Pretreatment with salicylic acid primes parsley cells for enhanced ion transport following elicitation

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Abstract Pretreatment with salicylic acid (SA), an inducer of plant disease resistance, enhanced the capacity of parsley cells for the induction of a rapid K^+/pH response and the subsequent coumarin (phytoalexin) secretion. In SA-primed cells, a low elicitor dose induced these two responses to a similar extent as did a high elicitor dose in non-primed cells. These observations suggest that the SA-mediated augmentation of the early K^+/pH response may contribute to the enhancement of subsequent coumarin secretion. As the amphotericin B-induced K^+/pH response was not enhanced in SA-primed cells, it is concluded that signaling components that are improved by priming are located between elicitor signal perception and the plasma membrane transporters mediating the K^+/pH response.

Key words: Elicitor signal amplification; Ion transport augmentation; Parsley (*Petroselinum crispum*); Priming; Salicylic acid; Systemic acquired resistance

1. Introduction

Upon infection with necrotizing pathogens many plants develop an enhanced resistance to various pathogens both in the tissue surrounding the primary infection site and often also in formerly uninoculated organs [1,2]. This so-called systemic acquired resistance (SAR) requires the endogenous accumulation of salicylic acid (SA; [1,3]). SAR is associated with an enhanced capacity for the quick and effective activation of cellular defense responses that are locally induced once the systemically protected tissue becomes attacked by a pathogen [4,5]. According to the terminology for a similar phenomenon in mammalian monocytes [6], this state of enhanced ability to activate cellular defense responses has been called the 'primed' state of the plant [7]. Though the phenomenon has been known for years, little is known about the molecular and/or biochemical mechanism(s) that mediate(s) priming.

The interaction of cultured parsley cells with a cell wall preparation from *Phytophthora sojae* (the so-called *Pmg* elicitor) and an elicitor-active 13-amino acid oligopeptide (Pep-13) derived from the *Pmg* elicitor, has proven useful for investigating the activation of various cellular plant defense responses [8]. These include rapidly induced alterations in K^+, H^+, Cl^-, and Ca^{2+} transport across the plasma membrane [8,9]. Induction of these ion fluxes is an absolute requirement for the subsequent accumulation of extracellular H_2O_2 (the so-called 'oxidative burst'), the activation of a mitogen-activated protein kinase [10], the expression of a complex set of defense genes and the synthesis and secretion of coumarin phytoalexins and cell wall phenolics [8,11].

Though activation of another prominent cellular defense reaction, the hypersensitive response (HR), is not a feature of the parsley suspension cell/Pep-13 elicitor interaction [8], induction of the early K^+/pH response has often been assumed to also play a major role in pathogen-induced host cell necrosis and hypersensitive cell death [12,13]. For example, the HR of cultured tobacco cells inoculated with incompatible *Pseudomonas syringae* pathovars is preceded by an efflux of K^+ and a concomitant alkalinization of the culture media [14]. Also, tobacco plants that constitutively accumulate bacterio-opsin, a proton pump from *Halobacterium halobium*, displayed spontaneous hypersensitive cell death [15]. And furthermore, the *Arabidopsis defense, no death 1* mutant, which is defective in the HR response to avirulent *P. syringae* pathogens, carries a mutation in a gene encoding a cyclic nucleotide-gated ion channel [16].

Kauss et al. [17], Thulke and Conrath [18] and Katz et al. [7] reported that, though not being suited to elucidate SAR, the parsley cell culture/elicitor model system is useful to study molecular and biochemical aspects associated with priming and the resulting augmentation of cellular defense responses. Pretreating parsley cells with SA or synthetic inducers of SAR, resulted in enhanced elicitation of the oxidative burst [19] and augmented secretion of both cell wall phenolics [20] and coumarin phytoalexins [7,17]. The increase in phytoalexin response was associated with enhanced activity of coumarin biosynthetic enzymes [17] and improved activation of genes encoding enzymes involved in coumarin production [17,18]. So far, however, there is no knowledge about whether the rapid elicitation of ion flux alterations across the plasma membrane is also affected by cell priming. In the present paper, we investigated the influence of pretreatment with SA on the elicitation of the rapidly induced K^+/pH response as a next step toward elucidating the physiology of priming in parsley culture cells.

