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Involvement of G-proteins in chitosan-induced Anthraquinone synthesis in *Rubia tinctorum*

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We have previously shown that chitosan stimulates anthraquinone synthesis in *Rubia tinctorum* L. cells through activation of the PLC/PKC, PI3K, MAPK and Ca^{2+} messenger systems. In view of this evidence, we have now investigated whether guanine nucleotide-binding G-proteins are part of the signal transduction mechanism which mediates the elicitor action. The G-protein agonists mastoparan, AlF_4^- and GTPyS increased anthraquinone levels to the same extent as chitosan. No additive effects were observed when cultured *R. tinctorum* cells were treated with agonist and the elicitor together. In agreement with these observations, the G-protein antagonists suramin and GDPβS abolished the increase in anthraquinone synthesis induced by chitosan. Furthermore, elicitation was not affected in the presence of pertussis toxin. Consistent with this result, when cell cultures were preincubated with a monoclonal anti-Gαq/11 antibody, the chitosan-dependent increase in anthraquinone levels was fully inhibited. Moreover, the presence of an immunoreactive protein of the expected size for Gαq/11 (42 kDa) was observed in *R. tinctorum* microsomal membranes by Western blot analysis using the same antibody. These results indicate that chitosan stimulates anthraquinone synthesis in *R. tinctorum* cells through a heterotrimeric G-protein, most likely belonging to the Gαq family.

Introduction

Plant cells respond to a wide variety of environmental stimuli including light, plant hormones, changes in external conditions, pathogen aggressions and elicitors. To achieve the appropriate response, the external signals must be rapidly transformed into an internal event that induces biochemical and molecular changes. The exact plant transduction mechanism of external information to intracellular signals is often unclear but, in many cases, there are similarities to signal transducing pathways in animals. As an example, all the components of a calcium-based signal transduction pathway are found in plant cells (Drobak 1993). Also, more recent evidence shows that phosphatidylinositol-specific phospholipase C (Apone et al. 2003), phospholipase D or calcium channels (reviewed in Jones 2002) are targets of a heterotrimeric G-protein, as also in animals

Transmembrane signalling systems present in mammals distinguish three functional different proteins: the receptor, which interacts with the extracellular signal, the effector, which alters second messenger levels and finally the transducer, which links the signal between

Abbreviations – Aqs, anthraquinones; PTX, *Bordetella* pertussis toxin; GDPβS, guanosine 5′-O- (2-thio)-diphosphate); GTPγS, guanosine 5′-O- (3-thio)-triphosphate; MAPK, mitogenic activated protein kinase; PI3K, phosphoinositide 3′-OH-kinase; PKC, protein kinase C; PLC, phospholipase C.

receptor and effector (Hepler and Gilman 1992). In this scheme, the transducer is a GTP-binding protein (G-protein). Heterotrimeric G-proteins are GTPases composed of α, β and γ subunits. Mammals have 23 different Gα, 6 Gβ and 12 Gγ subunits. These GTPases are classically associated with plasma membrane receptors or G-protein-coupled receptors (GPCRs) containing seven transmembrane domains. Receptor activation induces the exchange of GTP at the binding site of Gα thus dissociating the complex leading to the binding of the different subunits to effector proteins. Endogenous GTPase activity eventually returns the α subunit to its inactive, GDP-bound form, resulting in the reassociation of the trimer (Klein et al. 2000).

Although several aspects are still unknown, the existence of GTP-binding proteins in plants is well established. Different approaches have been used to demonstrate their presence in plants and recent reviews have focused on the plant physiological responses linked to G-protein action (Ma 1994, Fujisawa et al. 2001, Assmann 2002, Jones 2002). In addition, these findings show that plant G-proteins have similar properties to those found in other organisms. Signal-transducing GTPases in plants include small G-proteins, heterotrimeric G-proteins and, potentially, various unique types of GTP-binding proteins that are not members of these classes (Assmann 2002). Moreover, there may be several molecular species of α and β subunits in some plant species (reviewed in Fujisawa et al. 2001), indicating the complexity involved in the action of these proteins.

