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Research article

Arsenic stress induces changes in lipid signalling and evokes the stomata closure in soybean

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ABSTRACT

Soybean (*Glycine max*) is often exposed to high arsenic (As) level in soils or through irrigation with groundwater. In previous studies on As-treated soybean seedlings we showed deleterious effect on growth, structural alterations mainly in root vascular system and induction of antioxidant enzymes. However, there are not reports concerning signal transduction pathways triggered by the metalloid in order to develop adaptive mechanisms. Phosphatidic acid (PA), a key messenger in plants, can be generated via phospholipase D (PLD) or via phospholipase C (PLC) coupled to diacylglycerol kinase (DGK). Thus, changes in PA and in an enzyme involved in its metabolism (PLD) were analysed in soybean seedlings treated with 25 μM AsV or AsIII. The present study demonstrated that As triggers the PA signal by PLD and also via PLC/DGK mainly after 48 h of As treatment. DGPP, other lipid messenger produced by phosphorylation of PA by PAK increased in As treated roots. Arsenic also induced rapid and significant stomatal closure after 1.5 h of treatment, mainly with AsIII, probably as an adaptive response to the metalloid to reduce water loss by transpiration. This report constitute the first evidence that shows the effects of As on lipid signalling events in soybean seedlings which would be crucial in adaptation and survival of soybean seedlings under As stress.

1. Introduction

Plants are constantly exposed to different environmental stresses and they can survive by modulating their metabolism and physiological response. Heavy metal contamination is one of the major abiotic stresses for plants. Arsenic (As) is a highly toxic metalloid and a non essential element for plants (Zhao et al., 2009). Inside cells, As stress causes adverse effects in several metabolic processes including photosynthesis, respiration, growth regulation and reproduction (Stoeva et al., 2003/2004). These toxic effects result in reduced germination and plant growth (Abedin and Meharg, 2002; Rahman and Naidu, 2009; Talano et al., 2013), and/or in the generation of reactive oxygen species (ROS), with the consequent peroxidation of membrane lipids and finally cell death.

Arsenic signal is sensed and transferred through pathways that eventually modulate transcription factors which help plant survival under As stress. Plants ability to survive with As depends on its capacity to sense stress signals and on its translation into suitable responses (Sarwat et al., 2013; Okazaki and Saito, 2014; Lin et al., 2014). In this sense, there are several signalling pathways involved in signal transduction in response to heavy metals (DalCorso et al., 2010). The signal transduction starts by sensing the heavy metal, leading to activation of metal responsive genes which results in reduction of toxic effects in plants (Maksymiec, 2007). Activation of stress related genes are known to involve certain signal transduction pathways such as MAPK phosphorylation cascade, calcium–calmodulin system, ROS signalling, and hormones (Islam et al., 2015).

However, early signalling events that involve lipid membrane signals remain unexplored to date. It has been shown that plants exposed to high metals/metalloids concentration increased stomata resistance, decreased transpiration rate and altered water relations (Singh, 2005). In addition, in cadmium and As stressed plants, a reduced water transport was caused by the obstruction of

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xylem vessels due to cell wall degradation product deposition. Furthermore, reduced water absorption is also due to a decrease of root growth and increased structural damage.

During stress response, the abscisic acid (ABA) plays an important role in mechanisms leading to the acclimation of changing environments. Under unfavourable environmental conditions, ABA accumulation induces stomata closure in the leaf epidermis and modules the expression of numerous genes in different tissues. Both, As stress and ABA have been associated (Huang et al., 2012) and they affected the behaviour of stomata apparatus, as ecological adaptations for plant protection (Gupta and Bhatnagar, 2015 and references therein). As a consequence, the transpiration rate and stomatal conductance is reduced and, hence, the photosynthetic efficiency of plants is affected (Stoeva et al., 2003/2004). ABA-signalling pathways are complex (Finkelstein, 2013). ABA, perceived by several receptors, induces the phosphorylation and dephosphorylation of many target proteins, relaying the signal via through multiple second messengers (Nakashima and Yamaguchi-Shinozaki, 2013). Among these, ABA-response messengers are lipid molecules, including phosphatidic acid (PA) and diacylglycerol pyrophosphate (DGPP). In *Arabidopsis* guard cells, PA can bind to ABI1 thus inhibiting its protein phosphatase activity (Zhang et al., 2004). Besides, genetic studies have shown that PA is involved in ABA signalling. Antisense phospholipase Dα (PLDα) and knockout PLDα1 constructs were used to show that PLDα participates in ABA-promoted stomata movements (Zhang et al., 2004; Okazaki and Saito, 2014). In plants, PA plays a key role as a valuable signal-transducing molecule (Testerink and Munnik, 2011). It may be generated by the hydrolytic activity of phospholipases, such as phospholipase D (PLD), or phospholipase C (PLC) followed by diacylglycerol kinase (DGK) (Wang et al., 2006; Arisz et al., 2013; Pokotylo et al., 2014). On the other hand, PA removal can be produced mainly by lipid phosphate phosphatase (LPP) and phosphatidate kinase (PAK) that forms diacylglycerol pyrophosphate (DGPP) (Racagni et al., 2008; Villasuso et al., 2013).

