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Source: The Arabidopsis Book, 2011(9)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0156

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First published on December 20, 2011: e0156. doi: 10.1199/tab.0156

Salicylic Acid Biosynthesis and Metabolism

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Salicylic acid (SA) has been shown to regulate various aspects of growth and development; it also serves as a critical signal for activating disease resistance in *Arabidopsis thaliana* and other plant species. This review surveys the mechanisms involved in the biosynthesis and metabolism of this critical plant hormone. While a complete biosynthetic route has yet to be established, stressed Arabidopsis appear to synthesize SA primarily via an isochorismate-utilizing pathway in the chloroplast. A distinct pathway utilizing phenylalanine as the substrate also may contribute to SA accumulation, although to a much lesser extent. Once synthesized, free SA levels can be regulated by a variety of chemical modifications. Many of these modifications inactivate SA; however, some confer novel properties that may aid in long distance SA transport or the activation of stress responses complementary to those induced by free SA. In addition, a number of factors that directly or indirectly regulate the expression of SA biosynthetic genes or that influence the rate of SA catabolism have been identified. An integrated model, encompassing current knowledge of SA metabolism in Arabidopsis, as well as the influence other plant hormones exert on SA metabolism, is presented.

INTRODUCTION

Salicylic acid (SA, 2-hydroxy benzoic acid) is one of a diverse group of phenolic compounds, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesized by plants. Until fairly recently, these phenolic compounds were presumed to be non-essential for critical processes common to all organisms, and were therefore relegated to the category of "secondary metabolites" (Hadacek et al, 2011). However, the concept that these compounds are of lesser importance is coming under growing criticism, since phenolics are involved in various plant processes, including lignin biosynthesis, regulation of responses to abiotic stresses, allelopathy, and disease resistance (Malamy and Klessig, 1992; Raskin, 1992; Métraux and Raskin, 1993; Humphreys and Chapple, 2002). Indeed, SA has been shown to regulate various aspects of plant growth and development, as well as play key signaling roles in thermogenesis and disease resistance (Vlot et al, 2009).

The first suggestion that SA is an endogenous signal was based on the discovery that SA is present in the phloem of flowering, but not vegetative, *Xanthium strumarium* L. and is capable of inducing flowering in *Lemna gibba* G3 (Cleland, 1974; Cleland and Ajami, 1974). Subsequent studies have yielded conflicting results regarding SA's role in flowering induction. However, the recent demonstrations that i) flowering is delayed in SA-deficient *Arabidopsis thaliana* mutants (Columbia [Col] background; Martínez et al, 2004), ii) UV-C light accelerates flowering transition in an SA-dependent manner (Martínez et al, 2004) and iii) the early flowering phenotype of the SUMO E3 ligase mutant *siz1* is dependent on elevated levels of SA (Jin et al, 2008) suggest that SA does impact this process in Arabidopsis.

Conclusive evidence that SA is a plant hormone initially came from the demonstration that SA regulates thermogenesis (heat production) in the reproductive structures of cycads and the flowers of certain angiosperms (Raskin, 1992; Vlot et al, 2009). SA stimulates thermogenesis primarily by inducing the expression of alternative oxidase, which in turn enhances the capacity of the mitochondrial alternative respiratory pathway. This pathway, unlike the cytochrome respiratory pathway, produces ATP at only one step; the remaining potential energy is released as heat. Interestingly, SA treatment also regulates alternative oxidase expression and/or alternative respiration in non-thermogenic plant species, including Arabidopsis (Clifton et al, 2005) and *Nicotiana tabacum* (tobacco; Norman et al, 2004 and references therein).

A few years after SA was shown to regulate thermogenesis, analyses of plants responding to microbial pathogen infection revealed another function: SA is a key signal for the activation of disease resistance. Currently, the model for plant disease resistance

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can be envisioned as a 'zig-zag', in which the interplay between plant and pathogen leads to continued escalation of defenses and counter-defenses, respectively (Chisholm et al, 2006; Jones and Dangl, 2006; Nishimura and Dangl, 2010). After a potential pathogen has penetrated the leaf or root surface and cell wall, it encounters extracellular surface receptors that recognize pathogen-associated molecular patterns (PAMPs). If recognition occurs, a variety of defenses are activated in association with PAMP-triggered immunity (PTI, formerly called basal resistance). While PTI is sufficient to prevent further colonization by many microbes, some pathogens have evolved effectors which suppress PTI. Whether such a pathogen can successfully infect the host plant is then determined by whether the plant expresses a resistance (R) protein that directly or indirectly recognizes one of these effectors (also termed avirulence (avr) factors) to induce effector-triggered immunity (ETI). In the absence of this avr – R protein interaction, or if the pathogen produces additional effectors that suppress ETI, the virulence of the pathogen on its host will be promoted.

Following PAMP- or *R* gene-mediated pathogen recognition, a variety of defense responses are activated in the inoculated leaf, including production of reactive oxygen species (ROS), accumulation of SA, and increased expression of pathogenesis-related (*PR*) genes (Tsuda et al, 2008 and references therein; Vlot et al, 2009). ETI also is frequently associated with the development of a hypersensitive response (HR), in which necrotic lesions form at the site(s) of pathogen entry. Subsequent to these responses in the inoculated leaf, both PTI and ETI can trigger systemic SA accumulation and *PR* gene expression, as well as a long-lasting, broad-based resistance to subsequent pathogen infection known as systemic acquired resistance (SAR; Durrant and Dong, 2004; Mishina and Zeier, 2007; Vlot et al, 2009). Although it is currently unclear how substantially the signaling pathway(s) leading to PTI and ETI overlap, SA plays a critical role in activating both forms of resistance (Mishina and Zeier, 2007; Tsuda et al, 2008;Vlot et al, 2009).

The first suggestion that SA functions as an endogenous signal for plant disease resistance was made by White and coworkers (White, 1979; Antoniw and White, 1980), who demonstrated that injecting SA into the leaves of tobacco plants induced PR protein accumulation and enhanced resistance to infection by Tobacco Mosaic Virus (TMV). SA treatment has since been shown to induce *PR* gene expression and/or enhance resistance in many plant species, and increased levels of endogenous SA correlate with the activation of local and/or systemic defense responses (Raskin, 1992; Klessig and Malamy, 1994; Vlot et al, 2009). Stronger evidence supporting SA's role in signaling resistance came from analyses of Arabidopsis and tobacco that accumulated little to no SA due to i) expression of the bacterial *nahG* gene, which encodes SA-degrading salicylate hydroxylase or ii) altered expression/mutation of genes that impact SA biosynthesis (see below; Gaffney et al, 1993; Delaney et al, 1994; Mauch-Mani and Slusarenko 1996; Pallas et al, 1996; Nawrath and Métraux, 1999; Dewdney et al, 2000). These plants exhibited enhanced susceptibility to virulent and avirulent pathogens, and resistance was restored by treatment with SA or its analogs.

In addition to local resistance, systemic *PR* gene expression and/or SAR development were suppressed in SA-deficient tobacco and Arabidopsis (Gaffney et al, 1993; Delaney et al, 1994; Vernooij et al, 1994; Pallas et al, 1996; Nawrath and Métraux, 1999). This observation raised the possibility that SA is a mobile signal that is generated in the inoculated leaves and transmitted via the phloem to the systemic leaves, where it induces SAR. To assess this possibility, chimeric tobacco containing rootstocks and scions derived from wild type (wt) or SA-deficient plants were constructed (Vernooij et al, 1994; Pallas et al, 1996). SAR was fully suppressed in chimeric plants containing an SA-deficient scion, indicating that SA must accumulate in the uninfected tissue to signal systemic defenses. However, SA does not appear to be the mobile signal because SAR developed in wt scions grafted on to SA-deficient rootstocks. Recent studies have indicated that a methylated derivative of SA, methyl salicylate (MeSA), serves as a critical phloem-mobile SAR signal (see below).

Efforts to elucidate the mechanism(s) through which SA signals disease resistance have uncovered a complex network of upstream and downstream components. In this review, we discuss the mechanisms of SA biosynthesis and modification, as well as the upstream components that regulate SA accumulation in Arabidopsis. For a discussion of signaling processes downstream of SA, the reader is referred to several recent reviews (Garcion and Métraux, 2006; Lu, 2009; Vlot et al, 2009). The authors also note that a variety of plant species have been, and continue to be, used to investigate SA metabolism and SA's role in mediating responses to (a)biotic stress.While a full discussion of SA research in plants other than Arabidopsis is beyond the scope of this review, we have included important findings from other species in each section.

SA BIOSYNTHESIS

Efforts to elucidate the SA biosynthetic pathway in plants have uncovered evidence for two distinct pathways, the isochorismate (IC) pathway (Route 1) and the phenylalanine ammonia-lyase (PAL) pathway (Route 2). Both of these pathways originate from chorismate, the end product of the shikimate pathway (Figure 1; Table 1). However, to date neither biosynthetic route has been fully defined. Here, we provide historical context for our current understanding of the relative contributions of the IC and PAL pathways to SA synthesis in Arabidopsis.

The PAL Pathway To SA

Phenylalanine ammonia-lyase (EC 4.3.1.5), the first enzyme in the phenylpropanoid pathway, converts phenylalanine (Phe) to *trans*-cinnamic acid (*t*-CA) and NH_3 via a non-oxidative deamination reaction (Raes et al, 2003; Rohde et al, 2004). *t*-CA is a precursor for the biosynthesis of diverse phenolic compounds; in Arabidopsis, these include lignin, lignans, flavonoids (which include UV-absorbing, anti-microbial, and anti-oxidant compounds), volatile benzenoid esters and benzoylglucosinolates (Figure 1). Thus, by removing Phe from the pool of aromatic amino acids to generate *t*-CA, PAL serves as a critical regulator between primary and secondary metabolism. Arabidopsis has been shown to contain four *PAL* genes that encode enzymes with varying kinetic and physical properties (Cochrane et al, 2004).

Early feeding studies with radio-labelled Phe, *t*-CA, or benzoic acid (BA) suggested that SA is synthesized from Phe via *t*-CA, which is then converted to SA via two possible intermediates: *ortho*-

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coumaric acid (Route 2a) or BA (Routes 2b&c), depending on the plant species (Klämbt, 1962; El-Basyouni et al, 1964; Chadha and Brown, 1974). In the 1990s, the discovery that SA plays a critical role in signaling defense responses and thermogenesis brought renewed interest in elucidating the SA biosynthetic pathway (Malamy and Klessig, 1992; Raskin, 1992; Dempsey et al, 1999). Using pathogen-inoculated tobacco and cucumber, elicitor-treated potato, or healthy rice seedlings, radio-labelling studies suggested that SA was synthesized from Phe via BA (Yalpani et al, 1993; Meuwly et al, 1995; Silverman et al, 1995; Mölders et al, 1996; Coquoz et al, 1998). It should be noted, however, that while incorporation of a radio-labelled precursor allows one to determine whether a predicted pathway is possible, this result may be misleading as it may not reflect *in planta* metabolism. Additional evidence supporting a role for PAL in SA biosynthesis came from the combined demonstrations that tobacco and Arabidopsis resisting pathogen infection exhibit increases in *PAL* expression (Pellegrini et al, 1994; Mauch-Mani and Slusarenko, 1996) and endogenous SA levels (Dempsey et al, 1999). Furthermore, loss of PAL activity, due to sense-suppression or treatment with the PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP), reduced pathogen-induced SA accumulation in tobacco, cucumber and Arabidopsis (Meuwly et al, 1995; Mauch-Mani and Slusarenko, 1996; Pallas et al, 1996). Based on these combined results, it was commonly accepted that SA synthesis occurred via the PAL pathway (Raskin, 1992; Dempsey and Klessig, 1995; Lee et al, 1995; Coquoz et al, 1998).

Subsequent studies investigated the conversion of *t*-CA to BA. As shown in Figure 1, plants can potentially utilize three biosynthetic routes to BA, including a β-oxidation route from cinnamoyl Co-A (designated Route 2c-1), a non-oxidative route from cinnamoyl Co-A (Route 2c-2), and a non-oxidative route from *t*-CA to BA (Route 2b; Wildermuth, 2006 and references within). Radio-labelling studies using Phe or putative pathway intermediates performed in TMV-infected tobacco, smoke-treated *N. attenuata* (Torrey; coyote tobacco), or cucumber detected incorporation of radio-label into BA and SA but not benzaldehyde, suggesting that SA is synthesized by the cinnamoyl-CoA β-oxidation route (Route 2c-1; Ribnicky et al, 1998; Jarvis et al, 2000). Similar studies have not been used to probe downstream components of SA synthesis via PAL in Arabidopsis. However, investigation of BA production in developing seeds identified an Arabidopsis Aldehyde Oxidase 4 (AAO4, encoded by *At1g04580*) that catalyzes the conversion of benzaldehyde to BA, which is then incorporated into benzoylglucosinolates (Ibdah et al, 2009). Further studies will be required to assess whether benzaldehyde serves as an intermediate for SA synthesis in Arabidopsis.

