TECHNICAL ADVANCE

Imaging-Based Resistance Assay Using Enhanced Luminescence-Tagged *Pseudomonas syringae* Reveals a Complex Epigenetic Network in Plant Defense Signaling Pathways

Dinesh S. Pujara,¹ Sung-Il Kim,¹ Ji Chul Nam,¹ José Mayorga,¹ Isabella Elmore,² Manish Kumar,¹ Hisashi Koiwa,³ and Hong-Gu Kang^{1,†}

¹ Department of Biology, Texas State University, San Marcos, TX 78666, U.S.A.

² San Marcos High School, San Marcos, TX 78666, U.S.A.

³ Department of Horticultural Sciences, Texas A&M University, TX 77843, U.S.A.

Accepted 17 May 2021.

High-throughput resistance assays in plants have a limited selection of suitable pathogens. In this study, we developed a *Pseudomonas syringae* strain chromosomally tagged with the Nanoluciferase (NL) from the deep-sea shrimp *Oplophorus gracilirostris*, a bioluminescent marker significantly brighter than the conventional firefly luciferase. Our reporter strain tagged with NL was more than 100 times brighter than *P. syringae* tagged with the *luxCDABE* operon from *Photobacterium luminescens*, one of the existing luciferase-based strains. In planta imaging was improved by using the surfactant Silwet L-77, particularly at a lower reporter concentration. Using this imaging system, more than 30 epigenetic mutants were analyzed for their resistance traits because the defense signaling pathway is known to be epigenetically regulated. SWC1, a defense-related chromatin remodeling complex, was found to be a positive defense regulator, which supported one of two earlier conflicting reports. Compromises in DNA methylation in the CG context led to enhanced resistance against virulent *Pseudomonas syringae* pv. *tomato*. Dicer-like and Argonaute proteins, important in the biogenesis and exerting the effector function of small RNAs, respectively, showed modest but distinct requirements for effector-triggered immunity and basal resistance to *P. syringae* pv. *tomato*. In addition, the transcriptional expression of an epigenetic component was found to be a significant predictor of its immunity contribution. In summary, this study showcased how a high-throughput resistance assay enabled by a pathogen strain

with an improved luminescent reporter could provide insightful knowledge about complex defense signaling pathways.

Keywords: *Arabidopsis*, defense epigenetics, imaging-based assay, phenomics, plant responses to pathogens, *Pseudomonas syringae*

The scale and scope of genomic characterization have been significantly expanded, thanks to technical advances and cost depreciation in next-generation sequencing (Koboldt et al. 2013). This advancement has facilitated extensive genomic characterization of a wide range of crop plants, leading to a wealth of genomics knowledge and its application in advanced breeding (Harfouche et al. 2019). However, profiling the phenome associated with genetic variations (e.g., visual symptoms, pathogen growth, and weight or height alteration in response to infection) remains a significant challenge, despite substantial improvements in imaging, sensor, and data analysis (Tardieu et al. 2017).

Several high-throughput quantitative assays for resistance traits in plants have been developed. Most of these assays rely on indirect changes such as visual and molecular characteristics in plants responding to infection (Chakravarthy et al. 2009; Laflamme et al. 2016; Lloyd et al. 2014; Nguyen et al. 2010). For instance, disease progression was estimated by measuring leaf yellowing (Laflamme et al. 2016) or cell-death-triggered defense responses (Chakravarthy et al. 2009) by *Pseudomonas syringae*. However, these approaches require starting inoculums well over a conventional dose or using an indirect measurement of resistance in plants, making them inherently less practical and accurate than conventional resistance assays, which directly and manually quantify the growth of pathogens from infected tissues.

Bioluminescence is a chemiluminescent reaction that takes place in living cells. The main strength of bioluminescence as a bioreporter is its high sensitivity, because luminescence is measured in the dark. Among the bioluminescent reporters, beetle luciferases are most extensively used in high-throughput assays (Roda et al. 2016). For instance, a luciferase from a firefly produces luciferin, a light-emitting chemical, in an ATP-dependent fashion. The requirement of ATP by luciferase is often exploited for ATP quantitation (Chappelle and Levin 1968). The *Photobacterium luminescens luxCDABE* operon was engineered to be self-luminescent without ATP and an external substrate (Winson et al.

Current address for J. C. Nam: Department of Molecular Biosciences, University of Texas, Austin, TX 78712, U.S.A.

†Corresponding author; H.-G. Kang; kang@txstate.edu

Funding: Support was provided by the National Science Foundation (grant IOS-1553613).

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary figures and supplementary tables are published online.

The author(s) declare no conflict of interest.

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1998). A *Pseudomonas syringae* pv. *tomato* DC3000 strain that chromosomally tagged the *luxCDABE* operon (hereafter *Pst_Lux*) was successfully used to investigate natural variation in *Arabidopsis* ecotypes (Fan et al. 2008), presenting an exciting opportunity for plant pathologists to run large-scale assessments of resistance traits.

