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Salicylic acid-induced superoxide generation catalyzed by plant peroxidase in hydrogen peroxide-independent manner

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It has been reported that salicylic acid (SA) induces both immediate spike and long lasting phases of oxidative burst represented by the generation of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet -}$). In general, in the earlier phase of oxidative burst, apoplastic peroxidase are likely involved and in the late phase of the oxidative burst, NADPH oxidase is likely involved. Key signaling events connecting the 2 phases of oxidative burst are calcium channel activation and protein phosphorylation events. To date, the known earliest signaling event in response to exogenously added SA is the cell wall peroxidase-catalyzed generation of $O_2^{\bullet -}$ in a hydrogen peroxide (H_2O_2)-dependent manner. However, this model is incomplete since the source of the initially required H_2O_2 could not be explained. Based on the recently proposed role for H_2O_2-independent mechanism for ROS production catalyzed by plant peroxidases (Kimura et al., 2014, Frontiers in Plant Science), we hereby propose a novel model for plant peroxidase-catalyzed oxidative burst fueled by SA.

Keywords: alkalization, auxin, Compound III, oxidative burst, peroxidase, superoxide

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Introduction

It has been reported that salicylic acid (SA) induces both immediate and long lasting phases of oxidative burst represented by generation of reactive oxygen species (ROS), chiefly superoxide anion radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2).[1-3] Early studies have indicated that SA is an active signal accompanying oxidative burst by which development of systemic acquired resistance (SAR) against a wide range of pathogens is induced, as reviewed in several articles by our group.[2-6] In 1990s, it has been proposed that SA signaling paths leading to SAR require the accumulation of ROS derived from H_2O_2,[7] based on the observation that SA binds and inhibits 2 types of H_2O_2-detoxifying enzymes, namely catalase[8,9] and ascorbate peroxidase.[10] In addition to these passive modes of ROS production by SA, more active modes of SA action involving extracellular peroxidase and NADPH oxidase that directly generates ROS in the presence of SA was reported later.[1,11,12] Interestingly, multiple roles for ROS confirmed to date include (i) activation SAR associated with systemic propagation of the oxidative burst, (ii) induction of intracellular signaling pathway such as the further synthesis and release of SA and activation of mitogen-activated protein kinase (MAPK) cascade, (iii) strengthening of cell wall through oxidative cross-linking of glycoproteins, and (iv) direct microbicidal actions.[2,7]

In general, in the earlier phase of oxidative burst, apoplastic peroxidase are likely involved and in the late phase of the oxidative burst, NADPH oxidase activity is likely involved.[13] The key signaling events connecting 2 phases of oxidative burst are calcium channel activation and protein phosphorylation events.[6,11,13,14] Real-time detections of both SA-induced ROS generation (especially of $O_2^{\bullet -}$) and SA-induced increase in cytosolic calcium concentration were first performed by our group by using *Cypridina* luciferin analog (CLA)-treated and aequorin-expressing model plant cells (tobacco BY-2 cells).[11]

CLA is an $O_2^{\bullet-}$-specific chemiluminescent probe and aequorin is a calcium responsive luminescence protein. Treatment of tobacco BY-2 cells with SA (sub-mM) resulted in rapid and transient generation of $O_2^{\bullet-}$ and in turn, $O_2^{\bullet-}$ stimulated the influx of extracellular Ca^{2+} into the cytosolic space in the tobacco cells in a ROS scavenger-sensitive manner. Here, we need to emphasize that SA-induced extracellular $O_2^{\bullet-}$ generation was shown to be catalyzed by apoplastic free and cell wall-bound peroxidases.[11,15]

To date, known earliest signaling event in response to exogenously added SA is the cell wall peroxidase-catalyzed generation of $O_2^{\bullet-}$ in a H_2O_2-dependent manner.[11] Interestingly, peroxidase-mediated $O_2^{\bullet-}$ generation and secondarily induced calcium signaling are important events in the early signaling phase of SA-induced rapid stomatal closure in *Vicia faba*[16] and *Arabidopsis thaliana*.[17]

