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Selective desensitization of jasmonate- and pH-dependent signaling in the induction of benzophenanthridine biosynthesis in cells of *Eschscholzia californica*

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Dedicated to Professor Dr. Meinhart H. Zenk on the occassion of his 70th birthday

Abstract

The biosynthesis of benzophenanthridine alkaloids, phytoalexins of *Eschscholzia californica*, in cultured cells can be induced by a glycoprotein preparation from yeast, methyljasmonate, artificial acidification with permeant acids, or mild osmotic stress. Each of these stimuli strongly attenuated the subsequent response to the same stimulus (homologous desensitization). Elicitor contact and artificial acidification mutually desensitized the cells for either signal. In contrast, elicitor-treated cells maintained their responsiveness to methyljasmonate or hyperosmolarity (sorbitol). Elicitor concentrations that nearly saturated the alkaloid response did not cause a detectable increase of jasmonate content. Transient acidification of the cytoplasm is a necessary step of signaling by low elicitor concentrations but was not detectable after jasmonate treatment. Seen together, the data indicate the existence of a jasmonate-dependent and jasmonate-independent (ΔpH controlled) signal pathway towards the expression of benzophenanthridine biosynthesis. Selective desensitization allows either stimulus to activate a distinct share of the biosynthetic capacity of the cell and limits the accumulation of toxic defense metabolites.

Keywords: *Eschscholzia californica*; Papaveraceae; Cell biology; Benzophenanthridine alkaloids; Jasmonates; Signal transduction; pH signaling; Desensitization

1. Introduction

The overproduction of phytoalexins in response to microbial and other stressors provides species-specific components for the complex series of plant defense reactions. In challenged tissues and plants phytoalexin biosynthesis is usually induced in concert with the ubiquitious elements of hypersensitive response (oxidative burst, polyphenol formation, cross-linking of cell wall proteins, localized apoptosis) and several other proteins of plant defense, e.g. chitinases (e.g. Heath, 2000). Obviously, there are integrative control mechanisms and cross-talk within a network of signal transfer reactions that may obscure individual signal paths and their molecular components (Feys and Parker, 2000; Thomma et al., 2001; Kunkel and Brooks, 2002). Cultured plant cells often underly a limited complexity of regulatory constraints and thus may allow to induce secondary metabolic pathways independent of the hypersensitive response or other associated stress reactions (Jakobek and Lindgren, 1993; Kuchitsu et al., 1997; Roos et al., 1998). Recently, considerable progress has been made in the identification of transcription factors and protein kinases that are involved in the signal transduction towards various stress responses including phytoalexins (Mememlink et al., 2001; Eulgem et al., 2000; Romeis, 2001). However, one important reason for divergence, cross talk and other kinds of complexity of signal transfer is still poorly understood: the involvement and mode of action, respectively interaction, of low MW second messengers as Ca^{2+}, pH, NO, systemins, octadecanoids, salicylic acid or ethylene (e.g. Kunkel and Brooks, 2002). Among them is jasmonic acid, a long-known, multifunctional stress signalling molecule (Wasternack and Parthier, 1997; Reymond and Farmer,

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1998). Its involvement in the expression of various phytoalexin biosynthetic pathways has been demonstrated by showing jasmonic acid dependent gene activation at the mRNA level (Blechert et al., 1995; Sharon et al., 1998; Menke et al., 1999) and was much substantiated by the identification of the ORCA transcription factors, members of the AP2/ERF-domain family, that activate a number of jasmonate-regulated genes encoding e.g., enzymes of terpenoid alkaloid bioynthesis and its precursor pathways (van der Fits and Memelink, 2000; Memelink et al., 2001).

At the same time evidence accumulates that also jasmonate-independent signal paths essentially contribute to pathogen defense. For instance, separated modules of jasmonate-, salicylate- and ethylene-controlled signal transfer have now been identified and actual research is aimed to understand their coordination and the resulting plasticity of defense mechanisms (Thomma et al., 2001; Kunkel and Brooks, 2002).

The present paper presents data from cell biological experiments that imply the existence and advantagous coordination of jasmonate-dependent and -independent signal transfer pathways in the expression of alkaloid phytoalexins. The object of study are cultured cells of *Eschscholzia californica* that retained the plant's ability to overproduce benzophenanthridine alkaloids in response to microbial elicitors (Schumacher et al., 1987; Müller et al., 1993). Jasmonates proved to be an efficient inducer of alkaloid biosynthesis, as shown at the levels of product accumulation (Collinge and Brodelius, 1989; Gundlach et al., 1992) and transcription of genes of biosynthetic enzymes (Blechert et al., 1995; Pauli and Kutchan, 1998; Haider et al., 2000).

In parallel we have shown that the expression of benzophenanthridine biosynthesis can be elicited by the "yeast glycoprotein elicitor" (fraction of 30–100 kD) at concentrations well below those required for the hypersensitive response and its associated reactions as loss of K^+, external alkalinization and polyphenol accumulation. The latter response, but not alkaloid formation, was prevented by the presence of catalase in the culture medium (Roos et al., 1998) indicating that the induction of benzophenanthridine bioynthesis in *Eschscholzia* cells does not necessarily involve the formation of H_2O_2 or related reactive oxygen species.