Several newly improved luminescent proteins have meanwhile been developed as reporters (Roda et al. 2016). In particular, the Nanoluc luciferase (NL), cloned from the deep sea shrimp *Oplophorus gracilirostris*, is one of the smallest (19 kDa) and brightest luminescent proteins to date (approximately 150-fold brighter than the firefly luciferase) that catalyzes furimazine, a coelenterazine derivative with low background autoluminescence (Hall et al. 2012). Moreover, NL, with the emission wavelength of 460 nm, is highly stable and belongs to a glow-type luminescence with a signal half-life of 2 h or longer without requiring ATP, making it one of the most promising luminescent reporters.

Plant resistance comprises distinct layers of defense components (Cui et al. 2015; Jones and Dangl 2006; Peng et al. 2018). The first line of defense starts with the perception of pathogen-associated molecular patterns (PAMPs) by the innate immunity, which induces PAMP-triggered immunity (PTI). Pathogens counter plant immune responses, including PTI, by delivering their effector proteins to host plants to promote disease development; these phenomena are collectively known as effector-triggered susceptibility (ETS). Under this condition, susceptible plants display mostly basal resistance. Plant resistance (R) proteins recognize these effectors and oppose ETS with effector-triggered immunity (ETI) mechanisms. Interestingly, the mechanistic difference between PTI and ETI is primarily quantitative rather than qualitative, suggesting that both immunities essentially involve common signaling components (Tao et al. 2003; Tsuda et al. 2009), and that an R protein, upon recognizing a corresponding effector, functions to expedite and amplify defense signaling.

Transcriptional reprogramming in plant defense signaling requires a diverse group of molecules in the nucleus, including transcription factors, small RNAs (sRNAs), histone/DNA modifiers, and chromatin remodeling proteins (Huang et al. 2016; Lämke and Bäurle 2017; Ramirez-Prado et al. 2018). For instance, RNA-directed DNA-methylation (RdDM) is a well-characterized silencing mechanism with direct consequences on chromatin by methylating target DNAs. The canonical RdDM in *Arabidopsis* uses 24-nucleotide (nt)-long sRNAs, which are mainly generated by Dicer-like 3 (DCL3) and loaded to Argonaute 4 (AGO4), to direct methylation. DNA methylation under biotic stress is dynamic, and its misregulation leads to changes in resistance (Dowen et al. 2012; Yu et al. 2013), highlighting the importance of changes in chromatin. The SWR1 chromatin remodeling complex, which substitutes canonical H2A histones with the histone variant H2A.Z, is also a defense regulator with an impact on chromatin topology. A loss of function mutation of the SWR1 subunits alter resistance and expression of defense genes (Berriri et al. 2016; March-Díaz et al. 2008). However, it is debatable whether the SWR1 complex is a positive or negative regulator because the two aforementioned studies reported opposite outcomes.

We reasoned that a high-throughput platform is critical to characterize the plant defense signaling pathway on a systemic level because the labor-intensive conventional resistance assay can only examine a limited number of plants. Thus, a new luminescent *P. syringae* pv. *tomato* carrying *nanoluc* (hereafter *Pst_NL*) was developed to enhance the throughput and sensitivity of the resistance assay. This new strain, in comparison with *Pst_Lux* (Fan et al. 2008), was more than 100 times brighter. In addition, Silwet L-77 enhanced in planta imaging analysis through consistent substrate administration for NL. Combined with a do-it-yourself automation table developed earlier (Makhija et al. 2020), our high-throughput analysis demonstrated that epigenetic components play distinct

roles in ETI and basal resistance in *Arabidopsis* and that stomata-mediated bacterial resistance is likely age sensitive.

RESULTS

Generation of *P. syringae* tagged with NL.

P. syringae pv. *tomato* was tagged with the NL gene to generate a brighter luminescence reporter strain for high-throughput resistance analysis. NL1.1 carries an original clone (Hall et al. 2012) and NL1.2, among several NL variants, contains the PEST domain, which triggers autodegradation (Rogers et al. 1986). Thus, NL1.2 has a shorter half-life with less protein accumulation in cells over time, which may provide more uniform luminescence across young and old cells than the stable NL1.1. These NL genes under the constitutive *ProD* promoter (Davis et al. 2011) were inserted into the attTn7 site of the *P. syringae* pv. *tomato* chromosome via a Tn7-based bacterial cloning system (Choi and Schweizer 2006; Choi et al. 2005b), resulting in *Pst_NLI.1* and *Pst_NLI.2*. Chromosomal integration of the reporter genes was used to maintain a stable copy number of the reporter instead of episomal tagging; a single copy insertion of the NL genes was verified via PCR and genome sequencing (Supplementary Fig. S1). Viability and infectivity of these reporter strains in *Arabidopsis* were compared with the parental strains by measuring the growth of *Pst_NLI.1* and *Pst_NLI.2* at 3 days postinfection (dpi). The growth assay in Figure 1 revealed a growth pattern comparable with the parental strains except for *Pst_NLI.1*, which showed a slight reduction, consistent with an earlier report that insertion at the attTn7 site had no adverse phenotypes (Chakravarthy et al. 2018). Note that, in addition to *P. syringae* pv. *tomato*, an avirulent *P. syringae* pv. *tomato* strain carrying *AvrRpt2* (*Pst_AvrRpt2*) was generated, which displayed significant growth retardation in the *Arabidopsis* Columbia (Col-0) ecotype (Fig. 1) due to its cognate *RPS2* R gene.

