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SHORT COMMUNICATION

Effects of Phosphatidic Acid on Cytokinin Signal Transduction in Periwinkle Cells

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Abstract In periwinkle cell suspensions, the accurate quantification of gene expression through real-time RT-PCR showed that two type-A response regulators (*RR-A*), considered primary cytokinin (CK)-responsive genes, were differentially regulated after CK treatment. Specific inhibition of phospholipase D (PLD)-dependent phosphatidic acid (PA) production by primary alcohols reduced significantly the transcript level of one gene in response to CK, although the other gene was unaffected. Moreover, this inhibitory effect on gene transcript level could be antagonized by exogenous supply of PA. These results suggest that PA, likely released from the membrane by PLD activity, could operate in the early steps of CK signalling in periwinkle cells.

Keywords Cytokinin signalling · Phosphatidic acid · Response regulator · Real-time PCR · *Catharanthus roseus*

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Introduction

Although the central roles of cytokinins (CK) in the control and regulation of major processes involved in proliferation, development, and differentiation of plant cells have been recognized for a long time, the molecular mechanisms that govern the transduction of the hormonal signal have been addressed only in the past decade. The generally accepted model is that CK signalling utilizes elements similar to the two-component multistep pathways used by bacteria, that is, histidine kinase receivers (HK), histidine phosphotransferases (HPt), and response regulators (RR). In *Arabidopsis thaliana*, the CK signalling pathway consists of 3 HK (AtHK2-3, AtHK4/CRE1), 5 HPt (AHP1-5), and 23 RR. The latter are grouped into two classes, 10 type-A *Arabidopsis* response regulators (ARR), which act mainly as negative regulators of CK responses, and 13 type-B ARR, which are transcription factors that play a positive role in CK-regulated gene expression (Hwang and others 2002). The roles of these different elements in the signal transduction mechanisms have been widely investigated in recent years (Ferreira and Kieber 2005) and the emerging picture suggests that extensive redundancy exists in each of these multigene families (Nishimura and others 2004; Mason and others 2005). Thus, the main question is how these overlapping functions allow specific responses to CK. Moreover, additive elements functioning in tandem with the canonical ones may exist (Rashotte and others 2006) and other classical transduction pathways could also participate in CK signalling (Romanov and others 2002).

For several years we have studied an experimental model in which a CK-habituated cell suspension of periwinkle responds to exogenously applied CK by a dramatic increase of monoterpene indole alkaloids (MIA) accumulation (Décendit and others 1992). To better understand the

role of CK in this model, several elements putatively involved in a two-component multistep signalling pathway have been isolated and tentatively characterized; although fully habituated to CK, the cells in vitro still sense the CK signal because the transcription of a gene (*CrRR1*) encoding a type-A response regulator is rapidly and specifically induced by exogenous CK. Moreover, blocking the signalling pathway downstream of a putative histidine kinase receiver (CrCKR1), either by using chemical inhibitors or through RNA interference, prevents *CrRR1* transcripts accumulation (Papon and others 2003, 2004).

In this article we first compared more precisely the expression of *CrRR1* with that of *CrRR3*, another type-A response regulator, in periwinkle cells submitted to CK. We showed that both genes are induced rapidly by CK but with significant differences with respect to their transcript levels and kinetics of expression. Then we investigated if other signalling components, either previously reported to modulate MIA accumulation in CK-treated periwinkle cells (Mérillon and others 1993) or likely involved in CK signalling in *Arabidopsis* (Romanov and others 2002), could also affect the transcript level of these genes and therefore the early steps of CK signalling in periwinkle. We show that treatments with primary alcohols, considered specific inhibitors of PLD-dependent PA production, differentially affect the expression of each gene: *CrRR3* transcripts levels are strongly reduced whereas *CrRR1* gene expression is not affected. Moreover, exogenously supplied PA entirely abolishes the inhibitory effects of primary alcohols on *CrRR3* expression in CK-treated cells.

