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Effects of Ethylene and Ca^{2+} on Activity of Phenylalanine Ammonia-Lyase in Glucan-Treated *Daucus carota*

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Involvement of ethylene and Ca^{2+} on the induction of phenylalanine ammonia-lyase (PAL) was investigated in *Daucus carota* L. suspension culture system. Ethylene production started to increase about 3 h after glucan treatment. And the maximal induction of ethylene was preceded by PAL induction by 30 min. After the treatment of ethrel, PAL activity was increased. When cells were treated with glucan and Co^{2+}, PAL activity was simultaneously reduced. Ethylene production was reduced dramatically in calcium-free medium, even though glucan was treated. PAL activity and ethylene production was inhibited conspicuously when ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) was treated with glucan. Verapamil and trifluoperazine also inhibited PAL activity. When cells were treated with calcium ionophore A23187, PAL activity was increased in nontreated medium. We report here PAL activity is increased in related to ethylene production and involvement of Ca^{2+} in glucan-treated carrot suspension cells.

Keywords: phenylalanine ammonia-lyase, elicitor, calcium, ethylene, plant defense response

Plants sensitively recognize the change of environment and convert them into internal signals. Many environmental stress on plants can cause increase in the production of stress hormone, ethylene (Yang and Pratt, 1978). When plants were attacked by pathogen, ethylene production increases and this event is known as a sign of early defense mechanism (Yang and Hoffmann, 1984; Boller, 1990a).

The material that causes defense mechanism in plants is called elicitor. Among them glucan is well known as a very effective elicitor which induces accumulation of phytoalexin (Albersheim and Valent, 1978). Various kinds of elicitors are known as an inducer of phenylalanine ammonia-lyase (PAL) which is the key enzyme of phenylpropanoid pathway (Riov *et al.*, 1969). When plants were treated with elicitor, ethylene production increases in a few h. But it is not certain that PAL induction is related to ethylene production in all plant species and there is much to be investigated (Chappell *et al.*, 1984; Boller, 1990b). These reports may suggest that ethylene production is merely a sign of response to external stress and does not have a role of second messenger in defense response (Paradies *et al.*, 1980).

When plants were treated with chitosan, it is reported that calcium has a role in phytoalexin accumulation (Kohle *et al.*, 1985). In animal, it is well known that increase of calcium concentration in cytosol regulates cellular process directly or indirectly (Williamson and Monck, 1989) and has a role of second messenger. According to Pelissier (1986) elicitor causes the change of cell permeability and membrane potential in a few min. And this event suggests that H^+-ATPase may have a role in membrane depolarization.

We report here that ethylene is related to PAL induction in glucan-treated carrot suspension cells.

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We also examined the role of calcium as a mediator of the PAL induction and whether this reponse is ethylene dependent or not.

MATERIALS AND METHODS

Plant material

Carrot (*Daucus carota* L.) cell cultures were grown in the B5 medium which contains 0.1 mg/mL of 2,4-D at 27℃ in the total darkness. After 5 d of subculture, rapidly growing cells were transfered to B5 liquid medium and shaked at 120 rpm, 27℃ in the darkeness for 12 d. Cells of 0.2 g transferred into 10 mL B5 medium in 50 mL Erlenmeyer flask were used as plant material.

Glucan treatment

Carrot suspension cells were treated with 10 μg/mL of glucan (Sigma) as an elicitor. Cells were harvested after glucan treatment using filter paper at the time indicated.

Ethylene determination

Samples of the gas phase were removed from the flasks at the time indicated and injected 1 mL into gas chromatograph (Shimazu, model GC3BF). Ethylene was identified and quantified according to the retention time and peak height.

