Calcium Mobilization in Salicylic Acid-Induced *Salvia miltiorrhiza* Cell Cultures and Its Effect on the Accumulation of Rosmarinic Acid

Hongbo Guo · Nan Zhu · Michael K. Deyholos · Jun Liu · Xiaoru Zhang · Juane Dong

Abstract Ca^{2+} serves as a second messenger in plant responses to different signals, and salicylic acid (SA) has been recognized as a signal mediating plant responses to many stresses. We recently found that SA treatment led to the cytoplasmic acidification of *Salvia miltiorrhiza* cells and alkalinization of extracellular medium. Here, we demonstrate that SA can rapidly induce Ca^{2+} mobilization in protoplasts, but the induction can be blocked with a channel blocker of either plasma or organellar membranes. Following SA, A_{23187}, or 10 mmol/L Ca^{2+} treatment, rosmarinic acid (RA) accumulation reached the highest level at 16 h, whereas the peak was found at 10 h if plasma membrane channel blockers were used. By contrast, the highest accumulation of RA occurred at 16 h when organellar channels were blocked, exhibiting the same tendency with SA-induced cells. In agreement with these observations, both phenylalanine ammonia-lyase (PAL) activity and its gene expression detected by real-time PCR also showed the same patterns. These results indicate that SA treatment firstly results in calcium release from internal stores, which in turn leads to PAL activity increase, RA accumulation, and a large amount of Ca^{2+} influx from apoplast after 10 h of SA induction.

Keywords *Salvia miltiorrhiza* · Calcium mobilization · Cell culture · Salicylic acid · Rosmarinic acid · Secondary metabolism

Introduction

Plants provide a wide variety of secondary metabolites useful to mankind, such as pharmaceuticals, food additives, and other industrial materials. Often, high-value secondary metabolites are found in low abundance in nature [1], such as tanshinone and rosmarinic acid (RA)

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and its derivative salvianolic acid B, in *Salvia miltiorrhiza* Bunge [2–4]. *S. miltiorrhiza* is a well-known medicinal plant, and its root has remarkable and reliable pharmaceutical functions [2, 4, 5]. Although several reviews have discussed how to increase the production of secondary metabolites from cell cultures [6, 7], the mechanism involved in the synthesis has generally not been elucidated.

Salicylic acid (SA), a phenolic compound broadly distributed across plant species, has been recognized as a regulatory signal mediating responses to many abiotic stresses [6]. Most research on SA has focused on its role in systemic acquired resistance [8–10] and in plant growth and development [6]. However, its role in secondary metabolites synthesis is poorly understood.

Calcium ion (Ca^{2+}) is considered a versatile second messenger in responses to different stimuli like plant hormone, drought, or salt stresses [11]. The Ca^{2+} signal is effected by modulating activities of channels and transporters at different membranes and cell organelles [12–14]. While the functions of Ca^{2+} extrusion system are rather well understood in plant cells, the specific influx channels remain unknown [11].

Rosmarinic acid (RA), one of the water-soluble phenolic acids, has been found to have significant bioactivities including as an antioxidant and in anti-ischemia reperfusion [3]. RA is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid (3,4-DHPLA), the biosynthesis of which involves both the phenylpropanoid pathway (for caffeic acid production) and a tyrosine-derived pathway (for 3,4-DHPLA production) [15]. However, the mechanism involved in RA accumulation is unknown as is the role of Ca^{2+} in SA responses in cell cultures. The aim of this investigation is to reveal the mechanism Ca^{2+} mobilization in SA-induced *S. miltiorrhiza* cell cultures and its effect on the accumulation of RA.

Materials and Methods

Cell Culture and Treatment

Fresh seeds of *S. miltiorrhiza* were inoculated on autoclaved Murashige and Skoog (MS) media that contained 30 g/L sugar and 5.5 g/L agar with pH 5.8 after sterilization and then cultured in illumination incubator under 2000–3000-lx light intensity at 25 ± 2 °C for 12–16 h per day. The axenic leaves collected from 2-month-old seedlings were cut into 0.5×0.5-cm segments and inoculated on the autoclaved MS media supplemented with 1 mg/L NAA, 1 mg/L 6-BA, 1 mg/L 2,4-D, 5.5 g/L agar, and 30 g/L sugar. The segments were grown at 24 ± 2 °C under a 12-h photoperiod for induction of calli [5].

The calli were transferred to a 100-ml flask and cultured on liquid hormone-free MS media at 25 °C in darkness on an orbital shaker with shake speed 125 rotations per minute (rpm). Each flask contained 30-ml MS media and 1.3-g fresh calli collected from 20-day-old flask cultures. The calli were inoculated to new MS media every 20 days for subculturing for three times (60 days), when the morphology and growth speed of calli were stable. At that time, the calli were collected from culture media through filtration to harvest fresh weight after blotting dry with paper towels. The dry weight was obtained by drying them at 47 °C in a vacuum oven till to constant weight.

