Salicylic Acid Glucoside Acts as a Slow Inducer of Oxidative Burst in Tobacco Suspension Culture

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Salicylic acid β-glucoside (SAG) is a storage form of a defense signal against pathogens, releasing free salicylic acid (SA), to meet the requirements in plants. Since excess SA induces locally restricted cell death following oxidative burst and Ca^{2+} influx in plants, the effects of SAG on cell viability, Ca^{2+} influx, and generation of superoxide ($O_2^{\bullet-}$) were examined in suspension-cultured tobacco BY-2 cells expressing aequorin. Among SA-related chemicals tested, only SAG induced the slow and long-lasting $O_2^{\bullet-}$ generation, although SAG was less active in acute $O_2^{\bullet-}$ generation, Ca^{2+} influx and induction of cell death. The prolonging action of SAG is likely due to gradual release of SA and the data suggested that a peroxidase-dependent reaction is involved. Notably, pretreatment with low-dose SA (50 μM) enhanced the response to SAG by 2.5-fold. There are four possible secondary messengers in early SA signaling detectable in the BY-2 culture, namely $O_2^{\bullet-}$, H_2O_2, Ca^{2+} and protein kinase (PK). If these messengers are involved in the low-dose SA-dependent priming for SAG response, they should be inducible by low-dose SA. Among the four SA-inducible signaling events, PK activation was excluded from the low-dose SA action since a much higher SA dose (> 0.4 mM) was required for PK activation.

Key words: Priming, Salicylic Acid, Salicylic Acid β-Glucoside

Introduction

Salicylic acid (SA) has been claimed to be essentially involved in development of systemic acquired resistance (SAR) against various pathogens with various natures (Gaffney *et al.*, 1993). A certain number of reports has indicated that Ca^{2+} is essential for the action of SA during plant defense, since Ca^{2+} plays key roles as a secondary messenger for certain processes in plant defense mechanisms (Knight *et al.*, 1991; Sanders *et al.*, 1999). For instance, removal of Ca^{2+} or blockade of Ca^{2+} signaling inhibits the induction of chitinase accumulation by SA in tobacco leaves (Raz and Fluhr, 1992) and carrot cell culture (Schneider-Müller *et al.*, 1994). It has been proposed that SA signal transduction leading to SAR may be mediated by reactive oxygen species (ROS) derived from H_2O_2, since SA binds and inhibits catalase (Chen *et al.*, 1993). While inhibition of ROS-degrading enzymes by SA is a passive mechanism supporting the increases in ROS, the active mechanism directly generating ROS in response to SA has not been revealed until recently.

The first report on successful detections of SA-induced ROS and the SA-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), directly connecting the action of ROS and Ca^{2+} signaling, has been documented by us, using the aequorin-expressing tobacco cells (Kawano *et al.*, 1998). It has been shown that SA induces the generation of superoxide ($O_2^{\bullet-}$) detected with a specific chemiluminescence (CL), and in turn $O_2^{\bullet-}$ triggers the influx of Ca^{2+} into the cells (detected with aequorin luminescence). Our previous works (Kawano *et al.*, 1998; Kawano and Muto, 2000) have shown that the SA-induced extracellularly occurring generation of $O_2^{\bullet-}$ is catalyzed by extracellularly localized peroxidase. The mechanism of plant peroxidase actions for SA-induced oxidative bursts has been exposed to intensive studies both in tobacco cell culture and *in vitro* enzyme preparations, with CL (Kawano *et al.*, 1998; Kawano and

Muto, 2000), electron spin resonance (Kawano and Muto, 2000), and spectroscopic analysis of enzyme oxidation states (Kawano, 2003). The mechanism for SA-dependent early ROS production is solely depending on the action of peroxidase thus independent from the well documented ROS-generating system involving NADPH oxidase gp91^{phox} homologs (Yoshioka *et al.*, 2003), although SA eventually induces NADPH oxidase in the late stages of SA action (Yoshioka *et al.*, 2001). The SA-induced peroxidase-dependent $O_2^{\bullet-}$ generation was also observed in *Vicia faba* epidermis during induction of stomatal closure which is a Ca^{2+}-dependent process (Mori *et al.*, 2001).