Soybean (*Glycine max*) is a legume of worldwide economic importance. In Argentina, it is often cultivated in soils with high As levels. Sometimes, it is irrigated with groundwater containing this metalloid because of the crop expansion to regions with low rainfall regime (Smedley et al., 2008; Bundschuh et al., 2012). In previous studies related to As-treated soybean seedlings, we showed a deleterious effect on growth, structural alterations in roots, such as dark deposits in vascular system and induction of antioxidant enzymes. We argue that these facts constitute strategies to avoid As translocation to the aboveground tissues as well as to efficiently scavenge ROS accumulation that could be related to adaptation and detoxification mechanisms induced by this metalloid (Armendariz et al., 2016). Although PA signal was associated with abiotic stress response in other plants (Xue et al., 2009), little is known about the dynamic of phospholipids (PLs) involved in signal transduction pathways induced by heavy metals or metalloids in soybean. Therefore, the aims of this study were to analyse changes in lipid kinases and PLD activities of soybean roots treated with As, as well as to determine whether As affects the stomata apparatus as a possible adaptive mechanism.

2. Materials and methods

2.1. Plant material and growth conditions

Soybean seeds (*G. max*) cv. DM 4670 were used. Seeds were disinfected using 70% (v/v) ethanol for 1 min and then 30% (v/v) sodium hypochlorite for 10 min, washed thoroughly with sterile distilled water, submerged in distilled water and incubated at 28 ± 2 °C with agitation for 24 h. After this process, seeds were kept for germination in Petri dishes with moistened filter paper at 28 ± 2 °C in the dark. After radicle emergence, seeds were placed in pots with perlite and irrigated by capillarity as they need with ½ Hoagland for 8 d. After that, seedlings were irrigated with distilled water (control) or with a single dose of solutions containing 25 μM sodium arsenate ($AsHNa_2O_4$ $7H_2O$) (SIGMA) (AsV) or sodium arsenite ($NaAsO_2$) (SIGMA) (AsIII) and then, they were harvested at 12, 24 and 48 h after treatment. Roots were separated and immediately frozen in liquid nitrogen to be used for further studies.

2.2. Preparation of membranes

Approximately 0.5–0.9 g of *G. max* roots (control and treated with AsV or AsIII) were thawed and homogenized in 10 volume of 50 mM HEPES (pH 7.4) containing: 0.25 M sucrose, 5 mM KCl, 1 mM EDTA and protease inhibitors. This suspension was frozen at −80 °C and thawed three times, homogenized in a glassy Teflon homogenizer and centrifuged at 1000 g for 15 min to remove unbroken cells and cell debris. Then, supernatant was centrifuged at 105,000 g for 60 min to obtain the membrane fraction. Membranes were washed and resuspended with 50 mM HEPES, pH 7.4. These membranes were used as a source of PLD and lipid kinases. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.3. Lipid kinase assay

The lipid kinase activities were simultaneously assayed using endogenous lipids as substrates, unless otherwise stated. The membrane fraction isolated (60 μg of proteins) was added to thermally equilibrated 50 mM HEPES buffer (pH 7.4) containing: 0.1 mM EDTA, 0.5 mM DTE, 10 mM $MgCl_2$, 0.2 mM sodium *o*-vanadate, 1 mM Mg^{2+}-ATP and [γ-^{32}P]ATP (specific activity 450 cpm/pmol) and protease inhibitors. Endogenous lipid phosphorylation was allowed to proceed for 2 min at 30 °C in a final volume of 100 μL. The reaction was subsequently stopped with 1.5 μL of chloroform/methanol (1:2, v/v).

2.3.1. Phospholipid extraction and separation

Lipids were extracted from membranes according to Racagni-Di Palma et al. (2002) and the PLs were separated by TLC. The samples were spotted on silica gel plates impregnated with potassium oxalate solution and heated at 110 °C for 60 min just before use. The TLC was developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). The radiolabeled lipids were visualized by autoradiography on Kodak film. The intensity of spots was quantified by densitometry of autographs using an image analyzer software (ImageJ).

2.4. PLD activity

PLD activity was measured as the production of phosphatidylbutanol (NBD-PtdBt) related to NBD-PA and NBD-PC (1-acyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine) levels and determined by TLC, as described below (Ibañez et al., 2016). NBD-PC was provided by Avanti Polar Lipids and stored at −80 °C in chloroform. Prior to use, it was dried under a stream of N_2 and suspended in an assay mixture. The standard assay mixture contained 20 mM MES-NaOH (pH 6.5), 50 mM $CaCl_2$, 0.25 mM SDS, 5 mL fluorescent substrate (NBD-PC, 10–50 μg), 1% (v/v) 1-butanol and 60 μg of proteins, in 40 μL of total volume. The reaction was initiated by the addition of the substrate and incubated at 30 °C for 30 min with shaking (100 rpm). The reaction was stopped by the addition of 150 μL chloroform:methanol (1:2, v/v), 40 μL chloroform and 40 μL 2 M

KCl. Then, the mixture was mixed and centrifuged at 15,000 g for 2 min. The phases were separated and 100 μL chloroform was added to the aqueous phase, mixed, and centrifuged at 15,000 g for 2 min, and the lower chloroform phases from each step were pooled. Each sample was dried under a stream of N_2 and resuspended in the minimum volume of chloroform:methanol (95:5, v/v) added and spotted onto TLC plates (silica gel G, Fisher Scientific) and developed with 2,2,4-trimethylpentane:acetic acid:H_2O:ethyl acetate (2:3:10:13, v/v). Fluorescence (excitation 460 nm, emission 534 nm) from lipids was measured in a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream Molecular Imaging) and quantified with ImageJ.

