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Review

Controlled salicylic acid levels and corresponding signaling mechanisms in plants

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Abstract Salicylic acid (SA) is a natural signaling molecule involved in plant defense response against pathogen infection. This article covers the recent key works contributing to our understanding of SA signaling and biosynthesis leading to a controlled SA level in plants.

Key words: Reactive oxygen species, salicylic acid.

Salicylic acid (SA) that was named after *Salix* plant (willow), was first discovered as a major component in the extracts from willow tree bark that had been used as a natural anti-inflammatory drug from the ancient time to the 18th century (Rainsford 1984; Weissman 1991). Acetylsalicylic acid which is widely known as aspirin is the world first synthetic drug that had been produced by Bayer Company as anti-inflammatory agent in 1897 (Weissman 1991). Since then aspirin became one of the most popular drugs among the people and has been widely used for over 100 years, and pharmacological actions of aspirin and related salicylates in animal system have been intensively studied while only little about the action of SA in plants has been elucidated. White (1979) was the first plant biologist paid attention to salicylates as disease resistance-inducing chemicals. He and his colleague demonstrated that injection of aspirin into tobacco leaves enhanced the resistance to subsequent infection by tobacco mosaic virus (TMV) (White 1979; Antoniw and White 1980). Later, it has been shown that this treatment induces the accumulation of pathogenesis-related (PR) proteins (Kessmann and Ryals 1993; Malamy et al. 1990; Métraux et al. 1990).

Recent intensive studies have shown that SA biosynthesis and signaling required for plant defense should not be regarded as a linear pathway but rather as a complex network (Shah 2003). This article describes the recent progress in our understanding of SA signaling and biosynthesis leading to a controlled level of SA in plants, by covering historical milestone works.

Until now, involvement of SA signaling was commonly confirmed by the phenotypes of plants carrying the *NahG* transgene encoding bacterial salicylate hydroxylase which converts SA to catechol (Delaney et al. 1994; Hunt et al. 1996; Alvarez 2000). Lowered non-host resistance to a *Pseudomonas syringae* pv. *phaseolicola* strain is one of the representative *NahG* phenotype reported for *NahG Arabidopsis* plants, but a recent report presented the data questioning above effect of *NahG* and also reliability of *NahG* experiments judging the involvement of SA was very much weakened (van Wees et al. 2003). The report uncovered that the by-product of *NahG* action, catechol may be the actual player lowering the non-host resistance to the pathogen. The report propounded that any conclusion about SA requirement based solely on phenotypes of *NahG* plants should be reevaluated. Further investigations on *NahG* side effects are urgently required for evaluation of the earlier works using *NahG* plants. In contrast, the works directly blocking the SA biosynthesis for examining the SA requirement in defense response (e.g. Nawrath and Métraux 1999; Wildermuth et al. 2001) must receive

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; *as-1*, activation *sequence-1*; BA2H, benzoic acid 2-hydroxylase; $[Ca^{2+}]_c$, cytosolic free calcium concentration; CaM, calmodulin; CDPK, calcium-dependent protein kinase; CNGC, cNMP-gated channels; ESR, electron spin resonance; HR, hypersensitive response; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; ICS, isochorismate synthase; iGluR, ionotropic glutamate receptor, IPL, isochorismate pyruvate-lyase; JA, jasmonic acid; LRR, Leu-rich repeat; NBS, nucleotide-binding site; NO, nitric oxide; *NPR1*, *non-expressor of pathogenesis-related gones 1*; O_2^-, superoxide; PAL, phenylalanine ammonia-lyase; POX, peroxidase; PR gene/protein, pathogenesis-related gene/protein; R gene, resistance gene; rboh, respiratory burst oxidase hormolog; ROS, reactive oxygen species; SA, salicylic acid; SABP, SA binding protein; SAG, SA β-glucoside; SAGase, SA β-glucosidase; SAGT, SA 3-O-glucosyltransferase; SAR, systemic acquired resistance; SIPK, SA-induced protein kinase; TIR, toll-interleukin-2 receptor; TMV, tobacco mosaic virus; TNV, tobacco necrosis virus; *TPC1*, *two-pore channel 1.*

more attentions meanwhile. Therefore, in the present review article, the works based on *NahG* experiments are less emphasized.

Many excellent reviews on SA signaling, feedback regulation of SA biosynthesis and recent discovery in SA biosynthetic pathways have been published (Christiane and Métraux 2002; Métraux 2002; Shah 2003; Eckardt 2003; Métraux and Durner 2004; Singh et al. 2004). Readers are encouraged to read them for further information.

SA Biosynthesis 1: Earlier works emphasizing cytoplasmic pathways in tobacco and cucumber plants

In plants, an array of compounds, some of which have been implicated in chemical and physical defense against pathogens, are produced from *trans*-cinnamic acid, the first metabolite in the phenylpropanoid pathway. These compounds include lignin, flavonoids, phenolics and phytoalexins. SA is also formed from *trans*-cinnamic acid *via* either benzoic acid or *o*-coumaric acid, although the synthesis of SA *via* *o*-coumaric acid is not clearly worked out (Ward et al. 1991). In tobacco, production of *trans*-cinnamic acid from phenylalanine by phenylalanine ammonia-lyase (PAL) contributes to resistance against fungal invasion mainly *via* production of benzoic acid thus leading to increased SA production (Figure 1A), but not *via* production of *p*-coumaric acid from which lignin, flavonoids, and phytoalexins are to be produced in the downstream paths (Serino et al. 1995; Mauch-Mani and Sulsarenko 1996).

Benzoic acid 2-hydroxylase (BA2H) is an enzyme responsible for conversion of benzoic acid to SA, and it is known that this key enzyme is induced by virus inoculation to tobacco leaves (Léon et al. 1993). Injection of ^{14}C-labeled benzoic acid to cotyledons of cucumber reportedly results in accumulation of ^{14}C-labeled SA in phloem, and conversion of benzoic acid to SA requires the inoculation of tobacco necrosis virus (TNV), suggesting that SA biosynthesis and its transport take places before development of systemic acquired resistance (SAR) (Mölders et al. 1996).

SA Biosynthesis 2: Recent research progress highlighting the chloroplastic pathway in *Arabidopsis*

There has been accumulation of data questioning the role of above pathways in *Arabidopsis thaliana*, although early studies have suggested that plants synthesize SA from phenylalanine. Especially in *Arabidopsis*, SA could be produced even when the phenylalanine pathway was inhibited, and radio-labeling experiments often indicated that the rate of conversion of SA precursors in the phenylalanine pathway to SA may unexpectedly low (Wildermuth et al. 2001). Interestingly, bacterial siderophore studies have shown that SA production in bacteria is free from the phenylalanine-pathway, and instead some bacteria produce SA by coupling isochorismate synthase (ICS) and isochorismate pyruvate-lyase (IPL) (Mercado-Blanco et al. 2001). Such SA-producing bacteria include *Pseudomonas* spp. (Ankenbauer and Cox 1988; Anthoni et al. 1995; Meyer et al. 1992; Visca et al. 1993), *Mycobacterium* spp. (Ratledge and Winder 1962), *Azospirillum lipoferum* (Saxena et al. 1986), and *Burkholderia cepacia* (Sokol et al. 1992).

