Examination of Elicitor-induced Plant Defence Response

PhD Thesises

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Summary

The number of profitable (pharmaceutical) molecules produced by plant cell cultures is growing because of the several advantages of the applied methods.

In our work, we studied the callus formation and the features of reprogrammed cells, and we also evaluated particular steps of the elicitor induced plant defence response in cell cultures. The applied methods allowed us to examine the in vitro cultures of *Rubia tinctorum* and *Capsicum anuum* and their behaviour from several points of view after elicitor treatments. The structural investigations were carried out by microscopy and electron microscopy, while the appearance of particular steps of the plant defence response was followed by applying quantitative methods (spectrofluorimetry, spectrophotometry, HPLC-DAD-MS).

In our work, we observed that the callus formation from non-wounded parts of leaves of *Rubia tinctorum* was initiated by the transfer cells of vascular bundles. In the transfer cells significant structural changes were observed before the callus formation. In the mesophyll cells similar changes were also started, but these cells were not able to divide. The accumulation of anthraquinone derivatives during the callus formation exhibited two-step kinetic process.

The elicitor induced H_2O_2 production (which is a known part of the plant defence response) exhibited several maximum values, and the appearance of these maxima (duration, time-course, amplitude) depended on the applied elicitor. The electron microscopic investigations affirmed the phenomenon of H_2O_2 production, and we found that this H_2O_2 production had local characteristics independent of the direction of the signal. By using inhibitors during the experiments, we pointed out that the presence of the calcium signal had important role in the elicitor induced H_2O_2 production. Direct linkage with the IP_3 signalization is presumable.

We confirmed by using liquid chormatographic method that application of different elicitors in *Rubia tinctorum* cell cultures resulted different antraquinone compositions. This fact may give the opportunity to develop more selective anthraquinone-producing strategies in the in vitro systems.

Our results contribute to better understanding of the elicitor induced plant defence response. In this way, the influence on signaling pathways gives an effective tool to change the quantity and quality of the selected products.

Introduction

Plant organisms have evolved a sophisticated defence response during their evolution coping with the harmful biotic and abiotic components of their environment. This defence response is a complex phenomenon.

From pharmaceutical point of view, the most important parts of the plant defence response are generally those processes, which are involved in the increased or changed production of secondary metabolites. Molecules orginating from secondary metabolism are applied in the medicine after direct extraction or after semi-synthetic modification.

The applied plant biotechnological methods introduce several approaches to increase the level of production of secondary metabolites. One of these approaches is the formation, maintenance and exploitation of plant cell suspension cultures. However, several successful methods have been developed and applied to increase the production of secondary metabolites in plant cell suspension cultures, insufficient knowledge exists about the regulation of the signal transduction and cell physiological processes. The effective exploration of the mentioned regulational processes may provide more selective, designable and more efficient production of natural ingredients leading to the significant decrease of the production costs.

One of the methods (which has been applied in plant suspension cultures for a long time) is elicitation. It is observed that for induction of the plant defence response plants do not need the presence of the whole biotic and abiotic environment. A particular segment of the abovementioned environmetal factors, which could be recognized by the plant, is enough to induce the response. Effects of elicitors became the object of scientific attention and several new discoveries were published in this field during the last decades. Application of these new results may enlarge the number of profitable and highly productive cell cultures in the near future.

According to our current knowledge, elicitors are recognized by plant receptors, which are in the plasma membrane or in the cytoplasm. After binding and recognizing the elicitor, receptors activate their effectors and through second messenger molecules they modulate the activity of particular genes. Finally, the altered gene expression may manifest in increased production of secondary metabolites.

Naturally, the plant defence response is influenced by several endogen factors, for example the level of differentiation of cells, which gives the opportunity to the cells to produce higher amount of special metaboltes.

In our work, we followed the differentiational/dedifferentiational/redifferentiational changes at structural and chemical alteration levels during the formation of *Rubia tinctorum* callus cultures. We also examined some particular components of the elicitor induced plant respone by using microscopic and analitical methods (formation of the oxidative stress, its connection with the other signaling pathways and alterations of secondary product formation after elicitor treatments).

Aims of the work

The object of our work was to examine the plant cell dedifferentiation and to reveal certain signal transduction events (and results of their modulation) in the applied model plant (*Rubia tinctorum*).

