Review

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Research review

Molecular perspectives on age-related resistance of plants to (viral) pathogens

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Summary

Age-related resistance to microbe invasion is a commonly accepted concept in plant pathology. However, the impact of such age-dependent interactive phenomena is perhaps not yet sufficiently recognized by the broader plant science community. Toward cataloging an understanding of underlying mechanisms, this review explores recent molecular studies and their relevance to the concept. Examples describe differences in genetic background, transcriptomics, hormonal balances, protein-mediated events, and the contribution by short RNA-controlled gene silencing events. Throughout, recent findings with viral systems are highlighted as an illustration of the complexity of the interactions. It will become apparent that instead of uncovering a unifying explanation, we unveiled only trends. Nevertheless, with a degree of confidence, we propose that the process of plant age-related defenses is actively regulated at multiple levels. The overarching goal of this control for plants is to avoid a constitutive waste of resources, especially at crucial metabolically draining early developmental stages.

Introduction

The disease triangle is a model used to illustrate the significance of compatibility between the host, pathogen, and environment when considering plant disease, and is one of the earliest concepts taught to fledgling pathologists (Agrios, 2005; Scholthof, 2007). However, an often overlooked yet significant factor is host age and development. The plant immune system consists of a complex set of surveillance networks that cooperatively function to perceive, respond to, and defend against biotic and abiotic threats. Numerous cellular processes combine with preformed and inducible defense signals to form the plant immune system. While responses to a diverse battery of invading pathogens must be swift and decisive, defense signaling must also be integrated with critical pathways such as growth, development, and reproduction. Hence, host age or developmental stage often influences the outcome of plant–pathogen interactions.

Developmental transitions throughout the plant lifecycle are coupled with genetic and morphological changes that have inevitable impacts on disease resistance. While this age-related resistance (ARR) phenomenon has been observed and exploited in agricultural practices for decades, our understanding of the molecular and cellular functions remains highly incomplete. Moreover, the role of ARR in plant virus infection has been insufficiently explored, and sometimes avoided all together. To provide that perspective, this review will evaluate the functional mechanisms and interconnectedness of complex pathways that underpin ARR, with an emphasis toward viral diseases. Additionally, multiple noteworthy advancements have been made toward understanding this phenomenon, which warrant recognition, including evidence of a link between ARR and RNA silencing during viral infection. Finally, outstanding questions and considerations surrounding ARR will be highlighted. Understanding how age impacts plant–microbe interactions and disease (i.e. ARR) will become increasingly more important in our current era of rapidly advancing molecular methods and tools, to bring forth both new challenges and novel solutions toward sustainable control.

What is age-related resistance?

While it has been known by many names, including adult plant resistance (APR), mature plant resistance (MPR), developmental

resistance, and ontogenic resistance, the term 'age-related resistance' (ARR) broadly encompasses phenomena in which the characteristic of disease resistance changes with host age, and therefore will be used in this review (Lazarovits *et al.*, 1981). Commonly, observations of ARR have referred to the acquisition of or increase in resistance as plants age, generally resulting in mature plants being less susceptible to disease than young plants. However, many examples of ARR exist in which the plant becomes more susceptible with age, exemplifying the underlying diversity and variability of the response (Huang *et al.*, 2020).

For over a century, reports of ARR have been made in a broad range of plant systems and have been exploited for studies in both basic genetic research and applied agriculture. For instance, while studying tobacco mosaic virus (TMV) resistance conferred by the *N* gene, F. O. Holmes observed that young *Nicotiana rustica* plants developed systemic necrosis upon TMV infection, while older plants developed localized necrosis (Holmes, 1934). This contrasted with observations made in other *Nicotiana* species, such as the age-independent local lesions developed by *N. glutinosa*. Following this, age- or development-related changes in immunity have been reported in a wide range of plant–microbe interactions and across the plant kingdom, including in economically significant crops (Panter & Jones, 2002).

Age-related resistance has a history of being adapted for disease management programs, where it is recognized as a key factor associated with reduced infection and yield losses in the field, such as with late blight of potatoes, stem rust of oat, multiple fungal diseases of wheat, and a variety of viral diseases (Broadfoot, 1933; Peturson, 1944; Peterson & Mills, 1953; Knutson & Bishop, 1964; Ross, 1969; Demski & Chalkley, 1974; Scott & Benedikz, 1977; Pasko *et al.*, 1984; Agrios *et al.*, 1985; Lot *et al.*, 1998; Dinglasan *et al.*, 2022; Eigenbrode & Gomulkiewicz, 2022). As an illustration, wheat breeders have been targeting ARR-associated genes for over a century to confer stem rust resistance (Dinglasan *et al.*, 2022). Cultivation practices often incorporate ARR by adjusting planting to avoid exposing crops at susceptible ages to a seasonally active pathogen. This is particularly useful for the management of many of the most agriculturally and economically significant plant viruses, which are commonly vectored by insects. Growers can optimize planting and insecticide spraying to allow the onset of ARR before optimal vector season. Furthermore, genes related to ARR could offer plant breeders an additional pool to supplement resistance. For instance, resistance to papaya ringspot virus increases with age in multiple varieties of transgenic papaya (Tennant *et al.*, 2001), and potato cultivars are bred to exhibit ARR to potato virus Y depending on the dominant viral strain of the region (Sigvald, 1985; Gibson, 1991; Dupuis, 2017; Chikh-Ali *et al.*, 2020).

Furthermore, viruses are often exploited as biotechnological tools, in the form of gene vectors for silencing experiments and delivery of heterologous cargo, including gene editing materials (Cody & Scholthof, 2019; Chiong *et al.*, 2021). Results could be unintentionally altered or misinterpreted due to host age influencing the functionality of the experimental tool. Therefore, understanding ARR could have significant implications on the contemporary use of recombinant viruses as biotechnological tools and experimental design. Significant questions remain related to ARR that could influence our understanding of pathology, genetics, biotechnology, and experimental design.

