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ANAC055 and ANAC092 contribute non-redundantly in an EIN2-dependent manner to Age-Related Resistance in *Arabidopsis*☆

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ABSTRACT

As *Arabidopsis* matures it becomes resistant to virulent *Pseudomonas syringae* pv. *tomato* (*Pst*), a defense response known as Age-Related Resistance (ARR). The contribution of two NAC transcription factors (ANAC055 and ANAC092) and jasmonic acid/ethylene (JA/ET) signaling to ARR was examined by comparing *Pst* growth in wild-type plants, *nac* mutants, an *ANAC092*-overexpressing line, *lox2*, and *ein2-1*. *PDF1.2* expression and *anac055anac092* double mutant analysis suggests that ANAC055 and ANAC092 play non-redundant roles in ARR. Additionally, ANAC092 contributes to the initiation of flowering in short day-grown plants. *ANAC055* and *ANAC092* expression was reduced in partially ARR-defective *ein2-1* suggesting that regulation of *ANAC055* and *ANAC092* by EIN2 contributes to ARR.

1. Introduction

Plants use a number of strategies to combat infection by pathogens. One of the first responses of plants to pathogen attack includes basal resistance or PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI) [1]. PAMPs are general features common to a variety of microbes, such as bacterial flagellin proteins, that are recognized by plant pattern recognition receptors (PRRs) to initiate PTI [2]. PTI signaling results in a number of local physiological, biochemical, and molecular changes in the plant that slow the infection process [1–3].

In *Arabidopsis thaliana*, defense signaling pathways that promote resistance to (hemi)biotrophs often lead to repression of resistance to necrotrophs and visa versa. For example, plants inoculated with hemibiotrophic *Pseudomonas syringae* pv. *tomato* (*Pst*) display increased susceptibility to necrotrophic *Alternaria brassicicola* [4]. This is thought to occur as a result of a mutually antagonistic relationship between SA, which is a key signaling molecule in defense against (hemi)biotrophs, and jasmonic acid (JA) and ethylene (ET), which promote defense mainly against necrotrophs [5,6]. In some circumstances SA and JA/ET signaling act in a cooperative manner to promote resistance to biotrophs and necrotrophs in solanaceous plants such as tobacco [7] and tomato [8,9].

Some pathogens are thought to have evolved a battery of weapons that contribute to the development of plant disease and inhibit plant immunity [10]. For example, *Pseudomonas* species possess a type III secretion system (TTSS) that delivers effector proteins into host cells [11], some of which suppress defense responses [10,11]. *Pseudomonas* species also produce a number of phytotoxins, such as coronatine, that promote pathogenicity and suppress plant defense responses [12]. Some of these molecules inhibit immune responses to *Pseudomonas* by up-regulating JA/ET signaling, which subsequently represses SA signaling, thus promoting susceptibility to *Pseudomonas* [13–20].

Resistance to pathogen infection can vary over the life of a plant. In some plant–pathogen interactions resistance increases as plants mature, and in other cases plants become more susceptible as they develop. Pathogen resistance that develops as plants age is known as Age-Related Resistance (ARR). The onset of ARR competence in some plants is associated with flowering, senescence, or stressful growing conditions [21–25]. The molecular mechanism of ARR has been studied in only a few plant species including tobacco and *Arabidopsis*. As tobacco matures, it becomes more resistant to *Peronospora tabacina* [24], *Phytophthora parasitica* [25], and Tobacco Mosaic Virus (TMV) [26–28]. ARR in tobacco is associated with flowering,

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accumulation of *PATHOGENESIS RELATED* (*PR*) gene transcripts and anti-microbial compounds in older leaves [24–26,28,29]. Mature tobacco plants inhibit *P. parasitica* at early stages of infection in an SA-independent manner and utilize SA-dependent mechanisms to affect later stages of infection [25].