SA presents mainly as SA β-glucoside (SAG; 2-*O*-β-D-glucosylbenzoic acid) in plants (Klick and Herrmann, 1988). Enyedi *et al.* (1992) and Malamy *et al.* (1992) have reported that SAG increases in parallel with free SA in the tobacco leaves that had acquired resistance to infection by TMV. SAG contents in potato leaves and cell culture are reportedly elevated following interaction with *Phytophthora infestans* and *P. infestans*-derived elicitors (Keller *et al.*, 1996). Above reports are indicative of a possible role for SAG in plant defense mechanism. Both endogenous and exogenously applied SAG are hydrolyzed and yield SA, and SAG is likely active only after hydrolysis to SA in the induction of PR-1 gene in tobacco leaves (Hennig *et al.*, 1993). Thus SAG seems to be the storage form from which physiologically active free SA is released when necessary (Seo *et al.*, 1995). Consistently, we have previously reported that SAG is less active than free SA in the immediate action inducing $O_2^{\bullet-}$ generation and $[Ca^{2+}]_c$ elevation in tobacco cell culture (Kawano *et al.*, 1998). It has been shown that the hydrolysis of SAG in tobacco leaves occurs in the extracellular spaces (Hennig *et al.*, 1993) where considerable activities of SA β-glucosidase were found (Seo *et al.*, 1995), despite the formation of SAG from SA catalyzed by SA 3-*O*-glucosyltransferase occurs inside the cells (Hennig *et al.*, 1993). Taken together, it is most likely that SAG is excreted to the intercellular space where the SAG-degrading and ROS-generating enzymes are abundant, thus free SA could be released from SAG, and utilized for generation of $O_2^{\bullet-}$.

As previously shown, the induction of oxidative burst by injections of SA and related compounds was only transient lasting only up to 1 min (Kawano *et al.*, 1998). However, in nature, it is likely that SAG may function as a slow SA-releasing agent enabling the sustainable, prolonging oxidative action of SA. In this study, the effect of SAG on $O_2^{\bullet-}$ generation, Ca^{2+} signaling and cell viability were examined in tobacco BY-2 cell suspension culture. The effect of SA pretreatment on the level of SAG-inducible oxidative burst was also examined.

Materials and Methods

Plant material

Tobacco suspension culture cells (*Nicotiana tabacum* L. cv. Bright Yellow-2, cell line BY-2), which express apoaequorin specifically in the cytosol (Takahashi *et al.*, 1997), were used 3 d after subculturing. Cells were propagated as described (Kawano *et al.*, 1998).

Chemicals

SA, 3-hydroxybenzoic acid (3-HBA), 4-hydroxybenzoic acid (4-HBA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA), catechol, and luminol were obtained from Wako Pure Chemical Industries (Osaka, Japan). Salicylhydroxamic acid (SHAM) and fluorescein diacetate were from Sigma (St. Louis, MO, USA). 2-*O*-β-D-Glucosylbenzoic acid (SAG), 3-*O*-β-D-glucosylbenzoic acid (3-HBAGlc), 4-*O*-β-D-glucosylbenzoic acid (4-HBAGlc), SA glucose ester and 4-HBA glucose ester were prepared as described (Umetani *et al.*, 1990; Tanaka *et al.*, 1990). Chemically synthesized coelenterazine was a generous gift from Prof. M. Isobe, Nagoya University. A *Cypridina* luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

SA was dissolved in ethanol and diluted with water to appropriate concentrations keeping the ethanol concentration at 2% (v/v). To the cell suspension, 1/20 volumes of chemical solutions were injected with a microsyringe (final ethanol fraction, 0.1%).