2. Materials and methods

The parsley (*Petroselinum crispum*) cell culture was grown as described [9,17]. For a given priming experiment on ion fluxes, a 3-day-

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Abbreviations: Pep-13, 13-amino acid oligopeptide elicitor; SA, salicylic acid; SAR, systemic acquired resistance

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old cell culture was split into two halves. One half of the culture was treated with water while the other half was incubated with SA for 24 h (if not stated otherwise). On the fourth day after subculturing, respectively treated cells were washed on a funnel and suspended at a density of 60 mg/ml in 10 mM bis-Tris/Mes buffer (pH 5.6) containing 4% (v/v) B5 growth medium and 3% (w/v) sucrose. 50 ml aliquots of this cell suspension were allowed to adapt in 250 ml Erlenmeyer flasks by shaking for at least 1 h at 120 rpm.

10 ml aliquots of adapted cell suspension in 15 ml beakers were used to continuously monitor, after addition of the Pep-13 oligopeptide elicitor or the polyene antibiotic amphotericin B, changes in external [K^+] and pH value with a K^+ electrode [9] and a conventional glass electrode, respectively. Each electrode was connected to a Methrom 605 pH-Meter and the signals recorded with a Kipp and Zonen BD 41 printer.

For assaying the dose–response relationship of the Pep-13-induced K^+/pH response, 4-day-old parsley cell cultures were harvested, washed, resuspended, adapted and used in the experiments as described above.

For the determination of coumarin secretion, a 3-day-old cell culture was split into two halves. One half was treated with water while the other half was treated with SA. 24 h later, both subcultures were supplemented with the indicated concentrations of Pep-13 or amphotericin B. Coumarin derivatives were extracted from the culture medium 24 h after elicitation and quantified photometrically as described [7].

SA was bought from Sigma, dissolved in water as a 10 mM stock solution (pH 5.8) and stored at 4°C. The Pep-13 oligopeptide elicitor (VWNQPVRGFKVYE) was custom synthesized by Sigma-Genosys, dissolved in water as a 100 μM stock solution and stored at 4°C. Amphotericin B was from Sigma, dissolved as a 20 mM stock solution in dimethyl sulfoxide (DMSO) and stored at −20°C.

All experiments presented in this paper were performed at least three times with similar results.

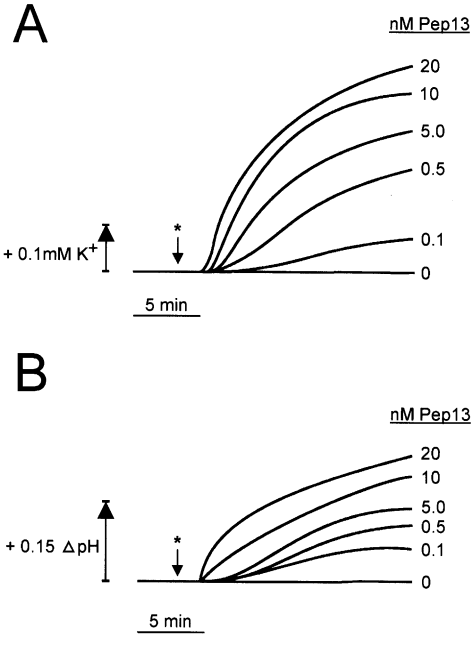

Fig. 1. Dose–response relationship for Pep-13-induced K^+ release (A) and external alkalinization (B) in routinely grown parsley cell cultures. 4-day-old cells were washed and suspended in diluted B5 growth medium. After adaptation, 10 ml aliquots of cell suspension were supplied with the indicated concentrations of Pep-13 (asterisks). Changes in external [K^+] (A) and pH value (B) were continuously monitored with a K^+ electrode and a conventional glass electrode, respectively.