Materials and Methods

Plant Material and Treatments

Cells suspension cultures (line C20D) were maintained on a 7-day growth cycle in B5 medium (Gamborg and others 1968) supplemented with 58 mM sucrose and 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4D), in 250-ml Erlenmeyer flasks containing 50 ml of medium on a rotary shaker (100 rpm) at 25°C, in the dark.

For experimental use, the cells were subcultured in 2,4D-free B5 medium for 3 days then treated as follows: (1) For kinetic studies, cells were supplied with 5 μM 6-benzylaminopurine (BA) (dissolved in water) or none (mock) and incubated for 15, 30, 60, or 120 min prior harvest; (2) for inhibitor assays, serial cultures were pretreated for 30 min with none (mock) or 1% butan-1-ol, 1% butan-2-ol, 1% propan-1-ol, or 1% propan-2-ol, then for an additional 30 min with 5 μM BA before harvest. PA (catalog number P9511, purity ≥98%, prepared from L-α-phosphatidycholine by hydrolysis with cabbage phospholipase D, according to the manufacturer) or linoleic acid (dissolved in distilled water and sonicated) was added for 30 min to the reaction mixture before cell harvest. All chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

RNA Extraction and Reverse Transcription

Harvested cells were immediately ground to fine powder in liquid nitrogen. Total RNAs were extracted with RNAeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA samples were treated with 2 U of DNAse RQ1 (Promega, Madison, WI, USA) at 65°C for 10 min and subsequently purified on a column. First-strand cDNA was synthesized from 5 μg of total RNA using 100 ng of random hexamers and 15 U of Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 10 min at 25°C, 50 min at 50°C, and 5 min at 85°C, the cDNA was treated with 2 U of RNAse H (Invitrogen) at 37°C for 20 min.

Real-Time PCR

The following genes were amplified: *CrRR1* (GenBank AF534888, forward primer: AGAGCCTCATTCACCTC GAA, reverse primer: GATTCAGCACCACTGTCCAA, amplicon size: 158 bp); *CrRR3* (GenBank AF534890, forward primer: CCTAATTCATTCACAGGAAAACC CA, reverse primer: TCATCTGTGTGCTGCTTCCTTTT, amplicon size: 152 bp); *RpS9* (GenBank AJ749993, forward primer: TTACAAGTCCCTTCGGTGGT, reverse primer: TGCTTATTCTTCATCCTCTTCATC, amplicon size: 107 bp). Real-time PCR was run on an ABI Prism 7000 SDS light cycler (Applied Biosystems, Foster City, CA, USA) using the SYBR® Green I technology. Each PCR mixture was performed in a total reaction volume of 25 μl containing 125 ng of cDNA template, 2× Platinum Quantitative PCR SuperMix-UDG (12.5 μl) (Invitrogen), and 2 μM forward and reverse primer. The reaction was initiated by a decontamination (50°C for 2 min) and denaturation step (95°C, 2 min), followed by 40 cycles at 95°C for 20 s and either 64°C for 40 s (for *CrRR1* and *CrRR3* transcripts) or 60°C for 40 s (for *RpS9* transcripts). The melting curve was analyzed for each sample to check PCR specificity (48 cycles at 70°C for 40 s with increasing set point temperature after cycle 2 by 0.5°C). PCR efficiencies were always above 98%. Specific standard curves were established from serial dilutions (from 2×10^6 to 2×10^2 copies) of purified PCR fragments (239 bp for *CrRR1*; 152 bp for *CrRR3*, and 200 bp for *RpS9*). *RpS9* was used as a control gene to allow the normalization of the gene copy numbers in each sample (Menke and others 1999). Each assay was performed in triplicate from three

independent experiments. Data correspond to average values ± SD (standard deviation) ($n = 3$) and relative transcripts levels in each sample are expressed as a ratio of the abundance of the *RpS9* transcripts.