Stock solutions of SA (Sigma, USA), A_{23187} (calcium ionophore from apoplast to cytoplast, Sigma, USA), verapamil and $LaCl_3$ (blockers of calcium channel on plasma membrane, Sigma, USA), 2-APB (IP_3R antagonist, which can be against the reaction of IP_3 with IP_3R, and thus inhibiting calcium release mediated by IP_3, Sigma, USA), LiCl (inhibitor of IP_3 cyclase, which can block IP_3 synthesis, and thus inhibiting calcium release mediated by IP_3,

Sigma, USA), and CaCl$_2$·2H$_2$O (exogenous calcium, Sigma, USA) were prepared in distilled water and then sterilized after filtration through 0.22-μm membrane. Each inhibitor was added separately to the cell cultures 30 min before SA treatment. All experiments were performed with three replications.

Determination of Intracellular Free Calcium

The cellulose, pectase, and macerozyme were dissolved in a mixture solution including 0.4 mol/L mannitol, 20 mmol/L KCl, and 20 mmol/L 2-morpholinoethanesulfonic acid (MES) and maintained at 1.5, 0.3, and 0.5 %, respectively. The pH value of this mixture was modulated to 5.7 by using 1 mol/L KOH. Ten milliliters of mixed solution was incubated at 55 °C in a water bath for 10 min, followed by filtration by 0.45-μm membrane after cooling to room temperature and sterilization. Fresh cell cultures (1.5 g) were added to the sterilized solution and cultured at 25 °C on an orbital shaker in darkness with a rotation speed of 40 rpm for 12 h. The mixture was centrifuged at 80 rpm for 1 min, followed by filtration by 600-mesh (23 μm) nylon net. The filtrate was collected in a 50-ml centrifuge tube, to which equal volume of W5 solution (154 mmol/L NaCl, 125 mmol/L CaCl$_2$, 5 mmol/L KCl, 2 mmol/L MES-K, pH 5.7) was added. The mixture was centrifuged at 600 rpm for 5 min, and another 10-ml W5 solution was added to the deposit to repeat centrifugation at 600 rpm for 5 min. Two-milliliter W5 solution was added to the deposit, and the new mixture was put on ice for 30 min, followed by centrifugation at 600 rpm for 3 min. Another 1.5-ml W5 solution (0.4 mol/L mannitol was added) was added to the deposit, and the purified protoplast of *S. miltiorrhiza* was obtained.

Before observation under a laser scanning confocal microscope, the purified protoplast was fixed on a cover glass that was coated with 0.02 g/ml poly-L-lysine hydrobromide with 300,000 molecular weight (Sigma, USA). At first, Fluo-3/AM, a visible wavelength calcium probe, was dissolved in dimethylsulfoxide (DMSO) to obtain 1 mol/L stock solution, and it was stored at −20 °C. The purified protoplast was incubated in Fluo-3/AM working solution (20 μmol/L) for 1 h, which was obtained from dilution of stock solution by using W5 solution (containing 0.4 mol/L mannitol). After washing twice by W5 solution, the protoplast was put at room temperature for 1 h, followed by fixation on the cover glass after different treatments. Fluorescence intensity was determined and photographed every 2 s within the 20-min duration period after that treatment was carried out. The excitation and emission wavelengths were 488 and 510 nm, respectively. The control did not add any inhibitors, calcium, or calcium ionophore except deionized water.

Analysis of PAL Activity

Fresh calli (2 g) were collected to put in a precooled mortar at 4 °C after being washed by deionized water and removing water on the surface by suction filtration. The calli were homogenized with 5 ml extraction buffer (0.05 mol/L boric acid buffer, 5 mmol/L mercaptoethanol, 1 mmol/L EDTA-2Na, 5 % glycerol, PVP, pH 8.8) that was precooled at 4 °C. The homogenate was filtrated through four layers of cheesecloth after grinding on ice. The filtrate was centrifuged at 4 °C for 15 min with 4000 rpm. The supernatant was crude enzyme, which was used to determine the phenylalanine ammonia-lyase (PAL) activity. The 3-ml reaction mixture contained 0.5 ml crude enzyme, 16 mmol/L L-phenylalanine, 50 mmol/L of Tris-HCl buffer (pH 8.9), and 3.6 mmol/L of NaCl, which was incubated at 37 °C for 60 min. The reaction was stopped by using 500 μl 6 mol/L HCl. The reaction mixture was then centrifuged for 10 min at 12,000×g. The absorbance was measured at 290-nm wavelength, and

one enzyme activity unit (U) was defined when the OD value varied 0.01 within 1 min. The formula of calculating PAL activity was as follows:

$$\mathrm{PAL}\left(\mathrm{U\ g^{-1}\,FW\,h^{-1}}\right) = \frac{A_{290} \times v_t \times v}{0.01 \times V_s \times \mathrm{FW} \times t}$$

where V_T represented total volume of enzyme (ml), FW represented fresh weight (g), V_s represented the volume of enzyme used to determine activity (ml), v represented total volume of reaction mixture (ml), and t represented reaction time (h).