How is the onset of ARR controlled?

When considering plant age, determination and quantification are not necessarily as straightforward as one may assume. While perhaps the most direct approach could be chronological measurement, such as weeks postgermination (wpg), ontogeny of individual organs may warrant consideration as well. Throughout plant development, individual organs undergo physiological changes in size, shape, and structure via cell division and differentiation, which may offer forms of ARR. This is evident in cucumber and winter squash, where changes in the structure and chemical content of the fruit exocarp confer ARR (Ando *et al.*, 2015; Alzohairy *et al.*, 2020). Moreover, a tissue-specific transcriptome analysis revealed candidate ARR-associated genes that implicate pathways such as flavonoid and terpenoid biosynthesis, oxidative stress, and innate immunity (Ando *et al.*, 2015; Mansfeld *et al.*, 2017, 2020). These findings are an excellent reflection of a reoccurring theme in ARR – the coordination of multiple lines of reinforcement, including physical barriers, innate immunity, and chemical defenses to confer ARR.

Most flowering plants also undergo a series of predictable and successive developmental stages throughout their lifecycle, the transitions to which often correspond to changes in defense. These may be defined by the appearance of characteristic physiological features, such as germination or flowering. Details of these stages and transitions can be found in previous reviews (Bäurle & Dean, 2006; Huijser & Schmid, 2011). In order, they begin with the transition from the embryonic to the juvenile vegetative stage, followed by transition to an adult vegetative state, which then enters the reproductive phase. These transitions are often associated with changes in pathogen resistance. For example, in Arabidopsis, insect resistance is induced during the maturation stages (Mao *et al.*, 2017), and resistance to common rust and European corn borer is associated with a corresponding transition in maize (Abedon & Tracy, 1996). Similarly, the transition to flowering coincides with TMV and blue mold resistance in tobacco (Yalpani *et al.*, 1993), and *Pseudomonas syringae* resistance in *Nicotiana benthamiana* (Saur *et al.*, 2016). Resistance to multiple geminiviruses in *N. benthamiana* depends on whether the plant is in the vegetative or floral stage, as does resistance against cauliflower mosaic virus (CaMV) in Arabidopsis and turnip (Leisner *et al.*, 1992, 1993; Jackel *et al.*, 2015). In Arabidopsis, a delay in flowering results in the delayed onset of resistance to *P. syringae* pv. *tomato* (*Pst*) (Rusterucci *et al.*, 2005; Patharkar *et al.*, 2017).

Molecular players involved in ARR may be uncovered by identifying defense-related genes and metabolites that exhibit changes in expression during developmental transitions. Recent transcriptomic analyses have identified many ARR-associated genes and pathways that could function as potential signaling cascades to integrate disease resistance and developmental control (Gusberti *et al.*, 2013; Ando *et al.*, 2015; Mansfeld *et al.*, 2017; Zou *et al.*, 2018). Yet, a longstanding question has been whether ARR

associated with those transitions represent an independent program or merely reflect a physiological consequence. With regard to the floral transition specifically, studies in Arabidopsis infected with bacterial or fungal pathogens have supported that the molecular programs regulating the timing of ARR onset can be decoupled from flowering (Wilson *et al.*, 2013; Lyons *et al.*, 2015). Specifically, photoperiod-induced flowering and multiple flowering-time Arabidopsis mutants were used to separate flowering from other developmental events that occur as plants age (Wilson *et al.*, 2013). Under short-day conditions, late-flowering plant lines acquired ARR to *Pst* at approximately the same time as Col-0. Photoperiod-induced transient expression of Flowering Locus T (FT), a positive regulator of flowering, triggered early flowering in short-day grown Arabidopsis, but again, the timing of ARR competence was unaffected.

On the contrary, an excellent example of coordination between ARR and the flowering developmental transition is represented by the Short Vegetative Phase (SVP) transcription factor. Short Vegetative Phase is responsible for integrating thermoresponsive pathways and vernalization to repress flowering, and *svp* mutants exhibit early flowering phenotypes. Interestingly, *svp* mutants are unable to launch an effective ARR response due to the inability to sufficiently accumulate intracellular SA (Zheng *et al.*, 2015; Wilson *et al.*, 2017). However, studies utilizing SVP expressed under tissue-specific promotors demonstrated that while meristematic SVP controls flowering time, leaf SVP is responsible for ARR. This suggests that the leaf pool of SVP protein is responsible for activating defense, and ARR is not merely a secondary consequence of the developmental transition to flowering (Wilson *et al.*, 2017). Therefore, tissue-specific functions of proteins such as SVP may impact ARR, in addition to other changes in defense-related gene and metabolite expression associated with developmental transitions.

The aforementioned studies were able to utilize powerful genetic tools and mutants to distinguish and decouple age-dependent innate immunity from the secondary physiological consequences of developmental transitions. However, similar resources are not yet available or as easily accessible for many plants. Therefore, genetic tools and markers to distinguish the expression of ARR in non-model plants will require further development and exploration.

Explored mechanisms

Physiology

The clear diversity in the onset of ARR depending on the pathosystem in question suggests a wide range of potential underlying mechanisms. Development-associated changes in resistance could reflect changes in host physiology, or the expression or availability of genetic determinants. The contribution of development-related physiological changes to ARR is exemplified in Arabidopsis plants that are more susceptible to tomato spotted wilt virus (TSWV) as they age, which is correlated with increased expression of pectin methylesterase (PME) and β-1,3-glucanase (BGL) (Huang *et al.*, 2020). These enzymes regulate the size exclusion limit of plasmodesmata and cell-to-cell trafficking (Chen *et al.*, 2000; Huang *et al.*, 2020). As intercellular parasites, viruses require plasmodesmal-mediated transport to facilitate systemic spread throughout the host plant, and studies have demonstrated that PME and BGL mediate cell-to-cell movement of multiple different plant viruses (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Iglesias & Meins Jr, 2000; Bucher *et al.*, 2001). In the case of young Arabidopsis plants, susceptibility to multiple TSWV isolates gradually increased with age starting at 4 wk, eventually reaching 100% infectivity in 7- or 8-wk-old plants. However, while this age-dependent susceptibility to TSWV was observed in two different ecotypes of Arabidopsis, it was not observed in any of the Solanaceous species tested, including tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), and *N. benthamiana* (Huang *et al.*, 2020). This represents both an intriguing question and potential hurdle in the study and application of ARR, and adds a new dimension to an existing scientific dilemma – *How does ARR vary by species, and how well can potential breakthroughs made in one plant type be applied to others*?