Statistical Analysis

Data were analyzed with Statistica v6.0 (StatSoft Inc., Tulsa, OK, USA). Experiments were tested with one-way ANOVA followed by Tukey HSD post-hoc comparisons at the $P < 0.05$ level.

Results and Discussion

Quantification of *CrRR1* and *CrRR3* Transcripts by Real-Time PCR

To investigate the early steps of CK signalling in periwinkle cells, we established a real-time PCR method to analyze and compare the transcript levels of *CrRR1* and *CrRR3*. Our results confirmed and refined previous data concerning the induction of *CrRR1* transcription by CK treatment. Although the expression of both genes was significantly enhanced by CK after 15 min of treatment with a maximum reached at 30 min, the subsequent transcription profiles of each gene differed: Transcripts of *CrRR1* returned to basal level after 2 h (Figure 1a), whereas that of *CrRR3* remained unchanged for the same period (Figure 1b). Moreover, although the constitutive expression of *CrRR3* in untreated cells was 10-fold lower than that of *CrRR1*, the impact of CK on *CrRR3* was more pronounced, with a 45-fold level change after 30 min of treatment, whereas *CrRR1* showed only a 20-fold change during the same time.

Search for a Role of Phospholipase D (PLD) and Phosphatidic Acid (PA) in CK Signalling

PLD activity has been previously reported to play a role in the early steps of CK signalling in *Arabidopsis* (Romanov and others 2002). To address whether this enzymatic activity could modulate the response of periwinkle cells to the CK signal in the same manner, we studied the effects of primary alcohols, considered specific inhibitors of PLD-dependent PA production (Wang 2000), on *CrRR1* and *CrRR3* transcript levels. We found that the primary alcohols differentially affected the upregulation of each gene in response to CK treatment: butan-1-ol and propan-1-ol did not modify the *CrRR1* transcript levels, whereas it significantly reduced (by approximately 65% and 50%, respectively) those of *CrRR3* (Figure 2). Under the same

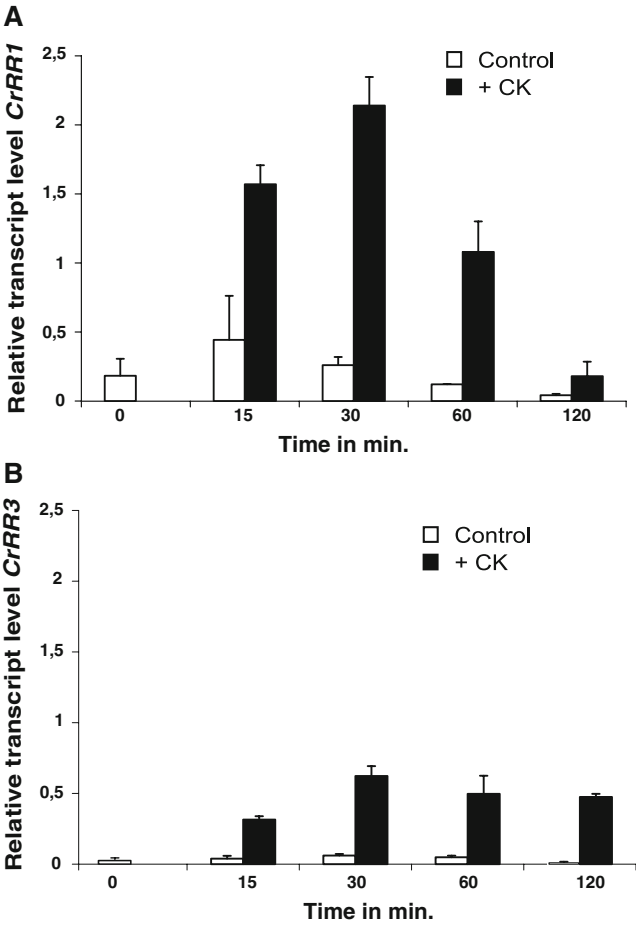

Fig. 1 Time-course expression of *CrRR1* (**a**) and *CrRR3* (**b**). Three-day-old cell cultures were treated with 5 μM BA (black bars) or mock-treated (open bars). Relative transcript levels are normalized to the abundance of the *RpS9* transcript (mean values ± SE; $n = 3$)

