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Shikonin Accumulation is Related to Calcium Homeostasis in *Onosma paniculata* Cell Cultures

By

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With 1 Table and 3 Figures

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Summary

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The involvement of calcium homeostasis and calmodulin in shikonin accumulation in *Onosma paniculata* Bur. & Franch cell cultures was studied by using pharmacological method. The results showed that the addition of excess Ca^{2+} and chelator, EGTA, to M_9 production medium resulted in a reduction of shikonin formation, and shikonin production was completely suppressed by EGTA at 6 mM. Ca^{2+} channel blockers, verapamil and nifedipine, and Ca^{2+} ionophore A23187, exhibited a partial inhibition of shikonin accumulation. Furthermore, the addition of CaM an-

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tagonist, W-7 at 80 μM, completely inhibited shikonin accumulation. In contrast to W-7, the relatively inactive analog W-5 at equivalent concentration did not apparently influence shikonin production. These results indicated that calcium homeostasis and CaM-mediated Ca^{2+} signal sensing might be involved in shikonin accumulation in *O. paniculata* cell cultures.

Zusammenfassung

Liu Z., Li Y., Yang T., Su J., Zhang M., Tian R., Li X., Pang Y., Qi J. & Yang Y. 2011. Shikonin accumulation is related to calcium Homeostasis in *Onosma paniculata* cell cultures. [In *Onosma paniculata* Zellkulturen ist eine Shikonin Anreicherung mit einer Kalzium-Homeostase verbunden]. – Phyton (Horn, Austria) 51 (1): 103–113, with 3 figures.

Anhand pharmokologischer Methoden wurde die Rolle der Kalzium-Homeostase sowie die Rolle von Calmodulin in der Shikonin Produktion in *Onosma paniculata* Bur. & Franch Zellkulturen untersucht. Die Resultate zeigten, dass bei einem Überschuss von Ca^{2+} und dem Chelator EGTA im M_9 Kultur-Medium weniger Shikonin gebildet wird. Bei einer Konzentration von 6 mM EGTA war die Shikonin Produktion komplett gehemmt, während die Ca^{2+} Kanalblocker Verapamil und Nifedipin und die Ca^{2+} Ionophore A23187 eine teilweise Hemmung der Shikoninbildung bewirkten. Das Hinzufügen des Calmoldulin Antagonisten W-7 in einer Konzentration von 80 μM hemmte die Shikoninbildung komplett. Im Gegensatz zu W-7 zeigte bei gleichen Konzentrationen das relativ inaktive Analogon W-5 keinen sichtbaren Einfluss auf die Shikoninproduktion. Diese Ergebnisse implizieren, dass die Kalzium Homeostase zusammen mit Calmodulin, als Calcium-bindendes regulatorisches Protein, möglicherweise an der Shikonin Produktion in *O. paniculata* Zellkulturen beteiligt sind.

Introduction

Calcium homeostasis plays an important role in regulation of plant growth, development, accumulation of secondary metabolites, and other physiological responses (Gilroy & al. 1993). Plant cells usually keep a cytosolic calcium concentration at 100–200 nM, which is maintained by calcium pumps and channels located in the plasma membrane and the membranes of cellular organelles (Gilroy & al. 1993). Changes in cytosolic free calcium can be evoked by a wide variety of abiotic and biotic signals including light, low and high temperatures, touch, stress, hormones, fungal elicitors, pathogens attacking, and nodulation factors (Bush 1993, Zhao & al. 2005, Kim & al. 2009).

The roots of boraginaceae plants, such as *Lithospermum erythrorhizon* and *O. paniculata*, produce red naphthoquinone compounds, shikonin and its derivatives. These secondary metabolites have been used medicinally for antibacterial, anti-inflammatory, anti-tumor, and inhibition of HIV virus, and also been used as colorants for cosmetics, fabrics, and foods (Efferth & al. 2007, Ma & al. 2008). The two-stage cell culture system of these plants was used, including a growth stage for cell pro-

liferation in B5 medium and a production stage in M_9 liquid medium, for the formation of shikonin and its derivatives (FUJITA & al. 1981, NING & CAO 1995). At present, the metabolic pathway of shikonin biosynthesis has been well-characterized, and some progresses have been made in the biochemical and molecular mechanisms of regulation of shikonin production (TABATA & al. 1974, FUJITA & al. 1981, HEIDE & al. 1989, BUSH 1993, YAZAKI & al. 1999).