Accumulation of RA

The dried cells (0.05 g) were extracted ultrasonically with 3 ml of methanol-water solution (7:3 v/v) for 45 min. The mixture was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered through a 0.45-μm membrane. The content of RA was quantified by HPLC analysis that was performed using Shimadzu (Japan) system with UV/visible absorbance detector (DAD). The 5-μm C18 reverse-phase column of 4.6×250-mm dimension was used with column temperature of 30 °C. The mobile phase was acetonitrile, distilled water, and phosphoric acid (25:75:0.1, v/v/v) with flow rate 1.0 ml/min. RA was detected at 285 nm and quantified by co-chromatography with a RA standard. The injection volume was 10 μl. RA content was calculated based on the formula, $C=3.399x\times10^{-8}/0.05$, in which C represented RA content (mg/g DW), x represented the peak area of RA, and 0.05 was the dry weight of cells.

Total RNA was extracted from 200-mg cell cultures by using TRIzol method (Qiagen, Beijing) according to the protocol provided by the manufacturer. The integrity and quantity of RNA were detected by protein nucleic acid analyzer (BioPhotometer Plus, Eppendorf, Germany). First-strand complementary DNA (cDNA) was synthesized from 2 μg RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Primers used to amplify PAL for real-time quantitative PCR are listed in Table 1, and the actin gene was employed as control. The qPCR amplification was performed in a total 20-μl reaction system, which contained appropriate cDNA, 0.2 μmol/L of each primer, 10 μl 2× qPCR Mix (SYBR Green II was included), and RNase-free water. Negative control missing cDNA template was also included in this experiment. qPCR was performed with the condition as 1 cycle of 94 °C for 1 min, 35 cycles of 94 °C for 10 s, 60 °C for 20 s, and 82 °C for 30 s [16]. The products of qPCR were detected on 1.5 % agarose gel electrophoresis, and the equal-sized band was observed as predicted. Quantification of *PAL* gene expression was done with comparative CT method. Each data represents the average of three replications.

PAL Gene Expression

Total RNA was extracted from 200-mg cell cultures by using TRIzol method (Qiagen, Beijing) according to the protocol provided by the manufacturer. The integrity and quantity of RNA

Table 1 Oligonucleotide primers used for phenylalanine ammonia-lyase (PAL) gene cDNA amplification and cloning from *Salvia miltiorrhiza* cell cultures	Primer	Primer sequence (5′→3′)
	PAL-forward	GATAGCGGAGTGCAGGTCGTAC
	PAL-reverse	CGAACTAGCAGATTGGCAGAGG
	Actin-forward	AGGAACCACCGATCC
	Actin-reverse	GGTGCCCTGAGGTCCTGTT

were detected by protein nucleic acid analyzer (BioPhotometer Plus, Eppendorf, Germany). First-strand cDNA was synthesized from 2 μg RNA using RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Primers used to amplify PAL for real-time quantitative PCR are listed in Table 1, and the actin gene was employed as control. The qPCR amplification was performed in a total 20-μl reaction system, which contained appropriate cDNA, 0.2 μmol/L of each primer, 10 μl 2× qPCR Mix (SYBR Green II was included), and RNase-free water. Negative control missing cDNA template was also included in this experiment. qPCR was performed with the condition as 1 cycle of 94 °C for 1 min, 35 cycles of 94 °C for 10 s, 60 °C for 20 s, and 82 °C for 30 s [17]. The products of qPCR were detected on 1.5 % agarose gel electrophoresis, and the equal-sized band was observed as predicted. Quantification of *PAL* gene expression was done with comparative CT method. Each data represents the average of three replications.

Results

Calcium Mobilization Induced by SA in Protoplast

SA (22 mg/L) significantly promoted calcium mobilization in protoplasts of *S. miltiorrhiza*. The increase began from 43 s after SA treatment and reached the highest level at 12 min (Fig. 1), at which point mobilization was 4.3-fold of H_2O control. After that time, the calcium fluorescence intensity decreased gradually until 20 min post-treatment, at which point intensity was the same as at the beginning of the experiment.

The compounds 2-APB (15 μmol/L) and $LaCl_3$ (0.5 mol/L) were used to inhibit calcium mobilization mediated by IP_3 and on the plasma membrane, respectively. Both could significantly inhibit the calcium mobilization induced by SA, and $LaCl_3$ showed stronger and significant inhibition from the fourth minute when compared with 2-APB (Figs. 1 and 2). The calcium fluorescence intensity in $LaCl_3$-treated protoplasts was equal to or lower than that of H_2O (Figs. 1 and 2).

Exogenous calcium ($CaCl_2$) was used to determine the capacity of A_{23187} (calcium ionophore from apoplast to cytoplast). A_{23187} addition did not cause calcium mobilization in protoplasts if no calcium was added to its culture media (Fig. 3). Meanwhile, if protoplasts were cultured in the
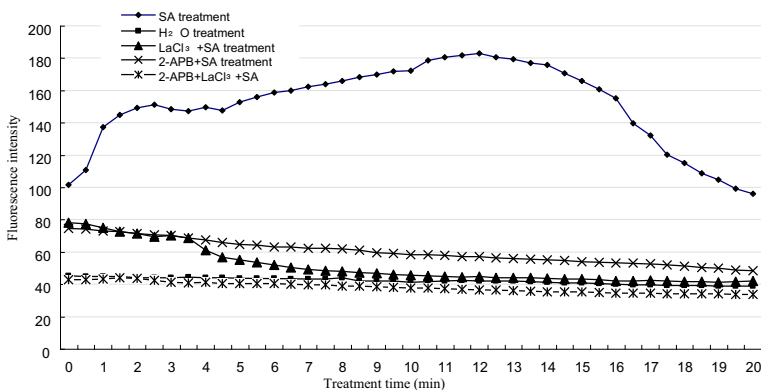

