Elongator Plays a Positive Role in Exogenous NAD-Induced Defense Responses in *Arabidopsis*

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Submitted 7 January 2015. Accepted 21 February 2016.

Extracellular NAD is emerging as an important signal molecule in animal cells, but its role in plants has not been well-established. Although it has been shown that exogenous NAD⁺ activates defense responses in *Arabidopsis*, components in the exogenous NAD⁺-activated defense pathway remain to be fully discovered. In a genetic screen for mutants insensitive to exogenous NAD⁺ (*ien*), we isolated a mutant named *ien2*. Map-based cloning revealed that *IEN2* encodes ELONGATA3 (ELO3)/AtELP3, a subunit of the *Arabidopsis* Elongator complex, which functions in multiple biological processes, including histone modification, DNA (de)methylation, and transfer RNA modification. Mutations in the *ELO3/AtELP3* gene compromise exogenous NAD⁺-induced expression of pathogenesis-related (*PR*) genes and resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326, and transgenic expression of the coding region of *ELO3/AtELP3* in *elo3/Atelp3* restores NAD⁺ responsiveness to the mutant plants, demonstrating that ELO3/AtELP3 is required for exogenous NAD⁺-induced defense responses. Furthermore, mutations in genes encoding the other five *Arabidopsis* Elongator subunits (ELO2/AtELP1, AtELP2, ELO1/AtELP4, AtELP5, and AtELP6) also compromise exogenous NAD⁺-induced *PR* gene expression and resistance to *P. syringae* pv. *maculicola* ES4326. These results indicate that the Elongator complex functions as a whole in exogenous NAD⁺-activated defense signaling in *Arabidopsis*.

NAD and its derivative NADP are pyridine nucleotides serving as ubiquitous redox carriers and coenzymes in both anabolic and catabolic pathways (Noctor et al. 2006). In addition to participating in fundamental metabolic reactions, NAD(P) has been implicated in intracellular signaling by acting as ADP-ribose (ADPR) donors, acetyl group acceptors, or precursors for cyclic (c)ADPR and nicotinic acid adenine dinucleotide phosphate (NAADP) to trigger release of calcium (Ca²⁺) from intracellular

compartments in different organisms (Bürkle 2001; Denu 2003; Hunt et al. 2004; Jacobson and Jacobson 1999; Lee 2001; Ziegler 2005). Upon environmental stress, cellular NAD(P) can be released into the extracellular compartment by active exocytosis or diffusion through transmembrane transporters in living cells or passive leakage across membrane in dying cells (Billington et al. 2006). Recent studies have established extracellular (e)NAD(P) as a signal molecule for diverse physiological and pathological responses (Billington et al. 2006; Judkins et al. 2006).

In animal cells, eNAD(P) is metabolized by ectoenzymes or perceived by cell-surface receptors or channels to activate downstream signaling pathways. CD38, CD157, and mono(ADP-ribosyl) transferases (ART) are NAD(P)-metabolizing ectoenzymes located on the extracellular surface of the plasma membrane (Malavasi et al. 2006; Morabito et al. 2006; Partidá-Sánchez et al. 2007; Zolkiewska 2005). CD38 is a multifunctional enzyme that utilizes NAD(P) as substrates to produce the secondary messengers cADPR and NAADP (Ceni et al. 2003; De Flora et al. 2004; Krebs et al. 2005; Partidá-Sánchez et al. 2007). ART are glycosylphosphatidylinositol-anchored or secreted ectoenzymes that utilize NAD to ADP-ribosylate lipid raft-associated signaling proteins (Bannas et al. 2005; Han et al. 2000; Nemoto et al. 1996; Seman et al. 2003). Furthermore, activation of the purinoceptor P2Y₁₁ by eNAD functionally activates human granulocytes (Bruzzone et al. 2006; Moreschi et al. 2006) and interaction between eNADP and purinoceptors can cause contraction of mouse aorta (Judkins et al. 2006).

In plants, NAD and its derivatives have been shown to be involved in several signaling pathways that are mostly associated with stress tolerance or defense responses (Adams-Phillips et al. 2010; Berger et al. 2004; Djebbar et al. 2012; Dutilleul et al. 2003, 2005; Ford et al. 2010; Noctor et al. 2006; Pétriacy et al. 2013). Increasing intracellular NAD levels by overexpressing the bacterial NAD biosynthesis gene *nadC* leads to enhanced defense-gene expression and resistance to bacterial pathogens (Pétriacy et al. 2012), whereas mutations in the de novo NAD biosynthesis gene *FLAGELLIN-INSENSITIVE4* compromise stomatal immunity (Macho et al. 2012). Moreover, changing intracellular NAD(H) levels by overexpressing *AtNUDT6*, which encodes an ADP-ribose/NADH pyrophosphohydrolase or knockout of *AtNUDT6*, *AtNUDT7*, or *AtNUDT8* alters salicylic acid (SA)-mediated defense responses (Adams-Phillips et al. 2008; Bartsch et al. 2006; Fonseca and Dong 2014; Ge et al. 2007; Ishikawa et al. 2010). Furthermore, we recently provided the first line of evidence that eNAD(P) may play a signaling role in plant immunity (Zhang and Mou 2009). We showed that pathogen-induced hypersensitive response causes leakage of NAD(P) into the extracellular fluid at concentrations sufficient to induce pathogenesis-related (*PR*) gene expression and disease resistance. More importantly, exogenous NAD(P)-induced *PR* gene expression appears to be associated with Ca²⁺ signaling and SA (Zhang and Mou 2009). However, NAD(P)