Determination of PAL activity

Determination of PAL activity in carrot suspension cells was done according to the method of Chaluts (1973) as modified. Zero point five g of glucan-treated suspension cells were harvested using filter paper at the time indicated. Cells were homogenized in a pestle and mortar with 2 mL of cold acetone. Filter the homogenized cells at the vacuum condition and dried for 24 h. The dried powder was added with 3 mL 0.1 M borate (Fluka) buffer (pH 8.8) and incubated at 4℃ for 1 h and centrifuged at 18,000*g* for 30 min. The supernatant was assyed for PAL activity. The reaction mixture was incubated for 1 h at 40℃ which consisted of 0.75 mL supernatant and 0.5 mL 0.05 M L-phenylalanine (Sigma) made up also in the borate buffer, and 0.125 mL 0.15 M borate buffer (pH 8.8). Reaction was stopped by adding 0.1 mL 5 N NaOH. Extract reaction product by adding 5 mL of ether and add 2 mL of 0.05 N NaOH to the dried reaction product. The trans-cinnamate produced was determined by measuring the absorbance at 269 nm.

Treatments of ethrel, cobalt ion, EGTA, verapamil, TFP and A23187

Adding 1 mL of 1 μM cobalt ion (Fluka), or 5 ppm ethrel into the medium, PAL activity was determined at the time indicated. To know the effect of calcium on PAL activity, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (Sigma) was added to the culture medium at concentration of 1 mM, and A23187 (Sigma) was added with a varying concentration. After each treatment cells were harvested under vacuum filtration and PAL activity was dertermined. Twelve-day old cells were treated with various concentrations of trifluoperazine (TFP) (Sigma) or verapamil (Sigma) and cells were harvested as described before and PAL activity was dertermined.

Protein dertermination

Protein was measured by the method of Lowry *et al.* (1957) with BSA (Sigma) as a standard reference.

RESULTS AND DISCUSSION

Change of PAL activity

When carrot suspension cells were treated with various concentrations of glucan, PAL activity was increased according to the concentration of glucan (Fig. 1). According to this results the elicitor preparation was used at concentration to achieve approximately half maximal induction of PAL activity.

Effects of ethylene on PAL activity

After 30 min of glucan treatment ethylene production started to increase and reached maximal level in 5 and half an h (Fig. 2). After its maximal level ethylene production decreased. Glucan caused approximately 20-fold increase in ethylene production.

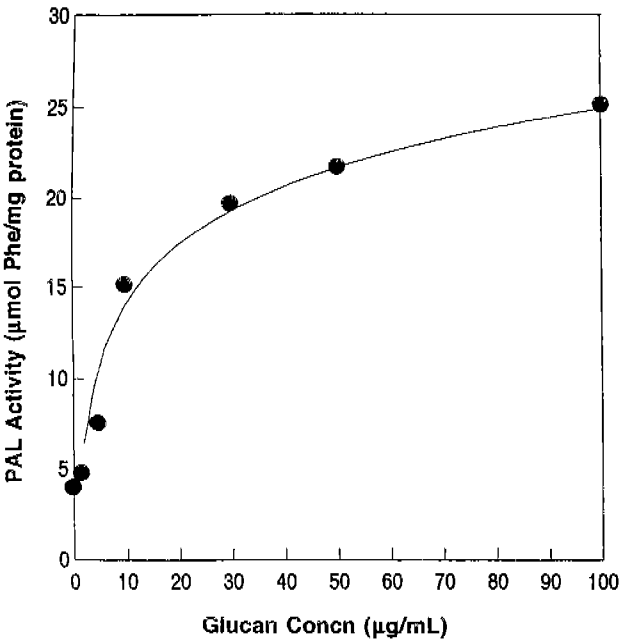

Fig. 1. Induction of phenylalanine ammonia-lyase in carrot suspension culture cells by glucan. Cells were treated with different amount of glucan.

