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RESEARCH ARTICLE

Nicotinamide mononucleotide confers broad-spectrum disease resistance in plants

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Highlights

- Nicotinamide mononucleotide (NMN) confers broad-spectrum resistance in plants.
- NMN displays effectiveness within the concentration range of 50–600 μmol L^{-1}, with 75 μmol L^{-1} NMN exhibiting the most pronounced effect.
- NMN-induced plant immunity is not entirely dependent on nicotinamide adenine dinucleotide (NAD).

Abstract

Nicotinamide mononucleotide (NMN), a precursor in nicotinamide adenine dinucleotide (NAD) biosynthesis, has long been recognized for its pivotal role in medicine. Recent investigations have suggested its potential as a plant immunity inducer for controlling fungal diseases. However, whether NMN confers plant broad-spectrum resistance against diverse phytopathogens, and its underlying mechanisms remain ambiguous. In this study, we investigate the effect of NMN against multiple phytopathogens in tobacco. Our results demonstrate that tobacco pretreated with NMN exhibits enhanced resistance against *Ralstonia solanacearum* CQPS-1, *Pseudomonas syringae* DC3000 Δ*hopQ1-1*, *Phytophthora parasitica*, and tobacco mosaic virus (TMV). NMN displays effectiveness within the concentration range of 50–600 μmol L^{-1}, with 75 μmol L^{-1} NMN exhibiting the most pronounced effect. The impact of NMN pretreatment could persist for up to 10 days. Beyond tobacco, NMN pretreatment enhances disease resistance in tomato and pepper plants against diverse pathogens, underscoring NMN's capacity to confer broad-spectrum disease resistance in crops. Moreover, RT-qPCR analysis reveals that NMN significantly upregulates the expression of the pattern-triggered immunity (PTI) marker gene *NbCYP71D20* and salicylic acid (SA) marker gene *NbPR1a*. This suggests that NMN enhances plant resistance by inducing both PTI and SA-mediated immunity. Interestingly, the positive impact of NMN on plant disease resistance is not significantly compromised in both *NMN adenylyltransferase* (*NMNAT*)-silenced plants and NAD receptor mutant *lecrk-I.8*, suggesting the existence of NAD-independent signaling pathways for NMN-induced plant immunity. In conclusion, our study establishes that the bioactive molecule NMN imparts broad-spectrum disease resistance in plants, offering a simple, environmental-friendly, and promising strategy for safeguarding crops against diverse phytopathogens. These findings also provide valuable insights for future in-depth studies into the functional mechanisms of NMN.

Keywords: nicotinamide mononucleotide, immunity induction, broad-spectrum resistance, crops, NMNAT, LecRK-I.8

1. Introduction

Plants face continuous threats from diverse phytopathogens, including bacteria, fungi, oomycetes, and viruses. These persistent attacks result in substantial losses every year

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in agricultural production, with a pronounced impact on developing countries in particular (Yang *et al.* 2022; Zhu *et al.* 2024). The predominant method of disease control has been the utilization of chemical pesticides, but their prolonged use raises concerns about potential environmental hazards and risks to human health (Asghar *et al.* 2016). Consequently, there is an urgent need for sustainable and eco-friendly approaches to bolster the plant defense system, thereby mitigating the economic impact of plant diseases. Recognizing this challenge, the pursuit of green and effective methods to induce plant immunity has gained increasing attention. These strategies aim to minimize the influence of phytopathogens on agricultural production without causing adverse effects (Zhu *et al.* 2024). Plant-derived metabolites are appealing for these approaches owing to their environmentally friendly characteristics and their ability to induce plant immunity.

Inducers of plant immunity serve to fortify plant defenses against phytopathogens by activating the plant immune system without causing pollution. The utilization of plant-derived immunity inducers is considered safe and environmentally friendly due to their biodegradability (Choi *et al.* 2014; Burketova *et al*. 2015). Some immunity inducers are recognized or detected by plant receptors (Liang and Zhou 2018). Subsequently, these receptors transmit signals to downstream components through multiple signaling pathways. Ultimately, the downstream signal pathways enhance resistance against phytopathogens by triggering various defense responses. These responses include cellular Ca^{2+} influx, reactive oxygen species burst, salicylic acid (SA) production, hypersensitive response, expression of defense-related genes, callose accumulation, and more (Tang *et al.* 2017; Zhou and Zhang 2020; Zhu *et al.* 2024).

Nicotinamide mononucleotide (NMN) has garnered attention in the field of human medicine and healthcare due to its remarkable properties and potential therapeutic applications. This naturally occurring compound is found in various dietary sources such as vegetables, fruits, milk, and meat (Mills *et al.* 2016; Trammell *et al.* 2016). As an intermediate in the synthesis of nicotinamide adenine dinucleotide (NAD), a crucial coenzyme integral to metabolic processes, NMN plays a pivotal role in cellular energy production. Research demonstrates NMN's significance in addressing NAD deficiency, and its potential impact on aging-related conditions and diverse diseases. Studies suggest that NMN supplementation may contribute to anti-aging effects and overall health benefits (Hou *et al.* 2018; Okabe *et al.* 2019). In the field of medicine, NMN has exhibited positive effects in treating a variety of diseases, including age-induced type 2 diabetes, Alzheimer's disease, cerebral and cardiac ischemia, as well as corneal injuries (Yoshino *et al.* 2011; Mills *et al.* 2016; Hong *et al.* 2020; Nadeeshani *et al.* 2022). The versatility of NMN in influencing diverse physiological processes has positioned it as a subject of interest for further exploration and potential therapeutic interventions.