The biosynthesis of shikonin was found to be highly affected by many environmental factors, such as light, plant growth regulators, medium nutrients, and fungal elicitors (TABATA & al. 1974, ZHAO & al. 2005, WU & al. 2009). However, little is known about the involvement of calcium homeostasis in the accumulation of shikonin and its derivatives. In the present study, a pharmacological approach with the reagents specifically involved in regulation of cytosolic calcium concentration was used to investigate the possible roles of calcium in shikonin production in *O. paniculata* cell cultures. The results provided further understanding of the accumulation and regulation of shikonin and its derivatives in the cultured cells.

Materials and Methods

Plant Materials and Cell Culture Conditions

The callus used is a somatic line YN12, derived from young shoots of *O. paniculata* BUR. et FRANCH (YANG & al. 1999). The two-stage culture system was used, including a growth stage for cell proliferation, in which the calli were maintained in a B5 solid medium (8 g/L agar) at 25°C in light (80 μmol m^{-2} s^{-2} 8 h/day) and the subculture was carried out every 16–18 days before the cells reached stationary phase (NING & CAO 1995), and a production stage for the formation of shikonin and its derivatives in M_9 liquid medium (FUJITA & al. 1981, NING & CAO 1995). IAA at 0.05 mg/L and BAP at 1 mg/L were added to B_5 medium as a basic growth regulator combination, while IAA at 0.1 mg/L and BAP at 1 mg/L were added to M_9 production medium (NING & CAO 1995, YANG & al. 1999, YANG & al. 2003). To produce shikonin, about 2 g of 16–18 days old calli produced during the growth stage in B_5 medium was inoculated in a 250 mL Erlenmayer flask containing 50 mL of M_9 medium and cultured on a rotary shaker at 120 rpm at 25 ± 1°C in the dark. The calli were harvested after 18–20 days to measure shikonin production (YANG & al. 1999).

Preparation of Chemicals and Experimental Treatments

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Mo., USA). Chemicals were dissolved in water or dimethyl sulfoxide (DMSO) to make stock solutions, and all aqueous solutions were sterilized by filtration. EGTA [Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid], a Ca^{2+} chelator, was dissolved in distilled water to make a 0.2 M stock solution, and the solution pH was adjusted to 7.5 with 10 N NaOH. Compound A23187 (calcimycin), a Ca^{2+} ionophore, was dissolved in DMSO to make a 1 mM stock solution. Ca^{2+} channel blockers nifedipine [1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl este] and verapamil [5-(N-(3,4-Dimethoxyphenylethyl) methylamino) -2-

(3,4-dimethoxyphenyl)-2-isopropylvaleronitril] were dissolved in DMSO to make 0.2 and 0.1 M stock solutions, respectively. The following CaM antagonists were used to examine the role of CaM in shikonin production. Compounds W-7 [N-(6-Aminohexyl)-5-chloro-1-naphthalene sulfonamide] and W-5 [*N*-(6-aminohexyl)-1-naphthalene sulfonamide] were dissolved in DMSO and distilled water, respectively, to make 10 mM stock solutions.

An obvious color alternation was observed in *O. paniculata* cells and M_9 production medium when cells were cultured in the dark for several days. The striking red pigment facilitates primary screening for the effects of chemical inhibitors on the accumulation of shikonin and its derivatives. The live and pigment-producing cells become a little stiff during the process of cultivation, while the harmed cells become soft, rotten then dead, and nearly no color alternation occurs in production medium. All the chemicals were added to M9 production medium together with inoculation at designed concentrations. The pre-treatments proved that these chemicals were harmless to *O. paniculata* cell cultures at the range of applied concentrations in the present experiments. The final concentrations of solvent DMSO added to the medium was adjusted to less than 1% and had no discernible effects on cultured cells and shikonin accumulation. The controls were treated with equivalent amounts of distilled water or DMSO. All treatments were performed at least twice with three replicates each.

Measurement of Shikonin and its Derivatives Content

The shikonin and its derivatives in the cells were determined as described by Heide & Tabata 1987 with slight modification as follows: These metabolites were extracted from both cells and the culture medium using petrol ether, and measured at 520 nm with 2800 UV/VIS spectrophotometer (Unico, Shanghai, China). Total amounts of these metabolites were calculated after adjustement to a standard curve (shikonin content = $41.66 \times OD_{520} \times$ dilution fold), and reported in mg/g cell fresh weight (FW) (Liu & al. 2006, Zhang & al. 2010).