Pst_NL was significantly brighter than its conventional counterpart, *Pst_Lux*.

Pst_Lux (Fan et al. 2008) has been used in more than 30 studies for quantitating pathogens since its development (Supplementary Table S1), by using either a luminometer or a camera. To deduce the sensitivity of *Pst_Lux*, the initial inoculum of *Pst_Lux* in these studies was analyzed. Interestingly, most of the initial inoculums were significantly higher than what is considered an optimal starting dose of approximately 10^5 CFU/ml (Clarke et al. 2000; Shah et al. 1997) (Fig. 2; Supplementary Table S1). Furthermore, image-based detection using a camera used an even higher initial

Fig. 1. Nanoluc luciferase (NL)-tagged *Pseudomonas syringae* reporter strains were as virulent to *Arabidopsis* as their parental strains. Wild-type *Arabidopsis* Columbia ecotype plants (3.5 weeks old) were syringe infiltrated with the indicated *P. syringae* pv. *tomato* (*Pst*) strains at 10^5 CFU/ml. Bacterial growth was measured by a conventional leaf disc assay (Kang et al. 2008) at 0 and 3 days postinfection (dpi); the mean $\pm$ standard error ($n = 20$) is presented; an asterisk (*) indicates $P < 0.05$ (t test). Another independent experiment was performed with a similar result.

inoculum than detection by a luminometer, suggesting that *Pst_Lux* is too dim to be used at a conventional inoculum.

We examined in vitro whether the luminance of *Pst_NLI.1* and *Pst_NLI.2* was enhanced relative to *Pst_Lux*, and found that the newly engineered strains displayed significant improvements in brightness by a magnitude of approximately two to three orders over several different concentrations (Fig. 3). With this significant improvement, we tested whether a camera-based quantitation method could benefit from these new reporter strains. To this end, *Arabidopsis* plants were infected with *Pst_NLI.1* with the starting inoculum at 10^5 CFU/ml and imaged with an electron-multiplying, charge-coupled device (EMCCD) camera. The *Pst_NLI.1* strain was readily detectable at 1 dpi (Fig. 4A, luminescence image). These infected plants were then reassessed with a conventional leaf disc-based manual quantitation. These parallel experiments revealed comparable outcomes (Fig. 4B), suggesting that this enhanced reporter strain provided sufficient luminance for an image-based quantitation. In contrast, *Pst_Lux*, when imaged with *Pst_NLI.2* side by side, displayed barely detectable luminescence even at 2 dpi (Supplementary Fig. S2).

NL utilizes coelenterazine or its derivative for its substrates (Hall et al. 2012). Furimazine, a coelenterazine derivative, provides approximately 30-fold higher luminance than coelenterazine (Hall et al. 2012). Furthermore, furimazine is more stable in cell culture media and less autoluminescent than coelenterazine. Thus, we examined whether these substrates would show comparable outcomes for *Pst_NL*. Furimazine gave off significantly more luminescence than coelenterazine-h (Supplementary Fig. S3). In particular, coelenterazine-h failed to produce notable luminance when *Pst_NLI.1* at 10^5 CFU/ml was syringe infiltrated. These observations indicate that, consistent with the earlier report (Hall et al. 2012), furimazine is the superior substrate compared with coelenterazine for NL-based detection.

Fig. 2. Initial inoculum of the *luxCDABE*-tagged *Pseudomonas syringae* pv. *tomato* strains was significantly higher than a conventional dose in both imaging- and luminometer-based methods. In total, 33 articles citing Fan et al. (2008) utilized the *luxCDABE*-based reporter in either luminometer- or imaging-based detection for pathogen quantitation. The initial inoculum of these studies in $\log_{10}$ (CFU/ml) was presented as a conventional boxplot, with a diamond and a bar representing a mean and a median, respectively.

Silwet L-77 helped consistent application of *Pst_NL*.

A substrate application is a potential variable in pathogen quantification because NL requires an external substrate. Surfactant, known as a wetting agent, often enhances the biological performance of agrochemicals via increasing the foliar uptake and enhancing even spread (Castro et al. 2014). Thus, we examined five commonly used surfactants for their ability to enhance a uniform substrate application. To this end, the wetting property (i.e., water spread on leaves) was first examined at different concentrations. Among the tested detergents, Silwet L-77 spread water several-fold more than the rest, with 1% being the most effective concentration (Supplementary Fig. S4). Therefore, we examined whether Silwet L-77 at 1% could enhance consistency in the image-based assay. Although bioluminescence showed high correlation with bacterial counts ($r^2 = 0.886$) (Fig. 4B), the correlation was further improved with addition of Silwet L-77 ($r^2 = 0.934$) (Fig. 4C). In particular, Silwet L-77 improved luminescence notably at the lower dose of *Pst_NLI.1* (compare the relative luminance unit at 1 dpi in the absence and presence of Silwet L-77 in Fig. 4B and C, respectively). Note that Silwet L-77 was also effective with *Pst_NLI.2* (Supplementary Fig. S5) and had little growth impact on *P. syringae* pv. *tomato* (Supplementary Fig. S6). However, we observed chlorosis from *Arabidopsis* by Silwet L-77 within 2 h after application. Thus, if plants are subject to another experiment, running the resistance assay without Silwet L-77 or washing it off soon after imaging should be considered.

