Dissecting the Role of SAG101 in Extracellular NAD(P)-Mediated Immune Signaling

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Abstract
Plants are the primary source of food for all animals, but they are often infected by microbial pathogens, which reduces crop yield and threatens global food security. Therefore, plants have evolved sophisticated defense mechanisms to fend off microbial infections. EDS1, PAD4, and SAG101 are well-characterized plant immune regulators. While the EDS1-PAD4 complex was proven to be necessary for extracellular NAD(P) [eNAD(P)]-mediated immune signaling, the function of the EDS1-SAG101 complex in this pathway has yet to be determined. This research aimed to determine the necessity of SAG101 in the process of eNAD(P) signaling. Our hypothesis was that SAG101 formed a complex with EDS1 to carry out a necessary role in eNAD(P) signaling. Arabidopsis thaliana T-DNA insertion lines SALK_022911C and SALK_030411C were genotyped, and SALK_022911C was identified as a sag101 homozygous mutant. NAD(P)-induced immune responses in the sag101 mutant were compared to those in the eds1 and pad4 mutants. Exogenously applied NAD(P) induced expression of the defense marker genes, FMO1 and ALD1, as well as resistance to the bacterial pathogen Pseudomonas syringae pv. maculicola ES4326 in sag101. However, this induction was completely blocked in eds1 and pad4. These results demonstrate that the EDS1-SAG101 complex is not involved in the eNAD(P) signaling pathway and supports the model that the EDS1-PAD4 and EDS1-SAG101 complexes function differently in plant immunity.

Keywords: plant immunity, eds1, pad4, sag101

Introduction
Plants are susceptible to various harmful microbes, such as bacteria and viruses. In response to the constant exposure to these pathogens, plants developed inducible defense mechanisms to build immunity and prevent further infection (Jones et al., 2016). The first layer of resistance is called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), where conserved microbial elicitors are recognized by the membrane receptors. Successful pathogens have developed the ability to deliver virulence effector proteins into the host cell. In response to this, plants have evolved intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) to detect the effector proteins or their perturbation, triggering the second layer of resistance called
effector-triggered immunity (ETI) (Sun et al., 2021). These immune responses are typically initiated by the activation of a signaling pathway, which is then followed by interactions among other signal transduction pathways. Enhanced Disease Susceptibility 1 (EDS1), Phytoalexin Deficient 4 (PAD4), and Senescence-Associated Gene 101 (SAG101) are proteins in this signaling network that are well-known for their roles as regulators in ETI. (Dongus & Parker, 2021). In the model plant Arabidopsis thaliana, EDS1 interacts with both PAD4 and SAG101 to form the EDS1-PAD4 and EDS1-SAG101 complexes, which operate independently of one another (Wagner et al., 2013). Additionally, Activated Disease Resistance 1 (ADR1) and N Requirement Gene 1 (NRG1), two types of helper NLRs, function downstream of EDS1-PAD4 and EDS1-SAG101 complex respectively, to trigger immunity and defend against infection (Sun et al., 2021). Nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) are electron carriers that are exceedingly prevalent in metabolic reactions in plants and animals (Noctor, Queval, & Gakière, 2006). NAD(P) has been shown to be present in the extracellular compartments of plants during pathogen infection, inducing pathogenesis-related gene expression and disease resistance (Zhang & Mou, 2009). In addition to ETI, it has been found that EDS1 and PAD4 also play an important role in NAD(P)-triggered immunity (Wang et al., 2019). It has yet to be determined whether SAG101 is also necessary for triggering the eNAD(P)-mediated immune signaling. This study was therefore designed to address this question. Two Arabidopsis T-DNA insertion lines, SALK_022911C and SALK_030411C, were characterized. SALK_022911C was confirmed to be a sag101 homozygous mutant through a genotyping process conducted with polymerase chain reaction (PCR). Exogenous NAD(P)-induced resistance to the bacterial pathogen Pseudomonas syringae pv. maculicola ES4326_luxCDABE (Psm ES4326_lux) in the sag101, eds1, and pad4 mutants was compared. Furthermore, quantitative real-time PCR (qPCR) was performed to analyze the expression levels of Flavin-Dependent Monoxygenase 1 (FMO1) and AGD2-Like Defense Response Protein 1 (ALD1), two key marker genes of eNAD(P)-mediated immunity (Wang et al., 2019). While sag101 mutant plants exhibited significant resistance to pathogen infection as well as increased FMO1 and ALD1 expression level, eds1 and pad4 mutant plants exhibited largely diminished resistance to pathogen infection. These results revealed that while EDS1 and PAD4 are required for NAD(P)-induced immune responses, SAG101 is dispensable for this induction.
Materials and Methods

