Age-Related Resistance in *Arabidopsis thaliana* Involves the MADS-Domain Transcription Factor SHORT VEGETATIVE PHASE and Direct Action of Salicylic Acid on *Pseudomonas syringae*

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Arabidopsis thaliana exhibits a developmentally regulated disease-resistance response known as age-related resistance (ARR), a process that requires intercellular accumulation of salicylic acid (SA), which is thought to act as an antimicrobial agent. ARR is characterized by enhanced resistance to some pathogens at the late adult-vegetative and reproductive stages. While the transition to flowering does not cause the onset of ARR, both processes involve the MADS-domain transcription factor SHORT VEGETATIVE PHASE (SVP). In this study, ARR-defective *svp* mutants were found to accumulate reduced levels of intercellular SA compared with wild type in response to *Pseudomonas syringae* pv. *tomato*. Double mutant and over-expression analyses suggest that SVP and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) act antagonistically, such that SVP is required for ARR to alleviate the negative effects of SOC1 on SA accumulation. In vitro, SA exhibited antibacterial and antibiofilm activity at concentrations similar to those measured in the intercellular space during ARR. In vivo, *P. syringae* pv. *tomato* formed biofilm-like aggregates in young susceptible plants, while this was drastically reduced in mature ARR-competent plants, which accumulate intercellular SA. Collectively, these results reveal a novel role for the floral regulators SVP and SOC1 in disease resistance and provide evidence that SA acts directly on pathogens as an antimicrobial agent.

Many plants exhibit enhanced resistance to disease at later developmental stages (Develey-Rivière and Galiana 2007; Whalen 2005). This phenomenon is often referred to as age-related resistance (ARR). The model plant *Arabidopsis thaliana* displays ARR to *Pseudomonas syringae*, resulting in reduced bacterial growth in the leaf intercellular space of mature plants compared with young plants (Kus et al. 2002). ARR is a

salicylic acid (SA)-dependent process, as several SA-deficient mutants are ARR-defective (Cameron and Zaton 2004; Carviel et al. 2009; Kus et al. 2002). However, SA signaling mutants are ARR-competent, suggesting that SA-mediated defense signaling is not essential for ARR (Carella et al. 2015; Kus et al. 2002). Instead, it is thought that SA acts directly on the pathogen as an antimicrobial agent during ARR. Evidence supporting this hypothesis includes the observation that antibacterial activity and SA are both detected in intercellular washing fluids (IWFs) of mature plants after inoculation with *P. syringae* pv. *tomato* DC3000 but not in IWFs of untreated or young plants inoculated with *P. syringae* pv. *tomato* (Cameron and Zaton 2004; Kus et al. 2002). Moreover, addition of SA to the intercellular space of SA-deficient mutants restores ARR and reduction of intercellular SA levels in wild-type plants by pressure-infiltrating with SA hydroxylase compromises ARR (Cameron and Zaton 2004). Finally, SA exhibits antimicrobial activity against a variety of phytopathogens in vitro (Amborabé et al. 2002; Brown et al. 2007; Cameron and Zaton 2004; El-Mougy 2002; Georgiou et al. 2000; Martín et al. 2010; Prithiviraj et al. 1997; Lowe-Power et al. 2016; Yuan et al. 2007); however, concentrations greater than 1 mM are often required and it is unclear whether these concentrations are reached in planta.

ARR coincides with the transition to flowering in both short-day (9 h of light) and long-day (16 h of light) photoperiods (Rusterucci et al. 2005). Despite the co-occurrence of these developmental events, analysis of flowering-time mutants and wild-type plants that were induced to flower early by transient exposure to long days demonstrated that the transition to flowering is neither sufficient nor required for the onset of ARR (Wilson et al. 2013). Interestingly, the same study also revealed that SVP (SHORT VEGETATIVE PHASE) plays a role in both processes, since mutants for this well-known flowering-time regulator were found to be ARR-defective.

The transition to flowering is a highly regulated process affected by numerous environmental and endogenous signals (Amasino 2010; Fornara et al. 2010). A few key genes act to integrate these signals, including SVP, a major negative regulator of flowering (Hartmann et al. 2000). SVP is a MADS-domain transcription factor that delays flowering, in the absence of inductive signals, through direct repression of positive regulators of flowering such as *SOC1* (SUPPRESSOR OF OVEREXPRESSION OF CO 1) (Gregis et al. 2013; Li et al. 2008). *SOC1* functions primarily at the shoot apical meristem, in which it activates the floral meristem identity gene *LFY* (LEAFY) to initiate

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flowering in response to inductive signals such as long days, gibberellic acid, and aging (Lee et al. 2008; Liu et al. 2008; Moon et al. 2005). SVP also represses *SOC1* in leaves (Li et al. 2008); however, the biological significance of this interaction is unclear.

In this study, investigation of the role of SVP during ARR revealed that SVP is required for intercellular SA accumulation and identified *SOC1* as an additional MADS-domain transcription factor that affects both flowering and ARR. The potential for direct action of SA on *P. syringae* pv. *tomato* was assessed by antibacterial and biofilm assays in minimal medium that reflects the conditions in the intercellular space. Low concentrations of SA ($\geq 2 \mu\text{M}$) reduced biofilm formation, while higher concentrations ($\geq 100 \mu\text{M}$) reduced bacterial growth. Moreover, accumulation of SA in the intercellular space during ARR was associated with reduced formation of biofilm-like aggregates of *P. syringae* pv. *tomato*, suggesting that intercellular SA directly impacts either pathogen growth, biofilm formation, or both in mature *Arabidopsis*.

RESULTS

SA accumulation is reduced in *svp* mutants responding to *P. syringae*.