Spraying inoculation revealed different resistance levels between young and old leaves against *P. syringae* pv. *tomato*.

In the *Arabidopsis*–*Pseudomonas* pathosystem, the main methods of pathogen delivery are syringe injection, spraying or dipping inoculation, and vacuum infiltration (Katagiri et al. 2002). Spray

Fig. 3. *Pseudomonas syringae* pv. *tomato* carrying *nanoluc* (*Pst_NL*) reporter strains displayed significantly brighter bioluminescence than their conventional counterpart, *luxCDABE*-tagged *P. syringae* pv. *tomato* (*Pst_Lux*). Luminance emitted by 50 μ l of bacterial cultures at 1 through 10^5 CFU/ μ l in vitro was measured by using a luminometer. *Pst_Lux* was tested with and without furimazine, a substrate for Nanoluc luciferase (NL). The mean $\pm$ standard error ($n = 2$) was presented. RLU = relative luminance unit. Another independent experiment was performed with a similar result. All strains except those indicated had furimazine applied.

inoculation, in particular, has appeal for high-throughput applications, because it does not involve a tedious inoculation process. Thus, we investigated whether *Pst_NL* is also compatible with spray inoculation. Interestingly, the imaging assay showed significant variation in resistance levels between young and old leaves within the same plants against virulent and avirulent *P. syringae* pv. *tomato* (Fig. 5A and B). At 1 and 3 dpi, both virulent and avirulent *Pst_NL1.2* grew significantly better in old leaves than

in young ones (Fig. 5C and D). Both conventional and imaging methods showed comparable outcomes (Fig. 5E and F), raising the possibility of development-dependent bacterial resistance when *P. syringae* pv. *tomato* spray infected *Arabidopsis*. These observations suggest that our NL-based imaging method is also compatible with the spraying method, and its visual capacity to inspect an individual sample likely provides previously overlooked insight.

Fig. 4. Imaging-based resistance assay using *Pseudomonas syringae* pv. *tomato* carrying *nanoluc* (*Pst_NL*) produced an outcome correlated with a conventional leaf-discs-based resistance assay. Leaves from 3.5-week-old wild-type (WT) *Arabidopsis* plants were syringe-infiltrated with the *Pst_NL1.1* strain. Progress in infection was analyzed by both imaging-based and conventional resistance assay at 1, 2, and 3 days postinfection (dpi). **A**, Infected plants were imaged with (normal light) and without (luminescence) dim light. Images for luminescence are presented in pseudocolor. Bars = 3 cm. **B** and **C**, Quantitated values from imaging-based and conventional resistance assays were plotted in a scattered plot. The x-axis represents RLU (relative luminance unit) for the image-based assay, while the y-axis shows CFU/cm² for the conventional leaf-disc-based quantitation. The comparison was made without (**B**) and with (**C**) Silwet L-77. WT plants were inoculated with *Pst_NL1.1* at 10⁵ CFU/ml and incubated for the indicated time. Each dot represents the mean of three replicates. A broken line shows a linear regression line.

Fig. 5. Spray inoculation of *Pseudomonas syringae* pv. *tomato* carrying *nanoluc* (*Pst_NL*) revealed development-dependent resistance in *Arabidopsis*. Leaves from 3.5-week-old wild-type plants were spray inoculated with *Pst_NL1.2* at 2×10^7 CFU/ml. Representative images of plants **A**, infected with *Pst_NL1.2* and **B**, *Pst_NL1.2_AvrRpt2* at 1 day postinfection (dpi) are shown with (left) and without (right) dim light. Old and young leaves were marked with blue and red arrows, respectively. Luminescence is presented in pseudocolor; bar = 3 cm. Bacterial growth was measured by both **C**, conventional and **D**, imaging-based assay at 0, 1, and 3 dpi. For each time point, old and young leaves from four plants were analyzed separately. The mean $\pm$ standard error ($n = 8$) is presented; statistical difference between old and young leaves at the same day postinfection is indicated; an asterisk (*) indicates $P < 0.05$ (t test). Quantitated values from imaging-based and conventional resistance assays with **E**, *Pst_NL1.2* and **F**, *Pst_NL1.2_AvrRpt2* were plotted in a scattered plot. The x-axis and y-axis represent quantitation by the image-based and conventional resistance assay, respectively. RLU = relative luminance unit. Four plants with two older and younger leaves were assessed for each day postinfection. A broken line shows a linear regression line. Another independent experiment was performed with a similar result.

Roles of epigenetic components in ETI and basal resistance against *P. syringae* pv. *tomato*.

Plant defense signaling involves a wide range of epigenetic components (Ramirez-Prado et al. 2018). To gain insight into the role of these components in plant resistance, the imaging-based resistance analysis was performed on 32 epigenetic mutants, together with 2 defense-related mutants: *npr1* (Cao et al. 1997) and *rps2* (Bent et al. 1994). All 4 *dcl* and 10 *ago* genes essential in the biogenesis and action of sRNAs in *Arabidopsis* (Vaucheret 2008), four DNA methyltransferases (MET1, CMT3, DRM1, and DRM2) for de novo or maintenance DNA methylation (Zhang et al. 2018), and four SWR1 chromatin remodeling components (March-Díaz and Reyes 2009) (ARP6, SWC2, SWC5, SWC6, and PIE1) were tested. In addition, histone modifiers (SUVH2/9) (Kuhlmann and Mette 2012) and a few additional players in RdDM (RDR2, NRPD, NRPE, and MORC1/2/6) (Matzke et al. 2015) were also included.

