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Signal transduction events mediating chitosan stimulation of anthraquinone synthesis in *Rubia tinctorum*

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Abstract

The signalling mechanism by which chitosan increases anthraquinones in *Rubia tinctorum* L. is largely unknown. We recently showed that the effects of the elicitor require activation of the PLC/PKC cascade. In view of the intrincate network of pathways mediating extracellular stimuli, in this study we investigated whether mitogen activated protein kinase (MAPK), a pathway known to be a PKC target, also participates in chitosan action. Immunoblot analysis revealed a marked stimulation of the MAPK in elicited cells. In presence of the PKC inhibitor calphostin C, lack of activation of MAPK paralleled by a 90% suppression of the chitosan-dependent increase in anthraquinones were observed. Moreover, the elicitor action was decreased 65% by MAPK kinase inhibitor PD 98059. Also, we tested whether the adenylyl cyclase (AC)/cAMP/PKA messenger system plays a role in elicitation. Forskolin, which stimulates AC, and the PKA activators adenosine 3′,5′-cyclic monophosphorothioate-Sp (Sp-cAMPS) or 8-Br-cAMP, were not able to mimic the induction of anthraquinone synthesis in *R. tinctorum* cells by the elicitor. In addition, the PKA inhibitor adenosine 3′,5′-cyclic monophosphorothiolate-Rp (Rp-cAMPS) did not block chitosan effects. On the other hand, phosphoinositide 3′-OH-kinase (PI3K) inhibitors LY204002 and wortmannin blocked chitosan stimulation of PKC activity and anthraquinone synthesis. These results involve PI3K-mediated activation of PKC and in turn of MAPK in chitosan elicitation.

Keywords: *Rubia tinctorum* L.; Chitosan; Anthraquinone; PI3K/PDK-1/PKC/MAPK

1. Introduction

Plants are a source of a large variety of secondary metabolites. These compounds do not appear to be involved in the basic plant cellular processes but many of them are endowed with economical importance, such as pigments, lignin and drugs, among others. In general, the synthesis of secondary metabolites in vitro systems is feasible although not at significant levels thus limiting their commercial application [1]. Elicitation is an interesting strategy largely employed to enhance productivity of secondary metabolites [2,3]. Elicitors are compounds that are able to trigger defense mechanisms like hypersensitive response, production of reactive oxygen species, activation of defense-related genes as well as phytoalexin synthesis [4,5]. The last event is a classic example of secondary metabolism activation by elicitor treatment because phytoalexins are secondary metabolites of low molecular weight with antimicrobial activity, formed and accumulated in plant cells in response to pathogenic attack [6,7].

Chitosan is an effective elicitor that is extensively used [2,3,8,9]. It induces cell wall lignification [10,11]. Production of phytoalexins and the generation of hydrogen peroxide, a reactive oxygen species, are also responses of plants elicited with chitosan [12,13].

Moreover, by treatment of cultures of *Rubia tinctorum* L. with chitosan we have recently shown enhanced of production of anthraquinones [14]. *R. tinctorum* produces various kinds of anthraquinones colorant, such as purpurin, xanthopurpurin and alizarin, the latter being the most abundant of them [15]. Anthraquinones are metabolites of commercial and pharmacological interest and their biosynthesis in elicited cultures would represent an interesting approach due to lower cost than synthetic production, renewed interest in natural dyes and environmental concerns.

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Although the effects of chitosan on plants have been thoroughly studied, there is scarce evidence on the signal transduction pathways involved in the elicitor actions. It has been reported that chitosan stimulates the accumulation of jasmonic acid, a signal molecule related to defense-gene regulation [9].

The transduction of elicitor signals in plant cells may utilize a mechanism similar to the process reported in animal cells in response to extracellular stimuli, where second messengers are generated, leading to the activation of protein kinase cascades which may activate the biosynthetic ability for specific plant products [16,17].

