Environmental and Experimental Botany 77 (2012) 141–145

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Phospholipid turnover and phospholipase D activity in tobacco hairy roots exposed to phenol

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ARTICLE INFO

Article history:
Received 10 March 2011
Received in revised form 8 September 2011
Accepted 9 November 2011

Keywords:
Phospholipid turnover
Phenol
Phospholipase D
Lipid signal transduction

ABSTRACT

Plants are exposed to a great variety of environmental factors, which can affect their survival. In addition, the rapid urbanization and the increased release of different pollutants, such as phenol, to the environment, produce another stressful condition to the development and growth of the plants. In this work, we studied the effects on the [^{32}P]$P_i$phospholipid turnover and phospholipase D (PLD) activity after phenol treatment, using tobacco hairy roots (HRs), double transgenic (DT) for two peroxidase genes (tpx1 and tpx2) and wild type (WT) ones. In both HRs, the [^{32}P]phospholipid turnover of the most abundant phospholipids (PLs), such as phosphatidylcholine (PC), phosphatydilethanolamine (PE), phosphatydilglycerol (PG) and cardiolipin (CL) did not show any changes after phenol treatment. However, modifications in the minor PLs of both HRs were observed. Phenol treatment significantly increases the turnover of phosphatidic acid (PA) and phosphatydilinositol (PI), in WT HRs. In DT HRs, phenol produced significant increase in the turnover of PI, lisophosphatidic acid (LPA), diacylglycerolpyrophosphate (DGPP) and PA with a concomitant decrease in the phosphatidylinositol monophosphate (PIP). Moreover, phosphatidylinositol bisphosphate (PIP_2) was detected, but its level did not change in presence of the pollutant. Phenol treatment significantly increased the PLD activity of both HRs. In WT HRs the increase was 100% higher than the control, whereas in DT HRs it was about to 50%. These results suggest the participation of minor PLs, mainly PA, and the PLD pathway as one source of PA production in the activation of intracellular mechanisms that might be important in the response of these plant tissues to phenol treatment.

1. Introduction

Plants are constantly exposed to a wide variety of stressful conditions, which are not only environmental factors (high or low temperatures, high salt levels, water availability, pathogen attack, etc.), but also organic pollutants, such as phenol. This compound is frequently found in the wastewater of different industries at variable concentrations, from 5 to 1000 mg l^{-1} (Paisio et al., 2009) and released into the environment generating a severe threat to the human health as well as to the ecosystem. Due to its high toxicity and persistence in soil and water, it has been classified as a prioritary pollutant by the United States Environmental Protection Agency (USEPA). In plant tissues relatively little is known about phenol effects, its metabolism and the full range of enzymes involved in its transformation. Several plant-based experimental systems are available to study and elucidate the interaction between plant cells and environmental pollutants. Among them, hairy root cultures have been applied in numerous studies aimed at identifying the capacity of plant cells to tolerate, assimilate, detoxify, metabolize and store a wide variety of inorganic and organic pollutants (Nedelkoska and Doran, 2000; Eapen et al., 2003; Araujo et al., 2006; Coniglio et al., 2008). In this sense, Singh et al. (2006) have reported that *Brassica juncea* hairy roots (HRs) treated with phenol showed induced peroxidase activity and an increase in the hydrogen peroxide (H_2O_2) contents. Furthermore, in previous works performed in our laboratory, tobacco HRs (WT and DT) were exposed to different phenol concentrations (100–800 mg l^{-1}) and 100 mg l^{-1} phenol was the concentration at which these HRs showed the highest phenol removal efficiencies and non significant changes in their growth index respect to the control were found (unpublished data). In addition, phenol treatment (100 mg l^{-1} at different times) was able to induce the antioxidant response (enzymatic and non enzymatic defense systems) in both HRs, after 120 h of treatment. Besides, DT HRs were more tolerant to phenol treatment than WT HRs (Sosa Alderete et al., 2011). It is important to note that plant tissues need to monitor changes in the external environment, to transfer rapid and appropriate signals inside and consequently to adapt and survive. There are multiple signal