A transient decrease of the cytoplasmic pH has been identified as an essential step in the signal chain triggered by low elicitor concentrations (Roos et al., 1998). This shift is most probably mediated by lysophosphatidylcholine that is produced by an elicitor-activated, G-protein controlled phospholipase A_2 (Roos et al., 1999) and in turn activates a H^+/Na^+ exchanger at the vacuolar membrane (Viehweger et al., 2002). The present study addresses the question of whether jasmonate and intracellular pH shifts operate in a common signaling sequence or represent separated pathways of information transfer towards the induction of benzophenanthridine biosynthesis. One of our experimental approaches made use of the desensitization phenomenon, a distinct feature of G-protein controlled signal modules known from animal cell systems (Freedman and Lefkovitz, 1996): contact with effectors not only triggers the predetermined downstream events but also sets the signal path to a refractory state, i.e. it strongly attenuates subsequent responses within a distinct period of time. Rapid desensitization has also been found in some plant signal perception systems that initiate e.g. responses to chitin oligomers or ergosterol (Felix et al., 1993; Boller, 1995) as well as to oligogalacturonides and the harpin elicitor (Binet et al., 1998; Chandra et al., 2000).

The present study provides first evidence for a selectice desensitization of the phytoalexin response that supports the coexistence of jasmonate- and pH controlled signal pathways.

2. Results

2.1. Elicitor contact triggers both alkaloid formation and desensitization

Cultured cells of *E. californica* challenged with the yeast glycoprotein elicitor overproduce benzophenan-

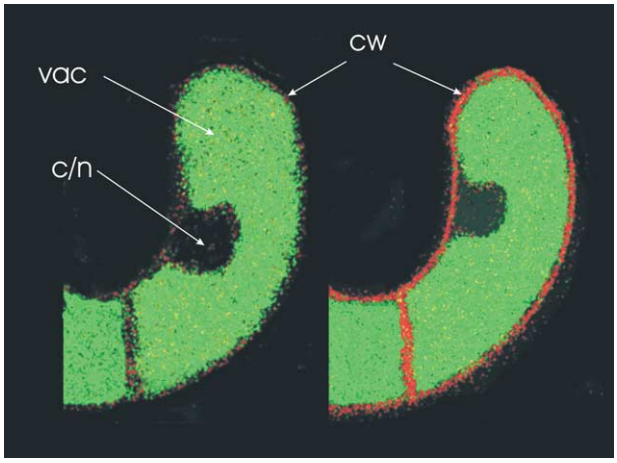

Fig. 1. Accumulation of benzophenanthridine alkaloids in the cell wall region of elicitor-treated cells (confocal laser scanning microscopy). 1 ml cell suspension was preincubated for 2 h with 5 ìM DM-NERF (a rhodol based fluorescence indicator, Molecular Probes) and received elicitor (1 µg /ml) for 30 min. After washing with phosphate-free culture liquid a 5 µl sample was spotted onto an agarose film and placed in a 140 µl cell chamber that was mounted at the confocal microscope stage and perfused with control medim (300 µl/min). Confocal images were scanned 30 min (left image) and 12 h (right image) after elicitor contact. Red color: benzophenanthridine alkaloids, visualized by their emission at $\lambda_{EM} = 565–640$ nm. Green colour: DM-NERF, emission at $\lambda_{EM} = 493–525$ nm. The vacuolar accumulation of this compound proves the integrity and proton accumulating capacity of the vacuole and therefore serves as an indicator of cell viability during microscopic examination.

thridine alkaloids, most of them are excreted into the outer medium. A distinct portion of these alkaloids accumulates in the cell wall region, where they can be detected microscopically due to their genuine fluorescence (Fig. 1).

Under the used culture conditions and cell densities, elicitor concentrations around 1 μg/ml nearly saturated the alkaloid response (around 85% of maximum, cf. Fig. 2). As such concentrations neither triggered browning (polyphenol formation) nor impaired the viability of the cells (as indicated by fluorescence indicators, cf. Fig. 1, and the growth rate) they were used throughout in this study.

When the increase of total alkaloid content was followed in a challenged culture it became obvious that a 30 min contact with an elicitor-containing medium was sufficient to evoke nearly the same rate of alkaloid formation as the permanent presence of the elicitor during 24 h (Fig. 3, upper curves). It can be fairly excluded that the availability of active elicitor was diminished by rapid deactivation or binding: the culture filtrate obtained after 24 h retained >90% of its original potency to induce alkaloid formation in untreated cultures (data not shown). Hence, most or all cells that are susceptible to elicitor stimulation received this signal within 30 min and then remained insensitive to further elicitation.

In another set of experiments the response to repeated elicitor contacts was tested with cells obtained from elicited cultures. As shown in Fig. 3 (left arrow), after 30 min of elicitor contact the response to a second challenge decreased to around 65% of the original reactivity. Similar degrees of attenuation were seen after 2 and 3 h of elicitor treatment (data not shown). After a 24 h exposure to the elicitor the cells responded to a second contact with very low alkaloid production, if any (Fig. 3, right arrow). This strong attenuation of subsequent responses caused by the original elicitor contact persisted over several days of cultivation. As shown in Fig. 4, the second or any further elicitor contact did not cause an increase of alkaloid production irrespective whether gaps of 24, 48 or 72 h were allotted between the periods of elicitor treatment.

Taken together, these data indicate that the first elicitor treatment not only triggers the expression of alkaloid production but also establishes a refractory state for any subsequent elicitor challenge, at least within our test period of 72 h.