We have recently provided evidence on the participation of the PLC/PKC pathway in the anthraquinones response elicited by chitosan in *R. tinctorum*. Thus, the action of the elicitor could be greatly reduced with neomycin, U-73122, calphostin C, bisindolylmaleimide and PKC down-regulation with high concentrations of phorbol ester PMA. Moreover, PMA mimicked the effects of chitosan and increases in PKC activity and PKC α associated to the cell membranes were observed in response to the elicitor [14]. Although, no plant genes for PKC homologs have been cloned, the above mentioned results expand the list of proteins with PKC characteristics in higher plants [18–22]. For example, a PKC homolog in maize was purified and characterized, called ZmcPKC70. This protein, of 70 kDa approximately, has kinase activity in presence of calcium, oleyl acetyl glycerol (OAG) and phosphatidyl serine and showed other specific biochemical properties typical of mammalian PKC [23]. Moreover, a homolog of mammalian PKC has been involved in elicitor-induced defense response in potato [24].

Considering the intricate network of interacting cascades mediating extracellular stimuli in biological systems, the possibility that other signalling mechanisms may be associated with chitosan elicitation should be not excluded. In view of much evidence showing that mitogen activated protein kinase (MAPK) cascades are evolutionarily-conserved signaling systems with essential regulatory functions in eukaryotes, including yeasts, flies, worms, mammals and plants and which were involved in different plant signalling events like activation by stresses, pathogens, plant hormones and certain elicitors [17,25–31], we have investigated its participation in chitosan-induced anthraquinone production. Additionally, we searched for interactions of this pathway with PKC, phosphoinositide 3′-OH-kinase (PI3K) and adenylyl cyclase (AC)/cAMP/PKA messenger systems.

2. Materials and methods

2.1. Materials

Calphostin C, chitosan (from crab shells: β-(1,4)-2-amino-2-deoxy-D-glucose), forskolin, Immobilon P (polyvinylidene difluoride, PVDF) membranes and all the medium components were purchased from Sigma–Aldrich. Adenosine 3′,5′-cyclic monophosphorothioate-Sp (Sp-cAMPS), adenosine 3′,5′-cyclic monophosphorothiolate-Rp (Rp-cAMPS), PD 98059 (2′-amino-3′-methoxyflavone) and 8-bromo-cAMP were obtained from Calbiochem (San Diego, CA, USA). LY294002 ([2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] and wortmannin were provided by Alomone Labs (Jerusalem, Israel). The chemiluminescence blot detection kit (ECL) was provided by New England Nuclear (Chicago, IL, USA). Molecular weight colored markers were bought from BioRad Laboratories (Richmond, CA, USA). Phospho-p44/p42 MAP kinase (Thr202/Tyr204) E10 monoclonal antibody and secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP) were purchased from New England Biolabs (Beverly, MA, USA). PepTag non-radioactive PKC assay kit were from Promega Corp. (Madison, WI, USA). All the other reagents used were of analytical grade.

2.2. Cell cultures

Cell suspension cultures from *R. tinctorum* roots were obtained as previously described [32]. The cells were cultured in B5 medium [33] containing 2 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l 1-naphthaleneacetic acid (NAA), 0.5 mg/l indoleacetic acid (IAA) and 0.2 mg/l kinetin. After the pH was set at 5.70–5.80, the medium was sterilized by autoclaving (1 bar, 20 min). The cultures were grown in 250 ml Erlenmeyer flasks at 25 °C on a gyratory shaker (100 rpm), applying a photoperiod of 16 h. Cell cultures were subcultured every 7 days by three-fold dilution into fresh medium. Experiments were performed using 125 ml Erlenmeyer flasks with exponentially grown cells.

2.3. Sample preparation

Cells (7–10 g) were homogenized in TES buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, containing protease inhibitors [0.3 mM phenylmethylsulfonyl fluoride (PMSF); 20 μg/ml leupeptin; 20 μg/ml aprotinin]), with a manual homogenizer under ice (1 ml buffer/g cell). The total homogenate was filtered through two layers of nylon mesh. Protein concentration was measured by the method of Bradford [34] using bovine serum albumin as standard.

