The diversity of salicylic acid biosynthesis and defense signaling in plants: Knowledge gaps and future opportunities

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Abstract

The phytohormone salicylic acid (SA) is known to regulate plant immunity against pathogens. Plants synthesize SA via the isochorismate synthase (ICS) pathway or the phenylalanine ammonia-lyase (PAL) pathway. The ICS pathway has been fully characterized using *Arabidopsis thaliana*, a model plant that exhibits pathogen-inducible SA accumulation. Many species including *Populus* (poplar) depend instead on the partially understood PAL pathway for constitutive as well as pathogen-stimulated SA synthesis. Diversity of SA-mediated defense is also evident in SA accumulation, redox regulation, and interplay with other hormones like jasmonic acid. This review highlights the contrast between *Arabidopsis* and poplar, discusses potential drivers of SA diversity in plant defenses, and offers future research directions.

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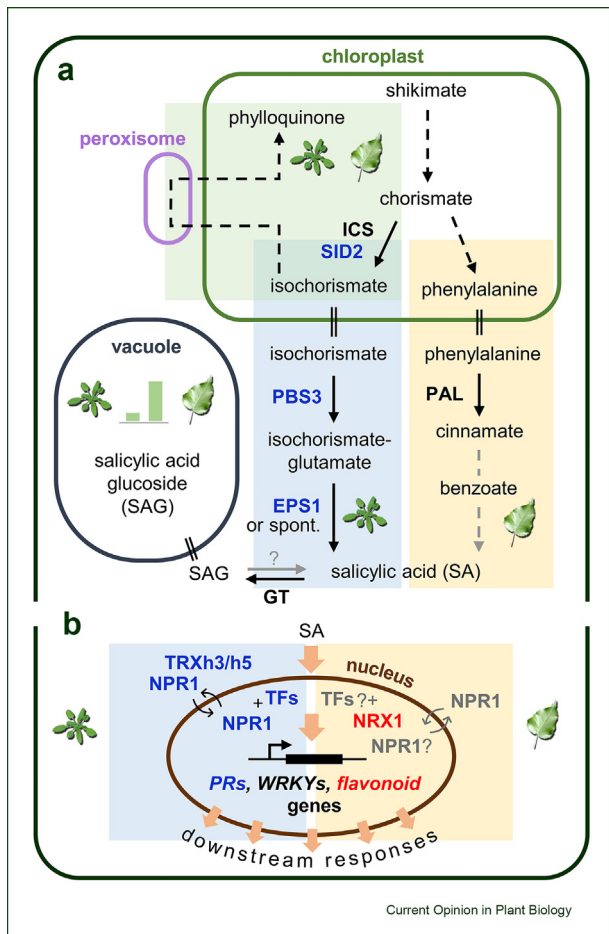Corresponding author: Tsai, Chung-Jui (cjtsai@uga.edu)

Introduction

Salicylic acid (SA) is a phytohormone and a central mediator of pathogen-induced plant defenses [1,2]. The discovery of SA has a storied past; it was first prepared from salicin, an analgesic produced in willow and poplar (family Salicaceae), and ultimately inspired the development of the blockbuster drug aspirin [3]. Nearly two centuries following its discovery, our understanding of SA biosynthesis in plants remains incomplete. Significant progress in understanding SA action in plant defense has been made using the powerful *Arabidopsis* model system. Translating these findings to crops and trees has not been straightforward, in part because of variation between species in SA biosynthesis, accumulation, and signaling. This review highlights recent advances in SA biosynthesis, redox regulation, and signaling crosstalk, with an added focus on the woody perennial *Populus* (poplar). Given the multifaceted roles of SA in plant responses to biotic and abiotic stresses, the underexplored diversity in SA biosynthesis and signaling presents a challenge to translational research for crop improvement in the face of climate change.

SA biosynthesis pathways: universal or lineage-specific?