Results

Effects of External Ca^{2+} and Ca^{2+} Chelator on Shikonin Accumulation

The salt of Ca^{2+} and Ca^{2+} chelator were added to M_9 production medium to determine the effect of external Ca^{2+} on the accumulation of shikonin and its derivatives. The original concentration of Ca^{2+} in M_9 medium (control) with $Ca(NO_3)_2{\cdot}4H_2O$ as calcium source was 0.1 mM, and other gradients of Ca^{2+} were applied with $Ca(NO_3)_2{\cdot}4H_2O$. The addition of Ca^{2+} resulted in a decreased accumulation of shikonin and its derivatives (Fig. 1). The inhibitory effect was significant when 0.8 mM Ca^{2+} was added to M_9 production medium and Ca^{2+} at 1 mM decreased shikonin content by about 43% compared with the control (0.1 mM Ca^{2+}). The depletion of external Ca^{2+} by chelator, EGTA, significantly reduced shikonin content (Fig. 2). Only a little of shikonin was detected when 4 mM EGTA was added to M9 medium and the accumulation of shikonin and its derivatives was completely repressed by 6 mM EGTA. These results indicated that extracellular calcium homeostasis was necessary for shikonin accumulation.

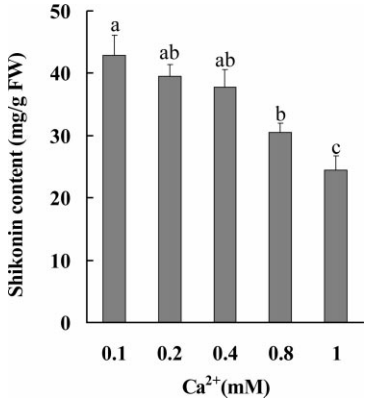

Fig. 1. Effect of Ca^{2+} at different concentrations on shikonin accumulation in cultured *O. paniculata* cells. The original concentration of Ca^{2+} in M_9 production medium (control) is 0.1 mM, and gradients of calcium were prepared with chemical $Ca(NO_3)_2 \cdot 4H_2O$. Significance is used LSR test at α=0.05 and showed by different letters up the bars. The bars indicate standard errors of the means with three replications.

Effects of Ca^{2+} Channel Blockers on Shikonin Accumulation

Some blocking agents, such as La^{3+}, Gd^{3+}, diltiazem, verapamil, and nifedipine, were widely used to inhibit Ca^{2+} transport through specific channels at the cytoplasmic membrane or at the internal cellular compartments. In the present study, two Ca^{2+} channel blockers, voltage-dependent Ca^{2+} channel blocker verapamil and nifedipine were used here to clarify the effects of Ca^{2+} ion channels on the accumulation of shikonin

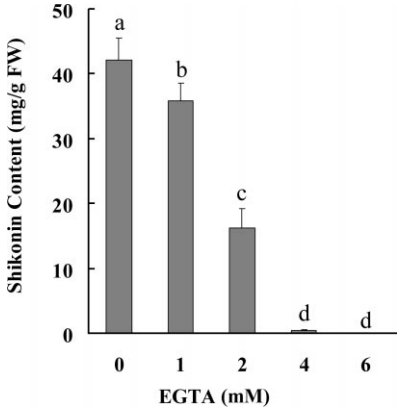

Fig. 2. Effect of Ca^{2+} chelator EGTA on shikonin accumulation in cultured *O. paniculata* cells. The sterilized EGTA was added in M_9 production medium together with inoculation. Significance is used LSR test at α=0.05 and showed by different letters up the bars. The bars indicate standard errors of the means with three replications.

and its derivatives (Table 1). The results showed that verapamil at 50, 100, and 200 μM, significantly decreased shikonin production by 32%, 47%, and 50%, respectively. Treatments of the cells with nifedipine at lower concentrations, 100 or 200 μM, had only a slight inhibitory effect on shikonin accumulation. However, the higher concentrations we employed at 500, 1000, and 2000 μM resulted in decreasing apparently shikonin production by 29%, 64%, and 66%, respectively.