We first became interested in *SVP* upon discovering that mutants for this gene were ARR-defective (Wilson et al. 2013) (Fig. 1A). Given that SA accumulation is a key component of the ARR response, we hypothesized that *SVP* might regulate SA accumulation, in which case *svp* mutants should be compromised in their ability to accumulate SA. Therefore, SA was measured in mature Col-0 and *svp-41* before and after inoculation with *P. syringae* pv. *tomato*. Specifically, SA levels

were measured in IWFs and leaf tissue (with IWFs removed) to assess intercellular and intracellular fractions of SA, respectively. As expected, Col-0 accumulated intracellular free SA and intracellular glucose-conjugated SA (SAG) in response to *P. syringae* pv. *tomato* (Fig. 1C and D). The *svp-41* mutant also accumulated intracellular free SA, albeit with a small but statistically significant 1.2-fold reduction, relative to Col-0, at 24 h postinoculation (hpi). Intracellular SAG accumulation was noticeably reduced in *svp-41* relative to Col-0 at 24 and 48 hpi (two- to fourfold reduction). While little to no intercellular SAG accumulates in response to *P. syringae* pv. *tomato* (Carviel et al. 2014), free SA accumulated to $2,093 \text{ ng ml}^{-1}$ in Col-0 IWFs at 24 hpi but was reduced to 711 ng ml^{-1} in *svp-41* (threefold reduction) (Fig. 1B). These data suggest that *SVP* function contributes to intracellular and intercellular SA accumulation during ARR.

Addition of SA to the intercellular space restores ARR to *svp* mutants.

If the ARR-defective phenotype of *svp* mutants is caused by reduced intercellular SA accumulation, then adding exogenous SA to the intercellular space should restore ARR in *svp* mutants. To test this, 0.1 mM SA or a mock solution was pressure-infiltrated into the leaves of mature plants either 4 or 24 h before inoculation with *P. syringae* pv. *tomato*. Bacterial levels were then measured at 3 days postinoculation (dpi). Col-0 served as a positive control for ARR, and the SA-deficient *sid2-2* mutant was included as a negative control for ARR and because application of exogenous SA restores ARR to *sid2* mutants (Cameron and Zaton 2004). Mock-treated *svp-32* and *sid2-2* supported higher bacterial levels than Col-0, consistent with the ARR-defective phenotypes of both mutants (Fig. 2). When SA was applied 4 h before inoculation with *P. syringae* pv. *tomato*, *svp-32* and *sid2-2*

Fig. 1. Age-related resistance-defective *svp-41* mutants have reduced salicylic acid (SA) accumulation in response to *Pseudomonas syringae*. **A**, Plants were inoculated with 10^6 CFU ml^{-1} *P. syringae* pv. *tomato* at either 4 or 6 weeks postgermination (wpg) and bacterial levels were quantified 3 days later. Different letters indicate significant differences (analysis of variance followed by Tukey's honestly significant difference test, $P < 0.05$). **B**, SA was measured in intercellular washing fluids (IWFs) collected from plants that were either untreated or inoculated with 10^6 CFU ml^{-1} *P. syringae* pv. *tomato*. **C**, SA and **D**, SA glucosides (SAG) were measured in the corresponding leaves (from which IWFs had been removed). Asterisks indicate significant differences between genotypes at the same timepoint (t test, $P < 0.05$). Each bar shows the mean of three sample replicates and error bars show standard deviation. These experiments were performed three times with similar results.

both supported lower bacterial levels relative to their mock-treated counterparts, whereas resistance was not enhanced in ARR-competent Col-0, as observed previously (Cameron and Zaton 2004). In contrast, when SA was applied 24 h before inoculation with *P. syringae* pv. *tomato*, all three genotypes supported similar bacterial levels relative to their mock-treated counterparts. Similar results were observed with the *svp-41* mutant (Supplementary Fig. S1). Previous work has shown that leaves pressure-infiltrated with 0.1 mM SA contain elevated SA in IWFs after 5 h, with a return to untreated levels after 24 h (Cameron and Zaton 2004). Together, these data suggest that exogenous intercellular SA restores ARR in *svp* mutants. These data link the ARR-defective phenotype of *svp* mutants to reduced intercellular SA accumulation and further demonstrate the importance of intercellular SA for ARR.

Direct action of SA on *P. syringae*.

In rich medium (King's B [KB]), *P. syringae* pv. *tomato* growth is reduced at SA concentrations of ≥ 1 mM (Cameron and Zaton 2004) and ≥ 2 mM (Fig. 3A). Since characteristics of the growth medium (e.g., pH) can influence bacterial growth rate, metabolism, and physiology (Kim et al. 2010), as well as the activity of antimicrobial agents (Amborabé et al. 2002), the antibacterial activity of SA was assessed in conditions similar to those found in the leaf intercellular space. To accomplish this, antibacterial assays were conducted using *hrp*-inducing minimal (HIM) medium (Huynh et al. 1989), which more closely resembles the composition of the leaf intercellular space. In HIM medium, the growth of *P. syringae* pv. *tomato* was reduced at 100 to 200 μ M SA and was completely inhibited at 1 mM (Fig. 3B). At ≥ 2 mM SA, no colonies were observed when the culture was transferred to solid KB medium without SA, suggesting that SA had bactericidal activity (Supplementary Fig. S2). Therefore, SA is a stronger antibacterial agent under conditions that better represent the intercellular space. It has been suggested that acidification of the culture medium by SA accounts for its toxic effects (Van Duy et al. 2007); however, in our experiments, SA had little effect on pH at the concentrations tested (Supplementary Fig. S3). Since some plant pathogens degrade SA and use it as a carbon source (Lowe-Power et al. 2016; Rabe et al. 2013), we speculated that *P. syringae* pv. *tomato* may also have this ability. However, *P. syringae* pv. *tomato* was unable to grow in HIM medium with SA as the sole

carbon source, suggesting that *P. syringae* pv. *tomato* DC3000 lacks the ability to degrade SA (Supplementary Fig. S4).