Monitoring of ROS and $[Ca^{2+}]_c$

Generation of $O_2^{\bullet-}$ and $[Ca^{2+}]_c$ elevation were monitored by $O_2^{\bullet-}$-specific CL of CLA and the Ca^{2+}-dependent luminescence of aequorin, respectively, as described (Kawano *et al.*, 1998). CLA-

CL specifically indicates the generation of $O_2^{\bullet -}$, and of singlet oxygen ($^1O_2$) to a lesser extent, but not of O_3, H_2O_2 or hydroxyl radicals (Nakano *et al.*, 1986). The signal for $^1O_2$ can be minimized by avoiding the use of high concentration of organic solvents such as ethanol not exceeding 2% (v/v), in the reaction mixture or culture media (Yokawa *et al.*, 2004). Thus the induced CLA-CL increases recorded in the normally cultured tobacco cell suspension must be representing the generation of $O_2^{\bullet -}$, as previously examined (Kawano *et al.*, 1998). H_2O_2 production was monitored by the luminol-dependent CL under pH controlled conditions (Kawano and Muto, 2000). The concentration of $O_2^{\bullet -}$ and H_2O_2 were estimated using calibration curves for KO_2-dependent CLA-CL and H_2O_2-dependent luminol-CL. Though luminol requires peroxidase for reporting the presence of H_2O_2, no peroxidase was added, since the BY-2 tobacco culture is rich in endogenous cellular, cell wall-bound and secreted free peroxidases (Kawano *et al.*, 1998).

To monitor $[Ca^{2+}]_c$ in the transgenic BY-2 cells, the apo-aequorin-expressing cells were incubated with 1 μM coelenterizine in darkness for 8 h to reconstitute the Ca^{2+}-responsive luminescence protein aequorin. Luminescence reflecting the increase in $[Ca^{2+}]_c$ induced by SA-derivatives was measured with a CHEM-GLOW Photometer (American Instrument Co., Md., USA) equipped with a pen recorder as previously described (Kawano *et al.*, 1998).

Cell viability test

The effect of SA and related chemicals on induction of cell death in tobacco cell culture was examined as reported previously (Kawano and Muto, 1998). After various treatments, 20 μl of 0.5% (w/v) fluorescein diacetate in acetone was added to 1 ml-aliquots of cell suspension. After incubation for 20 min at 28 °C, the cells were washed twice with fresh culture medium, and stained (viable) cells were counted under a fluorescence microscope. For each assay more than 100 cells were counted but actual number of cells counted on the slides varied from sample to sample in a range from 100 to 300 cells depending on the preparations. This assay was repeated 3 times using different cultures. A typical result from 3 replications with identical tendency was presented. As previously reported (Kawano *et al.*, 1998), the results expressed as % of live cells (stained cells) were calculated from a single set of assay which is typical and representative.

Protein kinase (PK) assay

An in-gel PK assay was carried out as previously reported (Yuasa and Muto, 1996), with minor modifications (Takahashi *et al.*, 1997). The tobacco cells were subjected to treatment with SA or H_2O_2 for 5 min and killed by addition of ¼ volume of chilled 50% (v/v) trichloroacetic acid, and kept on ice for 30 min. After centrifugation (15,000 × *g*, 15 min, 4 °C), the pellets were re-suspended and sonicated in 80% acetone and centrifuged again (repeated twice). The pellets were solubilized in SDS-PAGE sample buffer and exposed to SDS-PAGE using the assay gels (10% polyacrylamide containing 0.2 mg/ml of myelin basic protein). The gels were washed successively with 20 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol. Proteins were de-natured by shaking the gels in the washing buffer containing 6 M guanidine-HCl. In order to re-nature the PK, the gels were gently shaken in the washing buffer containing 0.05% Tween-20 at 4 °C for 15–20 h. After equilibrating with 20 mM Hepes-KOH (pH 7.6) containing 10 mM $MgCl_2$, 1 mM ethyleneglycol-bis-(β-aminoethylether) *N*,*N*′-tetraacetic acid (EGTA) and 1 mM dithiothreitol at room temperature for 30 min, the gels were further incubated in this reaction buffer supplemented with 25 μM [γ-^{32}P] ATP at 25 °C for 1 h. The reaction was terminated by transferring the gels into 5% (w/v) trichloroacetic acid with 1% (w/v) $Na_4P_2O_7 \cdot 10\ H_2O$. The gels were successively washed with the same solution and un-reacted ATP was removed. The gels were dried and the radioactive bands on the gels were visualized by a Bioimaging Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo, Japan).