Plant immunity

Changes in innate immune networks throughout development could also impact ARR. The canonic model defining plant immunity has generally described two layers of defense, including Pathogen-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI), which have been the subject of multiple comprehensive reviews (Thomma *et al.*, 2011; Ngou *et al.*, 2022). Briefly, while PTI contributes basal resistance to diverse adapted and nonadapted microorganisms, ETI confers defense against race-specific or host-adapted pathogens. PTI is triggered following perception of microbe-derived molecules known as MAMPs (Microbe-Associated Molecular Patterns) by host cell surface-localized pattern recognition receptors (PRRs). Most PRRs complex with an associated kinase, and upon activation by a MAMP, the PTI response is triggered.

Downstream events include activation of mitogen-activated protein kinase (MAPK) cascades, ion fluxes across the plasma membrane, reactive oxygen species (ROS) generation, cell wall fortification, hormone signaling, and dynamic transcriptional reprogramming of defense genes. Successful pathogens have evolved effector molecules capable of PTI suppression. In response, plants have coevolved ETI as an additional layer of resistance. ETI utilizes Resistance (R) proteins, such as the nucleotide binding leucine-rich repeat (NB-LRR; NLR) receptors, which are capable of recognizing pathogen effectors and effector products. Recently, a growing body of literature has significantly advanced our understanding of NLR-mediated immunity, including the formation of oligomers upon activation (Wang *et al.*, 2019, 2023; Lolle *et al.*, 2020). Additionally, downstream 'helper' NLRs (hNLRs) are often required to be activated by the initial effector-perceiving NLR (Jubic *et al.*, 2019). In addition to the initial sensor NLR that perceives the avirulent effector, ETI activation is associated with enhanced and sustained local and systemic responses, including the hypersensitive response (HR), which is a form of programmed cell death hypothesized to restrict further pathogen spread.

However, exactly how NLR activation functions to initiate ETI remains in question. ETI-associated defense responses often require crosstalk with other signaling molecules and phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and brassinosteroids (Feys & Parker, 2000; Nakashita *et al.*, 2003; Collum *et al.*, 2016; Alazem & Lin, 2020; Zhao & Li, 2021). While traditional models have illustrated PTI and ETI as distinct, yet separate pillars of immunity, their synergy, coordination, and mutualistic potentiation has become increasingly more evident (Ngou *et al.*, 2021, 2022). In parallel to local immune signaling, a broad spectrum of distal immune signaling is activated, such as systemic acquired resistance (SAR) and systemic RNA silencing (Kachroo & Kachroo, 2020; Lopez-Gomollon & Baulcombe, 2022). This allows systemic, distal tissues to be 'primed' in advance of pathogen spread, which manifests as enhanced resistance activity and reduced secondary invasion, proliferation, and disease. Clearly, many avenues are worthy of pursuit in molecularly tracking the influence of plant immunity components on ARR.

Our knowledge of pro-immune signaling for defense activation is considerably more advanced than our understanding of the converse – controlled immune signaling attenuation. In plants, many of the signal transduction pathways and their molecular players often have significant overlapping functions in both immunity and development. Since growth can be negatively affected by the considerable resources that must be devoted to activated immune pathways, induction, and attenuation of immunity signaling must be efficiently integrated with other major processes such as germination and flowering. This results in a precisely balanced growth-defense trade-off. While the broad range of defense responses described above must be rapidly launched upon pathogen perception, the attenuation of immune signaling is also a critical process of self-defense and allows energy to be redirected into growth. This poses an intriguing question at the forefront of understanding ARR – *How do plants efficiently integrate enhanced age-associated defense responses while avoiding negative impacts on growth and development*? This question is particularly intriguing given that most observed examples of ARR describe the host plant gaining resistance as a 'package deal' with maturity.

Phytohormonal signaling

In addition to functioning as key regulators of growth and development, phytohormones such as SA, JA, ET, ABA, auxin, and BR are essential for biotic and abiotic stress signaling and have been implicated in multiple ARR responses (Johnson & Ecker, 1998; Kus *et al.*, 2002; Mauch-Mani & Mauch, 2005; Develey-Rivière & Galiana, 2007; Shibata *et al.*, 2010; Al-Daoud & Cameron, 2011; Wilson *et al.*, 2017; Xu *et al.*, 2018; Zhang *et al.*, 2023). In addition to the well-known role of SA in immunity, it also has roles in growth and development, both independently and through crosstalk with other phytohormones and signaling molecules, and has been implicated as a factor in the expression of ARR (Wilson *et al.*, 2014; van Butselaar & Van den Ackerveken, 2020; Pokotylo *et al.*, 2021). For instance, Arabidopsis exhibits a distinct SA-dependent increase in resistance to *Pst* at approximately 6 wpg.