The conversion of BA to SA has been proposed to occur via an inducible BA 2-hydroxylase (BA2H; Figure 1). In tobacco, increases in BA2H activity paralleled or preceded SA accumulation induced by TMV infection, UV exposure, or treatment with BA or hydrogen peroxide (León et al, 1993; Yalpani et al, 1994; León et al, 1995a); partial purification of this activity indicated that it was a P450 monooxygenase (León et al, 1995b). Although there has been no subsequent publication describing a BA2H-encoding gene in plants, Arabidopsis leaves treated with the neonicotinoid metabolite 6-chloropyridinyl-3-carboxylic acid (CPA) exhibited a similar activity, converting CPA to the SA mimic 2-HO-CPA *in planta* (see Figure 1 in Ford et al, 2010). Alternatively, the glucose-conjugated ester of BA might act as an intermediate for the synthesis of the SA glucose ester and SA, as suggested by studies in poplar (Ruuhola and Julkunen-Titto, 2003) and tobacco (Chong et al, 2001).

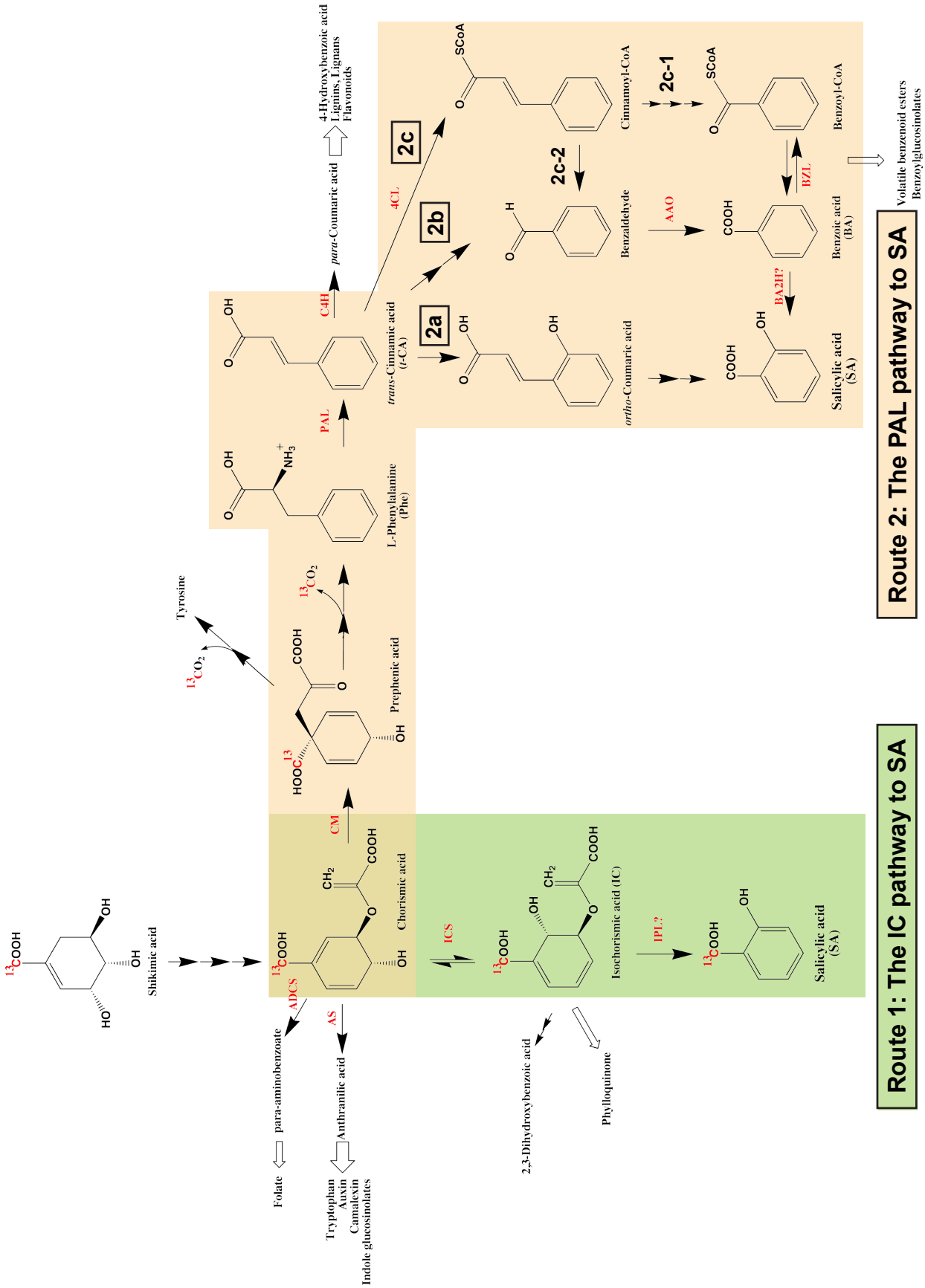
The IC Pathway To SA

In some of the radio-labelling studies described above, labelled precursor incorporation into SA was lower than expected, particularly following infection/induction (e.g. Chada and Brown, 1974; Yalpani et al, 1993; Coquoz et al, 1998). At the time, the reduced specific activity of [^{14}C]SA following pathogen/elicitor treatment was generally thought to be due to increased synthesis of unlabelled precursor (e.g. BA) with treatment. However, these results could also be explained by pathogen/elicitor induction of an alternate SA biosynthetic pathway. Consistent with this latter possibility, AIP-mediated inhibition of PAL reduced chemical- or pathogen-induced SA accumulation by several fold in potato or Arabidopsis, respectively, but did not fully suppress it (Mauch-Mani and Slusarenko, 1996; Coquoz et al, 1998).

Several genera of bacteria have been shown to synthesize SA, which is employed in the production of iron-chelating siderophores (Garcion and Métraux, 2006). In the bacterial pathway, chorismate is converted to SA through an IC intermediate (Verberne et al, 1999). In some bacterial species, such as *Pseudomonas aeruginosa* and *P. fluorescens*, a unifunctional enzyme, isochorismate synthase (ICS, EC 5.4.4.2), isomerizes chorismate to IC; IC is then converted to SA and pyruvate by another unifunctional enzyme, isochorismate pyruvate lyase (IPL; Serino et al, 1995; Mercado-Blanco et al, 2001). By contrast, SA synthesis in *Yersinia enterocolitica* and *Mycobacterium tuberculosis* is mediated by a single, bifunctional enzyme (termed SA synthase; SAS) that directly converts chorismate to SA via an isochorismate intermediate (Pelludat et al, 2003; Kerbarh et al, 2005; Harrison et al, 2006). The ICS and SAS enzymes are structurally very similar and contain conserved active sites, making an enzyme assignment based on sequence alone precarious (Harrison et al, 2006; Kerbarh et al, 2006; Kolappan et al, 2007; Parsons et al, 2008). ICS and SAS begin with nucleophilic attack at C2 of chorismate, with water as the nucleophile, concomitant with displacement of the C4 hydroxyl group in an S_N2 reaction (He et al, 2004). For SAS, this is followed by elimination of pyruvate and release of SA.

In plants, chorismate is synthesized in the plastid (Poulsen and Verpoorte, 1991; Schmid and Amrhein, 1995). This observation, plus the fact that many plastid-localized pathways are derived from prokaryotic endosymbionts, raised the possibility that plants also utilize a chorismate/IC pathway for SA synthesis (Verberne et al, 1999; Wildermuth et al, 2001). To assess whether plants contain an endogenous pathway that synthesizes SA from IC, Wildermuth et al. (2001) identified two putative *ICS* genes in the newly sequenced Arabidopsis genome. *ICS1* (*At1g74710*) and *ICS2* (*At1g18870*) share 83% identity at the amino acid level and are 57% identical to a *C. roseus* ICS whose activity was confirmed biochemically (van Tegelen et al, 1999a). Transcripts for *ICS1*, but not *ICS2*, accumulated in the inoculated leaves following infection with the fungal biotroph *Golovinomyces orontii* (formerly called *Erysiphe orontii*) or a virulent strain of the bacterial hemi-biotroph *P. syringae* pv. *maculicola* (*Psm*; Wildermuth et

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al, 2001). *ICS1* expression correlated with SA accumulation and expression of the SA-associated *PR-1* gene. Moreover, two Arabidopsis mutants, *SA induction-deficient* (*sid*)*2-1* (Nawrath and Métraux, 1999) and *enhanced disease susceptibility* (*eds*)*16-1/sid2-2* (Dewdney et al, 2000), which accumulate only 5-10% the level of SA detected in wt plants following infection by virulent or avirulent pathogens, were found to contain lesions in the *ICS1* gene (Wildermuth et al, 2001). Subsequent analyses revealed that *ICS1* transcripts also accumulate in response to a variety of (a)biotic stresses, including UV light, ozone, PAMPs, (hemi)-biotrophic pathogens and exogenous SA treatment (Ogawa et al, 2005; Nobuta et al., 2007; Harrower and Wildermuth, 2011; Okrent et al., 2011; AtGenExpress: Abiotic and Biotic Stress Experiments (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp; Kilian et al., 2007; Postel et al. 2010)). Interestingly, overexpression of bacterial ICS- and IPL-encoding

Table 1. Summary of key Arabidopsis enzymes discussed in SA synthesis and modification sections.

Locus number	Protein	Catalyzed reaction	Specific Activity[a] (nmol $min^{-1}mg^{-1}$)	K_m[b] (μM)
At1g74710	ICS1	Chorismate → Isochorismate	241	41.5
At1g18870	ICS2	Chorismate → Isochorismate	ND	ND
At2g37040	PAL1	Phenylalanine → *trans*-cinnamic acid	330	68
At3g53260	PAL2		630	64
At5g04230	PAL3		24	2560
At3g10340	PAL4		594	71
At2g43840	UGT74F1	SA → SAG	347.4	230
At2g43820	UGT74F2/ SGT1	SA → SGE SA → SAG	62.4 6	190
At3g11480	BSMT1	SA → MeSA	ND	16
At2g23620	MES1	MeSA → SA	2.89×10^4	57.3
At2g23600	MES2		1.77×10^3	ND
At2g23580	MES4		1.61×10^4	ND
At2g23560	MES7		1.28×10^4	ND
At4g37150	MES9		2.59×10^4	147.1
At4g27260	GH3.5/ WES1	SA → SA-Asp	ND	ND
At2g03760	SOT12	SA → SA-SO_3	0.16	440

[a]Specific activities were taken from publications (see text for references) and recalculated to nmol min^{-1} mg^{-1} assuming a linear ratio between activity per second and per minute and between activity per μg and per mg. [b]Apparent K_m values are given (see text for references). For SA modifying enzymes, activity on SA is shown; note that these enzymes can also use other substrates (see text). Abbreviations are as follows: isochorismate synthase 1 and 2 (ICS1/2), phenylalanine ammonia-lyase 1-4 (PAL1-4), UDP-glucosyltransferases 74F1 and 74F2 (UGT74F1/2), salicylic acid glucosyltransferase 1 (SGT1), salicylic acid (SA), SA 2-*O*-β-D-glucoside (SAG), salicylate glucose ester (SGE), benzoic acid/salicylic acid carboxyl methyltransferase 1 (BSMT1), methyl salicyliate (MeSA), methyl esterase 1, 2, 4, 7 and 9 (MES1/2/4/7/9), GH3 acyl adenylase family member 3.5 (GH3.5), WESO 1 (WES1), salicyloyl-L-aspartate (SA-Asp), sulphotransferase 12 (SOT12), not determined (ND).

Figure 1. Potential pathways for the biosynthesis of salicylic acid in plants.

The isochorismate (IC) pathway (Route 1, green) is the primary route for SA production in *Arabidopsis thaliana*. The phenylalanine ammonia-lyase (PAL) pathway from *trans*-cinnamic acid (Route 2, tan) has been implicated in SA synthesis in a number of species and plays a minor role, either directly or indirectly, in SA production in Arabidopsis. Pathway products branching from precursors and intermediates in the proposed SA biosynthetic pathway are shown, with focus on Arabidopsis compounds. Open arrows indicate flux to these pathways, with larger arrows indicating greater flux. Results from studies in *C. roseus* using labeled glucose are consistent with the bulk of induced SA being synthesized via the IC pathway, with retention of the ^{13}C label in SA (shown above). For the PAL pathway, there are a number of possible routes to SA (2a, 2b, 2c-1, 2c-2). Synthesis of SA from BA could also include glycosylated intermediates (not shown). Enzymes (red) are abbreviated as follows: aldehyde oxidase (AAO), 4-amino-4-deoxychorismate synthase (ADCS), anthranilate synthase (AS), benzoic acid 2-hydroxylase (BA2H), benzoyl-CoA ligase (BZL), cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), chorismate mutase (CM), isochorismate synthase (ICS), isochorismate pyruvate lyase (IPL), and phenylalanine ammonia-lyase (PAL). Enzymes involved in modification of SA are not included (see Figure 2). For details refer to the text.

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genes (either co-expressed or fused to form a novel SAS protein) in transgenic tobacco or Arabidopsis led to the accumulation of highly elevated levels of total SA (free SA and its glucoside) as compared to wt plants, provided the enzymes were targeted to the chloroplasts (Verberne et al, 2000; Mauch et al, 2001).