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doi:10.1016/j.envexpbot.2011.11.006

transduction pathways in the plant response to stressful conditions which include several molecules among them, the phospholipids (PLs). PLs are major and vital components of all biological membranes, and play a key role in the membrane trafficking, cytoskeletal rearrangement, synthesis of cell regulators and signal transduction. In the last years, the role of PLs in the plant growth and development and, the plant response to the biotic and abiotic stress has mainly concentrated on the signaling pathways (Cowan, 2006). Although the downstream targets and the specific action of lipid signal are not well known, several components such as phospholipase C (PLC), phospholipase D (PLD), phosphatidic acid (PA) are key in lipid signaling pathways in plants (Cowan, 2006). In animals, receptor-mediated activation of phospholipase C (PLC) leads to hydrolysis of PIP_2 to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 can diffuse into the cytosol, allowing the release of calcium from intracellular stores, while DAG remains in the membrane, and it can activate members of the protein kinase C (PKC) superfamily (Laxalt and Munnik, 2002). Unlike animals, plants seem to use two phosphorylated derivatives, instead of IP_3 and DAG, they use inositolhexaphosphate (IP_6) and PA. Moreover, PA can be formed indirectly through the sequential action of PLC and diacylglicerol kinase (DGK). PLC hydrolyzes polyphosphoinositides, such as PIP_2 into IP_3 and DAG. Then, both PLs are phosphorylated, IP_3 is converted to IP_6 while DAG is phosphorylated to PA by DGK. PA can be also produced through the action of the phospholipase D (PLD), by hydrolysis of structural PLs, such as PC (Darwish et al., 2009). Under stressful conditions, like hyperosmotic stress, PA levels are modified and others lipid signals such as diacylglycerolpyrophosphate (DGPP), phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol monophosphate (PIP) are modified as well (Darwish et al., 2009; Munnik and Vermeer, 2010). However, according to the current knowledge the lipid signaling response, the phospholipid turnover and the signal transduction pathways triggered by phenol treatment in plants are still unknown. Thus, based on the growing evidence on signaling phospholipids as an important component in response to stress, the purposes of the present study were to determine (i) the effect of phenol treatment on the tobacco HRs (WT and DT) phospholipid turnover, either structural or signaling ones and (ii) the influence of phenol in the PLD activity, as one of the possible pathways responsible for the PA formation.

2. Materials and methods

2.1. Plant material

Tobacco (*Nicotiana tabacum*) DT HRs which expressed two basic peroxidase genes from tomato (tpx1 and tpx2) and WT HRs were used (Sosa Alderete et al., 2009). The HR cultures were maintained for successive subcultures each 25–30 days on Murashige and Skoog (MS) (1962) medium supplemented with vitamins, at 25 ± 2 °C in the dark on an orbital shaker at 70 rpm.

2.2. Sample processing

HRs were treated with 100 mg l^{-1} phenol for 120 h after 15 d of growth on MS medium (exponential growth phase). Water treatment was used as control. Then, both HRs were harvested and the fresh tissue was dried and used for the further studies.

2.3. Incorporation of [^{32}Pi] Pi at the tobacco HRs

HRs were incubated with [^{32}Pi]Pi (18.5×10^4 Bq/100 mg of HRs) in 1 ml of 50 mM sodium succinate buffer, pH 6.5 supplemented with 10 mM of $CaCl_2$ at 30 °C for 24 h in a water bath. Then, the total lipids were extracted according to the procedure described by Bligh and Dyer (1959). After incubation time, 250 μl of 25% perchloric acid (v/v) ($HClO_4$) were added and incubated at room temperature for 30 min. Then, $HClO_4$ was discarded and 400 μl of the mixture chlroroform:methanol:fuming hydrochloric acid (50:100:1, v/v/v) were added and resuspended for 5 min. Samples were submerged at least three times in liquid N_2, and a solvent mixture of chlroroform:methanol:fuming hydrochloric acid (50:100:1, v/v/v) was recovered into a clean tube and 400 μl of chloroform and 214 μl of 0.9% NaCl (w/v) were added. The mixture was mixed and centrifuged at $90 \times g$ for 5 min. Then, the lower phase was recovered into a clean tube and 500 μl of the mixture chroroform:methanol:hydrochloric acid 1 N (3:48:47, v/v/v) was added. The mixture was mixed and centrifuged at $90 \times g$ for 5 min. The lower phase was recovered in a new tube, since it contained PLs radiolabelled with [^{32}P]P_i. In order to determine the number of counts per minute (cpm), 2 μl from each sample were taken and analyzed in the liquid scintillation counter Beckman LS-6001C according to Racagni et al. (1992).