Recently, drastic progress has been made in the search for biosynthetic pathway of SA, revealing that the bacterial type of SA biosynthesis takes place in plants as shown in Figure 1B. In 1999, van Tegelen et al. have reported the first isolation of a plant ICS, from the cell cultures of *Catharanthus roseus*, which shares homology with the bacterial ICS. At the same time, they have proposed a possible role for the plant ICS in SA biosynthesis *via* isochorismate production, despite massive evidences from tobacco supporting the SA biosynthesis from phenylalanine *via* benzoate (van Tegelen et al. 1999). In 2000, Verberne et al. have reported that overexpressions of the chloroplast-targeted bacterial enzymes (ICS and IPL) in transgenic tobacco plants overproduce SA at 500- to 1000-fold higher level compared to the control plants. In 2001, Mauch et al. have engineered a novel hybrid enzyme with SA synthase activity, by fusing two bacterial genes *pchA* and *pchB* isolated from *Pseudomonas aeruginosa*, encoding ICS and IPL, respectively. Then, the ICS-IPL fusion enzyme was overexpressed in *Arabidopsis* under control of CaMV 35S promoter. Targeting of the chimeric enzyme protein to the plastids resulted in notably high level of SA production and PR gene expression, while the protein targeting to cytosolic space resulted in relatively low enhancement of SA production (Mauch et al. 2001). These data suggest that the levels of substrate for ICS-IPL enzyme in the different compartments limit the level of products. Therefore, appropriate localization of the enzyme in the specific compartment is required. Wildermuth et al. (2001) have shown that SA is synthesized, in *Arabidopsis*, from chorismate by means of an *Arabidopsis* ICS equivalent, *ICS1*. Furthermore, it has been shown that this pathway is required for both local and systemic resistance responses. *ICS1* gene possesses a putative plastid-transit sequence and a cleavage site, indicating the use of ICS in the chloroplasts, utilizing chloroplast-synthesized chorismate as a substrate (Wildermuth et al. 2001). Thus, SA production may take place in the chloroplasts. However, discovery of chorismate pathway does not explain how the SA biosynthesis is stimulated by the presence of SA

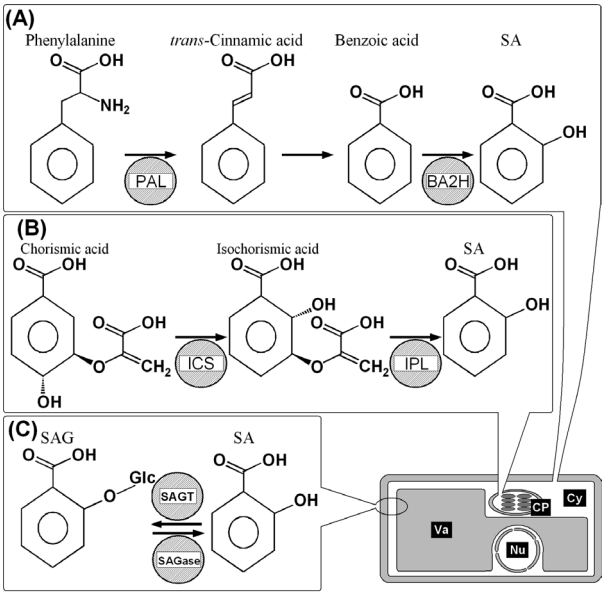

Figure 1. Proposed pathways for SA biosynthesis in plants. (A) Cinnamate pathway reported for tobacco plants, starting from phenylalanine. This pathway may take place in the cytosolic space where phenylalanine ammonia-lyase (PAL) and benzoic-acid-2-hydroxylase (BA2H) are active. (B) Bacterial type of SA biosynthesis found in *Arabidopsis*. This pathway may take place in the plastids. Two-step formation of SA from chorismic acid *via* isochorismic acid catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate-lyase (IPL) has been proposed. (C) Interconversion between SA and SAG. Conversion of SA to SAG catalyzed by SA 3-*O*-glucosyltransferas (SAGT) occurs inside the cells and most of SAG is stored in vacuoles. Degradation of SAG and release of free SA catalysed by SA β-glucosidase (SAGase) are likely to occur in the apoplast. Therefore, there must be a translocation of SAG from vacuoles to apoplast. Va, vacuole; Nu, nucleus; CP, chloroplast; Cy, cytosol.

itself, since the promoter region of *ICS1* gene does not contain any SA-responsive element (Wildermuth et al. 2001).

Storage form of SA and its use in emergency

In plants, interconversion between SA and SA β-glucoside (SAG; 2-*O*-β-D-glucosylbenzoic acid) occurs as illustrated in Figure 1C, and SA presents mainly as SAG rather than free form (Klick and Herrmann 1988). When the excess of SA is present in plants, it is likely that many portion of SA is converted to SAG and stored. Elicitors such as chitosan elevate the SAG content while free SA level is unchanged (Messner and Schroder 1999). Overproduction of SA in transgenic plants expressing SA-producing enzymes (Verberne et al. 2000; Mauch et al. 2001) and a TIR-NBS-LRR type R gene mutant *ssi4* (Shirano et al. 2002) reportedly result in increase in SAG content. In 1992, Enyedi et al. and Malamy et al. have reported independently 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 a pathogenic fungus *Phytophthora infestans* and elicitors derived from it (Keller et al. 1996), suggesting the roles for SAG in defense mechanism.

According to Hennig et al. (1993), both endogenous SAG and exogenously applied SAG are hydrolyzed to yield SA, and SAG is likely active only after hydrolysis to SA in the induction of *PR-1* gene in tobacco leaves. Thus SAG seems to be the storage form from which physiologically active free SA is released when necessary (Seo et al. 1995a). It has been shown that the hydrolysis of SAG in tobacco leaves occurs in the extracellular (intercellular) spaces (Henning et al. 1993) where considerable activities of SA β-glucosidase (SAGase) were found (Seo et al. 1995a), despite the formation of SAG from SA catalyzed by SA 3-*O*-glucosyltransferase (SAGT) occurs inside the cells and vacuole is considered as the major SA reservoir (Hennig et al. 1993). Taken together, it is likely that SAG is partially excreted by unidentified mechanism, to the intercellular space where the SAG-degrading enzyme (SAGase) and SA-utilizing enzymes (see below sections) are abundant, thus free SA could be released from SAG, and utilized for defense mechanisms such as oxidative burst.

As previously shown, in tobacco BY-2 cell culture, induction of oxidative burst by SA was only transient lasting only up to 1 min (Kawano et al. 1998). However, in nature, it is likely that SAG is buffering the action of SA behaving as a slow SA-releasing agent enabling the sustainable, prolonging oxidative action of SA, since oxidative burst induced by SAG lasted for notably longer period as long as 20–30 min (Kawano et al. 2004a). In addition, we observed the effect of SA pretreatment (50 μM) on the SAG-induced oxidative burst (Kawano et al. 2004a). With the best pretreatment timing (30 min), the SAG-induced superoxide ($O_2^{\cdot -}$) generation attained the level almost identical to that induced by free SA. It has been reported that pretreatment of tobacco leaves with SA results in sharp increase in extracellular SAGase activity that releases more SA from extracellularly stored SAG within 1 h (Seo et al. 1995a), indicating that SA-enhanced responsiveness to SAG may be due to increase in glucosidase activity. This process may contribute partially to SA-dependent increase in free SA level when required.

SA-dependent amplification circuits

Disease resistance (R) genes in plants provide a major mode of defense against a wide variety of pathogens and

pests (Ramakrishna et al. 2002). The most abundant class of R gene encodes proteins with a nucleotide-binding site (NBS) and a Leu-rich repeat (LRR) region, and the genome of *Arabidopsis thaliana* contains more than 120 NBS-LRR genes (Arabidopsis Genome Initiative 2000). From the NBS-LRR genes that have received functional analysis, it appears that the LRR region provides the specificity for recognition of a pathogen gene product, thereby leading to the initiation of a signal transduction cascade that activates several defense pathways (Ellis et al. 2000). It has been well documented that activation of R-gene-mediated defense signaling induces SA biosynthesis and downstream defense responses. In turn, addition of exogenous SA activates the expression of R genes of the toll-interleukin-2 receptor (TIR)-NBS-LRR type (Shirano et al. 2002). Accordingly, a gain-of-function mutation in a TIR–NBS–LRR–type R gene, *SSI4*, revealed that activation is SA-dependent, but NPR1-independent in *Arabidopsis ssi4* mutant (Shirano et al. 2002).