2.2. Different stimuli cause desensitization to specific groups of signals

Alkaloid biosynthesis can be evoked by a number of stimuli with no structural relation to the glyoprotein elicitor. Three of them have been optimized for their

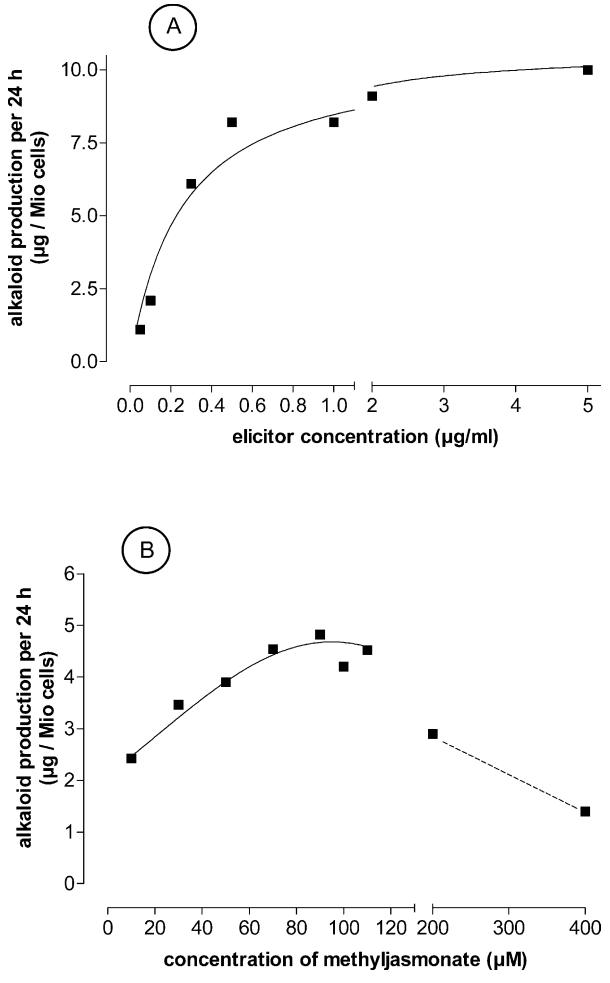

Fig. 2. Dependence of the alkaloid response on the concentration of yeast elicitor (A) and methyljasmonate (B). Yeast elicitor or methyljasmonate was present during each 3 h, alkaloid production was measured over the following 24 h. The alkaloid production of untreated control cultures was subtracted. Data are averaged from each three experiments with different cell culture batches, S.D. of the single experiments was 7–20%.

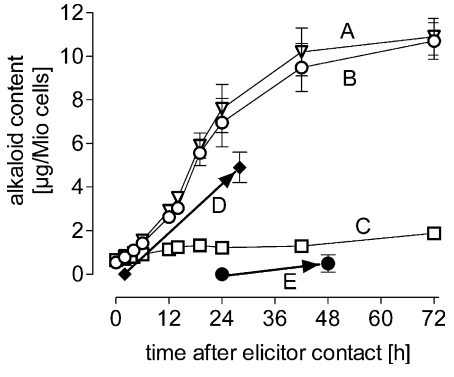

Fig. 3. Dynamics of alkaloid content after single, permanent and repeated contact with yeast elicitor. A (triangles): permanent presence of elicitor; B (circles): elicitor contact over 30 min; C (squares): control (no elicitor); D, E (arrows): alkaloid production per 24 h of cells that were pretreated with elicitor for either 30 min (D) or 24 h (E). The latter data (C, D) are corrected for the alkaloid content present at the beginning of the second elicitor contact.

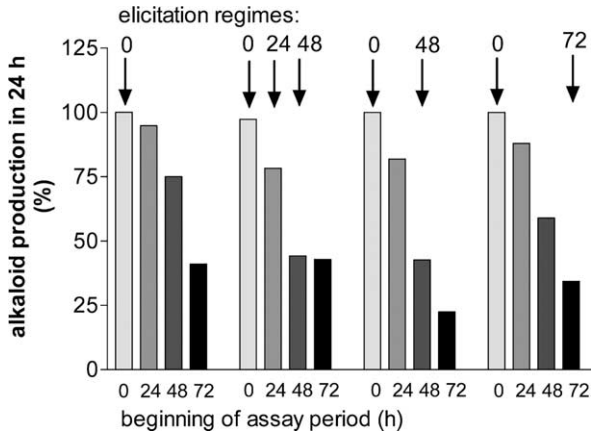

Fig. 4. Refractory state of elicited cells to elicitor challenge, monitored at long-term intervals 5 day cells were resuspended in control medium and received yeast elicitor at 24 h intervals as indicated by the arrows. Elicitor was present for each 30 min, followed by washing and resuspending the cells in elicitor-free medium. Samples were taken at 24 h distances and assayed for alkaloid content. Data are normalized to the alkaloid production triggered by the first contact to yeast elicitor which is set to 100%. S.D.: 8–25%, $n=3$.

stimulatory effects on alkaloid production in the present cell culture (concentration dependence, time of contact, little or no impairment of cell viability) and the resulting effects on alkaloid production are compiled in Fig. 5A.