Biochemical and molecular analyses of ICS1 further supported a role for this enzyme in SA biosynthesis. As would be expected for an enzyme whose substrate is largely present in chloroplasts, ICS1 contains a putative plastid transit sequence and cleavage site (Wildermuth et al, 2001). A stromal location for ICS1 was confirmed using chloroplast import assays and immuno-localization studies (Strawn et al, 2007). Following expression in *E. coli*, purified recombinant His-tagged mature ICS1 exhibited a specific activity of 241 nmol min^{-1} mg^{-1}, an apparent K_m for chorismate of 41.5 μM and a K_{cat} = 38.7 min^{-1} (Strawn et al, 2007). Importantly, this affinity is sufficient to allow ICS1 to compete with other pathogen-induced enzymes that utilize chorismate as their substrate, such as anthranilate synthase (see Figure 1). Since recombinant ICS1 converts chorismate to IC, but SA is not detected as a product of this reaction, ICS1 functions as a unifunctional ICS, rather than a bifunctional SAS. Additional analyses indicated that ICS1 exhibits an absolute and specific requirement for Mg^{2+}, and it displays maximal activity over a broad range of pH and temperature; these results suggest that ICS1 activity is not regulated by light-mediated changes in the stromal environment.

Since SA accumulation is not completely blocked by mutations in ICS1, the possibility that ICS2 has a partially redundant function was investigated. Complementation analyses, combined with localization studies in tobacco cells, indicated that ICS2 is a functional, chloroplast-localized ICS (Garcion et al, 2008). In addition, analysis of recombinant ICS2 confirmed it to be a unifunctional ICS that is imported into the chloroplast stroma (M. Strawn, K. Inoue, and M.C. Wildermuth, unpublished). However, genetic analyses revealed that ICS2 is responsible for only a marginal level of SA biosynthesis (Garcion et al, 2008). Total SA levels in the *ics2* single mutant were comparable to those in wt Arabidopsis both before (0.54 μg/gFW) and after an SA-inducing treatment with UV light (4.17 μg/gFW). In contrast, UV-treated *ics1* single mutants accumulated 0.47 μg/gFW total SA, similar to the levels detected in uninduced wt plants. Double mutant *ics1 ics2* plants exhibited an even lower level of total SA (0.12 μg/gFW), and induction by UV treatment was completely suppressed. These plants appear to be completely lacking plastidic ICS activity. Thus, the ability of the double mutant to accumulate even very low levels of SA suggests the presence of an IC-independent pathway (Garcion et al, 2008). Several lines of evidence suggest that the PAL pathway is responsible for this residual SA (presented below).

In addition to Arabidopsis, *ICS* homologs have been identified in a wide variety of plant species, including *C. roseus*, *Capsicum annuum* (pepper), *N. tabacum* (tobacco), *N. benthamiana*, *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), *Vitis vinifera* (grapevine), *Medicago truncatula* (barrel medic), *Sorghum bicolor* (sorghum), *Glycine max* (soybean), *Ricinus communis* (castor bean), and *Populus trichocarpa* (poplar) (van Tegelen et al, 1999a; Ogawa et al, 2005; Uppalapati et al, 2007; Catinot et al, 2008; Yuan et al, 2009 and references therein). Given their role in phylloquinone synthesis, it is highly likely that ICS homologs will be identified in all plant species. Therefore, identification of an *ICS* gene in a given plant species is not sufficient to indicate that SA synthesis occurs via IC. However, analysis of [1-^{13}C]-D-glucose incorporation in *C. roseus* cells, which tends to reflect *in planta* metabolism, revealed that most of the SA synthesized after elicitation with a *Pythium aphanidermatum* extract was generated via the IC pathway (Mustafa et al, 2009; see ^{13}C label in Figure 1). In addition, virus-induced gene silencing of *ICS* expression in *N. benthamiana* or tomato suppressed UV- and/or pathogen-induced SA accumulation, and in tomato, led to hyper-susceptibility to infection by virulent *P. syringae* pv. *tomato* (*Pst*; Uppalapati et al, 2007; Catinot et al, 2008).

While these findings argue that SA is synthesized via the IC pathway in various plant species (Figure 1), the mechanism through which IC is converted to SA is unclear. This conversion is assumed to occur enzymatically, since non-enzymatic synthesis of SA from IC was negligible when the reactants were incubated under conditions consistent with the chloroplast stroma (Strawn et al, 2007). Furthermore, it is expected that the enzyme(s) involved in SA synthesis from IC is plastid-localized, as transgenic Arabidopsis expressing *nahG* fused to a chloroplast localization sequence failed to exhibit pathogen- or UV-inducible SA accumulation (Fragnière et al, 2011). However, no plant genes encoding an IPL activity have been identified. Indeed, it was recently noted that the sequenced Arabidopsis genome does not contain any genes encoding proteins similar to the bacterial enzyme (Z. Chen et al, 2009). One possible explanation for this conundrum is based on the observation that *PchB*, which encodes an IPL in *P. aeruginosa,* may have evolved from a chorismate mutase (CM; Strawn et al, 2007). PchB exhibits residual CM activity, but its affinity for chorismate is 10-fold lower than for IC (Gaille et al, 2002). The Arabidopsis genome contains three confirmed CM-encoding genes and one putative *CM* gene. Since all but one of these gene products are predicted to be plastid localized, they are reasonable candidates for a plant IPL. Alternatively, the conversion from IC to SA may be mediated by a route different from that characterized in bacteria.

Function of the IC versus PAL Pathways

In Arabidopsis, the significance of the IC pathway for SA synthesis has been explored largely in response to (a)biotic stresses, although this pathway also has been implicated in other functions (e.g. modulation of flowering time). Unlike wt plants, the *ics1* mutant exhibits little to no increase in SA levels following exposure to UV light, treatment with ozone or PAMPs, or pathogen infection (Nawrath and Métraux, 1999; Dewdney et al, 2000; Wildermuth et al, 2001; Ogawa et al, 2005; Garcion et al, 2008; Tsuda et al, 2008). As discussed above, any small increase in SA detected in induced *ics1* plants is likely due to ICS2, as UV-induced SA accumulation is completely abrogated in the *ics1 ics2* mutant (Garcion et al, 2008). *ICS1* also has been shown to play a critical role in PTI, ETI and SAR, since mutation of this gene leads to increased susceptibility to virulent pathogens, decreased resistance to avirulent pathogens, reduced defense gene expression (e.g. *PR-1*), and failure to develop SAR in the systemic leaves of pathogen- or elicitor-treated plants (Nawrath and Métraux, 1999; Dewdney et al, 2000; Wildermuth et al, 2001; Tsuda et al, 2008). By contrast, the role of ICS1 in pathogen-induced HR formation is more complicated. HR-associated cell death was not altered in

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ics1 mutants inoculated with avirulent *Psm avrRpt2* (Dewdney et al. 2000) or high titers of avirulent *Pst* carrying *avrRpm1, avrRpt2, or avrRps4* (Nawrath and Métraux, 1999). However, subsequent studies revealed that for a certain class of R proteins (including RPM1 and RPS2, but not RPS4), the role of SA (via ICS1) is masked by the function of another component of the SA signaling pathway, Enhanced Disease Susceptibility 1 (EDS1*;* At3g48090; Venugopal et al, 2009; see SA Regulation). Additional hints that ICS1 may play a role in HR formation come from the combined observations that i) *ics1* mutant plants inoculated with *Hyaloperonospora arabidopsidis* (formerly called *Peronospora parasitica*) developed a trailing necrosis, rather than the discrete necrotic lesions displayed by wt plants (Nawrath and Métraux, 1999), and ii) the increased fungal resistance exhibited by the *enhanced disease resistance* (*edr)2* mutant was dependent on SA and *ICS1*, and was associated with enhanced cell death (Tang et al, 2005).

While the above results argue that the IC pathway is the predominant route for synthesis of both basal and induced SA in Arabidopsis, other evidence suggests that an IC-independent pathway, likely mediated by PAL, also is operational. For example, the *ics1 ics2* double mutant is able to accumulate 20% and 4% of the total SA detected in untreated and UV-treated wt plants, respectively (Garcion et al, 2008). Treatment with the PAL inhibitor AIP also was shown to reduce the accumulation of free and bound SA in Arabidopsis inoculated with avirulent *H. arabidopsidis*, and it reduced resistance to this pathogen (Mauch-Mani and Slusarenko, 1996). AIP-treated Arabidopsis also developed larger lesions than untreated plants following infection with the necrotrophic fungus *Botrytis cinerea* (Ferrari et al, 2003). Since AIP treatment of *nahG* plants did not further increase the size of *B. cinerea*-induced lesions, it was concluded that the SA signal for local resistance is generated via the PAL pathway. In addition to these inhibitor studies, analysis of Arabidopsis mutants in which all four *PAL* genes (*PAL1-4; At2g37040; At3g53260; At5g04230; At3g10340*) were knocked out revealed a ~75% reduction in the basal level of total SA as compared with wt plants (~0.4 μg/gFW vs. ~1.4 μg/gFW, respectively), and a ~50% reduction in total SA following infection with an avirulent bacterial pathogen (~12.5 μg/gFW in the mutant vs. ~25μg/gFW in wt; Huang et al, 2010); it should be noted that these quadruple mutants still retain 7-9% residual PAL activity. Consistent with the reduction in total SA levels, the quadruple *PAL* mutants displayed enhanced susceptibility to a virulent bacterial pathogen. In contrast, disruption of *ICS1* reduced total SA levels to a greater extent, with a ~93% reduction in the basal level (~0.1 μg/gFW) and a ~92% reduction in the pathogen-induced level (~2 μg/gFW) despite containing a functional *ICS2* gene (Garcion et al, 2008).

One way to reconcile the studies implicating PAL in SA synthesis with those indicating that the IC pathway predominates in Arabidopsis is to contend that dramatic disruption of the PAL pathway impacts chorismate flux, and in so doing, reduces chorismate available for SA synthesis via IC. Indeed, Arabidopsis *pal1* and *pal2* single mutants, and *pal1 pal2* double mutants exhibited substantially altered transcriptomes and metabolomes. In addition to dramatic changes in aromatic and other amino acids, flavonoids, lignin, and carbon metabolism, these plants exhibited enhanced transcript accumulation for CM and tryptophan synthase, which utilize chorismate as a direct or indirect substrate, respectively (Rohde et al, 2004; Figure 1). Although SA levels were not substantially altered in the *PAL* single and double mutants (Huang et al, 2010), the impact of quadruple *PAL* mutations on plant metabolism would likely be much more severe. Thus, it is possible that up-regulation of CM and tryptophan synthase in the *PAL* quadruple mutants reduces the pool of chorismate available for ICS-mediated production of IC, and thus SA. This impact on SA (via the IC pathway) in the quadruple PAL mutant is expected to dominate any small reduction in SA associated with diminished SA synthesis via a PAL pathway. It should be noted that other defensive compounds are synthesized from *t*-CA in Arabidopsis (see Figure 1). Inhibition of PAL expression or activity could impact synthesis of these compounds (as well as flux to SA synthesis via IC) and thereby further reduce resistance to pathogen infection. For example, pathogen-induced lignification contributes to resistance in *H. arabidopsidis*-inoculated Arabidopsis, and this response, as well as SA accumulation, was suppressed by AIP treatment (Mauch-Mani and Slusarenko, 1996).

SA MODIFICATIONS

Once synthesized, SA may undergo a number of biologically relevant chemical modifications including glucosylation, methylation, and amino acid (AA) conjugation (Figure 2, Table 1). Most modifications render SA inactive, while at the same time they allow fine-tuning of its accumulation, function, and/or mobility. Glucosylation inactivates SA and allows vacuolar storage of relatively large quantities of SA due to reduced toxicity. Methylation inactivates SA while increasing its membrane permeability, as well as its volatility, thus allowing more effective long distance transport of this defense signal. AA conjugation of SA is less well characterized, but may be involved in SA catabolism. In this section, we discuss enzymatic formation and hydrolysis of the different derivatives, as well as their biological relevance and regulation. We also note the possibility of SA sulfonation, as suggested in a recent report. Finally, we address the potential conversion of SA to the dihydroxybenzoates 2,3- and 2,5-DHBA.