In contrast to mammals, the Arabidopsis genome contains only single canonical Gα (GPA1) and Gβ (AGB1) subunits and possibly two Gγ subunits (Jones 2002). In addition, it has been shown by two hybrid analyses and *in vitro* translation assays that the plant γ subunit interacts with the plant β subunit (Mason and Botella 2000). However, there is no information on the interaction between the α subunit and the $\beta\gamma$ dimer in plants.

Elicitors are known to activate different mechanisms related to the production of second messengers (Van der Luit et al. 2000, Zhao et al. 2004), thereby modulating genes that encode for various enzymes of biosynthetic pathways, thus inducing secondary metabolism. The polysaccharide chitosan has been widely used as elicitor (Darvill and Albersheim 1984, Doares et al. 1995, Jin et al. 1999, Kneer et al. 1999). Chitosan is the deacetylated form of chitin, the major component of exoskeletons of insects and crustacea, and are found in the cell walls of some fungi. Chitosan is obtained by treating chitin under strong alkaline conditions (Brine et al. 1992).

We have previously demonstrated that chitosan enhances production of anthraquinones (Aqs) in *Rubia tinctorum* L. cells involving the activation of the phospholipase C (PLC)/protein kinase C (PKC) pathway (Vasconsuelo et al. 2003), phosphoinositide 3′-OH-kinase (PI3K) and the mitogenic activated protein kinase (MAPK) cascade (Vasconsuelo et al. 2004), as well as the participation of Ca^{2+} signalling mechanisms (Vasconsuelo et al. 2005).

There are experimental data implicating G-proteins in the response to elicitors (Legendre et al. 1993). For example, it has been demonstrated that elicitor-induced stimulation of PLC is mediated by G-proteins (Legendre et al. 1993). Other studies have reported evidence for activation of G-proteins, thus showing that elicitor treatment of a multimeric complex dissociates a 42 kDa protein that cross-reacts with Gα antibodies (Xing et al. 1997).

In view of the above findings, our aim was to investigate whether G-proteins are involved in chitosan-induced anthraquinone synthesis in *Rubia tinctorum* cells.

Materials and methods

Materials

Chitosan (from crab shells: β- (1,4)-2-amino-2-deoxy-D-glucose), minimum 85% deacetylated, *Bordetella* pertussis toxin (PTX), suramin, guanosine 5′-O- (2-thio)-diphosphate (GDPβS), guanosine 5′-O- (3-thio)-triphosphate (GTPγS) and all media components were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Gαq/11 rabbit monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit antibody (IgG) conjugated to horseradish peroxidase (HRP) was purchased from New England Biolabs (Beverly, MA, USA), and mastoparan from Calbiochem (San Diego, CA, USA). All other reagents were of analytical grade.

Cell cultures

Cell suspension cultures from *R. tinctorum* roots were obtained as described before (Schulte et al. 1984). Briefly, cells were cultured in B5 medium (Gamborg 1970) containing 2 g l^{-1} sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA), 0.5 mg l^{-1} indoleacetic acid (IAA) and 0.2 mg l^{-1} kinetin. After the pH was set at 5.7–5.8, 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 r.p.m), 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.

Elicitation

The elicitation process was carried out with chitosan. A stock solution was prepared by dissolving chitosan in 1% aqueous glacial acetic acid by stirring overnight and was then sterilized at 120°C for 20 min; the final pH was 5.6. The elicitor was added at a final concentration of 200 mg l^{-1} (and 50/100 mg l^{-1} in an experiment whose results are given in the text) during the exponential growth phase of cell cultures (4–5 days-old) and incubated for 24 h. No variations in final pH were detected in the culture medium. In these experiments, in which specific modulators (AlF_4^-, mastoparan or suramin) 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 performed to establish the optimum concentration. In some experiments, pertussis toxin was added to the cells cultures (2.5 μg ml^{-1}) 5 h before elicitation.