Previously proposed formulae for SA-dependent generation of $O_2^{\bullet-}$ in plants[11,15] and model enzyme system using horseradish peroxidase (HRP),[18,19] suggest that the byproducts of peroxidase-catalyzed oxidation of SA is involved in generation of $O_2^{\bullet-}$ as follows:

$$POXN^{(3)} + H_2O_2 \rightarrow CI^{(5)} + H_2O \quad (1)$$

$$CI^{(5)} + SA \rightarrow CII^{(4)} + SA^{\bullet} \quad (2)$$

$$CII^{(4)} + SA \rightarrow POXN^{(3)} + SA^{\bullet} \quad (3)$$

$$2SA^{\bullet} + 2O_2 \rightarrow 2SA^{+} + 2O_2^{\bullet-} \quad (4)$$

where POX N, CI, and CII stands for native ferric form, Compound I and Compound II of plant peroxidase, respectively. $SA^{\bullet}$ and SA^{+} are free radical species and the 2-electron oxidized intermediate products derived from substrate SA, respectively. The likely structures of $SA^{\bullet}$ and SA^{+} were proposed by Gozzo.[20] The formal oxidation states of the heme within the peroxidase enzyme are indicated by numbers in the small brackets. As above, SA is an e^{-} donating substrate while H_2O_2 is viewed as the e^{-} acceptor. Then, phenoxy radical species derived from SA (shown as $SA^{\bullet}$) released thereafter may react with molecular oxygen to form $O_2^{\bullet-}$. Since $O_2^{\bullet-}$ is readily transformed into H_2O_2 in biological systems, a single cycle of SA-oxidizing peroxidase reactions initiated by single unit of H_2O_2 results in yield of 2 units of $O_2^{\bullet-}$ which is equivalent to 2 units of H_2O_2, and therefore, by this way, ROS could be amplified.[1,11] Electron spin resonance spectroscopic analysis has shown that production of $SA^{\bullet}$ occurs in SA-treated plant cells and reaction mixture of purified HRP.[15] Since this mechanism requires the initial input of low level of H_2O_2 as one of starters, supplementation of low dose H_2O_2 reportedly enhances the SA-induced production of $O_2^{\bullet-}$ in cell suspension culture[11] and model enzyme system.[15]

However, above model is incomplete since the source of the initially required H_2O_2 could not be explained. Here, we propose a novel model for plant peroxidase-catalyzed oxidative burst fueled by SA.

H_2O_2-independent ROS production

When plants are threaten by pathogens or recognized the molecules derived from microorganisms, extracellular space alkalization is often induced, under which pH-dependent extracellular oxidative burst involving peroxidase reportedly proceeds, especially at the site of microbial challenge. However, direct stimulus involved in activation of peroxidase-catalyzed oxidative burst has not been fully understood. We have recently studied a likely role for free ferrous ion (Fe^{2+}) in reduction of ferric native enzyme of HRP (with heme at Fe^{III}) into ferrous enzyme intermediate (Fe^{II}) which H_2O_2-independently produces $O_2^{\bullet-}$ *via* mechanism involving Compound III (Fe^{III}-$O_2^{\bullet-}$), especially under alkaline condition, thus, possibly contributing to the plant mechanism combatting against the microbial invasion.[21] This H_2O_2-independent cycle of enzyme is now referred to as oxygenase-like cycle as molecular oxygen participates and binds to the enzyme. In addition to Fe^{2+}, indole-3-acetic acid (IAA) is an active inducer of $O_2^{\bullet-}$ production in H_2O_2-independent manner[1,22-24] possibly involving molecular oxygen[25] to form Compound III.[26]

Based on the views that formation of enzyme-substrate complexes such as [POX-IAA-O_2] equivalent to Compound III results in release of $O_2^{\bullet-}$,[24] medical application of HRP-labeled antibodies and IAA has been proposed as a novel $O_2^{\bullet-}$-generating system for cancer cell-targeted and controlled cell death induction, by designing the HRP-conjugated immuno-labeling of cancer-related molecules or expression of recombinant HRP in mammalian cells.[27-30]

Furthermore, we have previously proposed our view that nitric oxide (NO) is also one of candidate chemicals for reducing native plant peroxidase into ferrous intermediate to initiate the oxygenase-like cycle of plant peroxidases.[2] As summarized in **Figure 1A**, after completion of the oxygenase-like cycle, $O_2^{\bullet-}$ which could be converted to H_2O_2 via disproportionation can be released, suggesting that H_2O_2-dependent cycle of peroxidase reaction can be concomitantly achieved depending on the types and combination of the substrates or chemicals added to the system (**Fig. 1B**).