Results and Discussion

Prolonging $O_2^{\bullet -}$ generation induced by SAG

Both free SA and SAG induced the generation of $O_2^{\bullet -}$ in tobacco cells (Fig. 1A). The peak height of $O_2^{\bullet -}$-dependent CLA-CL was much higher in the free SA-treated cells than in that of SAG-treated cells, but the oxidative burst induced by SAG lasted for a notably longer period. The SAG-induced $O_2^{\bullet -}$ generation was a biphasic event composed of an immediate sharp peak and a secondary gently-sloping peak tailing for many min-

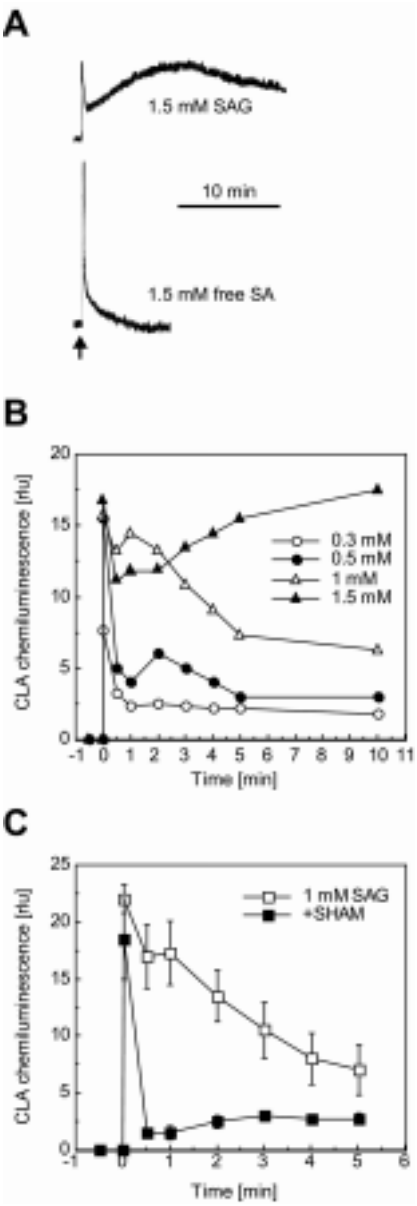

Fig. 1. SAG-induced $O_2^{\bullet-}$ generation in tobacco cell suspension culture. (A) Typical traces recorded for $O_2^{\bullet-}$ generation induce by 1.5 mM SAG (upper) and SA (lower). (B) Effects of SAG concentration on $O_2^{\bullet-}$ generation. (C) Effect of a peroxidase inhibitor, SHAM (5 mM) on SAG (1 mM)-induced $O_2^{\bullet-}$ generation. The indicated concentration of SAG was injected at zero time. Vertical bars represent SE (n = 4). rlu represents relative luminescence unit. CLA, *Cypridina* luciferin analog.

utes. The prolonging action of SAG became obvious when the concentration of added SAG was elevated to 1.5 mM (Fig. 1A, B). The $O_2^{\bullet-}$ generation showed a gradual long-lasting increase after the first immediate peak and the CL lasted as long as 20 min. Such biphasic $O_2^{\bullet-}$ generation is not induced by addition of 0.1–2.0 mM free SA (Kawano *et al.*, 1998).

It has been shown that the SA-induced generation of $O_2^{\bullet-}$ is sensitive to peroxidase inhibitors such as SHAM, as examined in tissue (Mori *et al.*, 2001), cell suspension (Kawano *et al.*, 1998), cell wall preparation (Kawano *et al.*, 1998), and horseradish peroxidase reaction mixture (Kawano and Muto, 2000). Here, we observed that the SAG-induced $O_2^{\bullet-}$ generation, especially the prolonging phase, was highly sensitive to SHAM, suggesting that SAG action on prolonging oxidative burst is due to gradual release of SA and peroxidase-dependent reaction (Fig. 1C).

According to calibration using KO_2, the level of $O_2^{\bullet-}$ induced by 1.5 mM SAG reached *ca.* 0.6 μM and was maintained at similar level for about 20 min. However, H_2O_2 production did not attain the detectable level using luminol (data not shown).

