Salicylic acid induces vanillin synthesis through the phospholipid signaling pathway in *Capsicum chinense* cell cultures

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Signal transduction via phospholipids is mediated by phospholipases such as phospholipase C (PLC) and D (PLD), which catalyze hydrolysis of plasma membrane structural phospholipids. Phospholipid signaling is also involved in plant responses to phytohormones such as salicylic acid (SA). The relationships between phospholipid signaling, SA, and secondary metabolism are not fully understood. Using a *Capsicum chinense* cell suspension as a model, we evaluated whether phospholipid signaling modulates SA-induced vanillin production through the activation of phenylalanine ammonia lyase (PAL), a key enzyme in the biosynthetic pathway. Salicylic acid was found to elicit PAL activity and consequently vanillin production, which was diminished or reversed upon exposure to the phosphoinositide-phospholipase C (PI-PLC) signaling inhibitors neomycin and U73122. Exposure to the phosphatidic acid inhibitor 1-butanol altered PLD activity and prevented SA-induced vanillin production. Our results suggest that PLC and PLD-generated secondary messengers may be modulating SA-induced vanillin production through the activation of key biosynthetic pathway enzymes.

Introduction

Phospholipids are structural components of the cell plasma membrane and are important messengers that regulate plant growth and development and cellular response to environmental change or stress.[1] Phospholipid-generated signal transduction involves a family of phospholipases that catalyze the hydrolysis of plasma membrane phospholipids to generate secondary messengers. Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to generate inositol 1,4,5 trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). In plants, Ins(1,4,5)P_3 may be converted instead to inositol hexaphosphate (IP$_6$), which has been shown to stimulate the release of Ca^{2+} from intracellular stores in guard cells.[2] Phospholipase D (PLD) hydrolyzes membrane phospholipids, generating structural phosphatidic acid (PA) and releasing the polar head of the phospholipid.[3] Phospholipid signaling is involved in plant response to phytohormones such as

salicylic acid (SA), an important endogenous signaling molecule in plant defense.[4-7] SA also regulates several plant physiological processes and is essential for the expression of some defense genes.[8,9]

Two spatially separated SA biosynthetic pathways of have been proposed in higher plants: the cytoplasmic and chloroplastic routes. The cytosolic pathway initiates from phenylalanine, whereas the chloroplastic one does it from chorismate, via isochorismate (IC).[10] SA accumulated in response to pathogens, is produced in the chloroplast in different species, such as Arabidopsis, *Nicotiana benthamiana*, and tomato (*Solanum lycopersicum*).[11-13] If this is the case in *C. chinense* has yet to be established however, since other members of the Solanaceae family, including tomato[12] use the chloroplastic pathway, a similar scenario can be inferred.