Fig. 1 Fluorescence intensity changes of calcium mobilization in *Salvia miltiorrhiza* protoplasts after adding plasma membrane channel blocker $LaCl_3$ and organellar membrane channel blocker 2-APB to the salicylic acid (SA)-induced cell cultures
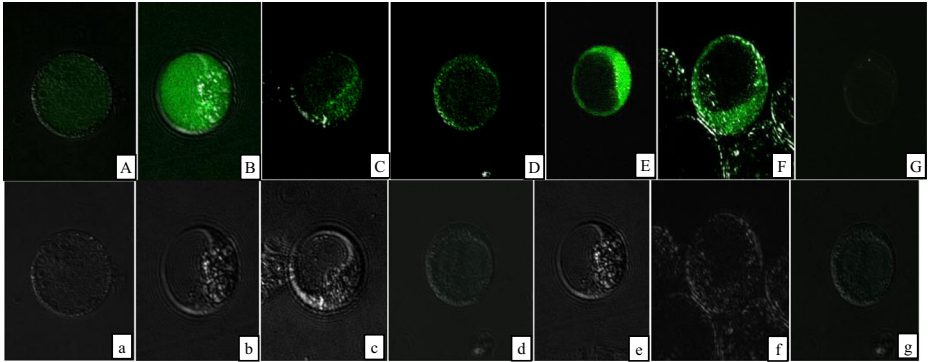
Fig. 2 The fluorescence photograph collected at the time point of 11 min based on the intensity tendency exhibited in Fig. 1 after being treated with different membrane channel blockers or calcium ionophore. The protoplasts were cultured in the medium treated with (*A*) H_2O, (*B*) SA, (*C*) $LaCl_3$ + SA, (*D*) 2-APB + SA, (*E*) A_{23187} + Ca^{2+}, (*F*) exogenous Ca^{2+}, and (*G*) $LaCl_3$ + 2-APB + SA. Those protoplasts from *a* to *g* were controls corresponding with treated protoplasts from *A* to *G*

media containing 10 mmol/L $CaCl_2$, A_{23187} would significantly increase the calcium in the protoplast (Fig. 2). The calcium fluorescence intensity reached the strongest level at 11 min, which was 3.5-fold higher than that of A_{23187} treatment without calcium in culture media.

Effects of SA, A_{23187}, and Exogenous Calcium on RA Accumulation in Cell Cultures

To investigate the effects of calcium mobilization on RA accumulation in cell cultures, treatments including SA, A_{23187} and exogenous calcium were employed. From 10 h post-treatment, RA content in SA-, A_{23187}- and exogenous calcium-treated cells was significantly higher than that of H_2O-treated controls (Fig. 4). This situation lasted for 14 h. RA accumulation
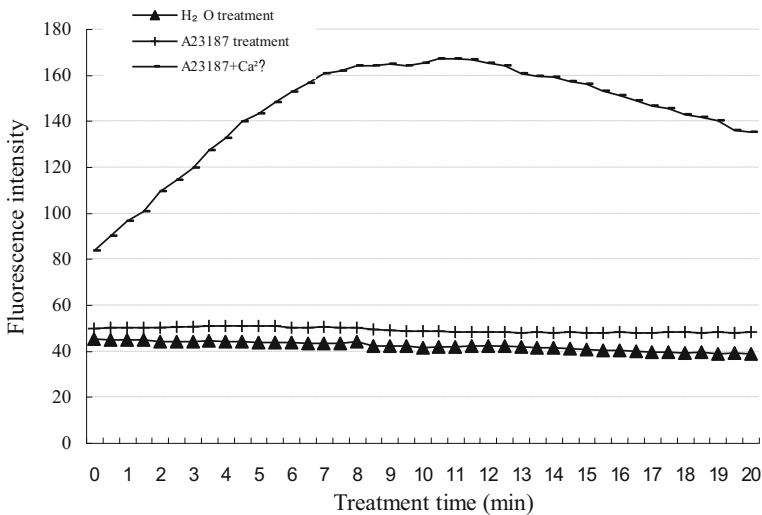

