–361.

Lanahan MB, Yen HC, Giovannoni JJ, Klee H. 1994. The never ripe mutation blocks ethylene perception in tomato. *The Plant Cell* 6, 521–530.

Laxalt AM, Riet BT, Verdonk JC, Parigi L, Tameling WIL, Vossen J, Haring M, Musgrave A, Munnik T. 2001. Characterization of five tomato phospholipase D cDNAs, rapid and specific expression of LePLDδ1 on elicitation with xylanase. *The Plant Journal* 26, 237–247.

Lee S, Suh S, Kim S, Crain RC, Kwak JM, Nam H-G, Lee Y. 1997. Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. *The Plant Journal* 12, 547–556.

Lee SH, Chae HS, Lee TK, Kim SH, Shin SH, Cho BH, Cho SH, Kang BG, Lee WS. 1998. Ethylene-mediated phospholipid catabolic pathway in glucose-starved carrot suspension cells. *Plant Physiology* 116, 223–229.

Lein W, Saalbach G. 2001. Cloning and direct G protein regulation of phospholipase D from tobacco. *Biochimica et Biophysica Acta* 1530, 172–183.

Li G, Xue H. 2007. *Arabidopsis PLDc2* regulates vesicle trafficking and is required for auxin response. *The Plant Cell* 19, 281–295.

Li K-T, Burns JK, Syvertsen JP. 2008. Recovery from phytotoxicity after foliar application of fruit loosening compounds to citrus. *Journal of the American Society for Horticultural Science* 133, 535–541.

Li WQ, Li MY, Zhang WH, Welti R, Wang XM. 2004. The plasma membrane-bound phospholipase D delta enhances freezing tolerance in Arabidopsis thaliana. *Nature Biotechnology* 22, 427–433.

Mishra G, Zhang W, Deng F, Zhao J, Wang X. 2006. A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. *Science* 312, 264–266.

Muday GK, Brady SR, Argueso C, Deruere J, Kieber JJ, DeLong A. 2006. RCN1-regulated phosphatase activity and EIN2 modulate hypocotyl gravitropism by a mechanism that does not require ethylene signaling. *Plant Physiology* 141, 1617–1629.

Pappan K, Zheng SQ, Wang XM. 1997a. Identification and characterization of a novel plant phospholipase D that requires polyphosphoinositides and submicromolar calcium for activity in *Arabidopsis*. *Journal of Biological Chemistry* 272, 7048–7054.

Pappan K, Qin WS, Dyer JH, Zheng L, Wang XM. 1997b. Molecular cloning and functional analysis of polyphosphoinositide-dependent phospholipase D, PLDδ1, from *Arabidopsis*. *Journal of Biological Chemistry* 272, 7055–7061.

Park C, Park HM, Chae Q. 1996. Identification and characterization of phytochrome-regulated phospholipase D in oat cells (*Avena sativa* L.). *Journal of Biochemistry and Molecular Biology* 29, 535–539.

Patterson SE. 2001. Cutting loose, abscission and dehiscence in *Arabidopsis*. *Plant Physiology* 126, 494–500.

Pozo L, Yuan RC, Kostenyuk IA, Alferez F, Zhong GY, Burns JK. 2004. Differential effects of 1-methylcyclopropene on citrus leaf and mature fruit abscission. *Journal of the American Society for Horticultural Science* 129, 473–478.

Pozo L, Malladi A, John-Karuppiah K-J, Lluch Y, Alferez F, Burns JK. 2007. Daily fluctuation in fruit detachment force of ‘Valencia’ orange is related to time of day, temperature, relative humidity, fruit weight and juice percentage. *Proceedings of the Florida State Horticultural Society* 120, 41–44.

Qin CB, Wang XM. 2002. The Arabidopsis phospholipase D family. Characterization of a calcium-independent and phosphatidylycholine-selective PLD zeta 1 with distinct regulatory domains. *Plant Physiology* 128, 1057–1068.

Qin WS, Pappan K, Wang XM. 1997. Molecular heterogeneity of phospholipase D (PLD). Cloning of PLDγ, -β, and -α by polyphosphoinositides and calcium. *Journal of Biological Chemistry* 272, 28267–28273.

Ritchie S, Gilroy S. 1998. Abscisic acid stimulation of phospholipase D in the barley aleurone is G-protein-mediated and localized to the plasma membrane. *Plant Physiology* 124, 693–702.

Romanov GA, Kieber JJ, Schmulling T. 2002. A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signaling. *FEBS Letters* 515, 39–43.

Sixton R, Roberts JA. 1982. Cell biology of abscission. *Annual Review of Plant Physiology* 33, 133–162.

Taylor EJ, Whitelaw JA. 2001. Signals in abscission. *New Phytologist* 151, 323–339.

Testerink C, Dekker HL, Lim ZY, Johns MK, Holmes AB, de Koster CG, Ktistakis NT, Munnik T. 2004. Isolation and identification of phosphatidic acid targets from plants. *The Plant Journal* 39, 527–536.

Testerink C, Larsen PB, Van der Does D, van Himeren JAJ, Munnik T. 2007. Phosphatidic acid binds to and inhibits the activity of *Arabidopsis* CTR1. *Journal of Experimental Botany* 58, 3905–3914.

Thain SC, Vandenbussche F, Laarhoven LJ, Dowson-Day MJ, Wang Z-Y, Tobin EM, Harren FJM, Millar AJ, Van Der Straeten D. 2004. Circadian rhythms of ethylene emission in *Arabidopsis*. *Plant Physiology* 136, 3751–3761.

Ueguchi-Tanaka M, Fujisawa Y, Kobayashi M, Ashikari M, Iwasaki Y, Kitano H, Matsuoka M. 2000. Rice dwarf mutant *dl*, which is defective in the α subunit of the heterotrimERIC G protein, affects gibberellin signal transduction. *Proceedings of the National Academy of Sciences, USA* 97, 11638–11643.

Wang XM. 2002. Phospholipase D in hormonal and stress signaling. *Current Opinion in Plant Biology* 5, 408–414.

Wang XM. 2004. Lipid signaling. *Current Opinion in Plant Biology* 7, 329–336.

Wang X. 2005. Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. *Plant Physiology* 139, 566–573.

Wang X, Wang C, Sang Y, Qui C, Welti R. 2002. Nwtoxing of phospholipases in plant signal transduction. *Physiologia Plantarum* 115, 331–335.
Welti R, Li WQ, Li MY, Sang YM, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang XM. 2002. Profiling membrane lipids in plant stress responses: role of phospholipase Dα in freezing-induced lipid changes in Arabidopsis. Journal of Biological Chemistry 277, 31994–32002.

Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni JJ, Klee H. 1995. An ethylene-inducible component of signal transduction encoded by never-ripe. Science 270, 1807–1809.

Yuan RC, Wu ZC, Kostenyuk IA, Burns JK. 2005. G-protein-coupled α2A-adrenoreceptor agonists differentially alter citrus leaf and fruit abscission by affecting expression of ACC synthase and ACC oxidase. Journal of Experimental Botany 56, 1867–1875.

Yuan RC, Burns JK. 2004. Temperature factor affecting the abscission response of mature fruit and leaves to CMN-pyrazole and ethephon in ‘Hamlin’ Oranges. Journal of the American Society for Horticultural Science 129, 287–293.

Zhao J, Wang XM. 2004. Arabidopsis phospholipase Dα1 interacts with the heterotrimeric G-protein α-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. Journal of Biological Chemistry 279, 1794–1800.