1. Methyljasmonate was selected because of its known inducer function in the expression of bezophenanthridine biosynthesis in the used cell culture (Blechert et al., 1995; Pauli and Kutchan, 1998; Haider et al., 2000, also cf. above).
2. A transient acidification with a permeable acid (butyric or pivalic) has been established as potential inducer as it mimicks in part the elicitor-triggered acidification of the cytoplasm (e.g. from pH 7.4 to 6.7, Roos et al., 1998) and under optimized conditions causes a significant increase of alkaloid production (Roos et al., 1998; Küppers, 2000).
3. Osmotic treatment has not yet been described as a stimulus of alkaloid biosynthesis in *Eschscholzia*. In our hands, a 3 h exposure of cell suspensions to culture liquids that contained additional KCl, NaCl or sorbitol to attain an osmotic pressure above 240 mOsM triggered a pronounced increase of alkaloid bioynthesis. Addition of 125 mM sorbitol (final osmotic pressure 256 mOsM) caused an optimum alkaloid response without detectable cell damage.

The optimized stimuli whose primary effects are shown in Fig. 5A caused entirely different responses when applied to elicitor treated cells (Fig. 5B): not only the response to elicitor treatment (as shown above) but also to artificial acidification was strongly attenuated. The same cells showed, however, an almost unimpaired reactivity towards methyljasmonate and hyperosmotic treatment. This result not only demonstrates a clear specificity of desensitization but also proves that the primary elicitor contact did not lead to the maximum expression of the alkaloid biosynthetic capacity.

Further characteristics of desensitization of the alkaloid response became apparent if cells pretreated by either of the forementioned stimuli were mutually exposed to the same or other treatment. The data shown in Figs. 5C–E confirm a pronounced selectivity of desensitization: pretreatment with yeast elicitor or artificial acidification desensitizes the cells to either of these stimuli, but still allows stimulation by jasmonate and hyperosmolarity (Figs. 5B and C). On the contrary, pretreatment with methyljasmonate or sorbitol attenuates a subsequent response to the same stimulus, but allows significant reactivity towards yeast elicitor and artificial acidification (the latter response was even amplified).

Seen together, the above data indicate that the attenuation of alkaloid responses by elicitor or methyljasmonate does not involve a decrease of the overall capacity of alkaloid biosynthesis as cells pretreated with either compound display the original rate of alkaloid production after subsequent challenge by at least one other stimulus (Figs. 5A and C). Also, preincubation with sorbitol or artificial acidification that seemed to cause some reduction of the total biosynthetic capacity, still retained at least 75% of the original level (Figs. 5C and E). Thus, the desensitizing effects are rather directed to elements of the signal transduction pathways that operate upstream of the biosynthetic level. The attenuation exerted by elicitor contact obviously effects only distinct signal elements that may be shared with the transduction of the pH/acidification signal (as suggested from the mutual desensitization of elicitor and acid treatment) but not with the signal pathways originating from jasmonate or hyperosmotic treatment. The different mode of desensitization exerted by jasmonate or sorbitol, i.e. deactivation of subsequent responses to the same signal but not to the other stimuli likewise argues for the existence of stimulus-specific intermediates that are subject to selective attenuation.

2.3. Jasmonate peak and pH shift require different elicitor concentrations

The simplest interpretation of the above data would conclude that elicitor- and pH dependent signalling use common signal elements that are not shared with the jasmonate dependent signal transfer. This idea could be tested by experiments aimed to find out

1. whether the used elicitor concentrations caused shifts of the jasmonate content and