2.4. Determination of phospholipase D (PLD) activity

PLD activity was estimated by the phosphatidylbuthanol (PBut) formation through transphosphatidylation reaction (Santander, 2004). A volume of n-buthanol (0.1%, v/v) was added to HR samples pre-labeled (24 h) with [^{32}P]Pi (18.5×10^4 Bq/100 mg) for 20 min. The reaction was finished by the addition of $HClO_4$ and lipids were extracted as described above using the organic upper phase of an ethyl acetate system (III) (Di Palma, 2009).

2.5. Separation, identification and quantification of phospholipids

Total lipids extracted were dried under nitrogen atmosphere and redissolved in a suitable chloroform:methanol (9:1, v/v). The phospholipids were routinely separated by thin-layer chromatography (TLC) on silica gel plates (Merck), previously impregnated with 1% potassium oxalate (w/v), 200 mM EDTA in methanol:water (2:3, v/v). Prior to use the plates were activated at 60–70 °C overnight. The chromatograms were performed using an acidic solvent system (I), chloroform:methanol:acetone:acetic acid:water (40:14:15:12:7, v/v/v/v/v), alkaline solvent system (II), chloroform:methanol:25% ammonium hydroxide:water (90:70:4:16, v/v/v/v) and the organic upper phase of an ethyl acetate system (III), ethyl acetate:*iso*-octane:formic acid:water (13:2:3:10, v/v/v/v). Structural and signal phospholipids were separated in the acidic and the alkaline system and, the ethyl acetate separations were use to analyze phosphatydilbutanol (PBut) levels. PLs were identified by co-migration with lipid standards. [^{32}P]P_iPLs were visualized by autoradiography on Kodak film. Spots were scrapped off the plates and quantified using a liquid scintillation counter Beckman LS-6001C (Racagni et al., 1992) or by densitometry of autographs using an image analyzer software (ScionImage).

2.6. Statistical analysis

The results of at least three independent experiments were statistically processed with the Duncan's test ($P < 0.05$) using software STATISTICA (version 6.0). The [^{32}P]$P_i$phospholipid turnover were expressed as relative percentages considering the water controls as the 100% of turnover activity.

3. Results

3.1. Analysis of tobacco HR phospholipid turnover after phenol treatment

Phenol could act directly on the plasma membrane of cells and probably trigger changes in the lipid patterns. In order to analyze