Glucosylation

Arabidopsis encodes at least two UDP-glucosyltransferases that glucosylate SA, UGT74F1 and UGT74F2, (also referred to as AtSGT1; Lim et al, 2002; Song, 2006; Dean and Delaney, 2008). UGT74F1, encoded by *At2g43840*, and UGT74F2, encoded by *At2g43820*, both catalyze the conjugation of SA at its hydroxyl group resulting in the formation of SA 2-*O*-β-D-glucoside (SAG). In addition, UGT74F2 catalyzes the conjugation of SA at its carboxyl group, resulting in the formation of salicylate glucose ester (SGE). *In vitro* recombinant UGT74F2 (SGT1) or extracts from transgenic plants over-expressing the corresponding gene convert the majority of radio-labelled SA into SGE and a minor proportion into SAG (Song et al, 2008). This corresponds to the specific activity of UGT74F2 for the different reactions, which is 1.04 nkatal/mg of protein for generating SGE and 0.10 nkatal/mg of protein for generating SAG (Lim et al, 2002); the K_m of this enzyme for SA is 0.19 mM (Song, 2006). In comparison, the specific activity of UGT74F1 for synthesis of SAG is 5.79 nkatal/mg of protein and it has a K_m for SA of 0.23 mM (Lim et al, 2002). Con-

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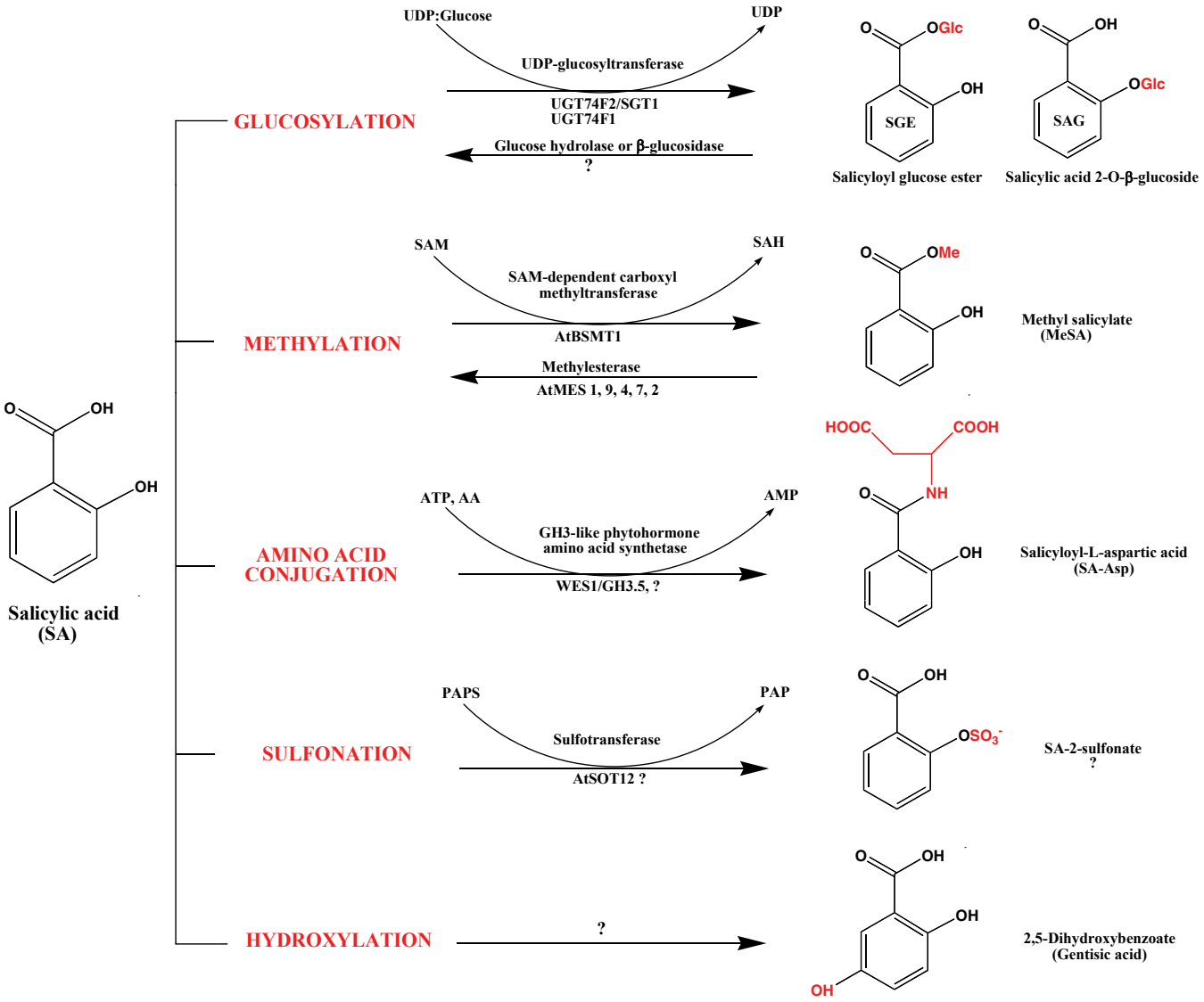


Figure 2. Modifications of SA in plants.

Arabidopsis proteins capable of catalyzing the described reaction are shown. Conversion back to SA is shown where evidence exists. All modifications excepting SA-2-sulfonate have been detected in plants including Arabidopsis. Note that while AtSOT12 is active on SA *in vitro*, it is also active on other substrates with much higher specific activity. While 2,3-DHBA (not shown) and 2,5-DHBA are synthesized via isochorismate in Arabidopsis, it is unclear whether SA is an intermediate in their biosynthesis. Refer to text for details. Abbreviations are as follows: uridine diphosphate (UDP), UDP-glucosyltransferases 74F1 and 74F2 (UGT74F1/2), salicylic acid glucosyltransferase 1 (SGT1), *S*-adenosyl methionine (SAM), *S*-adenosyl homocysteine (SAH), benzoic acid/salicylic acid carboxyl methyltransferase 1 (AtBSMT1), adenosine triphosphate (ATP), amino acid (AA), adenosine monophosphate (AMP), WESO 1 (WES1), GH3 acyl adenylase family member 3.5 (GH3.5), 3'-phosphoadenosine 5'-phosphosulphate (PAPS), 3'-phosphoadenosine 5'-phosphate (PAP).

sistent with these studies, while SAG formation is slightly reduced in the Arabidopsis *ugt74f2* mutant, SGE formation is abolished. By contrast, incorporation of radio-labelled SA into SGE in the *ugt74f1* mutant was not significantly altered, but SAG formation was delayed and reduced (Dean and Delaney, 2008). Thus, the formation of SGE and SAG in Arabidopsis depends on the intracellular levels of each enzyme and the concentrations of their relevant substrates. In addition to SA, UGT74F1 and UGT74F2 are also active on other substrates including benzoate, anthranilate (2-amino benzoate), and MeSA (Lim et al, 2002: Quiel and Bender, 2003; Song et al, 2008).

As discussed in the previous section, abiotic (e.g. ozone, UV-C) and biotic (e.g. *P. syringae*, powdery mildew, Turnip Crinkle Virus) stresses significantly induce the formation of free SA and SA glucose conjugates in Arabidopsis, with a 10-fold or more increase in total SA often observed (Uknes et al, 1993; Nawrath and Métraux, 1999; Summermatter et al, 1995; Wildermuth et al, 2001). Consistent with this induced response, both *UGT74F1* and *UGT74F2* expression are induced by SA and appropriate (a)biotic stresses (AtGenExpress Biotic and Abiotic Stress Series (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp); Harrower and Wildermuth, 2011); *UGT74F2* exhibits

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higher constitutive and more dramatically induced leaf expression than does *UGT74F1* (e.g. NASCArray (Craigon et al, 2004) Experiment 414 (*P. syringae*) and Experiment 123 (*Phytophthora infestans*)). In tobacco (Lee and Raskin, 1998) and soybean (Dean et al, 2003), where the specific glucose conjugates were examined, SAG was the dominant glucosylated form of SA. Current protocols widely used in the Arabidopsis community, however, may not distinguish SGE and SAG, as both acid hydrolysis (e.g. 4N HCl, 80°C, 1 hr) and β-glucosidase treatment can release SA or similar compounds from both conjugates (Harbone and Corner, 1961; Quiel and Bender, 2003; Song, 2006).

In vitro analysis of SA glucosyltransferase activity in uninduced Arabidopsis leaves revealed the presence of an SGE-forming activity, but not an SAG-forming activity (Dean and Delaney, 2008). SAG-forming activity was observed in Arabidopsis that received a pretreatment with SA, although at a much lower level than the SGE-forming activity. In comparison, incubation of Arabidopsis leaf strips with [^{14}C]SA for 12 hr led to similar incorporation of radio-label into SAG and SGE. Analyses of SA glucosyltransferase activity in tobacco similarly detected an SGE-forming, but not SAG-forming, activity prior to SA treatment (Lee and Raskin, 1998). Following SA treatment, SAG-forming activity and SAG levels continued to increase over time, while SGE formation occurred earlier and transiently (Lee and Raskin, 1998). It remains to be seen whether an early and transient accumulation of SGE occurs in response to relevant (a)biotic stresses in Arabidopsis.

Similar to tobacco (Dean et al, 2005) and soybean (Dean et al, 2003; Dean and Mills, 2004), SAG in Arabidopsis is thought to be produced in the cytosol and then (actively) transported into the vacuole as a non-toxic storage form of SA (Figure 3). Transport of SAG into the vacuole also ensures there is no end-product inhibition of the cytosolic glucosyltransferase, and can thus increase yield. It is currently unclear whether SAG is biologically active, since it is readily hydrolyzed to SA *in planta*. Several studies imply that SAG is inactive and must be converted to SA to induce defenses. For example, injection of tobacco leaves with SAG induced expression of the SA marker gene *PR-1*, but both uptake of SAG and induction of *PR-1* were preceded by conversion of SAG into SA by extracellular glucosidases (Hennig et al, 1993). In addition, a non-hydrolyzable chemical analog of SAG was not capable of inducing *PR-1* expression. Furthermore, Arabidopsis over-expressing *UGT74F2*, which should increase the conversion of SA to SGE and SAG, exhibited a compromised immune response, enhanced levels of MeSA and MeSAG, and severely decreased levels of both free and glucosylated SA compared to wt plants (Song et al, 2008). It should be noted, however, that interpretation of studies in which SA glucosyltransferase (SAGT) activity is altered can be complicated, as i) altering SAGT activity can also impact SA synthesis and ii) SAGTs can utilize substrates other than SA. Interestingly, while SAG itself appears incapable of inducing SA-mediated responses, experiments in rice showed that the conversion of SA to SAG is essential for immunity. Silencing of *OsSGT1* compromised SAG accumulation and chemically-induced resistance against the blast fungus *Magnaporthe grisea* (Umemura et al, 2009).

The mechanism through which SAG is hydrolyzed to SA in plants is currently unclear. The observed hydrolysis of SAG in the tobacco apoplast (Hennig et al, 1993) may be catalyzed by cell wall-associated β-D-glucan glucohydrolases (Hrmova and Fincher,

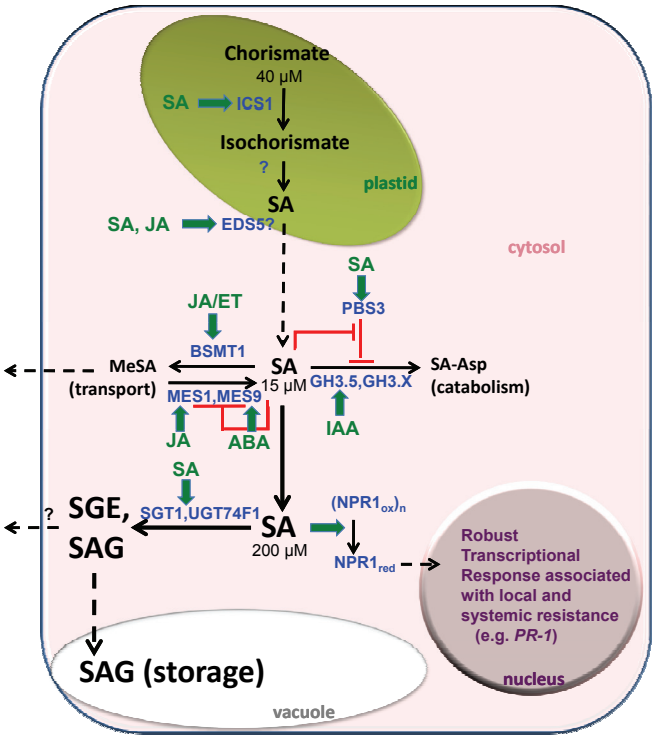


Figure 3. Model integrating current knowledge of biochemical and transcriptional regulation of SA synthesis, SA modification, and activation of robust defense-associated responses via reduced, nuclear-localized NPR1.

SA-dependent NPR1-independent processes are not indicated here. Although all processes are shown in one cell, it is likely that spatial and temporal separation of responses is critical to their function. Dashed lines indicate transport across membrane(s). Concentrations shown reflect the K_m of the enzymes for SA. Feedback inhibition of enzyme activity is shown in red. Hormone-induced gene expression is indicated by thick green arrows, based on data from AtGenExpress Hormone and Chemical data series, Exogenous SA treatment of Whole Leaves experiment (NCBI Gene Expression Omnibus Acc. No. GSE33402), and the literature (in text). Transcription factors regulating gene activity are not shown. Although several other AtMES enzymes are active on SA, only the two most active are shown for brevity. Abbreviations are as follows: abscisic acid (ABA), benzoic acid/salicylic acid carboxyl methyltransferase 1 (BSMT1), enhanced disease susceptibility 5 (EDS5), ethylene (ET), known and/or unknown members of the GH3 acyl adenylase family (GH3.5/GH3.X), indole-3-acetic acid (IAA), isochorismate synthase 1 (ICS1), jasmonic acid (JA), methyl esterase 1 and 9 (MES1/9), methyl salicylate (MeSA), oxidized or reduced forms of Non-expressor of Pathogenesis-Related genes 1 ($NPR1_{ox}$/$NPR1_{red}$), *avrPphB* susceptible 3 (PBS3), pathogenesis-related 1 gene (*PR-1*), salicylic acid (SA), salicyloyl-L-aspartate (SA-Asp), SA 2-*O*-β-D-glucoside (SAG), salicylate glucose ester (SGE), salicylic acid glucosyltransferase 1 (SGT1), UDP-glucosyltransferase 74F1 (UGT74F1). See text for details and supporting information. Adapted from Okrent et al. (2011).

2007). In support of this hypothesis, Seo et al. (1995) detected SA-inducible SA glucosylhydrolase activity in the intercellular space of tobacco leaves. However, whether SAG is transported from the vacuole to the apoplast for hydrolysis, and whether stress-induced cellular damage results in the release of vacuolar cargo bringing SAG in contact with its glucosylhydrolase has not been established. Alternatively, there may be intracellular enzymes that mediate the

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conversion of vacuolar SAG to SA. Once these activities are identified, analyses of the temporal relationship between the induction of the genes encoding the glucosyltransferases and glucosylhydrolases that inter-convert SA and SAG should provide insight into how the SA-SAG equilibrium is established and/or maintained, and how shifts in this equilibrium affect immunity.