Algebraic and graphical handling of enzyme behaviors

Graphs presented in **Figure 1B** are summary of redox state shifts in plant peroxidase upon treatment with various substrates or chemicals. These theoretical graph models are based on our previous bio-computational approach for algebraically expressing the cyclic behavior (redox cycling) of HRP among native enzyme and its 2 electron-oxidized and single electron-oxidized intermediates (Compounds I and II) as a cyclic additive group $Z_3 = \{C_0, C_2, C_1\} = \{C_0, 1C_2, 2C_2\} = \{0, 2, 1\}$, and a cyclic multiplicative group $Z_3^{*} = \{C_1, C_2\} = \{C_1, C_2^{1}\} = \{1, 2\}$, with C_2 as the common generator (generalized inputs of e-donating and e-accepting substrates); by viewing that the system is simply consisted of "states" and "transitions."[31]

Each of small graphs in **Figure 1B** is consisted of 5 vertices and directed edges corresponding to the enzyme intermediates and their transitions, respectively. Red and blue edges represent the steps with and without (direct and/or concomitant) release of $O_2^{\bullet-}$, respectively. Final state of the enzyme following various chemical treatments are highlighted with red-colored circles. Chemicals listed are H_2O_2, typical peroxidase substrates (AH)

such as phenolics, Na ditionate (an active reductant with deoxygenation effect), nitric oxide (NO), Fe^{2+}, IAA, and SA.

Additions of Fe^{2+} to HRP[21] and NO to soybean peroxidase (SBP),[32] reportedly result in arrest of the enzymes at Compound II suggesting that these enzymes enter the conventional peroxidase cycle after oxygenation cycle. Upon addition of peroxidase substrates such as phenolics or amines, the peroxidase cycle is completed and native enzyme was shown to be regenerated.[32] In case of IAA, multiple roles are played by IAA, primarily as an enzyme-reducing agent converting native enzyme to ferrous enzyme, and secondarily as a conventional peroxidase substrate being oxidized by compound I and II.[24] Thus, both reaction cycles are completed eventually leaving native enzyme. By analogy to the roles of NO, Fe^{2+} and IAA, modes of SA actions leading to release of $O_2^{\bullet-}$ catalyzed by plant peroxidase must be revised.

Proposed model

Among three potential inducers of $O_2^{\bullet-}$ (IAA, SA, Fe^{2+}), only Fe^{2+} induced an intense and long-lasting peak of CLA-CL in both HRP[21] and SBP (unpublished results). These data might be reflecting the facts that both IAA and SA present at excess level behave as suicide substrates by targeting Compound III (**Fig. 1A**).[19,33]

SA has affinity for various forms of heme proteins such as native form of catalase[8,9] and ascorbate