Among the AGO and DCL mutants, *ago1*, *ago2*, *dcl1*, and *dcl3* displayed compromised ETI, whereas *ago3*, *ago4*, *ago7*, and two other *dcl* mutants showed compromised resistance to both virulent and avirulent *P. syringae* pv. *tomato* (Fig. 6A), suggesting that these sRNA-associated components play an important role in plant resistance. MET1 maintains CG DNA methylation, and its mutation led to enhanced resistance to virulent *P. syringae* pv. *tomato* with little change to the avirulent counterpart. From the SWC1 complex, mutations of four components—ARP6, SWC2, SWC5, and SWC6—showed susceptibility to avirulent *P. syringae* pv. *tomato*, while *swc5* was the only one compromised in resistance to virulent *P. syringae* pv. *tomato*. These changes in bacterial resistance in the SWC1 complex mutants confirm the role of the histone variant H2A.Z in plant resistance found in two earlier reports (Berriri et al. 2016; March-Díaz et al. 2008). Although these reports found the opposite outcomes from comparable mutants, our outcomes are largely consistent with those reported by Berriri et al. (2016).

Several mutants, including *ago5*, *ago6*, *ago8*, and *ago9*, showed very little difference in resistance relative to the wild type. Although these epigenetic components may be involved in biological roles other than resistance, it is feasible that the lack of resistant phenotypes can be caused by little or no RNA expression of corresponding genes. There have been a few transcriptome studies investigating the induction of defense genes in *Arabidopsis* reacting to *P. syringae* pv. *tomato* (Howard et al. 2013; Lewis et al. 2015; Mine et al. 2018). Mine et al. (2018) provided the most detailed transcriptome dynamics using a set of *P. syringae* pv. *tomato* strains, including those used in our study, enabling us to assess whether the epigenetic components tested are expressed in response to pathogen infection. *AGO5*, *AGO6*, *AGO8*, *AGO9*, and *DRM1* showed little or no detectable expression over the 24-h period in response to virulent as well as avirulent *P. syringae* pv. *tomato* infection (Fig. 6B and 6C). Therefore, these RNA data may explain their insignificant changes in resistance in the corresponding mutants. Although only a higher-order mutant, *drm1/2*, for *DRM1* was analyzed, given that *DRM1* displayed little RNA expression, it is feasible that *DRM2* contributes to resistance with little involvement of *DRM1*. Together, these observations suggest that checking RNA transcription to predict phenotypic relevance is a good practice in the era of massively available genome and transcriptome data.

DISCUSSION

We engineered the brightest luminescent *P. syringae* strain (*Pst_NL*) to date, via a recently developed luminescent protein, NL. This new strain was 100 to 1,000 times brighter than a conventional Lux operon strain (*Pst_Lux*) (Fan et al. 2008). This enhanced luminance allowed a high-throughput imaging-based

resistance assay to run in conjunction with an automated table synchronized with a camera. Using this system, more than 30 epigenetic mutants were examined for the resistance trait against virulent and avirulent *P. syringae* pv. *tomato*, revealing differential involvement of these epigenetic players in ETI and basal resistance to *P. syringae* pv. *tomato* in *Arabidopsis*. The age of leaves was also found to play a role in bacterial resistance when *Arabidopsis* was spray inoculated.

Pathogen quantitation has involved multiple labor-intensive steps in which sampling, homogenization, extraction, titration, and plating would take several hands-on hours for a handful of plants. Therefore, a pathogen quantitation assay, while arguably the most direct method for analyzing resistance traits, has mostly been limited to small-scale tests. To overcome this limitation, we engineered a new reporter strain compatible with imaging-based quantitation and imaged more than a hundred *Arabidopsis* plants in a few minutes. This powerful tool, if used widely in the community, will likely expedite the progress in characterizing complex resistance signaling pathways in plants on a systemic scale.

Having a brighter reporter for pathogen quantitation also offers a few practical advantages. First, the time to scan infected plants is substantially shortened. To take full advantage of this speed, we built an automated table synchronized with the EMCCD camera so that a conventional flat carrying a large number of plants is automatically imaged without manual interruption (Makhija et al. 2020). These developments allowed the imaging of approximately 50 fully grown *Arabidopsis* plants on one flat in less than 2 min, allowing for resistance trait analysis on a much larger scale. Second, the imaging by an EMCCD camera can be operated at a non-maximum setting. We have used 3 s or shorter exposure at 10% of the maximum sensitivity of the EMCCD sensor. The EMCCD-based detection at the maximum or near-maximum setting is known to trigger gain aging (Dunford et al. 2018), in which the sensor loses its sensitivity over a short time. Thus, the brighter NL reporter strain provides a long-term benefit of protecting the camera, a vital tool in imaging analysis, from losing sensitivity. This stability is especially essential for a large-scale experiment that is carried out over multiple days, if not months.