As for the possible receptors of SA (see ref. 8 for a review), Fu et al.[14] reported a small family of pathogenesis-related genes (NPR1, NPR3, and NPR4) that might function as SA receptors in the immune response of *Arabidopsis thaliana*. However, besides these
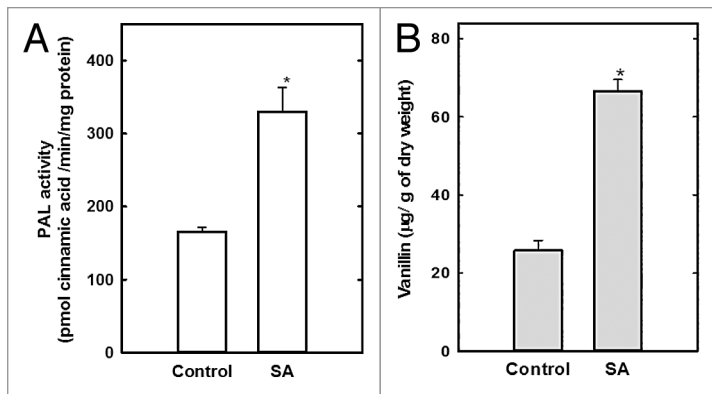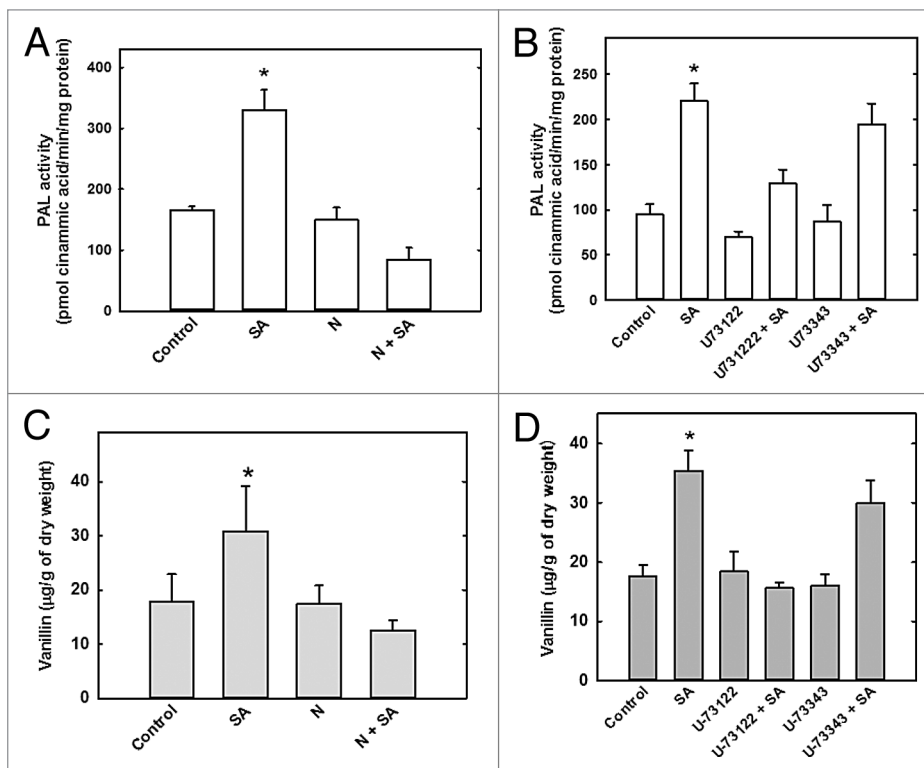
Figure 1. Salicylic acid increases PAL activity and vanillin content in *C. chinense* cells. After a 14-d culture cycle, cells were treated with 200 μM SA or untreated (control) and then PAL activity (**A**) and vanillin production (**B**) assessed. Results represent the mean of 3 independent experiments ± SE, *P < 0.001.

Figure 2. PLC-inhibitors suppress vanillin accumulation and PAL activity in *C. chinense* suspension cells. Cells were treated with 100 μM neomycin (N), 10 μM U73122 or 10 μM U73343 (inactive analog) for 15 min before SA addition and the PAL activity (**A** and **B**) and vanillin content (**C** and **D**) were evaluated. Data represent the mean of 3 independent experiments ± SE, *P < 0.001.

via branched-chain amino acids such as valine or leucine.[18] Phenylalanine ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway, forms cinnamic acid by the deamination of phenylalanine. PAL activity can be induced in response to various stress-inducing factors including freezing,[19,20] wounding,[21] UV light,[22] and phytohormones such as SA. Increased PAL activity is associated with the accumulation of secondary metabolites (e.g., anthocyanins, flavonoids, and other phenolic compounds) in the tissues of plants such as pear,[23] grape,[24,25] tomato,[26] apple,[27] strawberry[28] and tangerine.[29] This supports evidence suggesting that PAL is an environmental stress indicator in different plant tissues.[19,30] However, the relationship between phospholipid signaling, SA, and PAL has not yet been established.

Several inhibitory substances have been used to elucidate the role of phospholipid enzymes in cellular responses.[31] In pharmacological studies using the inhibitors of phosphoinositide-PLC (PI-PLC) neomycin and U73122, it was shown to play a role in cellular response in plant models such as rice, soybean, peas cell suspensions.[32-34]

An evaluation of PLD involvement in different plant cellular processes has been conducted using a primary alcohol such as 1-butanol (1-But), which can inhibit PLD-mediated PA production through competing with water by generating the phosphatidyl-butanol group.[35] This lipid is not normally present in cells but can be easily synthesized in vivo when cells are pre-incubated with low concentrations (0.1–0.5%) of 1-butanol.[36] In this method, PLD-induced PA production is required to regulate increased production of secondary metabolites such as silymarin in *Silybum marianum* cell suspensions[37] or scopoletin in tobaccum suspensions.[38] These studies suggest that the products of the phospholipid signaling cascade may function as secondary messengers during the stimulation of secondary metabolism in plants.[3,39]

