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## Biochemical characterization of phospholipases C from *Coffea arabica* in response to aluminium stress

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### ABSTRACT

Signal transduction in plants determines their successful adaptation to diverse stress factors. Our group employed suspension cells to study the phosphoinositide pathway, which is triggered by aluminium stress. We investigated about members of the PI-specific phospholipase C (PLC) family and evaluated their transcription profiles in *Coffea arabica* (Ca) suspension cells after 14 days of culture when treated or not with 100  $\mu$ M AlCl<sub>3</sub>. The four *CaPLC1-4* members showed changes in their transcript abundance upon AlCl<sub>3</sub> treatment. The expression profiles of *CaPLC1/2* exhibited a rapid and transitory increase in abundance. In contrast, *CaPLC3* and *CaPLC4* showed that transcript levels were up-regulated in short times (at 30 s), while only *CaPLC4* kept high levels and *CaPLC3* was reduced to basal after 3 h of treatment. *CaPLC* proteins were heterologously expressed, and *CaPLC2* and *CaPLC4* were tested for *in vitro* activity in the presence or absence of AlCl<sub>3</sub> and compared to *Arabidopsis* PLC2 (*AtPLC2*). A crude extract was isolated from coffee cells. *CaPLC2* showed a similar inhibition (30%) as in *AtPLC2* and in the crude extract, while in *CaPLC4*, the activity was enhanced by AlCl<sub>3</sub>. Additionally, we visualized the yellow fluorescent protein PH domain of human PLC $\delta$ 1 (YFP-PH<sub>PLC $\delta$ 1</sub>) subcellular localization in cells that were treated or not with AlCl<sub>3</sub>. In non-treated cells, we observed a polar fluorescence signal towards the fused membrane. However, when cells were treated with AlCl<sub>3</sub>, these signals were disrupted. Finally, this is the first time that PLC activity has been shown to be stimulated *in vitro* by AlCl<sub>3</sub>.

### 1. Introduction

Phospholipids are key structural components of biological membranes and have a prominent role in plant signalling cascades in response to adverse biotic and abiotic conditions [1,2]. The most abundant phospholipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [3], while phosphoinositides (PIs), such as phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] [4,5], are also present in large amounts. Phospholipids can be degraded by phospholipases, which hydrolyse them into various products, including diacylglycerol (DAG), phosphatidic acid (PA), free fatty acids (FFAs), and lysophospholipids (LPLs) by phospholipase C (PLC), phospholipase D (PLD) and phospholipase A (PLA), respectively, based on the site of glycerophospholipid hydrolysis [6,7]. Of these enzymes, only PLC and PLD are able to produce important second messengers that regulate various

plant responses [6–11].

Plant PLCs can be divided into three groups according to substrate specificity and cellular function: phosphoinositide-specific PLC (PI-PLC or PLC), which hydrolyses phosphoinositides [12], nonspecific PLC (NPC), which preferentially acts on the common phospholipids, such as PC and PE [13], and glycosylphosphatidylinositol (GPI)-PLC, which hydrolyses GPI-anchored phosphoinositides [14]. PI-PLCs produce inositol 1,4,5-trisphosphate [I(1,4,5)P<sub>3</sub>] and diacylglycerol (DAG) as the final products. DAG can subsequently be phosphorylated by a diacylglycerol kinase (DGK) to PA, which is subsequently phosphorylated to produce diacylglycerol pyrophosphate (DGPP), both of which have been identified as possible second messengers in plants [7].

Using *in vivo* labelling with <sup>32</sup>P<sub>i</sub>, it was previously demonstrated that aluminium stress reduced PA levels by almost 30% [15]. This was not due to the PLD pathway, as it had no effect on the formation of phosphatidylbutanol (PBut) in the presence of n-butanol, but was due to PLC