Fig. 3 Fluorescence intensity changes of calcium mobilization in *Salvia miltiorrhiza* protoplasts after being treated with calcium ionophore A_{23187}, exogenous Ca^{2+}, and combination of both in the culture medium
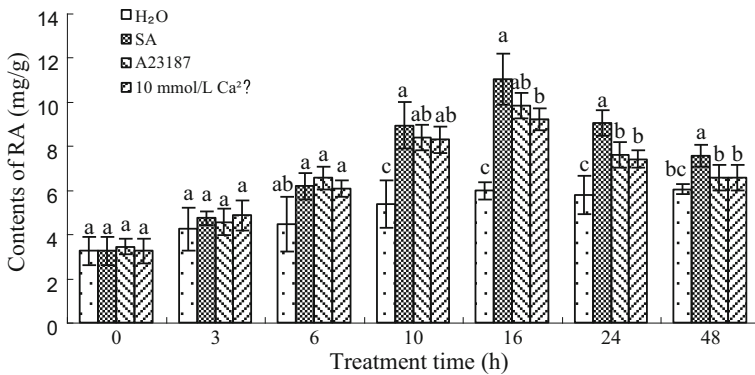
Fig. 4 Effects of salicylic acid (SA), A_{23187}, and exogenous Ca^{2+} on rosmarinic acid (RA) accumulation. Different *lowercase letters over columns* represented significance at 0.05

reached the highest level at 16 h post-treatment, when its content was 11.025 mg/g in SA-elicited cells and was 84.7 % higher than that of H_2O-treated controls.

Effect of Calcium Mobilization Inhibited by Plasma Membrane Channel Blockers on RA Accumulation

Both LaCl$_3$ (0.5 mmol/L) and verapamil (0.5 mmol/L) were added separately to cell cultures in order to inhibit calcium influx from extracellular culture media. Their effects on RA accumulation were evaluated, and no significance was found between each of them and H_2O (data not shown), indicating that such concentrations of both inhibitors were appropriate for subsequent treatments. RA contents in LaCl$_3$ + SA and verapamil + SA treatments were significantly lower than that of SA-induced cells, which began from 16 to 24 h (Fig. 5). At the time point of
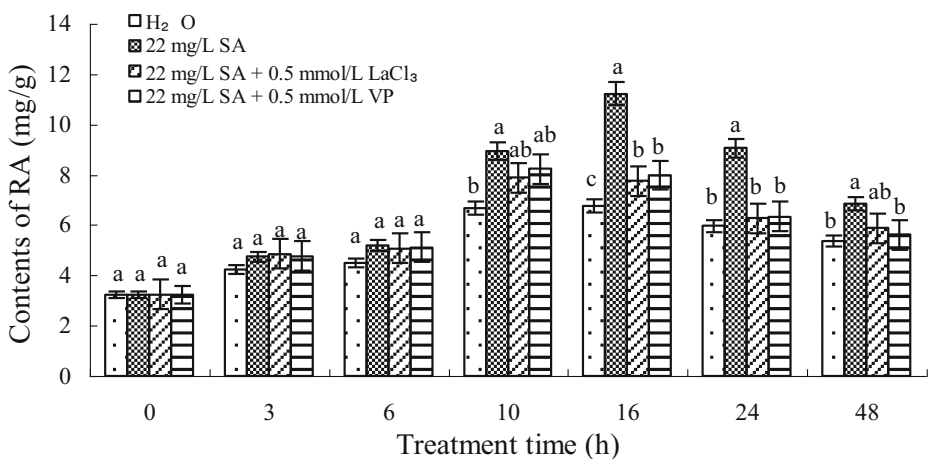

Fig. 5 Effects of calcium mobilization on rosmarinic acid (RA) accumulation when plasma membrane channel blockers, LaCl$_3$, and verapamil (VP) were added to salicylic acid (SA)-induced cell cultures. Different *lowercase letters over columns* represented significance at 0.05

10 h, RA accumulation reached the highest level in both $LaCl_3$ + SA and verapamil + SA treatments, while it increased to the peak till to 16 h in SA-induced cells.

Effect of Calcium Mobilization Inhibited by Organellar Channel Blockers on RA Accumulation

Both 2-APB and LiCl were used to inhibit calcium release mediated by IP_3, and the effects of both on RA accumulation were also evaluated. It was found that 15 μmol/L 2-APB and 7 mmol/L LiCl did not significantly affect accumulation of RA. However, RA contents in both 2-APB + SA and LiCl + SA treatments were significantly lower than that of SA-induced cells from the 10 h and lasted until 16 h (Fig. 6).

Effect of Calcium Mobilization Inhibited by Both Channel Blockers of Plasma and Organellar Membranes on RA Accumulation

The $LaCl_3$ and 2-APB treatments were used simultaneously to inhibit calcium mobilization on plasma membrane and calcium release mediated by IP_3. RA content in $LaCl_3$ + 2-APB treatment was not significantly different from that of $LaCl_3$ + 2-APB + SA-treated cells during all treatment times. But, RA contents in these two combined treatments were significantly lower than that of SA-induced cells from 10 to 48 h (Fig. 7). These results indicate that calcium mobilization was modulated by SA, which was also the key factor to regulate downstream genes to accumulate RA.