2.4. Anthraquinone determination

The concentration of anthraquinones produced by *R. tinctorum* was determined by spectrophotometry [35]. Cells (0.1 g) were extracted with boiling 80% aqueous ethanol usually twice until the tissue was colorless. The absorbance of the extract and the medium was then measured at 434 nm

using the molar extinction coefficient of alizarin ($\varepsilon_{434} = 5.5 \times 10^{-3}$). It has been shown by chromatographic analysis that the absorption spectrum at 434 nm is exclusively due to anthraquinone pigments [35]. The extinction coefficients of different anthraquinones do not vary significantly. For instance, the difference between the molar extinctions of alizarin, ruberythic acid or rubiadin are less than 5% under the measurement conditions used in the present work [35]. Results represent the total content of anthraquinones (medium and cells). The results were calculated as mmol anthraquinone/g cell fresh weight. When expressing anthraquinone levels with respect to cell dry weight the same relative changes in response to chitosan were observed.

2.5. Elicitation

The elicitation process was carried out with chitosan (from crab shells: linear homopolymer β-(1,4)-2-amino-2-deoxy-D-glucose), minimum 85% deacetylated. A stock solution was prepared by dissolving chitosan in 1% aqueous glacial acetic acid by stirring overnight and was then sterilized at 120 °C during 20 min, the final pH was 5.6. The elicitor was added at a final concentration of 200 mg/l during the exponential growth phase of cell cultures (4–5 days old) and incubated for 24 h; this treatment not induces variations in the pH of the culture medium. In those experiments in which specific modulators (forskolin, calphostin C, Sp-cAMPS, Rp-cAMPS, 8-Bromo-cAMP, PD 98059, LY294002 and wortmannin) were used to mimic or block elicitor effects, the stock solutions and dilutions were sterilized by filtration. The different modulators were added to the cultures 15–20 min before the elicitor. Dose-response studies for each compound were perfomed to establish the optimum concentration.

2.6. Western blot analysis

Protein samples (12 μg) were combined with one-fourth (v/v) of sample buffer (400 mM Tris/HCl pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT and 2 μg/ml bromophenol blue), boiled for 5 min and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [36]. Fractionated proteins were electrotransferred to PVDF membranes and then blocked for 1 h at room temperature with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were incubated with anti-active phospho-MAPK antibody (1:2000) overnight at 4 °C in PBS-T containing 5% non-fat dry milk. After several washings with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated antibodies, namely anti-rabbit IgG (1:10000) in PBS-T containing 5% non-fat dry milk. Immunoreactive proteins were developed by means of enhanced chemiluminescence (ECL). The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

2.7. Protein kinase C assay

Samples (12 μg) from the different experimental conditions tested were assayed for PKC using the PepTag nonradioactive kit (Promega) according to the manufacturer's conditions. This kit uses the brightly colored Glycogen Synthase peptide substrate (GS; PLSRTLSVAAKK). The hot pink color is imparted by a dye molecule conjugated to the substrate. The reaction (30 min, 30 °C) was performed in the presence of 3 μg/ml 1,2-dioleyl-rac-glycerol (DG). The phosphorylated peptide substrate was separated from the non-phosphorylated substrate by electrophoresis on 1% agarose gels, according to their migration to the anode and cathode, respectively. The gels were photographed on a transilluminator. PKC activity was estimated by the fluorescence intensity of the band corresponding to the phosphorylated substrate.

2.8. Statistical analysis

Statistical treatment of the data was performed using the Student's *t*-test [37]. Data are means±standard error of three independent experiments. Statistical significance of the data was evaluated using probability values below 0.05 ($P < 0.05$) were considered significant.

3. Results and discussion

Plants efficiently respond to a great number of ambient stimuli to survive. The activation of the mechanism to elaborate the adequate response could be triggered by the perception of a primary stimulus (probably through specific receptors). This generates different signals across the cell and finishes in a response, which results from the participation and intercommunication of various messenger systems.

Since a stimulation of mitogen activated protein kinase in response to different elicitors has been demonstrated in plant cells [17,38], chitosan modulation of MAPK activity in *R. tinctorum* cells was investigated by Western blot analysis using an antibody which specifically detects the active phosphorylated forms of MAPK (Erk1 and Erk2). This antibody is raised against the evolutionary-conserved TEY (Thr-Glu-Tyr) motif found on MAPKs and exclusively reacts if the two residues are phosphorylated simultaneously. The threonine and tyrosine residues whose dual phosphorylation is necessary for the activation of these kinases are conserved in plant MAPKs, as a Thr-x-Tyr motif between the VII and VIII subdomains of the catalytic core [39]. Moreover, this phospho- p44/p42 MAP kinase antibody has been used in other studies of MAPKs in higher plants [40,41].