Several studies suggest that biofilm formation contributes to the virulence of *P. syringae* on various hosts, including *Arabidopsis* (Aslam et al. 2008; Schenk et al. 2008; Yu et al. 1999). This led us to speculate that elevated intercellular SA levels during ARR may reduce *P. syringae* pv. *tomato* biofilm formation; therefore, we examined whether treatment with SA reduces *P. syringae* pv. *tomato* biofilm formation in vitro. The ability of *P. syringae* pv. *tomato* to form biofilms in vitro was assessed using a spectrophotometric microplate assay, in which staining of surface-adherent cells with crystal violet allows for quantification of biofilm formation (O'Toole 2011). In KB medium, *P. syringae* pv. *tomato* biofilm formation was negligible (Supplementary Fig. S5), whereas extensive biofilm formation was observed beginning at 24 h in HIM medium (Fig. 4A). Interestingly, biofilm formation was reduced by SA in a concentration-dependent manner, as shown by quantitative assays (Fig. 4A) and qualitative assays performed with test tubes instead of microplates (Fig. 4B). The effect was most dramatic at 24 h and onwards, once extensive biofilm formation had occurred in the SA-free controls. At the lowest effective concentrations of SA (2 to 5 μ M), biofilm formation was reduced by 1.3- to 2.3-fold. At higher concentrations (50 to 100 μ M), similar to the levels estimated to accumulate in the intercellular space during ARR (Fig. 1C), biofilm formation was reduced by 2.1- to 7.1-fold. Concentrations greater than 100 μ M were not tested, as they were observed to reduce the growth of *P. syringae* pv. *tomato* (Fig. 3B).

To determine whether intercellular SA accumulation during ARR plays a role in inhibiting biofilm formation in planta, a green fluorescent protein (GFP)-expressing strain of *P. syringae* pv. *tomato* DC3000 (carrying plasmid pDSK-GFPuv) (Wang et al. 2007) was used to monitor the formation of biofilm-like aggregates (groups of densely packed, stationary bacterial cells) in leaf tissue of young and mature Col-0 and *svp-32* via fluorescence microscopy. Aggregates were not observed in leaves immediately after inoculation nor in the inoculum itself (Supplementary Fig. S6). At 24 hpi, aggregates and individual free-swimming (planktonic) bacterial cells were visible in the spaces between mesophyll cells with autofluorescent chloroplasts (Fig. 5A and B; Supplementary Fig. S7). For young Col-0 and *svp* mutants, most fields of view contained both

Fig. 2. Exogenous intercellular salicylic acid (SA) restores age-related resistance (ARR) to *svp-32* mutants. Mature plants (7 weeks postgermination) were pressure-infiltrated with 0.1 mM SA or water (mock) either 4 or 24 h before inoculation with 10^9 CFU ml⁻¹ *Pseudomonas syringae* pv. *tomato*. Bacterial levels were quantified 3 days later. Each bar shows the mean of three sample replicates and error bars show the standard deviation. Asterisks indicate significant differences (analysis of variance, followed by Tukey's honestly significant difference test, $P < 0.05$). This experiment was performed three times with similar results.

free-swimming and aggregated cells (Fig. 5C). In contrast, most fields of view contained only free-swimming cells (66%) for mature Col-0 leaves, whereas most fields of view for mature *svp* leaves contained both free-swimming and aggregated cells (77%), similar to those for young plants. Taken together, these data support the hypothesis that mature, ARR-competent plants reduce the formation of biofilm-like aggregates through intercellular SA accumulation.

SA reduces growth and biofilm formation of *P. syringae* pv. *tomato* in a concentration-dependent manner in vitro. To gain insight into whether SA might function similarly in the intercellular space during ARR, it would be useful to know the concentration of SA in planta. While collection and examination of IWFs makes it possible to detect intercellular compounds, dilution of intercellular contents is a consequence of the collection procedure. To account for this, a dilution correction factor was calculated for mature Col-0 according to the indigo carmine method described by Husted and Schjoerring (1995). The dilution correction factor (6.73 ± 3.42 , average $\pm$ standard deviation of three independent experiments) was applied to the concentrations of SA measured in IWFs and suggests that SA is present at 40 to 100 μ M in mature Col-0 at 24 hpi with *P. syringae* pv. *tomato*. At these concentrations, SA has little effect on *P. syringae* pv.

tomato growth in vitro (Fig. 3B) but significantly reduces biofilm formation (Fig. 4).

The ARR-defective phenotype of *svp* is not related to early flowering.

It is possible that morphological changes associated with early flowering contribute to the *svp* ARR defect. To examine this idea, *KNAT1:SVP/svp-41* plants were assessed for ARR, since *SVP* expression from the meristem-specific *KNAT1* promoter delays flowering, while *SVP* is nonfunctional in the leaves (Andrés et al. 2014). Col-0 did not flower during the experiment and showed a strong ARR response (119-fold reduction in bacterial levels in mature plants relative to young plants [Fig. 6]). The two *KNAT1:SVP/svp-41* lines did not flower during the experiment and both lines displayed an ARR-defective phenotype similar to the *svp-41* parental line. Furthermore, this corresponded to reduced intercellular SA accumulation compared with wild type (Supplementary Fig. S8). Thus, the ARR-defective phenotype of *svp* mutants can be separated from early flowering, suggesting that the requirement of functional *SVP* for ARR is not an indirect effect of its role in flowering-time control.