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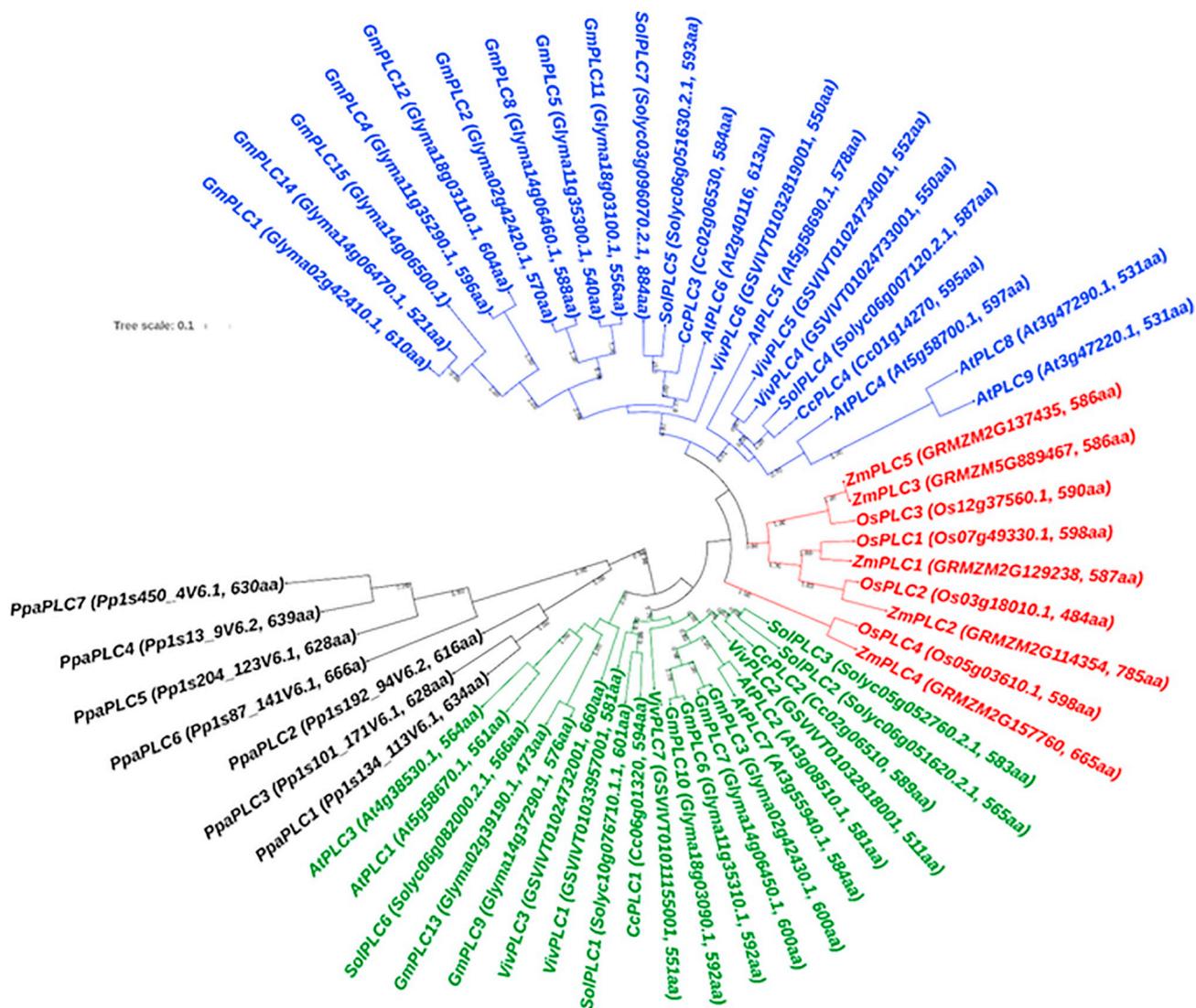
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**Fig. 1.** Phylogenetic relation between plants PLCs. The phylogenetic tree was made using the maximum parsimony method, tested with 1000 bootstrat replicates and displayed using MEGA 7. The tree shows four well-defined clades. In blue and green are the PLCs from dicotyledonous plants, separated by PLCs from monocotyledons plants (in red) or the outgroup (the moss *Physcomitrella patens* (black)). The numbers at the nodes are the bootstrap values (> 50%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

since the inhibitor U73122 (1-[6-[[[(17 $\beta$ )-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) was able to inhibit the same proportion of PA [15], suggesting a role for PLC in the signalling pathway triggered by aluminium stress.

Considering these precedents, we analysed the molecular and biochemical roles of PLC from *Coffea arabica* suspension cells in response to aluminium stress.

## 2. Materials and methods

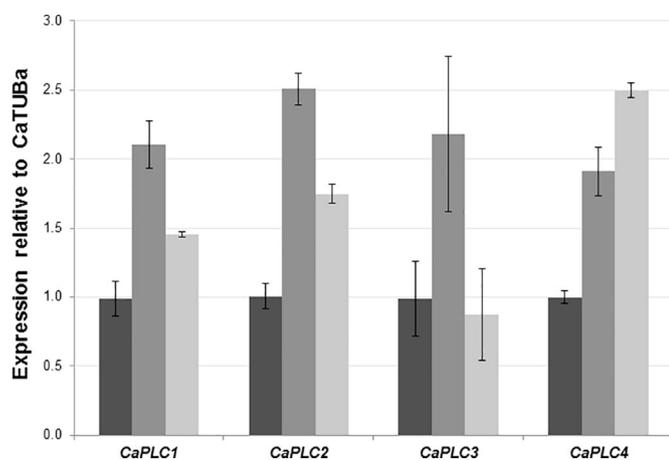
### 2.1. Cell culture and aluminium treatment