RPW8.1 and *RPW8.2* are members of *Arabidopsis* R genes that participate in recognition of powdery mildew pathogens subsequently inducing a localized necrosis or hypersensitive response (HR) (Xiao et al. 2001). Similarly to TIR–NBS–LRR type R gene activation, SA activates the expression of *RPW8*, in combination with other supportive data, above results led us to a conclusion that HR development requires the transcription of *RPW8.1* and *RPW8.2* regulated by SA-dependent feedback amplification (Xiao et al. 2003). SA also activates the expression of the *EDS1* gene, which is required for SA accumulation and resistance conferred by these R-gene-activated pathways, indicating the feedback regulation of these R genes and of *EDS1* by SA (Feys et al. 2001). *EDS1* plays a dual role in plant defense (Feys et al. 2001). *EDS1* is required for HR development, and it is also required for SA accumulation, in association with *PAD4*. Expression of *EDS1* and *PAD4* is reportedly activated upon pathogen attacks and addition of benzothiadiazole (a SA analog) (Feys et al. 2001). Moreover, expression of *EDS5* and *PAD4* genes, which regulate SA biosynthesis, and that are of the SA biosynthesis gene *SID2* (encoding ICS) activated by SA (Verberne et al. 2000; Wildermuth et al. 2001; Feys et al. 2001). Above studies suggest that at multiple points SA exerts regulatory positive feedback effects. Activation of the expression of multiple R genes by SA could be a part of mechanisms that activates broad-spectrum resistance, and likewise a feedback mechanism may be important in amplifying plant defense responses (Shah 2003).

In contrast to the positive feedback regulation discussed above, a negative feedback loop involving *NPR1* (*non-expressor of pathogenesis-related genes 1*) is also reported. *SID2* (*ICS1*) expression in pathogen-infected plants is negatively feedback-regulated by *NPR1* (Wildermuth et al. 2001). SA accumulation is reportedly higher in pathogen-inoculated *npr1* mutants than in wild-type plants after inoculation with pathogen (Shah et al. 1997; Delaney et al. 1995). In addition, SA accumulation in several lesion-mimic mutants is higher in the *npr1* mutant background than in the *NPR1* wild-type background (Clarke et al. 2000; Shah et al. 1999). The expression of *SID2* was also greater in pathogen-inoculated *npr1* plants than in wild-type plants (Wildermuth et al. 2001).

Since extremely high levels of SA, observed in several transgenic and mutant *Arabidopsis* plants are associated with dwarfing (Mauch et al. 2001; Shah et al. 1999; Clarke et al. 2000), and uncontrolled synthesis of SA may also compromise other defense pathways that are inhibited by SA (Kunkel and Brooks 2002; Feys and Parker 2000), it is important to regulate SA synthesis and signaling. Positive and negative feedback loops allow for the tighter regulation of SA accumulation and the fine-tuning of plant defense signaling (Shah 2003).

The positively controlled feedback leading to burst of biosynthesis of stress-related plant signaling molecules is not surprisingly new. We have experienced the era of ethylene molecular biology emerged in the late 1980s and developed in early 1990s. Ethylene biologists have described the phenomenon in which ethylene markedly enhances the production of ethylene itself as "*auto-catalytic*" (Yang and Hoffman 1984; Kende 1993). The similar phenomena in SA biology is now referred to as "*SA-dependent amplification circuit*" (Xiao et al. 2003) or "(*SA*) *feedback loop*" (Shah 2003). Understanding of "*autocatalytic*" ethylene biosynthesis was possible only after isolation and cloning of 1-amino-cyclopropane-1-carboxylate (ACC) oxidase (ethylene-forming enzyme) and ACC synthase (the rate-limiting enzyme), by studying the mode of regulation of these enzymes (Wang et al. 2002, 2004). By analogy, we can now move to the next stage for understanding the "*loop*" by revealing the mode of key enzyme activation since SA synthesizing pathway (chorismate pathway) has been identified in plants.

Roles for *NPR1*

The SA-dependent increases in SA production could be explained by crosstalk between SA and jasmonic acid (JA) too. Both JA and SA function in SAR signaling and disease defense accompanying with their cooperative and antagonistical interactions (Turner et al. 2002; Kunkel and Brooks 2002). For instance, it has been reported that JA and SA induce expression of PR-genes through antagonistical interactions (Niki et al. 1998).

Production of JA by wounding (Seo et al. 1995b) and inhibition of JA production by SA (Doares et al. 1995)

are well known phenomena in plants. In addition, SA production is also induced by wounding in tobacco plants only in the presence of excess of cytokinins (Sano et al. 1994; Sano and Ohashi 1995), and such induction of SA production was shown to be inhibited by methyl JA (Sano et al. 1996). Furthermore, induction of acidic *PR* gene expression by SA and that of basic *PR* gene expression by JA could be inhibited in the presences of JA and SA, respectively (Niki et al. 1998). According to above model, the SA synthesis may be drastically induced by breaking the balance of SA–JA levels by supplying certain level of SA.

Recent studies suggested that SA-dependent inhibition of JA action (lowered induction of JA-responsive genes) could be manifested *via* activation of *NPR1* (Pieter and van Loon 2004). In addition to its crucial role in regulation of SA-responsive *PR* gene expression by shuttling into nucleus from the cytoplasm, *NPR1* was shown to act within cytoplasm for participating the crosstalk between SA and JA. Activation of SA-mediated SAR suppresses the JA signaling, and thus SA-dependent resistance takes priority over JA-dependent defenses. According to Spoel et al. (2003), antagonistic effect of SA on JA-responsive gene expression is negatively regulated by SA-activated *NPR1* while nuclear localization of *NPR1* appeared to be unnecessary for opposing the JA action. Although the mode of *NRR1* action in inhibition of JA-responsive gene expression is still unknown, Pieterse and van Loon (2004) have proposed that cytosolic *NPR1* interfers with the SFC^{COI1} uniquitin-ligase complexes that plays a key role in JA response (Xu et al. 2002; Devoto et al. 2002).

In the SA signaling pathway, *NPR1* plays a central role in activation of defense-related genes by enhancing the binding of transcription factors belonging to TGA subclass of the basic leucine zipper family (TGAs) to SA-responsive elements in the promoter regions of *PR* genes (Zhang et al. 1999). Recently, Mou et al. (2003) has proposed a novel model of *NPR1* activation in which *NPR1* protein conformation changes in response to SA-induced changes in cellular redox status, based on the observation that *NPR1* proteins from various plant species share the conserved Cys residues capable of forming both intra- and inter-molecular disulfide bonding and that replacement of these Cys residues with other amino acids reproduced the mutant *npr1*-like phenotype. In addition, SA-mediated changes in redox status upon induction of SAR, reduces the Cys residues of *TGA1* too (Després et al. 2003). Only after reduction of disulfide bonds to free Cys residues, *TGA1* is capable to interact with *NPR1*. However, the mechanism of SA-dependent changes in cellular redox status is still unclear. SA-induced production of reactive oxygen species (ROS) (oxidative burst) and subsequently activated enzymes for removing ROS may have great impact on cellular redox status changes required for *NPR1* activation (Mou et al. 2003). In the following sections, the early upstream signaling events including SA perception and oxidative burst, possibly prior to *NPR1* activation, are discussed.