Plants have evolved two distinct routes for synthesizing SA (Figure 1). Classical radiolabeling studies in diverse plant species support a biosynthetic origin of SA from cinnamic acid and benzoic acid [4,5]. This route shares common steps with the central phenylpropanoid pathway initiated by phenylalanine ammonia lyase (PAL) in the cytosol (Figure 1A). Despite this early finding, several biosynthetic enzymes leading to benzoic acid and SA remain hypothetical [6]. Major breakthroughs have come from forward genetic screens and functional characterization of *Arabidopsis* SA mutants. The chloroplastic AtICS1 (isochorismate synthase1, also known as SA INDUCTION DEFICIENT2 or SID2) is central to pathogen-induced SA synthesis [7]. This ICS pathway (Figure 1a) resembles the eubacterial synthesis of SA-derived siderophores [8], but the plant homolog for bacterial isochorismate-pyruvate lyase remained elusive. In 2019, two groups independently identified the missing link as AtGH3.12, an acyl adenylase family

Figure 1

Diversity of SA biosynthesis and signaling. **a.** SA biosynthetic routes and vacuolar storage of SA conjugates. ICS and PAL pathways are shown in blue and yellow, respectively. The three Brassicaceae-specific proteins are shown in blue. Dashed lines indicate multi-step pathways and grey denotes unclarified steps. Ancestral ICS function in phylloquinone biosynthesis via chloroplast and peroxisome is shown in green box. **b.** Simplified representation of SA-sensitive redox signaling with key players shown. Responses reported for *Arabidopsis* and *Populus* are shown in red and blue, respectively.

[10−12]. *AtICS1* is derived from a Brassicaceae-specific genome duplication, and its exon-intron structure, basal expression, and pathogen-inducibility all differ from *AtICS2* [7,13]. *AtICS2* resembles the ancestral gene involved in the biosynthesis of phylloquinone (vitamin K1) essential for photosynthetic electron transport [11**] (Figure 1a). The phylloquinone pathway has an endosymbiotic origin and is similar to eubacterial menaquinone (vitamin K2) biosynthesis [14,15]. Interestingly, pathway diversification at ICS also occurs in eubacteria, with EntC and MenF differentially involved in the biosynthesis of SA-derived siderophores and menaquinone, respectively [8,16]. Poplar, like many genome-sequenced plants, harbors a single *ICS* that is transcriptionally insensitive to abiotic stress and SA [11**,17]. These data strongly suggest recruitment of AtICS1 to SA biosynthesis as a lineage-specific event. The recent findings that downstream enzymes AtGH3.12 and AtEPS1 for SA biosynthesis are also Brassicaceae-specific [10,12,18] lend credence to the ICS pathway as a taxon-restricted evolutionary novelty rather than a conserved mechanism.

PAL pathway involvement in SA synthesis (Figure 1a) by many crop and tree species remains under-studied even where evidence for ICS involvement is at best equivocal. In barley, for instance, characterization of a homozygous *ics* mutant derived from fast neutron mutagenesis unequivocally demonstrated ICS involvement in phylloquinone, but not SA, biosynthesis [19*]. In the absence of large-scale mutant collections, CRISPR-based approaches can be used to generate targeted knockout mutants to aid functional characterization. Alternatively, a genome-wide association study (GWAS) in conjunction with metabolite and expression profiling of 300 unrelated *Populus tomentosa* individuals identified putative pathway intermediates and candidate genes for SA biosynthesis [20]. Although that analysis did not discriminate between ICS and PAL pathways for relevance to poplar SA synthesis, similar approaches with a refined focus should be fruitful in the quest for missing genes in the PAL pathway.

SA redox signaling: the devil is in the details

Arabidopsis SA defense signaling requires AtNPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1), a redox-sensitive master regulator and SA receptor [21]. Upon pathogen stimulation or SA treatment, AtNPR1 undergoes conformational changes for translocation into the nucleus where it interacts with other transcription factors to activate downstream defense gene expression [22] (Figure 1b). Redox modification of cysteine residues in both the protein-protein interaction and the cryptic trans-coactivation domains of NPR1 is essential for its regulatory function [21,23,24]. For instance, mutations in the evolutionarily conserved

protein (also called AtPBS3 or avrPphB SUSCEPTIBLE3) involved in disease resistance [9,10]. AtGH3.12 is a cytosolic enzyme and catalyzes the conjugation of glutamate to isochorismate (IC) to form IC-glutamate [9,10] (Figure 1a). The IC-glutamate conjugate is then converted to SA either spontaneously or more efficiently by AtEPS1 (ENHANCED PSEUDOMONAS SUSCEPTIBILITY1), a BAHD acyltransferase-like protein [9,10].