Methylation

In Arabidopsis, BA/SA carboxyl methyltransferase 1 (BSMT1, At3g11480) catalyzes the formation of the SA methyl ester, methyl salicylate (Chen et al, 2003). The corresponding gene belongs to the *SABATH* family of methyltransferases, which contains 24 members. The apparent K_m of BSMT1 for SA is 16 μM, which is considerably lower than that of the glucosyltransferases discussed above. The respective K_m values indicate that MeSA is formed at >10-fold lower SA concentrations as compared to SGE/SAG (Figure 3). In normal growth conditions, *BSMT1* expression is higher in flowers than in leaves (Chen et al, 2003). This is in agreement with a role for MeSA in the development of floral scent, which plays a role in the attraction of pollinators (Effmert et al, 2005). In addition, *BSMT1* expression is induced in leaves in response to specific biotic or abiotic stresses (Chen et al, 2003; Koo et al, 2007; Attaran et al, 2009; Song et al, 2009; Liu et al, 2011a), and the resultant increase in MeSA is implicated in several aspects of plant defense signaling.

In addition to MeSA, its 2-*O*-β-D-glucoside, MeSAG, accumulates in *P. syringae*-infected Arabidopsis (Song et al, 2008). Production of MeSAG appears to be mediated by SGT1, as recombinant SGT1 converted MeSA to MeSAG *in vitro*. Soybean and tobacco cell suspension cultures also have been shown to convert radio-labelled SA to MeSA and/or MeSAG (Dean et al, 2003; 2005). The biological role of MeSAG remains unknown; one possible function is to serve as a non-volatile storage form of MeSA (Dean et al, 2003; 2005). In contrast to SAG, which is predominantly in the vacuole, only 50% or less of the MeSAG detected in soybean and tobacco was found in this organelle (Dean et al, 2003; 2005).

MeSA has been implicated in resistance to both microbial pathogens and insects. It mediates resistance to certain insects by attracting their respective predators (Van Poecke et al, 2001). By contrast, MeSA mediates resistance to microbial pathogens by serving as a key signal for SAR (Vlot et al, 2009). The ability of chimeric tobacco containing a *nahG*-expressing rootstock and a wt scion to develop SAR raises several questions concerning how MeSA is produced in the SA-deficient inoculated leaves, and whether it is degraded by NahG. Analysis of recombinant NahG failed to detect activity against MeSA (S. Park et al, 2007). Furthermore, *nahG*-expressing tobacco accumulated MeSA to nearly wt levels in both the TMV-inoculated and systemic leaves (S. Park et al, 2007). Based on this result, it was proposed that NtSAMT1, the tobacco BSMT1, has a higher affinity for SA and/or faster kinetics than NahG. Alternatively, NtSAMT1 and NahG may be localized in different cellular compartments, with NtSAMT1's location providing greater (or earlier) access to newly synthesized SA. MeSA itself does not appear to be biologically active, as its ability to induce *PR-1* gene expression and resistance to TMV in tobacco was abolished in transgenic lines expressing *nahG* (Seskar et al, 1998). Instead, MeSA likely needs to be converted to SA to be active. In support of this hypothesis, Arabidopsis over-expressing *O. sativa BSMT1* accumulated elevated levels of MeSA; however, they failed to express *PR-1* following SA or pathogen treatment and they exhibited enhanced disease susceptibility, presumably because their ability to accumulate SA and SAG was severely compromised (Koo et al, 2007). Interestingly, these *Os-BSMT1* over-expressors emitted vaporized MeSA at levels sufficient to induce *PR-1* expression in neighboring plants. Similarly, MeSA-containing gases collected from the headspace of TMV-infected tobacco induced *PR-1* expression and TMV resistance in untreated plants (Shulaev et al, 1997). Since 93% of the gaseous [^{14}C]MeSA supplied to healthy tobacco was converted to [^{14}C]SA (Shulaev et al, 1997), the biological relevance of MeSA may be to transport SA between or within (discussed below) plants and thereby signal/trigger defense responses to biotic or abiotic stresses (Shulaev et al, 1997; Baldwin et al, 2006; S. Park et al, 2007; Koo et al, 2007; Karl et al, 2008).

Evidence supporting a role for MeSA as an endogenous mobile signal that triggers SAR has come from studies in Arabidopsis, tobacco, and potato (S. Park et al, 2007; Vlot et al, 2008b; Manosalva et al, 2010). Analysis of tobacco initially suggested that SAR is activated via the following sequence: SA accumulates in the pathogen-inoculated leaf and is converted to MeSA by NtSAMT1; MeSA then travels to distal leaves via the phloem; in the systemic leaves, MeSA is converted to SA by the methyl esterase activity of NtSABP2; the resultant increase in SA triggers systemic defenses (S. Park et al, 2007). Subsequent analyses have indicated that SAR in Arabidopsis is activated by a similar process, as it is suppressed in plants that i) fail to accumulate pathogen-induced MeSA due to a knockout (KO) of *BSMT1* (Liu et al, 2010), ii) display reduced MeSA esterase activity due to either under-expression of certain AtMES family members (see below; Vlot et al, 2008b) or treatment with 2,2,2,2'-tetra-fluoroacetophenone (tetraFA), a specific inhibitor of MeSA esterases (Park et al, 2009), or iii) contain an elevated MeSA:SA ratio due to over-expression of BSMT1 (Liu et al, 2010). These findings may be further extended to potato, as SAR was suppressed by silencing of *StMES1* expression or tetraFA treatment (Manosalva et al, 2010).

Orthologs of NtSABP2 have been identified in Arabidopsis, which contains a family of 18 potential methyl esterases (AtMES; Vlot et al, 2008b), and *Solanum tuberosum* (potato), which contains a single ortholog (StMES; Manosalva et al, 2010). In Arabidopsis, at least five members of the AtMES protein family hydrolyze MeSA with varying efficiencies (Vlot et al, 2008b). AtMES1 (At2g23620) and AtMES9 (At4g37150) are the most efficient, with MeSA hydrolysis rates of 28.92 and 25.88 nmol/min/μg of protein and K_m values for MeSA of 57.3 and 147.1 μM, respectively. AtMES4 (At2g23580), AtMES7 (At2g23560), and AtMES2 (At2g23600) hydrolyze MeSA with decreasing hydrolysis rates of 16.07, 12.82, and 1.77 nmol/min/μg of protein, respectively. Characterization of AtMES, NtSABP2, and StMES enzymes revealed that they belong to the α/β hydrolase superfamily; these proteins share a conserved catalytic triad consisting of Ser, Asp, and His residues (Nardini and Dijkstra, 1999). The three-dimensional structure of NtSABP2 in complex with SA confirmed that NtSABP2 has the typical α/β fold and revealed that SA binds in its active site, which is defined by the catalytic triad (Forouhar et

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al, 2005). Indeed, SA strongly inhibits the MeSA esterase activity of NtSABP2, AtMES1, 2, 4, 7, and 9, and StMES1 (Forouhar et al, 2005; Vlot et al, 2008b; Manosalva et al, 2010); the K_i values for SA of AtMES1 and AtMES9 are 8.3 and 8.6 μM, respectively (Vlot et al, 2008b). Consistent with these findings, SA-mediated inhibition of NtSABP2 is required in the inoculated leaf, presumably to facilitate accumulation of MeSA for translocation to the distal tissue (S. Park et al, 2007).

There has been some controversy in the literature regarding the role of MeSA as an SAR signal in Arabidopsis. Two independent studies of *BSMT1* KO mutants detected reduced MeSA accumulation; however, only one also observed suppression of SAR (Attaran et al, 2009; Liu et al, 2010). The reason for this discrepancy was recently found to be related to differences in light conditions. In plants which received little exposure to light after infection (before the dark/night period), SAR development was compromised by inhibition of MeSA synthesis (Liu et al, 2010). In contrast, when the primary infection was followed by 5 hrs or more of light exposure, MeSA played only an auxiliary role by adding to the strength of the SAR (Liu et al, 2011b).

In addition to MeSA, one or more lipid-based mobile signals have been implicated in the activation of SAR (Vlot et al, 2008a; Jung et al, 2009; Shah, 2009; Chanda et al, 2011). Recent analyses of mutants in the lipid-transfer protein DIR1 (Maldonado et al, 2002) and of plants over expressing *AtBSMT1* suggest that SAR, under certain conditions, is activated via the interplay between at least two mobile signals, MeSA and a complex formed between DIR1 and a lipid or lipid derivative (likely a glycerol-3-phosphate-derived compound; Chanda et al, 2011). The function of this complex appears to be to suppress the expression and/or activity of AtBSMT1 in the systemic tissue; this facilitates the conversion of translocated MeSA to SA, which in turn leads to induction and potentiation of defense responses (Liu et al, 2011a).

Another possible function for MeSA may involve regulating cross-talk between the SA and jasmonic acid (JA) defense signaling pathways. Whereas SA is primarily involved in defense against biotrophic pathogens, another plant hormone, JA, is the main regulator of defenses against necrotrophic pathogens and insects. SA and JA signaling generally, but not always, antagonize each other (Robert-Seilaniantz et al, 2011). Several pieces of evidence suggest that the JA/SA interface is at least partially responsible for regulating MeSA production, which in turn, may influence the activity of these defense pathways. For example, volatile MeSA is released following infection with (hemi-)biotrophic pathogens in tobacco or Arabidopsis (Shulaev et al, 1997; Huang et al, 2003; Koo et al, 2007; Attaran et al, 2009), and also upon insect attack of tomato (Ament et al, 2004) and Arabidopsis (Van Poecke et al, 2001; Chen et al, 2003). In addition, data from various plant species suggest that JA or MeJA is essential and sufficient to induce the expression of BSMT/SAMT-encoding genes and/or the concomitant emission of MeSA (Ament et al, 2004; Chen et al, 2003; Filella et al, 2006; Koo et al, 2007; Attaran et al, 2009; Figure 3). In contrast to MeJA/JA, SA does not induce *AtBSMT1* expression in Arabidopsis leaves (Chen et al, 2003). Thus, one possible mechanism through which JA/MeJA antagonizes SA signaling could be by upregulating *BSMT* expression; this would result in increased conversion of SA into biologically inactive MeSA. Indeed, depletion of the local SA pool likely explains why SA-induced gene expression and SA-mediated suppression of MeJA-induced gene expression are partially compromised in Arabidopsis over-expressing *OsBSMT1* (Koo et al, 2007).

It remains to be established how MeSA (and/or BSMT) regulates and/or is regulated by SA-JA cross talk. The tobacco esterase SABP2, which hydrolyzes MeSA to SA, can also, although less efficiently, hydrolyze MeJA and the methyl ester of another hormone, indole-3-acetic acid (IAA; Forouhar et al, 2005). Consistent with this finding, six members of the Arabidopsis methyl esterase family, AtMES1, 2, 3, 9, 10 and 16, catalyze MeJA hydrolysis, and AtMES1, 2, 3, 7, 9, 16, 17, and 18 accept Me-IAA as a substrate (Vlot et al, 2008b; Yang et al, 2008). Taken together, the various members of the *AtMES,* and probably also the *AtSABATH,* family may fine-tune cross talk between SA and JA (and possibly other hormones, like IAA), via regulating the levels of their respective methylated derivatives.

AA conjugation

AA conjugates of JA and IAA have been detected in diverse plants; they have been shown to play important roles in modulating the activity and function of these hormones. For example, the Arabidopsis GH3 acyl adenylase protein JAR1 conjugates isoleucine to JA and its isomer (+)-7-*iso*-JA to activate the hormone (Fonseca et al, 2009; Suza et al, 2010). In contrast, conjugation of IAA inactivates this hormone, with distinct IAA-AA conjugates playing different functional roles in fine-tuning local auxin concentrations (Woodward and Bartel, 2005). Much less is known about SA-AA conjugates and their functional role(s). Salicyloyl-L-aspartate (SA-Asp) is the dominant stable SA-AA conjugate, as only SA-Asp conjugates have been detected in plants such as *Vitis* (grape) species (Steffan et al, 1988), *Phaseolus vulgaris* (bean; Bourne et al, 1991), and Arabidopsis (Zhang et al, 2007). As discussed below, Arabidopsis GH3 enzymes that modulate SA metabolism have now been identified, and a role for SA-Asp in SA catabolism has been proposed (Figure 3).