Search for SA receptors

Klessig and his colleagues have taken the great advantages in the search for SA binding proteins (SABPs), putative SA receptors. To date, 3 types of SABPs have been isolated. The first SABP isolated from tobacco (Chen et al. 1993a) was shown to be catalase (Chen et al. 1993b, Conrath et al. 1995). Since SA binding to catalase resulted in inhibition of H_2O_2-degrading reaction, Chen et al. (1993b) have proposed an idea that increases in H_2O_2 and/or other ROS derived from H_2O_2 may be the triggering events for SA signaling. The results with mammalian catalase were also likely supporting the SA action through catalase inhibition (Durner and Klessig 1996). By analogy, ascorbate peroxidase which consumes H_2O_2 during oxidation of ascorbate was also considered as an additional target of SA-dependent inhibition (Durner and Klessig 1995). However, inhibitory action of SA against ascorbate peroxidase is not supported by other researchers (Miyake et al. 1996; Kvaratskhelia et al. 1997). Tenhaken and Rübel (1997) also presented the data opposing the involvement of catalase and ascorbate peroxidase in SA action. Catalase activity was monitored by reading the decrease in given H_2O_2 concentration in the reaction mixture containing crude soybean enzyme preparations, and concluded that SA has no significant impact on catalase action. It seems to us, the detection of catalase inhibition based on H_2O_2 consumption is not appropriate especially in the *in vivo* and crude enzyme preparations possibly containing peroxidative activity. Kawano et al. (1998) detected the catalase inhibition by SA in BY-2 tobacco cell culture *in vivo* by monitoring the H_2O_2-dependent O_2 evolution. However, significance of SA-dependent catalase inhibition in early action of SA is uncertain.

The second SABP isolated from tobacco was a 25-kDa protein, designated as SABP2, showing much greater affinity for SA compared to catalase (Du and Klessig 1997). Based on sequence analysis, SABP2 was proposed as a lipase belonging to the α/β fold hydroxylase super family, and recombinant SABP2 expressed in *E. coli* was shown to have lipase activity (Kumar and Klessig 2003). Notably, lipase activity of the recombinant SABP2 was enhanced by 3.8-fold in the presence of SA. Furthermore, RNAi-based silencing of *SABP2* gene expression in tobacco resulted in suppression of local resistance to TMV, *PR-1* induction, and development of SAR (Kumar and Klessig 2003), indicating that SABP2 plays a key role as an SA

receptor. Further studies on the roles of SABP2 are eagerly expected.

Recently, Slaymaker et al. (2002) have reported the isolation of SABP3 from tobacco. SABP3 is identified as a chloroplast-targeted carbonic anhydrase that possesses antioxidative activity when examined in yeast. Since importance of chloroplasts as the site of SA biosynthesis is highlighted in *Arabidopsis* by the finding of *SID2* encoding chloroplast-localized ICS enzyme (Wildermuth et al. 2001), it is natural to expect that a SA-binding protein which is specifically present in chloroplasts plays some important roles. As Shah (2003) discussed in his review, chloroplasts and plastids might be the source of signals affecting the response to pathogens, by analogy to mitochondrial roles in animals.

The search for the proteins of great importance directly governing the SA signal transduction is still ongoing. There is no doubt that search for SABPs provided several key ideas such as involvement of ROS in SA signal transduction.

ROS and calcium signaling

Number of studies have indicated that SA may be an oxidative signal inducer which is essentially involved in development of SAR against various pathogens with various natures (e.g. Chen et al. 1993b; Rao et al. 1997). However, mechanism of SA signal transduction has not been fully uncovered. It has been proposed that SA signal transduction leading to SAR may be mediated by ROS derived from H_2O_2, since SA binds and inhibits catalase (Chen et al. 1993b). 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 had not been reported until recently. In addition to ROS, Ca^{2+} is another possible mediator of SA signals, and certain number of reports 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 cells and leaves (Raz and Fluhr 1992), and carrot cell culture (Schneider-Müller et al. 1994).

The first report on successful detection of SA-induced $O_2^{\cdot -}$ and the SA-induced increase in cytosolic free calcium 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). We showed that treatment of tobacco BY-2 cells with SA results in rapid and transient generation of $O_2^{\cdot -}$ detected with a specific chemiluminescence (using a *Cypridina* luciferin analog), and in turn $O_2^{\cdot -}$ likely 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 extracellular oxidative burst (generation of $O_2^{\cdot -}$) is catalyzed by extracellular free and cell wall-bound peroxidases (POXs).

According to calculations, the production of $O_2^{\cdot -}$ and H_2O_2 induced by 0.5 mM SA were estimated to be *ca*. 2.5 and 10 nmoles/ml culture, respectively; and the maximal $[Ca^{2+}]_c$ attained after addition of 0.5 mM SA was estimated to be *ca*. 600 nM (Kawano and Muto 2000). Since ROS production induced by SA in tobacco cells is very rapid and transient (except for SAG-induced oxidative burst), therefore high time-resolution is required for analysis. Other studies using fluorescence and chemiluminescence with much greater time scales spanning for several hours (e.g. Tenhaken and Rübel 1997) may hardly detect such early responses. It is noteworthy that Shirasu et al. (1997) and Rao et al. (1997) have successfully detected the accumulation of H_2O_2 after addition of SA to soybean cell suspension culture and *Arabidopsis* leaves, respectively. When the SA was applied alone (without pathogen inoculation), 0.2–1.0 mM (Shirasu et al. 1997) and 1.0–5.0 mM (Rao et al. 1997) of SA concentrations were required for induction of H_2O_2 production. However, these works lack the information on the earlier responses within 30 min after SA treatment, and notably the H_2O_2 production likely attained the peak levels after hours of incubation with SA, therefore H_2O_2 production reported in these works may not be reflecting the early phase of oxidative burst. The oxidative burst induced in longer time scale might be a consequence of early SA signaling events leading to expression of the genes involved in the later phase of oxidative burst as discussed later, thus SA signaling leading to acute gene expression requires much faster responses.

It has been shown that expression of certain genes can be rapidly induced by SA and pathogens. Such genes include *Arabidopsis* glutathione *S*-transferase genes and glucosyltransferase gene (Lieberkerr et al. 2003; Uquillas et al. 2004). According to Uquillas et al. (2004), immediate induction of such early genes by SA proceeds through an *NPR1*-independent pathway while SA-induced activation of late defense genes such as *PR-1* involves the participation of *NPR1* protein. Temporally distinct phases of oxidative burst (immediate Kawano model and later Rao-Shirasu model) and gene expression events (early genes and late defense genes) likely suggest the time-dependently regulated different modes of SA action. As suggested by *NPR1* works (Mou et al. 2003; Després et al. 2003), redox changes due to oxidative burst (represented by H_2O_2 production) may contribute to the late defense gene activation. It is reasonable to

assume that the early gene expression requires the earlier SA signaling mechanism other than late oxidative burst and *NPR1* involvement. The immediate oxidative burst (production of ROS) followed by Ca^{2+} movement or alternative ROS-independent early protein kinase activation event may form a possible signaling pathway corresponding to early gene activation. An earlier study showed that immediate early transcription activation by SA examined in transgenic tobacco plants carrying regulatory sequences derived from CaMV 35S promoter involves the activation of SA-responsive element known as *activation sequence-1* (*as-1*), and this immediate activation proceeds even in the presence of cycloheximide while the SA-induced transcription of PR-genes (late genes) was shown to be sensitive to cycloheximide treatment (Qin et al. 1994). Recent study is supporting the role for SA-induced early production of ROS such as $O_2^{\cdot -}$ (but not of H_2O_2) since the SA-induced *as-1*-like element-regulated expression of glutathione *S*-transferase gene in tobacco plants requires ROS excluding H_2O_2 (Garreton et al. 2002).

The mechanism of POX actions in SA-dependent induction of oxidative burst has been intensively studied both in tobacco cell culture and *in vitro* enzyme assay using enzyme cocktails, with chemiluminescence, electron spin resonance (ESR), and spectroscopic analysis of enzyme oxidation states, as recently reviewed and summarized (Muto and Kawano 2003; Kawano 2003). Data suggested that the mechanism for SA-dependent early ROS production is solely depending on the action of POX thus independent from the well documented ROS-generating system involving NADPH oxidase $gp91^{phox}$ homologs (Yoshioka et al. 2003). However, involvement of NADPH oxidase in the later stages of SA action is not excluded since it has been reported that SA eventually induces NADPH oxidase in potato tissues (Yoshioka et al. 2001).