These studies fully elucidated the ICS pathway for SA biosynthesis in *Arabidopsis*, but several questions remain. Perhaps the most underappreciated fact is that all three biosynthetic genes in this pathway, *AtICS1*, *AtGH3.12*, and *AtEPS1*, are only found in the Brassicaceae family

Cys[82] and Cys[216] resulted in monomerization and nuclear localization of NPR1 in both *Arabidopsis* and rice (*Oryza sativa* L.) and constitutive activation of defense gene expression [25,26]. However, SA-induced AtNPR1 monomerization and nuclear import is catalyzed by thioredoxins AtTRXh3 and AtTRXh5 via thiol-disulfide exchange reactions at Cys[156] [21]. Paradoxically, this AtNPR1-AtTRXh3/h5 interaction appears taxon-restricted because the regulatory Cys[156] is found in a small minority of the Viridiplantae NPR gene family in Phytozome v13 (13 out of 181 members), all belonging to Brassicaceae. The rice ortholog OsTRXh2 implicated in OsNPR1-dependent disease resistance [26] was recently shown to be a direct target of a bacterial effector for proteasomal degradation, which dampens host immunity [27**]. *Nicotiana benthamiana* defense against barley stripe mosaic virus (BSMV) also depends on SA activation and signaling, but involves the AtTRXh1 ortholog NbTRXh1 [28**]. BSMV γb protein physically interacts with NbTRXh1 to weaken its reductase activity, thereby allowing viral spread [28**]. Whether NbNPR1 has a role is not known. In rice, pathogen-induced SA response can be independent of OsNPR1, as the transcription factor OsWRKY45 bypasses the NPR1-mediated defense [29,30]. Similarly, in *Populus*, *NPR1* is poorly expressed, insensitive to *in planta* or exogenous SA manipulations, and lacks a defined role in pathogen defense [17,31**].

These studies support diversified redox signaling in SA-mediated defense depending on the species and/or cellular milieu, due in part to the functional multiplicity of plant TRX proteins [32,33]. For instance, AtTRXh5 but not its genome duplicate AtTRXh3 is involved in the *Arabidopsis* response to victorin, a fungal toxin [34]. *Populus* lacks *AtTRXh3/h5* orthologs but harbors a tandem array of *Nucleoredoxin1* (*NRX1*), a subfamily of the TRX superfamily [17]. Gene network modeling identified *PtaNRX1* as the only small redox protein family in poplar leaves that exhibits SA-dependent regulation [17]. The ortholog in cotton (*Gossypium barbadense*), GbNRX1, has been implicated in resistance against soil-borne pathogens via apoplast ROS modulation [35]. *Arabidopsis* AtNRX1 also modulates ROS scavenging; however, it acts as a negative regulator of disease resistance [36]. These data reinforce the complexity and diversity of redox signaling, as well as the challenges in translating results across species. With the advent of CRISPR technologies, precision gene editing at the allele, individual gene or gene family level can now be routinely achieved in a growing number of crop species. A recent study reported generation of null *nrx1* poplar mutants by CRISPR knockout of all seven tandemly duplicated genes [37]. Such mutants will be invaluable for functional characterization to address redundancy versus specificity of small redox protein (super)family members in SA signaling.