While many Arabidopsis GH3 enzymes are active on IAA, to date, only GH3.5 (also known as WES1) has been found to be active on SA (Staswick et al, 2002). GH3.5, encoded by *At4g27260*, exhibits even higher activity on IAA than on SA (P.E. Staswick, personal communication; Staswick et al, 2002) and can form a number of IAA-AA conjugates including IAA-Asp *in vitro* (Staswick et al, 2005). The *in vitro* AA preference of GH3.5 for SA has not been reported; however, it is likely to be similar to that for IAA, resulting in the formation of SA-Asp. Indeed, overexpression of GH3.5 results in a 3.5-fold increase in SA-Asp levels after pathogen infection, consistent with this role (Zhang et al, 2007). However, no significant difference in basal or induced SA, SA glucose conjugates, or SA-Asp levels was observed in the Arabidopsis *gh3.5-2* null mutant (J. Park et al, 2007; Zhang et al, 2007). This latter finding indicates that a GH3 protein other than GH3.5 also catalyzes the formation of SA-Asp. In addition, it raises the possibility that the dominant function of GH3.5 *in planta* is not its action on SA, but on IAA, which then impacts SA metabolism (Robert-Seilaniantz et al, 2011). Consistent with this hypothesis, the *gh3.5-2* mutant exhibits auxin-associated phenotypes (e.g. enhanced sensitivity to IAA in primary root inhibition assay) and reduced accumulation of IAA-Asp (J. Park et al, 2007). Conversely, basal IAA-Asp levels in the *GH3.5* overexpressor were

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elevated 7-fold compared to those in wt plants, as would be expected if GH3.5 acts directly on IAA (J. Park et al, 2007; Zhang et al, 2007). The impact of altered *GH3.5* expression on disease resistance and abiotic stress tolerance (e.g. to drought and temperature) could also result from cross-talk between IAA and other hormone (e.g. SA and ABA) signaling and response pathways (J. Park et al, 2007; Zhang et al, 2007).

In contrast to *gh3.5* mutants, null mutants in *GH3.12* (*At5g13320*) exhibited substantively reduced pathogen-induced SAG accumulation, *PR-1* expression, and resistance to *P. syringae* (Jagadeeswaran et al, 2007; Lee et al, 2007; Nobuta et al, 2007). GH3.12 (also known as PBS3, GDG1, and WIN3) does not utilize IAA or SA as acyl substrates (Staswick et al, 2002), but instead prefers 4-substituted benzoates such as 4-aminobenzoate and 4-hydroxybenzoate (4-HBA; Okrent et al, 2009). *In vitro* conjugation assays also indicated that Glu is a preferred AA conjugate, while Asp is not utilized (Okrent et al, 2009). Furthermore, GH3.12 activity was reversibly inhibited by SA (2-hydroxybenzoate) at physiologically relevant concentrations (IC_{50}= 15 μM). Since treatment with SA or an SA analog rescued the Arabidopsis *gh3.12* mutant phenotypes (Nobuta et al, 2007; Jagadeeswaran et al, 2007; Lee et al, 2007) and GH3.12 was not active on SA, this enzyme was predicted to function upstream of induced SA synthesis/accumulation, with feedback inhibition of its activity occurring in the presence of sufficient SA (Okrent et al, 2009). However, stress-induced *ICS1* transcript and protein expression were not compromised in *gh3.12* plants, despite a dramatic delay and reduction in total SA accumulation and *PR-1* expression (Okrent et al, 2011). Instead, SA-Asp was elevated in the stress-induced *gh3.12* plants. Exogenous treatment with SA-Asp did not induce *PR-1* expression, suggesting that SA-Asp is an inactive form of SA, which is likely targeted for catabolism, similar to IAA-Asp (Woodward and Bartel, 2005). Based on this result, Wildermuth and colleagues proposed that heightened conversion of SA to SA-Asp in the *gh3.12* mutant reduces the level of active SA below a threshold(s) required for both SAG formation and robust activation of SA-dependent responses (see SA Regulation).

Sulfonation

In mammalian systems, sulfonation is important for the activation or de-activation of various hormones. In Arabidopsis, flavonoids, glucosinolates, brassinosteroids, hydroxyjasmonate, and most recently SA have been shown to be sulfonated *in vitro* by members of the SOT family of sulphotransferases (Klein and Papenbrock, 2004; Baek et al, 2010 and references therein). A T-DNA insertion KO mutant lacking *SOT12* (*At2g03760*) displayed enhanced inhibition of primary root growth by SA and was severely compromised in pathogen-induced SA accumulation and resistance against *P. syringae*. By contrast, overexpression of *SOT12* enhanced SA accumulation and resistance both in infected and systemic leaves (Baek et al, 2010). Characterization of SOT12 revealed that it catalyzes the transfer of a sulphuryl group (SO_3^{-1}) to the 2-OH position of SA *in vitro*; thus, Baek et al. (2010) postulated that this protein functions as an SA sulfotransferase *in planta*. However, sulphonated SA was not detected *in planta* and sulphonated SA formation *in vitro* required high concentrations of the substrate (K_m= 0.44 mM for SA (Baek et al, 2010) compared with K_m= 7 μM for the brassinosteroid 24-epicathasterone (Marsolais et al, 2007)). Although the K_m of SOT12 for SA is in the range of the SA glucosyl transferases, it remains to be established if SOT12 acts on SA *in vivo* or primarily sulfonates another target.

Conversion to Dihydroxybenzoates

In addition to SA, a variety of substituted benzoates are formed in plants via products of the shikimate pathway, and they are subject to the same modifications, including glucosylation, methylation, and AA conjugation (Wildermuth, 2006; Mustafa and Verpoorte, 2007). Here, we focus our discussion on two dihydroxybenzoates, 2,3-DHBA and 2,5-DHBA (also known as gentisic acid), that are induced by pathogens and, based on early evidence, may be formed from SA (Ibrahim and Towers, 1959).

Previous studies have shown that SA can scavenge hydroxyl radicals (•OH), resulting in the non-enzymatic formation of both 2,3- and 2,5-DHBA *in vitro*, with the ratio of these products dependent upon iron ion concentrations and pH (Maskos et al, 1990; Chang et al, 2008). Although highly localized, non-enzymatic production of these compounds could occur during the activation of defences, Bartsch et al. (2010) argue that they are predominantly formed enzymatically as i) the levels of total (free plus sugar conjugated) 2,3-DHBA and 2,5-DHBA were not necessarily comparable in Arabidopsis of different ages or mutant backgrounds, and ii) mutations in the Arabidopsis *rboh* genes, which impact pathogen-inducible ROS generation and therefore would be expected to alter non-enzymatic 2,3- and 2,5-DHBA formation, did not affect total 2,3-DHBA accumulation (2,5-DHBA was not analyzed).

In Arabidopsis, 2,3-DHBA and 2,5-DHBA are synthesized largely, although not necessarily exclusively, via the IC pathway. A mutation in *ICS1* fully suppressed pathogen-induced accumulation of 2,3-DHBA, and substantially reduced that of 2,5-DHBA (Bartsch et al, 2010). Similarly, the labelling pattern of 2,3-DHBA in elicited *C. roseus* cells fed [^{13}C]glucose indicated that it is generated via the IC, rather than the PAL, pathway (Budi-Muljona et al, 2002; Mustafa et al, 2009). However, it remains unclear whether the direct precursor for 2,3-DHBA and 2,5-DHBA is SA or IC. [^{14}C]SA was shown to be incorporated into 2,3-DHBA, 2,5-DHBA, or their sugar conjugates in several plant species, with the percent incorporation varying dramatically between the species tested (Ibrahim and Towers, 1959). Later studies showed exogenously applied SA also was effectively converted to 2,5-DHBA glycosides in Arabidopsis (Dean and Delaney, 2008), *Glycine max* (soybean; Dean et al, 2003) and *C. roseus* (Shimoda et al, 2002). By contrast, Bartsch et al. (2010) found only a small fraction of exogenously supplied [^{2}H]SA was converted into 2,3-DHBA (detected as the pentoside) and another unidentified DHBA-pentoside, possibly that of 2,5-DHBA, in avrRpm1-expressing Arabidopsis transgenics. Currently, the plant genes/enzymes involved in 2,3-DHBA and 2,5-DHBA synthesis are unknown. However, based on bacterial pathways, it is possible that isochorismatase catalyzes the formation of 2,3-DHBA directly from IC (Rusnak et al, 1990), and hydroxylation of SA could be performed by a monoxygenase similar to those identified in *Pseudomonas* and *Ralstonia* (Hickey et al, 2001 and references therein).

The biological functions of 2,3-DHBA and 2,5-DHBA in Arabidopsis remain unclear. Total 2,3-DHBA levels were found to

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increase in Arabidopsis leaves in response to infection with avirulent pathogens or aging, and exogenously supplied 2,3-DHBA was a weak inducer of *PR-1* expression as compared with SA (Bartsch et al, 2010). These findings could be consistent with a role for 2,3-DHBA as a de-activated form of SA. Alternatively, it has been suggested that 2,3-DHBA serves as a protectant against oxidative stress, similar to its function in bacterial pathogens (Bartsch et al, 2010). Analysis of 2,5-DHBA has indicated that it is strongly induced in response to infection by systemic, non-necrotizing pathogens in a plant species- and pathogen-specific manner (Bellés et al, 1999; 2006). Although 2,5-DHBA has no reported function in Arabidopsis, its exogenous application in tomato, cucumber, and *Gynura* induces a distinct subset of *PR* genes from those induced by SA; this finding has led to the suggestion that 2,5-DHBA and SA play complementary signaling roles for activating plant defenses (Bellés et al, 1999; 2006; Dean and Delaney, 2008). 2,5-DHBA has also been reported to have antifungal activity (Lattanzio et al, 1994).

REGULATION OF SA ACCUMULATION

Research over the past 25 years has revealed many defense-associated signaling and regulatory processes upstream and downstream of SA. Due to space limitations, we will focus on those processes that occur upstream of SA in Arabidopsis, i.e. on regulation of induced SA accumulation, and refer to recent reviews for an overview on signaling mechanisms downstream of SA, including defense responses whose activation is dependent on Non-expressor of Pathogenesis-Related genes 1 (NPR1), a master regulator of SAR (Durrant and Dong, 2004; Loake and Grant, 2007; Vlot et al, 2009). In particular, we will discuss i) signaling/regulatory modulators that may trigger and amplify SA accumulation, ii) transcription factors presumed to have a direct role in *ICS1* expression, since (a)biotic stresses induce *ICS1* expression over a constitutive basal level, and iii) metabolic enzymes whose function impacts SA accumulation. We conclude with an integrated model of induced SA metabolism.

Signaling/Regulatory Modulators of Induced SA accumulation: NDR1, EDS1 and its Interaction Partners PAD4 and SAG101

The majority of characterized plant R proteins belong to the nucleotide binding-leucine-rich repeat (NB-LRR) class, which can be subdivided into two categories depending on whether the N terminus contains a toll-interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain (Collier and Moffett, 2009; Lukasik and Takken, 2009; Knepper and Day, 2010). Genetic analyses initially suggested that CC-type R proteins generally signal defenses via NDR1 (for Nonspecific Disease Resistance 1, At3g20600), whereas TIR-type NB-LRR proteins usually use EDS1 (Aarts et al, 1998). Subsequent studies identified two EDS1-interacting partners, PAD4 (for Phytoalexin Deficient 4, At3g52430), and SAG101 (for Senescence Associated Gene 101, At5g14930; Feys et al, 2001; 2005), that work co-operatively with EDS1 to regulate ETI triggered through TIR-type R proteins (Wiermer et al, 2005; Vlot et al, 2009). Although EDS1 was long thought to be fully dispensable for resistance responses mediated by CC-type R proteins, recent findings suggest that it acts redundantly with SA in this pathway; defenses triggered by CC-type R proteins were abrogated by simultaneous, but not single, mutations in *EDS1* and *ICS1* (also termed *SID2;* Venugopal et al, 2009). Furthermore, SAR induced by infection with *P. syringae* carrying avirulence genes recognized by CC-type R proteins was suppressed in *eds1* mutant plants (Truman et al, 2007; Rietz et al, 2011). In addition to R protein-mediated signaling, NDR1, EDS1, PAD4 and SAG101 have been implicated in PTI induced by virulent pathogens or PAMP treatment (Century et al, 1995; Wiermer et al, 2005; Vlot et al, 2009; Knepper et al, 2011).

A variety of studies have demonstrated that pathogen-induced SA accumulation is dependent on NDR1 and/or on EDS1 and its interaction partners (Wiermer et al, 2005; Vlot et al, 2009). The ability of SA treatment to rescue defense gene induction in *eds1* and *pad4* mutant plants, as well as the SAR-deficient phenotype of *ndr1*, indicates that downstream signaling is intact in the respective mutants. It also suggests that the corresponding genes act upstream of SA in the defense signaling cascade. Consistent with this possibility, global expression profiling of mutants in which SA and/or other defense-associated hormone pathways are impacted has placed *PAD4*, *EDS1*, and *NDR1* upstream of *ICS1* (Glazebrook et al, 2003; Wang et al, 2008; 2011). The observation that *EDS1* and *PAD4* expression are induced by SA suggests that together they may form a positive feedback loop that amplifies defense signaling.

In the cytoplasm, EDS1 forms both homodimers and what appear to be oligomers consisting of the EDS1 homodimer together with PAD4; in the nucleus, EDS1 forms molecularly and spatially distinct oligomers with PAD4 and heterodimers with SAG101 (Feys et al, 2005; Rietz et al, 2011). Recent findings suggest that EDS1 stabilizes PAD4 in the oligomer, and this oligomer plays a significant role in basal resistance and SAR. By contrast, low levels of dissociated EDS1 and PAD4 are sufficient for ETI triggered by TIR-type R protein signaling (Rietz et al, 2011). The functions of PAD4 and SAG101 appear to be partially redundant. Resistance to avirulent pathogens is suppressed to a lesser extent in *pad4* and *sag101* single mutants than in the *pad4 sag101* double mutant, which is as susceptible as the *eds1* mutant (Feys et al, 2005). EDS1 was recently shown to be shuttled between the nucleus and cytoplasm, and to be required in both cellular compartments to cover the full array of its defense-related functions (García et al, 2010).