Coffee (*C. arabica* L.) cv. Catuai suspension cells were obtained by callus desegregation and were subcultured every 14 days at 25 °C in the dark at 100 rpm in a modified Murashige and Skoog (MS) medium [16] using half the ionic strength and pH 4.3; the medium was supplemented with 3% sucrose, 140  $\mu$ M cysteine-HCl, 560  $\mu$ M myo-inositol, 30  $\mu$ M thiamine-HCl, 13.6  $\mu$ M 2,4-dichlorophenoxyacetic acid and 4.4  $\mu$ M 6-benzyl-aminopurine [17]. Cells from the 14th day of culture in MS medium pH 4.3 were then treated or not with the same solution used to dissolve AlCl<sub>3</sub> (control) or were challenged with 100  $\mu$ M AlCl<sub>3</sub>. All

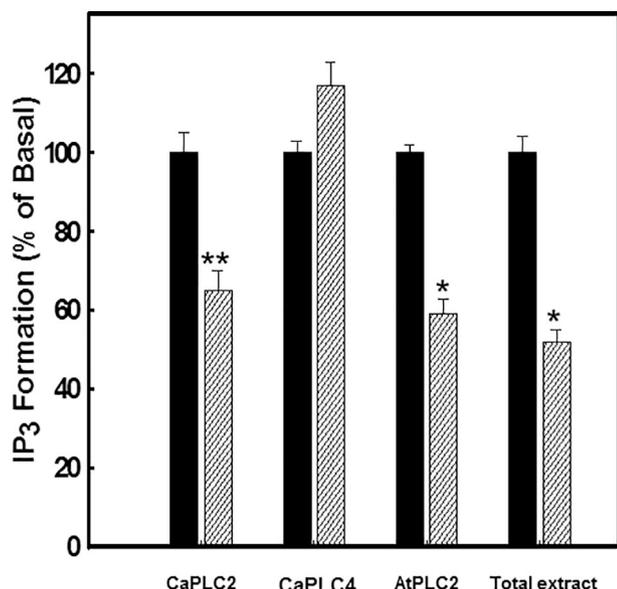
treatments were carried out at 25 °C on a shaker at 100 rpm in the dark for 3 h. After treatment, the cells were harvested, frozen in liquid nitrogen, and stored at -80 °C until RNA or protein extraction.

### 2.2. Phylogenetic analysis

For phylogenetic analysis, *Coffea* PLC sequences were obtained from the SOL Genomics Network database (<https://solgenomics.net>). The sequences were aligned using ClustalW and displayed with MEGA 7. The maximum parsimony method was employed, and a robustness of 1000 bootstrap replicates was tested. A phylogenetic tree was created for PLC proteins from dicotyledon plants, such as tomato (*Solanum lycopersicum*; SOL Genomics Network database, <https://solgenomics.net>), grapevine (*Vitis vinifera* L.; PlantGDB, [www.plantgdb.org](http://www.plantgdb.org)), soybean (*Glycine max* L.; PlantGDB, [www.plantgdb.org](http://www.plantgdb.org)) and *Arabidopsis* (*Arabidopsis thaliana* L.; TAIR, <https://www.arabidopsis.org/>), and from monocotyledons such as rice (*Oriza sativa* L.; PlantGDB, [www.plantgdb.org](http://www.plantgdb.org)) and maize (*Zea mays* L.; PlantGDB, [www.plantgdb.org](http://www.plantgdb.org)), as well as an outgroup—the moss (*Physcomitrella patens*; PlantGDB, [www.plantgdb.org](http://www.plantgdb.org)).



**Fig. 2.** PLC transcription profile in response to aluminium stress. Coffee suspension cells were incubated or not (■) for 30 s (▒) or 3 h (□) with 100  $\mu$ M  $\text{AlCl}_3$ . Their relative expression for CaPLC1 (Cc02g06510), CaPLC2 (Cc06g01320), CaPLC3 (Cc02g06530) and CaPLC4 (Cc01g14270) is shown in relation to TUBa as an endogenous control. The experiments were performed three-times independently, with similar results.