SAG is a safer and effective signal inducer

We compared the effects of SA and SAG on induction of $O_2^{\bullet-}$ generation, $[Ca^{2+}]_c$ elevation and cell death in tobacco cells (Fig. 2). For comparison, effects of structurally similar hydroxybenzoates and their glucose-conjugates were tested. The chemicals tested include SA, SAG, SA glucose ester, 3-HBA, 3-HBAGlc, 4-HBA, 4-HBAGlc, 4-HBA glucose ester, 2,3-DHBA, 2,5-DHBA, 2,6-DHBA and catechol. Catechol, 2,3-DHBA and 2,5-DHBA are well known metabolites of SA after reaction with hydroxyl radicals (Grootveld and Halliwell, 1986). 2,6-DHBA is a SA analog active in induction of SA 3-*O*-glucosyltransferase in tobacco leaves (Enyedi and Raskin, 1993).

Among the chemicals tested (0.5 mM each), SA and three DHBAs were highly active for inducing the acute generation of $O_2^{\bullet-}$ (Fig. 2A). SA and 2,3-DHBA were shown to be active in induction of $[Ca^{2+}]_c$ elevation (Fig. 2B). When the toxicity of the chemicals was examined by fluorescein diacetate staining (Fig. 2C), more than half the population was killed within 12 h in the cells treated with 0.5 mM of 2,3-DHBA (78.9% dead), 2,5-DHBA