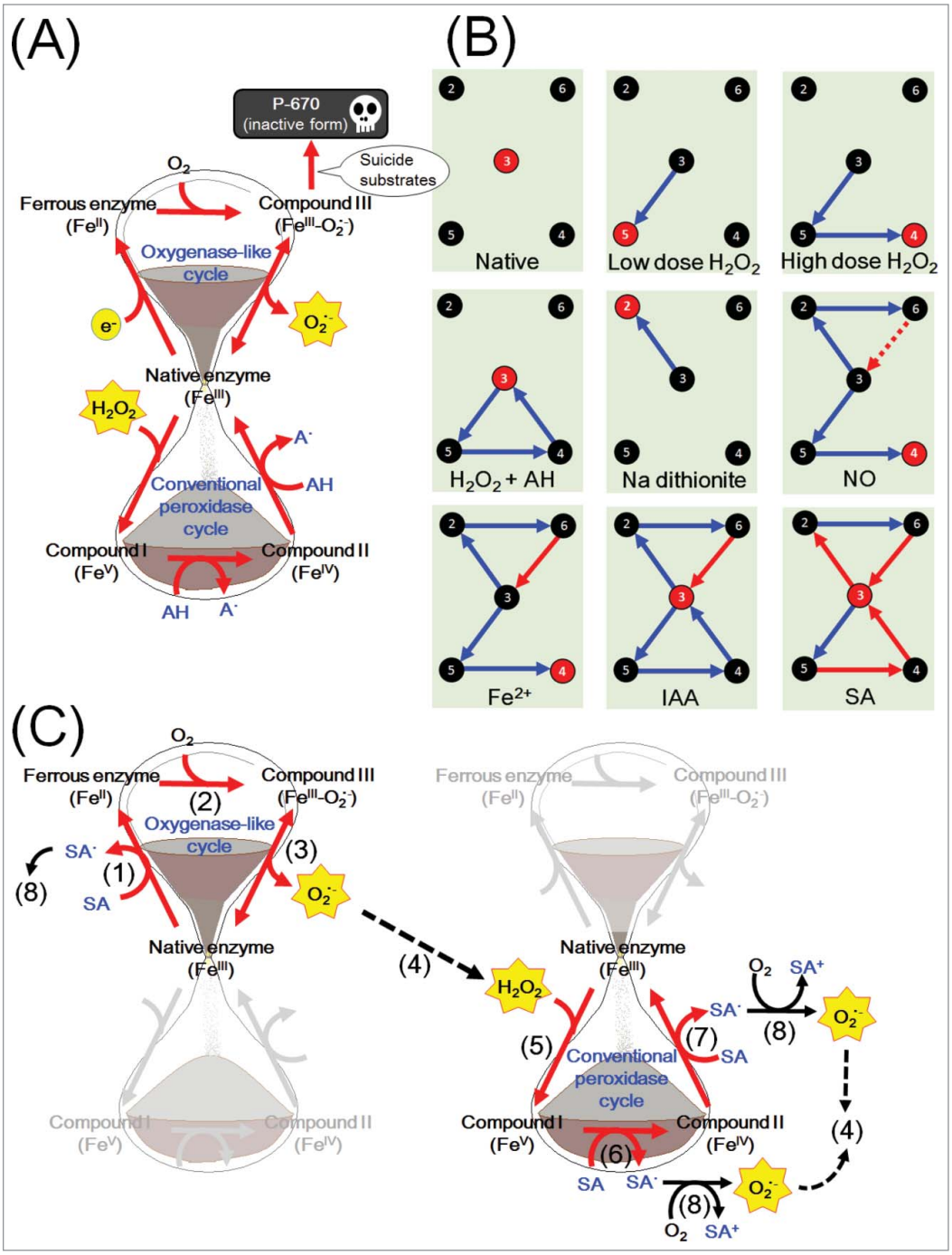

Figure 1. Mechanism of peroxidase-catalyzed ROS production. (**A**) Hourglass model which summarizes the inter-conversions among active and inactive forms of peroxidases involved in ROS generation. This model emphasizes that 2 distinct cycles are initiated by conversion of native peroxidase with e^- acceptor via conventional peroxidase cycle or with e^- donor via oxygenase-like cycle. (**B**) Graph theoretical summary of redox state shifts in plant peroxidase upon treatment with various substrates or chemicals. Each of small graphs is consisted with 5 vertices corresponding to the enzyme intermediates at different redox states, and directed edges (maximally 6 edges allowed). The formal oxidation states of the heme within the enzyme are indicated by numbers in the circles (vertices). Directed edges (arrows) indicate the transitions of the redox states. Red and blue edges represent the steps with and without direct and/or concomitant release of $O_2^{\bullet-}$. Final states of the enzyme following various chemical treatments are highlighted with red circle. Graphs in (**B**) were made based on the documented knowledge (Kawano, 2003a, 2003b, 2013; Kawano et al., 1998, 2001, 2002a, 2002b, 2004; Kawano and Bouteau, 2013a; Kawano and Muto, 2000; Kimura et al., 2014; Takayama et al., 2012). (**C**) Expanded model for SA-induced oxidative burst, based on the relay of H_2O_2-independnet and H_2O_2-dependent peroxidase actions. Series of reactions were numbered from (1) to (8).

