# Salicylic acid in plant defence—the players and protagonists

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Salicylic acid (SA) is synthesised by plants in response to challenge by a diverse range of phytopathogens and is essential to the establishment of both local and systemic-acquired resistance (SAR). SA application induces accumulation of pathogenesis-related (PR) proteins. Mutations leading to either reduced SA production or impaired SA perception enhance susceptibility to avirulent and virulent pathogens. However, our knowledge of the primary signalling components activating SA biosynthesis and linking to PR proteins accumulation is rudimentary. We review progress towards characterising key players (NPR1, MPK4) and processes (methylation, amino acid conjugation, *S*-nitrosylation) contributing to SA-signalling and perception pathways. Further, we examine emerging data on how pathogens have evolved strategies (e.g. ABA modulation and coronatine production) to suppress SA-mediated plant defence.

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## Introduction

The pharmacological properties of salicylate derivatives from willow bark were for centuries prized by ancient Greeks and American Indians, eventually leading to the invention and marketing of aspirin (acetylsalicylic acid), a mammalian cyclooxygenase inhibitor and the oldest, most widely used drug in history [1]. Treatment of plants with aspirin subsequently led to the demonstration that SA derivatives could induce both the resistance and the accumulation of PR proteins [2]. SA accumulation has subsequently been associated with a plethora of biotic and abiotic responses, but research is predominately polarised towards its role in plant defence responses and therefore this review primarily focuses on recent literature relating to the role of SA in biotic interactions.

## SA biosynthesis in defence

Plant resistance to biotrophic pathogens is classically thought to be mediated through SA signalling. By contrast resistance to nectrotrophic resistance is controlled by jasmonic acid (JA), ethylene (ET)-signalling pathways and genetically, SA and JA/ET defence pathways interact antagonistically (reviewed in references [3,4]).

Pathogen-derived SA is synthesised from chorismate by isochorismate synthase (ICS1). Chorismate itself is derived from the shikimate pathway whose components are strongly transcriptionally upregulated following pathogen challenge [5]. Recombinant ICS1 binds chorismate with high affinity, is active over a range of physiological parameters and is imported and immunolocalised to chloroplast stroma [6•]. The low Km of ICS1 would facilitate substrate competition in other chorismate-utilising enzymes; however, further biochemical characterisation of ICS1 and the related ICS2 is necessary to reveal their respective roles in stress and developmental responses.

## Bioactive SA conjugates

Modification of hormones by glucoslyation, methylation and amino acid conjugation are increasingly being recognised as integral to spatial and temporal control of plant regulatory processes. Most pathogen-induced SA is glucosylated by UDP-glucosyltransferase (UGT) to form non-toxic SA 2-*O*-β-D-glucoside (SAG) that is sequestered in vacuoles where it presumably forms a readily available hydrolysable source of SA [7]. However, recent advances suggest other SA modifications provide biological specificity in plant defence responses.

### Methylation

Methyl salicylate (MeSA), a volatile ester, is normally absent in plants but is dramatically induced upon pathogen infection [8,9].

MeSA is synthesised by SA carboxyl methyltransferase (SAMT), a member of the SABATH methyltransferase family, using the methyl donor *S*-adenosyl-l-Met and carboxylic acid containing substrates [10,11]. Arabidopsis plants overexpressing *Oryza sativa OsBSMT1* accumulated MeSA and MeBA (methyl benzoic acid) [12•]. Infection of *OsBSMT1* overexpressors with the fungal pathogen *Golovinomyces orontii*, or *Pseudomonas syringae* resulted in increased susceptibility and reduced accumulation of SA, the inactive SA glycoside (SAG) and *PR1* compared with wild-type plants [12•]. Strikingly, *OsBSMT1* overexpressors triggered *PR1* induction in neighbouring wild-type plants, which was not dependent upon ICS1-derived SA but was dependent upon non-expressor of PR1

(NPR1) a central positive regulator of systemic defence [12•,13]. Although an alternative route of SA production is conceivable, data suggest MeSA alone is ineffective in inducing a defence response but can function as a volatile signalling molecule.