Our research group has observed that the treatment of *C. chinense* cell suspensions with 100 and 200 μM SA modulates the in vitro enzymatic activities of PLC and PLD, resulting in increased vanillin content.[6] However, when vanillin content was evaluated in the presence of neomycin, the SA-induced vanillin production was inhibited. Therefore, we focused on elucidating the relationship between phospholipid signaling, PAL activity, and vanillin accumulation, which are all events closely related to the SA induction response. Our goal was to analyze biochemical evidence supporting PLC and PLD involvement in SA-induced signal transduction in the presence of

proteins, a plasma membrane receptor for SA has not been yet identified.

Salicylic acid has been applied in different plants to elicit the production of some secondary metabolites. In the genus *Capsicum*, SA has been used to induce secondary metabolites and increase capsaicinoid content in in vitro cultures.[15-17] Capsaicinoid formation can originate from the phenylpropanoid pathway and

neomycin, U73122, and 1-But and evaluate vanillin accumulation and PAL activity in *C. chinense* suspension cells.

Results

Effect of SA on PAL enzymatic activity and vanillin levels

SA-induced response and activity of PAL was studied in *C. chinense* suspension cells after culturing them for 14 d. Cells were harvested by transferring 1 g of the samples into flasks containing 25 ml fresh Murashige and Skoog (MS) medium. After a 15 min adjustment period, one sample was exposed to 200 μM SA for 30 min, and a control sample was left unexposed for the same period of time. Cells were then harvested by vacuum filtration and immediately frozen in liquid nitrogen.

PAL activity was found to be doubled in the 200 μM SA treatment compared with the control (**Fig. 1A**). Because increased PAL activity is associated with secondary metabolite accumulation, the present model was used to evaluate the effect of SA on vanillin accumulation. Addition of 200 μM SA to the cell suspensions 3 times stimulated vanillin production (**Fig. 1B**), suggesting that SA-induced vanillin biosynthesis yield is correlated with increased PAL enzyme activity.

U73122 and neomycin reduce PAL activity and SA-stimulated vanillin levels

Initially, the effect of inhibitors of PI-PLC (neomycin and U73122) and PLD inhibitor (1-But) on cell suspensions was evaluated, followed by analysis of the cellular structure using scanning electron microscopy. The treatments with SA and inhibitors did not cause any morphological damage to the cell structure that might compromise metabolic activity (data not shown).

To determine the role of PLC in the regulation of vanillin accumulation in *C. chinense* cells, the cells were first treated with neomycin and U73122 and then treated with SA. Cell suspensions were placed in 25 ml fresh MS medium containing neomycin and/ or U73122 for 15 min, and a sample without inhibitor treatment was used as the control. Salicylic acid (200 μM) was then added to some of the cell/inhibitor mixtures for 30 min, while the others were not treated with SA. Our results showed that upon neomycin-only treatment, PAL activity was similar to that of the control sample (**Fig. 2A**) but higher than the neomycin + SA treated sample, suggesting SA-induced stimulation. Treatment with U73122 lowered PAL activity only by 26% compared with the control. The U73122 + SA treatment further decreased PAL activity, especially compared with the SA only treatment (**Fig. 2B**). Treatment of cells with U73343, the inactive analog of U73122, did not alter PAL activity. These results suggest that SA-induced increases in PAL activity can be regulated by PLC-mediated signaling.