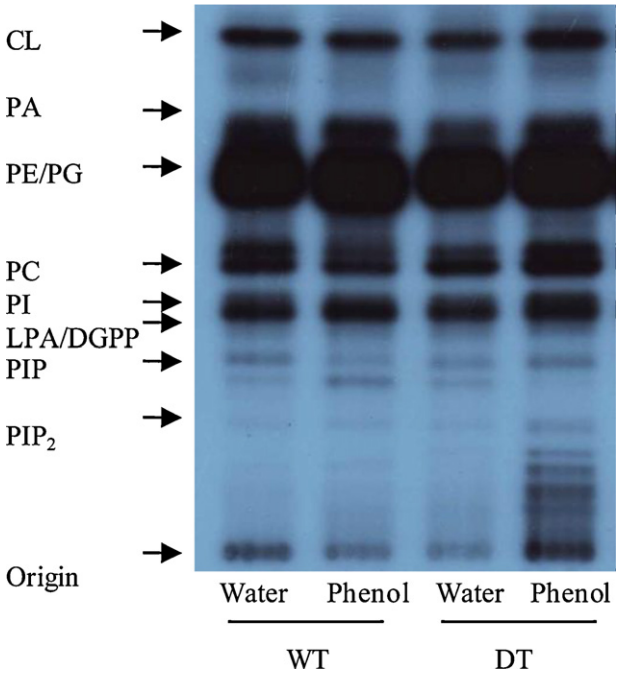

Fig. 1. Autoradiograph of total phospholipids prelabeled with [^{32}P]P_i–P_i for 24 h of WT and DT HR cultures, treated with phenol (100 mg l^{-1}) and water as control during 120 h. These samples were resolved by TLC, using solvent system (I) chloroform:methanol:acetone:acetic acid:water (40:14:15:12:7, v/v/v/v/v). The figure shows a representative experiment of three independent experiments (n = 3). CL: Cardiolipin, PA. phosphatidic acid; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PC: phosphatidylcholine; PI: phosphatidylinositol; LPA: lyso phosphatidic acid; DGPP: diglycerolpyrophosphate; PIP: phosphatidylinositol monophosphate; PIP_2: phosphatidylinositol biphosphate.

whether phenol treatment can produce a change at the PLs turnover (either structural or minor PLs), studies on the lipid profiles of both HR cultures (WT and DT) were carried out. Firstly, HRs were treated for 120 h with phenol (100 mg l^{-1}) and then labeled with [^{32}P]Pi for 24 h.

As shown in Fig. 1, the lipid profiles of both HRs treated with water showed no differences. However, in presence of phenol, clear differences were observed. In DT HRs, several PLs i.e. PIP_2, PI, PC and PA showed higher percentages of [^{32}P]P_i-incorporation than the control treated with water. Regarding WT HRs, the lipid turnover was similar to that of the control, with the exception of some PLs, such as PIP and PC which shown a decrease in the lipid turnover, while PA showed a higher [^{32}P]P_i-incorporation compared to the control. However, in this solvent system (I) some PLs could not be differentiated and identified due to overlap of bands. Therefore, to solve this problem an alkaline system solvent (II) was used. In this system, the lipid turnover of both HRs treated with phenol showed clear differences. In WT HRs, treated with water, it was determined that structural PLs had high percentage of [^{32}P]P_i-incorporation, being PC the most abundant ones and representing 23% of total PLs, followed by PE, PG and CL, with values of 17, 16 and 11%, respectively. When WT HRs were treated with the pollutant no changes in the ability to turnover of structural and some minor PLs (PIP and DGPP) were observed. However, other minor PLs, such as PA and PI, showed turnover rates of 6.1 and 5.1% respectively, being 27% and 41% higher than those obtained in the control (Fig. 2A).

In a similar manner, DT HRs also showed high distribution percentages of structural PLs (PC: 23%, PE: 16%, PG: 15% and CL: 10%), and no differences between the presence and absence of phenol were found (Fig. 2B). For minor PLs, some of them showed changes after phenol treatment, i.e. PIP showed a turnover rate 37.5% lower than the control. For the remaining minor PLs, the treatment significantly increased turnover rates. In DGPP and PA, the levels of turnover were 77 and 44% higher than the controls, respectively. PI and LPE showed also a similar behavior, exceeding 26 and 23% the values obtained in the controls. Although PIP_2 remained unchanged after phenol treatment, this minor phospholipid was only detected in DT HRs (Fig. 2B).

3.2. *In vivo* PLD activity in tobacco HRs after phenol treatment

In vivo PLD activity was measured by the transphosphatidylation assay. This methodology is based on the unique ability of the PLD to transfer the phosphatidyl moiety of a phospholipid to a primary alcohol rather than water, producing phosphatidyl alcohol rather than PA. Thus, in presence of low concentration of n-butanol, the production of phosphatidyl butanol (PBut) can be estimated as a relative measure of PLD activity.