Genotyping of T-DNA Insertion Lines

Genomic DNA of the two T-DNA insertion lines SALK_022911C and SALK_030411C (obtained from the Arabidopsis Biological Resource Center) were extracted using organic extraction media and cetyltrimethylammonium bromide (CTAB). Leaf tissues were collected and placed into 1.5 mL tubes, snap-frozen with liquid nitrogen, and ground into a fine powder using small metal spheres. Five hundred μL of CTAB extraction buffer was added to each tube. The samples were inverted to mix and incubated at 65°C for 15 min. Five hundred μL of chloroform was then added to each tube. After mixing, the samples were centrifuged at 10,000g for 5 min. The aqueous phase (400 μL) from each sample was transferred into a new 1.5-mL tube. Four hundred μL of isopropanol were added to each tube. The samples were inverted to mix, incubated at -20°C for 30 min to precipitate the DNA, and centrifuged at 10,000g for 5 min. The supernatant was discarded, and 500 μL of 70% ethanol was added to each tube. The samples were vortexed and centrifuged at 10,000g for 2 min. The supernatant was discarded, and the pellet was air-dried. The pellet was dissolved in 100 μL of water. The PCR amplification was performed in a 10 μL reaction volume consisting of 3.2 μL of water, 5 μL of Taq polymerase master mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 1 μL of DNA. The sequences of these primers are given in Table 1. This mixture solution was amplified by a PCR machine (Biorad). The thermal cycler was programmed for 5 min at 95°C as initial denaturation, followed by 35 cycles of 30 s at 95°C for denaturation, 30 s at 56°C for annealing, 90 s at 72°C for extension, and final extension at 72°C for 5 min. PCR products were examined using electrophoresis in a 1% (w/v) gel in 1 x TAE buffer at 200 V for 20 min.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>LBb1.3</td>
<td>ATTTTGCCGATTTCCGAAC</td>
</tr>
<tr>
<td>SAGgeno-F1</td>
<td>ATGTTGAACCTTTGCCTTTG</td>
</tr>
<tr>
<td>SAGgeno-F2</td>
<td>CTTGTGGTCCTCTGTGGTCTC</td>
</tr>
<tr>
<td>SAGgeno-R1</td>
<td>TGCATAAGGCACGTTTTAACG</td>
</tr>
<tr>
<td>SAGgeno-R2</td>
<td>TTGAACCGATCGTAGTAACCG</td>
</tr>
</tbody>
</table>
**Plant Growth Regimen**

Wild type, *sag101, pad4*, and *eds1* mutant seeds were sown on autoclaved soil. After germination, the seedlings were grown at 25 °C with a 14-hour-light/10-hour-dark routine for two weeks and then transplanted into new pots of autoclaved soil for optimal growth.

**Pathogen Infiltration and Treatment Variation**

A 1 mL needleless syringe was used to infiltrate plant leaves with solutions from the abaxial side. Three solutions were used in this process. The first was water as a mock treatment, the second was 0.4 mM NAD, and the third was 0.8 mM NADP. For each genotype, the plants were separated and labeled by the treatment they would receive. After the initial infiltration was done, the leaves were allowed to dry for 4 hr. For the pathogen infiltration, the leaves that had received the initial treatment were inoculated with the bacterial pathogen *Psm ES4326* _lux_ (OD$_{600}$ = 0.001) using the same method. Three days after the pathogen infiltration, the leaves were collected to examine pathogen growth. One leaf disk was obtained from each treated leaf and placed into a well with 100 μL magnesium chloride on a 96-well plate. The plate was then placed in a GloMax Luminometer (Promega) to obtain data for the pathogen level in each sample.

**RNA Extraction**

Leaf tissues were collected from the leaves of each genotype that received the same treatment. About 100 mg of leaf tissue was collected for each sample and placed into a 1.5 mL tube containing a small metal sphere. The leaf tissue was snap-frozen by submerging the tubes in liquid nitrogen and then ground into a fine powder. Five hundred μL of RNA extraction buffer were added to each sample, followed by 500 μL of phenol. The samples were vortexed and incubated at 65°C for 5 min. 500 μL of a new solution composed of 24 parts of chloroform and one part of isoamyl alcohol were then added. The samples were vortexed and centrifuged at 12,000 g at 4°C for 10 min. 500 μL and 50 μL of the supernatant were transferred for each sample, and then one tenth volume of 3.0 M sodium acetate (NaOAc) and two volumes of 100% ethanol were added. After mixing, the samples were incubated in a -80°C freezer for an hour and then centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was removed, and the pellets
were washed with 80% ethanol and finally centrifuged at 14,000 rpm for 5 min at 4°C to remove the residual ethanol. The pellets were left to air dry for 10 min and resuspended in DEPC-treated water by incubating at 4°C overnight to dissolve the RNA.