Effects of SA, A_{23187}, and Exogenous Calcium on PAL Activity

SA, A_{23187}, and exogenous calcium promoted RA accumulation in cell cultures (Fig. 4). PAL is a key enzyme at the beginning of the phenylpropanoid pathway. Therefore, the enzymatic activity of PAL was determined in response to calcium-mobilizing treatments. The enzyme activity was highest from the 10 to 16 h following treatment, and no significant difference was found among SA, A_{23187}, and exogenous calcium treatments (Fig. 8).
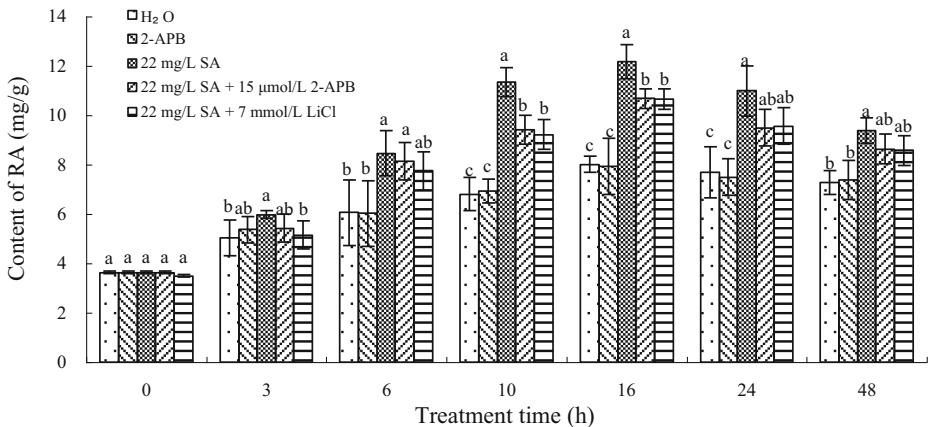

Fig. 6 Effects of calcium mobilization on rosmarinic acid (RA) accumulation when organellar membrane channel blockers, 2-APB and LiCl, were added to the salicylic acid (SA)-induced cell cultures. *Different lowercase letters over columns* represented significance at 0.05
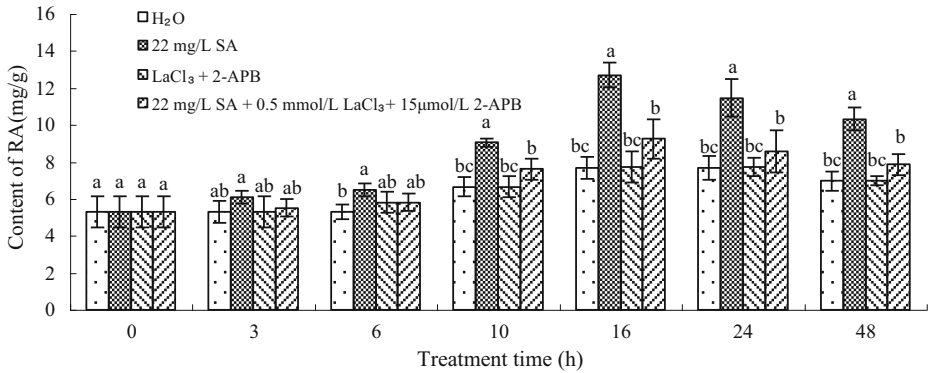
Fig. 7 Effects of calcium mobilization on rosmarinic acid (RA) accumulation when plasma and organellar membrane channel blockers, LaCl₃ and 2-APB, were added to the salicylic acid (SA)-induced cell cultures at the same time. Different *lowercase letters over columns* represented significance at 0.05

Effect of Calcium Mobilization Inhibited by Both Channel Blockers of Plasma and Organellar Membranes on PAL Enzyme Activity

Both $LaCl_3$ and verapamil were used to block calcium mobilization on plasma membrane, and 2-APB and LiCl were employed to block calcium release mediated by IP_3. PAL activities in $LaCl_3$ + SA and verapamil + SA treatments were significantly lower than that of SA-induced cells from 10 to 48 h (Fig. 9). The highest PAL activities in these two combined treatments occurred at 10 h. PAL activities in LiCl + SA and 2-APB + SA treatments continuously increased from the treatment beginning to 16 h when both reached the highest level, but no significance difference was found compared to SA-induced cells.

When $LaCl_3$ and 2-APB were applied simultaneously to inhibit calcium mobilization on plasma membrane and calcium release mediated by IP_3 at the same time, PAL activities in both $LaCl_3$ + 2-APB and $LaCl_3$ + 2-APB + SA treatments were significantly lower than that of SA-induced cells from 6 to 48 h (Fig. 10).
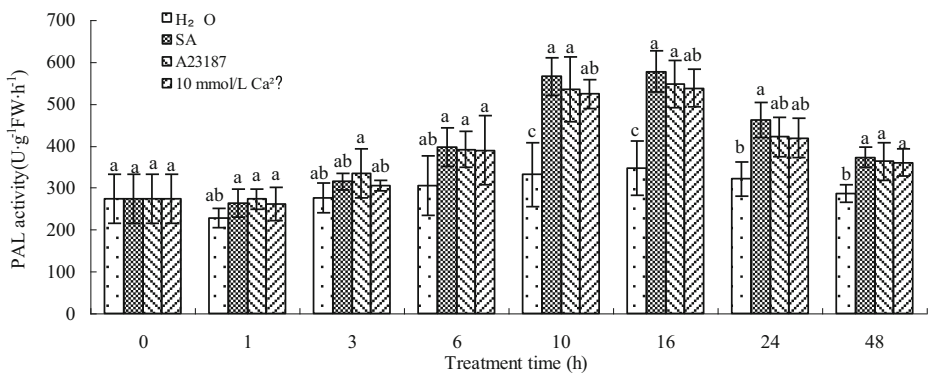