It has been shown that transcript accumulation of two isogenes of potato $gp91^{phox}$, designated as *StrbohA* and *B* was sensitive to protein kinase inhibitors but not affected by inhibitors of Ca^{2+} influx, suggesting that certain Ca^{2+}-independent protein kinase such as SA-induced protein kinase (SIPK) may be involved in induction of gene expression (Yoshioka et al. 2001). Actually, SA activates SIPK (Zhang and Kelssig 1997) and SIPK activation results in multiple defense responses (Zhang and Liu 2001), and such responses include SAR and oxidative burst (Yang et al. 2001; Ren et al. 2002). In addition, overexpression of constitutively active form of a genetically engineered potato ortholog of MAP kinase kinase (*StMEK1*) which phosphorylates and activates a potato SIPK ortholog (*StMPK1*), in *Nicotiana benthaniana* leaves reportedly results in increase in SIPK activity and ROS-mediated cell death (Katou et al. 2003). Increase in ROS production and cell death in above condition were shown to be due to induction of a *NbrbohB* since silencing of this gene negated the action of constitutive *StMEK1* (Yoshioka et al. 2003).

There are some other plant NADPH oxidases localized in the plasma membrane, of which the $O_2^{\cdot -}$-generating activity is directly and positively regulated by Ca^{2+} (Sagi and Fluhr 2001). It is possible that Ca^{2+} has a direct effect on the NADPH oxidase, since plant enzyme has an N-terminal sequence with two Ca^{2+}-binding EF-hand motifs (Keller et al. 1998; Torres et al. 1998).

Mechanism of POX reaction leading to oxidative burst

Land plants contain a large number of class III POXs (E.C., 1.11.1.7), for an instance, *Arabidopsis thaliana* genome harbors 73 genes encoding POXs (Valerio et al. 2004). In this class, all known isoforms possess a signal peptide, which targets the proteins into the secretary pathway via the endoplasmic reticulum (Valerio et al. 2004). Plants often react to various stimuli through the synthesis or induction of certain POX protein isoforms (Hiraga et al. 2001; Penel et al. 1992). In principle, POXs achieve a great deal of oxidation reactions essential for the cells, using H_2O_2 as an electron (e^-) acceptor and variety of substrates as e^- donors (Penel 2000). Many of apoplastic POXs are presumed to be bound to the cell wall by binding to polygalacturonic acid in the Ca^{2+}-induced conformation (Carpin et al. 2001). Recently, such a Ca^{2+}-pectate binding POX has been recognized among *Arabidopsis* POXs (Shah et al. 2004).

Figure 2A summarizes the possible overall interaction between SA and plant POX. As proposed earlier (Kawano et al. 1998; Kawano and Muto 2000), estimated reactions for the generation of $O_2^{\cdot -}$ are as follows:

(1) $N + H_2O_2 \rightarrow I + H_2O$
(2) $I + SA \rightarrow II + SA^{\cdot}$
(3) $II + SA \rightarrow N + SA^{\cdot}$
(4) $2SA^{\cdot} + 2O_2 \rightarrow 2SA^{+} + 2O_2^{\cdot -}$

where N, I, II are native ferric POX and its Compounds I and II, respectively. $SA^{\cdot}$ and SA^{+} are free radical species and the two-electron oxidized intermediate product, respectively. Here, SA acts as the e^- donor while H_2O_2 acts as the e^- acceptor. The released $SA^{\cdot}$ may then react with O_2 to form $O_2^{\cdot -}$. Evidence supporting the production of $SA^{\cdot}$ species has been obtained through an ESR study using ascorbate as a sensitive spin trapper (Kawano and Muto 2000). In Figure 2B, the likely structures of $SA^{\cdot}$ and SA^{+} proposed after our studies, by Gozzo (2003) are shown. Effect of SA on the POX oxidation state was spectroscopically examined using horseradish peroxidase (HRP) as a model enzyme (Kawano et al. 2002a, b) and finally the model presented in Figure 2A was proposed.

Other possible consequence of SA˙ formation is induction of lipid peroxidation. It has been shown that induction of *PR-1* gene expression by SA can be inhibited by diethyldithiocarbamic acid, a compound that converts lipid peroxides into hydroxyl derivatives, and addition of lipid peroxides induces the *PR-1* gene, and most importantly SA-induced production of lipid peroxides was observed *in vitro* (Anderson et al. 1998).

The reactions in the inactive POX (oxygenase) cycle (Figure 2A, left) can be attributed to the chemistry of heme thus common to many of heme proteins, while the active POX cycle (Figure 2A, right) involves Compound I in which the localization of second radical differs in different protein species (it can be on the hemes or amino acid residues). This variation may largely contribute to determination of the types of reaction catalyzed by each protein.

Compound III is the temporally inactive POX intermediate ($–Fe^{II}–O_2$) which is analogous to oxygen-bound hemoglobin that release ROS when auto-oxidized to met-hemoglobin (ferric protein analogous to native POX) (Alayash 1999; Kawano et al. 2002c). Compound III are likely formed from native POX in the presence of $O_2^{\cdot -}$ ($–Fe^{III}+O_2^{\cdot -} \rightarrow –Fe^{II}–O_2$, $–Fe^{III}–O_2^{\cdot -}$). POX can be inactivated also in the presence of high concentration of H_2O_2 *via* formation of Compound III (Kawano et al. 2002a, d). The formation of Compound III from native POX in the presence of ROS therefore mimics the reverse pathway of hemoglobin auto-oxidation. Typical feedback inactivation of POX by POX-mediated production of high concentration of H_2O_2 reported for many plant POXs (Barcelo 1999), might be attributed to this mechanism. When examined with HRP, this temporal inactive form is further converted to irreversibly inactive form known as P-670 in the presence of SA (Kawano et al. 2002a), indicating that SA inactivates the enzyme when the condition matches. The SA-dependent POX inactivation may be a possible explanation for termination of the POX-dependent ROS production in SA-treated tobacco cell culture (Kawano and Muto 2000).

Indole-3-acetic acid (IAA) has much greater activity in inactivation of HRP *via* Compound III (Kawano et al. 2002d), thus both SA and IAA are acting as suicide substrates in the inactive POX cycle, while the active POX cycle utilizes both of them as good substrates.

When the heme and oxygen dissociate from Compound III, $O_2^{\cdot -}$ and the native enzyme are released (Figure 2A). Most heme proteins including French bean POX are capable of generating H_2O_2 (derived from $O_2^{\cdot -}$) at higher pH by a mechanism that involves the formation of Compound III (Bolwell et al. 2002), thus very much different from the SA-dependent oxidative burst.