SA-JA interplay can be antagonistic and synergistic in plant defense

Research from *Arabidopsis* has established that SA-mediated defense is more effective against (hemi)-biotrophs while jasmonic acid (JA)-mediated defense is operative against necrotrophs. Furthermore, it is broadly accepted that these two hormones function in an antagonistic manner. Consistent with this dichotomy, certain pathogens have evolved virulence mechanisms that exploit the SA-JA antagonism to neutralize host defense [38,39]. The SA receptor AtNPR1 controls not only SA signaling but also SA-JA antagonism [40]. The JA receptor AtCOI1 (CORONATINE INSENSITIVE1) regulates JA-mediated inhibition of the SA pathway [41]. However, several recent studies suggest alternatives to SA-JA antagonism. For instance, the SA receptors NPR3 and NPR4 function oppositely to NPR1 [42] and can activate JA signaling during effector-triggered immunity with synergistic induction of both SA and JA defenses [43]. Under natural conditions, plants encounter numerous pathogens simultaneously, necessitating deployment of varied SA and JA defenses for ecological success. A meta-analysis of global transcriptome data revealed that a large number of defense genes activated by both biotrophy and necrotrophy are also induced by SA and JA [44]. Another study demonstrated spatial coordination of the SA and JA pathways during effector-triggered immunity in *Arabidopsis* [45]. The SA-JA interplay is likely modulated by additional factors, including the early signaling after pathogen recognition by the host, pathogen lifestyle, plant species and developmental stage, and resource availability, all of which deserve further research.
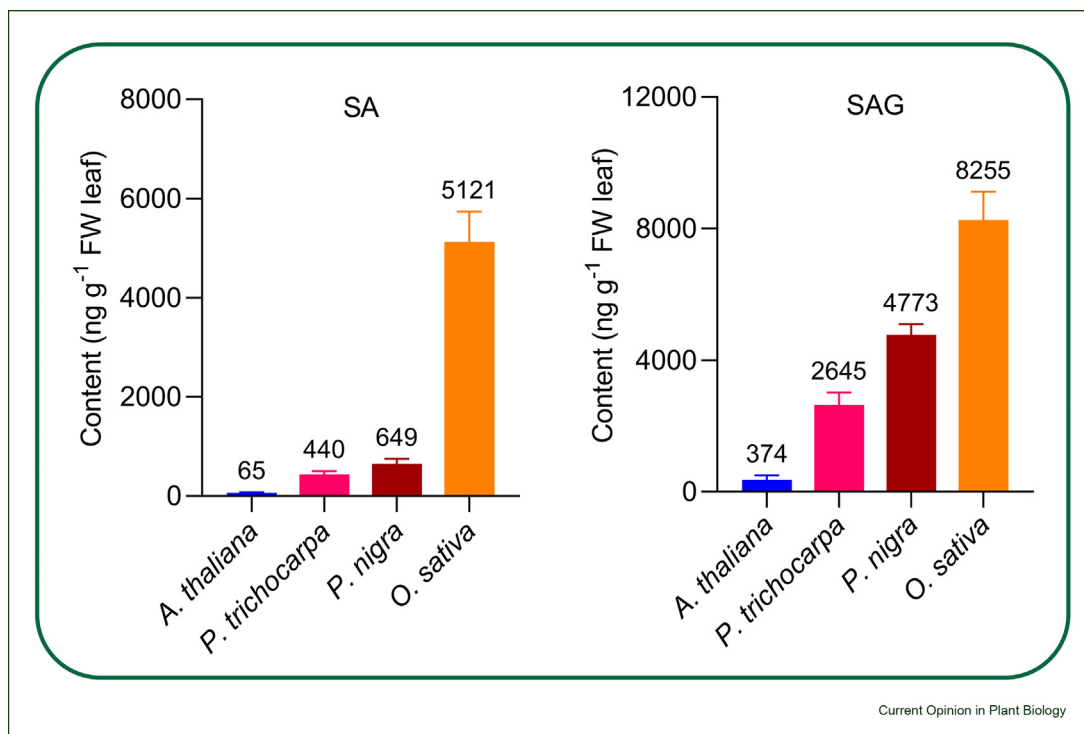
In *Populus*, SA concentrations increase markedly upon infection by multiple pathogens, including biotrophs [31**], hemibiotrophs [46], and necrotrophs [47]. Interestingly, JA-metabolites also increased in response to the rust fungus *Melampsora larici-populina*, an obligate biotroph [31**,48**]. Complex SA and JA signaling interplay may be especially relevant in long lived perennials because biotic pressures are varied and must be coped with year-round. In a recent study, poplars with constitutively elevated SA were found to accumulate higher levels of JA metabolites while exogenous JA applications increased the content of SA [48**.] Transgenic perturbation of the Salicaceae-specific salicinoid biosynthesis by CRISPR-knockout of a glycosyltransferase in poplar also resulted in elevated accumulation of both SA and JA-metabolites [49*]. SA was shown to positively regulate the biosynthesis of flavonoid phytoalexins, such as flavan-3-ols in poplar [31**,48**] and sakuranetin in rice [50]. JA also increases these antimicrobial flavonoids in both species [48**,51]. In rice, SA hydroxylase-knockout lines increased not only SA but also JA and sakuranetin contents, which resulted in broad-spectrum resistance