Anthraquinone determination

The concentration of anthraquinones produced by *R. tinctorum* was determined by spectrophotometry (Zenk et al. 1975). Cells (0.1 g) were extracted with boiling 80% aqueous ethanol, usually twice, until the tissue was colourless. The absorbance of the extract and the medium was then measured at 434 nm using the millimolar extinction coefficient of alizarin ($\varepsilon_{434} = 5.5$). It has been shown by chromatographic analysis that the absorption spectrum at 434 nm is exclusively due to anthraquinone pigments (Zenk et al. 1975). The extinction coefficients of different anthraquinones do not vary significantly. For instance, the differences between the molar extinctions of alizarin, ruberythic acid or rubiadin are less than 5% under the conditions used in the present work (Zenk et al. 1975). Results represent the total content of anthraquinones (medium and cells). The results were calculated as μmol anthraquinone/g cell fresh weight (μmol Aqs/g FWt).

Cellular permeabilization

Cellular permeabilization was performed as described previously (Tandon et al. 1999). Briefly, about 10 mg of *R. tinctorum* cells were exposed to 100 μl of permeabilization solution containing glycerol: sucrose: ethylene glycol: DMSO (20 : 5 : 20 : 5, wt/vol percentage) for 15 s at room temperature and immediately washed twice with 1 ml of ice-cold 1 M sucrose solution (plus 10 mM $CaCl_2$) and once with 1 ml of B5 medium. Cells were collected by centrifugation (780 g for 5 min at 0°C) between the washings.

Subcellular fractionation

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 phenylmethylsulphonyl fluoride (PMSF); 20 μg ml^{-1} leupeptin; 20 μg ml^{-1} aprotinin), with a manual homogenizer under ice using 1 ml buffer/g cell. The homogenate was filtered through two layers of nylon mesh and then centrifuged for 10 min at 9000 g. The supernatant was centrifuged again for 1 h at 105 000 g to obtain cytosolic and microsomal subcellular fractions. The pelleted microsomes were resuspended in the same buffer with 0.5% Triton X-100. Protein concentration was measured using bovine serum albumin as standard (Bradford 1976).

Western blot analysis

Protein samples (15 μg) were mixed with one-fourth of sample buffer (400 mM Tris/HCl pH 6.8, 10% SDS, 50% glycerol, 500 mM DTT and 2 mg ml^{-1} bromophenol blue), boiled for 5 min and resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1 h at room temperature with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated with anti-Gαq/11 antibody (dilution 1 : 200) overnight at 4°C in PBS-T containing 5% non-fat dry milk. After several washings with PBS-T, the membranes were incubated with antirabbit IgG horseradish peroxidase-conjugated antibodies (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.

Statistical analysis

Statistical treatment of the data was performed using the Student's *t*-test (Snedecor and Cochran 1967). Data are means ± standard deviation (SD) of not less than three

independent experiments. The data were considered statistical by significant when probability values were below 0.05 (* $P < 0.05$).

Results

In the present paper we have studied the involvement of G-proteins in chitosan stimulation of anthraquinone (Aq) synthesis in *R. tinctorum* cell suspensions. The first step was to investigate the effects of suramin (1 mM) on the elicitor-induced production of Aqs. This hexasulphonated naphthylurea compound uncouples G-proteins from its associated receptors, presumably by blocking their interaction with intracellular receptor domains (Chandi et al. 1998) and it is widely used as an inhibitor of G-protein coupled systems in plants (Rajasekhar et al. 1999, Kurosaki et al. 2001). Cell cultures were incubated for 15–20 min with suramin, followed by incubation with chitosan (200 mg l^{-1}) for 24 h as described under Methods. As shown in Table 1, suramin abolished the stimulatory effect of the elicitor.

Mastoparan is commonly used as a diagnostic tool testing for receptor-coupled G-proteins, since it stimulates GTPase activity by mimicking the intracellular loop of the receptor that interacts with them in animal cells as well as in plants, independently of a ligand–receptor interaction (Vera-Estrella et al. 1994, Cho et al. 1995, Gelli et al. 1997, Allen et al. 1999). At micromolar doses mastoparan acts like a G-protein activator (Higashijima et al. 1988) and it has been effective in other plant species at the same conditions employed in the present work (Irving 1998). In contrast to the inhibitory action of suramin, mastoparan (10 μM) increased Aq levels to the same extent as chitosan. In addition, when the cells were simultaneously treated with mastoparan and chitosan, no additive effects on anthraquinone synthesis were observed (Table 1).