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Nucleotide sequence data can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: ELO2/AtELP1 (At5g13680), AtELP2 (At1g49540), ELO3/AtELP3 (At5g50320), ELO1/AtELP4 (At3g11220), AtELP5 (At2g18410), AtELP6 (At4g10090), PR1 (At2g14610), PR2 (At3g57260), PR5 (At1g75040), and UBQ5 (At3g62250).

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

treatment induces not only SA-dependent but also SA-independent *PR* gene expression and disease resistance, suggesting that SA signaling is not the sole target for exogenous NAD(P) in activating defense responses (Zhang and Mou 2009).

Since no proteins with significant homology to animal CD38/CD157, ART, and purinoceptors have been identified in any available plant genome database (Sánchez et al. 2004; Zolkiewska 2005), it is not clear whether plants use similar mechanisms to process or perceive eNAD(P) and to transduce eNAD(P)-activated signals (Hunt et al. 2004). Expression of the human NAD(P)-metabolizing ectoenzyme CD38 partially compromises systemic acquired resistance (SAR) in *Arabidopsis* (Zhang and Mou 2012), which strongly suggests that plants may use different mechanisms to perceive eNAD(P). Identifying components of the eNAD(P)-activated signaling pathway in *Arabidopsis* is, therefore, a crucial step for understanding these mechanisms. To this end, our recent identification of *Arabidopsis* mutants insensitive to exogenous NAD⁺ (*ien*) revealed the Mediator complex subunits MED14/STRUWELPETER (SWP) and MED16/SENSITIVE TO FREEZING6 (SFR6)/INSENSITIVE TO EXOGENOUS NAD⁺1 (IEN1) as downstream regulators that transduce exogenous NAD⁺-activated signals to the RNA polymerase II (RNAPII)-mediated transcription initiation (Zhang et al. 2012, 2013).

The transition from transcriptional initiation to elongation is associated with changes in the factors that are associated with RNAPII. Elongator (ELP) is originally identified as an interactor of hyperphosphorylated RNAPII, a marker of transcriptional elongation in yeast and is subsequently purified from mammalian and plant cells (Hawkes et al. 2002; Nelissen et al. 2010; Otero et al. 1999). A fully functional Elongator complex consists of two copies of each of its six subunits, with ELP1 and ELP2 serving as scaffolds for complex assembly, ELP3 being the catalytic subunit, and ELP4-ELP6 forming an accessory complex. Elongator has been shown to function in multiple cellular processes such as histone modification, exocytosis, α -tubulin acetylation, zygotic paternal genome demethylation, and transfer RNA (tRNA) modification (Creppe et al. 2009; Hawkes et al. 2002; Huang et al. 2005; Okada et al. 2010; Rahl et al. 2005). Although the regulatory roles in both transcription and translation seem to be conserved, it becomes clear that Elongator has multiple kingdom-specific functions in diverse organisms (DeFraia and Mou 2011). In yeast, mutations in Elongator subunits cause resistance to the zymocin γ -toxin and sensitivity to salt, caffeine, and temperature (Jablonowski et al. 2001; Krogan and Greenblatt 2001; Otero et al. 1999). Elongator deficiency in humans results in defective neuron development, manifested as familial dysautonomia disease (Anderson et al. 2001; Slaughter et al. 2001).

The *Arabidopsis* Elongator complex also contains six subunits: ELONGATA2 (ELO2)/AtELP1, AtELP2, ELO3/AtELP3, ELO1/AtELP4, AtELP5, and AtELP6 (Nelissen et al. 2010). Mutations in ELO2/AtELP1, AtELP2, ELO3/AtELP3, ELO1/AtELP4, and AtELP6 lead to pleiotropic effects including hypersensitivity to abscisic acid, resistance to oxidative stress, severely aberrant auxin phenotypes, altered cell cycle progression, abnormal root development, and compromised disease resistance (Chen et al. 2006; Jia et al. 2015; Nelissen et al. 2005, 2010; Xu et al. 2012; Zhou et al. 2009). Additionally, AtELP2 and ELO3/AtELP3 have been shown to regulate the induction kinetics of defense genes of the SA pathway (DeFraia et al. 2010, 2013). Further characterization indicated that AtELP2 is an epigenetic regulator required for the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000/avrRpt2-induced rapid transcriptome reprogramming, likely by maintaining histone acetylation levels, modulating the genomic DNA landscape, and influencing pathogen-induced dynamic DNA methylation changes (Wang et al. 2013).