As shown in Fig. 1, marked MAPK activation was observed, using immunological methods, in elicited cultures. In addition, measurements of MAPK activity in vitro with [γ-^{32}P] ATP (2.5 μCi per assay) in presence of the specific substrate myelin basic protein also showed that chitosan

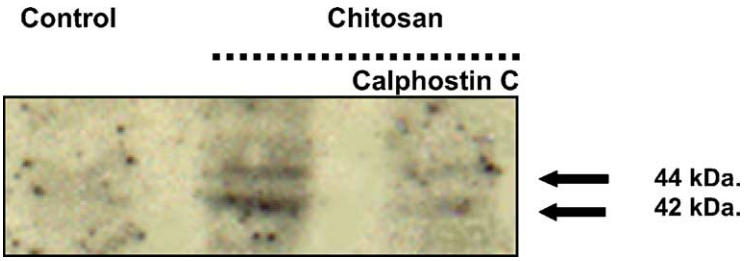

Fig. 1. Chitosan stimulation of MAPK phosphorylation in *R. tinctorum* cell cultures and its inhibition by calphostin C. Cell suspensions of *R. tinctorum* were treated with chitosan (200 mg/l) in the presence or absence of calphostin C for 24 h followed by homogenization. MAPK immunoreactivity was assayed in the total homogenate by Western blot analysis using an anti-active phospho-MAPK antibody as described under Section 2. The bands detected correspond to doubly phosphorylated in threonine and tyrosine (at the sequence TEY) in p42 and p44 MAP kinases (Erk1 and Erk2). Western blot representative of at least three independent experiments is shown.

activates MAPK, strengthening the Western blot results (data not given). Moreover, the effect of chitosan was blocked by 65–70% with 10 μM PD 98059, an inhibitor of MAPK kinase (MEK), and therefore of the subsequent phosphorylation and stimulation of MAPK (Table 1).

Numerous studies indicate that PKC acts as an upstream activator of MAPK in animal cells, stimulating Raf activity [42–44]. With the aim of finding a possible connection between both pathways in elicited cultures, we investigated whether PKC could mediate MAPK activation in *R. tinctorum* treated with chitosan. In previous work we obtained firm evidence for the participation of the PLC/PKC pathway in the response to the elicitor. When the cultures were elicited in presence of calphostin C (1 μM), a PKC inhibitor, no activation of MAPK by chitosan was observed (Fig. 1). In addition, calphostin C suppressed the increase in anthraquinone levels induced by chitosan by 90% (Table 1). This compound, at the concentration used in this work, has been shown to act as effective and specific inhibitor of PKC in plants [24]. We discard unspecific inhibition of other kinases as PKA or CDPK because the concentration required for 50% inhibition of PKC is 50 nM and the IC_{50} for PKA is higher than 50 μM [45]. Thus, the concentration used in our assays (1 μM) is insufficient to inhibit PKA. On the other hand, calphostin C interacts with the PKC regulatory domain by competing at the binding site of diacylglycerol (DAG) and phorbol esters, thus this inhibitor does no affect CDPK as it does not require DAG for its activation [46–48].

Table 1
Effect of calphostin C and PD 98059 on anthraquinone production by *R. tinctorum* cultures elicited with chitosan

	−Calphostin C; −PD 98059	+Calphostin C	+PD 98059
Control	14.75 ± 8	23.89 ± 1.1	20.65 ± 7.6
Chitosan	32.47 ± 2.43	24.9 ± 0.16	26.95 ± 10*

Cells were preincubated for 10–15 min with PD 98059 (10 μM) or calphostin C (1 μM) before elicitation with chitosan (200 mg/l) for 24 h. Anthraquinone levels in cells and culture medium were measured as described under Section 2. The results are expressed in mmol anthraquinones/g Fwt. Each value represents the mean of three independent determinations ± S.D.

* $P < 0.05$, with respect to the effect of chitosan in the absence of inhibitors.