2.5. Effect of As on stomata total size and closure

Eight-days-old soybean seedlings, grown as mentioned before, were treated with distilled water (control) or with a single dose of 25 μM AsV or AsIII solution. For stomata analysis, a layer of acrylic (synthetic nail coating) was brushed onto the adaxial and abaxial sides of the leaf after 1.5, 4 and 24 h of treatment. Then, the acrylic layer was carefully extracted and mounted for microscopic analysis with glycerol/distilled water 1:10 (D'ambrogio de Argüeso, 1986). Three leaf blades were processed for each treatment. Histological preparations of stomata were assessed using a standard Zeiss model 16 microscope. Photomicrographs were taken with a Zeiss Axiophot microscope equipped with image capture and digitization (AxioVision 4.3, with camera AxioCam HRc 200× magnification). Stomata total area and ostiole size were counted using an Iproplus program for image analysis.

To estimate absorption rate of control and As-treated seedlings, the final time to absorb 250 mL of solution placed in a lower tray was registered, and the results were expressed as mL/h absorbed by each plant.

2.6. Statistical analysis

Results are the average of at least 3 independent replicates, performed in triplicate. Mean and standard errors of the evaluated parameters were calculated and plotted using the Microsoft Excel 2007 program. Analysis of variance test (ANOVA) was used to determine the statistical difference between at least one pair of means. To determine significant differences between treatments, *Duncan* test was applied, with a significance level of 0.05 ($p < 0.05$). The statistical program used was InfoStat (2012e version).

3. Results and discussion

3.1. Effect of As on lipid kinase activities

Plants develop different responses for adaptation to the surrounding environment and, for that, the signals coming from the outside must pass through the cell membrane to generate a response. Environmental stresses cause rapid and transient changes in the composition of membrane lipid signalling (Munnik and Testerink, 2009; Xue et al., 2009; Munnik and Vermeer, 2010). In order to know whether As stress affects the PLs dynamic in soybean roots, the formation of radiolabelled PLs such as PIP_2, PIP, DGPP and PA was determined after 12, 24 and 48 h of treatment. The effect of As was evaluated with both AsV and AsIII, since it has been shown that AsV is reduced to AsIII when taken by roots (Zhao et al., 2009).

As shown in Fig. 1A, three main PLs, PIP, DGPP and PA were observed, which result from lipid kinase activities such as PIKs (phosphoinositide kinases), PAK (phosphatidate kinase) and DGK (diacylglycerol kinase). In presence of As, activities of the above

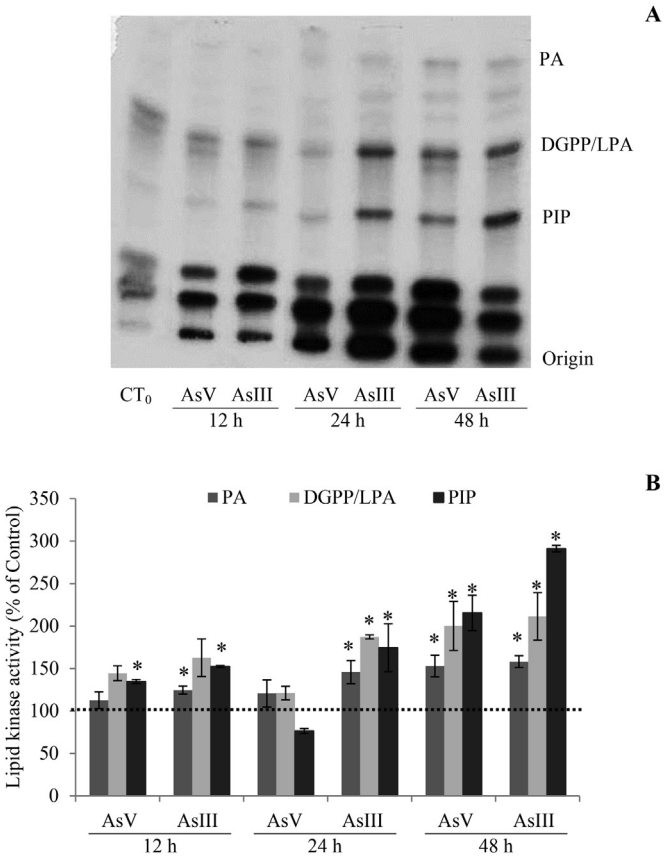

Fig. 1. A) Lipid products formed in the phosphorylation of soybean root membranes treated with AsV or AsIII (25 μM) and water as a control (CT_0) for 12, 24 and 48 h. Lipids were separated by TLC using an acid solvent system: chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). The radioactive lipids were detected by autoradiography. The figure shows a representative experiment of three repetitions carried out independently. B) Lipid kinase activities in membranes of soybean roots treated with AsV or AsIII (25 μM) and water for 12, 24 and 48 h. The lipid kinase activities were tested by determination of lipid products phosphorylated with [γ-^{32}P] ATP (arbitrary units) by image analysis with ImageJ software. PIP: phosphatidylinositol monophosphate; LPA: lysophosphatidic acid; DGPP: diacylglycerol pyrophosphate; PA: phosphatidic acid. The data represent the mean ± SE of three independent biological replicates (n = 3). Asterisks (*) represent significant differences from the control according to *Duncan's test* ($p < 0.05$).

mentioned enzymes increased and this was proportional to incubation time with the metalloid. At short exposure time (12 h), labelled PIP significantly increased under both As treatments, and PA level increased only with 25 μM AsIII (Fig. 1B). After 24 h of treatment, there were no significant changes in lipid kinase activities of AsV treated soybean roots, whereas in AsIII treated roots DGK, PAK and PIKs activities increased 46%, 87% and 74%, respectively. Finally, a marked increase in DGK, PAK and PIKs activities was observed after 48 h, corresponding to 53% and 58%, 100% and 111%, and 115% and 191% for AsV or AsIII treated roots, respectively, compared to control (Fig. 1B). It is important to note that there was a similar increase in PA, DGPP and PIP levels between seedlings treated with AsV and AsIII after 48 h. This behaviour could be explained by the fact that inside the cell, AsV is reduced to AsIII by arsenate reductase and hence, response at longer exposure time is the same for both As species because the prevalent ion would be AsIII.