conditions, the corresponding secondary alcohols butan-2-ol and propan-2-ol had no effect on the upregulation of both genes. These experiments suggested that in periwinkle cells, PLD activity could exhibit different effects on the transcript level of early-responsive genes in CK signalling. To gain further insight on the role of PLD, we examined whether exogenously supplied PA could reverse the inhibitory effect of primary alcohols on the induction of *CrRR3* expression by CK. The effect of increasing amounts of PA on *CrRR3* transcript level showed a bell-shaped curve: A 25-μM PA concentration antagonized partially the inhibitory effect of butan-1-ol, whereas a 50-μM PA concentration was sufficient to completely restore the upregulating effect of CK. At higher concentrations (up to 100 μM), PA was less efficient but *CrRR3* transcript levels remained still higher than that obtained with butan-1-ol alone (Figure 3). In the same experiment, 50 μM linoleic acid (LA) could not reverse the inhibitory effect of butan-1-ol, indicating that PA is unlikely to operate through its

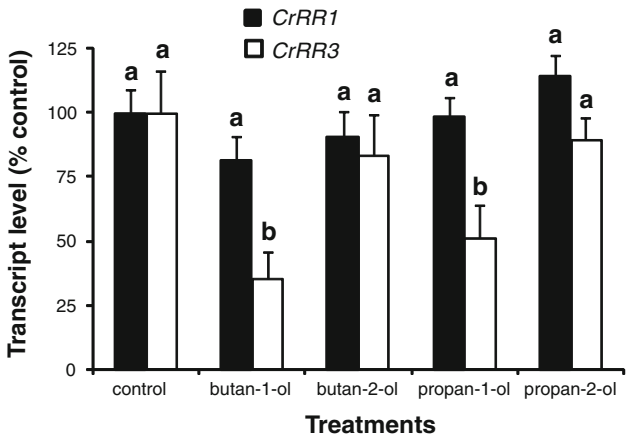

Fig. 2 Effects of PLD activity inhibition on the transcript level of *CrRR1* and *CrRR3*. Three-day-old cell cultures were preincubated for 30 min with 1% primary alcohols butan-1-ol or propan-1-ol (as specific inhibitors of PLD activity), or 1% secondary alcohols butan-2-ol or propan-2-ol (as a control of PLD activity), or untreated (control). The cells were subsequently treated for 30 min with 5 µM BA. *CrRR1* (black bars); *CrRR3* (open bars). Transcripts levels are in percent of the control (mean values ± SE; $n = 3$). Letters indicate significant differences at $P < 0.05$ level

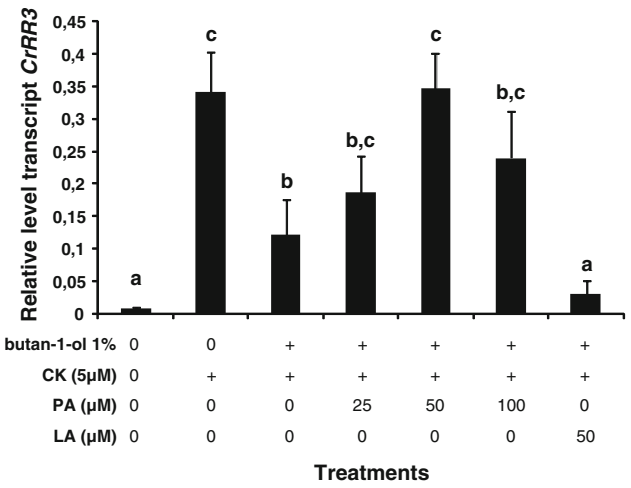

Fig. 3 Effect of of phosphatidic acid (PA) and linoleic acid (LA) on *CrRR3* transcript level in CK-treated cells under PLD inhibition. Three-day-old cell cultures were preincubated for 30 min with 1% butan-1-ol or not. The cells were subsequently treated for 30 min with 5 µM BA (CK) alone or in combination with 25, 50, 100 µM PA; 50 µM linoleic acid (LA) (mean values ± SE; $n = 3$). Letters indicate significant differences at $P < 0.05$ level

