Induction of H_2O_2 synthesis by β-glucan elicitors in soybean is independent of cytosolic calcium transients

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Abstract Soybean cell suspension cultures have been used to investigate the role of the elevation of the cytosolic Ca^{2+} concentration in β-glucan elicitors-induced defence responses, such as H_2O_2 and phytoalexin production. The intracellular Ca^{2+} concentration was monitored in transgenic cells expressing the Ca^{2+}-sensing aequorin. Two lines of evidence showed that a transient increase of the cytosolic Ca^{2+} concentration is not necessarily involved in the induction of H_2O_2 generation: (i) a *Bradyrhizobium japonicum* cyclic β-glucan induced the H_2O_2 burst without increasing the cytosolic Ca^{2+} concentration; (ii) two ion channel blockers (anthracene-9-carboxylate, A9C; 5-nitro-2-(3-phenylpropylamino)-benzoate, NPPB) could not prevent a *Phytophthora soja* β-glucan elicitor-induced H_2O_2 synthesis but did prevent a cytosolic Ca^{2+} concentration increase. Moreover, A9C and NPPB inhibited *P. sojae* β-glucan-elicited defence-related gene inductions as well as the inducible accumulation of phytoalexins, suggesting that the *P. sojae* β-glucan-induced transient cytosolic Ca^{2+} increase is not necessary for the elicitation of H_2O_2 production but is very likely required for phytoalexin synthesis

Key words: Cytosolic calcium; β-Glucan; Plant defence; Soybean

1. Introduction

For many years, studies have focused on the putative role of Ca^{2+} as a mediator in elicitor-induced signal transduction cascades. Ca^{2+} has been shown to be involved in the elicitation of defence responses in several plants [1]. Using transgenic tobacco plants expressing aequorin, it has been demonstrated that elicitor treatment causes a rapid transient increase in the cytosolic Ca^{2+} concentration [2]. This alteration of cytosolic Ca^{2+} concentration may trigger a cascade of as yet unknown biochemical events which finally result in appropriate defence responses. In soybean, a genus-specific β-glucan elicitor from the phytopathogenic oomycete *Phytophthora sojae* (*Ps*-β-glucan) is specifically recognized by a high-affinity β-glucan receptor located on the plasma membrane [3]. Recently, it has been shown for soybean cell suspension cultures that *Ps*-β-glucan can induce both, a transient cytosolic Ca^{2+} increase as a prerequisite for phytoalexin synthesis [4], as well as an accumulation of reactive oxygen species (ROS) [5].

The rapid release of ROS, termed oxidative burst, has been reported for several plants as an inducible reaction upon treatment with elicitors or pathogen invasion [6]. However, it is not clear whether ROS have direct antimicrobial activity [7]. As the synthesis of ROS represents a part of the inducible hypersensitive response, a signaling pathway between elicitor binding and ROS production is very likely involved. Although ROS might contribute significantly to immediate defence reactions, a role for these compounds as second messengers in elicitor-induced defence responses, marked by the production of phytoalexins, remains controversial [1].

Up to now, pharmacological studies with various inhibitors suggested a close correlation between early responses to elicitors, such as Ca^{2+} fluxes and ROS production, and the later defence responses such as phytoalexin synthesis [1,8,9]. Such compounds are valuable tools if their interference with a specific target in non-toxic concentrations correlates well with the inhibition of downstream elicitor-induced responses. Therefore, they may contribute to our picture of the function of the elicitor-induced processes. Among other compounds, anion channel blockers such as anthracene-9-carboxylate (A9C) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) are efficient inhibitors of anion fluxes in plant tissues [9–11] and typical elicitor-induced defence responses [8,9].

Cyclic (1,3)-(1,6)-β-glucans derived from *Bradyrhizobium japonicum* (*Bj*-β-glucans), the symbiotic partner of soybean, have been described as modulators of *Ps*-β-glucan elicitor-inducible reactions [12]. This activity is thought to act by competition with the *Ps*-β-glucan at the β-glucan-binding site of soybean membranes. Therefore, these cyclic *Bj*-β-glucans are useful in exploring the signaling events initiated at the β-glucan receptor. The application of both, the biological modulators and the pharmaceutical active compounds on soybean cells expressing the Ca^{2+} sensor aequorin, enabled us to discriminate between Ca^{2+}-dependent and Ca^{2+}-independent defence response pathways in soybean.