Resistance to a number of pathogens changes as *Arabidopsis* plants age [23]. *Arabidopsis* ecotype Columbia (Col-0) grown under short day conditions begins to display ARR to virulent *P. syringae* pv. *tomato* DC3000 (*Pst*), pv. *maculicula* and *Hyaloperonospora arabidopsidis* at ~ six weeks of age [30–33]. *Arabidopsis* ARR is characterized by a 10- to 100-fold decrease in virulent *Pst* growth in mature six week-old plants compared to young plants at three to four weeks post germination (wpg) [30]. Eriksson et al. [34] demonstrated that flowering marker genes, including *APETALA1* (*AP1*), were expressed in the shoot apical meristem (SAM) of six week-old short day-grown *Arabidopsis* (Col-0) [34]. They concluded that short day-grown *Arabidopsis* undergo the transition from vegetative to reproductive growth at six wpg [34]. Additionally, Rusterucci et al. [32] demonstrated that plants grown under conditions that promote early flowering (long days) display ARR earlier than plants grown under conditions that promote late flowering (short days). Collectively, these experimental results suggest that the transition from vegetative to reproductive growth in *Arabidopsis* is associated with the ability to manifest ARR.

Arabidopsis SA accumulation-deficient plant lines are defective in the ARR response to *Pst*, including *NahG*, *SA induction deficient1* and *2* (*sid1*, *sid2*), *phytoalexin accumulation-deficient4* (*pad4*), *enhanced disease susceptibility1* (*eds1*), and *important for ARR pathway1-1* (*iap1-1*), indicating that SA accumulation is necessary for ARR [30,31,33]. A number of studies indicate that SA acts as a signal during basal resistance and Systemic Acquired Resistance to up-regulate expression of *PR* genes, including *PR1*, through activation of Non-expressor of PR1 (NPR1; reviewed in [35]). During ARR in *Arabidopsis*, *PR1* expression is reduced in ARR-competent leaves of mature plants compared to ARR-incompetent leaves of young plants after inoculation with *Pst* [30,32]. This observation combined with the ARR-competent nature of SA signaling-deficient *npr1* [30] suggests that SA does not play its usual signaling role during ARR [30,32]. Intercellular washing fluids (IWFs) extracted from the intercellular space of *Arabidopsis* undergoing ARR display anti-microbial activity and contain elevated levels of SA compared to IWFs collected from young plants inoculated with *Pst* [30,31]. A number of studies indicate that SA possesses anti-microbial properties *in vitro* [31,36–39], providing further evidence that SA may contribute to the anti-microbial activity observed in IWFs of mature plants undergoing ARR. Additionally, the ARR response was reduced by infiltrating the SA-hydrolyzing enzyme, salicylate hydroxylase, into the intercellular space of ARR-competent mature plants. Moreover, infiltration of SA into the intercellular space of the SA accumulation-compromised mutants *sid2*, *pad4-1*, *eds1-1*, and *iap1-1* partially rescued their ARR defects [31,33]. These various experimental approaches led to the idea that SA acts in the intercellular space, perhaps as an anti-microbial agent, during ARR in *Arabidopsis* [31,33].

Additional genes that contribute to *Arabidopsis* ARR were identified in a microarray experiment in which gene expression was compared between mature plants that were either mock-inoculated or responding to *Pst* with ARR (12 hpi) [33]. A number of JA/ET-associated genes were up-regulated, including two *NO APICAL MERISTEM CUP-SHAPED COTYLEDON* (*NAC*) genes, *ANAC055* and *ANAC092*. T-DNA insertion mutants *anac055* and *anac092* display reduced ARR to *Pst* and *H. arabidopsidis*, suggesting that ANAC055 and ANAC092 play a role in ARR [33]. Furthermore, *ANAC055* and *ANAC092* are differentially expressed in leaves of young compared to mature plants after inoculation with *Pst* [33], suggesting that these NACs play different roles in young ARR-incompetent compared to mature ARR-competent plants.