Here, we report another *ien* mutant, *ien2/Atelp3-11*, which was isolated in the previously described genetic screen (Zhang et al. 2012). Characterization of *ien2/Atelp3-11* together with *elo2/Atelp1*, *Atelp2*, *elo1/Atelp4*, *Atelp5*, and *Atelp6*, mutants of the other five Elongator subunits, revealed that Elongator as a whole plays a positive role in exogenous NAD⁺-activated defense responses in *Arabidopsis*.

RESULTS

The *ien2* mutation compromises exogenous

NAD⁺-induced *PR* gene expression and disease resistance.

The *ien2* mutant was isolated in the previously described genetic screen (Zhang et al. 2012). After backcrossing twice with wild-type Columbia (Col-0) plants, the *ien2* mutant was further analyzed. Compared with the wild-type Col-0, exogenous NAD⁺-induced expression of *PR1*, *PR2*, and *PR5* was significantly inhibited in the *ien2* mutant plants (Fig. 1A). Consistent with the reduced *PR* gene induction, NAD⁺-induced resistance to the bacterial pathogen *P. syringae* pv. *maculicola* ES4326 was also compromised in the *ien2* plants (Fig. 1B and C). These results indicate that *IEN2* plays an important positive role in exogenous NAD⁺-induced defense responses.

Morphologically, *ien2* leaves were serrated, curly, and a lighter shade of green than the wild-type Col-0 (Fig. 1D), which is reminiscent of the previously described Elongator mutants (Chen et al. 2006; DeFraia et al. 2010, 2013; Nelissen et al. 2005, 2010; Zhou et al. 2009). Moreover, similarly to the *Atelp2* and *elo3/Atelp3* mutants (DeFraia et al. 2010, 2013), *ien2* exhibited enhanced susceptibility to *P. syringae* pv. *maculicola* ES4326 (Fig. 1E and F), indicating that *IEN2* also positively contributes to basal resistance against this bacterial pathogen.

IEN2 encodes the Elongator subunit 3.

During backcrosses with wild-type Col-0 plants, we found that all F₂ progeny with *ien2* morphology were susceptible to *P. syringae* pv. *maculicola* ES4326, indicating that the morphological and disease susceptibility phenotypes of *ien2* were caused by the same mutation or two tightly linked mutations. Additionally, the *ien2* morphology is controlled by a single recessive nuclear mutation, as the F₁ progeny displayed wild-type Col-0 morphology and the F₂ population segregated into wild-type-Col-0-like and *ien2*-like plants in a 3:1 ratio. We therefore used the morphological phenotype to map the *ien2* mutation. To this end, we crossed *ien2*, which is in the Col-0 genetic background, to the polymorphic ecotype Landsberg erecta. The F₁ plants were selfed to produce an F₂ population. Linkage analysis of 107 F₂ plants with *ien2* morphology placed the *ien2* mutation between the molecular markers CIW9 and CIW10 on chromosome 5 (Fig. 2A), an interval that contains the *ELO3/AtELP3* gene (DeFraia et al. 2013). Further analysis did not find any recombinants for markers at the genetic loci At5g50270 and At5g50360 (Fig. 2A). Since *ien2* morphologically resembled the previously described *elo3-6* and *Atelp3-10* mutants (Supplementary Fig. S1) (Nelissen et al. 2010; DeFraia et al. 2013), the *ien2* mutation might be located in *ELO3/AtELP3* (At5g50320). To test this, the *ELO3/AtELP3* coding region was amplified from *ien2* and was sequenced. A single base substitution of A for G was detected at the 5' splice site of the sixth intron of At5g50320 (Fig. 2B). Based on the mutation, a cleaved amplified polymorphic sequences marker was developed to genotypically distinguish *ien2* from the wild-type Col-0 (Fig. 2C). To test whether the mutation affected mRNA splicing, wild-type Col-0 and *ien2* cDNA were amplified by polymerase chain reaction (PCR), using a pair of primers spanning the mutation site. As shown in Figure 2D, while a single band was amplified from the wild-type Col-0 cDNA, multiple bands with different sizes

were amplified from the *ien2* cDNA, indicating that *ien2* is a new allele of *elo3/Atelp3*. Therefore, *ien2* was renamed *Atelp3-11* (Fig. 2B).

Next, we analyzed exogenous NAD^+ -induced *PR* gene expression in two previously reported *elo3/Atelp3* mutant alleles, *elo3-6* and *Atelp3-10* (DeFraia et al. 2013; Nelissen et al. 2010). Compared with the wild-type Col-0, NAD^+ -induced expression of *PR1*, *PR2*, and *PR5* was also significantly reduced in *elo3-6* and *Atelp3-10* (Fig. 2E), indicating that mutations in *ELO3/AtELP3* suppress NAD^+ -induced defense responses.