Table 1. Effects of G-protein modulators on anthraquinone synthesis in *Rubia tinctorum* cells. *R. tinctorum* cells were treated with chitosan (200 mg l^{-1}) for 24 h in the presence or absence of mastoparan (10 μM) or suramin (1 mM). Modulators were added 15–20 min prior to chitosan treatment. The control corresponds to cells treated with vehicle (acetic acid 1%) alone. Total content of Aqs was determined as described under Methods. Results are expressed as mean (μmol Aqs/g FWt) ± (SD) of five independent experiments. * $P < 0.005$ with respect to control

		Aqs (μmol/g FWt)
Control		16.7 ± 0.8
Chitosan		42.5 ± 0.9*
Suramin	1 mM	16.9 ± 0.4
	1 mM + chitosan	17.3 ± 0.8
Mastoparan	10 μM	41.0 ± 0.2*
	10 μM + chitosan	41.7 ± 0.3*

In the next set of experiments, another direct activator of G-protein was investigated. Cells were exposed to AlF_4^-, which resembles the presence of GTP, thus resulting in G-protein activation (Higashijima et al. 1991). At a concentration of 10 mm, AlF_4^- was as effective as chitosan in stimulating anthraquinone biosynthesis (Fig. 1). Lower doses (3.5 and 5 mM) of AlF_4^- caused no significant elevations in Aq levels (18.3 ± 2.8 and 19.1 ± 5.3 μmol Aqs/g FWt, respectively) in comparison to the control (16.7 ± 3.0 μmol Aqs/g FWt). Again, the combined treatment with the elicitor and AlF_4^- did not increase Aq production further with respect to either compound alone.

To provide additional evidence for the involvement of a heterotrimeric G-protein in chitosan elicitation of *R. tinctorum* cultures, we also tested the effects of membrane-impermeable and non-hydrolysable analogues of GTP, GDPβS and GTPγS. Cells were permeabilized in the presence or absence of GTPγS or GDPβS followed by elicitation with chitosan as described under Methods. These compounds have proved to be valid tools for the study of G-protein systems in plants used

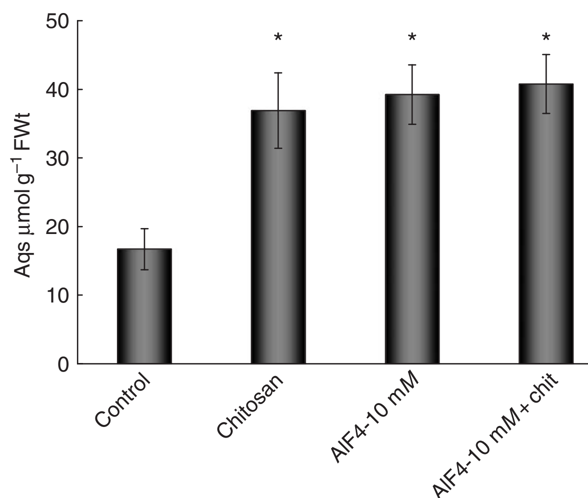

Fig. 1. Effects of AlF_4^- on chitosan-induced anthraquinone biosynthesis in *Rubia tinctorum* cells. *R. tinctorum* cell cultures were treated with acetic acid 1% for 24 h (control), chitosan (200 mg l^{-1}) for 24 h (chitosan), AlF_4^- (10 mM) for 15–20 min and then exposed to acid 1% for 24 h (AlF_4^-10 mM) or AlF_4^- (10 mM) for 15–20 min before elicitation (AlF_4^-10 mM + chit). Total content of Aqs was determined as described under Methods. Results are expressed as mean (μmol Aqs/g FWt) ± (SD) of three independent experiments. * $P < 0.005$ with respect to the control.