NL, while much brighter than the Lux operon counterpart, requires the addition of a substrate. This additional substrate can become another variable for the reporter assay because a luminescence reaction changes its kinetics over time. Luminescent reactions are generally classified as either a flash or a glow type, depending on their emission decay kinetics. For a luminescent protein with fast decay kinetics, a uniform measurement can be challenging to obtain for a large number of plants. NL, however, maintains comparable luminance over 2 h (Hall et al. 2012). We also found that the luminance from NL was steady for more than 2 h after the substrate application (data not shown). Thus, our engineered strain likely performs steadily for an extended time after the substrate application without losing notable luminance. This steadiness will likely be an important characteristic when this system is used on an even bigger scale than this report.

Although age-related resistance has been investigated (Hu and Yang 2019; Kus et al. 2002; Wilson et al. 2017) for almost two decades, revealing that plants develop more resistance as they age, resistance in leaves at different stages of maturation in the same plant lacks exploration. This knowledge gap is partly due to limitations in traditional resistance analysis, in which leaves were pooled from an individual plant to reduce experimental variation. Therefore, imaging-based resistance assays have an advantage in which visual inspection can be performed on an individual leaf basis. Spray inoculation that showed significant variation between young and old leaves (Fig. 5) has been utilized to assess resistance mediated by stomata (Melotto et al. 2006). We speculated that more rapid and robust stomata closure in response to pathogens in young leaves might lead to differential resistance

Fig. 6. Resistance and RNA expression analysis of select epigenetic mutants. Mutants were highlighted in different colors based on annotated or known biological functions. Mutants *rps2* and *npr1* were used as control. **A**, Wild-type (WT) and indicated mutant plants were subject to imaging-based resistance analysis using avirulent and virulent *Pseudomonas syringae* pv. *tomato* carrying *nanoluc* (*Pst_NL*) *Pst_NL1.2*. Leaves of 3.5-week-old plants were syringe infiltrated with virulent (lighter color) and avirulent (darker color) *Pst_NL1.2* at 10⁵ CFU/ml, and the bacterial population was quantified 2 days postinfection (dpi). AGO = Argonaute and DCL = Dicer-like. The means $\pm$ standard error (SE) ($n = 6$) are presented. Another independent experiment was performed with similar results. Statistical difference from WT is indicated; asterisks * and ** indicate $P < 0.05$ and 0.01 , respectively (t -test). **B** and **C**, RNA expression analysis of indicated genes from the publicly available dataset (Mine et al. 2018). WT plants were challenged with avirulent or virulent *P. syringae* pv. *tomato* or mock control. The means $\pm$ SE ($n = 3$) are presented. **B**, Total sum in log₂ of normalized reads of indicated genes in the x-axis across all the time points, from 1 to 24 h postinfection, are presented in the y-axis. Avirulent, virulent, and mock treatment are represented in darker, intermediate, and lighter colors, respectively. **C**, RNA expression over the indicated time points as a line graph was presented. Avirulent, virulent, and mock treatments are presented as blue, red, and black lines, respectively. The mean value of normalized reads in log₂ of indicated genes and the corresponding time points are presented in the y-axis and x-axis, respectively.

in the same plants compared with the old counterparts. Consistent with this view, stomatal closure was shown to be more robust in young leaves in wheat plants in response to abiotic stress (Chen et al. 2013).

Transcriptional reprogramming under biotic stress requires a wide range of epigenetic changes on chromatin (Ding and Wang 2015). Mutant *met1* displayed enhanced resistance to virulent *P. syringae* pv. *tomato*, of which the growth matched that of avirulent *P. syringae* pv. *tomato* (Fig. 6), raising an intriguing possibility that dynamic CG methylation is important in the robust defense responses such as ETI. A genome-wide methylome study found that hypomethylation occurs significantly more in the CG context and within 1 kb 5' upstream of protein-coding sequences (Downen et al. 2012). ROS1-dependent active removal of DNA methylation is part of an induced defense response (Yu et al. 2013), supporting DNA methylation's active role. Defense genes induced as early as 1 or 2 h postinfection include chromatin remodeling factors (Lewis et al. 2015). Thus, active chromatin demethylation is likely one of the drivers leading to defense-associated transcriptional reprogramming, while the absence of the methylation leads to constitutive elevation of defense genes.

Mutant *drm1/2* showed compromised ETI (Fig. 6A), suggesting the role of CHG and CHH methylation (H = A, T, C) in this type of resistance. Because AGO4 is involved in bacterial resistance and RdDM (Agorio and Vera 2007), DRM1 and DRM2, as the main RdDM enzymes, seem to be obvious players in plant defense signaling. It is noteworthy, however, that *DRM1* showed no RNA expression (Fig. 6B and C). *DRM1* and *DRM2* are tightly linked genes, and have often been studied together (Cao and Jacobsen 2002), although no function has been assigned to *DRM1* to date (Johnson et al. 2008). Thus, the absence of RNA expression in this study may suggest that *DRM1* is a pseudogene.