Table 1. Effects of Ca^{2+} channel blockers verapamil and nifedipine and Ca^{2+} ionophore A23187 on shikonin production in *O. paniculata* cell cultures.

Treatment	Concentration (μM)	Shikonin production (mg/g FW), mean ± SD
None (control)		39.6 ± 4.1 a
Verapamil	50	27.1 ± 3.8 b
	100	20.8 ± 2.9 c
	200	19.7 ± 3.5 c
Nifedipine	500	28.3 ± 3.6 b
	1000	14.1 ± 2.1 d
	2000	13.5 ± 1.8 d
A23187	1	38.8 ± 2.9 a
	2	37.3 ± 4.0 a
	4	29.2 ± 2.7 b

Note: The data are the twice with three replicates of each treatment and standard deviation. Significance is used LSR test at α=0.05 and showed by different letters followed the means.

Effects of Ca^{2+} Ionophore on Shikonin Accumulation

To confirm that extracellular Ca^{2+} influx is required for shikonin production, the Ca^{2+} ionophore, A23187, which can release Ca^{2+} from intracellular calcium stores by acting as a Ca^{2+} carrier, was applied to *O. paniculata* cell cultures. The shikonin accumulation was significantly reduced by 26% compared with the control when A23187 was added at 4 μM (Table 1). When M_9 medium was also supplemented with 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, the reduction of shikonin content was much more than that of treatment with A23187 alone(unpublished data). Therefore, the results indicated that disturbance of the calcium homeostasis by an increase of cytosolic calcium affected shikonin accumulation.

Effects of Calmodulin (CaM) Antagonists on Shikonin Accumulation

CaM, a potent Ca^{2+} binding protein, is the most common calcium signal sensor in plant cells. Because the above experiments indicated a requirement for calcium in shikonin accumulation, we investigated whether CaM was involved in the process by using the CaM antagonists, W-7, in the

present study. As a control, the effects of the relatively inactive analog W-5, less specific for CaM, were also tested. Figure 3 showed that shikonin accumulation was dramatically reduced at increasing concentrations of W-7. The production of shikonin was completely suppressed when W-7 was added at 80 μM. In contrast, treatments with W-5 at equivalent concentrations did not apparently affect shikonin accumulation. These results indicated that CaM-mediated Ca^{2+} signal sensing might be involved in shikonin accumulation in *O. paniculata* cell cultures.

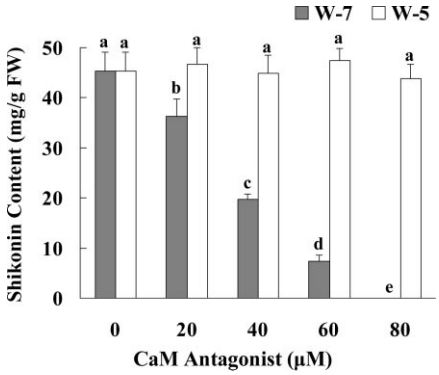

Fig. 3. Effect of calmodulin antagonist W-7 on shikonin accumulation in cultured *O. paniculata* cells. The relatively inactive analog W-5 was used as control. The sterilized W-7 and W-5 were added in M9 production medium together with inoculation. Significance is used LSR test at a=0.05 and showed by different letters up the bars. The bars indicate standard errors of the means with three replications.

Effects of Different Chemicals on Shikonin Excretion Ratio

In addition, the ratio of shikonin excreted into M_9 medium from cells was also measured. Although shikonin excreted into medium was decreased along with the reduction of those accumulated in the cultured cells treated with above chemicals, We found that the ratio of shikonin excretion into the medium to cellular accumulation kept at about 30%, which seems to be unaffected by different chemical treatments (data not shown).

Discussion

There are mounting evidences showing that calcium homeostasis plays an important role in physiological processes (Gilroy & al. 1993). For positive regulation, external calcium availability and calcium influx were necessary for production of secondary metabolites (Mahady & Beecher 1994, Ishihara & al. 1996, Zhao & Sakai 2003, Zhao & al. 2004). Calcium also negatively affected the production of some compounds like alkaloid and cardenolide in plant (Ning & al. 1998, Cacho & al. 1999, Paranhos & al. 1999, Moreno-Valenzuela & al. 2003). Despite the paradoxical roles in

different plants, the change of cytosolic calcium concentration happened in all cases.