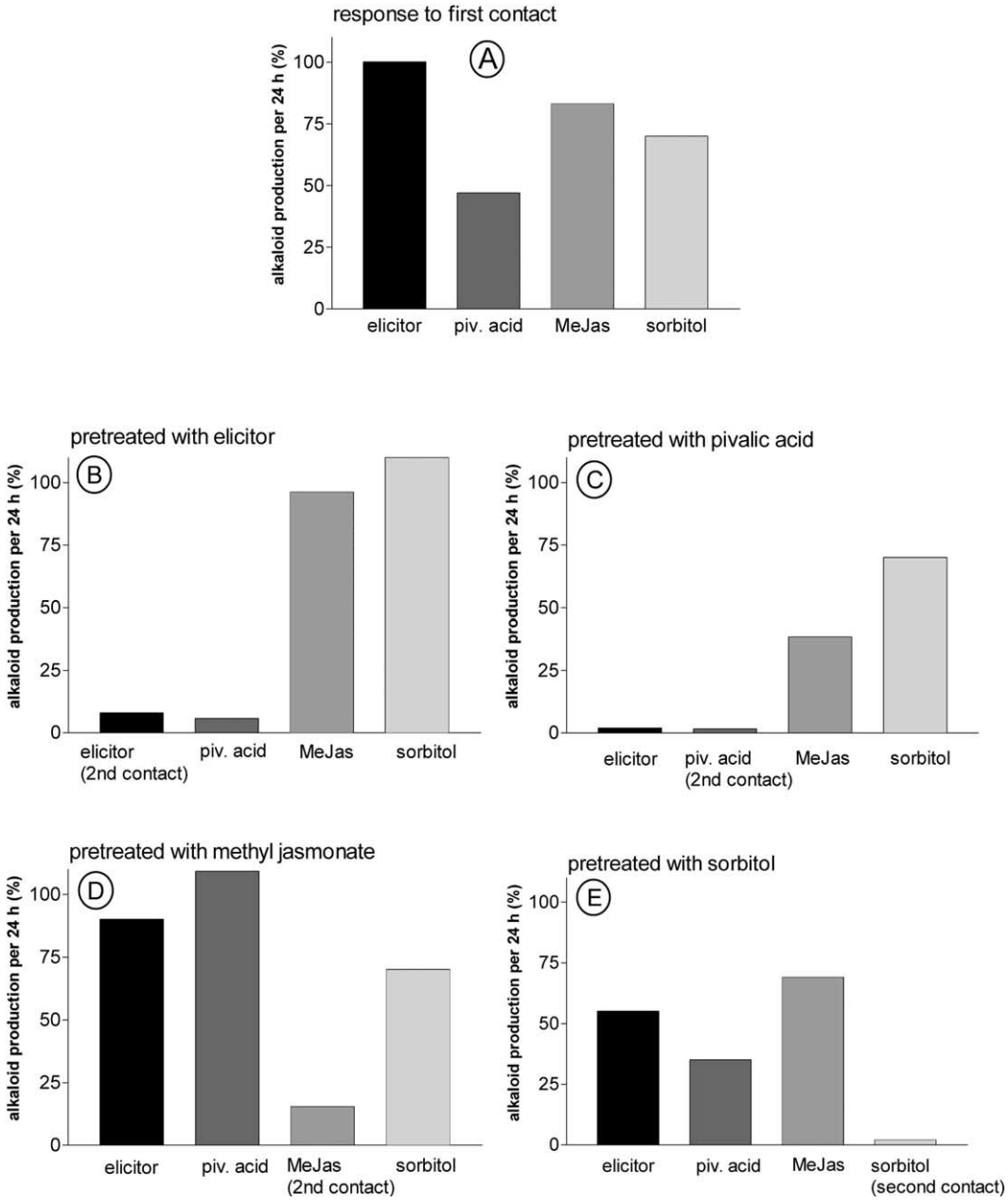

Fig. 5. Induction and attenuation of alkaloid responses by different effectors. A: alkaloid production per 24 h caused by yeast elicitor (1 μg/ml, 3h), pivalic acid (5 mM, pH 4.0, 2 min), methyljasmonate (100 μM, 3h) and sorbitol (125 mM, 3h). B–E : alkaloid reponses to the same effectors of cells pretreated for 24 h with either of these compounds, as indicated on top of each figure. Data are normalized to the alkaloid production triggered by the first contact to yeast elicitor which is set to 100%. This response was averaged from a large number of different batches as 6 μg/Mio cells. The alkaloid content present at the beginning of a new challenge was subtracted.

2. whether methyljasmonate caused cytoplasmic pH shifts of a duration and magnitude that allows induction of alkaloid biosynthesis.

First, at the used elicitor concentration of 1 μg/ml no significant increase of the jasmonate content could be found within 3 h of elicitor treatment (Table 1). However, at high elicitor concentrations the expected increase of jasmonate was detectable which proved the ability of the used culture to raise the cellular jasmonate content in response to external stimuli and thus confirmed earlier experiments by Müller et al. (1993).

Hyperosmotic (sorbitol) treatment also caused a significant increase of the jasmonate content but was less effective than high elicitor concentrations (Table 1).

9,10-Dihydrojasmonic acid that was detectable in most samples did not increase in parallel with the jasmonic acid content but rather decreased during elicitor treatment. This is consistent with the very low or lacking biological activity as it is suggested by the (few) data

Table 1
Jasmonate content and alkaloid production triggered by yeast elicitor and hyperosmotic (sorbitol) treatment

Treatment	Alkaloid production in 24 h (μg/g dwt)	Jasmonic acid (9,10-dihydrojasmonic acid) (ng/g dwt)
Control cells	40±12	1.2±1.3 (2.8±3.4)
	Fold increase:	Fold increase in 3h:
Elicitor (1 μg/ml)	58	1.0 (0.2)
Elicitor 50 (μg/ml)	65	5.3 (1.7)
Sorbitol (125 mm)	44	2.5 (0.3)

Five day cell suspensions in control medium received yeast elicitor or sorbitol as indicated. Jasmonate content was assayed in samples taken at 30 min intervals over 3 h. The peak content found in this period is given as the factor of increase over the corresponding control culture. In parentheses: content and increase factor of 9,10-dihydrojasmonic acid determined in the same samples. Alkaloid production is given as the increase of benzophenanthridine content in parallel samples during 24 h after effector contact. Data are averaged from three independent experiments.

available on this molecule (Gundlach and Zenk, 1998; Miersch et al. 1999).

Second, concentrations of methyl jasmonate that caused a well detectable alkaloid response (50–150 μM, maximum near 100 μM) were tested for their ability to induce intracellular pH shifts. For this purpose, confocal pH topography of individual cells was applied. A typical experiment is documented in Fig. 6. No measurable pH shift could be detected during the time jasmonate was present in the perfusion medium (14 min). After exchanging methyl jasmonate with the yeast elicitor the expected intracellular pH shift, i.e. an acidification of the cytoplasm, was observed within less than 4 min. Thus, methyljasmonate is unlikely to be involved in the generation of elicitor-triggered pH shifts.