Transcriptomic studies in young and mature plants have revealed interesting genes exhibiting early upregulation during ARR, including MAMP receptors, SA biosynthesis genes, and players in SAR establishment (Gusberti *et al.*, 2013; Ando *et al.*, 2015; Mansfeld *et al.*, 2017; Zou *et al.*, 2018; Shields *et al.*, 2022; Yildiz *et al.*, 2023). Specifically, NPR1 (nonexpressor of pathogenesis related −1) was identified, which is commonly dubbed the 'master regulator' of plant immunity and is a required activator of SA signaling (Shields *et al.*, 2022). SA biosynthesis and downstream signaling are closely intertwined with that of N-hydroxy-pipecolic acid (NHP) and its biosynthetic precursor pipecolic acid (Pip), which have been identified as important metabolites involved in plant immunity. Local and systemic accumulation of NHP and Pip can trigger defense gene expression, induce HR, and synergistically cooperate with SA to potentiate SAR establishment after pathogen attack (Hartmann *et al.*, 2018). However, in addition to roles in immunity, multiple studies have suggested that the SA/NHP interplay may mediate growth and development (Cai *et al.*, 2021; Lim, 2023; Yildiz *et al.*, 2023). The transcriptional reprogramming observed upon SAR activation or treatment with Pip includes a vast array of genes related to maintaining the equilibrium between growth and immunity. Given the significance of NHP in plant growth and SAR establishment, future studies investigating this transcriptional landscape during infection and throughout development could reveal important connections to ARR.

With regards to plant–virus interactions, regulation by SA has been demonstrated for three main stages of infection, including intercellular trafficking, long-distance movement, and viral replication (Zhao & Li, 2021). Therefore, it is not surprising that similar contributions of SA and elevated basal resistance have been demonstrated in ARR in multiple viral pathosystems. In tomato infected with tomato yellow leaf curl virus, the ARR response was more evident in cultivars with higher basal resistance, which correlated with an age-related increase in SA (Zhang *et al.*, 2021). In a variant of *N. edwardsonii*, SA levels substantially increased between 6 or 7 wk of age, which resulted in correspondingly elevated levels of pathogenesis related 1 (PR1) protein accumulation and enhanced ETI against TMV and tobacco necrosis virus (Cole *et al.*, 2004). Also, ARR to TMV in tobacco is SA-dependent and strongly correlated with the onset of flowering and increased expression of the antimicrobial PR proteins PR-1, -2, and -3 (Yalpani *et al.*, 1993). However, while SA accumulation and signaling appears not only to underpin the expression of systemic acquired resistance but also ARR, the question remains: *How exactly does SA modulate resistance in an age-dependent manner? Also, how are key inducers of SAR, including SA and NHP, involved in modulating ARR*?

Pathosystem specificity

Metabolite specificity

While SA seems to be a vital player in some ARR studies, others present contradictory conclusions, which highlights how ARR is often pathosystem-dependent. For instance, *Pst* infection in

Arabidopsis is the most extensively well-characterized with regard to ARR. However, *N. benthamiana* is also a powerful model plant and is commonly used in virological experiments due to its high susceptibility to most viral pathogens (Cauz-Santos *et al.*, 2022). In both the Arabidopsis-*Pst* and *N. benthamiana-Phytothora infestans* pathosystems, ICS1 and EIN2 are important for ARR, but not certain players in JA signaling (Rusterucci *et al.*, 2005; Al-Daoud & Cameron, 2011; Wilson *et al.*, 2014; Mao *et al.*, 2017). However, unlike in Arabidopsis, SA is involved in HR formation during ARR in *N. benthamiana*. Instead, it has been suggested that although HR does not occur during Arabidopsis ARR, SA still functions as an antimicrobial agent by accumulating in the intercellular space of mature plants (Wilson *et al.*, 2014). Additionally, while SA is required for ARR to the Emco5 ecotype of *H. parasitica*, it is not required for the Noco2 ecotype (McDowell *et al.*, 2005). Furthermore, NPR1 is required for ARR in the Arabidopsis-*H. parasitica* Emco5 pathosystem, but is dispensable in the Arabidopsis-*Pst* and *N. benthamiana-P. infestans* pathosystems (Kus *et al.*, 2002). Intriguingly, in Arabidopsis infected with the oomycete *P. parasitica*, activation of the SA cascade alone is not sufficient for induction of all the features of ARR, and while SA is required for the control of intracellular colonization, mechanisms controlling infection efficiency utilize an SA-independent pathway (Hugot *et al.*, 1999). While recognizing that plant growth conditions in reports may vary, it appears that individually observed differences illustrate that we may not achieve a consensus as to the underlying mechanistic descriptor of ARR.

Plant organ specificity

The importance of the experimental pathosystem goes beyond SA-related observations. For example, the extent to which leaf stage-dependent ARR is pathosystem-dependent was shown in *N. benthamiana* and Arabidopsis, where nonhost resistance to *Xanthomonas oryzae* pv. *oryzae*, and Arabidopsis non-host resistance to *Pst* was stronger in juvenile leaves than in adult leaves or those in the reproductive stage (Xu *et al.*, 2018). A similar trend was observed in the Arabidopsis-*Pst* pathosystem when ETI was triggered via the NLR RPS2. However, basal resistance to *Sclerotinia sclerotiorum* in *N. tabacum* and *S. sclerotiorum* and *Pst* in Arabidopsis displayed the opposite effect, where adult and reproductive stage leaves exhibited heightened resistance. Expression profiling of these pathosystems revealed genes necessary for the biosynthesis and signaling of phytohormones such as SA, JA, ET, and ABA, and multiple regulators of SAR, that were differentially expressed between leaf stages, revealing a vital, but pathosystem-dependent role. These findings align with previous reports (Kus *et al.*, 2002; Chang & Hwang, 2003; Sharma *et al.*, 2010; Steimetz *et al.*, 2012; Wilson *et al.*, 2017; Hu & Yang, 2019; Li *et al.*, 2020), and collectively highlight the ubiquitous significance of leaf stage- and developmental-associated resistance. This highlights an interesting question regarding the ubiquitous yet pathosystem-dependent requirement of phytohormone signaling – *Do the phytohormone signaling pathways that exhibit reoccurring roles in ARR share an upstream node to control or initiate development- or age-associated immunity*?