Finally, we tested NAD^+ responsiveness in the previously generated transgenic *Atelp3-10* plants expressing a *35S:AtELP3* transgene, which complemented the morphological and disease susceptibility phenotypes of the *Atelp3-10* mutant (DeFraia et al. 2013). We treated wild-type Col-0, *Atelp3-10*, and *Atelp3-10 35S:AtELP3* plants with NAD^+ and analyzed NAD^+ -induced

expression of *PR1*, *PR2*, and *PR5*, as well as resistance to *P. syringae* pv. *maculicola* ES4326. As shown in Figure 3A to C, both *PR* gene expression and *P. syringae* pv. *maculicola* ES4326 resistance were induced in the wild-type Col-0, and the induction was significantly inhibited in the *Atelp3-10* mutant plants. Importantly, NAD^+ -induced *PR* gene expression and *P. syringae* pv. *maculicola* ES4326 resistance were completely restored in the *Atelp3-10 35S:AtELP3* plants (Fig. 3A to C). These results further unambiguously demonstrate that *ELO3/AtELP3* is required for NAD^+ -induced defense responses.

Mutations in the other five Elongator subunits also inhibit exogenous NAD^+ -induced *PR* gene expression and disease resistance.

To test whether the integrity of the Elongator complex is required for exogenous NAD^+ -activated defense signaling, we

Fig. 1. Characterization of the *ien2* mutant. **A**, Exogenous NAD^+ -induced expression of *PR1*, *PR2*, and *PR5* in *ien2* and wild-type Col-0 plants. Leaves of four-week-old soil-grown plants were treated with 1 mM NAD^+ solution. Total RNA was extracted from leaf tissues collected at the indicated time points and was subjected to real-time quantitative polymerase chain reaction analysis. Expression was normalized against constitutively expressed *UBQ5*. Data represents the mean of three independent samples with standard deviation (SD). Asterisks indicate that the difference between the induction (the indicated time point versus 0 h) of the gene in *ien2* and the wild-type Col-0 plants was significant (two-way analysis of variance [ANOVA]; * = $P < 0.05$, ** = $P < 0.01$). The experiment was repeated twice with similar trends. **B**, Exogenous NAD^+ -induced resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 in *ien2* and wild-type Col-0 plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (–) 1 mM NAD^+ solution. Five hours later, the infiltrated leaves were inoculated with a *P. syringae* pv. *maculicola* ES4326 suspension (optical density at 600 nm [OD_{600}] = 0.001). The in planta bacterial titers were determined at 3 days postinoculation (dpi). Data represent the mean of eight independent samples with SD. The asterisks indicate that the difference between the induction (NAD^+ treated versus nontreated) of resistance to *P. syringae* pv. *maculicola* ES4326 in *ien2* and the wild-type Col-0 plants was significant (two-way ANOVA, $P < 0.01$). The experiment was repeated three times with similar trends. **C**, Disease symptoms caused by *P. syringae* pv. *maculicola* ES4326 on *ien2* and wild-type Col-0 leaves treated with (+) and without (–) NAD^+ . The experiment was carried out as in B. Photos were taken at 3 days postinoculation (dpi). **D**, Morphology of four-week-old soil-grown *ien2* and wild-type Col-0 plants. **E**, Susceptibility of *ien2* and wild-type Col-0 plants to *P. syringae* pv. *maculicola* ES4326. Four-week-old soil-grown plants were inoculated with a low dose of *P. syringae* pv. *maculicola* ES4326 (optical density at 600 nm = 0.0001). The in planta bacterial titers were determined immediately and at 3 dpi. Data represent the mean of eight independent samples with SD. The asterisk shows that *P. syringae* pv. *maculicola* ES4326 grew significantly more in *ien2* than in the wild-type Col-0 plants (Student's *t* test, $P < 0.05$). The experiment was repeated three times with similar trends. **F**, Disease symptoms caused by *P. syringae* pv. *maculicola* ES4326 on *ien2* and wild-type Col-0 leaves. The experiment was carried out as in E.

attempted to compare exogenous NAD^+ -induced defense responses in mutants of all six Elongator subunits. To this end, we used the mutant alleles *elo2/Atelp1* (SALK_004690), *Atelp2-2* (SALK_106485), *elo3-6* (GABI_555H06), *elo1/Atelp4* (SALK_079193), *Atelp5* (GABI_700A12), and *Atelp6* (SAIL_621_B08) for the six subunits, respectively. All these mutants are in Col-0 genetic background. *Atelp2-2* (SALK_106485), *elo3-6* (GABI_555H06), and *elo1/Atelp4* (SALK_079193) have previously been described (DeFraia et al. 2010; Nelissen et al. 2010; Zhou et al. 2009). *elo2/Atelp1* (SALK_004690) and *Atelp6* (SAIL_621_B08) are new alleles of the reported *elo2/Atelp1* and *Atelp6* mutants, respectively (Chen et al. 2006; Nelissen et al. 2005). Mutants for the fifth subunit *AtELP5* have not previously been characterized. We isolated a homozygous T-DNA insertion *Atelp5* (GABI_700A12) mutant for this study. Real-time