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2. Materials and methods

2.1. Chemicals, β-glucans and plant material

A9C, 5-nitro-2-(3-phenylpropyl-amino)benzoate, 3,3′-dimethoxybenzidine dihydrochloride (*o*-dianisidine) were purchased from Sigma, Deisenhofen, coelenterazine was from Molecular Probes Inc., Leiden, The Netherlands.

Oomycete β-glucans from *P. sojae* Kaufmann and Gerdemann were prepared from purified cell walls as described earlier [13]. Due to limited amounts of β-glucans with a degree of polymerization of 7–15 (DP 7–15) we could use this *Ps*-β-glucan on a molar basis only in experiments aimed to modulate the cytosolic Ca^{2+} concentration. For other experiments a *Ps*-β-glucan fraction with a degree of polymerization of >50 was used. This *Ps*-β-glucan elicitor fraction was as active as DP 7–15. Cyclic bacterial β-glucans from the *B. japonicum* strains USDA 110 and AB-1 were extracted from cell pellets as described [14].

Cell suspension cultures of soybean (*Glycine max* L.) line 6.6.12 carrying the stably integrated plasmid *pGNAequ/neo2* and expressing apoaequorin, and cv. Harosoy 63 were grown as described previously [4,15].

2.2. Aequorin-dependent luminescence measurements

Transgenic 6.6.12 cell lines were used to reconstitute aequorin in vivo with 10 μM synthetic coelenterazine on a shaker (125 rpm) in the dark for 24–48 h. The Ca^{2+}-specific luminescence (470 nm) was measured as described [4]. Treatments with various compounds were performed by adding 1–10 μl of different concentrations of stock solutions to the cell suspension culture. The concentration of the *Ps*-β-glucan elicitor with a degree of polymerization of 7–15 (DP 7–15) was 100 μM. In pre-treatment experiments, no second compound was added until the base-line luminescence was reached. Mixing time for the addition of any compound was 5–7 s. In each experiment, the concentration of reconstituted aequorin was not limiting under any of the experimental conditions, with a maximal consumption not exceeding 10%. The residual aequorin was completely discharged by adding 100 μl of 25% (v/v) DMSO containing 25 mM $CaCl_2$ (final concentration 12.5% and 12.5 mM, respectively). The resulting luminescence was used to estimate the total amount of aequorin present in various experiments in order to determine the rate of aequorin consumption. This enabled us to calculate the cytosolic Ca^{2+} concentrations. According to Moyen et al. [16] we used the equation $pCa = 0.332588(-\log k) + 5.5593$ where k is a rate constant equal to luminescence at any time point divided by total remaining luminescence counts. None of the used compounds interfered with the aequorin-dependent luminescence which was tested by the addition of 10% (v/v) of DMSO to destroy the membranes.

2.3. H_2O_2 determination

The assay was performed with 5–7 day old soybean cell suspension cultures (cv. Harosoy 63) using the dye *o*-dianisidine [5]. In typical experiments, the difference in absorbance between the time of addition of any compound (set as time 0) and after 20 min of incubation was determined. For control experiments, H_2O was used instead of elicitor solution. The *Ps*-β-glucan elicitor concentration used for the analysis of inhibitor effects was 50 μg/ml inducing around 80% of the maximal response (38.4 ± 6.1 μM H_2O_2) which was obtained by 200 μg/ml. For co-incubation experiments, studying the effects of cyclic β-glucans from different strains of *B. japonicum*, the *Ps*-β-glucan elicitor concentration used was 20 μg/ml evoking around 60% of the maximal response. The concentration of H_2O_2 was calculated from a standard curve [5] showing a linear correlation between H_2O_2 concentration and absorbance at 450 nm.

2.4. Northern blot analyses

Before induction soybean suspension cells were precultured in the dark for at least 12 h as described [17]. Internal control experiments were carried out with both soybean cell lines (cv. Harosoy 63 and 6.6.1.2, respectively) with nearly similar results. Total RNA of soybean cells cv. Harosoy 63 was prepared [18] and 10 μg per lane were blotted onto Biodyne-A membranes (Pall, Dreieich). Hybridization probes used were: bean chalcone synthase (CHS1) cDNA [19], and soybean 3,9-dihydroxypterocarpane 6a-hydroxylase (D6aH) gene-specific probe, generated by PCR from the cDNA as described [20]. Probes were radioactively labeled by use of the random prime procedure (Prime-a-Gene, Promega, Heidelberg). Hybridization and washing conditions of Biodyne-A membranes were according to Fliegmann and Sandermann [21]. Hybridization signals were visualized by either phosphorimaging or exposition to X-ray film at −80°C.