Under long-day conditions, the onset of ARR is accelerated such that it occurs at 4 weeks postgermination (wpg) (Rusterucci et al. 2005). To determine whether *SVP* is required for ARR in long days, the *KNAT1:SVP/svp-41* lines were used, since they flower later and produce more rosette leaves than *svp-41*, making them more amenable to disease-resistance assays performed with long-day-grown plants. While Col-0 showed a 15-fold decrease in bacterial levels between 3 and 4 wpg, the *KNAT1:SVP/svp-41* lines supported similarly high bacterial levels at 3 and 4 wpg (Supplementary Fig. S9). Therefore, meristem-specific expression of *SVP* does not rescue the ARR defect of *svp-41* under long or short days, suggesting that functional *SVP* in leaves is required for the ARR response.

SOC1 negatively influences ARR.

SVP directly represses expression of *SOC1* in both the meristem and leaves (Li et al. 2008). To test whether *SVP* function during ARR is associated with its repression of *SOC1*, *svp-32 soc1-2* double mutants were created and assayed for ARR. As shown previously (Wilson et al. 2013), *soc1-2* single mutants display wild-type ARR (Fig. 7A). Interestingly, the *svp-32 soc1-2* double mutant was ARR-competent, indicating that *SVP* function is not required for ARR in a *soc1-2* mutant background, and thus, the requirement of *SVP* for ARR may be due to its repression of *SOC1*. This suggested that *SOC1* may have a negative impact on ARR, and indeed, the *SOC1*-overexpressing plant line *soc1-101D* (Lee et al. 2000; Moon et al. 2005) was ARR defective and failed to accumulate intercellular SA (Fig. 7A and B). To further assess the potential involvement of *SOC1* in ARR, transgenic plants with β -estradiol-inducible overexpression of *SOC1* were created (*XVE:SOC1/soc1-2*, discussed below). Maximum *SOC1* expression levels were reached by 24 h after treatment with β -estradiol and were sustained for at least 3 days thereafter (Fig. 7E). To determine whether overexpression of *SOC1* impedes the ARR response, mature plants were pressure-infiltrated with β -estradiol or a mock solution 24 h before inoculation with *P. syringae* pv. *tomato* and bacterial levels were measured at 3 dpi. ARR was observed in the *soc1-2* parental line and β -estradiol treatment had no effect on bacterial levels in *soc1-2*. In contrast, two independent *XVE:SOC1/soc1-2* lines consistently supported higher bacterial levels after β -estradiol treatment, although the difference was not always statistically significant (Fig. 7D). Together, these data suggest that *SOC1* negatively impacts the ARR response downstream of *SVP* and upstream of intercellular SA accumulation. *SOC1* was recently identified in a yeast one-hybrid screen for transcription factors that interact with the promoter of

Fig. 3. Antibacterial activity of salicylic acid (SA) in vitro. **A**, *Pseudomonas syringae* pv. *tomato* was grown in King's B medium or **B**, hrp-inducing minimal medium, along with the indicated concentrations of SA in a 96-well plate with shaking. Each data point shows the mean of four wells and error bars indicate standard deviation. These experiments were performed at least three times with similar results.

the SA biosynthesis gene *ICS1* (Zheng et al. 2015). To test whether the negative impact of SOC1 on ARR could be explained by repression of *ICS1*, expression of *ICS1* was measured in mature Col-0 and *soc1-101D* plants before and after inoculation with *P. syringae* pv. *tomato*. While Col-0 displayed a 5.5-fold induction of *ICS1* expression at 24 hpi with *P. syringae* pv. *tomato*, induction of *ICS1* expression was not observed in *soc1-101D* (Fig. 7C). Together, these data suggest that SOC1 negatively impacts ARR by repression of *ICS1* expression and SA accumulation. A proposed model for the contribution of SVP and SOC1 to intercellular SA accumulation during ARR is presented in Figure 8.

Since *soc1-101D* plants also displayed enhanced susceptibility compared with wild type at the young-plant stage (Fig. 7A), XVE: *SOC1/soc1-2* plants were used to further assess the possibility that *SOC1* overexpression negatively impacts disease resistance in young plants. Young plants pressure-infiltrated with β -estradiol or a mock solution 24 h before inoculation with *P. syringae* pv. *tomato* supported similar in-planta bacterial levels at 3 dpi (Supplementary Fig. S10), suggesting that *SOC1* overexpression has a greater impact on disease resistance in mature, compared with young, plants.

DISCUSSION

SVP and SOC1 contribute to ARR.

Consistent with the hypothesis that SVP regulates SA accumulation during ARR, the ARR-defective phenotype of *svp* mutants is associated with a reduced ability to accumulate intercellular free SA in response to *P. syringae* pv. *tomato* (threefold reduction relative to Col-0). Moreover, exogenous SA applied to the intercellular space 4 h before pathogen inoculation restored ARR in *svp* mutants; however, no effect on ARR was observed

when SA was applied 24 h before inoculation, since it has been demonstrated that SA is taken up by cells by 24 h after SA application (Cameron and Zaton 2004).

Since SVP directly represses *SOC1* expression in seedlings (Gregis et al. 2013; Li et al. 2008), the ARR phenotype of *svp-32 soc1-2* mutants was tested to determine whether the function of SVP in ARR is associated with its repression of *SOC1*. The ARR-competent phenotype of *svp-32 soc1-2* mutants suggested that SOC1 acts downstream of SVP during ARR and that the requirement of SVP for ARR is related to its repression of *SOC1*. In support of this idea, constitutive *SOC1* overexpressors were fully ARR-defective and failed to accumulate intercellular SA. β -estradiol-inducible *SOC1* overexpression lines displayed a modest ARR-defective phenotype, which may be explained by higher *SOC1* expression levels in constitutive overexpressors, as supported by the dose-dependent effect of *SOC1* on flowering time (Lee et al. 2000).