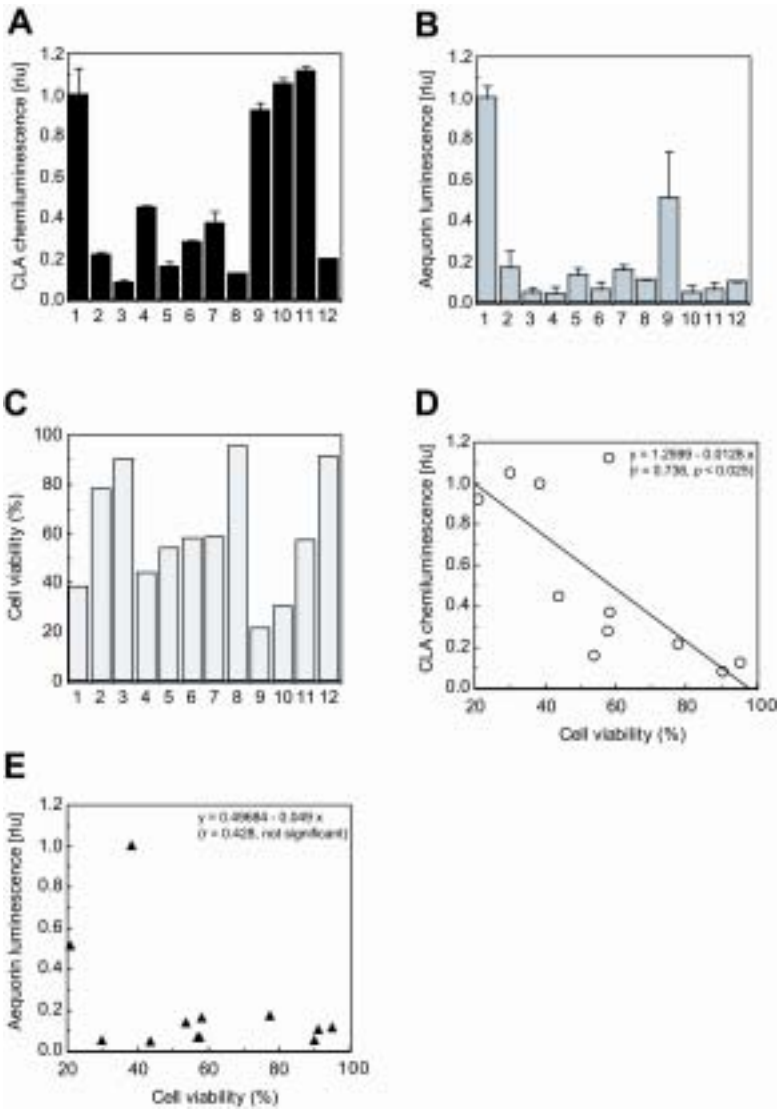

Fig. 2. Induction of $O_2^{\bullet -}$ generation, $[Ca^{2+}]_c$ elevation, and cell death in tobacco cells treated with SA-related chemicals. (A) Induction of $O_2^{\bullet -}$ generation. (B) Induction of $[Ca^{2+}]_c$ elevation. (C) Induction of cell death. (D) Relationship between $O_2^{\bullet -}$ generation and cell death. (E) Relationship between $[Ca^{2+}]_c$ elevation and cell death. The chemicals used were (1) SA, (2) SAG, (3) SA glucose ester, (4) 3-HBA, (5) 3-HBAGlc, (6) 4-HBA, (7) 4-HBAGlc, (8) 4-HBA glucose ester, (9) 2,3-DHBA, (10) 2,5-DHBA, (11) 2,6-DHBA, and (12) catechol. Vertical bars represent SE (n = 4). rlu represents relative luminescence unit.

(70.0% dead), SA (61.7% dead), and 3-HBA (56.2% dead). SAG was shown to be less active in induction of acute oxidative burst, Ca^{2+} influx, and cell death. However, it is noteworthy that SAG was the only compound inducing the prolonging oxidative burst. Although only SAG among the chemicals tested induced the prolonging oxidative bursts, SAG was less toxic to the cells (9.9% dead). For unknown reason, 2,5-DHBA and 2,6-DHBA were active only in induction of $O_2^{\bullet -}$, but not in induction of Ca^{2+} influx and cell death.

To our knowledge, SA is an inducer of $O_2^{\bullet -}$ generation, Ca^{2+} influx and hypersensitive cell death (Kawano *et al.*, 1998). To elucidate the key factor involved in the induced cell death, the relationships between the induced cell death and the two cellular events preceding the cell death, namely acute oxidative burst (Fig. 2D) and Ca^{2+} influx (Fig. 2E), were statistically analyzed by the least square method. The results clearly showed that the induction of $[Ca^{2+}]_c$ elevation and cell death have no significant relationship (r = 0.428). On the other hand, the relationship between the level of acute $O_2^{\bullet -}$ generation and cell death is highly significant (r = 0.736, $p < 0.025$). It is conclusive that acute induction of oxidative burst is a

key event required for the cell death, while elevation of $[Ca^{2+}]_c$ is not required. We propose a model that excess of ROS produced in response to SA triggers the cell death, while a moderate level of ROS induced by lower SA concentrations or SA analogs with lower activity stimulates the influx of Ca^{2+} without damaging the cells. On the other hand, SAG induces the prolonging low-level oxidative burst without leading to cell death. In nature, SAG may be a safer inducer of oxidative burst without causing the cell death.

Priming effect of low-dose SA

We tested the effect of low-dose SA pretreatment on the SAG-induced oxidative burst (Fig. 3A, B). Treatment with 50 μM SA resulted in a detectable level of $O_2^{\bullet-}$ generation and $[Ca^{2+}]_c$ elevation lasting for *ca.* 0.5–1 min, respectively, as previously reported. After incubating the cell suspension with 50 μM SA over 30 min, the cells were treated with 0.5 mM SAG. While the level of $O_2^{\bullet-}$ generation induced by 0.5 mM SAG in the control cells without any pretreatment was as low as ¼ of the free SA treatment, the SA-pretreated cells showed enhanced response to SAG which was 2.5-fold larger than that of non-pretreated cells. With best SA-pretreatment timing, the SAG-induced $O_2^{\bullet-}$ generation attained the level almost identical to that induced by free SA (Fig. 3B).

SAR is associated with a process called "priming", inducing cellular defense responses more rapidly and to a greater extent than in noninduced plants (Kohler *et al.*, 2002). According to the terminology for a similar phenomenon in mammalian monocytes (Hayes and Zoon, 1993), the enhanced ability to activate defense responses in plant cells is also named "priming" (Katz *et al.*, 1998). Although the pathogenesis-related (PR) proteins and their possible roles in SAR have been the object of thorough researches, the biochemical and genetic basis of priming remain largely unclear (Kohler *et al.*, 2002). According to Kauss *et al.* (1992), priming induction in parsley cells for stronger but low-dose elicitation of various cellular defense responses, by pre-incubation with SA, 2,6-dichloroisonicotinic acid, and benzothiadiazole, is a strictly time-dependent process. In this study, the SA-induced enhancement in SAG-dependent prolonging $O_2^{\bullet-}$ generation was also shown to be a time-dependent process, showing optimal enhancement within 30 min (Fig. 3).