at the same concentration range as in this work (Gelli et al. 1997, Ma et al. 1999). As expected, when the elicitation was performed in the presence of 1 mM GDPβS, the increment induced by chitosan in Aq levels was abolished (Fig. 2). In contrast, when elicitation was carried out in the presence of 300 μM GTPγS, this analogue did not interfere with chitosan (200 mg l^{-1}) stimulation of anthraquinone production in *R. tinctorum* cells. Cultures exposed to GTPγS alone had a similar elevation in anthraquinone levels as cultures treated with chitosan (Fig. 3), which was maximal as no further increases were observed at higher concentrations of either compound (not shown). At various doses tested the action of the elicitor was mimicked by the analogue (control: 17 ± 3 μmol Aqs/g FWt; chitosan: 20 ± 6, 25 ± 3 and 41 ± 4 μmol Aqs/g FWt for 50, 100 and 200 mg l^{-1}, respectively; GTPγS: 21 ± 7, 27 ± 2 and 42 ± 5 μmol Aqs/g FWt for 100, 200 and 300 μM, respectively).

The permeabilization procedure itself did not modify anthraquinone levels in either control or elicited cells (Fig. 2, centre bars). Moreover, we evaluated the effectiveness of the transient cell permeabilization procedure applied by using the fluorescent probe FITC conjugated to BSA (FITC-BSA). As shown in Fig. 4, only the permeabilized cells incorporated FITC-BSA (fluorescence in cytoplasm). In non-permeabilized cells, the fluorescence remained outside the cell. We also observed, using trypan blue and fluorescein diacetate (FDA) staining, that cell viability was not affected by permeabilization.

To obtain evidence on which type of G-protein might be involved in elicitation, permeabilized cells were

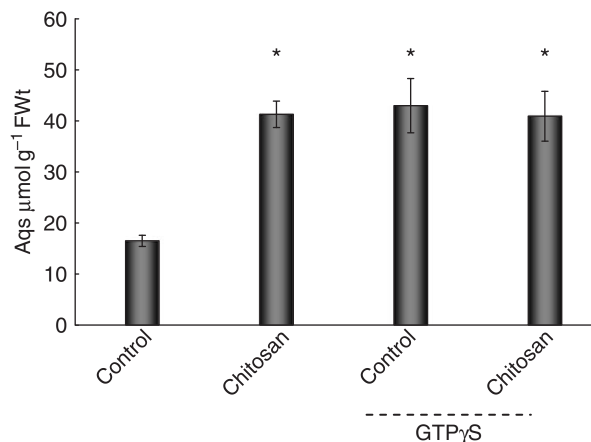

Fig. 3. The non-hydrolizable analogue GTPγS stimulates anthraquinone biosynthesis in *Rubia tinctorum* cells. Cells permeabilized in presence of GDPβS (300 μM) were exposed to chitosan (200 mg l^{-1}) or vehicle (acetic acid 1%) for 24 h. Total content of Aqs was determined as described under Methods. Results are expressed as mean (μmol Aqs/g FWt) ± (SD) of three independent experiments. * $P < 0.005$ with respect to the control.

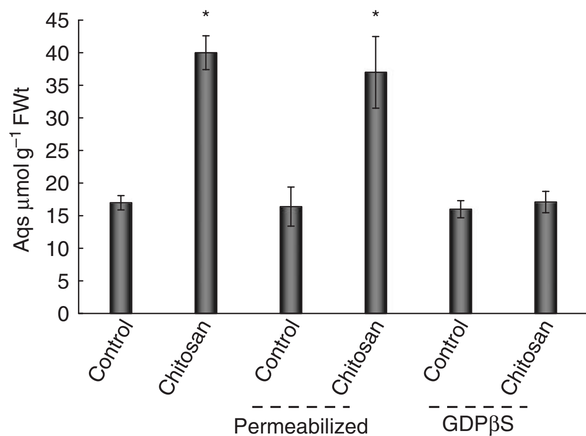

Fig. 2. The non-hydrolizable analogue GDPβS abolishes chitosan-dependent anthraquinone biosynthesis. Cells permeabilized in presence of GDPβS (1 mM) as described under Methods were used in elicitation assays with chitosan (200 mg l^{-1}) or vehicle (acetic acid 1%) for 24 h. The centre bars indicate that the permeabilization procedure itself did not modify Aqs values neither in control nor in elicited cells. Total content of Aqs was determined by spectrophotometry as described under Methods. Results are expressed as mean (μmol Aqs/g FWt) ± (SD) of three independent experiments. * $P < 0.005$ with respect to the control.