quantitative (q)PCR analysis revealed that, compared with the wild-type Col-0, expression of *ELO2/AtELP1*, *AtELP2*, *ELO3/AtELP3*, *ELO1/AtELP4*, *AtELP5*, and *AtELP6* was significantly reduced in the corresponding T-DNA insertion lines (Supplementary Fig. S2), indicating that they are either knockout or knockdown mutants.

The morphological phenotypes of *elo2/Atelp1*, *Atelp2*, *elo3/Atelp3*, *elo1/Atelp4*, and *Atelp6* have been separately described in previous studies (Chen et al. 2006; DeFraia et al. 2010, 2013; Nelissen et al. 2005, 2010; Zhou et al. 2009). When grown side by side, these mutants and *Atelp5* all exhibited similar morphological phenotypes including elongated and curly leaves with a lighter shade of green than the wild-type Col-0 (Supplementary Fig. S3). Compared with each other, the *elo1/Atelp4* and *Atelp5* mutants displayed the most and least

Fig. 2. Map-based cloning of *ien2*. **A**, A schematic diagram of the map-based cloning process. A total of 107 F_2 progeny from a cross between *ien2* and ecotype Landsberg erecta, which exhibited *ien2* morphology, were used to determine the approximate position of the *ien2* mutation by bulked segregant analysis. The *ien2* mutation was linked to molecular markers CIW9 and CIW10 on chromosome 5. No recombinants were found for two markers located at the genetic loci At5g50270 and At5g50360. **B**, The structure of the *ELO3/AtELP3* gene (At5g50320) and the positions of the *gns2/Atelp3-10*, *elp3-6* (GABI_555H06), *elo3-1*, and *ien2* mutations. Boxes denote exons and lines between boxes denote introns. **C**, A cleaved amplified polymorphic sequences marker for the *ien2* mutation. Polymerase chain reaction (PCR) products amplified with a pair of primers (5'-TACACAAAGGCACATGCTTAC-3' and 5'-TATCCACCCCTTGATCCG-3') from *ien2* and wild-type Col-0 genomic DNA were digested with *DpnII* and were separated in a 2% agarose gel. Two bands (621 and 198 bp) and three bands (340, 281, and 198 bp) were detected for Col-0 and *ien2*, respectively. **D**, Abnormal splicing in the *ien2* mutant. A pair of primers (5'-GATGCCTGATCTTCCTAATG-3' and 5'-CATGAGTTCTGGACACGTTAC-3') spanning the mutation site was used to amplify from wild-type Col-0 and *ien2* cDNA. The resulting PCR products were separated in a 2% agarose gel. Whereas a single band (544 bp) was amplified from the Col-0 cDNA, three bands with different sizes were amplified from the *ien2* cDNA. **E**, Exogenous NAD^+ -induced expression of *PR1*, *PR2*, and *PR5* in wild-type Col-0, *elo3-6*, *Atelp3-10*, and *ien2* plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (-) 1 mM NAD^+ solution. Total RNA was extracted from infiltrated leaf tissues collected at 16 h postinoculation and was subjected to real-time quantitative PCR analysis. Expression was normalized against constitutively expressed *UBQ5*. Data represents the mean of three independent samples with standard deviation. An asterisks indicates that the difference between the induction (NAD^+ treated versus nontreated) of the *PR* gene in the mutant and the wild-type Col-0 plants was significant (two-way analysis of variance, * = $P < 0.05$, ** = $P < 0.01$). The experiment was repeated twice with similar trends.

severe morphological phenotypes, respectively, suggesting some differences among the *eloAtelp* mutants.

To test whether mutations in each of the Elongator subunits affect exogenous NAD^+ -induced defense responses, we monitored NAD^+ -induced *PR* gene expression and resistance to *P. syringae* pv. *maculicola* ES4326 in *eloAtelp* and wild-type Col-0 plants. As shown in Figure 4A, exogenous NAD^+ -induced expression of *PR1*, *PR2*, and *PR5* was significantly reduced in all *eloAtelp* mutants compared with the wild-type Col-0. Similarly, exogenous NAD^+ -induced resistance to *P. syringae* pv. *maculicola* ES4326 was also significantly suppressed in all *eloAtelp* mutants compared with the wild-type Col-0 (Fig. 4B and C). These results demonstrate that all six subunits of the Elongator complex are required for exogenous NAD^+ -induced defense responses in *Arabidopsis*.