2.5. Determination of glyceollins

Soybean cell suspension cultures (cv. Harosoy 63, 5 days old) were subcultured in fresh medium for at least 15 h (4 g fresh mass per 40 ml medium). Treatment was done in 8 ml fractions of the cell suspension on a rotary shaker at 110 rpm and 26°C in the dark. The *Ps*-β-glucan elicitor concentration was 200 μg/ml. After 48 h cells were extracted and analyzed by HPLC [5]. Glyceollins were identified and quantified by using reference substances.

3. Results

Cyclic (1,3)-(1,6)-β-glucans produced by the bacterial symbiont of soybean, *B. japonicum*, share some structural features with the non-cyclic, elicitor-active (1,3)-(1,6)-β-glucans from the cell wall of *P. sojae*. Recently, they have been shown to suppress *Ps*-β-glucan-elicited defence responses in soybean, including the increase of the cytosolic Ca^{2+} concentration [4] and the synthesis of phytoalexins [12]. We now investigated the effects of these bacterial *Bj*-β-glucans on the inducible H_2O_2 production. Treatment of soybean cell cultures with cyclic (1,3)-(1,6)-β-glucans from the *B. japonicum* wild-type strain USDA 110 resulted in the induction of H_2O_2 production (Table 1). This response displayed the same kinetics with a maximum around 20 min after application as described for the *Ps*-β-glucan [5]. Cyclodecakis-(1,3)-β-glucosyl from the mutant strain AB1, a cyclic *Bj*-β-glucan lacking β-1,6-glycosidic linkages [14], was incapable of stimulating the accumulation of H_2O_2. Co-incubation experiments with *Ps*-β-glucan confirmed the lack of suppressor activity of the wild-type cyclic *Bj*-β-glucans on H_2O_2 synthesis (Table 1). At a concentration of 200 μg/ml the addition of cyclic *Bj*-β-glucans did not influence this *Ps*-β-glucan-elicited response as it did e.g. in the case of the inducible accumulation of phytoalexins [12].

Although cyclic *Bj*-β-glucans induced the production of H_2O_2, they could not elicit an increase of cytosolic Ca^{2+} con-

Table 1
β-Glucan-induced accumulation of H_2O_2 in soybean cell cultures

β-Glucan source	Concentration (μg/ml)	H_2O_2 (μM)
Control (H_2O)	–	6.3 ± 5.3 ($n=5$)
P. sojae	200	38.4 ± 6.1 ($n=3$)
P. sojae	20	24.2 ± 4.6 ($n=7$)
B. japonicum USDA 110	200	21.5 ± 7.4 ($n=7$)
B. japonicum AB-1	200	6.8 ± 6.5 ($n=5$)
B. japonicum USDA 110+ *P. sojae*	200+20	28.5 ± 9.6 ($n=7$)
B. japonicum AB-1+ *P. sojae*	200+20	27.9 ± 5.2 ($n=5$)

The *P. sojae* β-glucan elicitor showed maximal induction of H_2O_2 at 200 μg/ml. H_2O_2 production was monitored in the culture medium after 20 min of incubation. The results are the mean ± S.D., the number of independent experiments are indicated in brackets.