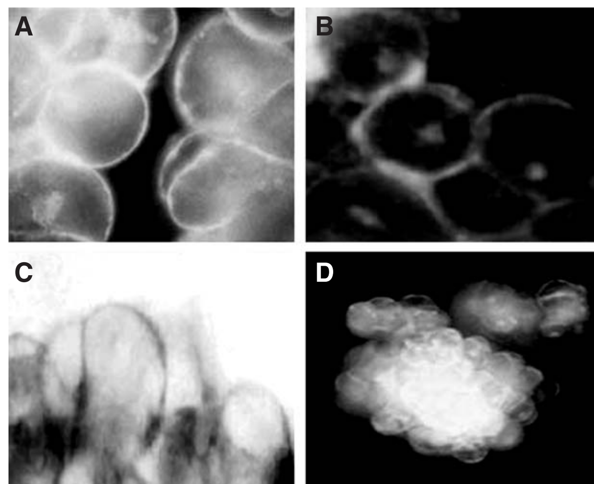

Fig. 4. Evaluation of *Rubia tinctorum* cell permeabilization using BSA-FITC, trypan blue and fluorescein diacetate (FDA). A. Fluorescence microscopy of cells permeabilized as described under Methods that were incubated with 10^{-6} M BSA-FITC for 25 min at 37°C shows strong fluorescence from FITC localized intracellularly. B. In non-permeabilized cells BSA-FITC was not incorporated and fluorescence was localized outside the cells. C. Permeabilized cells were stained with 0.4% trypan blue during 5 min at room temperature and the viable cells were counted. D. Fluorescence image of permeabilized R. tinctorum cells stained with FDA.

exposed to chitosan in presence of a monoclonal antibody against Gαq/11, considering the sensitivity of the elicitor effects to the specific PLC inhibitor U-73122 (Vasconsuelo et al. 2003; see Discussion). Preincubation with the antibody abolished Aq synthesis elicited by chitosan (Fig. 5). In cells incubated with antirabbit IgG or anti-Gαq/11antibody alone, no changes in Aq concentration were observed. In agreement with these observations, an immunoreactive band of the expected size for Gαq/11 (42 kDa) was observed in the microsomal fraction from *R. tinctorum* cells by Western blot analysis using the same anti-Gαq/11antibody (Fig. 6).

Incubation of cell suspension cultures with pertussis toxin (PTX; 2.5 μg ml^{-1}, 5 h) before the stimulation with chitosan, did not significantly affect elicitation (Fig. 7). In agreement with this, it has been shown that the Gα subunit of oat aleuron, like that of many other plant species, is insensitive to PTX (Jones et al. 1998).

Discussion

Plants efficiently respond to a great number of environmental stimuli to survive. The activation of a mechanism that elaborates the adequate response to each specific input signal, is probably initiated by perception of the stimulus through specific receptors. Findings such as the presence of guanine nucleotide-binding G-proteins and many classical downstream signalling elements such as phospholipases C and A_2, suggest that G-protein coupled receptor (GPCR)-mediated signalling operates in plant cells analogous to that in animal and yeast cells. In agreement with this hypothesis, previous reports have

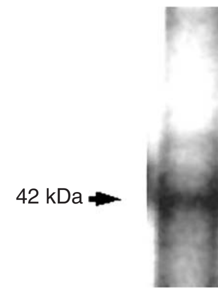

Fig. 6. Immunoblot detection of Gαq/11 in membranes from *Rubia tinctorum* cells. Gαq/11 immunoreactivity was assayed in the microsomal fraction isolated from cultured *R. tinctorum* cells by differential centrifugation as described under Methods. The membranes were resolved by SDS-PAGE and then subjected to Western blot analysis using a monoclonal anti-Gαq/11 antibody. The arrow indicates the Gαq/11 cross-reacting band of ~ 42 kDa. A representative immunoblot is shown.