The goal of our study was to investigate some early phenomena of dedifferentiation/differentioation after callus induction from leaves durig a 14-days period. We wished to follow the structural and ultrastructural changes, the chlorophyll pigment content alterations and the qualitative and quantitative changes of anthraquinone derivatives to reveal connection between the differentiational level and the anthraquinone production of the cells.

In order to get to know the background mechanisms of the secondary product formation, we aimed at exmamining some parts of the elicitor/hormone induced plant signal transduction in plant cell cultures. Our goals were to reveal the viability of the treated cells during the elicitation procedure, to make inquiries about the dynamism (short and long term; in the cells and in the supernatant) of the elicitor induced H_2O_2 production, to examine the localization of the H_2O_2 production at ultrastructural level, and to investigate the influence of specific inhibitors on the elicitor induced H_2O_2 production.

One of our main research was to reveal the effects of elicitors and plant hormones on the production of anthraquinone derivatives in cell cultures. Within the framework of this topic we would like to examine the qualitative and quantitative composition of anthraquinone derivatives of suspension cultures (in comparison with the pigment compositions of rhizome/root and calli).

Methods

The fully developed Rubia tinctorum plants derived from the Botanical Garden of the Research Institute of Medicinal Plants. During the evaluation of the dedifferentiation/differentiation steps of callus formation the applied leaves were obtained from the middle region of the stems. The surface of these leaves were sterilized, then they were cut into 1-2 cm^2 pieces. The callus induction was made in Petri-dishes, and on solid MS culture medium (containing 0.6 % agar, 30 g L^{-1} sucrose, 1 mg L^{-1} IAA, 0.2 mg L^{-1} NAA, and 0.2 mg L^{-1} kinetin) at natural light and room temperature.

The maintenance of the earlier initiated, rhizome-derived callus was carried out on the abovementioned MS culture medium at room temperature, in dark. Callus cultures were subcultured in every 2 months. Suspension cultures, which were started from these calli, were maintained in 500 mL Erlenmeyer flasks on liquid MS medium at natural light, room temperature and 125 rpm on a rotary shaker. Suspensions were subcultured in every 14 days.

The applied plant hormones (jasmonic acid and salicylic acid) were obtained from the trade, the elicitors were prepared in our laboratory. The phytopathogenous *Botrytis cinerea* Pers. Ex. Pers. cultures were maintained on 3% (v/v) malt culture medium. The BC elicitor, containing cell wall oligosaccharides, was prepared by purification and hydolysis. The necrotroph-parasitic *Coriolus versicolor* (Fr.) Quel. fungi derived from Buda hills. The water-soluble polysaccharide content (CV elicitors) were extracted by hot water from the fruit bodies. The polysaccharides were precipitated with 96% (v/v) ethanol and the obtained polysaccharide mixture was fractioned by gel filtration. The obtained three fractions (with different molecular weights) were used as separate elicitors.

The effective/optimal concentrations of the applied elicitors and plant hormones were established during the preliminary experiments. The sterile hormones/elicitors were dosed aseptically. Hormones were applied in four different concentrations during the experiments. We made stock solutions from the *Coriolus* elicitors (80 mg mL^{-1}), and 1 mL from these solutions were given into every 100 mL of suspension cultures, while from the BC elicitor 1.25 mL was added into every 100 mL of suspension cultures.

In control treatments, we introduced 1 or 1.25 mL of sterilized distilled water into the suspension cultures.

We examined the influence of different specific inhibitors (LiCl; $LaCl_3$; nifedipine, neomycine, 2-aminoetil diphenilborinate) on the H_2O_2 production of the cells. The used

concentrations of the metioned inhibitors were based on data from literature after checking their effectiveness through the preliminary experiments. During the experiments with these drugs, we used the inhibitors alone (as mock controls) or with elicitors applying inhibitors for 15 minutes pretreatments.

During the examination of callus formation we investigated the 0-14 days old samples with daily sampling. To keep under observation the structural changes along the differentiation process of the callus induction, we applied light and electron microscopic methods.

We followed the quantitative changes of chlorophyll a+b pigments in the leaves during the callus induction using TLC and spectrophotometric methods.