In tobacco, conversion of MeSA to SA appears to be catalysed by the high SA affinity cytosolic SA methyl esterase SABP2 (salicylic acid binding protein 2; [14]), SABP2-silenced tobacco plants had attenuated local resistance to tobacco mosaic virus (TMV), reduced *PR1* expression and were compromised in SAR [15••]. MeSA increases in both primary infected and systemic tissue following TMV inoculation, which is attenuated by overexpressing a mutant SABP2 with unregulated MeSA esterase activity in the primary infected tissue. Moreover, grafted tobacco plants silenced for *SABP2* in scions but not rootstocks show attenuated SAR, suggesting that MeSA is required for SAR (D. Klessig per. com). Collectively, these data implicate MeSA as a mobile or volatile inducer of SAR.

By contrast, recent studies implicate lipid signals such as JA-derived molecules are required for SAR [16,17••], and a putative lipid transfer protein is required in challenged tissue to initiate a mobile signal [18]. Previously the observation that JA-induced *AtBSMT1* provided a mechanistic explanation for how JA pathways may antagonise SA pathways by depleting the SA pool in plants [10]. However, it is equally conceivable that both SA and JA derivatives corroborate in temporally and spatially distinct modes of action to activate SAR. Owing to the inherent complexity of biotic signalling networks, detailed time-resolved studies are imperative to advance our understanding of the dynamics of host pathogen interactions leading to SAR.

### Amino acid conjugation

SA amino acid conjugates are emerging as bioactive inducers of defence responses. The jasmonate-resistant protein JAR1 is a member of the auxin-induced soybean GH3 acyl adenylate/thioesterase family and catalyses JA conjugation to isoleucine [19]. Functional analyses of this 19-member family in Arabidopsis identified At4g27260, which adenylates SA *in vitro* [19]. Recently, Arabidopsis PBS3 (*avrPphB* susceptible) was identified as a likely GH3 family phytohormone–amino acid synthase [20•]. *pbs3* plants exhibit enhanced susceptibility to both virulent and avirulent pathogens, attenuated expression of *PR1* and pathogen-induced accumulation of SAG, yet accumulated twofold the SA levels of wild-type plants following challenge with *P. syringae* expressing *avrRpt2*. These data imply elevated SA is not sufficient to activate *PR1*, or a threshold level of total SA (free SA and SAG) is necessary to activate *PR1* expression. Nobuta *et al.* speculate that an SA-aa may be exported to adjacent cells and subsequently hydrolysed to free SA that activates defence responses. Alternatively, the SA-aa conjugate may be targeted for the degradation pathways [20•]. Ubiquitin-mediated degradation of negative regulators of hormone-signalling pathways appears fundamental to hormonal control (reviewed in reference [21]). Indeed, mutations in the small ubiquitin modifier E3 ligase, SIZ1, regulate SA-mediated innate immunity [22].

## Have pathogens evolved to suppress SA signalling?

Plant responses to different environmental stresses are achieved through integrating shared signalling networks and mediated by the synergistic or antagonistic interactions with the phytohormones SA, JA, ET, abscisic acid (ABA) and reactive oxygen species (ROS). How particular stresses are decoded and translated to provide the output specificity remains largely unknown; it is likely both temporal and spatial hormonal balances contribute significantly [23]. As hormone-based defences enable rapid global activation of a broad spectrum of physiological responses it is unsurprising pathogens have evolved multiple strategies to suppress SA-based plant defences.

### JA/SA antagonism

Genetic evidence for JA antagonism of SA-signalling pathways has been well documented using jasmonate-signalling mutants [4], but emerging data suggest a more complex signalling network evoking both positive and negative regulatory interactions. Simultaneous application of SA and JA at low concentration results in synergistic expression of *PR1* and classical JA defence markers. By contrast, higher phytohormone concentrations are antagonistic and induce apoplastic reactive oxygen production and cell death [24]. Similarly, JA or a derivative thereof is necessary for full development of SAR [17••]. It is notable that SA and allene oxide synthase (AOS; the first committed enzymatic step in JA biosynthesis) share a chloroplastic location for synthesis and activity, respectively. SA has been reported to either activate [25] or inhibit [26] AOS activity. SA levels in the chloroplast are likely to be substantially higher than those measured in total leaf, and are therefore potentially capable of mediating local JA antagonism by inhibition of AOS. These conflicting reports may simply reflect different adaptations in different plants but highlight the lack of temporal and spatial knowledge of these pathway components during defence responses.