**Fig. 3.** Effect of  $\text{AlCl}_3$  treatment on *in vitro* PLC activity. Purified PLCs expressed in *E. coli* from *Coffea* (Ca), *Arabidopsis* (At), or total extract from *C. arabica* cells (crude extract) were incubated in the presence (▨) or absence (■) of 100  $\mu$ M  $\text{AlCl}_3$ . The PLC activity was measured after 3 min of exposure. The results are expressed as a percentage of PLC activity without treatment, which were 0.039, 0.108, 1.7 and 1.4 nmol/min/mg for CaPLC2, CaPLC4, AtPLC2 and crude extract from suspension cells. Each data point represents the mean  $\pm$  SE of duplicate assays from 3 to 5 independent preparations, \* $p < 0.001$ , \*\* $p < 0.05$ .

### 2.3. PLC transcript evaluation and data analysis

RNA was isolated using TRIZOL<sup>®</sup> RNA reagent (Invitrogen) and subjected to treatment with DNase I (Thermo Scientific), in accordance with the manufacturer's instructions. For RT-qPCR, cDNA was synthesized using 200 ng of total RNA with Revert Aid Reverse Transcriptase (Thermo Scientific), and the amplification was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) in a PikoReal 24 real-time PCR system (Thermo Fisher Scientific, Ratatsie 2, FI-01620 Vantaa, Finland). Primers were based on coffee sequences (*Coffea canephora* CDS v1.0) reported in the Sol Genomics Network database. The mRNA integrity was validated, its concentration running

in agarose gels was readjusted and all PCR products were stable in each sample. The PCR protocol was as follows: 1) initial denaturation step at 95 °C for 10 min, 2) two-step cycling at 95 °C for 40 s, followed by the  $T_m$  (°C) for 40 s for a pre-determined number of cycles in accordance with each gene (Table S1), and 3) a final melting curve step from 60 °C to 95 °C. We utilized a two-step method for RT-qPCR amplification, and the relative amount of each transcript was calculated by the  $2^{-\Delta\Delta CT}$  method using TUBa as an internal control.

### 2.4. Protein isolation and enzymatic activity from heterologously expressed PLCs

The PLC coding sequences were subcloned into the pColdII vector (Takara, Japan) using primers with *NdeI*/*BamHI* or *EcoRI*/*SaI* restriction sites (see Table S1), with an N-terminal polyhistidine tag (6  $\times$  His). Coffee PLC proteins were heterologously expressed using *E. coli* BL21star. The cells were transformed with each plasmid and grown in LB medium containing 100  $\mu$ g/mL ampicillin at 37 °C until an  $\text{OD}_{600}$  of 0.6 was reached. Cells were then induced by adding 1 mM isopropyl-1- $\beta$ -thiogalactoside (IPTG). After 12 h at 18 °C, the cells were collected by centrifugation and resuspended on ice in lysis buffer (50 mM Tris/pH 7.9, 0.5 M NaCl, 8% sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% triton X-100) to which 50  $\mu$ g/mL lysozyme and phenylmethylsulfonyl fluoride (PMSF) 1 mM were added and incubated at 4 °C for 30 min. The cells were put in freeze-thaw cycles twice using liquid nitrogen and sonicated ten cycles by 30 s with 1 min stands on ice, followed by centrifugation at 20,000  $\times g$  for 45 min at 4 °C. The supernatant fraction was purified using a Ni-NTA (Nickel-nitriloacetic acid) agarose column equilibrated previously with lysis buffer. The column was sequentially washed with 5 mL of lysis buffer containing 10, 50 and 100 mM imidazole, and the proteins were eluted in fractions using 1 M imidazole and adding immediately 1 mM EDTA. The fractions were dialysed in storage buffer (20 mM Tris-HCl pH 4.7, 100 mM NaCl, 1 mg/mL Leupeptin, 1 mM dithiothreitol (DTT), 2 mM EDTA) and then analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the bicinchoninic acid (BCA) method [18].

PLC activity was assayed as reported by [19] De los Santos-Briones et al. (1997) with slight modifications. The hydrolysis of [ $^3\text{H}$ ]-PI(4,5) $\text{P}_2$  was measured in a reaction mixture of 50  $\mu$ L containing 35 mM  $\text{NaH}_2\text{PO}_4$  (pH 6.8), 70 mM KCl, 0.8 mM Ethylene Glycol Tetraacetic Acid (EGTA), 0.8 mM  $\text{CaCl}_2$  (final concentration 25  $\mu$ M), 200  $\mu$ M PI(4,5) $\text{P}_2$  (containing between 30 and 50  $\mu$ g of protein and approximately 333 Bq of [ $^3\text{H}$ ]-PI(4,5) $\text{P}_2$ ) and 0.08% deoxycholate. After incubation at 30 °C for 10 min, the reaction was stopped with 100  $\mu$ L of 1% (w/v) bovine serum albumin (BSA) and 250  $\mu$ L of 10% (w/v) trichloroacetic acid (TCA). The precipitate was removed by centrifugation (13,500  $\times g$  for 10 min), and the supernatant was collected to quantify the [ $^3\text{H}$ ]-I(1,4,5) $\text{P}_3$  by liquid scintillation counting (ACS by Amersham Ltd.) [20]. Heterologously expressed PLC or a total protein extract from *C. arabica* suspension cells was incubated with water (mock) or 100  $\mu$ M  $\text{AlCl}_3$  for 3 min after which the reaction was initiated by adding the substrate (PI(4,5) $\text{P}_2$ ) as described above.