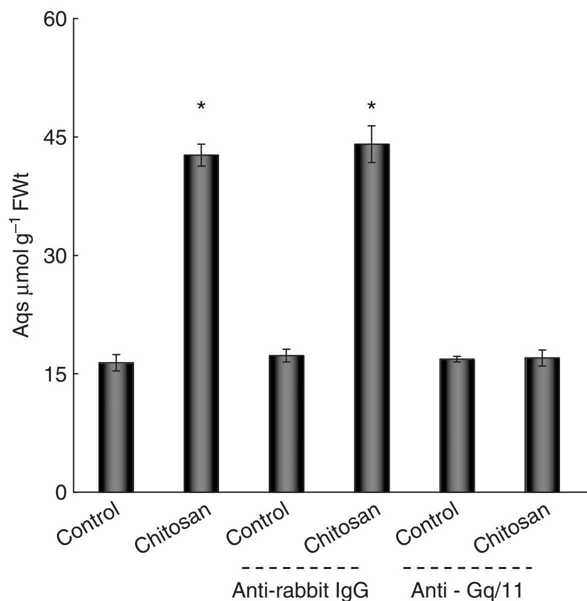

Fig. 5. Blockade of chitosan-induced elicitation by Gαq/11 antibody in *Rubia tinctorum* cell cultures. *R. tinctorum* cell cultures were incubated with permeabilization solution containing a monoclonal antibody raised against the Gαq/11 subunit of G-proteins (20 μg ml^{-1}) or antirabbit IgG as indicated under Methods. Then the cultures were treated with chitosan (200 mg l^{-1}) or vehicle (acetic acid 1%) for 24 h. Total content of Aqs was determined as described under Methods. Results are expressed as mean (μmol Aqs/g FWt ± SD) of three independent experiments. * $P < 0.005$ with respect to the control.

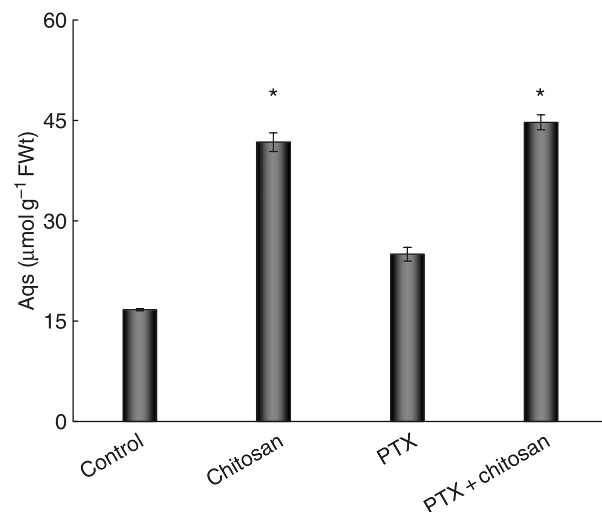

Fig. 7. Effect of bacterial pertussis toxin (PTX) on chitosan-induced anthraquinone production in *Rubia tinctorum* cells. *R. tinctorum* cell cultures were incubated during 5 h in the presence of PTX (2.5 μg ml^{-1}) before the treatment with chitosan (200 mg l^{-1}) or vehicle (acetic acid 1%) for 24 h. Total content of Aqs was determined as described under Methods. Results are expressed as mean (μmol Aqs/g FWt ± SD) of three independent experiments. * $P < 0.005$ with respect to the control.

demonstrated the presence of GPCR in plants (Millner and Causier 1996, Irving 1998).