In plants, PA plays a key role as a precursor in the biosynthesis of glycerophopsholipids (GPL) and triacylglycerols (TAG), and as an important signal-transducing molecule. In un-stimulated cells, PA levels are maintained low but in response to stress, its

accumulation can be increased by two main routes: through the hydrolysis of PC/PE catalysed by PLD or by the combined action of the phospholipase C (PI-PLC) and DGK (Peters et al., 2010; Arisz et al., 2013; Pokotylo et al., 2014). PA accumulation detected in soybean roots under As treatment, mainly after 48 h, indicates its involvement in signal transduction pathways during the response to the metalloid. Recently, an increase in PA levels through both pathways has also been reported in plants treated with an organic contaminant such as phenol (Sosa Alderete et al., 2012; Ibañez et al., 2016). In other reports, PA has been associated with induction of genes coding for antioxidant compounds and activation or inactivation of various target proteins (protein kinases, phosphatases, lipid kinases and lipid phosphatases, transcription factors, NADPH oxidase, etc.) upon biophysical effects on the plasma membrane. PA also serves as a substrate to other lipid regulators or it also regulates traffic or membrane biogenesis (Wang, 2004; Wang et al., 2006; Li et al., 2009; Testerink and Munnik, 2011). Moreover, different pathways responsible of attenuating PA signal have been described, such as phosphorylation of PA by catalytic action of PAK producing DGPP, which is other PL that increases in As treated roots. It has been described that DGPP increases under salt stress acting as a lipid messenger and modulating the response to ABA in rice and tobacco plants and in tomato cell suspensions ((Racagni et al., 2008; Arisz et al., 2009; Jeannette et al., 2010). DGPP role in PA switching off or its role as lipid signal has not yet been fully elucidated. Besides, genes coding for PAK, a key enzyme for the synthesis of DGPP have not been identified until now (Munnik and Vermeer, 2010).

We cannot rule out if PA could come from PLC activity since there is a large increase in PIK activity, which correlates with a PIP increase. The latter is phosphorylated by PIP4-K or PI4P5-K to PIP_2, that is substrate for PLC and finally produces IP_3 and DAG being then phosphorylated by DGK to PA. Since PIPs increased and there was no PIP_2 species, we inferred that a fraction of PA would come from this way. However, as mentioned above, PA increase may also be a consequence of PLD activity (Villasuso et al., 2013). Therefore, to determine whether this enzyme is involved in this response, PLD activity was determined by using NBD-PC as substrate on the same membrane fractions.

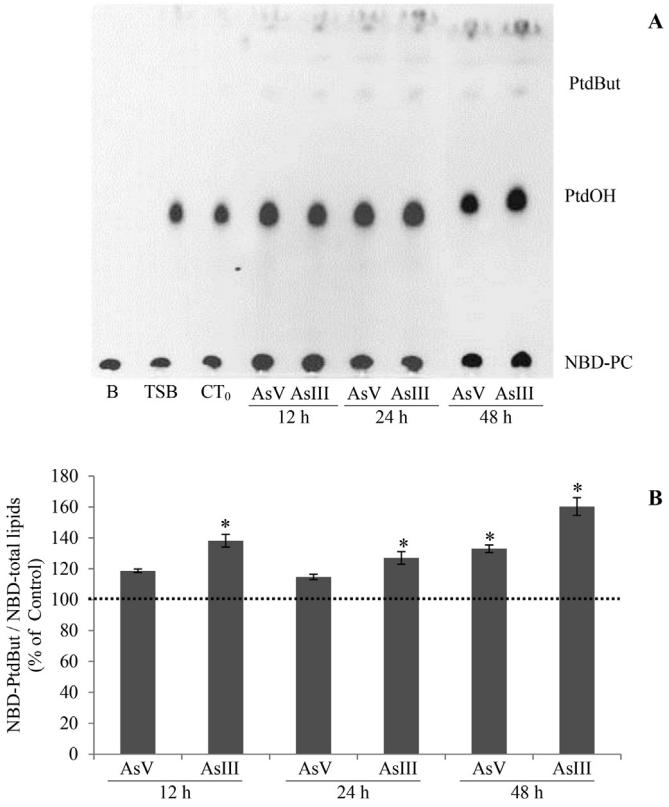

Fig. 2. A) Lipid products formed by PLD in membranes of soybean roots treated with AsV or AsIII (25 μM) and water as a control (CT_0) for 12, 24 and 48 h. B: control without proteins; TSB: control without 1-butanol,The reaction was determined with NBD-PC, lipids were extracted, plated on TLC and separated with 2,2,4-trimethylpentane: acetic acid: H_2O: ethyl acetate (2:3:10:13, v/v). PLD activity was determined as the formation of PtdBut. The figure shows a representative experiment of three repetitions carried out independently. B) PLD activity of soybean roots treated with AsV or AsIII (25 μM) and water as a control for 12, 24 and 48 h. It was expressed as NBD-PtdBut regarding to NBD-total lipids (% of control). The data represent the mean ± SE of three independent biological replicates (n = 3). Asterisks (*) represent significant differences from the control according to *Duncan's test* ($p < 0.05$).