The production of the *P. syringae* phytotoxin coronatine (COR), a jasmonoyl-isoleucine (JA-Ile) mimic, by the conjugation of coronafacic acid (CFA) to coronamic acid (CMA) by coronafacic ligase [27] provides a compelling example of how a pathogen has exploited negative interactions to suppress plant defences. COR is believed to activate or modulate JA signalling to suppress SA defences [28•], and consistent with these data mutants impaired in jasmonate signalling exhibit enhanced resistance to *P. syringae* original [28•,29]. Strikingly, the bac-

terial necrotroph *Erwinia caratovora* also appears to have horizontally acquired a pathogenicity island containing genes for CFA (but not CMA) synthesis [30]. CFA may be coupled to a variety of amino acids, providing another potential strategy for modulating host defence responses.

Bacterial effectors appear to utilise similar signalling networks to promote disease. Enhanced growth of a severely compromised *P. syringae hrp* mutant by conditional expression of the bacterial effector *AvrB*, in the absence of its cognate R protein, RPM1, requires COI1 (coronatine insensitive 1; a key component of JA-signalling pathways). These data suggest effector-mediated enhanced disease susceptibility can interfere with SA defence through JA pathways [31].

It has recently been demonstrated that JA responses are regulated through the F-box COI1 SCF (Skip/Cullin/F box) E3 ubiquitin ligase complex. Analogous to auxin signalling through $SCF^{TIR1}$ and AUX/IAA repressors, rapidly induced *JAZ* (*jasmonate ZIM-domain*) transcripts encode repressors of the $SCF^{COI1}$ E3 ubiquitin ligase complex. JAZ proteins are proteasome degraded following jasmonate treatment, and at least one JAZ protein negatively regulates a key transcriptional activator of jasmonate responses, MYC2 [32••,33••]. It will therefore be intriguing to determine whether SA responses are regulated in a similar manner. SA antagonism of JA signalling could be achieved by blocking JA-mediated degradation of JAZ proteins. Alternatively sharing of, or interference with, SCF E3 ligase components and associated adaptors may well explain the strong impact hormonal balances play in influencing the outcome of plant–pathogen interactions [23]. These data suggest that coronatine-mediated virulence and antagonism of SA signalling is most likely achieved through promoting $SCF^{COI1}$-mediated degradation of JAZ repressors.

### SA/ABA antagonism

ABA has recently emerged as a key determinant in the outcome of plant pathogen interactions [34]. Exogenous application of ABA-attenuated expression of Arabidopsis genes associated with aromatic amino acid biosynthesis and restricted lignin and SA accumulation during the incompatible interaction between *P. syringae* expressing *avrRpt2* [35•]. More remarkably, bacterial T3Es hijacked ABA biosynthesis to promote virulence. Ectopic expression of the effector, AvrPtoB, mimicked ABA induction and suppression of defence genes [36••]. We have since shown that ABA produced by virulent *P. syringae* suppresses SA accumulation. SA levels are enhanced in an ABA biosynthetic mutant, suggesting the earlier T3E-modulated ABA accumulation suppresses SA levels (de Torres & Grant, unpublished). As ABA is also emerging as central in resistance to JA defence pathways [37,38] the mechanistic basis for cross-talk between this triad of stress phytohormones represents a significant future challenge.

### Antagonism of SA signalling via *S*-nitrosylation

Nitric oxide (NO) is a key redox-signalling molecule in plants. In the plant defence response NO is thought to regulate both gene expression and hypersensitive cell death [39]. Like in animals, *S*-nitrosylation is emerging as a prototypic redox-based post-translational modification that underpins NO signal function during many plant cellular responses [40]. This post-translational modification entails the addition of an NO moiety to a reactive cysteine thiol to form an *S*-nitrosothiol (SNO). Recently, *S*-nitrosoglutathione reductase (GSNOR), central to endogenous SNO turnover has been identified in bacteria [41] and plants [42••]. Loss-of-function mutations in *Arabidopsis thaliana GSNOR* (*AtGSNOR1*) resulted in increased cellular levels of SNOs, while a gain-of-function mutation, which resulted in enhanced AtGSNOR1 activity, decreased endogenous SNO levels [42••]. Importantly, loss of *AtGSNOR1* function compromised non-host resistance against the wheat powdery mildew pathogen, *Blumeria graminis* f.sp *tritici*. Furthermore, the absence of AtGSNOR activity also compromised protection mediated by distinct R gene subclasses and basal resistance [42••]. Conversely, overexpression of *AtGSNOR1* resulted in strikingly enhanced basal resistance against a broad spectrum of pathogens. Thus, AtGSNOR1 is required for multiple modes of plant disease resistance. This enzyme regulates both SA biosynthesis and SA signalling, suggesting that at least two nodes of the SA-signalling network may be controlled by *S*-nitrosylation.