It was found that phenol treatment, significantly increased PLD activity in both tobacco HRs. In WT HRs, the increase in the PLD activity was 100% higher than the control, whereas DT HRs, the increase was 47% higher than the control (Fig. 3). However, under

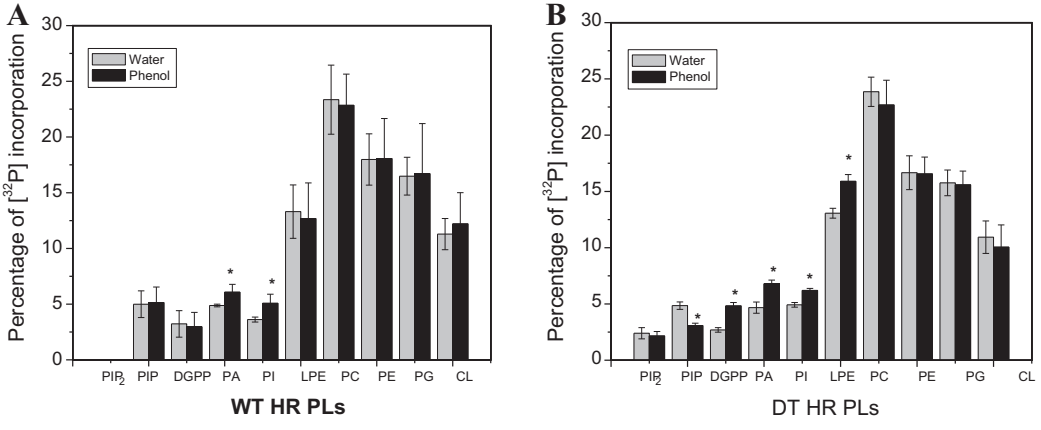

Fig. 2. Quantification of total PLs labeled with [^{32}P]P_i for 24 h of WT (A) and DT (B) HRs treated with phenol (100 mg l^{-1}) and water as control for 120 h. Samples were separated by TLC using alkaline solvent system (II), chloroform:methanol: 25% ammonium hydroxide:water (90:70:4:16, v/v/v/v). Lipids were analyzed by densitometry using an image analysis software (ScionImage) and quantified as a percentage of total radiolabeled lipids of at least three independent experiments (n = 3). CL: Cardiolipin, PG: phosphatidylglycerol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; LPA: lysophosphatidylethanolamine; PI: phosphatidylinositol; PA: phosphatidic acid; DGPP: diglycerolpyrophosphate; PIP: phosphatidylinositol monophosphate; PIP_2: phosphatidylinositol bisphosphate. Asterisks **(*)** represent significant differences according to Duncanĭs test (P < 0.05).

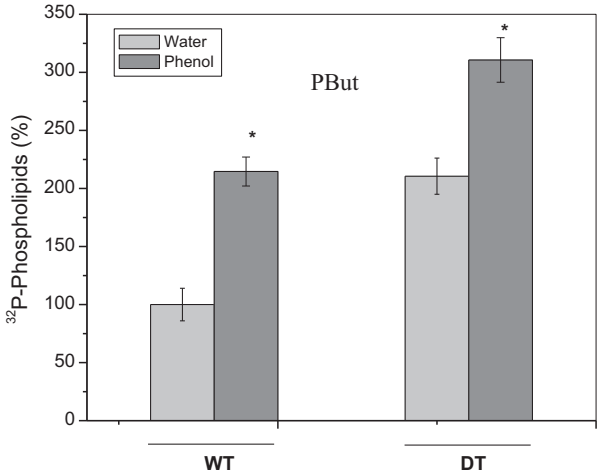

Fig. 3. *In vivo* PLD activity of WT and DT HRs after 120 h of phenol treatment (100 mg l^{-1}) and water as control. Samples were prelabeled with [^{32}P]P_i for 24 h. PLs were separated by TLC using the organic upper phase of an ethyl acetate system (III), ethyl acetate:*iso*-octane:formic acid:water (13:2:3:10, v/v/v/v) and revealed by autoradiography. Relative values of PBut were obtained using image analysis software (ScionImage) of at least three independent experiments ($n = 3$) and considering WT HRs control as the representative of 100% activity. Asterisks (*) represent significant differences according to Duncan's test ($P < 0.05$).

control conditions, it was noted that the PLD activity of DT HRs was 2-fold higher than that of WT HRs.