DISCUSSION

Although our previous work has shown that exogenous NAD^+ induces defense responses in *Arabidopsis* (Zhang and Mou 2009), the signaling components in NAD^+ -induced defense signaling pathways remain to be fully discovered. In a genetic screen for *Arabidopsis* mutants insensitive to exogenous NAD^+ (*ien*), we isolated a mutant named *ien2*. Map-based cloning

revealed that the *ien2* mutation is located in the *ELO3/AtELP3* gene, which encodes a subunit of the Elongator complex. Previously, we have shown that the Elongator subunits AtELP2 and ELO3/AtELP3 are required for basal resistance against both virulent and avirulent bacterial pathogens (DeFraia et al. 2010, 2013). We found that the other four subunits, ELO2/AtELP1, ELO1/AtELP4, AtELP5, and AtELP6, are also required for resistance to the avirulent bacterial pathogen *P. syringae* pv. *tomato* DC3000/*avrRpt2* (Supplementary Fig. S4A) and that mutations in all six Elongator subunits reduced *P. syringae* pv. *tomato* DC3000/*avrRpt2*-induced ion leakage, indicating that the Elongator complex is required for full induction of a hypersensitive response. Furthermore, we have recently reported that ELO2/AtELP1, AtELP2, ELO3/AtELP3, ELO1/AtELP4, and AtELP5 are essential for resistance to necrotrophic fungal pathogens (Wang et al. 2015). These results suggest that the Elongator complex is likely important for general defense responses in plants. In this study, by characterizing different Elongator subunit mutants, we demonstrate that the Elongator complex plays a critical positive role in exogenous NAD^+ -induced defense responses in *Arabidopsis*.

Elongator is a six-subunit protein complex and loss of any subunit generally renders the complex inactive (Glatt et al. 2012; Versées et al. 2010). However, it has been reported that

Fig. 3. Complementation of the *Atelp3-10* mutation restores NAD^+ responsiveness. **A**, Exogenous NAD^+ -induced expression of *PR1*, *PR2*, and *PR5* in wild-type Col-0, *Atelp3-10*, and *Atelp3-10 35S:AtELP3* plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (-) 1 mM NAD^+ solution. Total RNA was extracted from the infiltrated leaf tissues collected at 16 h postinoculation and were subjected to real-time quantitative polymerase chain reaction analysis. Expression was normalized against constitutively expressed *UBQ5*. Data represents the mean of three independent samples with standard deviation (SD). Asterisks indicate that the difference between the induction (NAD^+ treated versus nontreated) of the *PR* gene in *Atelp3-10* and the wild-type Col-0 or *Atelp3-10 35S:AtELP3* plants was significant (two-way analysis of variance [ANOVA], * = $P < 0.05$, ** $P < 0.01$). The experiment was repeated twice with similar trends. **B**, Exogenous NAD^+ -induced resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 in wild-type Col-0, *Atelp3-10*, *Atelp3-10 35S:AtELP3* plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (-) 1 mM NAD^+ solution. Five hours later, the infiltrated leaves were inoculated with a *P. syringae* pv. *maculicola* ES4326 suspension (optical density at 600 nm = 0.001). The in planta bacterial titers were determined at 3 days postinoculation (dpi). Data represent the mean of eight independent samples with SD. The asterisks show that the difference between the induction (NAD^+ treated versus nontreated) of resistance to *P. syringae* pv. *maculicola* ES4326 in *Atelp3-10* and the wild-type Col-0 or *Atelp3-10 35S:AtELP3* plants was significant (two-way ANOVA, $P < 0.01$). The experiment was repeated three times with similar trends. **C**, Disease symptoms caused by *P. syringae* pv. *maculicola* ES4326 on wild-type Col-0, *Atelp3-10*, and *Atelp3-10 35S:AtELP3* leaves treated with (+) and without (-) NAD^+ . The experiment was carried out as in B. Photos were taken at 3 dpi.

mutations in the core subcomplex subunits ELO2/AtELP1 and AtELP2 but not in the accessory subcomplex subunits ELO1/AtELP4 and AtELP6 lead to abscisic acid hypersensitivity in stomatal closure (Zhou et al. 2009), suggesting independent roles for the two subcomplexes in *Arabidopsis*. Previous studies have shown that mutants of ELO2/AtELP1, AtELP2, ELO3/AtELP3, ELO1/AtELP4, and AtELP6 have similar morphological phenotypes including chlorosis and developmental alterations (Chen et al. 2006; DeFraia et al. 2010, 2013; Nelissen et al. 2005, 2010; Zhou et al. 2009), indicating that the Elongator complex is essential not only for immunity but also for general plant fitness. The *Atelp5* T-DNA insertion mutant isolated in this study is also morphologically similar to other *elo/Atelp* mutants, although its morphology is less severe than others, suggesting that the Elongator complex functions as a whole in plant growth and development. Since exogenous NAD⁺-induced *PR* gene expression and resistance to *P. syringae* pv. *maculicola* ES4326 were similarly inhibited in mutants of all six Elongator subunits (Fig. 4A to C), the integrity of Elongator is also crucial for exogenous NAD⁺-induced defense responses.