### 2.5. Protoplast isolation and transient transformation

Protoplasts from *C. arabica* suspension cells were prepared as described previously [21], with minor modifications. Briefly, after 14 days of culture, the cell suspensions were collected by filtration and were washed twice with fresh MS. Next, one gram of suspension cells was gently shaking in 10 mL of enzyme solution A (1% macerozyme R-10 (Sigma Aldrich), 1% cellulase (Sigma Aldrich), 0.2% pectinase (Sigma Aldrich), 450 mM sorbitol, 123 mM KCl, 87  $\mu$ M sucrose, 6 mM  $\text{CaCl}_2$ , 10 mM  $\text{NH}_4\text{NO}_3$  and 624  $\mu$ M  $\text{KH}_2\text{PO}_4$  at pH 4.3) and then incubated at 25 °C on a shaker at 100 rpm in the dark for 16 to 18 h. Protoplasts were collected by slow decantation and gentle shaking with

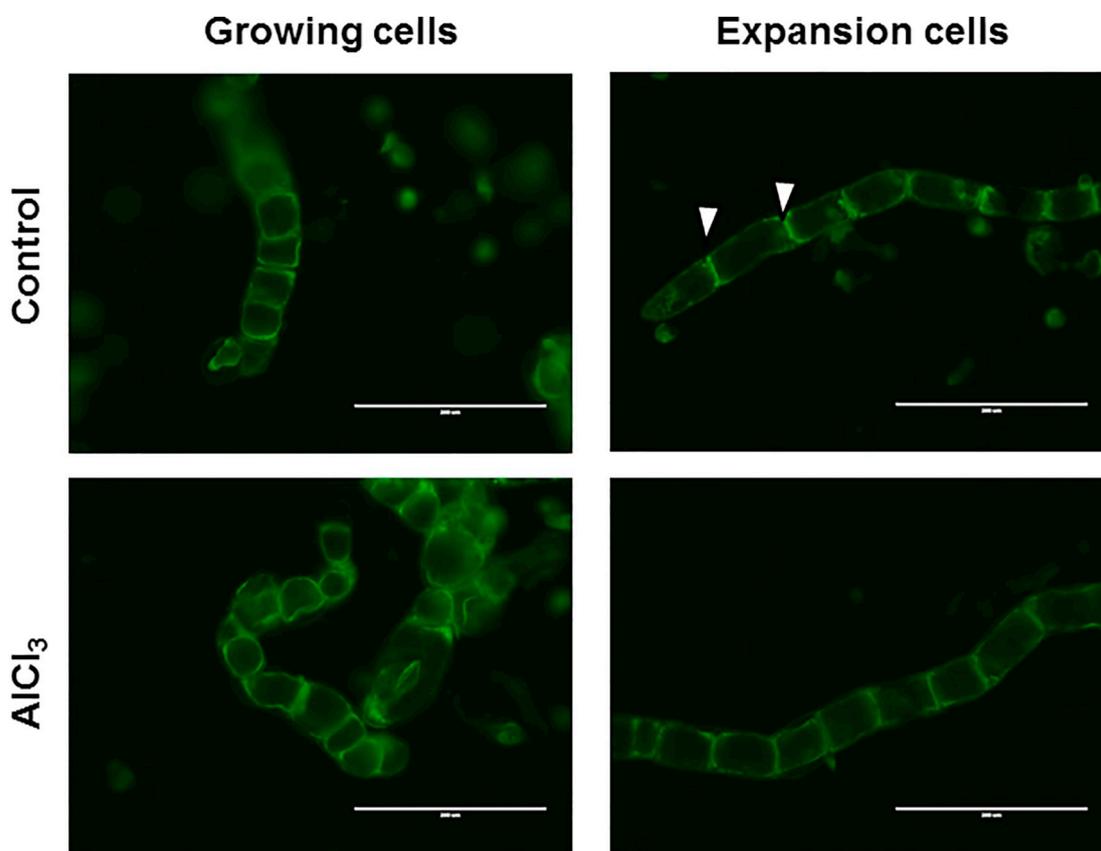


Fig. 4. YFP-PHPLC<sub>S1</sub> distribution in response to AlCl<sub>3</sub> treatment. *C. arabica* suspension cell protoplasts were transfected with the PIP<sub>2</sub> biosensor YFP-PHPLC<sub>S1</sub>. At 3 days post-transfection, they were exposed or not to 100 μM AlCl<sub>3</sub>. The arrow denotes the polar extreme in the cellular expansion. Bar = 200 μm.