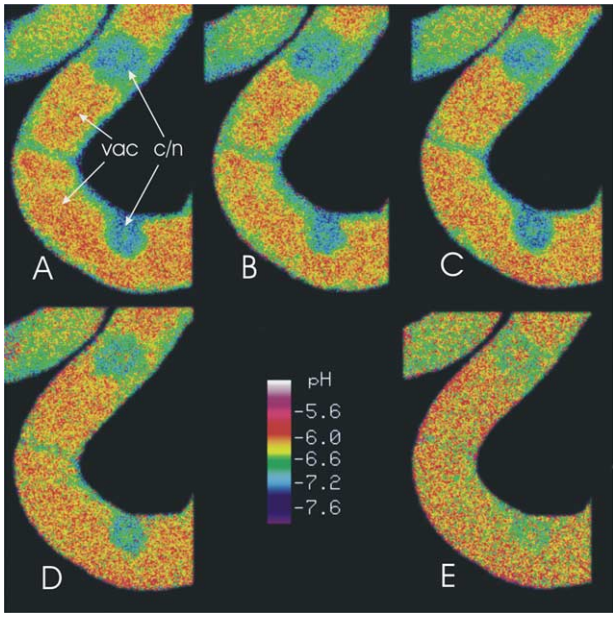

Fig. 6. Intracellular pH distribution of cells treated with jasmonate and elictor (confocal pH topography). Confocal pH maps with the pH-probe carboxySNARF1 were obtained as described in Section 4. 100 μM methyljasmonate was present in the perfusion medium over 14 min and then replaced by 1μg/ml yeast elicitor. Upper row: cells at $t=0$ (A), 2 min (B) and 6 min (C), after addition of methyljasmonate. Lower row: the same cells 1 min (D) and 4 min (E) after addition of yeast elicitor.

Summarizing, elicitor concentrations that trigger an intracellular pH shift and mobilize the major part of the benzophenanthridine biosynthetic capacity do not cause a significant raise of the cellular jasmonate content. On the other hand, jasmonate concentrations that are able to induce alkaloid biosynthesis do not evoke a transient shift of the cytoplasmic pH. These findings are consistent with the demonstrated selectivity of desensibilizing effects of jasmonate and yeast elicitor and support the concept of two non-interacting signal pathways towards the induction of benzophenanthridine biosynthesis.

2.4. Yeast elicitor and jasmonate can trigger additive expression of alkaloid biosynthesis

Finally we proved whether the independence of elicitor- and jasmonate- triggered induction of benzophenanthridine biosynthesis can be demonstrated in the same cell suspension, i.e. without the removal of culture filtrates (which contained most of the alkaloids produced after the original elicitor contact). Under such conditions, that might come closer to the signal events observed by a pathogen-attacked cell in its natural environment it became obvious that the contact with methyljasmonate after elicitor challenge activates a considerable additional capacity of alkaloid production (Fig. 7). This finding also demonstrates that the inducing effect of jasmonate is not impaired by the presence of the elicitor. It is further shown in Fig. 7 that high elicitor concentrations (50 μg/ml) cause similar effects as the addition of methyljasmonate, which supports the idea that the inducing effect of such concentrations is mediated via increased jasmonic acid levels (cf. Table 1).

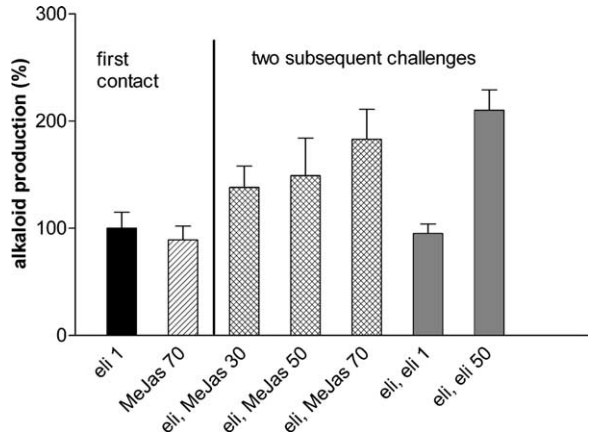

Fig. 7. Alkaloid production caused by the subsequent addition of yeast elicitor and methyljasmonate to the same cell suspension. After 24 h treatment with yeast elicitor (1 μg/ml), the cell suspension was divided into aliquots that received different concentrations of methyljasmonate or yeast elicitor without separating cells and outer medium. Left columns: alkaloid production of the first 24 h, triggered by yeast elicitor (1 μg/ml) or methyljasmonate (70 μM). Right columns: alkaloid production during 48 h; at $t=0$ cells received yeast elicitor (1 μg/ml), after 24 h the indicated concentrations of methyljasmonate (30, 50 or 70 μM) or of yeast elicitor (1 or 50 μM) were added ($n=4$).

3. Discussion

3.1. Jasmonate-dependent and -independent signaling towards benzophenanthridine biosynthesis

The present data established significant differences between the inducing effects of yeast elicitor and methyljasmonate on the expression of alkaloid biosynthesis in cultured cells of *E. californica*. They support the existence of two signal transfer pathways that are independent of each other at least in their early events.

(1) The signal character of jasmonate in the expression of benzophenanthridine biosynthesis is well evidenced for *Eschscholzia* cell cultures. A transient increase of this molecule was demonstrated after contact with >5 μg/ml yeast elicitor (Müller et al., 1993). Two enzymes of benzophenanthridine biosynthesis, i.e. berberine bridge enzyme (Blechert et al., 1995) and (*S*)-*N*-methylcoclaurine 3′-hydroxylase (Pauli and Kutchan, 1998) proved to be jasmonate-inducible on the mRNA level. Furthermore, structure–activity relationships have indicated that jasmonic acid rather than its precursor 12-oxo-phytodienic acid (which is involved in the transduction of stress signals in other plants (Blechert et al., 1995; Koch et al., 1999), is the likely signal molecule in *Eschscholzia* (Haider et al., 2000).