3.2. Effect of As on PLD activity

PLD and PA have been linked to various cellular processes in plants, including: 1) abiotic stress, such as freezing, dehydration, drought, salinity, nutrient limitation, injuries or wounds and ROS; 2) biotic stress, such as pathogenic fungi and bacteria attacks and nodule formation, and 3) growth and plant development during seed germination, leaf senescence, seed ageing, pollen tube growth, root growth, etc (Li et al., 2009). However, little is known about effects of metals or metalloids on PLD activity and PA levels. To analyse PLD activity, we use the transphosphatidylation reaction, in which the presence of small amounts of a primary alcohol, like 1-butanol, determines the production of novel phosphatidylalcohol (PBut). Using this unique enzyme property, PA signalling can be uncoupled from PLD activity and the cumulative PLD activity assessed by quantifying the formation of PBut (Ghars et al., 2012).

Formation of PA (NBD-PtdOH) and phosphatidylalcohol (NBD-PtdBut) was detected in control and As stressed roots (Fig. 2A). As shown in Fig. 2B, PLD activity increased 38%, 27% and 60% at 12, 24 and 48 h after AsIII exposure compared with the control (100%). By contrast, in AsV treated roots, PLD activity increased significantly (33%) only after 48 h exposure (Fig. 2B). These results indicate that PLD activity of *G. max* roots was stimulated by As, mainly AsIII, and this activity increased at longer exposure time to the metalloid. Consistent with our results, Navari-Izzo et al. (2006) described that wheat seedlings treated with Cu^{2+} showed an increase in PA production via PLD, detecting enzyme activation after 1 min of contaminant exposure, followed by a doubling of PA levels after 15 min exposure and with a maximal accumulation after 2 h. Contrarily, Ramos-Díaz et al. (2007) found no effect on PtdBut formation in *Coffea arabica* suspension cells incubated with different Al^{3+} concentrations, indicating that this metal did not affect PLD activity. This result suggests that a differential response to metal/metalloids stress involving probably different PLD isoforms and PA molecular forms could exist in plants.

As it is well known, there are different isoforms of PLDs that are activated under diverse conditions of growth and development, thus presenting particular functions. In addition, studies employing PLD deficient plants or those over-expressing specific genes encoding for different PLDs have provided evidence of the implication of certain PLDs in specific physiological responses. For instance, *Arabidopsis* plants deficient in PLDα1 showed less degradation of PLs and an increased cold tolerance compared with wild type plants under freezing stress, suggesting a degradative role for PLDα1 under these conditions (Welti et al., 2002). Later, Wang et al. (2006) suggested that PLDδ from *Arabidopsis* is activated by H_2O_2 and the resulting PA produces a decrease in H_2O_2-promoted programmed cell death. In addition, both *Arabidopsis* PLDs (PLDα1 and PLDδ) have been reported to be involved in ABA-induced stomatal closure signalling in guard cells, showing a cooperative activity but

not completely overlapped functions (Uraji et al., 2012). Recently, we demonstrated that As-treated soybean seedlings showed an increased activity of antioxidant enzymes [total peroxidases (Px) and superoxide dismutase (SOD)] in roots harvested 2 and 5 d after treatment. In addition, root structural alterations were observed which could be related to the increase of both PLD activity and PA content (Armendariz et al., 2016). Similarly, Galvan-Ampudia and Testerink (2011) described the participation of different PLD isoforms in adjusting root system architecture in response to abiotic stress. Thus, our previous results and these new findings constitute, to our knowledge, the first evidence which shows the effects of As on lipid signalling events.

3.3. Analysis of stomata total size and closure under As stress

The stomata perform an important function in plant responses to changing environmental conditions (Assmann, 2003). In the formation and structural differentiation of guard cells as well as in opening-closing mechanism of stomatal pore, microtubules play a key role. Heavy metals produced several stomata abnormalities in different plant species (Neelu et al., 2000; Tomar et al., 2000; Han et al., 2004) and, in particular, alteration/distortion on microtubules of guard cells, as was observed under cadmium (Xu et al., 2009), lead (Eun et al., 2000), tungsten (Adamakis et al., 2010) and other treatments (Patra et al., 2004). Also, As is assumed to have a possible effect on stomatal apparatus (Stoeva et al., 2003/2004; Vromman et al., 2011). Thus, we analysed the stomatal total size and ostiole closure in soybean seedlings treated with As.

In our present work, histological studies indicated that total area of stomata and stomata ostiole were influenced by As, mainly by AsIII. Regarding the stomata total area, a significant reduction was observed in seedlings treated with AsIII after 1.5 and 4 h of treatment in the adaxial side of leaves (Fig. 3A) and in all tested times in leaf abaxial side (Fig. 3B). AsV induced a significant reduction in total area of stomata from leaf abaxial side of soybean seedlings only after 24 h (Fig. 3B). Fig. 4 shows some representative microphotographs of acrylic blades from control soybean leaves (Fig. 4A, D) or those from treated seedlings with AsV (Fig. 4B, E) and AsIII (Fig. 4C, F) after 1.5 h. At this time, the major differences in stomata total area and aperture were observed.

On the other hand, size of stomata ostiole was also influenced by the metalloid. It was statistically reduced by AsIII in both leaf sides and at all analysed exposure times, indicating an evident and quick response of stomata closure under AsIII exposure. In AsV-treated seedlings a significant decrease in stomata ostiole was observed after 1.5 h of treatment in both leaf sides while for longer exposure times the stomata aperture did not differ from control seedlings. Similarly, stomata of *Vigna radiata* (K851) treated with As were adversely affected. In these plants, stomatal size was reduced and stomatal ostioles were permanently closed at the highest As concentration used (30 mg/kg soil), although stomatal index increased in a dependent manner with As increasing concentration. Also, frequency of arrested, fused and abnormal stomata as well as guard cells abnormalities increased (Gupta and Bhatnagar, 2015).