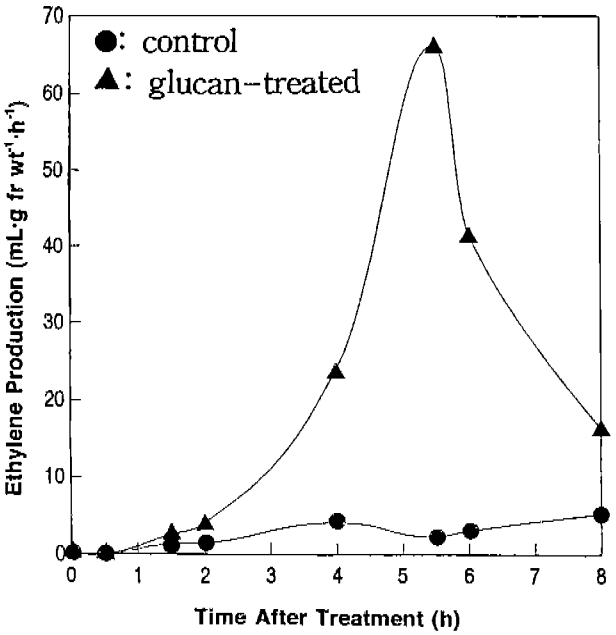

Fig. 2. Effect of glucan on ethylene production. Aliquots of 12-d-old cells were incubated in 50 mL flask and the accumulation of ethylenewas determined at the time indicated. Glucan (10 μg/mL) was added just before flasks were sealed. Controls were treated with distilled water.

Ethylene production was very low in control. This result is in accordance with the report that ethylene is a plant hormone which reponds sensitively to the external stimulus (Hogsett *et al.*, 1981; De Laat and Vanloon, 1982). PAL activity was also rapidly induced in response to glucan-treatment and reached the maximal level at 6 h. It gradually decreased after 6 h (Fig. 3). This result is in accordance with the other reports that PAL activity reached maximal level whithin a few h after elicitor treatment and also indicates that glucan can cause a response in plants whithin a very short time. The pattern of induction of ethylene production was similar to that of induction of PAL activity. The maximal level of ethylene production was preceded the maximal level of PAL activity only about 30 min. So, we can suggest that ethylene may have a role in PAL activity.

Next, we tried to know whether PAL activity would be changed or not, if ethylene was treated directly to the plant cells. Ethylene produced by 5 ppm of ethrel caused an increase of PAL activity. At this time PAL activity was about 25 folds of control (Fig. 4). It shows a similar pattern of glucan-treatment. When cells were treated simultaneously with glucan in the presence of Co^{2+}, known as an ethylene production inhibitor, ethylene production

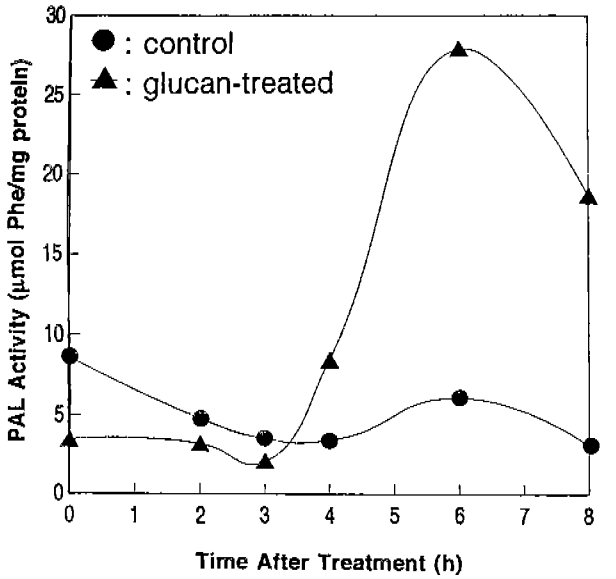

Fig. 3. Activity of PAL in carrot cell cultures. Aliquots of 12-d-old cell cultures were treated with glucan (10 μg /mL) and harvested after 6 h.

was decreased (Fig. 5). At the same time PAL activity also decreased about 14 folds in compared with glucan-treatment (Fig. 6). These data are in contrast to the report of Paradies *et al.* (1980) and show ethyl-