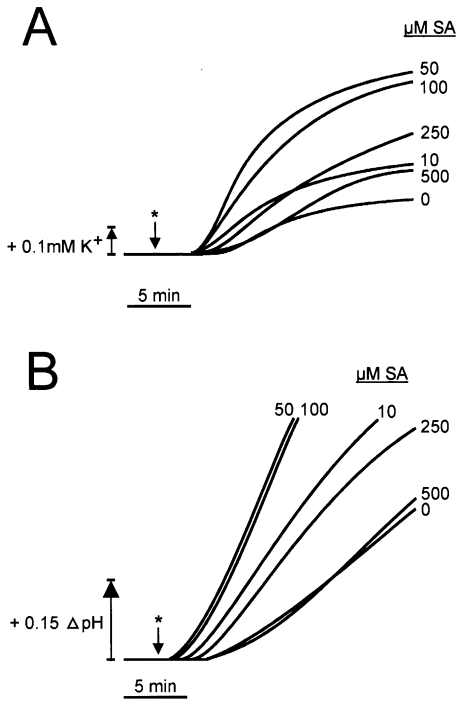

Fig. 2. Potentiation of the Pep-13-induced K^+/pH response after pretreatment with SA. 3-day-old parsley cell cultures were incubated in the absence (0 μM SA) or presence of the indicated concentrations of SA (A,B). 24 h later, the cells were washed, suspended and adapted in diluted growth medium. The resulting cell suspensions were treated with 0.5 nM Pep-13 (asterisks) and K^+efflux (A) and external alkalinization (B) assayed with respective electrodes.

3. Results

3.1. Dose–response relationship of Pep-13-induced K^+ release and external alkalinization

As a first step toward investigating the possible influence of priming with SA on the elicitation of early ion fluxes in routinely grown parsley cell cultures, the dose–response relationship for the Pep-13-induced K^+/pH response was determined. As is shown in Fig. 1, the Pep-13 oligopeptide caused a slight induction of both these responses already at 0.1 nM. The K^+/pH response was about half maximally induced at 0.5 nM Pep-13 and was highly induced at 10 and 20 nM Pep-13 elicitor (Fig. 1).

3.2. SA pretreatment augmented the elicitation of the K^+/pH response

To assay whether priming of cultured parsley cells with SA enhances their Pep-13-induced K^+/pH response, the parsley cell culture was pretreated with water or various SA concentrations before analysis of K^+ efflux and external alkalinization after addition of 0.5 nM Pep-13, representing a sub-optimal elicitor dose (Fig. 1). The endogenous level of free, biologically active SA has previously been calculated to be about 40 μM at the 24 h time-point post-addition of 250 μM SA to the parsley cell culture [18]. This value is consistent with the concentrations of SA found in leaves of induced plants [21].

As shown in Fig. 2, preincubating parsley cells with SA enhanced the K^+/pH response elicited by 0.5 nM Pep-13. The augmentation by SA was already obvious upon preincu-

bation with 10 μM SA and was most pronounced at 50–100 μM SA during preincubation (Fig. 2). At higher SA doses, the potentiation of the K^+/pH response was less prominent, and it was almost gone when the cells had been pretreated with 500 μM SA (Fig. 2). The reason for the decrease in potentiation of the K^+/pH response at the higher SA concentrations (250 μM and 500 μM SA) remains unclear. As there was no increase in the percentage of Evan's Blue positive cells in respectively treated suspension cultures (data not shown), enhanced cell death can be excluded as a cause of this phenomenon.

3.3. Augmentation by SA was best at low Pep-13 concentrations

To elucidate whether the pretreatment of parsley cells with SA also enhances their K^+/pH response to other Pep-13 concentrations, cell cultures pretreated with water or SA were supplied with various Pep-13 doses and then assayed for the K^+/pH response. The result of this experiment (Fig. 3) demonstrates that the augmentation of the K^+/pH response by pretreatment with SA was most striking at low Pep-13 doses (0.1–0.5 nM). At a higher elicitor concentration (5.0 nM Pep-13), the SA-induced enhancement of K^+ release and external alkalinization was not clearly seen anymore (Fig. 3).

3.4. Augmentation of the elicited K^+/pH response correlates with enhancement of coumarin secretion

The *Pmg* (Pep-13) elicitor-induced rapid alterations in ion transport across the plasma membrane are an absolute requirement for subsequent activation of various defense genes