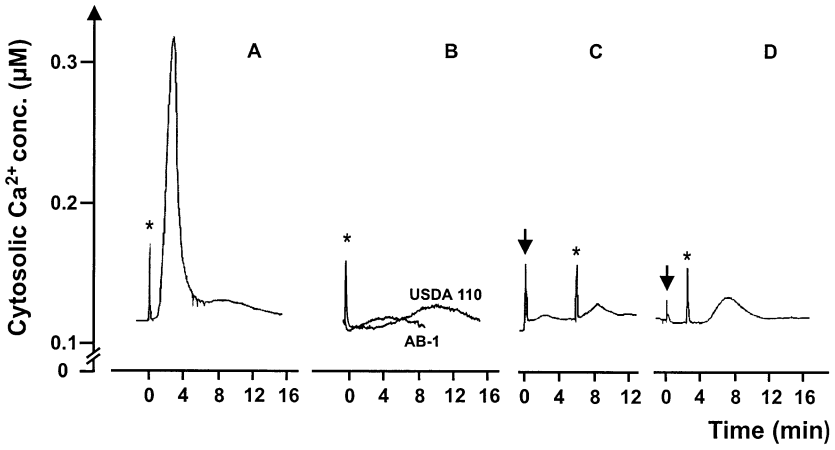

Fig. 1. Monitoring of cytosolic Ca^{2+} concentration in soybean cell suspension cultures. A: Increase of the cytosolic Ca^{2+} concentration upon treatment with 100 μM *Ps*-β-glucan. B: Effects of cyclic β-glucans (100 μM) from *B. japonicum* strains USDA 110 and AB-1, respectively, on the cytosolic Ca^{2+} concentration in soybean cell suspension cultures. C: Pre-treatment of the cells with NPPB (100 μM) followed by 100 μM *Ps*-β-glucan. D: Pre-treatment of the cells with A9C (100 μM) followed by 100 μM *Ps*-β-glucan. Addition of *Ps*-β-glucan is indicated by asterisks, addition of A9C or NPPB by arrows.

centration in aequorin-expressing soybean cells (Fig. 1B). This result indicated that the Ca^{2+} signal and cellular defence responses are not necessarily connected in soybean. To pursue this phenomenon in more detail we chose anion channel blockers as tools to modulate the Ca^{2+} regulation in soybean cells because they have been suggested to affect the *Ps*-β-glucan elicitor-mediated uptake of $^{45}Ca^{2+}$ [8]. Both compounds, A9C and NPPB, strongly diminished the *Ps*-β-glucan-induced transient increase of the Ca^{2+} concentration in the cytosol monitored by aequorin-dependent luminescence (Fig. 1C,D) although in this particular experiment (Fig. 1A) the second Ca^{2+} peak in the control was not as pronounced as it was observed earlier [4]. The anion channel blockers were used in non-toxic concentrations (100 μM, [8]) in combination with a *Ps*-β-glucan concentration (100 μM) which has been described to induce about 50% of the Ca^{2+}-mediated luminescence response and which was sufficient to induce glyceollin accumulation [4]. By contrast, in co-incubation experiments with the elicitor, the *Ps*-β-glucan-induced H_2O_2 production was not affected by the anion channel blockers in a concentration range between 1 and 100 μM (Fig. 2). A9C and NPPB per se had no stimulating effect on H_2O_2 production in soybean cell suspension cultures.

These results, indicating that the strong reduction of the cytosolic Ca^{2+} signal by the anion channel blockers affected cellular responses concerning the synthesis of phytoalexins but not the production of H_2O_2, prompted us to further investigate the role of Ca^{2+} in the elicitation of different defence responses in soybean. One of the reactions downstream of the *Ps*-β-glucan elicitor-induced Ca^{2+} response is the induction of CHS activity. This enzyme is involved in an early step of the biosynthesis of the pterocarpan phytoalexins of soybean and the up-regulation was described to be inhibited by both anion channel blockers, A9C and NPPB [8]. To analyze whether the inhibition of enzyme activity was depending on the inhibition of gene induction, the *Ps*-β-glucan-induced transcript accumulation for defence-related genes was examined (Fig. 3). The elicitor-induced increase of the steady state levels of transcripts for CHS and for a specific, late enzyme of the glyceollin-biosynthesis pathway, D6aH [20], was clearly reduced by NPPB. However, A9C showed somewhat unex-

Table 2
Inhibition of *Ps*-β-glucan elicitor-induced reactions in soybean suspension cultures by anion channel inhibitors

Ps-β-glucan-induced reactions	IC_{50} (μM)	
	A9C	NPPB
Cytosolic Ca^{2+} increase	6	5
CHS activity[a]	20	5
Phytoalexin accumulation	9	3
H_2O_2 production	–	–

–, no effect.
Values for inhibitor concentration to exert a half-maximal effect (IC_{50}) were calculated from dose–response curves for the inhibition of individual elicitor-induced reactions. For each data point of the curves at least three independent experiments have been done. The Ca^{2+} increase was measured as the increase of cytosolic Ca^{2+} concentration immediately after addition of *Ps*-β-glucan elicitor, the H_2O_2 production after 20 min. CHS activity was determined 12 h after elicitor treatment, and the phytoalexin contents were analyzed after 48 h.
[a]Data from [8]

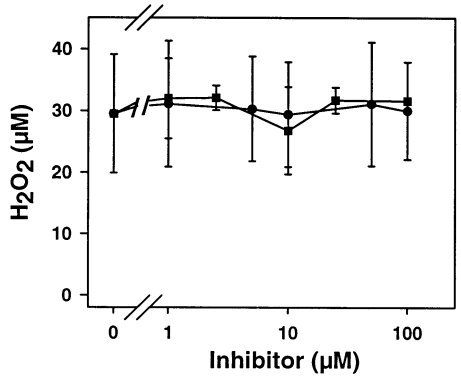

Fig. 2. Effect of the anion channel blockers A9C (●) and NPPB (■) on the *Ps*-β-glucan elicitor-stimulated H_2O_2 production. The *Ps*-β-glucan concentration was 50 μg/ml resulting in around 80% of the maximal H_2O_2 response. The results are the mean ± S.D. ($n = 3$ or 4).