This rapid response of stomata closure would be a result of water status distortion in leaves that affects transpiration rate and water potential of plants, as a consequence of exposure to heavy metals and other types of stress (Poschenrieder and Barceló, 1999). This effect was reinforced by the different absorption rates of solutions by capillarity when water, solution containing AsV and that

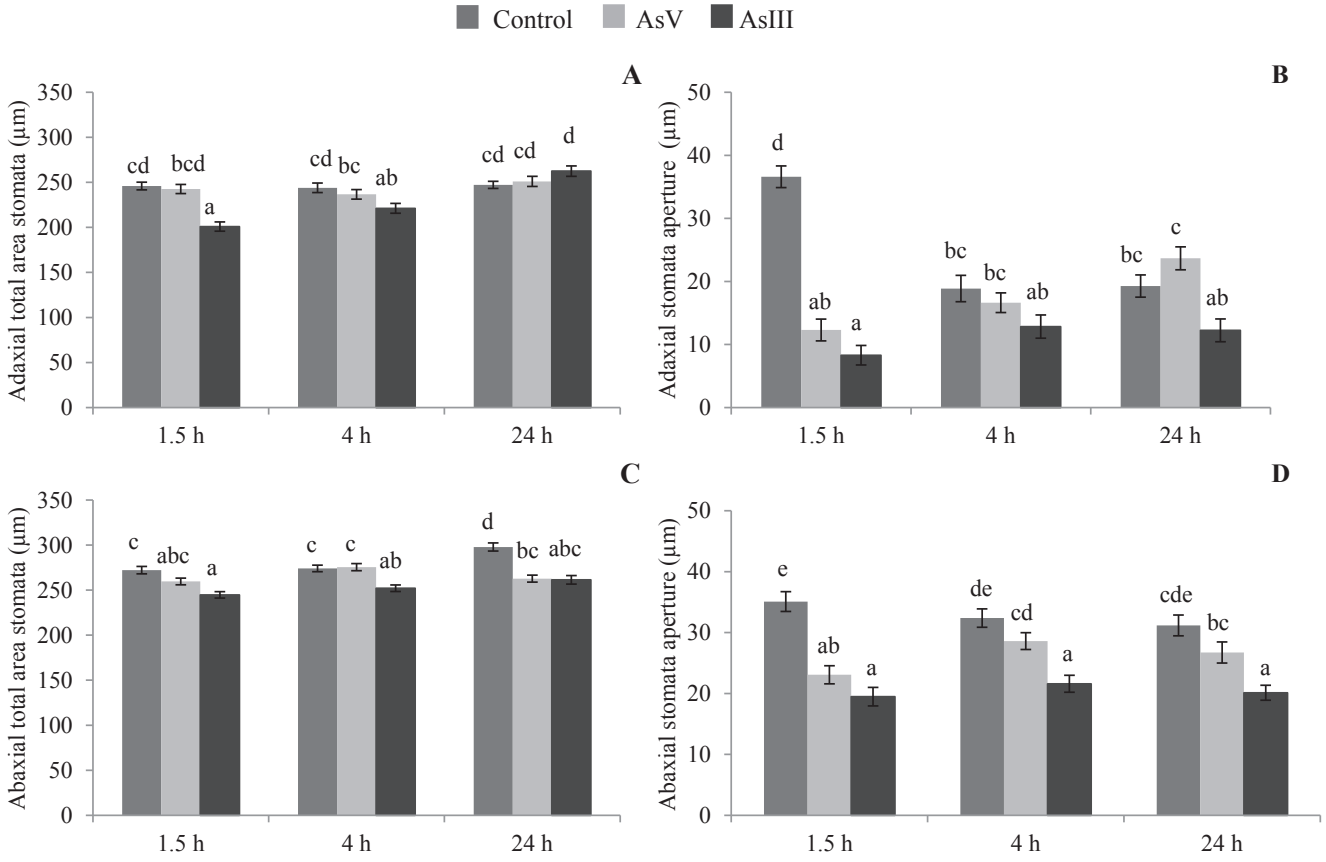

Fig. 3. Effect of As on stomatal total area (A, C) and aperture area (B, D) of adaxial (A, B) and abaxial leaf side (C, D) in soybean seedlings treated during 1.5, 4 and 24 h with 25 µM AsV or AsIII and water (control). Different letters indicate significant differences (*Tuckey's test*, $p \leq 0.05$).