The minimum concentration of the yeast elicitor preparation that raised the jasmonate content (around 5 μg/ml, cf. Müller et al., 1993) surpasses the threshold above which the expression of alkaloid biosynthesis is accompanied by elements of the hypersensitive response like polyphenol formation (browning), loss of K^+ and external alkalinization (Roos et al., 1998). Though still a matter of debate, the putative involvement of jasmonate in the expression of hypersensitive cell death (e.g. Asai et al., 2000) might establish a link between this ubiquitious defense mechanism and the induction of phytoalexin biosynthesis.

(2) A considerable part of the alkaloid biosynthetic capacity can be elicited under conditions where no measurable increase of jasmonate content occurs, i.e. with elicitor concentrations $\leqslant 1$ μg/ml or experimental acidification of the cytoplasm. The involvement of a cytoplasmic pH shift (Roos et al., 1998) appears as a typical and essential feature of the underlying signal transduction. Early events most probably include the elicitor-activation of phospholipase A_2 of the plasma membrane via a heterotrimeric G-protein (Roos et al., 1999). This enzyme produces the second messenger lysophosphatidylcholine that triggers an efflux of vacuolar protons via an H^+/Na^+ exchange process (Viehweger et al., 2002). The search for signal steps downstream of ΔpH, especially for pH induced regulatory proteins is in progress in this laboratory. ΔpH dependent inductions are known for the secondary metabolic enzymes PAL and HMGCoA reductase (Lapous et al., 1998).

The two signal pathways characterized by the above data are triggered by either low or high concentrations of the "yeast elicitor preparation". As long as the active components of this widely used preparation (cf. Section 4) are not yet purified to homogeneity it remains open whether both signals originate from two different elicitor compounds or represent concentration dependent effects of the same signal molecule. Attempts to purify the most active component of the yeast glycoprotein mixture are under way in this laboratory.

3.2. Desensitization of plant defense responses

The phenomenon of desensitization in the regulation of plant pathogen defense has not yet been investigated in molecular details. The available data refer solely to responses that are associated with the hypersensitive response, as external alkalinization, influx of Ca^{2+} and production of reactive oxygen.

Tobacco cells treated with oligogalacturonides became refractory to the same stimulus but remained responsive to cryptogein. In contrast, cryptogein-treated cells still responded weakly to a second application of cryptogein or oligogalacturonides (Binet et al., 1998). The oxidative burst of soybean cells is subject to both homologous and heterologous desensitization (Chandra et al., 2000): oligogalacturonides, the harpin protein or the abiotic elicitor fensulfothion not only attenuated a subsequent oxidative burst by the same stimulus but also left the cells refractory to the effect of the other

chemical elicitors. The duration of the refractory state was relatively short (10–20 min) and depended of the magnitude of the original oxidative burst. It may thus be concluded that the desensitization serves the protection of the cell against the toxic, yet short-lived reactive oxygen species.

To our knowledge, desensitization of a phytoalexin responses has not been reported so far. Our finding, that artificial acidification, i.e. a signal event that is triggered by the elicitor contact, has the same desensitizing activity as the elicitor argues that the attenuated target reaction is located downstream of the pH shift. The attenuating effects of methyljasmonate and hyperosmotic treatment might be mediated via different mechanisms as sorbitol treated cultures—despite the increase of jasmonic acid (cf. Table 1)—allow a full response to jasmonate (Fig. 5). Interestingly, jasmonate treatment amplifies the alkaloid response of cells pretreated by artificial acdification (Fig. 5D) which might suggest some cross talk between both signal pathways.

The future characterization of molecular details of the desensitization mechanism might open the use of this phenomenon as an experimental tool for the dissection of complex signal mechanisms, i.e. to identify sites of attenuation that are specific for distinct stimuli or responses as can be assumed from the present example.

The selective attenuation of phytoalexin production appears advantageous for plant cells if their biosynthetic potential can be activated by several stimuli:

The ability to generate a phytoalexin response can be maintained over a longer period of time as with a one-shoot strategy. The cells remain responsive to multiple stimuli if either of them can mobilize only a limited portion of the total biosynthetic capacity. In our example, attenuation of the elicitor-triggered response allows the Eschscholzia cells to ''reserve'' a considerable part of their benzophenanthridine biosynthetic capacity for responses that are transduced via jasmonate dependent signalling (cf. Fig. 7). Thus, selective desensitization might allow to manage responsiveness to multiple stressors even at a limited biosynthetic capacity.

Attenuation of the phytoalexin response might also be an element of protection against self toxicity of the defense compounds as it keeps their local concentrations below tolerable limits. The benzophenanthridine alkaloids produced by the present culture are potent toxins (Dzink and Socransky, 1985) due to their ability to intercalate into dsDNA, to uncouple oxidative phosphorylation and bind to cytoskeletal proteins (Sen et al., 1996; Faddeeva and Beliaeva, 1997; Wolff and Knipling, 1993). Preliminary data from our laboratory show that any damage to the plasma membrane which functions as a critical permeability barrier against external benzophenanthridines causes their immediate uptake and intercalation into DNA. High concentrations of the alkaloids act as membrane compromizing agents (Faddeeva and Beliaeva, 1997) and would thus create a non-tolerable self toxicity to the producing cells.

4. Experimental

4.1. Plant cell cultures

Suspension cultures of *E. californica* were grown in a medium according to Linsmaier and Skoog (1965) with the hormones 2,4-dichlorophenoxyacetic acid and α-naphthaleneacetic acid (1 μM each). Cultivation was performed on a gyrotary shaker (100 rpm) at 24 °C in continuous light (ca. 7 μmol m^{-2} s^{-1}) in a 10 day growth cycle. Cells from 5 or 6 day cultures were used for experiments.