The negative impact of SOC1 on ARR appears to be due to repression of *ICS1* expression, since constitutive *SOC1* overexpressors are defective for pathogen-induced *ICS1* expression and intercellular SA accumulation. Furthermore, repression of *ICS1* expression by SOC1 may be direct, since SOC1 interacts with the *ICS1* promoter in yeast one-hybrid experiments (Zheng et al. 2015). Young and mature wild-type plants express *ICS1* to similar levels in response to *P. syringae* pv. *tomato* (Rusterucci et al. 2005), suggesting that SVP and SOC1 do not regulate the onset of ARR competence in wild-type plants. Perhaps SOC1 only inhibits ARR when expressed at the high levels found in *svp* mutants or *SOC1* overexpressors (Lee et al. 2000; Li et al. 2008).

The ARR-defective phenotype of *svp* mutants is not related to their early flowering phenotype, since *KNAT1:SVP/svp-41* plants expressing functional SVP only in the meristem did not

Fig. 4. Salicylic acid (SA) reduces biofilm formation in vitro. **A**, *Pseudomonas syringae* pv. *tomato* was cultured in *hrp*-inducing minimal medium along with the indicated concentration of SA at room temperature, without shaking, in a 96-well plate. At the indicated timepoints, plates were rinsed to remove unattached planktonic cells and biofilms were quantified by staining with crystal violet. Each bar shows the mean of four wells and error bars show standard deviation. Asterisks indicate significant differences relative to the 0- μ M control at the corresponding timepoint (analysis of variance, followed by Tukey's honestly significant difference test, $P < 0.05$). **B**, Similar assays were conducted in test tubes that were photographed after staining with crystal violet following 30 h of incubation. These experiments were performed three times with similar results.

flower during short-day ARR experiments but remained ARR-defective. Similarly, the early flowering and ARR-defective phenotypes associated with constitutive *SOC1* overexpression could be separated by inducible overexpression of *SOC1* during infection, which did not accelerate flowering but still negatively impacted ARR. Consistent with these results, the altered disease-resistance phenotypes of several late-flowering mutants were unchanged by mutations or treatments that accelerated flowering (Lyons et al. 2015; Singh et al. 2013). Therefore, rather than the timing of flowering affecting disease resistance, some flowering-time regulators appear to have distinct effects on disease-resistance

Fig. 5. Formation of bacterial aggregates in the leaf intercellular space. At 24 h after inoculation with 10^6 CFU ml^{-1} of *P. syringae* pv. *tomato* carrying pDSK-GFPuv, the lower epidermis was removed from the leaves and bacterial cells were imaged by epifluorescence microscopy. **A**, Individual, free-swimming (planktonic) cells and **B**, stationary aggregates were observed. **C**, The proportion of bacterial cell types in plants of different ages (4 and 7 weeks post-germination) and genotypes was scored by assessing 8 to 10 fields of view from at least six plants for the presence of free-swimming cells, aggregated cells, or a mixture of both. The asterisk indicates a significant difference (Kruskal-Wallis test followed by Dunn's test). This experiment was performed three times with similar results. Scale bars = 5 μm .

responses, such as systemic acquired resistance (FLD) (Singh et al. 2013), resistance to *Fusarium oxysporum* (FVE and FPA) (Lyons et al. 2015), and ARR (SVP and SOC1) (this work).

Antibacterial activity of SA.

Many studies have shown that SA exhibits antimicrobial activity against phytopathogens in vitro (Amborabé et al. 2002; Brown et al. 2007; Cameron and Zaton 2004; El-Mougy 2002; Georgiou et al. 2000; Martín et al. 2010; Prithiviraj et al. 1997; Lowe-Power et al. 2016; Yuan et al. 2007), with the range of effective concentrations spanning several orders of magnitude, probably due in large part to the different organisms and experimental conditions used. We found that SA displayed approximately 10-fold greater antibacterial activity in minimal medium, which is a better representation of the conditions in the leaf intercellular space compared with rich media. This difference in SA antibacterial activity could be due to differential effects of these media on bacterial growth rate, physiology, and metabolism (Kim et al. 2010), or alternatively, on SA itself. For example, HIM medium has a pH of 5.7, similar to intercellular fluids (Jia and Davies 2007), whereas KB medium has a pH of 7.2. At lower pH levels, a greater proportion of SA exists in its protonated, lipophilic form, enhancing its ability to cross cell membranes, which may be a prerequisite for its antimicrobial activity. In support of this idea, Amborabé et al. (2002) showed that antifungal activity of SA against *Eutypa lata* increased

Fig. 6. The early flowering and age-related resistance (ARR)-defective phenotypes of *svp-41* are not linked. **A**, Percentage of plants with visible inflorescence stems was scored each week starting at 2 weeks postgermination (wpg, $n \geq 9$). **B**, Plants were inoculated with 10^6 CFU ml^{-1} *Pseudomonas syringae* pv. *tomato* at either 4 or 6 wpg and bacterial levels were quantified 3 days later. Each bar shows the mean of three sample replicates and error bars show standard deviation. Different letters indicate significant differences (analysis of variance, followed by Tukey's honestly significant difference test, $P < 0.05$). This experiment was performed three times with similar results and was part of the experiment shown in Figure 1A.

at lower pH levels and was associated with increased SA uptake by fungal hyphae. The mechanism of action of SA as an antimicrobial agent is not fully understood; however, several studies suggest that it may dissipate the transmembrane proton gradient required for ATP production (Gutknecht 1990; Jørgensen et al. 1976; Smith 1959;

Norman et al. 2004; Stenlid and Saddik 1962) or inhibit respiration (Norman et al. 2004) or catalase activity (Chen et al. 1993). In HIM medium, *P. syringae* pv. *tomato* growth was reduced at SA concentrations as low as 100 to 200 μ M, suggesting that SA may be effective at similar concentrations in planta. Application of an