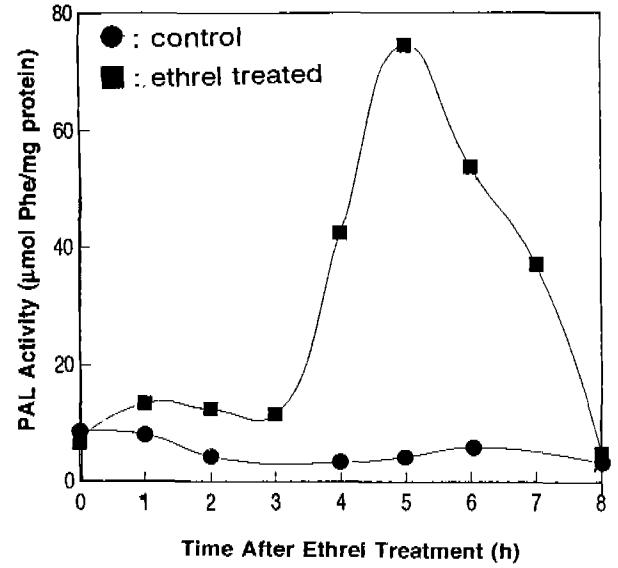

Fig. 4. Effect of ethrel on PAL induction. 12-d-old cell cultures were added with 5 ppm ethrel and incubated for indicated times. PAL activity was determined as above procedures. Controls were added with distilled water.

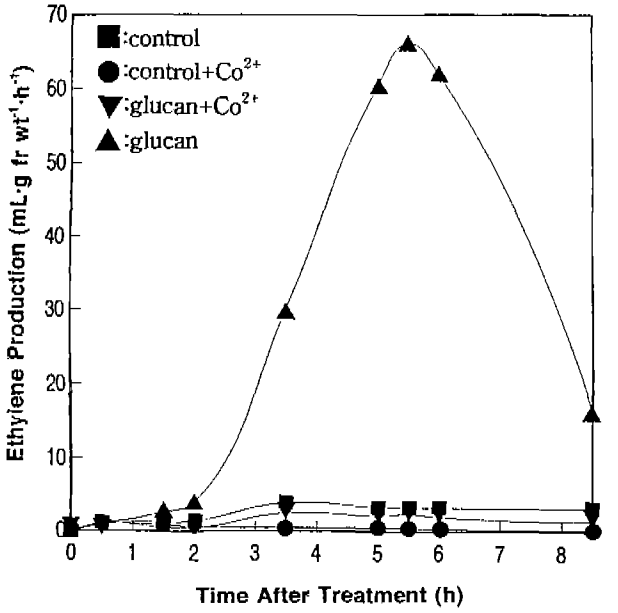

Fig. 5. Effect of glucan and Co^{2+} on ethylene formation Aliquots of 12-d-old cells were incubated in the absence or presence of Co^{2+} (1 μM) and glucan (10 μg/mL). Controls were added with same amounts of distilled water.

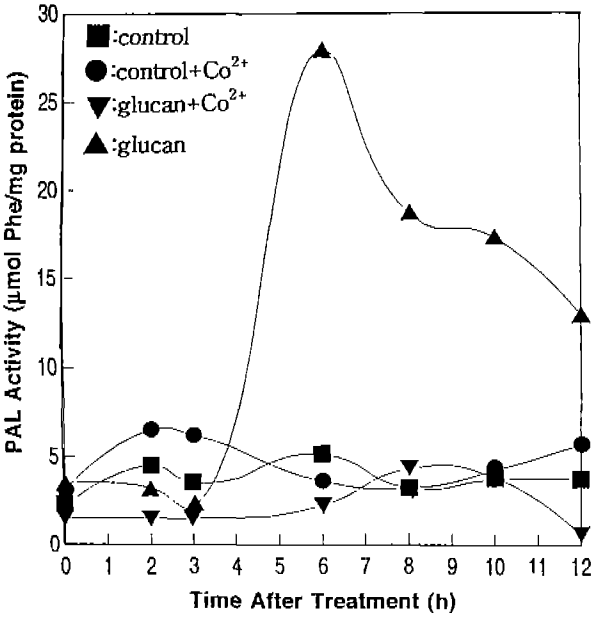

Fig. 6. Effect of Co^{2+} (1 μM) and glucan (10 μg/mL) on PAL inductiion. Aliquots of 12-d-old cells were incubated in the absence or presence of Co^{2+} and glucan. Controls were added with samc amounts of distilled water.