NPR1 is a potential target for *S*-nitrosylation. NPR1 is normally present in the cytoplasm and shuttles to the nucleus in response to changes in cellular redox tone during the establishment of disease resistance. NPR1 cytoplasm-to-nucleus shuttling is controlled by the presence of a number of redox responsive cysteines, which could potentially be targets for *S*-nitrosylation. In an unbiased biochemical screen for proteins that become specifically *S*-nitrosylated during the plant defence response Loake and co-workers have demonstrated that SA-Binding Protein 3 [43] is *S*-nitrosylated on a single reactive cysteine (Wang, Loake *et al.* unpublished). *S*-nitrosylation of SABP3 has been shown to modulate SA binding, and also the cognate carbonic anhydrase activity of this protein, which has been implicated in disease resistance [44].

## SA signalling

Significant progress has been made towards linking SA signalling and defence responses. Plants activate basal defences following perception of PAMPs. In Arabidopsis, recognition of the archetypal PAMP, bacterial flagellin activates a mitogen-activated protein kinase (MAPK) module leading to expression of basal defence-related genes [45]. The mitogen-activated protein kinase kinase kinase MEKK1 has recently been shown to be required for

flg22-induced activation of MPK4 but in contrast to a previous report [45] not MPK3 or MPK6 [46•,47•]. MAPK4 acts as a negative regulator of SA signalling but is required for induction of JA defence markers, suggesting it represents a key node mediating antagonism between SA/JA signalling [48]. Similar to other mutants with constitutive activation of SA-dependent defences, both *mpk4* and *mekk1* mutants are severely dwarfed. *mekk1* dwarfism can be alleviated by expression of *nahG* indicating MEKK1 is required for suppression of SA signalling, in agreement with its proposed upstream signalling role in flg22-induced activation of MPK4 [47•]. Additionally, MPK4 negatively regulates temperature-sensitive cell death and cell-specific cell death [46•] highlighting the global role of SA in plant stress responses. Intriguingly, a kinase-impaired version of MEKK1 (K361M) also restored wild-type phenotype and flg22-induced activation of MPK4 [47•] indicative of a scaffolding, rather than direct, signalling function for MEKK1. This is consistent with the MEKK1 N-terminal regulatory domain interaction with MPK4, and MEKK1's interaction with MKK1 [49] the most likely MAPKK candidate for a MEKK1/MPK4 complex. MKK1 can phosphorylate MPK4 *in vitro*, and following flg22 treatment of protoplasts MKK1 activates MPK4 [50•]. Moreover, *mkk1* mutants are compromised in both flg22 activation of MPK4 (and MPK3 and MPK6) and resistance to virulent and avirulent *P. syringae*. Notably, unlike *mpk4* and *mekk1*, *mkk1* mutants show no morphological anomalies [50•].

Downstream targets of MAPK4 have recently been revealed. The MAPK4 substrate MKS1 interacts, in a JA-independent manner, with two WRKY transcription factors, WRKY 25 and WRKY 33, which are both *in vitro* substrates of MAPK4 [51••]. A knockout of WRKY 33 results in elevated *PR1* expression indicative of a link between SA-mediated MAPK4 signalling. Consistent with these data an independent study showed WRKY25 was a negative regulator of SA-mediated defence responses to *P. syringae* [52]. Unexpectedly, genetic dissection of MAPK4 signalling revealed *PAD4/EDS1* (*phytoalexin deficient4/enhanced disease susceptible1*) mutations also act downstream of MAPK4. PAD4 and EDS1 are proposed to alleviate JA/ET antagonism and partially abrogate dwarfism through activating SA and repressing JA/ET defences [53,54•]. Significantly, these data suggest overlapping, or independent roles for MEKK1 and MAPK4 in SA-mediated signalling and link, for the first time, PAD4/EDS1, central mediators of SA/JA signalling to a kinase cascade.