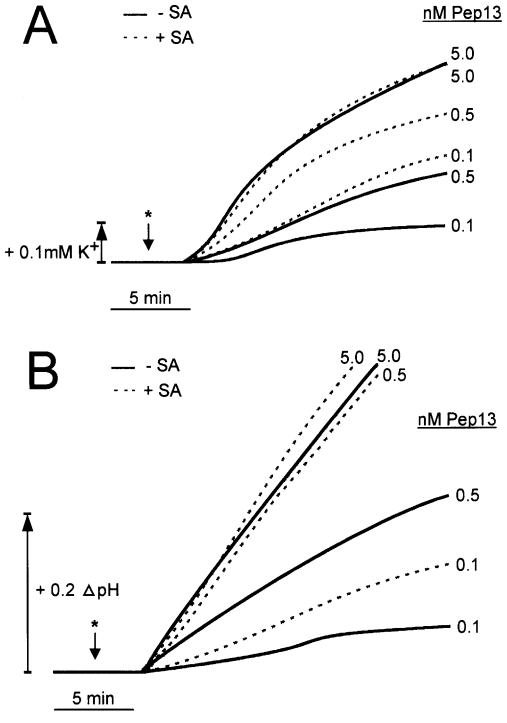

Fig. 3. K^+/pH response induced by various Pep-13 concentrations in SA-primed and non-primed parsley cells. 3-day-old cell suspensions were incubated in the absence (solid lines) or presence (dashed lines) of 100 μM SA. 24 h later, aliquots of cells were washed, suspended and adapted in diluted growth medium. The resulting cell suspensions were supplied with the indicated concentrations of Pep-13 (asterisks) and K^+ efflux (A) and external alkalinization (B) were monitored.

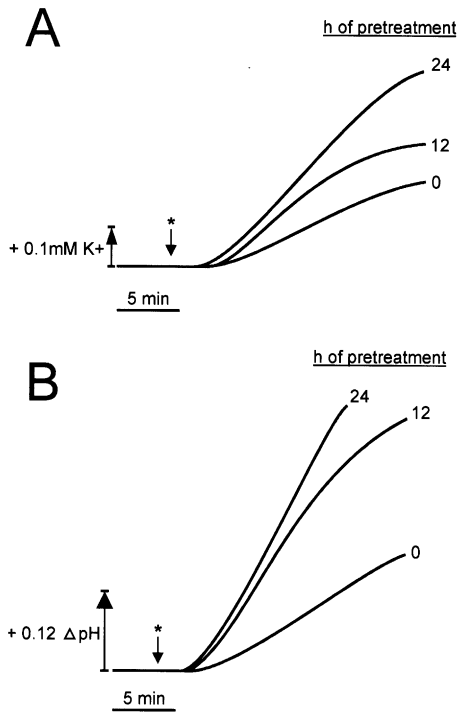

Fig. 4. Length of SA preincubation determines the extent of potentiation of the K^+/pH response. 4-day-old parsley cells were supplied with 100 μM SA and either immediately washed, suspended and adapted in diluted growth medium (0 h) or pretreated with 100 μM SA for 12 h and 24 h before washing, suspending and adapting in diluted growth medium. After addition of 0.5 nM Pep-13 (asterisks), K^+ release (A) and external alkalinization (B) were continuously monitored with respective electrodes (solid lines).

and the synthesis and secretion of antimicrobial coumarins [8,11]. This suggested to us that the potentiation of early ion flux alterations (Figs. 2 and 3) might be related to the observed augmentation of coumarin secretion [7,17]. If this were true, one could expect that the potentiation of elicited coumarin secretion in primed cells were also more pronounced at low Pep-13 concentrations than it were at high concentrations of the elicitor.

To address this issue, aliquots of the same cell culture as for Fig. 3 were incubated in the absence or presence of 100 μM SA. 24 h later, the suspensions were treated with various concentrations of Pep-13 and assayed for coumarin secretion after 24 h. As is shown in Table 1, the SA-mediated potentiation of Pep-13-induced coumarin secretion was high at 0.1 and 0.5 nM Pep-13 (56-fold and 27-fold, respectively), while

Table 1
Potentiation of coumarin secretion induced by various Pep-13 concentrations in SA-primed and non-primed parsley culture cells

Pep-13 (nM)	Coumarin secretion (nmol/ml)		Potentiation (-fold)
	−SA	+SA	
0.1	3	168	56
0.5	7	189	27
5.0	149	205	1.4

An aliquot of the same 3-day-old cell culture as for Fig. 3 was incubated in the absence (−SA) or presence (+SA) of 100 μM SA. 24 h later, aliquots of cells were treated, in their original growth medium, with the indicated concentrations of Pep-13 elicitor and assayed for coumarin secretion after 24 h.