DNase Treatment and DNase Inactivation

A DNase treatment was made in a total volume of 10 μL consisting of 5 μL of the dissolved RNA, 1 μL of DNase buffer, 0.5 μL of DNase I, and the remainder as water. The samples were left at room temperature for 25 min. Two and half μL of DNase inactivation reagent were added to the 10 μL reactions. These samples were left at room temperature for 5 min, disturbed to prevent settling, and then centrifuged at 2,000 g for 5 min.

Reverse Transcription

For reverse transcription, 2.5 μL of the DNase inactivated reaction was used in addition to 0.5 μL of 10 mM dNTP (DEPC-treated), 0.5 μL of 0.5 mg/mL oligo dT (DEPC-treated), and 2.5 μL of DEPC-treated water. These mixtures were incubated at 65 °C for 5 min, and then placed in ice for 1 min. Each sample then received 0.375 μL of DEPC-treated water, 2 μL of reverse transcription buffer, 1 μL of 0.1 M dT, 0.25 μL of RNase inhibitor, and 0.125 μL of reverse transcription enzyme. The reactions were left at 50 °C for 1 hr, and then 70 °C for 15 min. The newly formed cDNAs were diluted 20-fold and aliquoted into new tubes.

qPCR

Each qPCR reaction solution contained 5 μL of 2x SYBR green mix, 1 μL of 5 μM forward primer, 1 μL of 5 μM reverse primer, and 3 μL of the 1/20 cDNA dilution mixture. The reaction solutions were placed in the QuantStudio3 qPCR machine (Applied Biosystems) to obtain data. This process was done twice, once for the FMO1 gene, and again for the ALD1 gene, and each time the UBIQUITIN5 (UBQ5) gene was used as an internal control. Three technical replicates were performed for each genotype/treatment.

Results

Identification of a Homozygous T-DNA Insertion Mutant of the SAG101 Gene

PCR results indicated that the T-DNA insertion line SALK_022911C is homozygous. Figure 1 illustrates the locations of the T-DNA insertions. Figure 2 and Figure 3 are images of
the PCR product bands in the electrophoresis gel. For SALK_022911C, PCR products with primers \textit{SAGgeno-F1} + \textit{SAGgeno-R1} were compared to those with primers \textit{SAGgeno-F1} + \textit{SAGgeno-LBb1.3}. For SALK_030411C, PCR products with primers \textit{SAGgeno-F2} + \textit{SAGgeno-R2} were compared to those with primers \textit{LBb1.3} + \textit{SAGgeno-R2}. For the forward and reverse genomic DNA primers, homozygous lines will produce no bands due to the large size of the T-DNA insertion. When the T-DNA primer \textit{LBb1.3} is combined with a genomic DNA primer, both homozygous and heterozygous lines will produce a band. With this understanding, PCR products amplified with each primer pair from the T-DNA insertion lines and the wild type were compared. Figure 2 shows the PCR products of SALK_022911C. For the forward and reverse genomic DNA primers, no PCR bands were produced from the five SALK_022911C samples, whereas a clear PCR band was obtained from the Col-0 sample, suggesting that SALK_022911C may be homozygous. For the \textit{SAGgeno-F1} + \textit{LBb1.3} primers, the presence of a clear PCR band from the five SALK_022911C samples (excluding sample #3) but not from the Col-0 sample confirmed the homozygosity of SALK_022911C (fig. 2). Figure 3 shows the PCR products of the SALK_030411C line. For the forward and reverse genomic DNA primers, the presence of a clear PCR band from all five samples indicates that this line is not homozygous. For the \textit{LBb1.3} + \textit{SAGgeno-R2} primers, the presence of the PCR band from all five samples indicates that this line is heterozygous containing the T-DNA insertion (fig. 3). Therefore, SALK_022911C was used for the study in this paper.

![Figure 1. The T-DNA insertion sites in the SALK lines. Both T-DNA insertions are located in exons.](image-url)
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Figure 2. Agarose gel image of PCR products amplified from the T-DNA insertion line SALK_022911C. Sample #3 did not produce PCR products for either primer combination, indicating that the DNA sample was inadequate. Presence of bands for primers SAGgeno-F1 + LBb1.3 and absence of bands for primers SAGgeno-F1 + SAGgeno-R1 indicate homozygosity of SALK_022911C.

Figure 3. Agarose gel image of PCR products amplified from the T-DNA insertion line SALK_030411C. Note that the PCR results from the wild-type Col-0 plants were not on the same gel and thus not presented in Figure 3. The presence of bands in both primer combinations indicates heterozygosity of SALK_030411C.