10 mL of fresh solution A. The samples were then pelleted by centrifugation (100 × g for 10 min at 18 °C) and washed twice in fresh solution A. Finally, protoplast viability was determined using a microscope (data not shown).

Protoplasts were transiently transformed using the polyethylene glycol (PEG)-mediated gene transfer method [22]. Freshly obtained protoplasts were incubated for at least 30 min on ice, pelleted by centrifugation (100 × g for 5 min, 18 °C), and gentle shaking in 10 mL of solution A. For transformation with the PI(4,5)P<sub>2</sub> biosensor (van Leeuwen et al. 2007) [23], 10 μL of plasmid DNA (1000 ng/μL YFP-PHPLC<sub>S1</sub>) was added to the sample, followed by gentle shaking and incubation on ice for 10–15 min. After incubation, the transfection reaction was initiated by adding 10 mL of a calcium-PEG solution (10% PEG 4000, 200 mM mannitol, 100 mM CaCl<sub>2</sub>) and mixed by gentle shaking. The process was enhanced by short (30–40 s) heat-shock pulses at 37 °C that were immediately followed by a cool water incubation (approximately 18 °C) with gentle shaking. Approximately 10 cycles of heat-shock followed by cool incubation were applied. To stop the transfection, 20 mL of MS medium (pH 5.8) was added, and the sample was pelleted by centrifugation (100 × g for 5 min at 18 °C) in a stepwise fashion to remove the PEG, followed by two additional washes. Transfected protoplasts were grown for 3 days so that cell walls reappeared, and the cells were then treated with water (mock) or 100 μM AlCl<sub>3</sub> for 1 h. The fluorescence was then observed with an epifluorescence microscope (EVOS Fluid Cell Imaging Station).

### 3. Results

#### 3.1. Current view on PLC transcription profiles versus AlCl<sub>3</sub> treatments

To analyse the effect of AlCl<sub>3</sub>, we first defined how many PLC genes exist in the coffee genome. Using BLAST, PLC sequences from

*Arabidopsis* were employed to query the genomes in the database (SOL Genomics Network database), and at least 4 homologs in *Coffea canephora* were found. Subsequently, the phylogenetic relationships among PLCs from coffee versus other plant PLC sequences were tested to rename the PLC genes as the outgroup; the moss *Physcomitrella paten* was used to accomplish this (Fig. 1). The generated phylogenetic tree showed four well-defined clades. The coffee PLC sequences showed high similarity with the tomato PLC sequences and were named according to tomato PLCs. An exception was CaPLC3, which is a homolog to SolPLC5 from tomato but was renamed as CaPLC3 to adhere to an enumeration order. The first clade contained almost all PLC4s, PLC5s and PLC6s (from *Arabidopsis*, tomato, grapevine and coffee; Fig. 1 in green), and in a second clade, almost all PLC1s, PLC2s, PLC3s and PLC7s were grouped (from *Arabidopsis*, tomato, grapevine and coffee, Fig. 1 in blue); these were from dicotyledon plants species. There were some exceptions, such as all soybean PLC sequences, SolPLC6, SolPLC7, AtPLC8, AtPLC9 and CaPLC3. Finally, two clades were grouped separately containing the PLCs from monocotyledon species (Fig. 1 in red) and from the moss *Physcomitrella paten* (Fig. 1 in black).