Evidence indicates that second messengers like Ca^{2+}, adenosine 3′,5′-cyclic monophosphate (cAMP), inositol 1,4,5-trisphosphate (IP_3), and 1,2-diacylglycerol, as in animal cells [49–51], play a role in plants (reviewed in reference [16,52]). In particular, cAMP is widely distributed from prokaryotes to eukaryotes as a signalling molecule. The low levels of cAMP in higher plants, near or below the detection limit of the standard methodology available for its measurement, has hindered experimental investigations on the processes in which it participates. As a consequence, a clear picture of the function of the cAMP pathway in plants is not totally understood yet, although the advent of sensitive spectrofluorimetric assays [53] have recently shown the involvement of this messenger in the regulation of certain plant cell metabolic events [54,55]. In addition, adenylyl cyclase, the source of cAMP, has been not clearly identified in plants yet, perhaps due to the diversity of known ACs which precludes a homology search. However, the use of analogs and modulators of the cAMP pathway has allowed the obtaining of evidence congruent with physiological effects of this messenger in plants [56,57]. In animal cells, the best studied downstream target of cAMP is protein kinase A (PKA) and various studies suggest the existence of PKA in plants [56,57–59]. In addition, the application of molecular biology has allowed the identification of cAMP response element-binding proteins (CREBs) [59] compatible with multiple possible roles for cAMP in plants.

We used modulators of the cAMP/PKA pathway to investigate whether it mediates elicitation by chitosan. Since it was previously shown that chitosan (200 mg/l, 24 h treatment) is capable of enhancing anthraquinone levels in *R. tinctorum* cultures (in a range of 100–150% respect to the control), the same conditions for elicitation were employed. Cultures were first incubated with the elicitor in presence or absence of different doses (10, 25, 50 and 100 μM), of Sp-cAMPS, a cAMP analog which activates PKA. Treatment with 10 and 100 μM of activator did not mimic the increase of anthraquinones levels raised by chitosan, without significantly altering secondary metabolite production

Table 2
Effect of 8-Br-cAMP, Sp-cAMPS and Forskolin on anthraquinone production by *Rubia tinctorum* cultures

	mmol anthraquinone/g Fwt.
Control	58.5 ± 12
Chitosan	128.3 ± 19*
Sp-cAMPS	
10 μM	59.8 ± 8.2
10 μM + chitosan	93.6 ± 17*
100 μM	69.3 ± 7.6
100 μM + chitosan	92.7 ± 18*
8-Br-cAMP	
10 μM	39.55 ± 12
10 μM + chitosan	79.37 ± 23
20 μM	62.23 ± 11.5
20 μM + chitosan	102.3 ± 14.1*
Forskolin	
20 μM	56.96 ± 3.3
20 μM + chitosan	100.6 ± 10.7*
80 μM	52.96 ± 6.4
80 μM + chitosan	113.2 ± 18.5*

R. tinctorum cells were treated with chitosan (200 mg/l) for 24 h in the presence or absence of 8-Br-cAMP, Sp-cAMPS or Forskolin, at the concentrations indicated. Total culture anthraquinone content was determined by spectrophotometry as described under Section 2. Each value represents the mean of three independent determinations ± S.D.

* $P < 0.05$, with respect to the control (neither chitosan nor modulators included).

when applied together with the elicitor (Table 2), although these doses have been effective in other plants system [60]. At the other doses of Sp-cAMPS tested, similar results were obtained (data no shown). Moreover, treatment with 10 and 20 μM 8-Br-cAMP, another PKA stimulator which at these conditions has proved to be active in plant systems [61], neither mimicked nor interfered with chitosan effects (Table 2). Forskolin activates adenylyl cyclase in non-plant eukaryotes systems resulting in an increase of intracellular cAMP. Exposure of the cells to forskolin (20 and 80 μM) did not induce production of anthraquinones (Table 2); 40 μM forskolin was also without effect (not shown). At higher doses of forskolin the viability of the cultures is affected. In agreement with the above data, compound Rp-cAMPS, an inhibitor of PKA, did not affect chitosan-induced synthesis of anthraquinones (data not shown). We observed that 10 μM 8-Br-cAMP causes certain interference with chitosan effects, that it is not observed neither at 20 μM 8-Br-cAMP nor with treatments using Sp-cAMPS and forskolin. The effect of 8-Br-cAMP (10 μM) may represent metabolic side effects not generated by Sp-cAMPS which is indeed a more specific modulator of PKA than 8-Br-cAMP as evidenced in various studies [62–67]. Altogether, these results indicate that the AC/cAMP/PKA cascade does not contribute significantly to the elicitation mechanism triggered by chitosan in *R. tinctorum*.