Fig. 7. SOC1 negatively impacts age-related resistance (ARR). **A**, Plants were inoculated with 10^6 CFU ml⁻¹ *Pseudomonas syringae* pv. *tomato* at either 4 or 7 weeks postgermination (wpg) and bacterial levels were quantified 3 days later. Each bar shows the mean of three sample replicates and error bars show standard deviation. Different letters indicate significant differences (analysis of variance [ANOVA] followed by Tukey's honestly significant difference [HSD] test, $P < 0.05$). **B** and **C**, Plants were inoculated with 10^6 CFU ml⁻¹ *P. syringae* pv. *tomato* at 6.5 wpg and salicylic acid (SA) levels were quantified in intercellular washing fluids (B) and *ICS1* expression was quantified in leaf tissue (C) collected from untreated or inoculated plants at 24 h postinoculation (hpi). **D**, Plants were pressure-infiltrated with 50 μ M β -estradiol or a mock solution at 7 wpg and were inoculated with a 10^6 CFU ml⁻¹ solution of *P. syringae* pv. *tomato* 24 h later. Bacterial levels were quantified 3 days after inoculation. Different letters indicate significant differences (ANOVA, followed by Tukey's HSD test, $P < 0.05$). **E**, *SOC1* expression was measured by reverse transcription-polymerase chain reaction in leaf tissue of *XVE:SOC1/soc1-2* plants at 24 and 96 hpi with 50 μ M β -estradiol (+) or a mock solution (-). These experiments were performed at least twice with similar results.

experimentally determined dilution correction factor to SA levels measured in IWFs suggested that SA accumulates to 40 to 100 μM in the intercellular space of mature plants 24 hpi with *P. syringae* pv. *tomato*, similar to the levels that accumulate in the intercellular space of tobacco in response to *Tobacco mosaic virus* (Huang et al. 2006). These results suggest that SA may indeed act as an antimicrobial agent during ARR, especially given the possibility that SA is incompletely recovered during IWF collection and may also reach higher concentrations in microenvironments of the leaf intercellular space, which cannot be assessed using currently available techniques. Moreover, it is possible that other antimicrobials act together with SA during ARR.

Biofilm formation by *P. syringae* and anti-biofilm activity of SA.

Many *P. syringae* genes that are expressed in planta are also expressed in minimal but not rich media (Boch et al. 2002; Huynh et al. 1989; Rahme et al. 1992). Similarly, we found that *P. syringae* pv. *tomato* formed biofilms in intercellular space-mimicking minimal medium but not rich medium, leading to the idea that biofilm formation might also occur in planta. Indeed, fluorescence microscopy of leaves inoculated with GFP-expressing *P. syringae* pv. *tomato* revealed the presence of biofilm-like aggregates in the intercellular space at 24 hpi. Intriguingly, SA reduced *P. syringae* pv. *tomato* biofilm formation in vitro, at concentrations that were insufficient to affect growth and were estimated to exist in the intercellular space of mature wild-type plants. Furthermore, reduced formation of bacterial aggregates was observed in mature wild-type plants compared with young plants and mature *svp* mutants, both of which are impaired in intercellular SA accumulation. In young plants, *P. syringae* pv. *tomato* suppresses intercellular SA accumulation using the phytotoxin coronatine (Carviel et al. 2014), which contributes to bacterial growth and disease. On the other hand, mature ARR-competent plants accumulate intercellular SA in response

to the same pathogen, leading us to speculate that ARR-competent plants circumvent coronatine-mediated suppression of intercellular SA accumulation. Taken together, these data suggest that SA accumulation in the intercellular space reduces either *P. syringae* pv. *tomato* growth, biofilm formation, or both during ARR (Fig. 8).

Biofilms are usually described as surface-adherent aggregates of cells embedded in an extracellular matrix. These structures have been observed in several plant-pathogen interactions, are thought to provide protection against environmental stresses such as desiccation and antimicrobials, and have been linked to several virulence strategies (Danhorn and Fuqua 2007; Ichinose et al. 2013). In *P. syringae*, biofilms are largely studied in the context of epiphytic populations, although the kiwi pathogen *P. syringae* pv. *actinidiae* forms biofilms on both the leaf surface and within the intercellular space (Renzi et al. 2012). *P. syringae* pv. *tomato* biofilm formation is not well described; however, in leaves of *Arabidopsis*, *P. syringae* pv. *tomato* was shown to express *algA* and *algD* (Boch et al. 2002; Keith et al. 2003), genes involved in the biosynthesis of alginate, the major extracellular polysaccharide produced by *P. syringae* pv. *tomato* in tomato (Fett and Dunn 1989). Intriguingly, *P. syringae* pv. *tomato* *algD* mutants exhibited reduced growth in *Arabidopsis* compared with wild-type *P. syringae* pv. *tomato* (approximately 10-fold) (Aslam et al. 2008), suggesting that biofilm formation contributes to *P. syringae* pv. *tomato* virulence.

Interestingly, SA treatment at concentrations that are insufficient to affect bacterial growth also reduce in vitro biofilm formation and virulence factor production in animal pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Prithiviraj et al. 2005a and b; Yang et al. 2009). Similarly, in vitro SA treatment has been shown to suppress *hrpA* promoter activity in the plant pathogen *Erwinia amylovora* (Khokhani et al. 2013), the *virA/G* regulon of *Agrobacterium tumefaciens* (Yuan et al. 2007), and virulence gene expression in *Ralstonia solanacearum* (Lowe-Power et al. 2016). Yang et al. (2009) demonstrated that SA treatment interferes with multiple quorum-sensing systems in *P. aeruginosa*. Furthermore, SA treatment activated the *attKLM* operon involved in degradation of quorum-sensing signals in *A. tumefaciens* (Khokhani et al. 2013). Given that quorum sensing mediates many virulence strategies, including production of toxins, virulence effectors, and biofilm formation (Danhorn and Fuqua 2007; Ichinose et al. 2013), the ability of SA to suppress virulence in a wide range of pathogens may be due to an ability to interfere with quorum sensing. The in vivo and in vitro data presented in this paper provide evidence that SA acts directly on pathogens in planta; however, it is possible that SA also interacts with one or more host proteins that directly or indirectly influence bacterial survival or virulence.