To test whether any PLC members could be related to aluminium stress events, PLC transcription profiles were evaluated in coffee suspension cells after 14 days of culturing were treated or not with 100 μM AlCl<sub>3</sub> for 30 s or 3 h. Previously, we validated TUBa as a stable internal control gene tested in other Tm and analysed all melting curves for each sample. We calculated the control normalization error in all points (TUBa untreated vs each treatment Fig. 2), and tested other genes like translation elongation factor 1-alpha (EF1α) (data not shown) [24]. Subsequently, RT-qPCR analysis was performed to determine the expression levels of the coffee CaPLC1-4 genes relative to expression levels of CaTUBa. Changes in differential expression patterns were observed for all CaPLC1-4 genes (Fig. 2); however, each individual CaPLC1-4 genes showed particular patterns. Changes upon a 30 s

exposure could be related to the coffee suspension cell manipulation or to a general stress response, thereby helping to discriminate those changes associated only with aluminium stress events. Both *CaLCL1* and *CaPLC2* genes showed dramatic and transitory changes in their transcript abundance (Fig. 2), where an increased expression of over two-fold for 30 s and half-fold for 30 h with 100  $\mu$ M  $AlCl_3$  treatments were observed, at different expression levels, compared to control (water). In contrast, the transcript levels of *CaPLC3* and *CaPLC4* showed that they were up-regulated in short times (at 30 s), while only *CaPLC4* kept these high levels (at 3 h), compared to control (Fig. 2). Both *CaPLC3* and *CaPLC4* genes showed a dramatic increase in expression of over 2-fold (at 30 s), but the transcript level of *CaPLC3* was reduced to basal after 3 h of  $AlCl_3$  treatment similar to the coffee suspension cell cultures that were untreated.

The changes in the PLC expression profile during  $AlCl_3$  treatment appeared to be consistent and substantial.

### 3.2. Specific activity of recombinant coffee PLCs

Previously, our group reported the  $PI(4,5)P_2/PLC$  activity using a total protein extract from coffee cell suspensions [17]. This activity has also been reported for extracts from the shoots and roots of *C. arabica* cv. Typica seedlings [25]. However, no studies have investigated the purified coffee PLC activities, until now. All coffee PLC genes were cloned, and the proteins expressed in *E. coli*. The proteins were affinity purified using a Ni-NTA column. To accomplish this, each PLC was subcloned into the pColdII vector via a double digestion with *NdeI/BamHI* or *EcoRI/SalI* (see Table S1), followed by ligation, transformation into *E. coli* DH5 $\alpha$  and PCR corroboration. Protein expression and affinity purification was successful for all CaPLCs, with *CaPLC3* being expressed at a low abundance (Fig. S1).

The effect of  $AlCl_3$  on the *in vitro* PLC activity of heterologous proteins, the recombinant *CaPLC1* and *CaPLC3* proteins had either lost or displayed poor activity after purification; hence, attention was focused on *CaPLC2* and *CaPLC4*. Compared to *Arabidopsis AtPLC2*, the specific activity of *CaPLC2* and *CaPLC4* was lower (Fig. 3). Performing activity assays in the presence of  $AlCl_3$  revealed that *CaPLC2* and *AtPLC2* were inhibited to a similar degree (showing an approximately 30% decrease). In contrast, *CaPLC4* showed an amelioration in the presence of  $AlCl_3$  (Fig. 3).

We next tested whether the distribution of the  $PI(4,5)P_2$  biosensor YFP-PH<sub>PLC $\delta$ 1</sub> [23] was affected by  $AlCl_3$ . As coffee cells are not suitable for *Agrobacterium* transformation, a transient PEG-mediated gene-transfer method was used, which is highly amenable to protoplast studies [26–28]. At 3 days post-transfection, cells were either treated or not with the same solution to dissolve  $AlCl_3$  (control) or they were challenged with 100  $\mu$ M  $AlCl_3$ . In the control cells, the YFP fluorescence was localized in the cellular membranes of growing cells. Upon changing to a cell expansion state, the fluorescence was principally visualized in the polar extremes (Fig. 4). [23] Van Leeuwen et al. (2007) reported that YFP-PH<sub>PLC $\delta$ 1</sub> can accumulate in the fused membranes of B-Y2 cells during cell division, and the  $PI(4,5)P_2$  biosensor was also localized in the polar extremes of growing root hairs, which is effectively similar to our results. The cells treated with  $AlCl_3$  did not reveal significant changes in the fluorescence of growing cells. In contrast, YFP polarization was disrupted in expanding coffee cells when treated with  $Al^{3+}$ , probably due to arrested cellular growth (Fig. 4). These data show that aluminium stress in coffee cells disrupts the  $PI(4,5)P_2$  distribution towards the fused membranes.

## 4. Discussion

When studying about aluminium toxicity, we show that aluminium stress is commonly associated with a phytotoxic role in plants, while used in low concentrations, it could show a stimulating effect on growth [25,29], or increase plant fitness [29]. With respect to PLC-DGK

pathways, it is well documented that PLC, when exposed to aluminium phytotoxic concentration, showed an inhibited PLC activity [17], while DGK has been reported to increase to abiotic factors like freezing [30], at low concentration of chemical elements [31] and  $AlCl_3$  treatment [17]. Taking into account this background in the present work, we focused only in aluminium effect into PLC proteins.