Several sRNAs are also involved in transcriptional reprogramming. miRNA393* and miRNA393 are a passenger and a guide strand, respectively, from the same *MIR* gene functioning in defense signaling (Si-Ammour et al. 2011; Zhang et al. 2011). miRNA393*, associated with AGO2, targets a Golgi-localized SNARE gene, *MEMB12*, which increases the exocytosis of the pathogenesis-related 1 protein (Zhang et al. 2011). Consistently, *ago2*, *ago3*, *ago7*, and their combined mutant displayed compromised ETI (Fig. 6), consistent with an earlier report (Zhang et al. 2011); note that AGO2, AGO3, and AGO7 belong to one of three AGO phylogenetic clades in *Arabidopsis* (Vaucheret 2008). *AGO2* was expressed most robustly and rapidly in response to avirulent *P. syringae* pv. *tomato* (Fig. 6C), suggesting that AGO2 is the most active player in ETI among *Arabidopsis* AGOs. Interestingly, our ongoing study identified the association of AGO2 with ETI-specific transfer RNA (tRNA)-derived sRNAs, which alone can trigger defense responses (unpublished), supporting this notion. miRNA393, associated with AGO1, on the other hand, is a PTI regulator that suppresses auxin signaling by inhibiting an auxin receptor. Given that auxin is antagonistic to defense signaling, it is surprising that *ago1* showed compromised ETI but stable basal resistance (Fig. 6). It is possible that virulent *P. syringae* pv. *tomato* sufficiently suppresses PTI by a myriad of its effectors and, therefore, loss of miR393 in *ago1* did not result in additional measurable susceptibility in basal resistance. Also, the susceptible ETI phenotype in *ago1* suggests the existence of unidentified AGO1-associated sRNAs that function in ETI. Earlier sRNA studies tended to remove sRNAs that originated from tRNAs and ribosomal RNAs (Zhang et al. 2011) and limited the size range (Li et al. 2010), likely due to the convenience of the bioinformatic analysis. This practice could miss additional players in defense responses. Indeed, diverse sRNAs ranging from 10 to 17 nt in size were found inside extracellular vesicles (Baldrich et al. 2019). In addition, we also found that tRNA-derived sRNAs are predominantly 16 and 31 nt in size, which would have been

missed by the practice in the earlier studies (unpublished). Thus, our observations with AGOs and DCLs suggest many missing components in defense signaling pathways waiting to be discovered.

The SWR1 chromatin remodeling complex, which substitutes canonical H2A histones by the histone variant H2A.Z, is known as a defense regulator. Two reports showed that mutations of SWR1 complex subunits led to altered resistance (Berriri et al. 2016; March-Diaz et al. 2008). However, these reports showed opposite outcomes in the resistance phenotype from the comparable SWR1 complex mutants: the earlier one found mutants of the SWR1 subunits mostly resistant, whereas the latter one showed them mostly susceptible. We found mutants associated with the SWR1 complex largely susceptible to both virulent and avirulent *P. syringae* pv. *tomato*, in agreement with Berriri et al. (2016). Thus, we concluded that H2A.Z is likely a positive regulator for defense responses, and the substitution of the histone variant H2A.Z is important in inducing defense genes.

In this study, we have engineered a pathogen with a much brighter luminescent reporter. In conjunction with an automated table developed earlier (Makhija et al. 2020), we performed a high-throughput resistance analysis on many epigenetic mutants under the same conditions, gaining insight into a complex defense signaling network. We are currently further streamlining this high-throughput analysis by developing an AI algorithm because we found that the remaining obstacle in achieving an even grander scale was the image analysis performed manually. Once completed, we anticipate that our high-throughput phenome pipeline will be able to provide critical information on how the plant defense signaling is orchestrated by a myriad of players on a systemic scale because the phenome data will integrate many other genomic-scale datasets together.

MATERIALS AND METHODS

Generating NL reporter constructs and strains.

The *NL1.1* or *NL1.2* genes were PCR-amplified from plasmids pNL1.1 and pNL1.2 (Promega Corp.). To confer a highly constitutive and stable expression, proD, a synthetic promoter from pBT1-proD-mCherry (Davis et al. 2011), and a T7 terminator sequence from pET28 were fused with the *NL* gene, which was transferred to miniTn7-based suicide plasmid pCPP6351. The resulting plasmids (pCPP-NL1.1 and -1.2) were transferred to *Escherichia coli* *RHO3* strain by electroporation and subsequently delivered to the *P. syringae* pv. *tomato* genome by triparental mating between *E. coli* *RHO3*/pCPP6351 (donor plasmid), *E. coli* *RHO3*/pTNS2 (helper plasmid), and their recipient strain *P. syringae* pv. *tomato* DC3000 with and without *AvrRpt2*. Electroporation and triparental mating were performed as described (Choi et al. 2005b). A list of primers used in the cloning process is shown in Supplementary Table S2.

Whole-genome sequencing and its analysis.

The whole-genome sequencing of *Pst NL* was carried out on a BGISEQ-500 platform (insert size, 200 to 400 bp; read length, paired-end 150 bp) at BGI, Shenzhen, China. After removing adapter sequences, reads containing the Tn7 sequence were aligned using STAR to identify the insertion location.

Arabidopsis mutant lines.