The qualitative and quantitative changes of the anthraquinone derivatives during the callus induction were examined by HPLC-DAD-MS.

Following the alterations of the microtubular system of elicited cells the experiments were carried out on *Capsicum annuum* cells (instead of Rubia tinctorum cells because of its high autofluorescency) after 0-48 h of the elicitation. The visualization of the microtubular system was based on protocol from literature using FITC conjugated antibodies.

Viability was tested 0-96 h after hormone/elicitor treatments by fluorescein diacetate staining with the application of the appropriate controls.

The H_2O_2 content of the cells and of the supernatants was monitored after BC elicitor, jasmonic acid and salicylic acid treatments and in the control samples during 0-48 h. The effects of inhibitor treatments were followed during 0-4 h period. The H_2O_2 production of the cells was measured by titanium-peroxo complex formation method (the complex was quantified spectrophotometrically), while the H_2O_2 content of the supernatant was measured by a fluorimeter on the basis of scopoletin-oxidation (fluorescence quenching) method.

The H_2O_2 production related structural changes were followed by TEM in the samples of the inhibitor pretreated BC elicited or hormone treated cells in a period of 0-8 h (in case of inhibitor treatmets 0-4h). To investigate the ultrastructure of the H_2O_2 production in the cells we applied Bestwick's cytochemical method, which is based on the cerium perhydroxide precipitate formation during the reaction of the Ce ions and H_2O_2. The results of the cytochemical reactions were observed with electron microscope. The element analyses of the precipitates and the Ce-element maps were made by electron energy loss spectroscopy (EELS).

We examined the anthraquinone derivatives of the cells qualitatively and quantitatively in optimized times after the treatment of hormones or elicitors with different

concentrations. The extracts of the samples were examined by HPLC-DAD-MS method (HPLC-DAD-1946A MSD system, DAD online spectra: 200-600 nm, quantitative measurement: 254 nm, MS detektor ESI ionization positive ion mode, scan 140-700m/z). A part of the antharquinone standards were obtained from trade, another part was produced in our laboratory.

Results

Examination of the dedifferentiation/redifferentiation

The different types of cells of the *Rubia* leaves reached various differentiation levels and they were able to follow new (which differ from each other) ways of development concerning redifferentiation. We examined in detail the initiation of the callus formation around the veins (the callus formation at wounded parts is well documented in the literature, therefore we did not examine this phenomenon). The differentiation status of epidermal cells of the non-wounded regions was not changed, while the mesophyllar cells of these regions reached a certain differention level after dedifferentiation. These cells were not able to divide, however, their microscopic features unambigously showed the started dedifferentional process. The dedifferentiation affected in the highest digree the transfer cells of the veins. These cells showed characteristic ultrastructural changes (membrane bordered-limited autolysis, inequal-like plastid division, ultrastructural changes of the cell wall), and became able to divide and to form new cells.

The chlorophyll pigment content of the leaves after callus initiation decreased rapidly and this reduction stopped at the value of 25 percent of the original pigment concentration, and it did not change until the end of the observations. Parallel to the changes of the chlorophyll pigment content, the accumulation of the anthraquinone derivatives started in the cells. The accumulation showed two-step kinetics, however, the time-course of production and the composition of the anthraquinones were not equal during the observed period.

Effect of the elicitation on the cell viability

Cell viability was not changed during the early period after the different elicitor or hormone treatments. Significant reduction of the cell viability was observed only after 96 h.

This fact allowed to suppose that degradation of the cells did not influence the results of our experiments.

Effect of the elicitor/hormone treatmets on the H_2O_2 accumultaion in the cells and culture medium

The oxidative stress dectected in the cells and medium were specific to the applied elicitor/hormone. The observed H_2O_2 production in the cells had two early (4-8 min, 30-50 min) and two subsequent maxima (8-12 h, 36 h), which maxima differed characteristically in timetable, duration, and amplitude according to the used treatments. The H_2O_2 accumulation in the medium also depended on the applied elicitor/hormone, in this case well-defined maxima and minima were also characteristic.