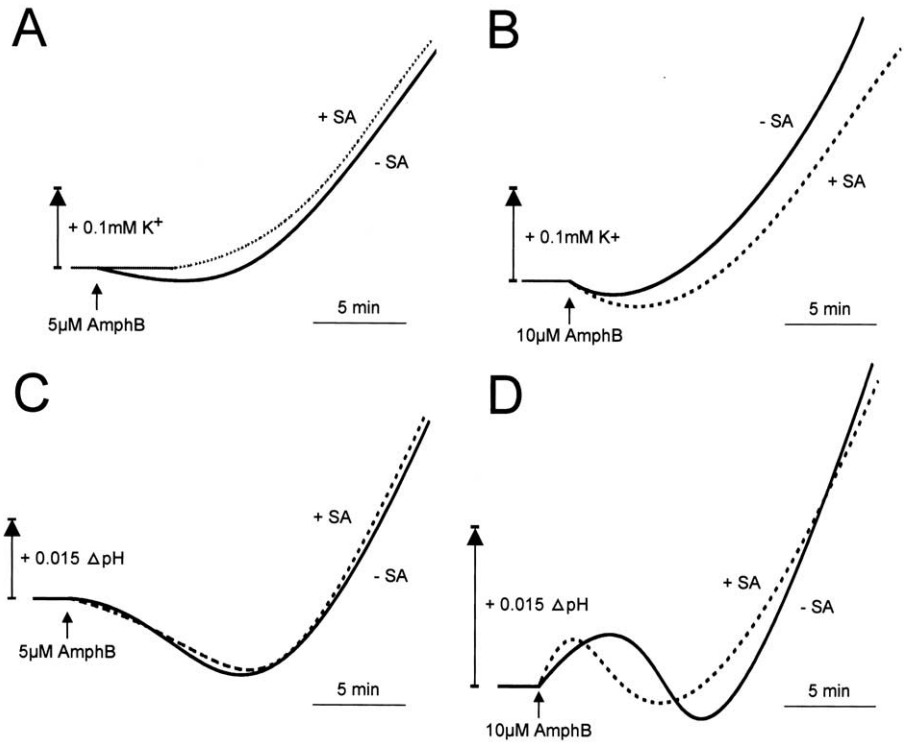

Fig. 5. Priming with SA does not affect the amphotericin B-induced K^+/pH response. Parsley cell cultures (3-day-old) were treated with water (solid lines) or 100 μM SA (broken lines) for 1 day. Subsequently, aliquots of the cells were washed, suspended and adapted in diluted growth medium. The resulting cell suspensions were supplied with amphotericin B (AmphB) at 5 μM (A,C) or 10 μM (B,D). K^+ efflux (A,B) and external alkalinization (C,D) were continuously assayed with respective electrodes. The final concentration of DMSO in the cell suspensions was 0.05% (v/v). At this concentration, DMSO had no effect on the K^+/pH response (data not shown).

it was only low (1.4-fold) when Pep-13 was used at 5.0 nM (Table 1). Thus, the potentiation of the K^+/pH response in primed parsley cell cultures is likely related to the enhancement of coumarin secretion in these cells.

3.5. Augmentation by SA of the K^+/pH response requires a preincubation period

SA may enhance the elicited K^+/pH response as a result of a synergistic action due to the simultaneous presence of SA and the elicitor or due to mediating priming in a time-dependent manner. Therefore, parsley cell cultures were pretreated with SA for only a few seconds (0 h), or for 12 h or 24 h, before addition of a low dose of Pep-13 and subsequent analysis of the K^+/pH response. Fig. 4 demonstrates that the enhancement of the K^+/pH response was best after 24 h of SA pretreatment and could also be detected, though to a lower degree, upon a 12-h preincubation period (Fig. 4).

3.6. Priming does not affect the amphotericin B-induced K^+/pH response

Next we investigated whether priming also augments the K^+/pH response when this reaction was induced by the polyene antibiotic amphotericin B which mimics elicitor-induced ion fluxes and induces coumarin secretion in cultured parsley cells [11]. As is shown in Fig. 5, amphotericin B was active at the induction of the K^+/pH response in both SA-primed and non-primed parsley cells. However, there was essentially no difference in the extent of the amphotericin B-induced K^+/pH response between SA-primed and non-primed parsley cells (Fig. 5).