fatty acid moiety. When tested alone, neither PA nor LA could strongly modify the *CrRR3* transcript level, the slight effect observed with 25- and 100-µM PA concentration considered weakly significant (Figure 4). Moreover, according to the results reported in Figure 2, the *CrRR1* transcript level was not significantly affected in the same conditions (Supplementary Figures S1 and S2).

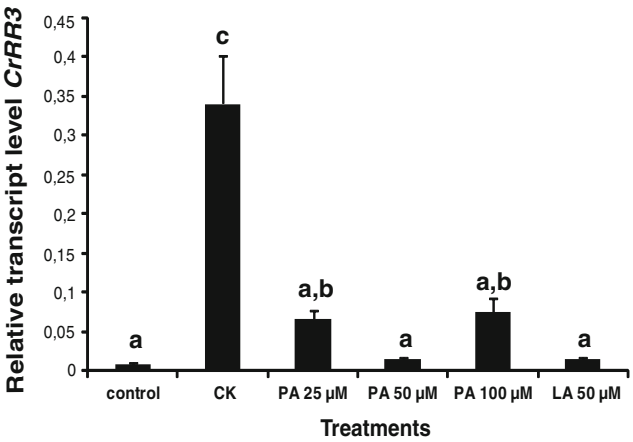

Fig. 4 Effect of of phosphatidic acid (PA) and linoleic acid (LA) on *CrRR3* transcript level under standard conditions. Three-day-old cell cultures were treated for 30 min with 25, 50, 100 µM PA; 50 µM linoleic acid (LA); 5 µM BA (CK; as a control of transcription under CK) or untreated (control) (mean values ± SE; $n = 3$). Letters indicate significant differences at $P < 0.05$ level

Discussion

Our data, obtained through accurate quantification of transcripts levels, show that two response regulators are differentially regulated in periwinkle cells treated by CK: *CrRR1* is strongly and transiently upregulated, whereas *CrRR3* exhibits a rather different expression profile, with a rapid but weaker increase of transcripts that remained unchanged for a longer period. Interestingly, these results are in accordance with those previously reported in *Arabidopsis* (D'Agostino and others 2000) where *ARR4-ARR7* and *ARR8-ARR9* showed transcript levels and kinetic expression profiles highly resembling that of *CrRR1* and *CrRR3*; moreover, CrRR1 and CrRR3 revealed sequence homologies and domain structure organizations closely similar to ARR4 and ARR8, respectively. In *Arabidopsis*, ten type-A response regulators have been identified, which fall in five pairs of highly related sequences, a consequence of a probable duplication event in the *Arabidopsis* genome (D'Agostino and others 2000). Data obtained through Northern blot experiments (Kiba and others 1999; D'Agostino and others 2000) or microarray analysis (Rashotte and others 2003; Brenner and others 2005; Lee and others 2007) indicate that most of the genes encoding type-A response regulators are rapidly induced by CK. Nevertheless, differences in the spatiotemporal expression patterns exist among the five pairs of type-A ARRs (To and others 2004). This suggests that although a partial functional redundancy may exist within the different members of the type-A response regulator family, precise control of their individual transcription in response to the CK signal could account for specific functions. The role of type-A ARRs remains poorly understood but most of them are considered negative

regulators of CK signalling (Hwang and Sheen 2001; To and others 2004). In this case, a strong and transient upregulation should account for an efficient feedback mechanism.