In several ecotypes of Arabidopsis, the expression of resistance to CaMV is correlated with the transition to flowering. The protein Terminal Flower 1 (TFL1) is a key negative regulator of flowering time and floral identity (Jin *et al.*, 2021). Inactivation of TFL1 in loss-of-function mutants results in both an early flowering phenotype and early onset resistance to CaMV (Leisner *et al.*, 1993). This appears to contradict previous findings in Arabidopsis where ARR to bacterial or fungal pathogens was not altered in mutant lines with altered flowering phenotypes (Wilson *et al.*, 2013; Lyons *et al.*, 2015). Importantly, *tfl1* mutants did not display early onset of ARR to *Pst*, despite the early flowering phenotype (Wilson *et al.*, 2013). These differing results are probably due to differences in pathogen type.

As an obligate intracellular parasite, the lifecycle of a virus can have major differences when compared to that of other pathogen types, such as *Pst*. In particular, host source/sink relationships can be especially important for systemic invasion of viruses, such as CaMV in Arabidopsis, that achieves long-distance transport via the phloem. Throughout development, source/sink dynamics change and regions of the plant that CaMV is capable of invading is progressively reduced, resulting in resistance (Nono-Womdim *et al.*, 1991; Leisner *et al.*, 1992, 1993). Since the rosette leaves are not invaded by the virus and therefore do not exhibit symptoms, early flowering plants may appear resistant to systemic viral infection, and the ability of CaMV to spread would parallel the early flowering phenotype seen in *tfl1* plants.

Theoretically, any factor capable of influencing the rate of development, such as environmental or genetic factors, could impact the severity of the viral infection and symptoms. Furthermore, source/sink dynamics directly impact the efficacy of antiviral RNA silencing, since systemic RNA silencing signals spread via phloem translocation, and environmental factors including light intensity and temperature can significantly impact the systemic movement of the silencing signal (Patil & Fauquet, 2015).

Molecular regulation of ARR in plant-virus interactions

RNA silencing is an evolutionarily conserved mechanism in eukaryotes and is a central regulator of gene expression (Alvarado & Scholthof, 2009; Ding, 2023). In plants, RNA silencing, which is mediated by noncoding short RNAs (sRNAs), is utilized for the spatiotemporal transcriptional and post-transcriptional regulation of development and immunity. The two major types of sRNAs that induce different pathways of RNA silencing are microRNAs (miRNAs) and short-interfering RNAs (siRNAs). In plants, RNA silencing involves the creation of miRNAs and siRNAs by Dicer-like (DCL) and RNA-dependent RNA polymerase (RdRP) proteins, which are utilized by Argonaute (AGO) proteins to cause degradation of the target. To combat host resistance, plant viruses have coevolved viral suppressors of RNA silencing (VSRs), which function to suppress or hinder antiviral RNA silencing through a variety of mechanisms. Given the central role of RNA silencing in both development and defense, and the modularity and redundancy of RNA silencing components, it is not surprising that it functions in the regulation of ARR.

miRNA-mediated ARR

In addition to the well-known role in development, miRNA-mediated RNA silencing is important for the functional regulation of defense-related gene expression by targeting host messenger RNAs (mRNAs) for cleavage or repression. A number of miRNA families have been identified that target R genes for PTGS in several plant species (Zhai *et al.*, 2011; Li *et al.*, 2012; Shivaprasad *et al.*, 2012; Boccara *et al.*, 2014; González *et al.*, 2015; Deng *et al.*, 2018). These miRNAs are usually conserved in the same species and target sequences encoding conserved R protein motifs (Zhai *et al.*, 2011). MiRNA156/157 (miR156/157) is an established master regulator of the developmental phase transition and targets the SPL (Squamosa Promotor Binding Protein-Like) family of transcription factors (TFs), including SPL9, for repression (Wu *et al.*, 2009; Yin *et al.*, 2019; Zheng *et al.*, 2019). SPL9 promotes the expression of miR172, which represses the TOE1/2 TFs. In Arabidopsis seedlings, high levels of TOE1/2 transcriptionally repress expression of the PRR FLS2, which when associated with the BAK1 co-receptor, can recognize the bacterial MAMP flg22 and trigger PTI (Heese *et al.*, 2007). However, after 6 d, miR156 decreases, ultimately resulting in increased miR172 levels, repression of TOE1/2 transcripts, and functional FLS2 levels (Zou *et al.*, 2018). While the flg22 MAMP can induce miR172 accumulation in 2-d-old and 8-wk-old Arabidopsis, miR172 levels remain unchanged in 4- or 5-wk-old plants (Li *et al.*, 2010; Zou *et al.*, 2018). This suggests that MAMP-dependent upregulation of miR172 is probably age-dependent. The repressive function of miR172 on TOE1/2 also regulates vegetative and floral transitions. Altered levels of miR172 have been observed as a result of pathogen infection in multiple plant species beyond the cotyledon stage, including during grapevine infection with leafroll-associated viruses (Alabi *et al.*, 2012), in addition to multiple other pathosystems (Gai *et al.*, 2014; Li *et al.*, 2014; Luan *et al.*, 2018).