Pathogens have evolved several strategies to resist the toxic effects of SA, including degradation of SA or its precursors (Tanaka et al. 2015). For example, *R. solanacearum* can degrade SA and use it as a carbon source (Lowe-Power et al. 2016). This ability confers enhanced SA tolerance in vitro and increased virulence on tobacco, compared with strains that cannot use SA as a carbon source (Lowe-Power et al. 2016). This ability does not appear to exist in *P. syringae* pv. *tomato* DC3000, which was unable to grow on SA as the sole carbon source.

Under biologically relevant conditions, SA concentrations in the micromolar range exhibit direct effects on pathogens, reducing their growth, virulence, or a combination of the two. We provide evidence that during ARR in *Arabidopsis*, SA accumulates in the intercellular space and contributes to suppression of *P. syringae* pv. *tomato* growth and biofilm formation. Furthermore, our data support the idea that the MADS-domain transcription factors SVP and SOC1 act antagonistically during flowering-time control and ARR, such that SVP is required for ARR to alleviate the negative

Fig. 8. Proposed model for the effect of SHORT VEGETATIVE PHASE (SVP) and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) on intercellular salicylic acid (SA) accumulation during age-related resistance (ARR). SVP is required for ARR to suppress the expression of *SOC1*, which codes for a MADS-domain transcription factor that negatively impacts ARR through repression of the SA biosynthesis gene *ICS1*. In the presence of functional SVP, mature plants accumulate intercellular SA, which limits either biofilm formation, bacterial growth, or both.

impact of *SOC1* on *ICS1* expression and SA accumulation. A better understanding of the interplay between development and defense, as well as the mechanisms of action of compounds that reduce growth and virulence of pathogens, will be useful for optimizing crop performance in the future.

MATERIALS AND METHODS

Plant lines and growth conditions.

All plant lines used are in the Col-0 background and have been described previously: *sid2-2* (Nawrath and Métraux 1999), *svp-32* (Lee et al. 2007), *svp-41* (Hartmann et al. 2000), *soc1-2* and *soc1-101D* (Lee et al. 2000), and *KNAT1:SVPSVP-41* (Andrés et al. 2014). Initial experiments used the *svp-32* allele, but later experiments with *KNAT1:SVPSVP-41* plants required that the *svp-41* allele be used. Similar results were obtained with both alleles. The *svp-32 soc1-2* mutant was created by crossing the corresponding single mutants. Seeds were surface-sterilized, were stratified for 2 days in darkness at 4°C, and were then plated on Murashige and Skoog medium. Approximately 1 week later, cotyledon-stage seedlings were transplanted to soil (Sunshine Mix #1) hydrated with 1 g of all-purpose 20-20-20 fertilizer per liter. Growth conditions were 22 ± 2°C, 80% ± 10% relative humidity, and 9 h of light, unless otherwise specified in the figure legend (mixed fluorescent and incandescent, 120 to 150 µE m⁻¹ s⁻¹).

To generate *XVE:SOC1/soc1-2* the *SOC1* coding sequence was polymerase chain reaction (PCR)-amplified from Col-0 cDNA, using primers 5'-TAAGCAGGCGCGCCATGGTGA GGGGCAAACTC (F) and 5'-TGCTTATTAATTAATCAC TTTCTTGAAGAACAAGGTAAC (R), and was cloned into the pER8 binary vector (Zuo et al. 2000) by restriction-ligation (*AscI* and *SacI* sites). The resulting construct was transformed into *A. tumefaciens* GV3101, *soc1-2* mutants were transformed via the floral dip method (Clough and Bent 1998), and transformants were selected on the basis of hygromycin resistance (25 µg ml⁻¹). β-estradiol (Sigma) was dissolved in dimethyl sulfoxide (DMSO), and a 50-µM solution (0.5% DMSO) was applied by pressure-infiltration with a needleless syringe.

Disease resistance assays.

Overnight cultures of *P. syringae* pv. *tomato* DC3000 were grown in KB medium to exponential phase. Cells were collected by centrifugation, were resuspended in 10 mM MgCl₂ to 10⁶ CFU ml⁻¹, and were pressure-infiltrated into fully expanded leaves using a needleless syringe. For quantification of in-planta bacterial levels, three sets of eight leaf disks (4 mm diameter) were collected and shaken at 200 rpm for 1 h in 10 mM MgCl₂ with 0.1% Silwet L-77. Serial dilutions were plated on KB medium with kanamycin (50 µg ml⁻¹) and rifampicin (100 µg ml⁻¹), and colonies were counted 2 days later.

IWF collection and dilution correction factor determination.

The technique for infiltration of leaves and collection of IWFs was adapted from Baker et al. (2012) and O'Leary et al. (2014). Three pools of eight to 12 leaves (or 30 to 40 leaves for *soc1-101D* plants, to account for their smaller size) were cut at the petiole and were vacuum-infiltrated with water inside a 60-ml syringe until they appeared completely water-soaked. Leaves were then blotted dry, were stacked between parafilm sheets, were rolled around a 1-ml pipette tip, were secured with a twist-tie, and were placed inside the bottom third of a 60-ml syringe fitted to a 1.5-ml centrifuge tube, with the petioles facing up. Leaves were then centrifuged using a swinging bucket rotor at 600 × g for 15 min at room temperature, to collect the IWFs. In

some cases, leaves were subsequently frozen in liquid nitrogen and were stored at -80°C for SA quantification. IWFs were centrifuged for an additional 5 min at 13,000 × g, were transferred to fresh tubes, were weighed, and were stored at -80°C. The remaining chlorophyll pellets (sometimes visible) were resuspended in 1 ml of ethanol and were measured spectrophotometrically at 664 and 700 nm, to assess contamination of IWFs with cellular contents (Baker et al. 2012). In a representative experiment, the chlorophyll levels in IWFs were 0.0085% of the corresponding leaf tissue, indicating that the IWFs had minimal cellular contamination.