In present study, we found that the accumulation of shikonin and its derivatives was significantly inhibited by the addition of Ca^{2+} in Ca $(NO_3)_2 \cdot 4H_2O$ (Fig. 1) and calcium ionophore (A23187) (Table 1). The results showed that high extracellular and intracellular Ca^{2+} concentrations both suppressed shikonin accumulation. Interestingly, specific calcium chelator (EGTA) also inhibited this physiological process (Fig. 2), which indicated that proper amount of calcium influx was required for shikonin accumulation. Previous work by NING & al. 1998 also revealed that calcium affected the accumulation of shikonin and its derivatives in *O. paniculata* cells when treated with elicitor or not. All these results indicated that severe disturbance of the optimum Ca^{2+} condition in cells might lead to reduction of shikonin accumulation. Thus we concluded that Ca^{2+} homeostasis, especially the endogenous Ca^{2+} level, was critical for the accumulation of shikonin and its derivatives.

Plants uptake calcium from environment to keep the balance between external and internal Ca^{2+} through Ca^{2+} channels (BUSH 1993, MARTINOIA & al. 2007). Several types of calcium channels in the plasma membrane, including endoplasmic reticulum, tonoplast, and plastid membranes of plant cells, have been identified and characterized (WHITE 2000, PAREKH & PUTNEY 2005). Due to the fact that two voltage-dependent Ca^{2+} channel blockers (verapamil and nifedipine) only had partially inhibitory effect in shikonin production (Table 1), it implies that other types of Ca^{2+} channels could also be involved in Ca^{2+} homeostasis-related accumulation of shikonin derivatives in *O. paniculata* cells.

Cytosolic calcium may function as a second messenger and regulate many physiological and chemical reactions (BUSH 1995, SANDERS & al. 1999, SANDERS & al. 2002, YANG & POOVAIAH 2003). Calcium signals are generated through the opening of ion channels that allow the downhill flow of Ca^{2+} from a compartment in which the ion is present at relatively high electrochemical potential (either extracellular or intracellular store) to one in which Ca^{2+} is at lower potential (SANDERS & al. 2002). The initial perception of a calcium signal occurs through the binding of calcium to many different calcium sensors. There are three types of proteins that respond to calcium ion fluxes, namely, calmodulin (CaM), calcineurin B-like proteins (CBL), and calcium-dependent protein kinases (CDPKs) (SANDERS & al. 2002). CaM is a highly conserved eukaryotic protein and a ubiquitous calcium sensor, while CBL and CDPKs are calcium sensors for specific signal response coupling in plants (LEE & RUDD 2002, LUAN & al. 2002). In plants, a complex Ca^{2+}/ CaM-mediated signal network is showed to affect many aspects of plant growth, development, and responses to environmental changes (YANG & POOVAIAH 2003). The Ca^{2+}/CaM signaling system

was proved to be involved in aflatoxin, melanin, and cercosporin toxin biosynthesis in bacteria and fungi (Kim & al. 1998, Rao & Subramanyam 1999, Chung 2003).

The antagonist of CaM action was employed in present study to study the effect of CaM in the accumulation of secondary metabolites in *O. paniculata* cell cultures. Significant suppression in shikonin accumulation was observed in the cell cultures treated with CaM inhibitor, W-7, but not with W-5, which indicated CaM was involved in regulating shikonin production. Further studies will be focused on to elucidate whether this regulation was related to the Ca^{2+}/CaM signaling pathway. It will also be interesting to investigate whether activities of key enzymes of shikonin biosynthesis are directly under the regulation of Ca^{2+}/CaM signaling pathway. Even though, we could not exclude the possibility that other sensors are also involved in detecting calcium change. Furthermore, recent identifications of cell-surface detectors of extracellular Ca^{2+} has prompted the possibility that Ca^{2+} may directly function as a signaling molecule (for review: Hofer 2005). Therefore it is really hard to say for sure that intracellular Ca^{2+} is the primary messenger that governs shikonin accumulation.

In general these present results indicated that Ca^{2+} homeostasis plays a pivotal role on the accumulation of shikonin and its derivatives in *O. paniculata* cell cultures. Ca^{2+}/CaM signaling pathway is probably involved in this process. However, further researches are needed to reveal more details and to test other possible pathways.

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