## Transcription factors participating in SA signalling

SA signalling is mediated by both NPR1-dependent mechanisms and NPR1-independent mechanisms [3], though the former pathway is better understood. SA-induced redox changes lead to the reduction of NPR1 from cytosolic, disulfide-bound oligomers to active monomers. NPR1 monomers nuclear localise and interact with the TGA class of basic leucine zipper transcription factors leading to the expression of a plethora of SA-dependent genes [55••]. The actual recruitment of NPR1 and TGA2 to the *PR1* promoter, however, is autonomous and independent of SA [56]. Rather SA application stimulates the formation of a TGA2/NPR1 transactivating complex capable of inducing expression from both heterologous and *PR1* promoters. The TGA2 coactivator function of NPR1 is mediated by its BTB/POZ protein interaction domain and requires further Cys-oxidation of NPR1 [56], consistent with the observation that overexpression of NPR1 alone does not activate PR1 expression. SA also induces the NPR1-dependent glutaredoxin GRX480, which interacts with TGA2 and probably catalyses thiol disulfide reductions that mediate alteration of TGA1-NPR1 redox states under inducing conditions [57•].

Emerging evidence suggests that WRKY transcription factors participate extensively in SA defence responses, downstream or concomitant with NPR1, both as activators and repressors of SA transcription [55••]. The combinatorial interactions of WRKY homodimers and heterodimers in modulating SA-mediated defence responses are extremely complex and are covered in a recent review [58].

## Conclusions

Progress has been made in identifying key components and bioactive derivatives of SA-signalling pathways. Effector-mediated suppression of SA defences are now well established, however understanding the mechanism by which these diverse effectors link to, and perturb SA signalling remains a significant challenge. There is a real need to move beyond end-point analyses such as the measurements of *PR1* and *PDF1.2* and their *carte blanc* attribution to SA and JA-dependent responses, respectively. SA signalling must be considered within the context of the local phytohormone balance and recognise temporally and spatially distinct (pathogen specific) phases associated with establishing defence against biotrophs. Revealing the inherent complexity of the SA defence signalling network requires resolving the dynamics of host pathogen interactions through detailed time-delimited analyses of infection, and infection compromised mutants, at all functional genomic scales. This includes recognising the contribution of volatile and conjugated SA derivatives as signalling molecules.

Maybe we are, after all, being too naive in trying to contain research under the umbrella of SA-dependent defence signalling.

## Acknowledgements

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Despite the importance of salicylic acid (SA) in plant defence, our understanding of SA biosynthesis and its transcriptional control is poor. Recently, the same authors showed the majority of SA was synthesised from chorismate, in a similar fashion to SA synthesis in bacteria. In this paper they demonstrate that isochorismate synthase 1 (ICS1), which catalyses the first step in SA biosynthesis is a plastid-localised stromal protein. This enzyme catalyses the production of isochorismate and does not produce SA directly, unlike a related enzyme in *Yersina*. Thus, in Arabidopsis a second, as yet elusive, enzyme is required for SA production. Furthermore, the Km of this enzyme suggests that ICS1 can compete effectively for chorismate with other pathogen-inducible chorismate-utilising enzymes.

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[32••] and [33••] represent landmark papers that demonstrate that JA, like auxin, signalling appears to be mediated through the rapid degradation of a class of repressor proteins termed JAZ (jasmonate ZIM-domain) proteins. JAZ proteins bind to the COI1 F-box protein, a component of the E3 ubiquitin ligase complex that is involved in ubiquitination of specific proteins that are subsequently degraded by the 26S proteasome. JAZ proteins appear to repress transcription of jasmonate responsive genes. *JAZ* transcripts are themselves JA inducible, thus can attenuate JA signalling. Critically Chini *et al.* demonstrated a negative feedback regulatory loop between the JA responsive MYC2 transcription factor and JAZ3. These studies provide a foundation to examine the mechanistic basis of JA-SA antagonism.

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