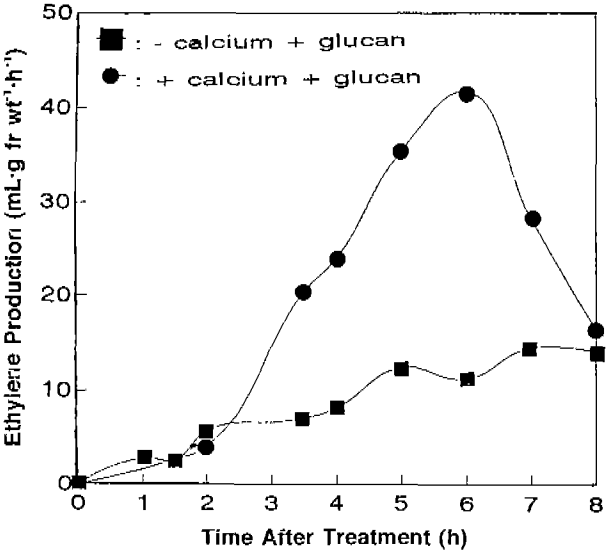

Fig. 7. Effect of Ca^{2+} on glucan-induced ethylene. To know the effect of Ca^{2+} on ethylene formation 12-d-old cells were treated with glucan (10 μg/mL). Cells were cultured in normal (+ calcium) or Ca^{2+}-free medium (− calcium).

ene has an effect on the increase of PAL activity.

Effect of Ca^{2+} on ethylene production

In order to identify the role of Ca^{2+} on ethylene production during plant defense response, we added glucan to Ca^{2+}-free medium and measured the ethylene production. In contrast to cells which have grown in normal Ca^{2+}-contaning medium, the cells in Ca^{2+}-free medium have produced conspicuously

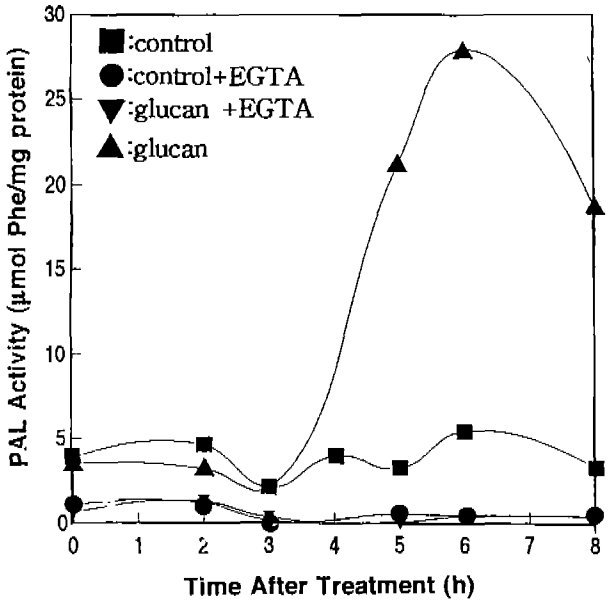

Fig. 8. Effect of EGTA on glucan-induced PAL. Aliquots of 12-d-old cell cultures were treated with 5 mM EGTA.

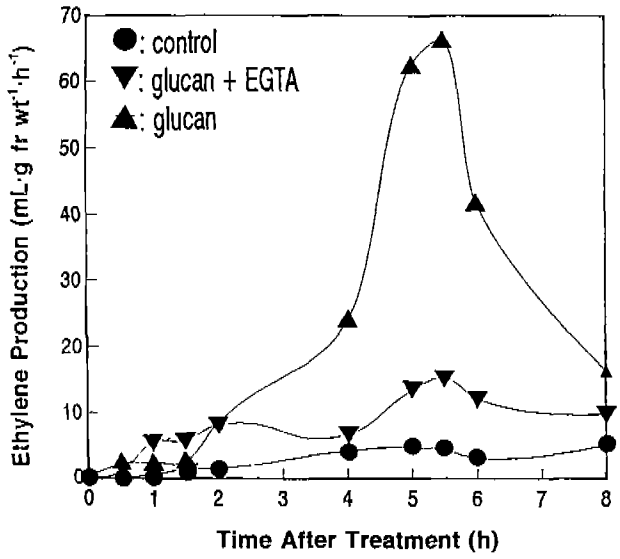

Fig. 9. Effect of EGTA on glucan-induced ethylene. Carrot suspension cells were treated with 5 mM EGTA and glucan (10 μg/mL). Controls were added with distilled water.

small amount of ethylene (Fig. 7). This data shows that Ca^{2+} is required for ethylene production.