The missing link in the inactive POX cycle is the mechanism for reduction of native ferric enzyme ($–Fe^{III}$) to ferrous enzyme ($–Fe^{II}$). It is likely that pH-dependent Compound III-mediated ROS production requires the formation of Compound III from ferrous enzyme after oxygenation ($–Fe^{II}+O_2 \rightarrow –Fe^{II}–O_2$), since the alternative path of Compound III formation from native enzyme requires $O_2^{\cdot -}$ or H_2O_2 and thus the sum of ROS consumption ($–Fe^{III}+O_2^{\cdot -} \rightarrow –Fe^{II}–O_2$) and production ($–FeII–O^2 \rightarrow –Fe^{III}+O_2^{\cdot -}$) becomes zero. Therefore, formation of ferrous enzyme from native enzyme is important for supporting the oxidative burst *via* Compound III. Here we would like to propose a likely mechanism for reduction of ferric enzyme to ferrous enzyme at alkaline condition during plant defense mechanism. When the plants are attacked by pathogens or treated with elicitors, extracellular alkalization occurs and thus pH-dependent extracellular POX-mediated oxidative burst proceeds, especially at the site of infection (Bolwell et al. 1998). On the other hand, nitric oxide (NO) also takes place in plant defense responses after pathogen attacks, especially at the site of infection too (Klessig et al. 2000). It is possible that interaction between NO and POXs occurs, since much of the mammalian biology of NO can be attributed to reactions with metal centers such as hemes (Fernandez et al. 2004). One of such reaction is reductive nitrosylation, a process whereby NO-dependent reduction of the metal proceeds at alkaline conditions (pH, >7) as examined in several ferriheme proteins such as met-hemoglobin,

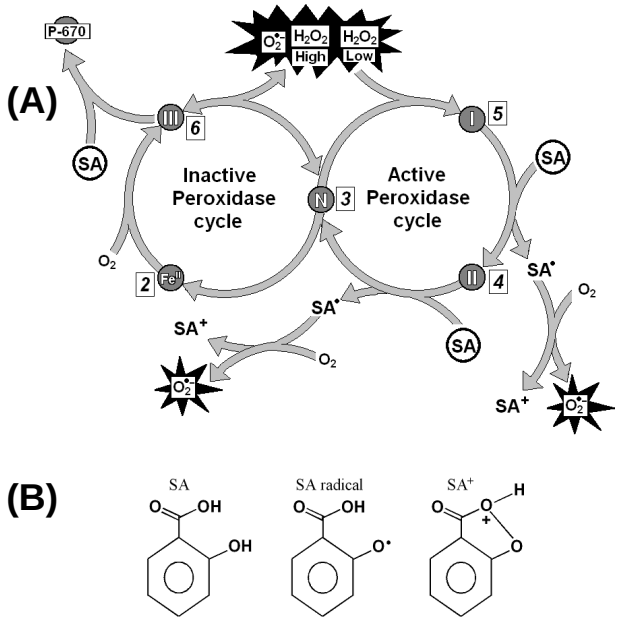

Figure 2. Possible interaction between SA and peroxidase. (A) Generation of ROS by peroxidase reactions and SA-dependent inactivation of the protein. (B) Intermediates of SA during peroxidase-mediated metabolism. N, native ferric enzyme; I, Compound I; II, Compound II; III, Compound III, Fe^{II}, ferrous enzyme; P-670, irreversibly inactive form (verdohemoprotein). Numbers in the squares (2–6), formal oxidation states of heme.

cytochrome *c* and laccase (Fernandez et al. 2004). Recent study showed that *Arabidopsis* nonsymbiotic hemoglobin also interacts with NO (Perazzolli et al. 2004). By analogy, native POX may react with NO and ferrous enzyme should be produced *via* three steps as follows, $-Fe^{III}+NO \rightarrow -Fe^{III}(NO)$ (step 1), $-Fe^{III}(NO)+(OH^-$, alkaline condition$) \rightarrow -Fe^{II}(NO_2H)$ (step 2), $-Fe^{II}(NO_2H) \rightarrow -Fe^{II}+NO^{2-}+H^+$ (step 3). This leads to oxidative burst *via* further two steps, (1) binding with O_2 to form Compound III and (2) dissociation to native POX and $O_2^{\cdot -}$. However, as discussed above, this mechanism may be sensitive to SA-dependent inactivation (Figure 2A).

Search for SA-responsive and ROS-regulated calcium channels

Similarly to SA signaling, some lines of studies have shown possible cascades of abscisic acid (ABA) signaling involving ROS (Apel and Hirt 2004) and calcium (Hetherington and Brownlee 2004). Recent advances in the study of ABA-induced stomatal closure have revealed that ABA action in guard cells is mediated with the generation of ROS and cytosolic free Ca^{2+} (Pei et al. 2000; Murata et al. 2001). In the proposed model worked out through biochemical and patch-clamping experiments, ABA elicits the production of H_2O_2 and, in turn, the resultant H_2O_2 stimulates the opening of Ca^{2+} channel(s), resulting in a rapid increase in $[Ca^{2+}]_c$ (Mori and Schroeder 2004). However, identification of the Ca^{2+} channel(s) responsible for ABA-stimulated (and ROS-dependent) Ca^{2+} current has not been reported to date, despite the search for ROS-responsive Ca^{2+} channel is of common interest to SA researchers.

Increases in $[Ca^{2+}]_c$ can be brought about by lifting up the Ca^{2+} influx rate and/or by lowering the rate of Ca^{2+} efflux, and in most of the cases, the former mechanism contributes to the increase in $[Ca^{2+}]_c$ (Hetherington and Brownlee 2004). Therefore, Ca^{2+}-permeable channels are the key entry points for Ca^{2+} into the cytosolic space, and for initiation of Ca^{2+} signaling (Sanders et al. 2002). As recently reviewed (Hetherington and Brownlee, 2004), the properties of the major groups of plant Ca^{2+}-permeable channels encoded by 41 genes have been surveyed in recent years. At molecular level, the channels reported to date likely belong the cNMP-gated channels (*CNGC*) family, ionotropic glutamate receptor (*iGluR*) family, or two-pore channel 1 (*TPC1*) family, the most likely group of Ca^{2+}-permeable channels involved in ROS-responses.

Recently, Furuichi et al. (2001) have cloned the plant's first gene of candidate encoding a putative voltage-gated channel with high affinity for Ca^{2+} permeation, from *Arabidopsis thaliana*. The channel possesses extremely high homology with a recently cloned *TPC1* (*two pore channel 1*) from rats (Ishibashi et al. 2000), thus designated as *AtTPC1*. It has been shown that *AtTPC1* is expressed in various tissues in *Arabidopsis*. Sense-antisense experiments in *Arabidopsis* and complementation tests in a Ca^{2+} uptake-defective yeast mutant have confirmed that *AtTPC1* functions as a Ca^{2+} channel (Furuichi et al. 2001). Recent studies have shown that *TPC1* channels can be found in other plant species, such as rice (Hashimoto et al. 2004; Kurusu et al. 2004) and tobacco BY-2 cells (Kadota et al. 2004). Tobacco BY-2 cells posses two *TPC1* orthologs highly identical to *AtTPC1*, designated as *NtTPC1A* and *B* (Kadota et al. 2004), and these channels are likely acting as elicitor-responsive Ca^{2+} channels.

AtTPC1 possesses two conserved homologous domains, each contains six transmembrane segments (S1–S6) and a pore loop between S5 and S6 (Furuichi et al. 2001) (Figure 3A), and shown to be localized on the plasma membrane (Figure 3B).

Recently, Kawano et al. (2004b) conducted a series of overexpression and cosuppression experiments using transgenic cell lines of tobacco BY-2 cells transformed with the *AtTPC1*-encoding vector construct, and