Fig. 8 Effects of salicylic acid (SA), A_{23187}, and exogenous Ca^{2+} on phenylalanine ammonia-lyase (PAL) activity. Different *lowercase letters over columns* represented significance at 0.05
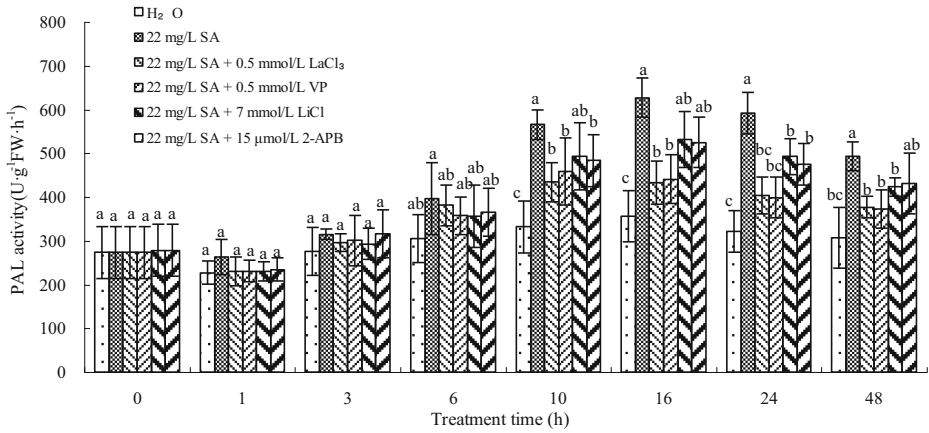
Fig. 9 Effects of calcium mobilization on phenylalanine ammonia-lyase (PAL) activity when plasma and organellar membrane channel blockers were separately added to the salicylic acid (SA)-induced cell cultures. Different *lowercase letters over columns* represented significance at 0.05

Effect of Calcium Mobilization on PAL Gene Expression in A_{23187} and Exogenous Calcium-Treated Cells

The real-time PCR result showed that both A_{23187} and exogenous calcium significantly promoted *PAL* gene expression when compared with its expression in those cells cultured in deionized H_2O. Meanwhile, the expression level in A_{23187} treatment was not significantly different than that exogenously Ca^{2+}-treated cells (Fig. 11).

Effect of Calcium Mobilization Inhibited by Both Channel Blockers of Plasma and Organellar Membranes on PAL Gene Expression

PAL gene expression levels in two combined treatments, $LaCl_3$ + SA and 2-APB + SA, were significantly lower than that of SA-induced cells, but significantly higher than both in $LaCl_3$ + 2-APB + SA and H_2O treatments (Fig. 12).
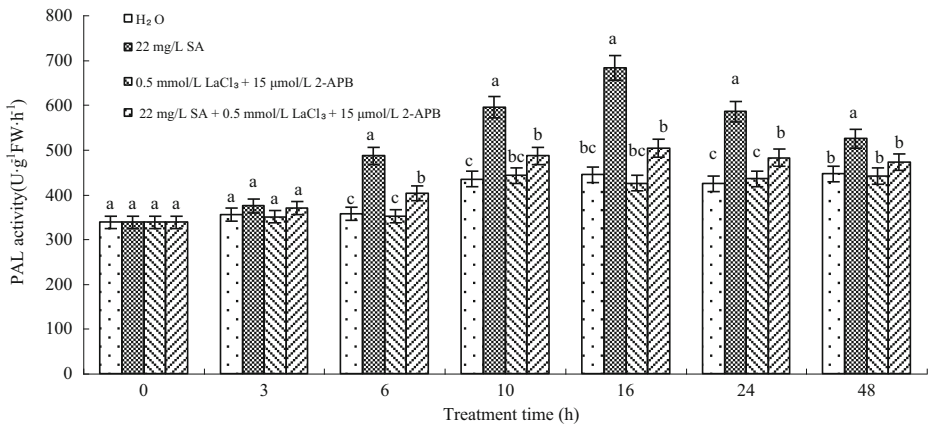

Fig. 10 Effects of calcium mobilization on phenylalanine ammonia-lyase (PAL) activity when plasma and organellar membrane channel blockers, $LaCl_3$ and 2-APB, were added to the culture medium at the same time. Different *lowercase letters over columns* represented significance at 0.05
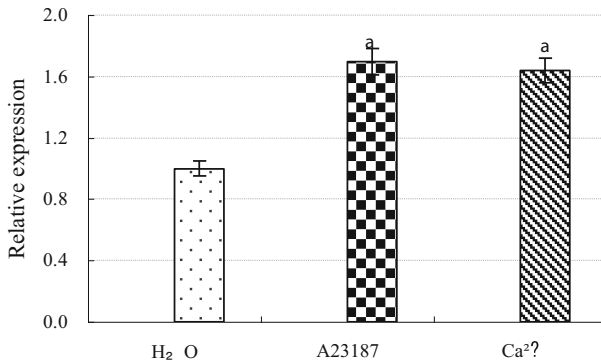
Fig. 11 Effects of calcium ionophore A_{23187} and exogenous Ca^{2+} on phenylalanine ammonia-lyase (PAL) gene expression. Different *lowercase letters over columns* represented significance at 0.05