We have previously shown that mutations in the Mediator complex subunits MED14/SWP and MED16/SFR6/IEN1 block exogenous NAD⁺-induced *PR* gene expression and compromise SAR, suggesting that the Mediator tail module may integrate exogenous NAD⁺-activated signals into the RNAPII transcription machinery in *Arabidopsis* (Zhang et al. 2012,

2013). Mediator mostly functions at the transcription initiation stage, and different Mediator subunits act as adaptors for specific transcription factors of various signaling pathways, whereas Elongator is a general transcription regulator functioning at the elongation stage of transcription through interacting with hyperphosphorylated (elongating) RNAPII (Conaway and Conaway 2011; Hawkes et al. 2002; Nelissen et al. 2010; Otero et al. 1999; Wittschieben et al. 1999). Therefore, Elongator-mediated transcription elongation may contribute to exogenous NAD⁺-induced defense gene transcription. These results together demonstrate the importance of the general transcription machinery in exogenous NAD⁺-induced defense signaling. Furthermore, it has previously been shown that, in plant cell proliferation, the Elongator complex is genetically positioned downstream of Mediator, which is consistent with their roles in the process of RNAPII-mediated transcription (Nelissen et al. 2005). As both Mediator and Elongator function in exogenous NAD⁺-activated defense gene transcription, it would be interesting to test whether the same epistatic relationship exists for Elongator and Mediator in NAD⁺-induced defense responses.

Elongator has been shown to function in histone acetylation and, one or both, DNA demethylation and methylation (Nelissen et al. 2010; Okada et al. 2010; Wang et al. 2013; Winkler et al. 2002). Although no intrinsic histone acetyltransferase and DNA demethylase activities have been reported for the *Arabidopsis* Elongator complex (Ding and Mou 2015), it has been shown

Fig. 4. NAD⁺-induced defense responses in mutants of all six Elongator subunits. **A**, Exogenous NAD⁺-induced expression of *PR1*, *PR2*, and *PR5* in wild-type Col-0 and *elo/Atelp* mutant plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (-) 1 mM NAD⁺ solution. Total RNA was extracted from the infiltrated leaf tissues collected at 16 h postinoculation and was subjected to real-time quantitative polymerase chain reaction analysis. Expression was normalized against constitutively expressed *UBQ5*. Data represents the mean of three independent samples with standard deviation (SD). Asterisks indicate that the difference between the induction (NAD⁺ treated versus nontreated) of the *PR* gene in the mutant and the wild-type Col-0 was significant (two-way analysis of variance [ANOVA], * = *P* < 0.05, ** = *P* < 0.01). The experiment was repeated twice with similar trends. **B**, Exogenous NAD⁺-induced resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 in *elo/Atelp* and wild-type Col-0 plants. Leaves of four-week-old soil-grown plants were infiltrated with (+) or without (-) 1 mM NAD⁺ solution. Five hours later, the infiltrated leaves were inoculated with a *P. syringae* pv. *maculicola* ES4326 suspension (optical density at 600 nm = 0.001). The in planta bacterial titers were determined at 3 days postinoculation (dpi). Data represent the mean of eight independent samples with SD. Asterisks show that the difference between the induction (NAD⁺ treated versus nontreated) of resistance to *P. syringae* pv. *maculicola* ES4326 in the mutant and the wild-type Col-0 was significant (two-way ANOVA, * = *P* < 0.05, ** = *P* < 0.01). The experiment was repeated three times with similar trends. **C**, Disease symptoms caused by *P. syringae* pv. *maculicola* ES4326 on *elo/Atelp* and wild-type Col-0 leaves treated with (+) and without (-) NAD⁺. The experiment was carried out as in C. Photos were taken at 3 dpi.

that histone acetylation levels and DNA methylation profiles at specific genomic regions are altered in several *Arabidopsis eloAtelp* mutants (Jia et al. 2015; Nelissen et al. 2010; Wang et al. 2013, 2015; Xu et al. 2012). We previously reported that a mutation in AtELP2 either reduces histone acetylation levels, alters DNA methylation landscape in several defense genes including *NONEXPRESSER OF PR GENES1 (NPR1)*, *PR2*, and *PR5*, or both (Wang et al. 2013). The altered chromatin structure may be responsible for the delayed or decreased induction of *PR* genes after NAD⁺ treatment and pathogen infection in *eloAtelp* mutants (Figs. 1A and 4A) (DeFraia et al. 2010, 2013; Wang et al. 2013). Therefore, Elongator-mediated histone acetylation and one or both DNA demethylation and methylation may also contribute to exogenous NAD⁺-induced defense gene expression. Additionally, given the multifunctional nature of Elongator, this protein complex might also contribute to exogenous NAD⁺-activated defense signaling at the translational level by regulating tRNA modification (Esberg et al. 2006; Mehlgarten et al. 2010; Versées et al. 2010). On the other hand, similarly to Elongator, several proteins regulating histone acetylation and DNA methylation have been shown to function in plant defense responses (Agorio and Vera 2007; Choi et al. 2012; Devoto et al. 2002; Downen et al. 2012; Kim et al. 2008; López et al. 2011; Luna et al. 2012; Wang et al. 2010; Yu et al. 2013; Zhou et al. 2005; Zhu et al. 2011). It would be interesting to test whether these epigenetic regulators play a role in exogenous NAD⁺-mediated defense pathways by using *Arabidopsis* histone acetylation and DNA methylation mutants.