against both hemibiotrophic and necrotrophic pathogens [52*].

These examples suggest synergistic SA-JA interactions may be more common in poplar and rice than in *Arabidopsis*. While the underlying molecular mechanism awaits further investigation, variations in SA biosynthesis, homeostasis, and signaling pathways likely contribute to the diversity. The basal levels of SA in poplar and rice leaves are substantially higher than pathogen-induced SA accumulation in *Arabidopsis* [31**,53] (Figure 2). Compared to *Arabidopsis*, levels of SA-glucoside (SAG) stored in poplar and rice leaves are 10- and 20-fold higher, respectively (Figure 2). SAG can be hydrolyzed to SA without *de novo* synthesis under stressed conditions [54], though the responsible enzyme has not been characterized. Both SA and SAG (and the dihydroxybenzoate derivatives) can activate defense response [55,56]. The low-SA accumulator *Arabidopsis* relies on the pathogen-inducible ICS pathway for defense and often displays a trade-off with JA signaling. In contrast, woody perennials and field crops accrue substantial amounts of SA and SAG via the PAL pathway, which shares common precursors for the biosynthesis of several classes of phytoalexins co-regulated by SA and JA (Figure 3). Furthermore, several *Pathogenesis-related* (*PR*) genes are frequently considered as SA markers in *Arabidopsis* [2]. However, *PR* gene expression remained largely unchanged in poplars with multifold differences in SA levels or following exogenous applications with an SA analog [48**], and *PR* genes were absent in the poplar SA-responsive gene network modules [17]. Instead, *PR* transcript abundances correlated positively with pathogen growth in infected leaves across multiple poplar genotypes with varying levels of rust resistance [31**,48**]. This is consistent with the original discovery of PRs as abundant antimicrobial proteins following pathogen infection [57]. Variable SA-responsiveness of the expanded *PR1* gene family has also been reported in rice [58]. The different transcriptional responses of *PR* genes to SA and other chemical stimuli may reflect differential adaptive evolution between species.
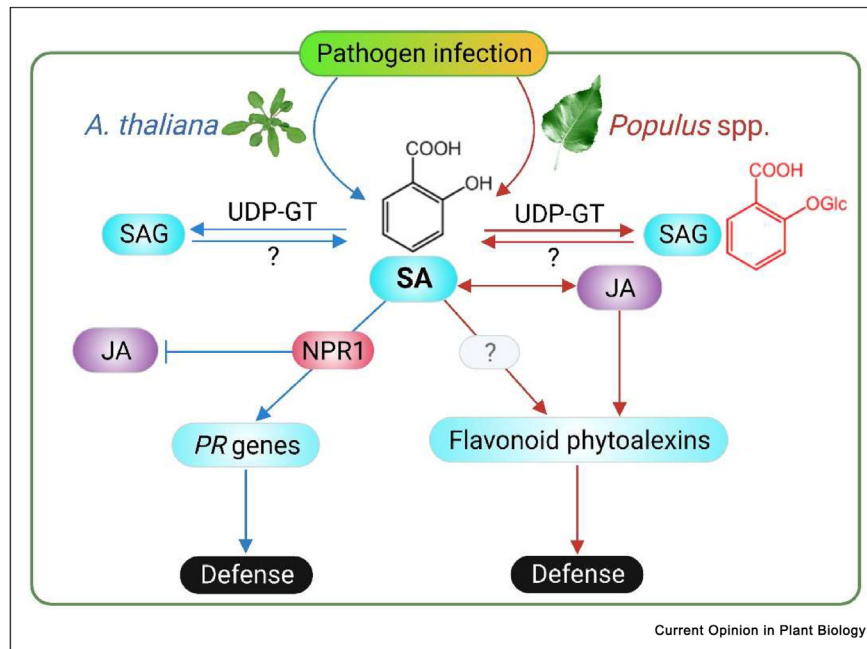
Figure 2