The above results also imply that PLC signaling and metabolite (i.e., vanillin) accumulation are both related to the SA induction response. To further evaluate the effect of neomycin and U73122 treatment on vanillin levels, cell suspensions were placed in 25 ml fresh MS medium containing neomycin or U73122 for 15 min before the addition of SA for 30 min (**Fig. 2C** and **D**). Neomycin-only treatment produced vanillin levels similar to control levels (**Fig. 2C**), but neomycin-SA treatment reversed any SA-stimulated
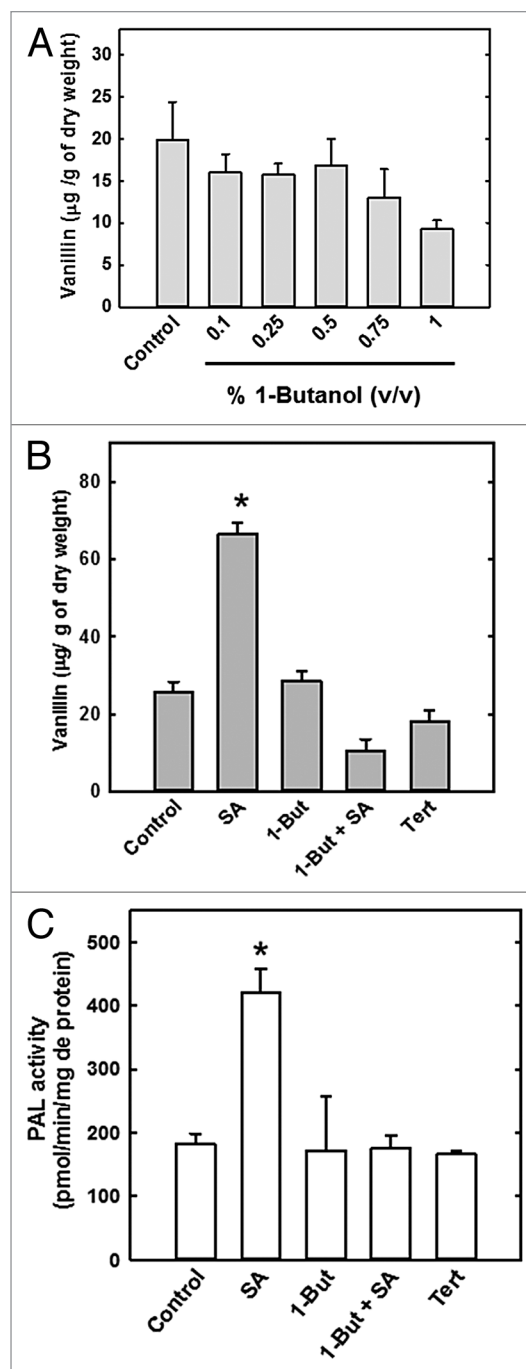
Figure 3. Reduction of vanillin accumulation and PAL activity in *C. chinense* cells in the presence of 1-butanol. Vanillin content was evaluated in cells treated with increasing concentrations of 1-butanol for 15 min (**A**). Cells were incubated in 1-butanol (1-But, 0.5%), 200 μM SA, 1-But + SA, or 0.5% Tert-butanol (Tert). Vanillin content (**B**) and PAL activity (**C**) were then evaluated. Data represent the mean of 3 independent experiments ± SE, *$P < 0.001$.

increases in vanillin accumulation. When treated with U73122 only, vanillin levels remained at basal levels similar to those observed in the neomycin-only treated cells (**Fig. 2D**). Combined U73122-SA treatment produced lower levels of vanillin than in the
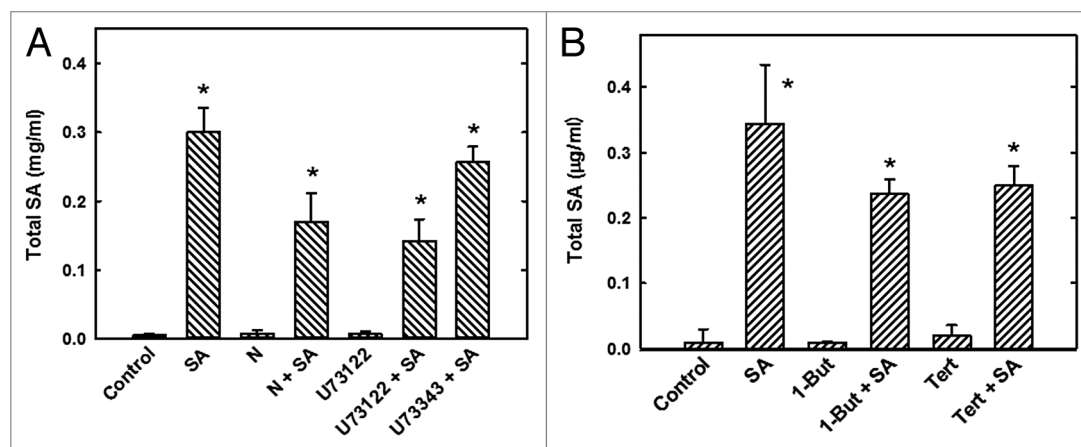
Figure 4. Effect of phospholipase inhibitors on total endogenous SA levels in *C. chinense* suspension cells. Cells were treated with the PLC inhibitors 100 μM neomycin (N), 10 μM U73122, or 10 μM U73343 with 200 μM SA (**A**), or the PLD inhibitor 0.5% 1-butanol or 0.5% Tert-butanol (Tert) with 200 μM SA (**B**). In all treatment groups, cells were pre-incubated with the inhibitors for 15 min before the 30 min SA treatment. The SA level in the cells after the different treatments was evaluated by HPLC (see **Materials and Methods**). The data represent the mean of 3 independent experiments ± SE, *$P < 0.001$