All *Arabidopsis* genetic stocks used in this study are in the Col-0 background. The following mutant lines were obtained from the *Arabidopsis* Stock Center: *ago2-1* (SALK_003380), *ago3-2* (SALK_005335), *ago5-1* (SALK_063806), *ago6-3* (SALK_106607), *ago8-1* (SALK_139894), *ago9-2* (SALK_112059), *ago10-2* (SALK_047336), *dcl2-1* (SALK_064627), *dcl3-1* (SALK_005512), *swc2* (SALK_091595), *swc5* (SALK_201865),

swc6 (SAIL_1142_C03), *piel* (SALK_013922), *cmt3-11t* (SALK_148381), *drm1-2* (SALK_031705), *drm2-2* (SALK_150863), *met1-7* (SALK_076522), *drd1* (SALK_132061), *npr1-1* (SALK_128428), *npr1-11* (SALK_029919), *rdr2-1* (SAIL_1277_H08), and *dcl4-2t* (GABI_160G05). The following mutant lines are as previously described: *ago4-2* (Agorio and Vera 2007), *arp6* (*suf3-1*) (Choi et al. 2005a), *morc1/2/6* (Kang et al. 2012), *svh2/9-1* (Kuhlmann and Mette 2012), *npr1-1* (Cao et al. 1994), *ago7* (*zip-1*) (Hunter et al. 2003), *rps2-201* (Kunkel et al. 1993), *ago1-27* (Morel et al. 2002), *dcl1-7* (Schauer et al. 2002), and *ago2/3/7* (Zhang et al. 2011).

Plant growth, bacterial inoculation or infection, and conventional resistance assay.

Arabidopsis plants were grown in soil at 22°C and 60% relative humidity, with a 16-h light period. Plants (3.5 weeks old) were infiltrated using a needleless syringe or sprayed with an indicated inoculum of *P. syringae* pv. *tomato* and grown for 2 days at 28°C in King's B medium with appropriate antibiotics. Inoculated leaves were harvested at the given time points for a conventional resistance assay and used for bacterial titer determination as described (Kang et al. 2008).

Luminometer-based quantitation of NL.

Bioluminescence from bacterial culture was measured in a Synergy H1 Hybrid Multi-Mode Reader (BioTek) at 460 nm. Bacterial cultures and NanoGlo master reagent (Promega Corp.) containing Furimazine were equally mixed (vol/vol); the NanoGlo master reagent was prepared by mixing the NanoGlo substrate and its buffer with a 1:50 ratio, which was then mixed 1:1 with phosphate-buffered saline with Tween (vol/vol).

Camera-based image quantitation of NL in planta.

ImageMx2, an EMCCD camera (Hamamatsu), was used to capture luminescence. The camera was cooled to -80°C and the image acquisition was made by using HCImageLive software (Hamamatsu) at the following parameters: binning at 4, sensitivity gain at 297, photon imaging mode at 1, and exposure time at 3 s. An automated mechanical table synchronized with the EMCCD camera (Makhija et al. 2020) was used to automate image acquisition. Lighted images of plants were captured without EM under a minimum light. Plants were dark acclimated for 30 min to reduce background luminescence, sprayed with the NanoGlo master reagent by using a spray (Preval), and imaged. In each image assay, standard curves generated from performing both conventional leaf-disc and imaging assays on *Arabidopsis* infected with *Pst_NL* for 1, 2, and 3 days were used to infer the bacterial population from luminescence images.

Testing surfactants for consistent substrate application and their potential cytotoxicity.

Triton X-100, Tween 20, Tween 80, sodium dodecyl sulfate, and Silwet L-77 were tested as spray surfactants for *Arabidopsis* leaves. Surfactant solution (3 µl) was spotted (two replicates per concentration) on the leaves, and the spreading area at 0 and 3 min was measured.

For the cytotoxicity assay, a surfactant was added to a liquid culture of *P. syringae* pv. *tomato* grown to an optical density at 600 nm of 0.2 and incubated for 1 h at room temperature. Culture (20 µl of 10⁵-fold diluted) was plated in Luria-Bertani medium, incubated at 28°C for 2 days, and counted. Bacterial culture with no surfactant was used as a control.

Luminescence quantitation.

Luminescence from images was quantitated by using ImageJ (Schneider et al. 2012). The mean gray value of luminescence was measured by selecting the infected area, which was used

to calculate actual luminescence by subtracting background luminescence.

Transcriptome analysis.

Multitime point RNA-sequencing (RNA-seq) data (Mine et al. 2018) were processed as outlined by Pertea et al. (2016). RNA-seq reads were aligned to the *Arabidopsis* genome (Araport11) using HISAT2 (Kim et al. 2019) with default parameters. Alignment results were fed to StringTie2 (Kovaka et al. 2019) to estimate the abundance of annotated transcripts. The read coverage tables produced by StringTie2 were fed to Ballgown (Frazee et al. 2015) to generate a normalized expression value in fragments per kilobase million.

Nucleotide sequence accession.

Deep genome sequencing data for *Pst_NL* have been deposited in NCBI, and its BioProject accession number is PRJNA725906.

ACKNOWLEDGMENTS

We thank A. H. Kang for critical comments on the manuscript, H. Jin for *ago2/3/7*, I. Lee for *arp6*, S. Y. He for technical advice and communication on spray infection, and a team of teachers from San Marcos High School (O. Maldonado, R. Randolph, and M. Stedman) for providing strong support for their students in this project.

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