Discussion

SA has been identified as a key component of the systemic acquired resistance (SAR) signal transduction pathway [18]. Upon pathogen attack, SA accumulates at the infection site to a high level, binds, and inactivates catalase activity [19], thereby leading to an increase of H_2O_2 release, which serves as a secondary messenger to induce the expression of genes and proteins [5, 20–23]. This stimulates the accumulation of secondary metabolites, such as indole gluco-sinolate in *Arabidopsis* [24], anthraquinone phytoalexin in *Rubia cordifo* cultures [25], and alkaloids in hairy root cultures of *Brugmansia candida* [26]. However, it is still not understood how SA stimulates ion transport and thus leads to accumulation of secondary metabolites.

Ion fluxes are immediate responses of plant cells in response to elicitors or stress, among which Ca^{2+} influx is regarded as one of the most significant events, since Ca^{2+} is a key second messenger for many diverse physiological changes and cellular processes [23, 27, 28]. In this paper, the Ca^{2+} influx in protoplasts increased drastically from 42 s after SA induction to 12 min when the level reached the highest (4.3-fold of H_2O, Fig. 1). It has been found that the increased Ca_{cyt} stimulates NAD(P)H oxidase activity, leading to extracellular O_2^- release and H_2O_2 production [29, 30]. The Ca^{2+} spiking not only mediates the subsequent accumulation of secondary metabolites [31], but also further amplifies Ca^{2+} signaling through Ca^{2+}-modulated production of H_2O_2 [32]. The Ca^{2+} influx/Cl^- efflux accompanied with K^+/H^+ exchange, derived from the depolarization of plasma membrane, leads to cytoplasmic acidification and

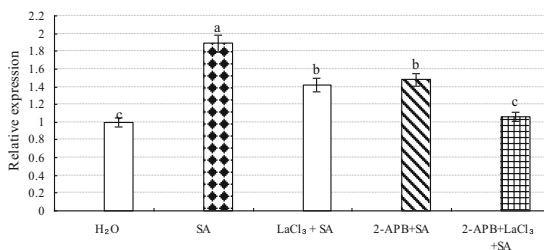**Fig. 12** Effects of calcium mobilization on phenylalanine ammonia-lyase (PAL) gene expression when plasma and organellar membrane channel blockers, $LaCl_3$, 2-APB, and combination of both, were added to salicylic acid (SA)-induced cell cultures. Different *lowercase letters over columns* represented significance at 0.05

alkalinization of extracellular medium [33–35]. Cytoplasmic acidification is regarded as an essential step in signal transduction, leading to the oxidative burst and accumulation of secondary metabolites [36]. The alkalinization of the culture medium also can cause an increase in phenylalanine ammonia-lyse (PAL) activity, accompanied by accumulation of isofavone glycosides [37], lignin [38], or phytoalexin [33].

Elevation of Ca_{cyt} in response to signals is due to Ca^{2+} influx from apoplast and/or Ca^{2+} release from intracellular stores (vacuoles, endoplasmic reticulum, mitochondria, chloroplasts, and nucleus), during which different signals use distinct Ca^{2+} mobilization means in elevating Ca_{cyt}. Ca^{2+} increase is inhibited by plasma membrane channel blockers in cold-induced tobacco cells but is not affected by organellar channel blockers [39]. In parsley cells, elicitor-induced Ca_{cyt} increase is also primarily due to the influx of extracellular Ca^{2+} [40]. However, internal Ca^{2+} stores contribute to wind- and chitosan-induced elevation of Ca_{cyt} [16, 41]. ABA-induced changes in Ca_{cyt} are found to be attributed to both Ca^{2+} release from internal stores and Ca^{2+} influx from apoplast [42], which is similar to SA-induced cells in this paper. In SA, A_{23187}, or 10 mmol/L Ca^{2+}-treated cells, RA accumulation reached the highest level at the 16th hour (Fig. 4), whereas the peak was found at 10 h if plasma membrane channel blockers were used (Fig. 5). By contrast, the highest accumulation of RA occurred at the 16th hour when organellar channels were blocked, exhibiting the same tendency with SA-induced cells (Fig. 6). These results indicate that SA induction firstly results in calcium release from internal stores, which in turn leads to PAL activity increase (Fig. 8) and a large amount of Ca^{2+} influx from apoplasts after 10-h SA induction.

Conclusions

SA can rapidly induce Ca^{2+} mobilization in protoplasts, but the induction can be blocked with either channel blocker of plasma or organellar membrane. In SA, A_{23187}, or 10 mmol/L Ca^{2+} treatment, rosmarinic acid (RA) accumulation reached the highest level at the 16th hour, whereas the peak was found at the 10th hour if plasma membrane channel blockers were used. By contrast, the highest accumulation of RA occurred at the 16th hour when organellar channels were blocked, exhibiting the same tendency with SA-induced cells. Both PAL activity and its gene expression also show the same tendency.

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