Sequence analyses have revealed that EDS1, PAD4, and SAG101 share homology in a novel C-terminal domain, originally designated EP for EDS1-PAD4-specific (Feys et al, 2001; 2005). In addition, EDS1 and PAD4 contain an α/β fold hydrolase-type catalytic domain similar to the MeSA esterases discussed above (Wiermer et al, 2005). This putative hydrolase activity does not appear to be required for resistance, as EDS1-mediated resistance responses were restored in the *eds1* mutant by complementation with versions of *EDS1* carrying mutations in the conserved catalytic triad (J.E. Parker, personal communication). By contrast, alteration of a conserved residue in the EP domain of EDS1 impacted EDS1 transcript accumulation, interaction with PAD4, and activation of resistance responses, including *PR-1* expression, following pathogen infection (Feys et al, 2001). It has been difficult to parse out the specific mechanism(s) by which EDS1 and its homologs

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impact SA accumulation and response. This is likely due to the variety of its known distinct molecular, functional, temporal and spatial interactions. In addition, the emerging prominent role of EDS1 and its partners in redox metabolism during defense responses (Mateo et al, 2004; Mühlenbock et al, 2008; Straus et al, 2010) suggests additional mechanisms through which EDS1 affects cell death, disease resistance, and possibly SA accumulation.

In comparison to EDS1 and its interacting partners, NDR1 is a post-translationally modified plasma membrane-bound protein that interacts with RIN4. RIN4 is a membrane-associated protein whose modification or destruction by pathogen-encoded virulence factors regulates CC-type R protein activation (Chisholm et al, 2006; Jones and Dangl, 2006). Recent efforts to elucidate NDR1 function have revealed that it shares a high degree of structural similarity with the integrin-like protein LEA14 and with mammalian integrins, which play established roles in cell adhesion and signaling (Knepper et al, 2011). Consistent with this observation, *ndr1* hypocotyls exhibit reduced cell wall adhesion compared to wt plants.

Transcriptional regulators that influence *ICS1* expression: EIN3 and EIL1, CBP60g and SARD1, WRKY28, and NPR1

The transcription factors Ethylene Insensitive 3 (EIN3, At3g20770) and EIN3-Like 1 (EIL1, At2g27050) have long been known as positive regulators of ethylene (ET)-dependent responses. However, a recent study has indicated that they also negatively regulate both ETI and PTI (H. Chen et al, 2009). The *ein3-1 eil1-1* double mutant constitutively expressed *PR-1*, *PR-2* and *ICS1*, accumulated elevated levels of free and total SA, and displayed enhanced resistance to virulent and avirulent *P. syringae,* whereas overexpression of *EIN3* enhanced disease susceptibility. These results raised the possibility that EIN3 and EIL1 regulate SA-mediated defenses by binding the *ICS1* promoter and repressing its expression. Using DNA electrophoresis mobility shift assays, H. Chen et al. (2009) found that EIN3 specifically binds the P5 fragment of the *ICS1* promoter sequence *in vitro*; this fragment (-117 to -324 bp upstream of the translational start site; J.-M. Zhou, personal communication) contains three EIN3 binding sites [A(C/T) G(A/T)A(C/T)]. Furthermore, removing the P5 sequence from the *ICS1* promoter led to enhanced expression of an *ICS1*-reporter gene in wt, but not *ein3-1 eil1-1* double mutant plants. Thus, EIN3 and EIL1 appear to be key regulators at the intersection of ET and SA signaling, positively regulating ET signaling while repressing SA synthesis and its associated defense responses.

On the other hand, two members of a newly defined plant-specific family of transcription factors, Calmodulin-Binding Protein 60-like g (CBP60g, At5g26920) and SAR-Deficient 1 (SARD1, At1g73805) appear to act as positive transcriptional activators of *ICS1* expression. Double *cbp60g sard1* mutants exhibited little if any accumulation of SA in response to avirulent or virulent pathogens, and were compromised in PTI, ETI, and SAR (Y. Zhang et al, 2010; L. Wang et al, 2011). By contrast, overexpression of *SARD1* resulted in a dramatic increase in constitutive free and total SA accumulation, SA-dependent gene expression, and enhanced disease resistance (Y. Zhang et al, 2010). CBP60g and SARD1 were found to bind a fragment of the *ICS1* promoter -1,110 to -1,290 upstream of the translational start site, with highest affinity for oligo-15 (GAAATTTTGG, -1,208 to -1,217). While this sequence contains no known motif, it is over-represented in genes whose expression is reduced in the *cbp60g sard1* double mutant following pathogen treatment, suggesting that it is the core motif bound by CBP60g and SARD1 (L. Wang et al, 2011). CBP60g seems to play a more important role in PTI and early defense responses, while SARD1 is more dominant later, with a discernable impact on ETI and SAR. However, there is significant functional overlap, as only the double mutant displays the dramatic phenotypes described above (Wang et al, 2009; Y. Zhang et al, 2010; L. Wang et al, 2011). Interestingly, CBP60g binds calmodulin, while SARD1 does not (Wang et al, 2009; Y. Zhang et al, 2010; L. Wang et al, 2011). Thus, CBP60g in particular may integrate early Ca^{2+} signals as part of the induction of SA synthesis and response. Transcriptional profiling studies at 24 hours post infection (hpi) with virulent *P. syringae* placed CBP60g/SARD1 downstream of PAD4/EDS1 (L. Wang et al, 2011). However, it cannot be ruled out that early events mediated by CBP60g (and SARD1) may function upstream of PAD4/EDS1.

The WRKY family of transcription factors has long been associated with plant response to (a)biotic stress, and many WKRYs that act downstream of SA accumulation have been defined (Eulgem and Somssich, 2007). WRKY proteins bind to the W-box (consensus motif: (C/T)TGAC(C/T)) and a number of W-box core motifs are present in the *ICS1* promoter. WRKY28 (encoded by *At4g18170*) was recently shown to bind two W-box core motifs at positions -445 and -460 in the *ICS1* promoter (van Verk et al, 2011). Over-expression of *WRKY28* in Arabidopsis protoplasts enhanced expression of *ICS1::GUS*, and mutation of these two W-box core motifs in the *ICS1* promoter reduced expression. *WRKY28* expression is induced rapidly by PAMPs and an avirulent *P. syringae* strain (Navarro et al, 2004; AtGenExpress Biotic Stress Series (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp)). Taken together, these findings suggest WRKY28 may act as a positive regulator of induced *ICS1* expression.

NPR1 (encoded by *At1g64280*) is an ankyrin-repeat containing protein with a BTB/POZ domain (Durrant and Dong 2004; Loake and Grant, 2007; Vlot et al, 2009). Cytosolic NPR1 mediates antagonistic cross-talk between SA and JA, whereas nuclear-localized NPR1 functions in SA-dependent expression of defense genes, including *PR-1*. Following pathogen infection, SA accumulation leads to altered cellular redox status; this, in turn, partially reduces NPR1 and facilitates its localization to the nucleus. Once in the nucleus, NPR1 interacts with specific transcription factors to regulate gene expression (Dong 2004; Pieterse and Van Loon, 2004). In addition to mediating these signaling events downstream of SA, NPR1 appears to negatively regulate SA accumulation by suppressing *ICS1* expression. Consistent with this finding, pathogen-induced *ICS1* expression and SA accumulation are enhanced in the *npr1* mutant background, and NPR1's nuclear localization is required for suppression of *ICS1* expression (Wildermuth et al, 2001; X. Zhang et al, 2010). Presumably NPR1-mediated down-regulation of SA occurs once resistance responses have been successfully activated, in order to prevent continued escalation of SA levels. However, the mechanism through which NPR1 mediates this suppression is currently undefined.

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Metabolic enzymes impacting SA accumulation: EDS5, PBS3, and EPS1

EDS5 (also known as *SID1*; *At4g39030*) was initially implicated in the defense signaling pathway because mutations in this gene conferred enhanced susceptibility to virulent pathogens; *eds5* mutants also exhibited impaired SAR development and accumulated reduced levels of free and conjugated SA after pathogen inoculation or ozone treatment (Rogers and Ausubel, 1997; Volko et al, 1998; Nawrath and Métraux, 1999). Although SA treatment induces *EDS5* expression, several lines of evidence argue that EDS5 functions upstream of SA in the SA signaling pathway. These include i) exogenously supplied SA or SA analogs induce similar levels of *PR-1* expression in *eds5* and wt plants (Volko et al, 1998; Nawrath and Métraux, 1999) , ii) SA treatment induces comparable levels of SA accumulation in *eds5* and wt plants (Nawrath and Métraux, 1999), iii) *EDS5* transcripts accumulate prior to increases in SA or *PR-1* expression in pathogen- or UV light-treated plants (Nawrath et al, 2002), and iv) SA is not essential for stress-induced *EDS5* induction (Nawrath et al, 2002). Since *EDS1*, *PAD4*, and *NDR1* are at least partially required for stress-induced *EDS5* transcript accumulation, EDS5 appears to function downstream of these regulators (Nawrath et al, 2002). In addition, expression of *EDS5*, like that of *ICS1*, appears to be negatively regulated by NPR1 as *EDS5* transcripts are slightly elevated in stress-induced *npr1* plants (Nawrath et al, 2002).

Although EDS5 impacts SA accumulation, its specific function remains unclear. EDS5 shows homology to transporters of the MATE (Multidrug And Toxic compound Extrusion; Kuroda and Tsuchiya, 2009) family, which transport small organic molecules (Nawrath et al, 2002). Given EDS5's location in the chloroplast (Ishihara et al, 2008), it is possible that this protein regulates SA accumulation by controlling transport of specific molecules across the plastid membrane. One possibility is that EDS5 transports plastid-localized IC to the cytosol for subsequent conversion to SA. However, this appears unlikely since analysis of transgenic tobacco expressing bacterial ICS and IPL indicated that both enzymes needed to be targeted to the plastid for dramatic SA accumulation (Verberne et al, 2000). Alternatively, EDS5 might transport a regulator of SA synthesis into the chloroplast, or export SA from the plastid (Figure 3). Supporting this latter possibility, given the likelihood that SA is synthesized in the chloroplast, SA would need to be translocated to the cytoplasm to mediate its cytosolic functions; such export also might prevent a possible feedback inhibition of SA synthesis.

PBS3 (*At5g13320*) also was identified through genetic screens for mutants exhibiting reduced disease resistance (Warren et al, 1999). Characterization of *pbs3* mutants in various Arabidopsis ecotypes has implicated *PBS3* (also termed *WIN3, GDG1* or *GH3.12*) in both PTI and ETI (Warren et al, 1999; Jagadeeswaran et al, 2007; Lee et al, 2007; Nobuta et al, 2007; G. Wang et al, 2011). In addition, *pbs3* mutants displayed reduced *PR-1* expression following pathogen infection and accumulated dramatically reduced levels of total SA, primarily due to a substantial decrease in SAG levels. By contrast, free SA levels were reduced in some pathogen-inoculated *pbs*3 mutants, but elevated in others. *PBS3* encodes the GH3 acyl adenylase thioester-forming enzyme GH3.12 (Nobuta et al, 2007). Analysis of *PBS3* expression revealed that it is induced by non-host, avirulent, and virulent *P. syringae* pathogens with kinetics that are generally similar to those of *ICS1* (Jagadeeswaran et al, 2007; Lee et al, 2007; Nobuta et al, 2007; AtGenExpress Biotic Stress Series (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp)). In addition, *PBS3* expression is induced by SA and, based on elevated transcript accumulation in pathogen-inoculated *npr1* plants, is inhibited by activated NPR1 (Jagadeeswaran et al, 2007).

As discussed in the SA Modifications section, PBS3 prefers 4-substituted benzoate substrates, such as 4-HBA, while 2-substituted benzoates, like SA, are strongly disfavored. In fact, SA acts as a competitive inhibitor of 4-HBA conjugation (see SA Modifications). These findings, combined with the observation that stress-induced *pbs3* mutants exhibited wt levels of *ICS1* transcripts and protein, but accumulated elevated levels of SA-ASP, led to the suggestion that PBS3 enhances SA accumulation by suppressing SA catabolism, possibly by inhibiting GH3.5 and/or an unidentified GH3 (GH3.X) that inactivates SA by conjugating it to Asp (Okrent et al, 2011; Figure 3). In this scenario, the resultant increase in SA would feedback inhibit PBS3 activity; this in turn would allow resumption of SA-Asp formation, thereby reducing free SA accumulation. While this model could explain how loss of PBS3, which is predicted to be localized in the cytosol, confers the reduced levels of SAG, *PR-1* expression, and disease resistance, several questions remain, including the identity of PBS3's *in planta* substrates, the mechanism through which PBS3 impacts SA-Asp accumulation, and whether this GH3 enzyme has other functions, such as regulating 4-HBA available for incorporation into lignin.