Thus far, we have identified Ca²⁺, SA, NPR1, two Mediator subunits (MED14 and MED16), and the Elongator complex as signaling components downstream of exogenous NAD⁺ (Fig. 4A to C) (Zhang and Mou 2009; Zhang et al. 2012, 2013). Since exogenously added NAD⁺ most likely acts in the extracellular compartment (Zhang and Mou 2009), these components should also function in endogenous eNAD-mediated signaling pathways. However, although we have shown that expression of the human NAD(P)-metabolizing coenzyme CD38 inhibits SAR induction in *Arabidopsis* (Zhang and Mou 2012), it is still uncertain whether endogenous eNAD functions as an elicitor in plants. In this regard, identification of plasma membrane-located eNAD receptors would help address the uncertainty.

MATERIALS AND METHODS

Plant material and growth conditions.

The wild type used was the *Arabidopsis thaliana* (L.) Heynh. Columbia (Col-0) ecotype and the mutant alleles used in this study are listed in Supplementary Table S1. The complementation line *Atelp3-10 35S:AtELP3* has previously been reported (DeFraia et al. 2013). Homozygous T-DNA insertion mutants were confirmed with primers flanking the T-DNA insertion sites (Supplementary Table S2) and the left border primers LBa1 and LB3 (Alonso et al. 2003; Sessions et al. 2002). *Arabidopsis* seeds were sown on autoclaved soil (Sunshine MVP, Sun Gro Horticulture, Agawam, MA, U.S.A.) and were vernalized at 4°C for 3 days. Plants were germinated and grown at approximately 23°C under a 16-h-light and 8-h-dark regime. Four-week-old plants were used for all experiments.

NAD treatment.

β-NAD sodium salt (Sigma-Aldrich, St. Louis) was dissolved in water solution (1 mM, pH 6.0, adjusted by 0.1 M NaOH). The solution was infiltrated into *Arabidopsis* leaves using a 1-ml needleless syringe as previously described (Zhang and Mou 2009). For determination of *PR* gene expression, infiltrated leaves were collected for RNA extraction 16 h after treatment or at indicated time points. For the disease resistance test, 5 h after

NAD⁺ treatment, the infiltrated leaves were inoculated with the bacterial pathogen *P. syringae* pv. *maculicola* ES4326.

Pathogen infection.

Inoculation of plants with *P. syringae* pv. *maculicola* ES4326 was performed by pressure infiltration with a 1-ml needleless syringe, as described previously (Clarke et al. 1998). For the susceptibility test, a suspension of *P. syringae* pv. *maculicola* ES4326 (optical density at 600 nm [OD₆₀₀] = 0.0001) was used, whereas for the NAD⁺-induced resistance assay, a suspension of *P. syringae* pv. *maculicola* ES4326 (OD₆₀₀ = 0.001) was used. Pathogen growth in the inoculated leaves was determined 2 to 3 days postinoculation.

RNA analysis.

RNA extraction, reverse transcription, and qPCR analysis were carried out as previously described (An and Mou 2012). The primers used for real-time qPCR analyses were listed in DeFraia et al. (2010) and Supplementary Table S3.

Statistical methods.

Statistical analyses were performed using data analysis tools in Excel, from Microsoft Office 2004 for Macintosh (Student's *t* test: two samples assuming unequal variances), and the one-way and two-way analysis of variance in Prism 5.0b (GraphPad Software, La Jolla, CA, U.S.A.).

Accession number.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ELO2/AtELP1 (At5g13680), AtELP2 (At1g49540), ELO3/AtELP3 (At5g50320), ELO1/AtELP4 (At3g11220), AtELP5 (At2g18410), AtELP6 (At4g10090), PR1 (At2g14610), PR2 (At3g57260), PR5 (At1g75040), and UBQ5 (At3g62250).

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center at The Ohio State University (Columbus, OH, U.S.A.) for SALK_004690, SALK_106485, SALK_079193, and SAIL_621_B08 seeds, and the European Arabidopsis Stock Centre at The University of Nottingham (Nottingham, U.K.) for GABI_555H06 and GABI_700A12 seeds. This work was supported by a grant from National Science Foundation (NSF IOS-0842716) awarded to Z. Mou.

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