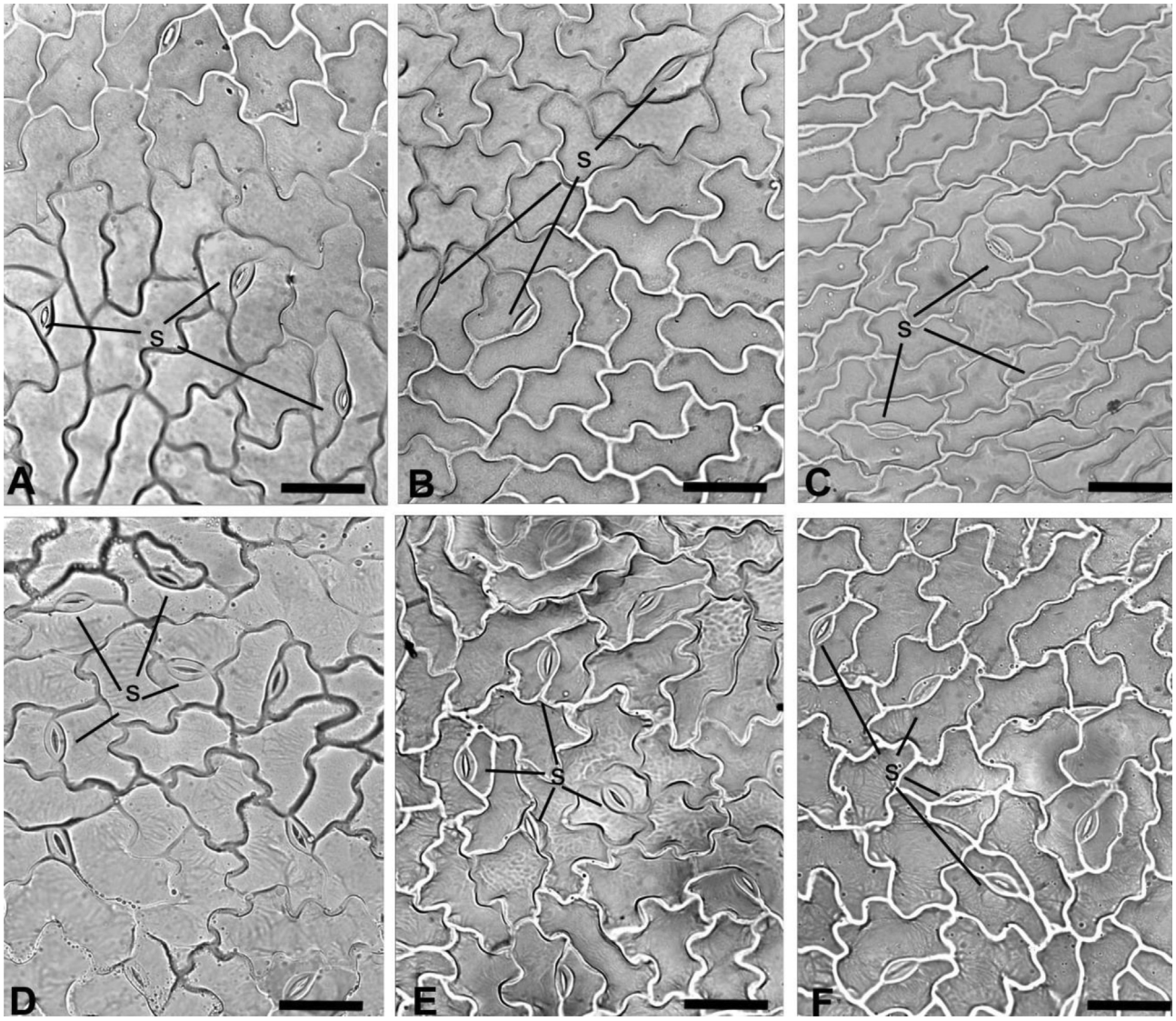

Fig. 4. Microphotographs of adaxial (A, B, C) and abaxial (D, E, F) epidermis from control soybean leaves (A, D) and from those seedlings treated during 1.5 h with 25 μM AsV (B, E) or AsIII (C, F). S, stomata. Scale 50 μm.

containing AsIII were supplied to soybean seedlings. For instance, seedlings irrigated with water showed an absorption rate value of 0.87 mL/h/plant, while for AsV and AsIII treatment, the solution was absorbed at 0.69 and 0.43 mL/h/plant, respectively. As already shown, the presence of As in solutions used for irrigation produced considerable reduction of absorption rate and these values were reduced proportionally according to the toxicity of As species, since AsIII has higher toxicity compared with AsV. This reduced absorption would be a consequence of minor root biomass and structural alterations, such as obstruction of xylem vessels elements caused by As (Rai et al., 2011; Piršelová et al., 2012). As a response to this reduced absorption, the plants would promote stomata closure to avoid loss of water and seedling dehydration. In this sense, dark deposits and entirely obliterated conduction elements were observed in soybean seedlings treated with As, mainly AsIII, in our previous work, and these findings were associated with a strategy to avoid As translocation to the aboveground tissues (Armendariz et al., 2016).

ABA pathway is mediated by PA signal and it is a phytohormone associated to osmotic stress, causing the closure of stomata and other important events in response to osmotic and other types of stress. Recently, Jiang et al. (2014) demonstrated that PA integrates calcium signalling and microtubules dynamic in the regulation of ABA-induced stomata closure in *Arabidopsis*. ABA induced microtubule depolymerisation and stomata closure in wild-type (WT) *Arabidopsis* plants, whereas these processes were impaired in a PLD mutant (*pldα*1). These results suggested that PLDα1 and PA regulate microtubules organization of guard cells and Ca^{2+} increases during ABA-induced stomatal closure. This cross-talk among signalling lipid, Ca^{2+} and microtubules is essential for ABA signalling. Thus, in this work, the synthesis of PA via PLD and PLC/DGK triggered by As could be, in part, responsible of ABA signal and consequently the stomata closure, since ABA has several ameliorative functions in response to stress. These include the rapid induction of stomatal closure to reduce water loss by transpiration and the activation of antioxidant defence to counteract oxidative stress, as was described by Armendariz et al. (2016) for soybean seedlings exposed to As. In *Arabidopsis*, PLDα1 and PLDδ expression and activity were increased by ABA (Uraji et al., 2012) and mutations in PLDs genes impair ABA effects on stomatal closure and germination inhibition. Likewise, PA could mediate ABA-induced production of both ROS and NO, leading to stomata closure (Jacob et al., 1999; Zhang et al.,