SA-only treatment. The inhibitory analog U73343 had no effect on vanillin levels (**Fig. 2D**).

PLD regulates PAL activity and accumulation of vanillin in *C. chinense*

To assess the role of PLD signaling in PAL regulation, 1-But was used as an inhibitor of PLD-induced PA formation. Because 1-But may have a toxic effect on metabolite production, different working concentrations (% v/v) were evaluated to identify the concentration that did not affect basal vanillin levels in cell suspensions. After 15 min treatment with 1-But, cells were filtered, lyophilized, and vanillin levels quantified. At the 1% concentration, 1-But reduced vanillin levels by 53% compared with the control, but concentrations < 0.75% had no effect on vanillin levels (**Fig. 3A**). Specifically, 1-But at 0.5% did not affect basal vanillin levels compared with the control and therefore, this concentration was used to evaluate the effect of 1-But on PAL activity and SA-stimulated vanillin accumulation. After 14 d in culture, cell suspension samples were placed in 25 ml fresh MS medium containing 0.5% 1-But for 15 min before the addition of SA. Tert-butanol, an inactive 1-But isomer, was used as a positive control (**Fig. 3B**). In the 1-But treatment, vanillin levels were similar to that of the control (**Fig. 3B**). In contrast, 1-But-SA treatment decreased the vanillin content by 84%, effectively reversing SA-stimulated vanillin accumulation. Tert-butanol had no effect on basal levels of vanillin content in the cell suspension.

In the 1-But-only treatment, PAL activity was similar to control (**Fig. 3C**). However, PAL activity was 58% lower in the 1-But + SA treatment than in the SA-only treatment. Tert-butanol did not modify PAL activity. These results suggest that SA-stimulated increases in PAL activity are mediated by PLD signaling.

Total endogenous SA production

Total endogenous SA level in the cells was quantified in the presence of neomycin, U73122 or 1-But to determine whether these inhibitors modify intracellular SA levels. Exogenous SA application produced a 3-fold increase in total endogenous SA content

compared with the basal content in cells (**Fig. 4A**). However, upon U73122 + SA and neomycin + SA treatments SA levels decreased by 43% and 53%, respectively (**Fig. 4A**), and by 43% after 1-But treatment (**Fig. 4B**). Cells treated only with the inhibitors did not affect basal levels of total SA.

Discussion

Elicitors increase secondary metabolite accumulation in cultured cells, thereby facilitating the study of metabolite biosynthesis regulation mechanisms.[40] Phytohormones such as SA increase the accumulation of metabolites such as capsaicinoids in cell suspensions of *C. annum C. frutescens*, and *C. chinense*.[6,15-17] This study evaluated the role of the phospholipase signaling pathway in SA-stimulated vanillin production in *C. chinense* cell suspensions. Pharmacological substances that inhibit PLC and PLD signaling were used and their effect on PAL activity and vanillin content quantified.