We showed that inhibition of PLD-dependent PA production differentially affected the expression of genes encoding two type-A response regulators after CK treatment, with an approximately 50-65% reduction of *CrRR3* transcript levels, whereas the *CrRR1* transcripts were not significantly changed. Of course, primary alcohols may modify other elements besides PLD in the membrane environment (Wang 2000) and PA is also produced through sequential action of phospholipase C (PLC) and diacylglycerol kinase (DGK) (Testerink and Munnik 2005). Nevertheless, our results indicate that as previously reported for *Arabidopsis* (Romanov and others 2002), PLD could participate in CK signalling in our model and suggests that multiple PLD-targeted elements may be involved. The mechanism(s) that could explain the implication of PLD in CK signalling remain(s) largely unknown, but it is conceivable that phosphatidic acids released from the membrane may contribute. Our data are consistent with this hypothesis and show for the first time the possible implication of PA in CK signalling. The effects are rapidly observed and dose-dependent, suggesting that the targets are likely pre-existing elements that do not require *de novo* synthesis in the cells. These elements could participate in either the canonical two-component multistep CK signalling pathway or another PLD-dependent pathway and have separate or coordinating effects on gene transcription. In addition, the targets of PLD activity should control the differential transcription of type-A response regulator genes, either by activating their promoters or by relieving them from a putative repressor. Previous data have reported that an increase in free linoleic acid could be associated with phospholipase D-mediated hydrolysis of phospholipids in wounded castor bean leaves (Ryu and Wang 1998), suggesting that other lipids besides PA may explain the effects of PLD activity on CK signalling. Our results do not corroborate these findings as exogenous supply of linoleic acid could not mimic the restoration of *CrRR3* transcription in periwinkle cells treated simultaneously with CK and butan-1-ol.

The role of PLD in plants has been documented mainly in stress and hormonal responses (Wang 2002; Bargmann and Munnick 2006). Absicisic acid (ABA) signalling has been investigated in more detail and it seems that membrane recruitment and/or modification of the activity of target proteins could represent two important modes of action of phosphatidic acids released through PLD activity (Testerink and Munnik 2005). Other works showed that PA tethers ABI1, a repressor of the ABA response, to the plasma membrane and prevents its shuttling to the nucleus (Zhang and others 2004); moreover, PLD targets GPA1, a G^{α} subunit of a heterotrimeric G protein likely involved in ABA perception, thus creating a bifurcation in the pathway (Mishra and others 2006). Ethylene is another hormone implicated in the responses of plants to various stresses. In ethylene signalling, the gaseous hormone is perceived by an HK receiver located at the reticulum membrane and immediately downstream the receiver, a MAPKKK (CTR1), functions as a negative regulator of the signalling pathway (Ouaked and others 2003). Recent works in *Arabidopsis* showed that PA binding to CTR1 resulted in almost complete inhibition of CTR1 kinase activity and disrupted the intramolecular interaction between the CTR1 kinase domain and the CTR1 N-terminal regulatory domain. In addition, PA blocked the interaction of CTR1 with ETR1, one of the ethylene receptors (Testerink and others 2007).

In CK signalling, the role of PLD activity remains to be elucidated. Interestingly, recent works in *Arabidopsis* show that the HK receivers not only perceive the CK signal but also mediate other stress responses, especially through negative regulation of ABA signalling, and that type-A ARR4 and ARR7 may operate in this feedback control (Tran and others 2007). This provides evidence that crosstalk exists between CK, ABA, and stress signalling pathways. As the first steps in CK signalling implicate the recruitment of HPt proteins at the plasma membrane where they are phosphorylated through interaction with the histidine kinase receivers prior to their transfer in the nucleus, it is conceivable that the membrane environment plays a central role during these early steps and that any variation of PA concentration would certainly affect the downstream events of the signalling cascade. We are currently addressing this hypothesis in periwinkle cells through RNA interference targeting *CrRR1* and *CrRR3*.

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