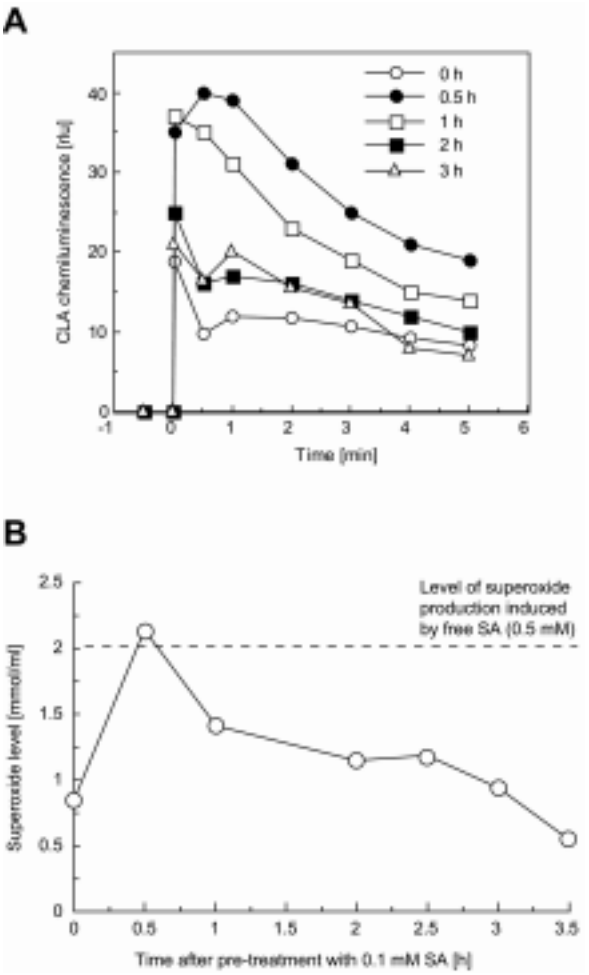

Fig. 3. Effect of SA-pretreatment on SAG-induced generation of $O_2^{\bullet-}$. (A) Typical records of SAG-induced generation of $O_2^{\bullet-}$ in the cell suspension culture pretreated with 50 μM SA (time-course). (B) Timing effect of SA-pretreatment (50 μM) on the level of $O_2^{\bullet-}$ generation induced by 0.5 mM SAG.

We observed that responses to other inert hydroxybenzoate-glucose conjugates such as 3-HBAGlc, 4-HBAGlc, SA glucose ester and 4-HBA glucose ester were not enhanced by the SA pretreatment (data not shown), thus the SA-induced priming mechanism is specific for SAG among hydroxybenzoate-related glucosides and glucose esters, supporting the possible involvement of SAG-specific enzyme (most likely SA β-glucosidase) in SA-induced priming for SAG response. Seo *et al.* (1995) have shown that treatment of tobacco leaves with low-dose SA results in a sharp increase

in extracellular SA β-glucosidase activity that releases more SA from extracellularly stored SAG within 1 h.

Possible signaling path mediating the low-dose SA-induced priming

There are two possible signaling pathways stimulated by low-dose SA leading to sensitization of the cells to SAG. The first likely path involves the production of $O_2^{\bullet -}$ followed by production of H_2O_2 and influx of Ca^{2+}. The second likely path is rather free from ROS, but involves the activation of PKs. SA is known to activate PKs such as a 48-kDa MAP kinase called SIPK (SA-induced protein kinase; Zhang and Klessig, 1997) or PMSAPK (plant multisignal-activated protein kinase; Usami *et al.*, 1995; Usami and Machida, 1997). SA-dependent phosphorylation events are reportedly independent from the action of ROS (Rao *et al.*, 1997) and the activation of PMSAPK by ROS (such as H_2O_2) is "not detectable" in both tobacco leaves and tobacco BY-2 cell culture (Usami and Machida, 1997).