PAL is the first enzyme in the activated phenylpropanoid biosynthesis pathway and is responsive to elicitor stimulation. Enzymatic activity was initially evaluated on day 14 of *C. chinense* cell suspension culture, and SA application was found to increase it. The use of neomycin and U73122 show that PLC signaling could be involved in the SA-stimulated vanillin production. This may occur as a result of the regulation of phenylpropanoid pathway (**Fig. 2A** and **B**). Induction of PAL may occur via a PLC-modulated signal, since neomycin is a phosphoinositide turnover and $PtdIns(4,5)P_2$ inhibitor.[41]

Kamada and Muto[42] evaluated the effect of the protein kinase inhibitors K252 and staurosporine on PAL activity and phosphoinositide turnover in *N. tobacum* cell suspensions after stimulation with an elicitor prepared from *Phytophthora nicotianae*. The inducing agent was found to stimulate phosphoinositide turnover and increase PAL activity, while the addition of K252 and staurosporine inhibited both these responses. The results suggested that phosphoinositide turnover plays an important role in stimulating PAL activity via kinases. Neomycin has been shown to a have a similar inhibitory effect on PAL elicitation when used in combination with an elicitor prepared from the cell wall of the pathogenic fungus *Fusarium oxysporum* that infiltrated *Pisum sativum* leaves[43] or *Larix decidua* cell suspensions.[44] Neomycin treatment reduced PAL activity when used in combination with the elicitor.

Effects of neomycin and U73122 on vanillin accumulation suggest that blocking one of the processes coupled to $Ins(1,4,5)P_3$
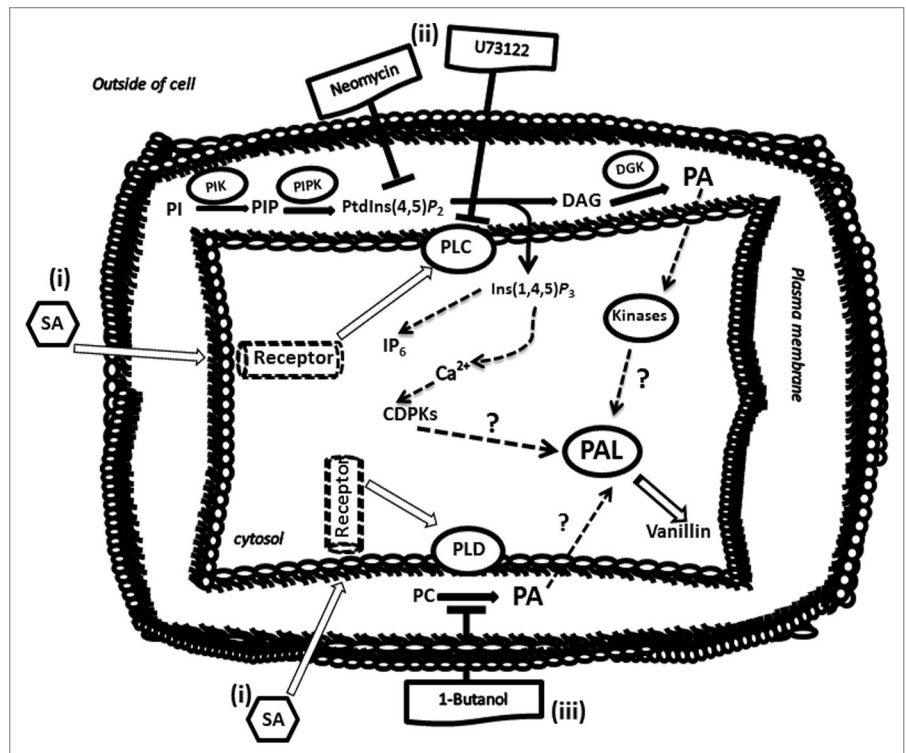
or IP_6, such is the cytosolic Ca^{2+} increment, could be affecting important signaling components responsible for PAL activation. This would affect vanillin levels in the *C. chinense* cell suspensions. This process was no identified in the present study, but there are reports of PAL activity regulation via phosphorylation,[45,46] although it is not clear if these effects occur either directly on PAL or on some regulatory element mediating the response to a stimulus.