Effects of EGTA, Ca^{2+} inhibitors and A23187 on ethylene production

In order to identify the role of Ca^{2+} on PAL activity we used various kinds of Ca^{2+} inhibitors. At first we treated EGTA with glucan to carrot suspension cells. As known before, EGTA can chelate Ca^{2+} effectively (Gilroy *et al.*, 1986). At this time glucan effect was remarkably decreased and PAL activity was reduced 5 folds compared to control (Fig. 8). It has been reported when tobacco cells were treated with 10 mM EGTA and glucan, ethylene production was decreased (Raz and Fluhr, 1992). In refer to this report we examined the ethylene production. As a result ethylene production was decreased evidently by EGTA (Fig. 9).

Next, we added Ca^{2+} antagonists, verapamil and TFP in the presence of glucan. In all cases PAL activity was decreased (Figs. 10 and 11). According to the results we can consider that Ca^{2+} has a role of mediator of response after a plant recognized the glucan. Finally, we treated various concentrations of Ca^{2+} ionophore. As a result PAL activity was increased as a dose dependent manner. But PAL activity was decreased about 5-6 folds in Ca^{2+}-free medium (Fig. 12) even though A23187 was added. These results indicate that low level of Ca^{2+} is sufficient to induce PAL activity and has a role in plant defense response.

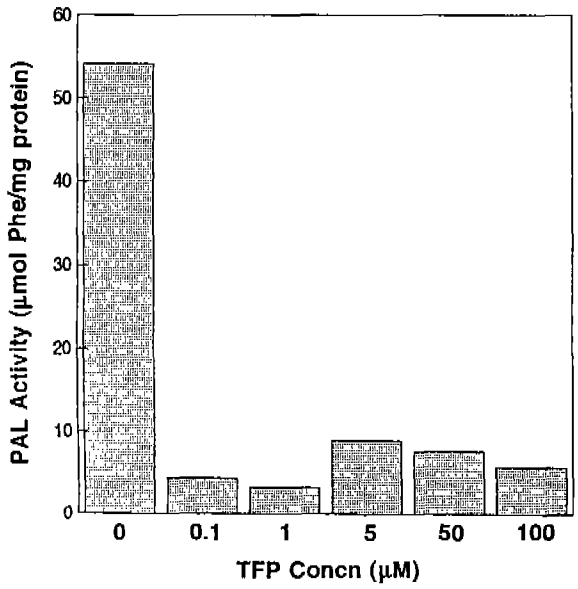

Fig. 10. Effect of Ca^{2+} antagonist, trifluoperazine (TFP) on glucan-induced PAL. Carrot cells (12-d-old) were treated with different amounts of TFP. All cells were treated with glucan (10 μg/mL) and harvested 6 h after the treatment.

It was reported that plant defense response was initiated by the receptor binding of elicitor (Cosio

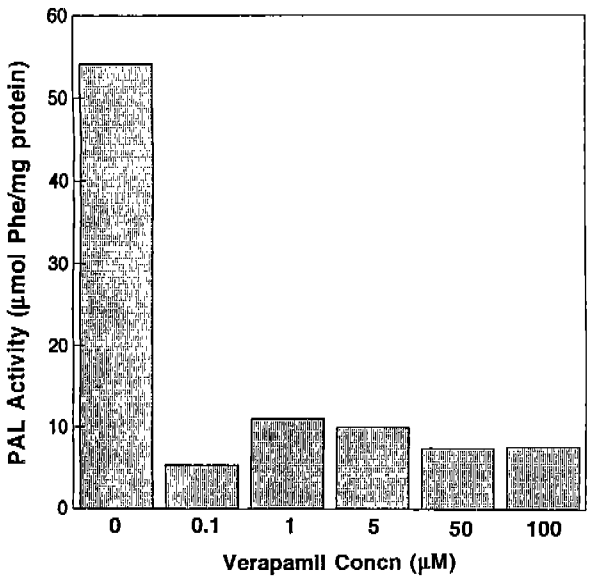

Fig. 11. Effect of Ca^{2+} antagonist, verapamil on glucan-induced PAL. Carrot cells (12-d-old) were treated with different amounts of verapamil. All cells were treated with glucan (10 μg/mL) and harvested 6 h after the treatment.