In mammalian cells, PKC comprises a family of serine/threonine protein kinases which are activated by different extracellular stimuli and play a key role in many physiological processes [68]. Most of the PKC isozymes need Ca^{2+} and DAG for their activation. Before interaction with both messengers PKC must be made competent by three sequential phosphorylations [69]. These modifications are required to stabilize the adequate enzyme conformation for full activity and to translocate the mature enzyme from the membrane to the cytosol to act on its downstream targets [70]. The first of these phosphorylations, the rate-limiting step, is regulated by a phosphoinositide-dependent kinase-1 (PDK-1) [71]. Recent reports present a model in which PKC activity could be regulated by PDK-1 mediating two phosphorylations, one in the activation loop and the other in the carboxyl terminus [72]. Although PDK-1 could be active in a phosphoinositide 3′-OH-kinase-independent manner, more recent studies indicate that PDK-1 function is partially mediated by the products of PI3K [73]. In addition, it has been shown that inhibition of the phosphorylation of certain PKC isozymes occurs in presence of the PI3K inhibitor wortmannin [74].

There is ample evidence showing similarities between mammalian and plant signal transduction mechanisms and the pathways so far involved in chitosan action in the present work are not an exception. Since these cascades play a critical role in numerous cellular events they have been evolutionary highly conserved. Moreover, PI3K, phosphoinositides and PDK-1 were found in plants [16,75]. Plant homologues of mammalian PDK-1 has been identified in *Arabidopsis thaliana* (AtPDK-1) and rice [75]. Furthermore, AtPDK-1 can bind PIP_3 and activate targets similar to those of the mammalian enzyme [75].

Initial studies were performed to obtain information on the possible participation of the above mentioned signalling proteins in chitosan-induced anthraquinone synthesis. Before elicitation, *R. tinctorum* cells were incubated with different doses of two structurally unrelated PI3K inhibitors, wortmannin (5 and 20 μM) and LY294002 (2.5, 5 and 10 μM). This treatment inhibited the chitosan effect, maximal blockade (90%) being observed when cells were preincubated for 15 min with 10 μM LY294002 and 5 μM wortmannin (Fig. 2), involving PI3K in the action of the elicitor. The remaining 10% of anthraquinone production observed in presence of these inhibitors may be due to chitosan effects mediated by additional cell signalling systems. These inhibitors were used in the micromolar range because in this concentrations have been previously reported to inhibit the activity of PI kinases in plant cells [76]. Furthermore, assays of PKC activity based on the estimation of fluorescence intensity of the phosphorylated substrate Glycogen Synthase peptide (PLSRTLSVAAKK) after its electrophoretic separation on an agarose gel revealed that chitosan stimulation of PKC is suppressed by prior addition of LY29004 (5 μM) to the cell cultures, indicating, as expected, that PI3K participates in elicitation through PKC (Fig. 3). Although, the conditions (GS substrate, 1,2-dioleyl-rac-glycerol, phosphatidilyserine and calcium) of this assay for non-radioactive