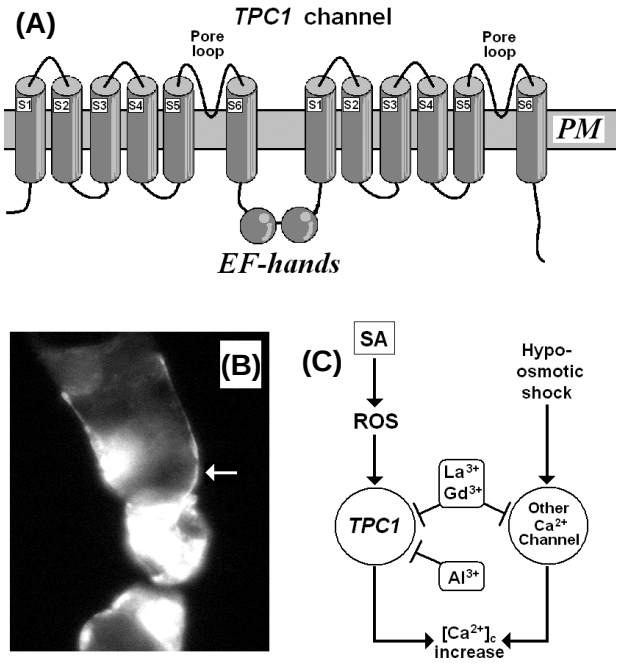

Figure 3. Possible involvement of *TPC1* channels in SA signaling in plant cells (modified from Kawano et al. 2004). (A) Likely structure of GFP-fused *AtTPC1* protein embedded in plasma membrane (PM). Transmembrane segments (S1–S6) and pore loops are indicated. (B) Localization of GFP-fused *AtTPC1* to plasma membrane of tobacco BY-2 cells (Kawano et al. 2004b). An arrow indicates the presence of GFP-fused *AtTPC1* in cell peripheral. *AtTPC1* was also abundant in the internal membranes. (C) A hypothetical model for explaining the *TPC1*-specific action of Al^{3+}. There may be two distinct groups of Ca^{2+} channels differed in sensitivity to Al^{3+}. The Al^{3+}-sensitive group represented by *TPC1* may contribute to the SA-induced and ROS-mediated Ca^{2+}-influx. Other Al^{3+}-insensitive group may participate in the hypoosmotic response.

demonstrated that plant *TPC1* channels are the only ROS-responsive Ca^{2+} channels, known in plants to date. In addition, it has been shown that Al^{3+} may be a novel channel blocker specific for ROS-responsive influx (Kawano et al. 2003) mediated by *TPC1* Ca^{2+} channels (Kawano et al. 2004b). The *TPC1* channel-stimulating ROS includes $O_2^{\cdot -}$ and H_2O_2 (Kawano et al. 2003). Therefore, Al^{3+}, in addition to lanthanides (such as $La^{3+}$and Gd^{3+}), may be a useful and strong tool to study the role of ROS-responsive *TPC1* Ca^{2+} channels. Use of Al enables the dissection of *TPC1*-mediated ROS-resoposive Ca^{2+} influx without affecting the Ca^{2+} influx stimulated by other stimuli (e.g. hypoosmotic shock, Figure 3C).

Above studies are highly implicative of the possible involvement of plant *TPC1* channels in SA-induced and ROS-mediated Ca^{2+} influx in plant cells. At present, we are accumulating the data for *TPC1*'s roles in SA signaling pathway based on the most likely working hypothetical model shown in Figure 3C. The inhibitory effect of $AlCl_3$, a putative and selective inhibitor of *TPC1* channels, acting against the SA-induced Ca^{2+} influx was confirmed in tobacco BY-2 cells (Kawano unpublished results). In addition, ineffectiveness of SA and H_2O_2 in induction of Ca^{2+} influx was confirmed in transgenic cell lines of *Arabidopsis* and tobacco BY-2 cells with suppressed *TPC1* expression (Furuichi, unpublished results). The roles of plant *TPC1* channels in overall SA signaling in regards to defense response against the pathogens, and their involvement in other redox-mediated Ca^{2+} signaling such as ABA signaling and oxidative stress responses remain to be determined.

Calmodulin (CaM) and calcium-dependent protein kinase (CDPK)

A possible mediator of SA-dependent Ca^{2+} signaling may be a class of CaM specifically induced by SA and HR-inducing treatment (Yamakawa et al. 2001). Similarly in soybean plants, expression of specific CaM isoforms (*SCaM-4* and *5*) among several *SCaM* genes were shown to be activated by infection or pathogen-derived elicitors, and participate in Ca^{2+}-mediated disease resistance responses (Heo et al. 1999). The induction of *SCaM-4* and *5* was shown to be highly dependent on the increase in $[Ca^{2+}]_c$. Since constitutive expression of these soybean CaMs in tobacco plants resulted in development of lesion and activation of SAR-associated genes without requirement for SA (examined with *NahG* experiments), their report concluded that SA is not involved in SAR gene activation by *SCaM-4* or *SCaM-5*, and these CaMs are components of a SA-independent signal transduction chain leading to disease resistance. Actually, the former part of conclusion is correct, but the latter part of conclusion sounds incorrect. For obtaining such conclusion, the tests to eliminate the possible involvement of SA in induction of CaM-dependent SAR must be carried out by showing inability of SA to induce such CaMs in soybean plants. Since Yamakawa et al. (2001) have shown examples of SA-inducible CaMs in tobacco, and Heo et al. (1999)'s work has clearly shown that induction of *SCaM-4* and *5* is mediated by $[Ca^{2+}]_c$ increase, it is tempting to speculate that such Ca^{2+}-inducible CaMs capable of inducing SAR may function downstream of SA-induced Ca^{2+} influx.

As discussed earlier, PAL is a key enzyme in SA production *via* cinnamate pathway (Figure 1A). It is known that SA induces the increase in PAL activity (e.g. Messner and Schroder 1999), and induction of PAL by various stimuli can be inhibited by Ca^{2+} channel blockers (Klessig et al. 2000). An attempt to place PAL in the regulatory circuit involving Ca^{2+} and protein phosphorylation has been conducted (Cheng et al. 2001). A CDPK from *Arabidopsis* (*AtCPK1*) but not other closely related protein kinases, is reportedly capable of PAL phosphorylation. Identification of this CDPK as a PAL kinase is indicative of the possible regulation of SA biosynthesis through the changes in $[Ca^{2+}]_c$. Therefore, SA-dependent modulation of SA production might be possible *via* induction of Ca^{2+} influx. The link between PAL activity and Ca^{2+} signaling must be further investigated.

SA and ROS, case studies

The SA-induced POX-dependent $O_2^{\cdot -}$ generation was also examined in *Vicia faba* epidermis (Mori et al. 2001). Addition of SA (Manthe et al. 1992; Lee 1998) and the SA-induced $O_2^{\cdot -}$ and the chemically generated $O_2^{\cdot -}$ (Mori et al. 2001) were shown to induce closure of stomata, which is known as a Ca^{2+}-dependently regulated event (Hedrich et al. 1990; Fell et al. 2000). Taken together, action of SA may be mediated both by cell wall POX-dependent ROS production and Ca^{2+} influx. Since some fungal and oligosaccharide elicitors capable of ROS induction are active in induction of stomatal closure when applied to epidermal peels (Lee et al. 1999), plants may respond to such defense-related signals by closing the stomata for limiting further fungal invasion (i.e. restricting the access of air-borne fungal spores to the internal tissues).

In induction of gene expression, SA targets the SA-responsive promoters, such as the *activation sequence-1* (*as-1*) like promoter element found in some redox-related genes such as several glutathione *S*-transferase genes, through induction of oxidative burst (Garreton et al. 2002). However, involvement of H_2O_2 was eliminated since treatment with H_2O_2 and/or 3-amino-1,2,5-triazole thought to mimic the action of SA by inhibiting catalase,

resulted in no activation of *as-1*-dependent gene expression, and instead, the inhibitor strongly inhibited the action of SA in induction of *as-1*-regulated gene expression (Garreton et al. 2002). Therefore, involvement of other ROS such as $O_2^{\cdot -}$ may have specific role distinct from that of H_2O_2. In addition, the mechanisms proposed in earlier days of SA research, emphasizing the SA-dependent catalase inhibition, was further questioned, while the importance of $O_2^{\cdot -}$ and other ROS in SA action was further confirmed. Interestingly, the *as-1* element is responsive to auxin too (Garreton et al. 2002). Since natural auxin IAA reportedly induces ROS production in some plant culture (Pfeiffer and Hoftinger 2001) and via a certain type of plant POX reaction (Kawano et al. 2001), the likely common intermediate signal for activation of *as-1* element by SA and IAA is ROS although the mechanism of POX reaction involving IAA is very differed from SA-related mechanisms (Kawano 2003).