Constitutive levels of SA and SAG in the leaves of *Arabidopsis*, poplar and rice. Hormone levels were determined using liquid chromatography coupled with tandem mass spectrometry as described previously [48**] using approximately 40 mg of fresh tissues. *A. thaliana* (ecotype Col-0) plants were grown under standard long day (16/8 h day/night cycle) growth conditions at 21°C, and four-week-old rosette leaves were collected for SA analysis.
P. trichocarpa (genotype 262/263) and *P. nigra* (genotype Firtzlar2) were grown as described previously [48**]. Three youngest fully-expanded leaves from trees around 80 cm in height were collected for hormone analysis. *O. sativa* (cv. Nipponbare) seedlings were grown under the conditions described previously [62] and leaf samples were harvested from 4-week-old seedlings. Data represent mean with standard error (n = 5). Average levels of SA and SAG are indicated above the bars.

Figure 3

Regulation of SA-mediated immunity in *Arabidopsis* and *Populus* against phytopathogens. In *Arabidopsis*, infection by biotrophic pathogens activates defense via the SA receptor NPR1, often with concomitant inhibition of JA signaling (blue lines). In poplar, both SA and JA pathways are induced simultaneously upon pathogen attacks, irrespective of their lifestyles (biotrophy or necrotrophy). Synergism between SA and JA pathways induces the accumulation of antimicrobial flavonoids, including catechins and proanthocyanidins (red arrows). Pathogen-inducible *PR* genes are not responsive to constitutively elevated SA in the absence of pathogens, and the involvement of NPR1 in poplar pathogen defense is unclear.

Conclusions and future directions

Multiple aspects of SA-mediated defense signaling from SA biosynthesis and homeostasis to redox modulation and hormonal crosstalk are more diverse than previously thought. Emerging data suggest that the key genes and regulators identified from the *Arabidopsis* model represent lineage-specific innovations and may not be directly translated across species. However, the SA signal relay and regulatory cascades defined in *Arabidopsis* provide a foundational framework to guide research efforts in crops. Elucidating missing steps in the PAL-mediated SA biosynthetic pathway will require integration of genetic, transcriptomic and metabolomic approaches as elegantly illustrated for the *Arabidopsis* ICS pathway [9,10]. For crop and tree species that lack genome-scale mutant collections, exploiting natural variations by GWAS and high-throughput phenotyping can be fruitful. This approach successfully identified multiple resistant and susceptible genes against the causal fungal pathogen of *Septoria* canker in poplar [59*]. Closing knowledge gaps in redox modulators and response markers will also be critical toward understanding SA signaling in diverse crop species. In the absence of SA mutants that have been instrumental in *Arabidopsis* research, ectopic expression of a bacterial SA synthase for constitutive (or inducible) accumulation of SA in non-model species can generate

novel insights for downstream processes. This approach has been used in different poplar species to facilitate discovery of NRX1 as an alternative to TRXh in SA-responsive redox cascades [17], SA regulation of flavonoid phytoalexins [31**], and synergistic SA-JA crosstalk in pathogen defense [48**]. Given the multifaceted role of SA in biotic and abiotic defense as well as growth and development [60,61], taxon-specific fine-tuning and evolutionary novelties in SA synthesis, activation and signaling cascades are perhaps the norm. Exploiting this diversity can lead to additional molecular targets to aid breeding, genomic selection and/or CRISPR-based crop improvement efforts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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* of special interest
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