For experimental purposes, PLD activity can be manipulated by the addition of 1-But. This strategy was previously used to show that PLD-induced PA production is necessary for increased production of the metabolite silymarin in *Silybum marianum* cell suspensions.[37]

In this way, the effect of inhibited PA derivative production on PLD-stimulated vanillin accumulation in *C. chinense* cell suspensions treated with SA was analyzed by adding 1-But. Both SA-stimulated PAL activity (**Fig. 3B**) and vanillin accumulation (**Fig. 3C**) were reduced by addition of 1-But. The same effect has been reported in *N. tobacum* cell suspensions treated with 1-But,[38] where it was shown to reduce riboflavin-stimulated scopoletin accumulation (activating defense response), whereas exogenous application of PA reversed the effect. This indicates that PLD and the PA products are important components in riboflavin-activated phytoalexin biosynthesis regulation. When 1-But was applied to mechanically injured

Figure 5. The conceptual model for salicylic acid role on vanillin synthesis involving phospholipid signaling pathway. **(i)** Salicylic acid can be sensed on or near the plasma membrane by a receptor and activate a signaling cascade through phospholipases (PLC and/or PLD) function. This is followed by the regulation of PAL enzymatic activity and increased vanillin content. **(ii)** In the presence of U73122 or neomycin (inhibitors of PLC signaling) the levels of DAG and $Ins(1,4,5)P_3$ (second messengers), reduced which lead to modifications of intracellular Ca^{2+} levels that may affect the activity of PAL as well as the promoter of reduced vanillin production. **(iii)** The inhibitory function of 1-butanol on formation of PA could be affecting phosphorylation processes through the regulation of protein kinases activities that may be responding to PA levels. This event also could be affecting PAL activity following vanillin production. Therefore, response to SA resulting in the production of second messengers such as DAG, $Ins(1,4,5)P_3$, and PA produced in phospholipid signaling pathway may be involved in regulating of PAL activity, and consequently vanillin production.

plants, both PAL activity and phenolic compounds were reduced.[47]

That the inhibition of PLD in SA-exposed *C. chinense* cells had abolished PAL and vanillin suggests that this signaling pathway participate in this response. PA is a vital molecule that has been characterized as a multifunctional phospholipid with direct and indirect impacts on many cellular processes.[48,49] For example, in a study of how PA may activate MAPK-type protein kinases in soybean (*Glycine max* L.) under stress, inhibition of PA production by 1-But resulted in wounding, and MAPK activation was also affected.[50] No reports exist in the literature on the direct role of PA in PAL activity, although some studies have suggested that Ca^{2+}, calmodulin (CaM) and ion channels are important components in the signal transduction pathway that stimulates PAL activity and that PA may be acting as a modulator of these signaling components.[51]

Capsicum chinense cells are sensitive to exogenous SA treatment and exhibit a significant increase in total SA in response to exogenous SA. These results are consistent with those from a study of SA stimulation in *Hypericum perforatum* L. shoots, callus,

and cell suspensions in which growth of callus or shoots in cell suspensions was facilitated by close contact between the cells and the elicitor.[52] In another study, treatment with SA was reported to induce de novo synthesis via activation of gene expression of protein involved in the SA biosynthesis pathway.[8,53]

Neomycin is widely used due to its affinity to form electroneutral complexes with PIP and $PtdIns(4,5)P_2$, thereby blocking the binding of PIP and $PtdIns(4,5)P_2$ with PLC.[54] This antibiotic has also been reported as an inhibitor of protein synthesis in bacteria and chloroplasts.[55] Chloroplast/plastids are important for lipid metabolism and the generation of lipid derived signal[9] and because neomycin effects in these organelles, we cannot rule out the possibility that it could have affected protein synthesis, affecting the chloroplast-localized SA pathway. However, we found that 100 μM neomycin did not affect the endogenous total SA. This result added to the effect of U73122 on total SA level support our hypothesis that neomycin is affecting the PLC signaling and not protein synthesis in chloroplast.

The results presented here suggest that PLC and PLD signaling inhibitors may be interfering at some level with SA biosynthesis, and consequently, with vanillin production.

Our results are integrated into a model (**Fig. 5**) that suggests that SA regulates PAL activity, and consequently, increasing vanillin content in the cell suspensions. However, treatment of cells with PI-PLC and PLD inhibitors prior to SA addition leads to a simultaneous decrease in PAL activity and vanillin.

Therefore, PAL may be an important enzyme in vanillin biosynthesis, which in turn suggests that signal molecules such as phosphoinositides (PI, PIP, and $PtdIns(4,5)P_2$) and PA are involved in regulating PAL activity. Overall, PLC and PLD inhibitors were found to reduce PAL activity and metabolite (vanillin) accumulation, suggesting that the PLC and PLD signaling pathways are involved in this SA-induced process.