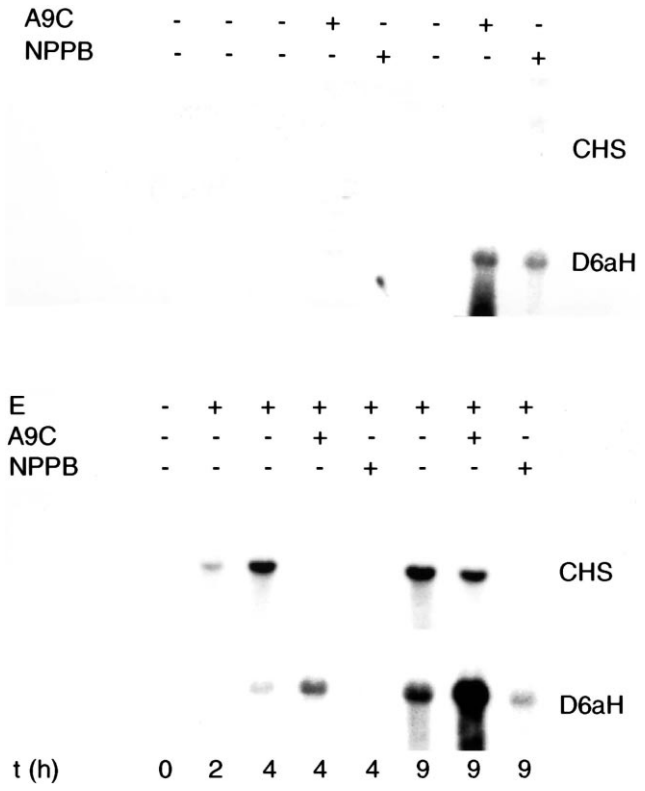

Fig. 3. Inhibition of the *Ps*-β-glucan-elicited increase in transcript levels for defence-related genes by anion channel blockers. Soybean cell cultures were treated for the indicated times (2, 4, 9 h) with A9C or NPPB (100 μM each) only, or together with *Ps*-β-glucan (E, 200 μg/ml). Equal loading of the blots was confirmed by control hybridization with a 28S rRNA probe.

pected effects because in control experiments, A9C alone induced an increase of transcript levels of D6aH (Fig. 3). An accumulation of phytoalexins was never observed upon treatment with A9C or NPPB alone.

Next, we analyzed the effects of the ion channel blockers on the *Ps*-β-glucan-induced phytoalexin production. Glyceollin accumulation of elicited soybean suspension cells was determined during treatment with A9C or NPPB, respectively, and found to be strongly inhibited at micromolar concentrations. With both compounds the maximal inhibition of glyceollin accumulation was obtained with an inhibitor concentration of 100 μM. The calculated IC_{50} values representing the concentrations necessary to obtain 50% inhibition of the different reactions analyzed were 3–5 μM for NPPB and 6–20 μM for A9C, respectively. In all cases, NPPB was slightly more effective than A9C (Table 2).

4. Discussion

The elevation of the cytosolic Ca^{2+} concentration is thought to be a central player in the regulation of elicitor-induced defence reactions in plants. This proposition was substantiated by experiments demonstrating that the presence of Ca^{2+} chelators as well as the Ca^{2+} channel inhibitor La^{3+} blocked elicitor-mediated reactions in several plants [1,22]. Plants expressing aequorin, a Ca^{2+}-sensing luminescent protein, now offer a valuable tool for a direct analysis of intracellular changes in Ca^{2+} concentration upon specific stimulation. This technique for monitoring Ca^{2+} fluxes is superior to the conventional use of $^{45}Ca^{2+}$ as discussed in detail [23]. Implementing this transgenic approach allowed us to examine the role of Ca^{2+} in different elicitor-inducible defence responses in soybean by using two different approaches, relying on both, biological effectors and pharmaceutical active compounds.