The culture used in this study consists of strings of 4–6 cells. Cell density, i.e. the number of such aggregates per ml, was routinely counted with a high frequency cell counter (Casy1, Schärfe Systems, Reutlingen).

4.2. Treatment of cells with effectors of alkaloid production

Cells were harvested by filtering without pressure through a 200 mesh nylon filter, washed by resuspension in the 5-fold volume of sterile 100 mM sorbitol solution and finally resuspended (50 mg fwt/ml) in 75% phosphate-free culture liquid which contained yeast elicitor (1 μg/ml if not indicated otherwise), methyljasmonate or sorbitol. After the indicated periods of contact, cells were again filtered, washed and resuspended as described. 75% phosphate-free culture liquid was used as the experimental medium in all control experiments (osmotic pressure around 135 mOsM).

Pivalic acid treatment: cells washed with 100 mM sorbitol were resuspended in phosphate free culture liquid that contained 5 mM Na-pivalate and was titrated with HCl to pH 4.0 prior to use. These suspensions were shaken for 2 min, filtered and the cells washed by resuspending and shaking for 10 min in control medium followed by final resuspension in the same liquid.

4.3. Determination of benzophenanthridine alkaloids

1 ml cell suspension was mixed with 1 ml MeOH (v/v) containing 5% HCl, extracted for 30 min at 40 °C and then centrifuged at 6000 *g* for 10 min. In the supernatant the alkaloids were determined by reading the fluorescence (λ_{EX} 460 nm, λ_{EM} 570 nm). Fluorescence intensities were converted into alkaloid concentrations via calibration curves obtained with the benzophenanthridine alkaloid sanguinarine dissolved in an analogous mixture of culture liquid, EtOH and HCl. The method was validated by HPLC of test extracts as described elsewhere (Roos et al., 1998).

4.4. Elicitor preparation

A glycoprotein fraction was prepared from commercial bakers yeast (DHW "Gold") as described by Schumacher et al. (1987). Concentrations given in the text refer to the dry weight of the crude elicitor preparation.

4.5. Confocal pH topography

The method was previously described in details (Viehweger et al., 2002). In brief, cells were loaded over 2 h with the pH probe carboxy-SNARF-1 and then spotted on gel disks (made from phosphate-free culture liquid with 2% agarose) which were fixed in a perfusion chamber and perfused with 75% phosphate-free culture liquid to which effectors were added as indicated.

Fluorescence images were obtained with the Leica TCS-SP confocal microscope (Mannheim, Germany) equipped with an argon ion laser that was operated in dual channel mode. Wavelength settings for SNARF were $\lambda_{EX} = 514$ nm; $\lambda_{EM} = 583 \pm 10$ nm (channel 1) and > 610 nm (channel 2). Signals from 8 frames were scanned simultaneously in both channels and the intensity ratio channel 1/channel 2 was calculated for each pixel. pH maps were obtained by color coding these intensity ratios according to self defined lookup tables.

4.6. Determination of jasmonate content

Fresh cells (6 g) were homogenized with 20 ml methanol and 50 ng ($^2H_6$)JA (prepared according to Miersch, 1991) was added as internal standard. The filtrate was loaded on a 3 ml DEAE-Sephadex A25 column and the column washed with 3 ml methanol. After washing with 3 ml 0.1 M acetic acid in methanol, eluents with 3 ml of 1 M acetic acid in methanol and 3 ml of 1.5 M acetic acid in methanol were collected, evaporated and separated on preparative HPLC and analyzed by GC–MS.

4.6.1. HPLC

Eurospher 100-C18, (5 μm, 250×4 mm), Solvent A: MeOH, Solvent B: 0.2% acetic acid in H_2O. Gradient: 40% A to 100% A in 25 min. Fractions between 12.30–14.00 min and 14.30–15.45 min were collected in 1 vial and evaporated.

4.6.2. Derivatization

Samples were dissolved in 200 μl $CHCl_3$ / *N*,*N*-diisopropylethylamine (1:1) and derivatized with 10 μl pentafluorobenzylbromide at 20 °C overnight. The evaporated samples were dissolved in 5 ml *n*-hexane and passed through a SiOH-column (500 mg; Machery-Nagel). The pentafluorobenzyl esters were eluted with 7 ml *n*-hexane/diethylether (2:1) evaporated, dissolved in 100 μl MeCN and analyzed by GC–MS.

4.6.3. GC–MS

(GCQ Finnigan), 70 eV, NCI, ionization gas NH_3, source temperature 140 °C , column Rtx-5w/Integra-Guard (Restek, Germany) (5 m inert precolumn;15 m×0.25 mm, 0.25 mm film thickness, injection temperature 250 °C, interface temperature 275 °C; Helium 40 cm s^{-1}; splitless injection; column temperature program: 1 min 100 °C, 25 °C min^{-1} to 200 °C, 5 °C min^{-1} to 300 °C , 20 min 300 °C; R_t of pentafluorobenzyl esters: ($^2H_6$)JA 10.30 min, ($^2H_6$)7-iso-JA 10.71 min, JA 10.36 min 7-iso-JA 10.76 min, 9,10-dihydo-JA 10.41 min, 9,10-dihydo-7-iso-JA 10.78 min. Fragments *m*/*z* 209 and 211 were used for quantification of JA and 9,10-dihydo-JA, respectively, against *m*/*z* 215 as a standard.

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