Materials and Methods

Materials

Neomycin sulfate, U73122, U73343, 1-But, tert-butanol, and sodium salicylate were purchased from Sigma-Aldrich. U73122 and U73343 were dissolved in dimethyl sulfoxide to make stock solutions. Bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co). All other chemicals were supplied by Sigma-Aldrich.

Cell culture and SA treatment

Capsicum chinense suspension cells were obtained by callus desegregation followed by culture in MS medium at pH 5.6.[56] The MS medium was supplemented with 0.5 mM myo-inositol, 0.02 mM thiamine, 0.2 mM cysteine, 4 µM 2,4-dyclorophenoxiacetic acid, and 3% sucrose. Cells were subcultured every 14 d. For the induction treatments, 1 g (fresh weight) cell suspension per flask was inoculated into 25 ml culture medium and maintained as described above for 14 d prior to SA exposure. After the culture period, 200 µM SA was added to the cell suspension, and water was added in the control sample. Both samples were kept at 25 °C on a rotary shaker at 100 rpm for 30 min. The cells were then harvested, frozen in liquid nitrogen, and stored at –80 °C until protein extraction.

Phospholipase inhibitor treatment

Before being added to cell suspensions, the inhibitors neomycin (100 µM), U73122 (10 µM), and 1-But (0.5%) were sterilized by filtration. Each inhibitor was added to a cell suspension at the end of the 14-d culture period and 15 min before SA addition.

Protein extract preparation

For protein extraction, 1 g of cells was homogenized in a mortar and pestle in 2 ml extraction buffer (50 mM Tris-HCl, pH 8.8, 15 mM β-mercaptoethanol) at 4 °C. The resulting mixture was centrifuged at 1747 × g for 30 min and the supernatant used as the PAL enzyme source in the activity assay. Sample protein concentration was measured by the BCA assay[57] using bovine serum albumin (BSA) as a standard.

PAL activity

Enzymatic activity assay was performed in 2 ml reaction volume containing 0.5 ml enzyme extract (5–20 µg protein), 1 ml 50 mM Tris-HCl (pH 8.8), and 0.5 ml 10 mM L-phenylalanine. After incubating this mixture at 37 °C for 1 h, 500 µl 6 M HCl was added to stop the reaction and then centrifuged at 10000 × g for 10 min. The supernatant was removed to quantify PAL activity in a spectrophotometer at 290 nm. The boiled extract was white in appearance and contained other reaction mixture components. A calibration curve was generated using cinnamic acid, and one unit of enzyme activity was designated as being equivalent to the amount required to produce 1 pmol cinnamic acid / min.[16]

Vanillin determination

Vanillin was acetone extracted from freeze-dried cultures and quantified by in situ TLC densitometry using a Shimadzu CS-930 dual wavelength chromatoscanner equipped with a DR 2 data collector (Shimadzu Corporation, Kyoto, Japan).[58]

Total SA Measurement

Endogenous total SA levels were measured in total extract by adding 800 µl buffer (50 mM NaCl, 1 mM EGTA, 250 mM sucrose, 10% glycerol, 50 mM Tris-HCl pH 7.4, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate), 1.24 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM β-mercaptoethanol per gram of plant tissue, homogenizing the mixture and centrifuging it at 1747 × g for 30 min at 4 °C. Organic phase extraction was performed by adding 1 ml 1M HCl to 0.5 ml protein extract, mixing for 20 s, adding 2 ml dichloromethane/isopropanol (9:1 v/v), stirring for 5 min and centrifuging at 3,351.04 × g for 5 min. The organic phase (bottom layer) was evaporated in a CentriVap (DyNA Vap) at 400 mbar for 1 h. The concentrate was resuspended in 50 µl mobile phase [pH 3.6 acetate buffer / methanol (72:28 v/v)]. SA separation was done by high performance liquid chromatography (HPLC) (Agilent 1100) using a 4.6 × 150 mm ion exchange column (Eclipse XDB-C18). SA was eluted with the mobile phase at a rate of 1 ml/min at room temperature and quantified SA by UV spectrometry at 280 nm.

Data presentation

All experiments were repeated at least 3 times using extracts prepared on separate occasions, and all produced similar results. Data were analyzed using a Student *t*-test. Analyses were run using the Sigma Stat ver. 3.1 program (2004).

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