4. Discussion

Tobacco HRs treated with 100 mg l^{-1} phenol for 120 h showed changes in the PLs turnover, mainly in the minor PLs. Despite the fact that some evidences suggest the involvement of certain lipids, such as PA, in signaling events associated with plant response to different stress conditions, such as osmotic stress, chilling, freezing injury, cold tolerance (Wang et al., 2006); the results of the present study constitute, to our knowledge, the first evidence which showed the effects of phenol on the lipid metabolism.

WT HRs only increased the turnover rates of PA and PI, whereas in DT HRs, several minor PLs increased the values of turnover, such as DGPP, PI and PA. However, a decrease in the turnover rates of PIP was observed. Moreover, PIP_2 was the only minor PLs detected in the DT HR remaining unchanged after pollutant treatment. These increases of [^{32}Pi]P_i turnover observed in PA and DGPP are consistent with those found in various studies that highlight the participation of minor PLs in the mechanisms of response to wide stressful conditions. For instance, increased levels of PA and DGPP were found in tomato plants and tobacco pollen tubes (Pedranzani et al., 2003; Zonia and Munnik, 2004), tomato cell suspensions, potato tubers and in rice leaves (Munnik et al., 2000; Munnik and Meijer, 2001; Cenzano et al., 2008; Darwish et al., 2009), in response to saline, hyperosmotic and biotic stresses.

Although PIP_2 remained unchanged in DT HRs after treatment, it has also been considered as a lipid signal, and its levels increased in response to hyperosmotic stress conditions. Previous works reported that cell suspensions from tobacco and *Arabidopsis* subjected to heat and hyperosmotic stresses showed a rapid increase at the PIP_2 levels, with a simultaneous decrease of PIP (Mishkind et al., 2009; Munnik and Vermeer, 2010). This is due to activation of kinases that phosphorylate the PIP which results in the PIP_2 formation. In addition, in double mutants of *Arabidopsis* for the two kinases (ATPI4Kβ1 and ATPI4Kβ2) which synthesize PIP, it was demonstrated that they were unable to increase PIP_2 levels under osmotic stress, highlighting the involvement of these enzymes in maintaining the pool of PIP, which will be converted into PIP_2, under stress conditions (Munnik and Vermeer, 2010). Furthermore, the PIP_2 is the substrate of PLC, an enzyme that has also been involved in various cellular processes, as well as in response to stress. The hydrolysis of PIP_2 by PLC would lead to the formation of IP_3, which is phosphorylated to IP_6. This compound has been implicated as the mediator of the release of calcium from intracellular stores, while DAG is phosphorylated to PA by the action of DGK (Ramos Díaz et al., 2007; Munnik and Vermeer, 2010).

In WT HRs, increases in the PA turnover were detected, without changes in PIP, or the presence of PIP_2. Thus, this increase in PA could be due to the activation of another pathway of biosynthesis, like the PLD pathway, which hydrolyzes structural PLs to form PA. Our results showed that phenol treatment increased significantly the PLD activity in both tobacco HRs. The increase in PLD activity was greater in WT HRs, compared to control, exceeding 100% of the control activity, while in DT HRs, the difference in the PLD activity was close to 50% compared with the control. However, it is important to note that under control condition, DT HRs showed a PLD activity 100% higher than that observed in WT HRs.