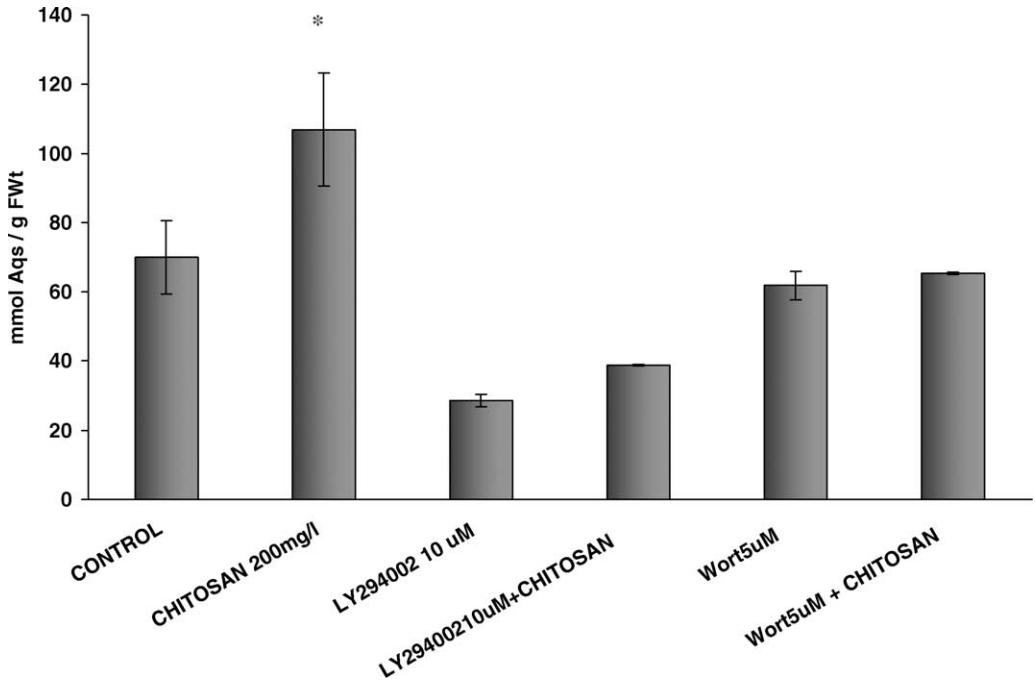

Fig. 2. Effect of LY294002 and wortmannin on chitosan-induced anthraquinone production by *R. tinctorum* cultures. Cell suspensions were treated with chitosan (200 mg/l) in the absence and presence of either compound LY294002 or wortmannin (10 and 5 μM, respectively; added 20 min before chitosan) for 24 h followed by determination of anthraquinone levels. Each value represents the mean of three independent determinations ± S.D. $^{*}P < 0.05$, with respect to the controls.

detection of PKC activity avoid non-specific phosphorylations, we compared this and the traditional assay which uses [γ-^{32}P] ATP obtaining congruent results. Besides, we also performed these assays with the PepTag non-radioactive kit

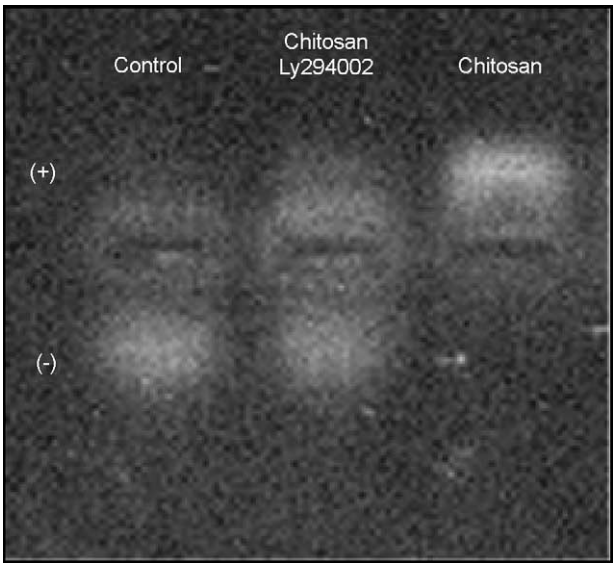

Fig. 3. Effects of PI 3-kinase inhibitor LY294002 on chitosan stimulation of PKC activity in *R. tinctorum*. Cell suspensions were treated with chitosan (200 mg/l) in the absence and presence of compound LY294002 (5 μM, added 20 min before chitosan) for 24 h followed by homogenization. PKC activity was estimated after electrophoretic separation of the phosphorylated substrate which migrated toward the anode (+) as described under Section 2. A photograph of a gel from three representative experiments is shown.

in the absence of the activators above mentioned and could not detect kinase activity (data not shown). Differently to plant PKCs so far characterized [23,24], CDPK does not require diacylglycerol for its activation [46–48]. The fact that in the absence of DAG no activity could be detected using the GS substrate argues in favor that the assay used was specific for PKC. Accordingly, PMA which fully substitutes DAG and activates a mammalian PKC homolog in plants [24], mimicked chitosan effects [14]. Further studies are necessary to confirm that PI3K regulates PKC through PDK-1. These results, in turn, are congruent with the fact that the PLC/PKC pathway mediates chitosan modulation of anthraquinone synthesis in *R. tinctorum* [14].