NAD(P)-Induced Resistance in the eds1, pad4, and sag101 Mutants

Infiltration of the leaves of the wild-type Col-0 and SALK_022911C (sag101) with 0.4 mM NAD and 0.8 mM NADP induced strong immunity against the bacterial pathogen Psm
ES4326_lux. Col-0 and sag101 leaves showed stronger resistance to the pathogen than the leaves of the eds1 and pad4 mutants (Fig. 4). This indicates that sag101 is not relevant in NAD(P)-triggered immune signaling, as the sag101 plants still mounted immunity to the pathogen despite the absence of functional SAG101. In contrast, the leaves of the eds1 and pad4 mutants showed compromised immunity to the pathogen, indicating that both EDS1 and PAD4 are necessary for NAD(P)-induced immunity. The symptoms shown in Figure 4 are consistent with the counting results displayed in Figure 5. The smaller log_{10} relative light values (RLU) of Col-0 and sag101 indicate greater induced immunity compared with the larger RLU values of eds1 and pad4. This corresponds with the leaf color variations observed (Fig. 4). Therefore, we conclude that SAG101 is not necessary for NAD(P)-triggered immunity, whereas EDS1 and PAD4 are. This conclusion is based on the result that NAD(P)-mediated induction can still occur despite the absence of sag101.

**Figure 4.** Disease symptoms on the leaves of wild-type Col-0, sag101, eds1, and pad4. More chlorosis, or yellowing of the leaves indicates higher pathogen growth. NAD(P) treatment induced resistance to pathogens in both Col-0 and sag101 mutants but not in eds1 or pad4 mutants.
Figure 5. Exogenous NAD(P)-induced resistance to *Psm*. Different numbers of asterisks above the bars denote different statistical significances (***p < 0.01, ****p < 0.001; Student’s t test). The values for *sag101* are comparable to those of Col-0, indicating that *sag101* was not significant in the eNAD(P)-induced resistance pathway.
NAD(P)-induced defense gene expression in *eds1*, *pad4*, and *sag101*

*FMO1* and *ALD1* are two widely used defense marker genes in *Arabidopsis*. 0.4 mM NAD and 0.8 mM NADP induced *FMO1* and *ALD1* expression in both Col-0 and *sag101* plants (Fig. 6). However, the NAD(P)-triggered induction of *FMO1* and *ALD1* was completely abolished in *eds1* and *pad4*. These results substantiate the conclusion that *SAG101* is not required for NAD(P)-induced immune responses in *Arabidopsis*.

**Figure 6.** Exogenous NAD(P)-induced expression of *FMO1* and *ALD1* in Col-0, *sag101*, *eds1*, and *pad4*. Different letters above the bars denote different statistical significances (Student’s t test). *eds1* and *pad4* both induced minimal expression, as compared with the expression in Col-0 and *sag101*, indicating their distinct roles in this pathway.

**Discussion**

Extracellular NAD(P) is an emerging immune signal that induces *Arabidopsis* resistance to *Psm*, one of the most common plant pathogens (Katagiri et al., 2002). Previous findings
suggested that during pathogen invasion, NAD(P) leaks out of the damaged cells into the extracellular compartment (Zhang and Mou, 2009; An et al., 2016). This allows it to be perceived by receptors on the plasma membrane, which induces immune responses in neighboring healthy cells to fight the infection (Wang et al., 2019). The EDS1 complex is a known mechanism involved in plant immunity (Cui et al., 2017). EDS1 forms two complexes, one with PAD4 and the other with SAG101. The EDS1-PAD4 pathway has recently proven to be integral to eNAD(P)-mediated immunity (Wang et al., 2019). However, the role of SAG101 in eNAD(P)-mediated immunity in Arabidopsis is undetermined. This study has shown that SAG101 does not play a role in eNAD(P)-mediated immunity, disproving our hypothesis. Figure 7 illustrates how eNAD(P) is perceived by the receptor complex in the cell membrane to transduce the signal to the EDS1 complex. The signal is passed to the EDS1-PAD4 pathway, but it is not transmitted to the EDS1-SAG101 pathway. Thus, the eNAD(P) immune responses are only mediated by the EDS1-PAD4 pathway. This is also demonstrated by sag101’s abilities to respond to exogenous NAD(P) in immune responses including resistance and defense gene expression. These findings regarding plant health and immunity can have future applications in agriculture and food security.
Figure 7. A model illustrating transduction of eNAD(P) signal through the EDS1-PAD4 module. After the plasma membrane receptors receive eNAD(P) signal, they transmit the signaling to the EDS1-PAD4 complex, but not the EDS1-SAG101 complex. The EDS1-PAD4 complex then activates immune responses through less understood pathways. (Created with BioRender.com.)

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References