Since viruses are obligate intracellular pathogens and are usually delivered directly into the cytoplasm by their vectors or through plasmodesmata, the existence of anti-viral PTI based on surface-localized receptors has been often disregarded. However, the last decade has demonstrated that PTI is indeed active against plant virus infection, and viral-derived nucleic acids can activate PTI, which is independent of the antiviral RNA silencing pathway (Niehl *et al.*, 2016; Amari & Niehl, 2020). In multiple studies, the significance of the previously mentioned BAK1 and BAK1-Like 1 (BKK1) coreceptors, which are essential PTI-mediating components for many systems, have been demonstrated in anti-viral defense. In summary, they are required or important for defense against turnip crinkle virus (Yang *et al.*, 2010), plum pox virus (PPV) (Nicaise & Candresse, 2017), and multiple diverse RNA viruses (Kørner *et al.*, 2013). Likewise, a negative regulator of PTI, MAPK4, was shown to suppress defense in soybean infected with bean pod mottle virus (Liu *et al.*, 2014). Furthermore, the coat protein (CP) of PPV functions to suppress antiviral PTI in Arabidopsis (Nicaise & Candresse, 2017). As previously mentioned, the FLS PRR required for *Pst* defense in Arabidopsis is developmentally controlled via miR172 repression (Zou *et al.*, 2018). *Could similar age-related repression of anti-viral PTI players contribute to ARR?* More research surrounding the significance of PTI in plant–virus interactions is required to answer this and similar questions.

The miRNA-mediated silencing of NLR genes also has a developmental component. In tobacco, suppression of the *N* NLR mediated by miR6019/miR6020 decreased with age, increasing N-dependent TMV resistance (Deng *et al.*, 2018). Additionally, *Brassica* miR1885 regulates the NLR TNL1 and development-related genes. Upon flowering, miR1885 levels spike, which is inversely correlated with the level of TNL1 (Cui *et al.*, 2020). As mentioned previously, miR156/157 is a regulator of the developmental phase transition, and the miR156/157-SPL module is a reoccurring player in development- and defense-related pathways. As the initially high levels of miR156/157 that suppress SPL transcript expression decrease throughout the juvenile phase, SPL proteins accumulate, allowing adult traits to develop (Poethig, 2013; He *et al.*, 2018). Genetic evidence exists linking the temporal reduction of miR156/157 to ARR in multiple systems, including during N- and RPS4-mediated ETI and JA signaling (Abedon & Tracy, 1996; Padmanabhan *et al.*, 2013; Reyes *et al.*, 2016; Mao *et al.*, 2017; Ge *et al.*, 2018). The age-related reduction of miR156/157 gene targets such as SPL6 can influence the integration of plant immune responses with the developmental phase transition. In young citrus shoots infected with citrus psorosis ophiovirus, miR156 is downregulated, corresponding with increased levels of SPL transcripts (Reyes *et al.*, 2016). In Arabidopsis, miR156, as well as miR164 are induced in response to infection with turnip yellow mosaic virus and its VSR (Kasschau *et al.*, 2003). In grape, a systematic analysis of miR156 binding sites also identified an NLR gene as a candidate target (Cui *et al.*, 2018).

The expression patterns of plant miRNAs can be ubiquitous, or differ by age, tissue type, or developmental stage (Khraiwesh *et al.*, 2012). In an analysis of miRNAs in *Brassica* and Arabidopsis, bra-miR172 was expressed exclusively in the flower, while ath-miR172 was expressed ubiquitously, but in higher levels in the stems and flowers. Bra-miR158 exhibited low silique accumulation, but high flower accumulation, is highly upregulated during infection with turnip mosaic virus (TuMV), and probably targets an R gene based on analysis. Contrastingly, no change in miR158 levels was observed during TMV, CaMV, or *S. sclerotiorum* infection in *Brassica*, suggesting that miR158 induction is highly specific to TuMV (He *et al.*, 2018). Additionally, viral proteins may directly interact with miRNAs, influencing their function and anti-viral capabilities (Reyes *et al.*, 2016). Post-transcriptionally, viral-encoded suppressors of RNA silencing (VSRs) can inhibit miRNA accumulation. However, even viruses that possess weaker VSRs, such as TMV and oil rapeseed mosaic virus are capable of miRNA alteration at the transcriptional level, and many plant viruses influence miRNA accumulation via currently unknown mechanisms (Bazzini *et al.*, 2011).

Taken together, these studies offer the possibility that pathogen interference with small RNA pathways such as host miRNA regulation could impact ARR, especially those forms of ARR associated with specific developmental transitions.

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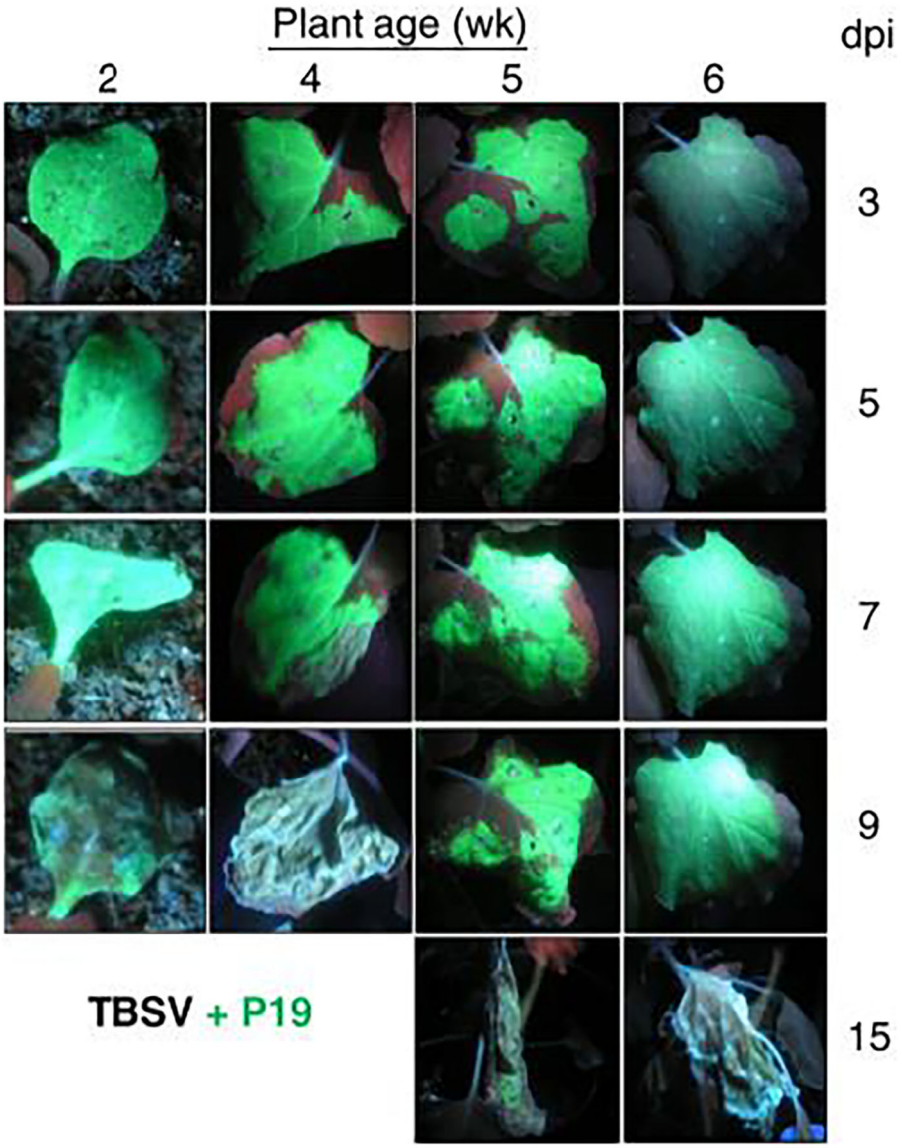