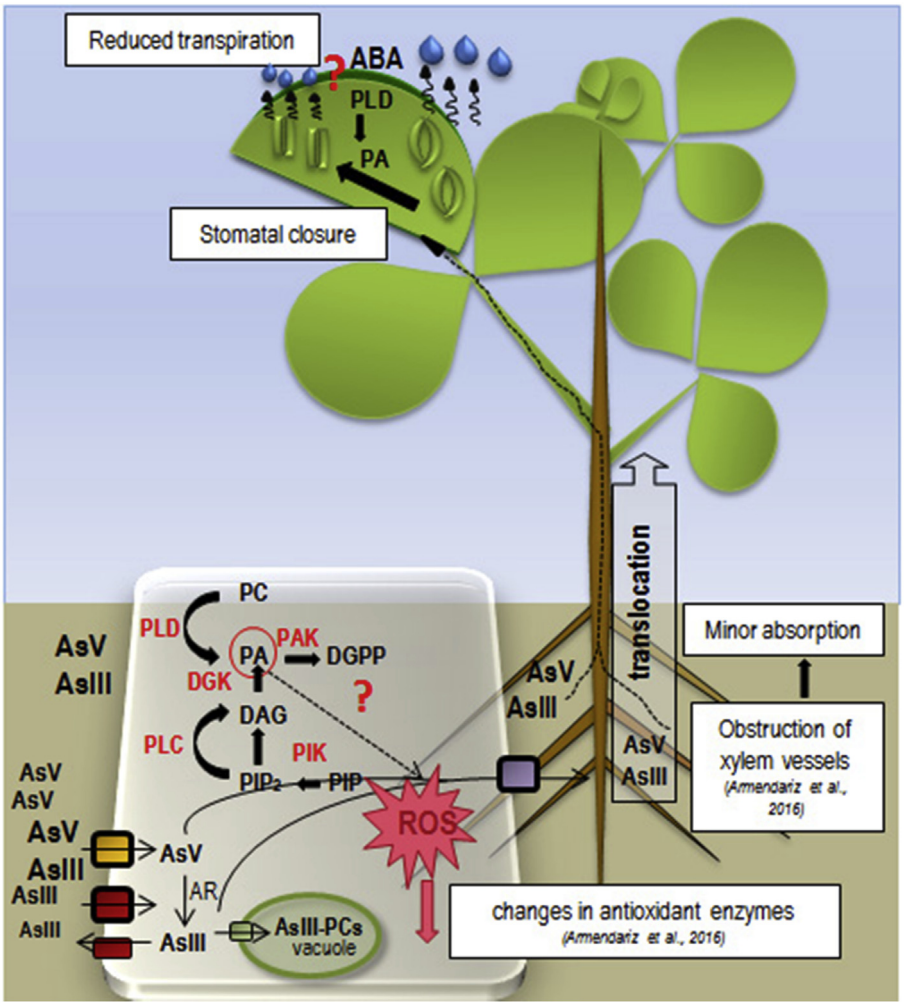

Fig. 5. Scheme of the already known mechanisms of As metabolism in soybean plants and the contribution of the present work, in particular, aspects such as PLs signalling pathways and its association with stomatal closure, as well as the relation with those responses to As (antioxidant response and structural changes in root anatomy) recently shown by Armendariz et al. (2016). Arsenate (AsV) is taken up by roots via phosphate (Pi) transporters while arsenite (AsIII) enters into root cells through aquaporins. AsIII efflux from roots to soil can occur. Once AsV enters the cells, it is reduced to AsIII by arsenate reductase (AR). In aerial parts, this metalloid could be reduced and accumulated in a similar way than in roots. Arsenic increases reactive oxygen species (ROS) concentration, which results in changes of the enzymatic antioxidant system. Regarding accumulation of this metalloid, AsIII forms complexes with reduced glutathione or phytochelatins (PCs) that are sequestered in vacuoles. Besides, some un-complexed AsIII and AsV could be translocated to the xylem and loaded to the aerial parts of the plant. The increase in PA along with ABA signal would be responsible of stomata closure, and this effect would be exacerbated by obstruction of xylem vessels and reduced root growth, since they decrease absorption and avoid transpiration as adaptive mechanisms to attenuate As stress.

2009). In soybean roots, As leading to PA signal, ROS production and stomata closure suggest that an ABA-dependent pathway could help to attenuate As stress. In Fig. 5, these events and those reported in our previous work have been represented in order to integrate different aspects of soybean seedling response to As.

4. Conclusion

The present study demonstrates that As triggers the PA signal by PLD and also via PLC/DGK. We also suggest that PA signal could be part of ABA-signalling pathway and these events could be the responsible of the stomata behaviour. The rapid stomata closure would reduce water loss by transpiration and this fact would evoke a minor absorption of As by roots. PA signal induced by As stress may also be associated with the modulation of the antioxidant system, helping with ROS dissipation, and with changes in root structural architecture. This cross-talk of signalling events would be crucial in the adaptation and survival of soybean seedlings under As stress. These new findings constitute, to our knowledge, the first evidence which shows the effects of As on lipid signalling events. We hope that further investigation will reveal the role of lipid, ROS signalling and the hormonal cross-talk as part of the machinery by which soybean plants respond and adapt to As stress.

Authors contribution

The research work was carried out with equal contribution from all the authors. ALA did all the experiments. CT and HR contributed with the ejecution and analysis of microscopical assays. ALA, MAT, ALV, GER and EA designed the experiments and analysed the results, and wrote the paper.

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