The dilution correction factor was determined according to the method described by Husted and Schjoerring (1995), where dilution correction factor = (V_{air} + V_{water})/V_{water}. IWFs were collected as described above, except that leaves were vacuum-infiltrated with 50 µM indigo carmine in 50 mM sodium phosphate (pH 6.2). The volume of intercellular air space (V_{air}) was estimated by calculating the difference in leaf weight before and after infiltration, and the volume of intercellular water (V_{water}) was calculated according to the equation V_{water} = D_{dye} × V_{air}/(1 - D_{dye}), where D_{dye} is the dilution of the dye after IWF collection. Indigo carmine was measured spectrophotometrically at 608 nm.

SA quantification.

SA was quantified using the ADPWH_{lux} SA biosensor (DeFraia et al. 2008) as described previously (Carviel et al. 2014). Similar results were obtained when SA was quantified by gas chromatography-mass spectrometry (Supplementary Fig. S11).

Antibacterial assays.

For antibacterial assays with pure SA, overnight cultures of *P. syringae* pv. *tomato* DC3000 were grown in KB medium to exponential phase. Cells were collected by centrifugation, were washed twice with fresh KB (20 g of proteose peptone per liter, 10 ml of glycerol per liter, 8.6 mM K₂HPO₄, 6 mM MgSO₄) or HIM (50 mM potassium phosphate buffer, 10 mM fructose, 7.6 mM (NH₄)₂SO₄, 3.6 mM MgCl₂, 1.7 mM NaCl, pH 5.7) medium, and were then resuspended in KB or HIM medium to an optical density at 600 nm of 0.05. Aliquots of 160 µl were added to a 96-well microplate (Corning) containing 3.2 µl of SA (Sigma) or ethanol (the solvent used for SA stock solutions, 0.02% final concentration). Plates were incubated with shaking at room temperature in a Tecan Sunrise with absorbance (600 nm) measured every 15 min. To determine bactericidal concentrations at the end of the experiment, well contents were transferred to microcentrifuge tubes and cells were collected by centrifugation, were resuspended in 10 mM MgCl₂, and were then plated on KB medium with kanamycin (50 µg ml⁻¹) and rifampicin (100 µg ml⁻¹). Bactericidal activity was assumed if no growth was visible after at least 3 days.

Biofilm assays.

In vitro biofilm assays were performed essentially as described by O'Toole (2011). Overnight cultures of *P. syringae* pv. *tomato* DC3000 were grown to exponential phase in KB medium, were diluted with fresh KB or HIM medium (1:100), and 150-µl aliquots were added to a 96-well microplate (Corning) containing SA or ethanol. The cultures were then incubated at room temperature and were stained at the indicated times. Prior to staining, microplates were inverted and were rinsed twice with water, to remove nonadherent cells. Each well was stained with 180 µl of 0.1% crystal violet for 10 min, followed by four washes with water to remove residual stain. For quantification of the stained biofilms, 200 µl of 30% acetic acid was added to each well and was incubated for 15 min, and then, absorbance was measured at 570 nm. For qualitative assays, a similar procedure was followed,

using a 500- μ l culture volume in polypropylene tubes that were photographed after the staining step.

Imaging of *P. syringae* pv. *tomato* in the intercellular space by epifluorescence microscopy.

Plants were inoculated with a 10^6 CFU ml⁻¹ solution of *P. syringae* pv. *tomato* carrying pDSK-GFPuv (Wang et al. 2007). After 24 h, leaves were cut at the petiole and sections of the lower epidermis were removed using invisible tape. Sections without the lower epidermis were isolated using a razor blade and were mounted in water on a glass slide with coverslip with the epidermis-less surface facing upwards. Slides were imaged immediately, using a Nikon Eclipse E800 microscope fitted with a Nikon DS-Fi1 camera head and the DS-U3 control unit using 60 $\times$ or 100 $\times$ oil immersion lenses and a B-2A filter cube. For comparing the proportion of cell types between young and mature plants of different genotypes, tissue preparation and imaging were performed by different individuals so that the scoring was blind.

Gene expression analysis.

RNA extraction and cDNA synthesis were performed as described previously (Carviel et al. 2014), except that M-MLV reverse transcriptase (Sigma) was used. Quantitative reverse transcription (qRT)-PCR was performed using a BioRad CFX-96 Touch real-time PCR detection system with 20- μ l reactions consisting of 2- μ l template (cDNA diluted twofold with water), 400 nM of each primer (annealing temperature 61°C), and Advanced qPCR mastermix (1 $\times$ final concentration; Wisent Bioproducts). For each sample, three biological replicates and two technical replicates were analyzed. Data analysis was performed using the BioRad CFX Manager 3.0 software, and *CUL4* (AT5G46210) and *SEC5A* (AT1G76850) were used as reference genes. The identity of each qRT-PCR product was confirmed by sequencing. RT-PCR was performed using *Taq* DNA polymerase (ABM). Annealing temperatures and cycle numbers were 60°C and 28 cycles for *ACTIN*, and 53°C and 32 cycles for *SOC1*. Supplementary Table S1 lists the primers used.

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