Fig. 1 TBSV-mediated expression of GFP independent of plant age in the presence of P19. *Nicotiana benthamiana* plants of different ages (in weeks) were agroinfiltrated with TBSV-GFP expressing P19 (+P19) using constructs and standard procedures previously described (Shamekova *et al*., 2014). At different days post-infiltration (dpi) images were taken from UV-illuminated leaves (unpublished data just for illustration of the age-related resistance (ARR) phenomenon).

siRNA-mediated ARR

Another form of RNA silencing, mediated by siRNAs, is a well-known mechanism of local and systemic antiviral defense, and is triggered by transgenes or viral nucleic acids (Yang & Li, 2018; Lopez-Gomollon & Baulcombe, 2022). The gene families that form the backbone of this antiviral pathway have expanded for the control of virus infection. For example, three of the four DCLs, 7 of the 10 AGOs, and three of the six RdRPs found in Arabidopsis are involved in antiviral RNA silencing (Lee & Carroll, 2018). Importantly, these components possess both functional specificities and redundancies, allowing multiple DCLs, AGOs, RDRs, and other modular proteins to function on each other's substrate or product depending on a variety of factors, including the plant species, protein availability, or the initial trigger (Alvarado & Scholthof, 2012; Lee & Carroll, 2018; Yang & Li, 2018). For example, in the absence of functional AGO1 in transgenic Arabidopsis, a more important role for AGO2 was observed, suggesting that if the primary AGO becomes unavailable, another player can step in to fulfill the role. This flexibility is assumed to allow a pathway to be more effective through adaptability, which may assist in age-related transition of otherwise developmentally important components towards antiviral contributions.

Age-related silencing against TBSV

Tomato bushy stunt virus (TBSV) is well-known for its native VSR, P19, which is highly effective at suppressing RNA silencing, and can be deployed in both plant and nonplant systems as a tool to enhance heterologous expression (Scholthof, 2006). In the context