At present, only a few plant PLCs have been biochemically characterized *in vitro*, including wheat [32], soybean [33], *Catharanthus roseus* [20], potato [34], *Arabidopsis* [35], *Nicotiana rustica* [36] and tomato [37]. Coffee was found to contain four PLC genes; these most closely resembled those of tomato. The transcript abundance of the 4 coffee PLCs were differently affected by  $AlCl_3$  and suggested that all coffee PLCs can potentially be rapidly activated by an environmental trigger without *de novo* transcription. This was described by Kollist [38], who suggested that mechanisms at a seconds-minutes timescale played an important role in plant acclimation stress responses [38,39]. These authors proposed that *Arabidopsis* genes increase their transcript abundance up to 100-fold within minutes following stimulation stress, similar to a rapid stomatal response, which requires minutes or less to initiate and 5–10 min to be completed when was mediated by ROS wave mechanisms [38]. In coffee, *CaPLC1* and *CaPLC2* showed a rapid and transitory expression, possibly a general response, suggesting that they do not play a relevant role in aluminium stress events. The *CaPLC4* transcript profile showed an increased expression in  $AlCl_3$  treatments, suggesting a more relevant role in aluminium toxicity acclimation, while *CaPLC3* could have a different role [9,39,40]. Biochemical activity was found for *CaPLC2* and *CaPLC4*; *CaPLC2* was inhibited (approximately 30% decrease), and *CaPLC4* showed an amelioration in the presence of  $AlCl_3$ . The transcription profile suggests a key role for *CaPLC4* in an aluminium stress survival mechanism [15]. According to earlier reports, *CaPLC* can be free in the cytosol and/or associated with the plasma membrane [20,41]. *CaPLCs* could potentially be associated with the plasma membrane in normal conditions or free in the cytosol upon  $AlCl_3$  treatment in accordance with the  $PI(4,5)P_2$  biosensor distribution in a similar way to that reported for *PLC1* from *Petunia inflata* [42] and *PLC2* from *Arabidopsis* [35].

In the presence of  $AlCl_3$ , the  $PI(4,5)P_2$  biosensor distribution was disrupted in the fused membrane (Fig. 4). In others studies, when drugs that disrupted vesicle trafficking (brefeldin A) were applied in *Arabidopsis* seedling plants, endocytosis was inhibited, membrane fluidity was reduced, and the roots were shorter as compared to the control [3,40]. The  $PI(4,5)P_2$  biosensor distribution in coffee cells and the reduced cell elongation suggested a disruption in vesicle trafficking within the coffee cells treated with  $AlCl_3$  (Fig. 4; [3]). Finally, the *CaPLC* amelioration of  $AlCl_3$  toxicity suggests that the effect of aluminium occurs at the protein level. It remains to be tested whether an interaction exists between proteins in high aluminium toxicity conditions and if, in this way, plants can adapt to soils with moderate to high aluminium concentrations.

## 5. Conclusion

Coffee PLCs were found to display distinct transcriptional and biochemical effects in response to aluminium stress that may contribute to the phospholipid signalling responses triggered by aluminium stress in coffee cell suspensions. These findings also revealed the potential use of a protoplast transfection system to gain knowledge about phospholipid signalling and other processes in response to abiotic stressors. Finally, this is the first time that PLC activity has been shown to be stimulated by  $AlCl_3$ , which may reflect an adaptation strategy for *Coffea* species, enabling them to survive in adverse aluminium conditions.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jinorgbio.2019.110951>.

## Abbreviations

AtPLC2	<i>Arabidopsis</i> PLC2
Ca	<i>Coffea arabica</i>
CaPLC1-4	<i>Coffea arabica</i> phospholipase C
DAG	diacylglycerol
DGK	diacylglycerol kinase
DGPP	diacylglycerol pyrophosphate
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-1- $\beta$ -thiogalactoside
MS	Murashige and Skoog
Ni-NTA	Nickel-nitriloacetic acid
PI4P	phosphatidylinositol 4-phosphate
PI(4,5)P <sub>2</sub>	phosphatidylinositol 4,5-bisphosphate
PA	phosphatidic acid
PBut	phosphatidylbutanol
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PEG	polyethylene glycol
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
EF1 $\alpha$	translation elongation factor 1-alpha
TUBa	tubulin a
YFP-PH <sub>PLC<math>\delta</math>1</sub>	yellow fluorescent protein-PH domain of human PLC $\delta$ 1

## Declaration of competing interest

The authors declare that they have no conflicts of interest.

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