Controlled SA excretion by ROS and calcium

Control of local and systemic SA levels requires the processes of SA movements in and out of the cells, tissues and organs. According to earlier works, in pathogen-infected plants, SA is thought to move to upper non-infected leaves from lower infected leaves through phloem (Métraux et al. 1990; Rasmussen et al. 1991; Yalpani et al. 1991). Mölders et al. (1996) showed that radioactivity of ^{14}C-labeled benzoic acid fed together with tobacco necrosis virus (TNV), to cotyledons of cucumber seedlings can be translocated through phloem to upper leaves only after conversion to ^{14}C-SA, indicating that SA rather than benzoic acid is the translocatable form. As a result, the SA derived from the site of virus inoculation to the upper young leaves induced the development of SAR.

Recent study has shown that translocation of SA is unexpectedly rapid when artificially applied onto the cut end of petiole from young and adult tobacco plants (Ohashi et al. 2004). When the spread of radioactive signal of ^{14}C-labeled SA was autoradiographically monitored after feeding of 1–10 μl of 60 μM–2 mM ^{14}C-SA to the petiole end of the adult plants with 13 expanded leaves, the signal reached the 6 neighboring upper leaves and 3 adjacent lower leaves in 10 min, and accumulated throughout the plant body in further 50 min in each plant treated.

As illustrated in Figure 4, Ohashi and her colleagues have demonstrated that the majority of SA may migrate as free form rather than glucosylated form, especially in the early phase of SA translocation examined within 10 min after SA addition.

When the ^{14}C-SA solution was placed onto the surface

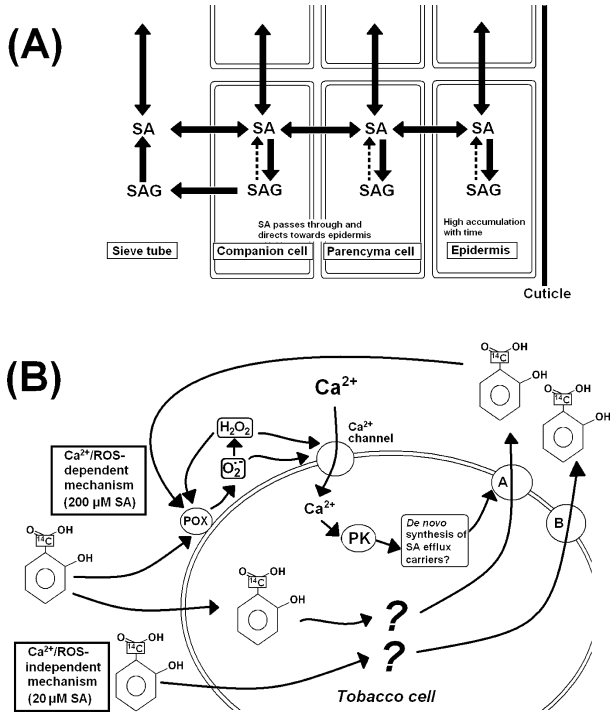

Figure 4. Possible mechanisms for SA translocation at cell level. (A) Models for SA translocation in plants, largely modified from Seo et al. (1995) and Ohashi et al. (2004). Vertical movement of SA involving vascular transport and cell-to-cell transport is faster than horizontal movement of SA. In horizontal SA movement, cell-to-cell exchange of SA with preferential direction towards epidermis takes place. The cuticle layer is hardly permeable to SA under the physiological pH. Thick arrows represent the massive and rapid movement or interconversion (to SAG) of SA. Thin and broken arrows indicate slower and minor SA movements. (B) SA-regulated and SA-independent excretion of SA. This model proposed by Chen et al. (1999, 2001) is mimicking the cells during cell-to-cell SA translocation in which SA pass-by each cell. When the cells are exposed to high dose of SA (200 μM), *de novo* induction of SA excretion mechanism takes place. This mechanism reportedly requires the generation of ROS and subsequent Ca^{2+} and protein phosphorylation signaling cascades. The SA excretion mechanism responsible for low-dose SA (20 μM), is constitutively active independent of ROS, Ca^{2+} and protein phosphorylation, requiring no de *novo* protein synthesis. PK, protein kinase.

of the tobacco leaves without piercing the cuticle layers, almost no signal was shown to be translocated within 1 h, indicating that the barriers at the surface of leaves are high enough for preventing the passive penetration of SA (Ohashi et al. 2004). Niederl et al. (1998) has shown that ^{14}C-SA can penetrate through the path on the cuticle that is utilized for water penetration, and this path allows no or negligible level of SA transport at normal physiological range of pH (>3, ≤ 6). In addition, the suggested form of SA active in cuticular penetration is methyl SA. Thus, conversion of SA to methyl SA which pH-independently diffuses in and out across the outer physical barriers of plants, may affect the level of total SA derivatives in plants.

Since SA is produced inside the cells, the first step in SA movement is excretion of SA by the cells. Chen et al. (1999, 2001) have reported that ^{14}C-labeled free SA (200 μM) added to tobacco cell suspension was readily taken up by the cells within 5 min, and interestingly majority (over 90%) of the radioactivity was released back to the extracellular medium as free SA in the following 5 h.

Possible mechanisms for SA-regulated and SA-independent excretion of SA at cell level were proposed by Chen et al. (1999, 2001) as shown in Figure 4B. This model is mimicking each component cell during cell-to-cell SA translocation in which SA pass-by each cell. When the cells are exposed to high dose of SA (200 μM), *de novo* induction of SA excretion mechanism reportedly takes place (Chen et al. 2001). This mechanism requires ROS production and subsequent Ca^{2+} and protein phosphorylation signaling cascades. On the other hand, there may be an alternative SA transport mechanism, when low-dose SA (20 μM) is applied. The SA excretion mechanism responsible for low-dose SA, is constitutively active independent of ROS, Ca^{2+} and protein kinase, requiring no *de novo* protein synthesis. These data indicate the presence of two distinct SA carrier(s) constitutively presents and newly produced in response to exogenous SA.

Interestingly, induction of a PR-protein (protein N) is differently regulated by the respective signaling mechanisms corresponding to high- and low-dose SA excretion controls (Chen et al. 2002). The higher SA (200 μM)-dependent mechanism is likely relaying the SA signal to induce Protein N through ROS, Ca^{2+} and protein phosphorylation, while lower SA (20 μM)-dependent mechanism induces protein N independently from above signaling events.

Since the works of Ohashi et al. (2004) used the ^{14}C-SA (60 μM-2 mM) for feeding plants, the cells closed to the feeding points were likely exposed to high range of SA concentration, thus it is possible that SA uptake and excretion may depend on the Ca^{2+}/ROS-regulated mechanisms (Chen et al. 1999, 2001). In the cells distant from the site of SA feeding may be responding to the low-dose SA *via* Ca^{2+}/ROS-independent mechanism.

In mammalian systems, presence of carrier-mediated SA absorption mechanism has been elucidated by tracing the fate of ^{14}C-SA (Utoguchi et al. 1999; Emoto et al. 2002). It was shown that the mechanism supporting the uptake of SA by animal cells and tissues both *in vivo* and *in vitro* are pH gradient-dependent, requiring low extracellular pH and higher intracellular pH, thus sensitive to protonophores and NaN_3. In the future, the mechanisms for SA uptake must be examined since above studies strongly suggest that plant cells possess such SA carriers.

Note added at the galleey proof

AtBT1-5, novel plant-specific CaM-binding proteins were shown to interact with transcriptional regulators belonging to fsh/Ring3 class, and nobably, all *AtBT* homologs are inducible by SA and H_2O_2 (Du and Poovaiah, 2004), thus supporting the view that early SA action involving ROS and Ca^{2+} may govem the trancscripsion via expression and activation of these proteins.

Acknowledgements

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