Cyclic (1,3)-(1,6)-β-glucans from *B. japonicum* USDA 110 are implicated to function as osmotically active solutes during hypoosmotic adaptation in the periplasmic space. Additionally, there is a growing body of evidence that cyclic β-glucans play a role during infection of the host plant soybean. These cyclic *Bj*-β-glucans are the only known bacterial ligands of the soybean β-glucan receptor and have been described as suppressors of *Ps*-β-glucan-induced defence responses [4,12]. Remarkably, these carbohydrates did not inhibit the elicited H_2O_2 accumulation in co-incubation experiments with *Ps*-β-glucan (Table 1). Instead, the cyclic β-glucans from *B. japonicum* wild-type themselves induced the generation of H_2O_2 (Table 1). The reason behind the different biological activities of the *P. sojae* and the *B. japonicum* β-glucan ligands, respectively, is still unknown. It is conceivable that slightly different structures of the β-glucans are responsible for turning on different signal transduction cascades after recognition. Basse et al. [24] demonstrated in tomato cells that a glycopeptide from yeast invertase acted as elicitor but the oligosaccharide moiety of that elicitor alone suppressed the elicited responses. Both molecules, the glycopeptide as well as the oligosaccharide, were ligands of the high-affinity binding site [25]. Results with polygalacturonic acid (PGA) as elicitor can be interpreted in the same way. PGA with a DP = 12 was most active in eliciting phytoalexin accumulation in soybean whereas PGA with a DP = 20 induced only the oxidative burst in soybean cells [26].

The ion channel blockers A9C and NPPB have been used as indirect tools to analyze the role of Ca^{2+} in the elicitation of soybean cells. Both compounds have been described to inhibit anion fluxes in plant cells [9–11]. Treatment of soybean suspension cells with these compounds inhibited typical elicitor-inducible responses such as the stimulation of CHS activity [8]. Extending that preliminary examination we showed that these ion channel blockers inhibited in non-cytotoxic concentrations other *Ps*-β-glucan elicitor-induced processes such as the synthesis of glyceollins (Table 2) or the transient increase of the cytosolic Ca^{2+} concentration (Table 2) with similar IC_{50} values in the micromolar range. Surprisingly, the inducible synthesis of H_2O_2 was not affected up to an inhibitor concentration of 100 μM (Fig. 2) indicating that neither the anion efflux itself, very likely causing membrane depolarization, nor the induced Ca^{2+} influx is necessary for the signal transduction pathway between perception of the *Ps*-β-glucan elicitor and the onset of the oxidative burst in soybean.

However, care must be taken to differentiate between real physiological responses and effects which can be unrelated to the addressed biochemical pathway if pharmacological agents are used to study specific cellular processes by modulating single reactions. The stimulating effect on the transcription of the D6aH gene by A9C and, to a minor extent, NPPB, in soybean cell cultures provides an example of such unexpected side effects because the elevation of transcript levels for the pterocarpan-specific enzyme D6aH by A9C did not coincide with glyceollin production. Thus, these results unambig-

uously demonstrated the necessity to examine different parameters of the elicitor-induced pathways.

Several elicitors of tobacco, including the carbohydrate oligogalacturonic acid, have been described to induce both, the oxidative burst as well as Ca^{2+} influx [23]. However, a peptide elicitor from *Erwinia amylovora*, harpin, exceptionally induced H_2O_2 production but no increase of the cytosolic Ca^{2+} level. These results and the data we obtained in soybean cells with carbohydrate elicitors strongly suggest that the induction of H_2O_2 accumulation is not necessarily depending on an elicitor-induced transient increase of the cytosolic Ca^{2+} concentration at least in some plants.

H_2O_2 can act as a signal upstream of the increase of the cytosolic Ca^{2+} concentration by activating Ca^{2+} channels as it has been demonstrated in guard cells of *Arabidopsis thaliana* [27]. In soybean cells, the *Ps*-β-glucan elicitor-induced transient Ca^{2+} increase was evident about 2 min after treatment and terminated after 20 min (Fig. 1A; [4]). The oxidative burst showed a delayed time course, with the maximum of H_2O_2 accumulation not before 20–30 min after treatment [5]. Therefore, elevated H_2O_2 levels very likely did not precede the cytosolic Ca^{2+} signal in soybean cell cultures as they do in abscisic acid signaling in *A. thaliana*.

We now have provided data for Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways in soybean by two independent lines of evidence. Using anion channel blockers as well as cyclic *Bj*-β-glucans, we presented data indicating for the first time that in soybean cell cultures the *Ps*-β-glucan-induced signaling cascade leading to the rapid production of ROS and the longer lasting phytoalexin response diverges at an early stage of elicitation.

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