These results of PLD activity and increases in the PA turnover are in agreement with those obtained in other studies, involving the participation of this enzyme, as one of the main pathways of PA formation in response to stressful conditions, such as, osmotic stress, water deficit, low temperatures and response to pathogens (Li et al., 2009). The involvement of the PLD as a mechanism of response to organic pollutants, like phenol, has not still been reported in the literature. Thus, to our knowledge, this is the first time that the phospholipid turnover and the PLD activity are related to phenol treatment. In addition, preliminary studies performed in our laboratory shown an increase in PA formation, after phenol treatment, by determining several products of the lipid kinase pathway (Sosa Alderete, unpublished data). Thus, we can suggest that the treatment with phenol could also activate the PLC/DGK pathway, which it would be responsible of the enhancement of PA especially in DT HRs. However, in order to confirm this, further studies would be required, for determining specific PLC activity.

Recently, we showed that after 120 h of phenol exposure (100 mg l^{-1}), there was an activation of the antioxidant defense system in tobacco HRs (WT and DT), i.e. a significant increase in the enzymatic (peroxidase, superoxide dismutase, ascorbate peroxidase) and non enzymatic defense systems (total glutathione) was detected (Sosa Alderete et al., 2011). However, the antioxidant response of DT HRs was more efficient than WT HRs, since no damage at the lipid membrane was found, whereas WT HRs showed a significant oxidative damage in the membrane lipids. This could be related to the increase of both PLD activity and [^{32}P]P_i turnover of PA observed in WT and DT HRs. These results suggested a possible network between different cell signaling pathways. In this context, it has been described that under conditions of environmental stress, the plants commonly suffer oxidative stress due to uncontrolled overproduction of reactive oxygen species (ROS). One explanation for these metabolic changes could be associated with the action of PLD. First, it has been shown that a specific PLD such as PLD α1 is activated by interaction with proteins located at plasma membrane, like G protein, which leads to an increase in the levels of PA. This phospholipid has been considered as a lipid signal, able to recognize specific domains and induce the catalytic activity of certain proteins, such as NADPH oxidase located at plasma membrane (Zhang et al., 2009). These enzymes are an important source of ROS production, which, despite the fact that they are tightly regulated by the antioxidant system, they can cause severe oxidative damage to biomolecules, like proteins, nucleic acids or membrane lipids. These alterations may eventually trigger programmed cell death (PCD) in affected plant cells (Wang et al., 2006). However, in *Arabidopsis*, Wang et al. (2006) suggested that another PLD, like PLDδ is activated by H_2O_2 treatment and the resulting PA leads to a

decrease in H_2O_2-promoted PCD. Moreover in rice cells it has been shown that H_2O_2 was able to induce not only the PLDδ activity but also the synthesis of compounds involved in the plant defense, such as phytoalexins. Nevertheless, the role of PA as inductor or attenuator of the PCD in plants could depend on several factors, such as the specific PLD activity, the location and timing of PA production, the PA concentration and the fatty acid composition, which esterifies the PA molecule (Wang et al., 2006). Furthermore, the survival of the plant will depend mainly on type and severity of stress and, the intrinsic ability of the plant tissues to interpret the exogenous signals to trigger intracellular signals that lead to an appropriate response to ensure their survival and adaptation to the stress condition.

5. Conclusion

Results found in this paper showed that phenol treatment induced changes in the lipid turnover and increased the PLD activity of both HRs. Moreover, in WT HRs, the increase in the PA levels could be associated with PLD activity, while in DT HRs this increase may involve either PLD or PLC/DGK pathways. In this context, preliminary results obtained in our laboratory suggest that phenol treatment may induce lipid kinase activities involved in the synthesis of signaling PLs such PIP, PIP_2, LPA, DGPP and PA in both tobacco HRs. However, in order to get a deeper insight into phospholipids, mainly the lower-abundant ones, it would be appropriate to make further determinations to improve a better understanding about the signaling mechanisms of tobacco in response to organic pollutants like phenol.

Acknowledgements

This work was supported by grants from Mincyt-Cba; SECYT-UNRC and CONICET. L.G.S.A. has a Mincyt-Cba-CONICET fellowship. E.A. is member of the Research Career of CONICET.

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