The *Arabidopsis* NAC family consists of 105 putative transcription factors with a conserved, highly charged N-terminal DNA-binding domain (NAC domain), and a variable transactivation domain at the C-terminus [40,41]. NACs are involved in many aspects of plant development and stress responses [41]. Over-expression and mutant studies suggest that ANAC055 and ANAC092 play roles in responses to drought and salt stresses [42,43]. ANAC055 is involved in defense responses to the necrotrophic fungus *Botrytis cinerea* [44], and ANAC092 is involved in leaf senescence [45,46]. *ANAC055* and *ANAC092* are induced in response to treatment with NaCl, abscisic acid (ABA), and *Pst* type III effectors [42,43,47,48]. Moreover, a number of studies indicate that ANAC055 and ANAC092 contribute to JA/ET signaling. For example, overexpression of *ANAC055* enhances expression of JA-associated genes, including *LIPOXYGENASE2* (*LOX2*) and *VEGETATIVE STORAGE PROTEIN1* (*VSP1*), in response to JA treatment [44]. In addition, *ANAC055* is expressed in response to methyl JA (MeJA) application [42,44] while *ANAC092* is expressed in response to ET treatment [49]. Furthermore, *ANAC055* and *ANAC092* are expressed in an *Ethylene Insensitive2* (*EIN2*)-dependent manner in leaves of young *Arabidopsis* plants (4 wpg in short day conditions) inoculated with *Pseudomonas* [50]. Expression of *ANAC092* also appears to be positively regulated by *EIN2* during NaCl treatment and leaf senescence [43,45]. EIN2 is a member of the Nramp transmembrane protein family and is considered to be a central component of JA/ET signaling [49].

Since ANAC055 and ANAC092 are involved in JA/ET signaling pathways in young *Arabidopsis*, this may also be true in mature *Arabidopsis* during ARR. However, mutant studies suggest that two JA signaling components, JIN1 and JAR1, are not required for ARR [33]. To further explore the relationship between ANAC055, ANAC092 and JA/ET signaling during *Arabidopsis* ARR to *Pst*, we characterized the ARR phenotypes of single and double *anac055* and *anac092* T-DNA insertion mutants, an *ANAC092*-overexpressing plant line, JA accumulation-deficient *lipoxygenase2* (*lox2*), and the JA/ET signaling-compromised *ein2-1* mutant and monitored expression of the JA/ET signaling marker gene *PLANT DEFENSIN1.2a* (*PDF1.2a*).

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana wild-type Columbia-0 (Col-0) plants were used along with *sid2* (C. Nawrath, University of Fribourg, Switzerland), *anac055* (SALK_014331), *anac092* (SALK_090154), *lox2* (CS3748), *ein2-1* (CS3071) (*Arabidopsis* Biological Resource Center, Ohio State University, USA, [49]), and 35S:*ANAC092* mutant plants (line 1, S-Y. Chen, Chinese Academy of Sciences, China [43]). Seeds were surface sterilized and germinated on Murashige and Skoog medium, and grown under continuous light (100 $\mu m^{-2}\ s^{-1}$) for five to seven days. The seedlings were then transferred to soil (Sunshine Mix No. 1, Sun Gro Horticulture, Bellevue, WA) moistened with 1 g L^{-1} 20-20-20 fertilizer and grown at 21–23 °C with a light intensity of 150–200 $\mu m^{-2}\ s^{-1}$, while maintaining humidity between 70 and 85% under short day conditions of 9 h light and 15 h of darkness.

2.2. Determination of Pst levels in planta

Leaves of *Arabidopsis* plants were inoculated with virulent *P. syringae* pv. *tomato* (*Pst*) strain DC3000 (rifampicin and kanamycin resistant) obtained from Dr. Andrew Bent (University of Wisconsin at Madison [51]). *Pst* was grown to mid-log phase in King's B media