Although a receptor specific for chitosan has not been shown yet, its existence cannot be ruled out since recent studies have identified oligosaccharide elicitor-binding proteins in plants (Yamaguchi et al. 2000). This as yet putative receptor could function coupled to a G-protein. In previous work we have demonstrated that anthraquinone levels in cultured *R. tinctorum* are markedly increased by elicitation with chitosan (Vasconsuelo et al. 2003). In this process we established the participation of the PLC/PKC-associated messenger system (Vasconsuelo et al. 2003), PI3K and MAPK cascades (Vasconsuelo et al. 2004) and the contribution of Ca^{2+} signalling mechanisms (Vasconsuelo et al. 2005). Within this context, the present study was carried out to ascertain whether G-proteins are involved in chitosan-induced elicitation in *R. tinctorum* cells. We first examined the effects of G-protein agonists and antagonists on chitosan-promoted anthraquinone synthesis. We observed that the G-protein agonists mastoparan, AlF_4^- and GTPγS increased anthraquinone levels to almost the same extent as chitosan. The combined simultaneous action of agonist and chitosan did not enhance chitosan-induced Aq production, suggesting that the compounds and the elicitor might act at the same level. Although mastoparan has commonly been used to indicate the presence of heterotrimeric G-proteins, it may also activate monomeric G-proteins (Koch et al. 1991). However, AlF_4^-, which has no effects on small monomeric G-proteins activity (Kahn 1991), generated a response similar to mastoparan. These experiments involve then heterotrimeric G-proteins in the signal transduction mechanism which mediates chitosan stimulation of Aq synthesis. The fact that antagonists of G-proteins, such as suramin or GDPβS, abolished the increase in anthraquinone induced by the elicitor, further supports this interpretation.

In accord with the finding that phospholipase C is activated by chitosan, compound U-73122, a specific PLC inhibitor, has been shown to block the effect of elicitor on anthraquinone synthesis (Vasconsuelo et al. 2003). Since this compound inhibits PLC activity and this impairs coupling of Gαq/11with the enzyme (Smith et al. 1990), we investigated whether the G-protein implicated in chitosan elicitation indeed belongs to the Gαq family. To that end, elicitation assays were performed in the presence or absence of PTX. Some members of the Gαq family of animals, as for example Gαq or Gα11, lack the recognition site for ribosylation by this toxin (Pang and Sternweis 1990, Stratmann and Simon 1990, Blank et al. 1991, Sternweis and Smrcka 1992). In contrast, $G\alpha_i$ and $G\alpha_0$ are pertussis-sensitive. It has been shown that plant Gα proteins also lack the cysteine residue near the C-terminus for ADP-ribosylation by PTX (Seo et al. 1995), although there are reports indicating that PTX affects G-protein containing signalling pathways (Irving 1998, Pan et al. 2005), an apparent discrepancy which remains unexplained. The fact that in the present study elicitation was not altered by the toxin, suggests nevertheless that the main G-protein involved in chitosan–induced anthraquinone production in *R. tinctorum* cultures is Gαq/11. Of importance for this interpretation, when cell cultures were preincubated with a highly specific anti-Gαq/11 rabbit monoclonal antibody raised against an evolutionary conserved region of Gαq/11, the chitosan-dependent increase in anthraquinone levels was fully inhibited. This specific antibody only reacts with Gαq and Gα11 and not with other subunits. Moreover, Western blot analysis of the microsomal fraction containing plasma membranes from *R. tinctorum* cells using this antibody showed the expected cross-reacting band of ~ 42 kDa for Gαq/11. Mammalian antibodies have been earlier employed in several plant studies to identify different G-protein components of signalling systems (Drobak et al. 1988, Wang et al. 1993). Moreover, this antibody is raised against the region essential for binding to GTP, which is highly conserved throughout all alpha subunits from higher plant species sequenced and mammals (Assmann 2002). Therefore, the immunoblot data suggest that this domain could be present in *R. tinctorum.*

In conclusion, our results suggest that chitosan elicit anthraquinone synthesis in *R. tinctorum* cells through a heterotrimeric G-protein, most likely belonging to the Gαq family.

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