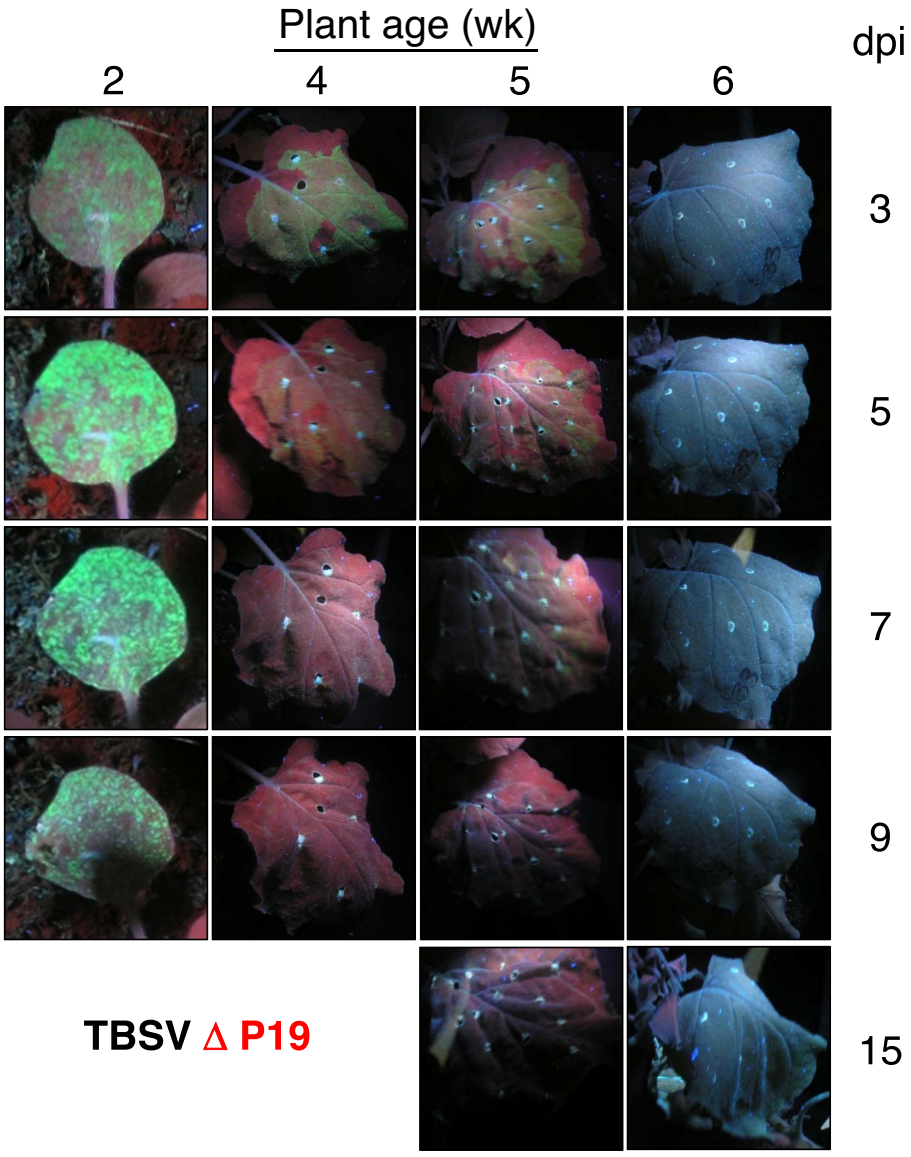

Fig. 2 TBSV-mediated expression of GFP is dependent on plant age in the absence of P19 (ΔP19). *Nicotiana benthamiana* plants of different ages (in weeks) were agroinfiltrated with TBSV-GFP not expressing P19, using constructs and standard procedures previously described (Shamekova *et al*., 2014). [Correction added after online publication 28 July 2023: the preceding sentence has been updated.] At different days postinfiltration (dpi) images were taken from UV-illuminated leaves (unpublished data just for illustration of the age-related resistance (ARR) phenomenon).

of age-related effects, TBSV expressing GFP (Shamekova *et al*., 2014) is fully capable of infecting 2–6-wk-old plants (Fig. 1). On the contrary, while a derivative devoid of P19 expression (ΔP19) can infect younger plants, it is unable to establish or maintain an infection in plants older than 3 wk as is illustrated in Fig. 2, where GFP expression is slightly visible at 3 dpi in most plants of any age but only maintained in the 2-wk-old plants. This is similar to earlier observations made using TBSVΔP19 without GFP (Chu *et al*., 2000).

Prolonged real-time RT-qPCR and immune detection-based attempts to elucidate a temporal molecular explanation for these observations have not yet revealed a definitive explanation of these observations. However, transcript accumulation of NbAGO2, which is the primary anti-TBSV AGO in *N. benthamiana* (Alvarado & Scholthof, 2012; Odokonyero *et al*., 2015), was at least twofold higher than that of other NbAGOs, and even further induced upon infection in younger plants, seemingly independent of P19. Together, this suggests that in young plants, activation of antiviral RNA silencing is either too late, still insufficient, or blocked. The latter could suggest a scenario in which NbAGO2 has a primary role in early development and is consequently not recruited until later for antiviral activities. That notion is further supported by studies showing that in transgenic plants downregulated for NbAGO2 expression, minor, but distinct deformative developmental defects can be observed at early stages, before 'snapping' out of it at later stages, when the phenotype disappears (Odokonyero *et al*., 2015). While these observations require further experimental investigation, they may indicate a scenario in which NbAGO2 has a supporting

role in the early development of *N. benthamiana* but can later be drafted into the antiviral defense arsenal when it is no longer essential elsewhere.

Conclusions and future directions

The existence and impacts of age-dependent defense responses have been become increasingly clear because of studies elucidating important molecular players and mechanisms of regulation. Identification of novel key components of ARR can provide molecular markers to study the ARR in future studies. These genes can also offer a new pool of targets for molecular breeding programs to confer disease resistance. Furthermore, if the age of infection influences eventual yield loss, rapidly spreading pathogens can have greater negative impacts than more slowly spreading pathogens, since higher proportions of the crop will be infected earlier in development (Eigenbrode & Gomulkiewicz, 2022). Therefore, understanding the onset of ARR could have important implications in disease management and could allow growers to alter planting practices and insecticide application to lessen the impact of disease. This is becoming increasingly relevant due to a rapidly and dynamically changing environment. Atmospheric changes such as temperature fluctuations and rising CO_2 levels are predicted to exacerbate the power of disease (Raza & Bebber, 2022).

The future of ARR research has important questions that must be addressed, many of which could have implications for understanding the basic mechanisms of immunity and development. Since many regulators of ARR, including specific genes, are also conserved players in development, how do plants integrate the growth-defense trade-off? Additionally, did the conserved molecular regulators originally evolve for functions in development, and were co-opted for defense, or vice versa? There are clear differences in ARR mechanisms and responses depending on the pathosystem in question. Can the findings established using model plants be successfully implemented in agricultural crops? Regarding SA specifically, it seems as though it may be playing different ARR-roles in different pathosystems. Could this be the case for other ARR-related components?

Furthermore, recent findings published here raise significant questions surrounding the role of RNA silencing in ARR. What component of the RNA silencing pathway against TBSV is age-dependent? Does this involve NbAGO2 expression or function? Are there similar mechanisms underlying ARR in other host species or to other viruses? In nature, co-infections by multiple plant viruses, different isolates of the same virus, or the presence of viral variants in the population are likely common in both cultivated and wild hosts. Does the expression of ARR alter these dynamics? Another agriculturally important area could be the impact of ARR on host association with non-pathogenic microbes. How does development and age impact the ability of the plant to form associations with and be colonized by beneficial microbes such as mycorrhizae and rhizobia, and are the interactions altered throughout growth by ARR-related mechanisms? Moreover, rather than RNA-seq or protein expression profiling, studies on partitioning of components with seemingly multiple roles might become more important to understand their contribution to ARR during development.

The many examples of relationships between pathogen resistance and plant age reflect an exciting avenue to study immunological and developmental processes. Understanding the fundamental systems that mediate the onset and perpetuation of the various forms of ARR will provide valuable insights and directions for future novel research.

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Competing interests

None declared.

Author contributions

AD researched literature and wrote the paper. VA initiated the project and provided input and data. HBS oversaw the project and helped write the paper.

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