The schematic diagram shown in Fig. 4, depicts the signal transduction events which have been so far involved in the stimulation of anthraquinone synthesis by chitosan in *R. tinctorum*. Probably, the final steps in the transduction of the elicitor's signal involves MAPK translocation to the nucleus to modulate transcription factors, resulting in a increased expression of genes coding enzymes which play a critical role in the biosynthetic pathway of anthraquinones in Rubiaceae [77,78], e.g. isochorismate synthase. In agreement with this interpretation, in *R. tinctorum* infected with *Pythium aphanidermatum* it has been shown that the increase in anthraquinones levels is preceded by an increase in isochorismate synthase activity and transcript levels [79]. Clearly, further investigations are required to identify other key steps participating in the signaling network activated by the elicitor. Such knowledge may be useful for manipulating the biosynthesis of anthraquinones in *R. tinctorum*.

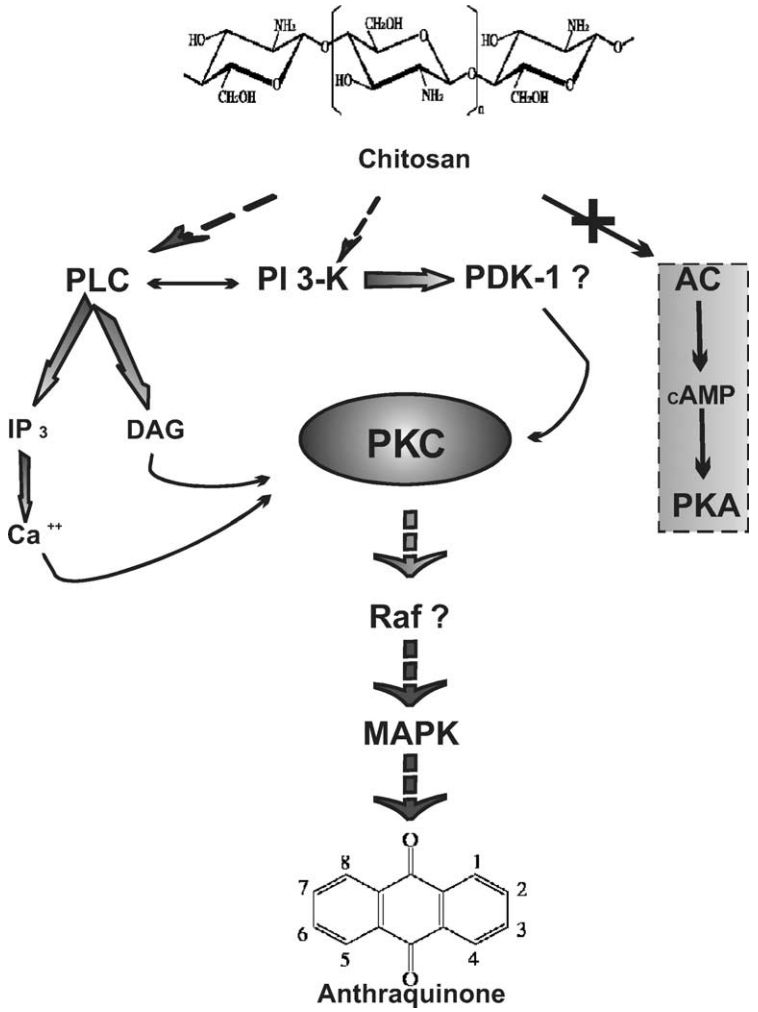

Fig. 4. Schematic diagram of signal transduction events involved in the stimulation of anthraquinone synthesis by chitosan in *R. tinctorum*. The solid arrows indicates strong evidences for the involved pathway; dashed arrows implicates more possible intermediates events not elucidate yet. The cross arrow discard the pathway.

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