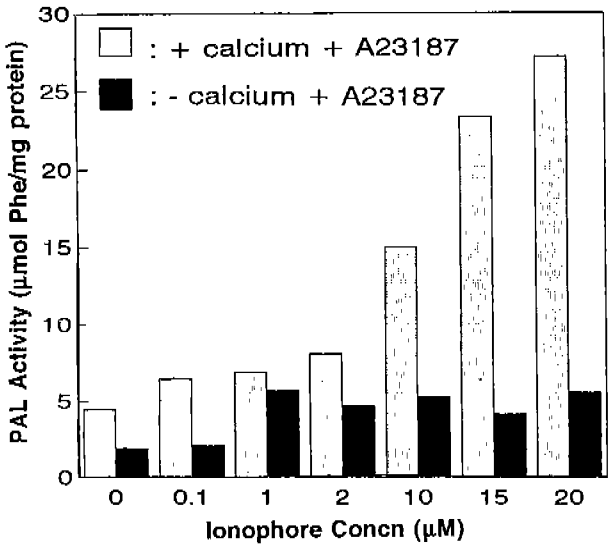

Fig. 12. Effect of Ca^{2+} ionophore A23187 on PAL induction. Cells were incubated in normal medium (+ calcium) or Ca^{2+}-free medium (− calcium). Cells were treated with different amount of Ca^{2+} as indicated above.

et al., 1988). According to Lotan and Fluhr (1990), there are at least 2 kinds of pathways that consist of plant defense response. The first pathway is regulated by ethylene and ethylene production is necessary in the process of response. The second pathway is independent and unaffected by ethylene, even though ethylene production is increased (Eyal and Fluhr, 1992). Our data also suggest that the increase of PAL activity by glucan is related to ethylene production and this response uses an ethylene-dependent pathway. According to Raz and Fluhr (1992), the ethylene-dependent response is dependent on Ca^{2+} concentration, and the transfer of stimuli is mediated by Ca^{2+}. Our results are in coincidence with this report. In conclusion glucan-induced PAL induction is an ethylene-dependent pathway and requires Ca^{2+} as a mediator of the response.

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Glucan 處理 당근에서 Phenylalanine Ammonia-Lyase의 活性에 미치는 에틸렌 및 Ca^{2+}의 效果

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적 요

Glucan 처리시 에틸렌의 합성 증가가 phenylalanine ammonia-lyase(PAL)의 활성에 미치는 영향과 이때 Ca^{2+}의 관여 여부에 대해 조사하였다. Glucan은 10 μg/mL씩 처리하여 반응을 유도하였다. Glucan 처리시 시간적으로 에틸렌의 합성 최대치는 PAL의 활성이 최대를 보이는 시점보다 선행되었다. 외부에서 ethrel을 처리하여 에틸렌을 발생시킨 경우에도 유사한 결과를 보여 PAL의 양상은 대조구에 비해 20배 가량 활성이 증가하였다. Ca^{2+}이 첨가되지 않은 배지에서 glucan을 처리한 경우, 에틸렌의 합성은 현저하게 감소하였다. 또한, 에틸렌 생성 효소 억제제인 Co^{2+}을 glucan과 함께 처리시 PAL의 활성을 감소시켰다. Glucan 처리시 Ca^{2+}의 영향을 조사하여 본 결과, PAL은 Ca^{2+} 억제제인 ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid(EGTA), verapamil, trifluoperazine 처리시 모든 경우에서 활성이 감소하였다. Ca^{2+} ionophore인 A23187을 Ca^{2+}이 첨가된 배지에 처리할 경우 처리 농도가 증가함에 따라 PAL의 활성이 비례적으로 증가됨을 확인하였다. 이상의 결과로부터 glucan 처리시 PAL의 활성 증가는 에틸렌 의존성 반응이며, Ca^{2+}이 매개하는 것으로 보인다.

주요어 : phenylalanine ammonia-lyase, elicitor, 칼슘, 에틸렌, 식물 방어 반응

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