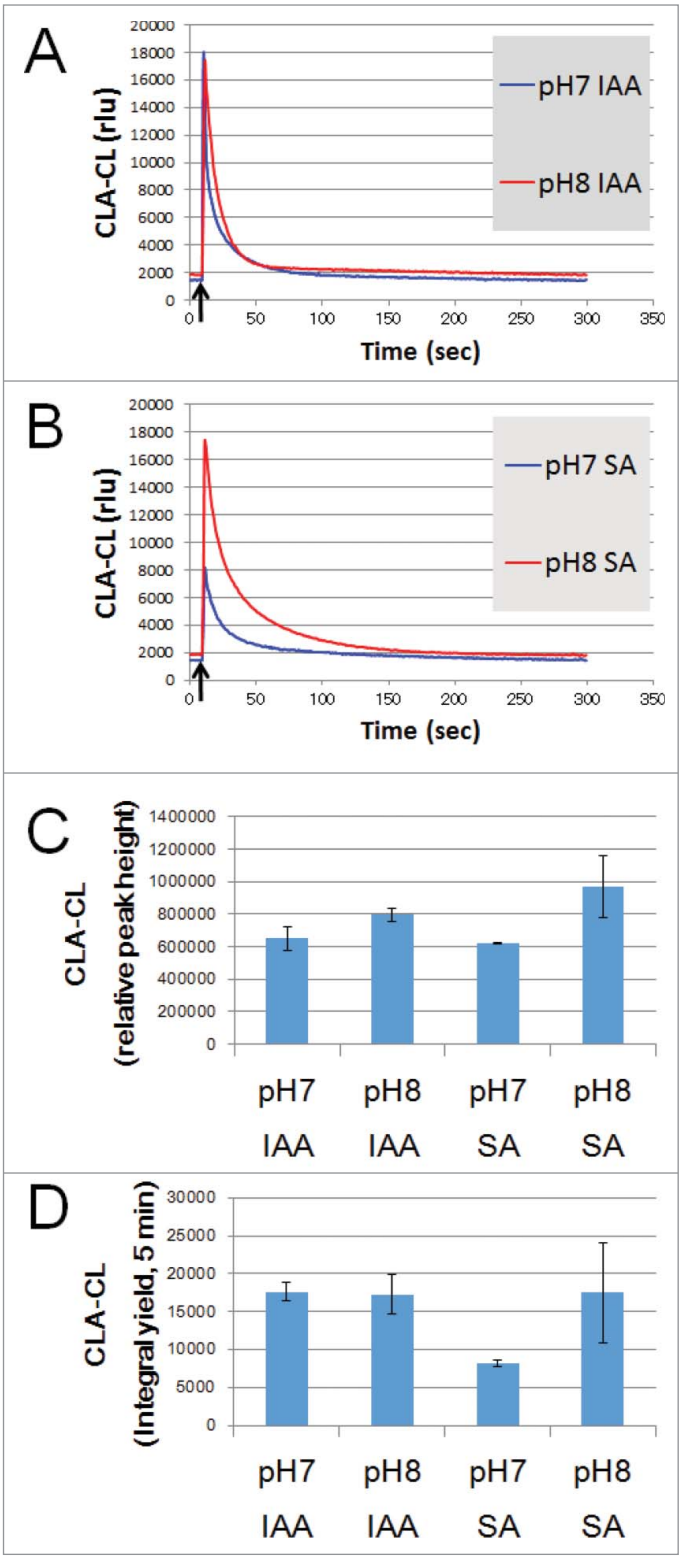

Figure 2. Effects of SA and IAA on $O_2^{\bullet-}$ generation in SBP reaction mixture. SA or IAA or water was added to SBP reaction mixture at pH 7.0 and 8.0. Temporal changes in H_2O_2-independent $O_2^{\bullet-}$-dependent CLA-CL upon addition of IAA (**A**) or SA (**B**) are compared. Arrows indicate the timing of chemical additions. Peak height (**C**) and integral yields (**D**) of CLA-CL induced by IAA and SA measured at pH 7.0 and 8.0 are compared. Bars, SD (n = 3). Conditions: total volume, 0.2 ml; K-phosphate, 25 mM (pH 7.0 or 8.0); SBP, 1.5 μM; CLA, 10 μM, reducing agents (IAA, SA), 100 μM.

peroxidase,[15] Compound II of HRP[18] and SBP,[32] and Compound III of HRP.[19] We can expect that SA may interact with native form of plant peroxidases. In fact, such preliminary data on the SA-dependent stimulation of native HRP[21] and SBP[32] leading to generation of $O_2^{\bullet-}$ have been shown in our previous articles. However, when we have reported such data, it was early for us to clarify our working hypothesis on the mode of SA action in the H_2O_2-independent peroxidase action leading to generation of ROS.

After publication of recent work by Kimura et al.,[21] we examined and confirmed that SA actually induces the generation of $O_2^{\bullet-}$ in the presence of model enzymes such as HRP and SBP, even in the absence of H_2O_2 supplementation. Here, typical data obtained with SBP are shown (**Fig. 2**). The data presented here simply but clearly supported our view that plant peroxidases catalyze the SA-dependent $O_2^{\bullet-}$ generation in H_2O_2-independent manner. Furthermore, comparison of the actions of SA and IAA at neutral and alkaline condition (**Fig. 2**) suggests that there is possibility that SA but not IAA contributes to the alkaline-responsive oxidative burst in plant defense mechanisms associated with microbe-associated molecular patterns, by involving the extracellular peroxidases as proposed by the group of Bolwell.[34-37]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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