It is natural to assume that the signaling pathway(s) involved in the low-dose SA-dependent "priming response" must be responsive to low range of SA concentration. We have examined the "priming-active" range of SA concentrations resulting in enhancement of SAG-dependent oxidative burst after 30 min of incubation. Then, we tested the effects of "priming-active" low SA concentrations (0.05–0.2 mM) on induction of four SA-inducible early signaling events namely productions of $O_2^{\bullet -}$ and H_2O_2, $[Ca^{2+}]_c$ increase and PK activation. The low-dose SA action on H_2O_2 production in tobacco BY-2 cells has been described in our previous work (Kawano and Muto, 2000). Detection of other SA-inducible early signaling events were carried out in this study. SA at 0.05 mM induced the detectable increases in CLA-CL and luminol-CL, and that at 0.1 mM induced the detectable increase in aequorin luminescence. Thus, a series of signaling events relayed from oxidative bursts ($O_2^{\bullet -}$ and H_2O_2) to $[Ca^{2+}]_c$ increments was shown to be responsive to "priming-active" low-dose SA. On the other hand, activation of PK was hardly detectable by treatments with low-dose SA (up to 0.2 mM). In BY-2 cells, activation of PK requires much higher concentrations of SA. Tobacco cells were treated with various concentrations of SA and the activation of PK was assayed by an in-gel PK assay using myelin basic protein as an artificial substrate. When treated for 5 min, narrow range of SA treatment (0.4–0.6 mM) resulted in activation of the 48-kDa myelin basic protein-phosphorylating PK (images of gel omitted). The present results showed that the range of SA concentrations for PK activation preferred in BY-2 cells is much higher than that reported for tobacco cell culture derived from *N. tabacum* cv. Xanthi (Zhang and Klessig, 1997).

Since a low dose of SA up to 0.2 mM hardly activates the 48-kDa PK, SA-responsive PK may not be the factor involved in the SA-inducible priming for SAG sensitization in tobacco BY-2 cells; while pretreatment with 50 μM SA does enhance the cellular response to SAG.

In addition to SA-dependent PK activation, activation of the same PK (48-kDa PK) by H_2O_2 (0.2–1.0 mM) was also tested here (image of the gels omitted). The data suggested that the SA-inducible extracellular oxidative bursts may not be functioning as the intermediate inducer of PK activation, since activation of the 48-kDa PK was detectable only when abnormally excess of H_2O_2 (0.6 mM) was added. This is consistent with the statement of Rao *et al.* (1997) that SA modulates the phosphorylation of several proteins and such action is independent of H_2O_2 requirement. Studies have also shown that a MAP kinase counterpart (a homolog of SIPK) in *Arabidopsis*, ATMOK6, is responsive to H_2O_2 (Yuasa *et al.*, 2001; Gupta and Luan, 2003). However, for experimental activation of ATMOK6, notably high concentration of H_2O_2 (1–10 mM) is required (Yuasa *et al.*, 2001; Gupta and Luan, 2003), and most importantly ATMOK6 is not significantly responsive to SA up to 2 mM (Yuasa *et al.*, 2001), thus excluded from the low-dose SA signaling. Although "priming" is inducible by low-dose SA (< 0.3 mM) in *Arabidopsis* (Kohler *et al.*, 2002), obviously ATMOK6 is not likely involved in such a low-dose SA response. By analogy, the tobacco counterpart of ATMOK6, SIPK/PMSAPK may not be involved in the low-dose SA action in tobacco cells.

Our knowledge on SAG action in induction of oxidative burst is now summarized as follows: (1) Production and decomposition of SAG may buffer the acute and toxic actions of SA in plants. (2) In nature, release of low concentration of SA may potentiate the plant's ability to utilize SAG in induction of prolonging oxidative burst, by the mechanism similar to the "priming" process. (3)

Involvement of PK activation may be excluded from the low-dose SA-dependent priming process leading to enhanced response to SAG.

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