The results obtained by electron microscope and EELS confirmed the dinamic feature of H_2O_2 production of the cells, and provided additional information as: the H_2O_2 production of the cells was local event (the early H_2O_2 appearance was observable at the area of the plasmamembrane of the cells, later it occured in the cell wall) inspite of the fact that the signals affecting the cells had not specific direction. The treatments with elicitors/hormones and inhibitors also supported this local appearance of the H_2O_2 production, although the appearance and the extension of precipitate deposition varied.

Effect of inhibitors on the elicitor induced H_2O_2 production

Signal transduction of the cells may be disturbed using inhibitors, so H_2O_2 production in the cells might be significantly decreased, but it could not be fully diminished. Our results suggest that availability of the calcium signals and undisturbed activity of phospholipase C enzyme are needed for the BC induced H_2O_2 production.

On the basis of treatments with different inhibitors we established that the regulation of the elicitor triggered early H_2O_2 production involves mainly the voltage dependent calcium channels and the phospholipase C enzyme, while regulation of the later production maxima (after 2 h) involves dominantly the role of L-type calacium channels, however, the results also indicate significant overlapping among the abovementioned regulational mechanisms.

The results of electron microscpic observation verify these data. The appearance of the obtained cytochemical results was in good accordance with the quantitative data in almost all cases.

Fate of the microtubular system after BC treatment

On the basis of our observations made at the timepoints when the H_2O_2 production was maximal, we can state that the used BC treatments did not alter morphology of the microtubular system in the cells, even not in those times, in which the oxidative status of the cells differed significantly from the control ones. The microtubular system of the elicited cells did not show polarization, in contrast with our expectation based on ultrastructural appearance of the H_2O_2 productions.

Effect of different elicitors/hormones on the athraquinone production of the cells

Ten anthraquinone derivatives were successfully identified and quantified in the treated and control suspension cultures by HPLC-DAD-MS.

We determined the effects of fungi-derived elicitors and the two applied hormones on the production of the anthraquinone derivatives in *Rubia tinctorum* cell cultures. The effectiveness of various treatments was different.

In case of application of the fungi-derived elicitors higher sum anthraquinone contents could be measured, which contents also exceed the anthraquinone contents of rhizome/roots samples (application of the *Coriolus* elicitor fraction with the highest molecular weight resulted the highest anthraquinone production).

The jasmonic acid treatment increased selectively the pseudopurpurin production of the cells, while the salicylic acid treatment increased selectively the alizarin production.

Our results showed that application of fungi-derived elicitor treatments and higher concentration of jasmonic acid is more effective then the compound extraction from the underground organs of the intact plants.

Similarly, application of the two plant hormones in the suspension cultures is more advantageous then the compound extraction from the intact plants because of their more selective effects on the anthraquinone production.

List of publications

Papers in English

1. Orbán N, Boldizsár I, Szűcs Z, Dános B. (2008)
Influence of different elicitors on the synthesis of anthraquinone derivatives in *Rubia tinctorum* L. cell suspension cultures.
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Book chapter

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1. Boldizsár Imre, Orbán Norbert, Dános Béla
Az antrakinon-összetétel befolyásolásának lehetősége *Rubia tinctorum* L. szuszpenziós tenyészetben. XII. Magyar Növényanatómiai Szimpózium Sárkány Sándor Emlékére 2006. június 22-23, pp.78-83 (Jate Press, Szeged, 2006; Szerk.: Mihalik Erzsébet; ISBN 963 482 767 5)

2. Orbán Norbert, Boldizsár Imre, Bóka Károly
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Dynamism and localization of H_2O_2 production in elicited plant cells.
The 14th Congress of the Federation of European Societies of Plant Biology, August 2004, Cracow, Poland.

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1. Kristóf Z, Orbán N, Bóka K. Hidrogénperoxid felszabadulás vizsgálata elicitált növényi sejtekben citokémia, EELS, és elektronmikroszkóp segítségével. A Magyar Mikroszkópos Társaság 2008. évi Konferenciája, Balatonalmádi, 2008. 05. 15-17.

2. Boldizsár I, Orbán N, Szűcs Z, Bóka K, Dános B, Füzfai Zs, Molnár-Perl I. Determination of anthraquinone derivatives in cell suspension cultures of *Rubia tinctorum* L.: elicitation's impact on the amount of the compounds formed.
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