4. Discussion

In the present paper we demonstrated that cultured parsley cells primed with SA have an enhanced capacity for the elicitor activation of certain ion fluxes (Figs. 2–4), the earliest events detectable upon elicitation of the cells [8,9,11]. Since Pep-13, even at high concentrations, does not induce the production of SA in cultured parsley cells (data not shown), we can exclude the possibility that pretreatment of the cells with SA allows a critical SA level to be reached, thus leading to augmented activation of the K^+/pH and coumarin responses upon further stimulation with Pep-13. Moreover, there was a close, though not quantitative, correlation between the appearance of potentiation of the elicited K^+/pH response and augmentation of the subsequent coumarin secretion in primed parsley cells (Fig. 3; Table 1). Therefore, the enhancement of the elicited K^+/pH response (Fig. 3) might contribute to the observed potentiation of coumarin secretion (Table 1; [7,17]). Since the latter (Table 1) was much more drastic than the enhancement of the K^+/pH response (Fig. 3), it is obvious that there is additional amplification of the Pep-13 elicitor signal within primed cells. This may explain why, although being low at the K^+/pH response (Fig. 3), there still is some potentiation of coumarin secretion when the cells were challenged with Pep-13 at 0.5 nM (Table 1).

The above conclusion that the enhancement of the K^+/pH response might contribute to the potentiation of coumarin secretion is consistent with the finding that in non-primed cells, induction of the early ion fluxes is a key regulatory step in defense gene activation and coumarin secretion [11].

As mentioned above (see Section 1), activation of the HR is not a feature of the parsley cell suspension/Pep-13 interaction [8]. However, in other model systems, SA-mediated potentiation of the K^+/pH response might causally be related to improved elicitation also of the HR response. This conclusion is supported by the finding that physiological concentrations of SA, in addition to defense gene activation and H_2O_2 accumulation, also enhanced hypersensitive cell death induced by an avirulent strain of *P. syringae* pv. *glycinea* in soybean culture cells [22].

Amphotericin B is assumed to interact with sterols and the lipophilic part of phospholipids in cell membranes and, thus, induces ion flux alterations independently of complementary binding sites of specific receptor molecules [23,24]. Our finding that the amphotericin B-induced K^+/pH (Fig. 5) and coumarin (data not shown) responses have not been augmented in SA-primed cells suggests that key regulatory components of the priming mechanism for improved induction of this response are located between the elicitor signal perception step and the induction of the K^+/pH response. As the enhancement of the K^+/pH and coumarin responses is maximal at non-saturating Pep-13 concentrations (Fig. 3; Table 1) it is unlikely that the augmentation of these two responses is due to increased presence of Pep-13 elicitor receptor molecules in the plasma membrane of primed cells.

The augmentation by SA-pretreatment of elicited K^+ release, external alkalinization and coumarin secretion increased with the length of SA-preincubation (Fig. 4; [7,17]). In accordance to earlier conclusions [7,17,18] we assume that SA, in a time-dependent process, increases the synthesis of one or more signal transduction components that shift the cells to an alerted state which allows better activation of the K^+/pH and coumarin responses once the cells are treated with Pep-13. The above proposed signaling components that are located between elicitor signal perception and the K^+/pH response as well as a SA-induced, but until elicitation inactive, mitogen-activated protein kinase (data not shown) are prime candidates for such a cellular component.

Treatment of parsley cells with SA or some of its biologically active, or inactive, analogs does not directly induce the K^+/pH response [25] or various subsequently elicited defense reactions [7,17–20]. Rather, a pretreatment with SA or its functional analog 5-chloro-SA primes the cells for better elicitation of various defense mechanisms [7,17–20], including activation of the K^+/pH response (Figs. 2–4; [25]), while preincubation with the biologically inactive SA analog 3-hydroxybenzoic acid was inactive at augmenting the K^+/pH [25] and subsequently elicited defense responses [7,18]. Thus, there is a strong correlation between the ability of SA analogs to prime parsley cells for an augmented K^+/pH response and their capability to enhance subsequently induced cellular defense responses in these cells. When extrapolating these results to whole plants that are able to develop SA-dependent SAR, SA and its functional analogs might enhance their disease resistance by improving the ability to induce various cellular defense responses that occur once the pathogen is recognized by the plant. Reports on the stronger induction of pathogen-activated defense responses in whole tobacco [5] and *Arabidopsis* [4,26] plants preincubated with inducers of plant disease resistance support this conclusion.

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