Similar to *pbs3*, the *enhanced Pseudomonas susceptibility 1* (*eps1*) mutant displays hyper susceptibility to virulent and avirulent *P. syringae*, accompanied by significantly lower levels of SAG and total SA after infection compared to wt plants (Zheng et al, 2009). By contrast, free SA levels in *eps1-1* were reduced following infection with virulent *P. syringae*, but slightly elevated in response to avirulent *P. syringae*. Because exogenous SA induced *PR-1* expression and restored defenses against virulent *P. syringae* in *eps1-1*, EPS1 appears to act upstream of SA. *EPS1* was constitutively expressed at low levels in untreated plants, and transcript levels increased following *P. syringae* infection (Zheng et al, 2009). This induction was dependent on the JA signaling pathway, as it was blocked in the *coi1* mutant. Moreover, unlike *PBS3*, *EPS1* expression was induced by MeJA, but not SA, treatment. Interestingly, *eps1-1* (No-0 background) displayed enhanced resistance to two necrotrophic fungi, and this phenotype correlated with enhanced pathogen-induced expression of the JA marker gene *PDF1.2* (Zheng et al, 2009). By contrast, *eps1-2* (Col-0 background) did not exhibit enhanced resistance to necrotrophic fungi, although these plants, like *eps1-1*, displayed strongly enhanced symptom formation in response to virulent *P. syringae*. Thus, *EPS1* may play a role in SA-JA cross talk, with aspects of this response dependent on the ecotype.

EPS1 (*At5g67160*) encodes a putative BAHD acyl transferase (Zheng et al, 2009). The large BAHD family of acyl transferases (88 family members in Arabidopsis; Gang, 2005) is named after the first four family members identified, and it is characterized by two conserved motifs: i) the catalytic HXXXD motif required for acyl transferase activity, and ii) the C-terminal DFGWG motif (D'Auria, 2006). BAHD acyl transferases catalyze the transfer of an acyl moiety from acyl-activated coenzyme A (CoA) to O or N atoms

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in various plant secondary metabolites (D'Auria, 2006). EPS1 is a member of Clade II, which is comprised of two subgroups: IIa and IIb. Subgroup IIa contains 13 Arabidopsis BAHDs of unknown function including EPS1, while subgroup IIb encompasses almost all the functionally characterized anthocyanin/flavonoid malonyl transferases and hydroxycinnamoyl transferases (Yu et al, 2009). EPS1 is highly unusual in that the conserved His of the HXXXD motif is substituted with Ser (Zheng et al, 2009). As this His is required for acyl transferase activity (see references within Ma et al, 2005), EPS1 may not have this transferase activity. Perhaps as a non-functional enzyme, it sequesters substrates from or interacts with and inhibits specific BAHD Clade II members. Alternatively, it may have a unique biochemical or regulatory function.

Integrated Model of Induced SA Metabolism

Based on our current knowledge in Arabidopsis, we present an integrated model to provide a framework for discussing important features of induced SA metabolism (Figure 3). According to this model, SA metabolism is primarily regulated at the transcriptional level. In the absence of an inducing stress or hormone, the genes involved in SA synthesis and modification are expressed at low levels (see respective sections). Following an (a)biotic stress, activation of *ICS1* expression likely requires both de-repression of negative transcriptional regulators, such as EIN3 and EIL1, and activation by positive regulators, including CBP60g, SARD1, and/or WRKY28 (not shown in Figure 3). Since exogenous SA induces the expression of genes associated with robust SA accumulation, including *ICS1, EDS5, PBS3, SGT1* and *UGT74F1*, a feed-forward amplification loop also is likely present (Figure 3). Once sufficient levels of SA have been generated, SA-mediated activation of NPR1 leads to feedback inhibition of *ICS1* expression, thereby preventing runaway SA accumulation. Transcriptional regulation of SA modifying genes such as *BSMT1* and *GH3.5* by the hormones IAA, JA and ET (but not SA) also may play a role in controlling cellular SA levels (see SA Modifications section, and AtGenExpress Hormone and Chemical Series (Goda et al, 2008)). In this manner, IAA, JA, and/or ET can limit free SA accumulation, which in turn supresses the activaton of SA-induced defense responses. In addition, ET and JA promote the expression/stabilization of EIN3, which negatively regulates *ICS1* expression and thereby supressess SA levels (see above).

Superimposed on transcriptional regulation, SA metabolism can be rapidly fine-tuned at the biochemical level, largely by modulating the activities of SA modifying enzymes (Figure 3; SA Modifications section). Several of the modifying enzymes are promiscuous, with activity on substrates other than SA or its derivatives, such as GH3.5 which also conjugates IAA, or some of the MESs which hydrolyze MeJA and MeIAA in addition to MeSA. Therefore, local hormone concentrations and the comparative catalytic efficiencies of these enzymes, as well as their level of expression, all play a role in determining the level of free SA. SA-mediated inhibition of enzymes such as PBS3 and MES, which promotes free SA accumulation in the cytosol, also can feedback inhibit SA accumulation, once sufficient SA concentrations have been achieved. Whether sulfonation and/or DHBA synthesis impact free SA levels by regulating SA metabolism and, if so, the mechanism through which these processes are regulated, is currently unclear.

It is interesting to note that the SA modifying enzymes have very large differences in their K_m for SA (3->10X); thus, their activity on SA will vary in concert with the temporal and spatial variations in free SA levels. For example, a local threshold of ~200 μM free SA appears to be required for glucosylated SA formation by SAGT1 and UGT74F1 and robust activation of defense gene expression. By contrast, enzymes that convert free SA to inactive forms for transport or catalysis appear to have a higher affinity for SA (e.g. AtBSMT1 K_m= 15 μM). While the K_m for the GH3 enzyme(s) responsible for converting SA to SA-Asp is unknown, this enzyme(s) is depicted in Figure 3 as acting on low levels of SA based on i) data supporting a role for PBS3 as an inhibitor of SA-Asp formation, and ii) the ability of low concentrations of SA to inhibit PBS3 activity (IC_{50}= 15 μM). The substantially lower K_m exhibited by this latter group of enzymes towards SA raises the possibility that their activity could interefere with cytosolic SA accumulation and defense response activation. Indeed, this phenomenon was observed in Arabidopsis overexpressing *OsBSMT1* or *AtBSMT1* (Koo et al, 2007; Liu et al, 2010). Both sets of transgenic plants constitutively accumulated high levels of MeSA that increased further upon pathogen infection, whereas, the levels of SA, SAG, and disease resistance were substantially reduced as compared to wt plants. However, since wt plants are able to accumulate MeSA and high levels of SA and its glucosides, as well as activate defense responses following infection with an avirulent pathogen, these findings suggest that the expression of SA-modifying enzymes, like BSMT1, is highly restricted so that their activity is insufficient to prevent SA accumulation following pathogen infection. Supporting this hypothesis, *BSMT1* expression in unstressed Arabidopsis is virtually absent (Chen et al, 2003; Koo et al, 2007; Attaran et al, 2009; Song et al, 2009; Liu et al, 2011a). In addition, the proper balance between SA synthesis and catabolism may involve temporal and/or spatial separation of these competing processes.

FUTURE DIRECTIONS

The use of *Arabidopsis thaliana* has greatly facilitated our understanding of the synthesis, modification, and regulation of the phytohormone SA, as well as helped elucidate SA's role in plant response to (a)biotic stress. However, there remain many unanswered questions. To date, there is no fully-defined SA biosynthetic pathway in plants. For example, what enzyme(s) is responsible for the conversion of IC to SA? Additionally, the enzymes involved in SA synthesis via PAL have not all been identified, and it remains unclear when this pathway is operational. In Arabidopsis, the PAL pathway does not appear to play a significant role in (a)biotic stress-induced SA synthesis in leaves. However, this does not exclude an important role for SA synthesis via PAL. Low level, perhaps highly localized, SA production in shoots and/or roots may play an important role(s) in cell death, growth, and/or other processes yet to be defined (Vanacker et al, 2001). By fully defining SA biosynthetic pathways, one can assess the relative contribution of these pathways to their functional output (e.g. robust defense gene induction, cell growth, etc.).

Furthermore, by examining SA metabolism and response in other species, shared and divergent components, regulatory

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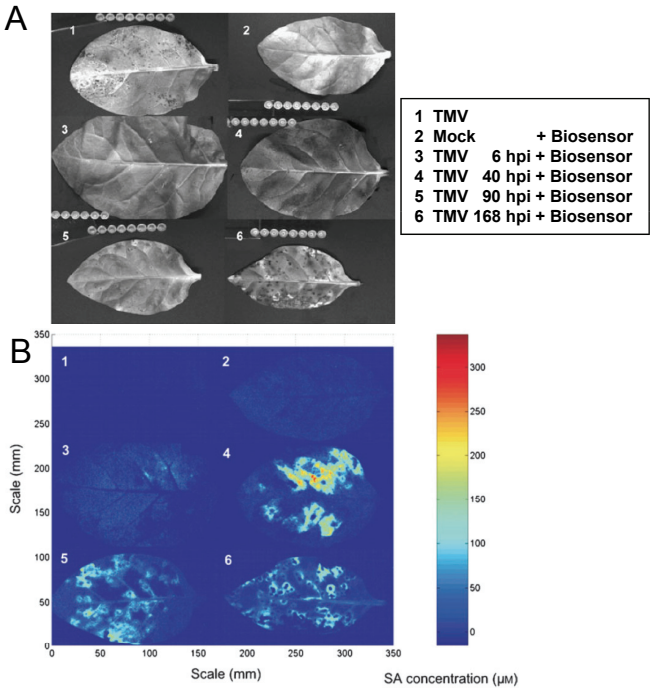


Figure 4. Spatial and temporal variation in free SA accumulation in TMV-infected tobacco.

Leaves of TMV-infected tobacco undergoing a hypersensitive response **(A)** photographed under room light or **(B)** imaged in the dark for SA-induced bioluminescence from the infiltrated *Acinetobacter* sp. ADP1 SA biosensor. A false color coded SA concentration map based on *in vitro* concentration ladders is shown in **(B)**. Dark spots in **(A)** are HR lesions. From Huang et al. (2006) Plant Journal 46: 1073-1083 with permission from John Wiley & Sons.

schemas, and functional outputs may be ascertained. For example, in some species, IC metabolism is more complex than in Arabidopsis, with significant flux of induced IC channeled to the formation of anthraquinones (van Tegelen et al, 1999b; Stalman et al, 2003). In other plants, such as rice and *Populus* species, although SA (and SA-derived compounds) play an important role in disease and herbivory resistance, SA synthesis is not dramatically induced by (a)biotic stress. Instead, basal SA plus SA glucoside levels are high, typically greater than those found in pathogen-induced Arabidopsis leaves (Silverman et al, 1995; Koch et al. 2000). Precursor feeding and PAL inhibition studies have implicated the PAL pathway for SA synthesis in rice (Silverman et al, 1995) and poplar (Ruuhola and Julkunen-Tiito, 2003). Thus, it is currently unclear whether ICS plays a role in SA synthesis in these high SA accumulating plants.

Although considerable progress has been made in identifying and characterizing key players in SA metabolism, spatially and temporally resolved analyses are now needed to understand how these enzymes function *in planta*. In addition, inter- and intra-cellular SA transport must be examined. We know SA can be transported as MeSA to distal tissue via the phloem. How and in what form(s) is SA transported within and between cells at the site of infection? Is free SA accumulation in cells neighboring an HR associated with transport of SA to those cells or with the induction of *de novo* SA synthesis? For these efforts, both compound and gene/protein reporters (of SA biosynthetic and modifying enzymes) are required for *in situ* visualization. Compound visualization ideally would be concentration- and locale (e.g. cytosol vs. plastid)-specific. An engineered *Acinetobacter* biosensor that produces SA-induced bioluminescence has provided important spatial and temporal data on apoplastic SA in tobacco (Figure 4; Huang et al, 2006); unfortunately, it does not work well with Arabidopsis. Synthetic SA-responsive promoters coupled to reporter proteins similar to those used in auxin research (Benková, et al, 2003), SA FRET nanosensors such as those developed to detect a range of glucose concentrations (Deuschle et al, 2006), and other SA reporters/sensors using emerging technologies (e.g. riboswitch sensor for B12 (Fowler et al, 2010)) would be invaluable components of an SA tool box. Markers that reflect a given SA functional output are also important, as evidenced by the widespread use of *PR-1*-GUS as a marker of robust NPR1-dependent defense gene expression.

Coupling visualization tools with cell-specific global analyses (e.g. expression profiling of laser micro dissected cells at the infection site (Chandran et al, 2010)) and computational models that incorporate and integrate disparate types of data will facilitate i) the identification of missing components in SA metabolism and ii) the integration and coordination of SA metabolism and response with that of other networks. For example, there is substantial cross-talk between SA and other hormones including, but not limited to, JA, ET, and IAA; additional layers of cross-talk are discussed in a recent review by Robert-Seilaniantz et al. (2011). Importantly, computational models allow one to describe a complicated system and develop testable hypotheses (e.g. in plant development (Roeder et al, 2011)). Future research on SA will allow us to better understand at the (sub)cellular level how plants respond to (a)biotic stress and will place this information in the context of other hormone and stress-response pathways. Comparing findings in Arabidopsis with those from other species will enable us to explore the selective pressures driving the evolution of SA synthesis, modification, and response in diverse plants.

ACKNOWLEDGEMENTS

We thank our colleagues for providing recent reprints and preprints of their studies. We apologize to those researchers whose work was not included in this review and/or was referenced indirectly through other publications. The grants supporting studies reported in this chapter from the group of D.F.K. were/are US National Science Foundation IBN-0241531, IOS-0525360 and IOS-0820405. NSF MCB-04202267 and IOS-0958100 support(ed) research in the laboratory of M.C.W. Work in the laboratory of A.C.V. is supported by the DFG as part of SFB924.

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