Recent research has indicated NMN's potential in eliciting disease resistance against fungal phytopathogens in barley, wheat, and Arabidopsis (Miwa *et al.* 2017; Sidiq *et al.* 2021). A critical inquiry arises regarding whether NMN's impact is specifically targeted at fungal diseases or if it exhibits a broader spectrum of effectiveness. In terms of the functional mechanisms, researchers have discovered that NMN treatment promotes the accumulation of antimicrobial compounds such as trigonelline, cystothiazole A, and picolinic acid in wheat (Sidiq *et al.* 2021). Notably, the enhanced resistance attributed to NMN is accompanied by the accumulation of NAD in Arabidopsis leaves, which has been reported as a plant defense activator (Pétriacq *et al.* 2013, 2016). NAD, derived from NMN and ATP through the action of NMN adenylyltransferase (NMNAT) (Cantó *et al.* 2015), can activate the plant immune response by binding to its receptor LecRK-I.8, a lectin receptor kinase (LecRK) (Mou 2017; Wang *et al.* 2017). NMNAT serves as the rate-limiting enzyme in NAD biosynthesis, and its over-expression has been linked to enhanced disease resistance in Arabidopsis (Miwa *et al.* 2017). These findings indicate that NMN potentially induces plant immunity by promoting NAD synthesis. RNA-seq data further supports this by indicating that, similar to NAD, NMN enhances plant immunity through the promotion of SA pathway while suppressing ethylene/jasmonic acid (JA) pathway in Arabidopsis (Miwa *et al.* 2017; Wang *et al.* 2017). However, intriguingly, the concentration of NMN and antimicrobial compounds in plants remains significantly elevated, while the content of NAD remains unchanged seven days after pretreatment with NMN in wheat (Sidiq *et al.* 2021). This observation implies that NMN may trigger plant immunity through an NAD-independent pathway. Therefore, further research is imperative to elucidate whether NMN treatment enhances plant disease resistance through its conversion to NAD and to decipher the intricacies of its functional mechanisms.

In this study, we delved into the role of NMN in inducing plant immunity against a diverse array of pathogens. Contrary to previous reports focusing solely on fungal pathogens, our results demonstrated that NMN exhibits the capacity to enhance tobacco resistance against bacterial, oomycete and virus pathogens. Notably, we established that the effectiveness of NMN pretreatment in triggering plant immunity extends for approximately 10 days. Intriguingly, our study revealed a broad effective concentration range for NMN, spanning from 25–600 μmol L^{-1}, with the lower concentration 75 μmol L^{-1} proving more efficacious than higher concentrations. More importantly, we assessed the application potential of NMN in conferring broad-spectrum resistance in crops by employing pathosystems involving *P. capsici*-pepper, *P. parasitica*-tomato, and *R. solanacearum*-tomato. Our findings underscored the significant promise of NMN as a tool to enhance crop resilience against diverse pathogens. Furthermore, an exploration of the functional mechanism revealed that NMN induces both pattern-triggered immunity (PTI) and SA signaling pathways. Notably, NMN-induced plant immunity remained in *NMNAT*-silenced plants and mutants lacking NAD receptor LecRK-I.8, suggesting the existence of independence from the traditional NAD-dependent immune signaling pathways. Our findings not only present a promising strategy for augmenting broad-spectrum disease resistance in plants but also lay a foundation for a deeper

understanding of the intricate mechanisms underlying NMN-induced plant immunity.

2. Materials and methods

2.1. Plant materials and bacterial strains

Arabidopsis thaliana, *Nicotiana benthamiana*, *Solanum lycopersicum*, and *Capsicum annuum* plants utilized in this study were cultivated under controlled conditions at 24°C with a photoperiod of 16 h light/8 h dark. *Agrobacterium tumefaciens* strain GV3101 with tobacco mosaic virus (TMV)-GFP was grown on LB agar plates with appropriate antibiotics at 28°C. *Ralstonia solanacearum* strains CQPS-1 and GMI1000 were cultured at 28°C on Bacto-agar and glucose (BG) medium. *Pseudomonas syringae* strains DC3000 Δ*hopQ1-1* and DC3000 were propagated in LM medium at 28°C. *Phytophthora parasitica* and *Phytophthora capsici* were cultured at 28°C in a V8 Juice Broth medium.

2.2. Vector construction

A 300-bp DNA fragment corresponding to the *NbNMNAT* gene was successfully cloned into the pTRV2 vector, and the integrity of the sequence was verified through Sanger sequencing (Sangon Biotech, Shanghai, China). For gene silencing, a mixture containing *Agrobacterium tumefaciens* carrying pTRV2-NMNAT and pTRV1 in a 1:1 ratio was prepared in infiltration buffer (150 mol L^{-1} acetosyringone, 10 mmol L^{-1} $MgCl_2$, and 10 mmol L^{-1} MES, pH 5.6) to achieve a final OD_{600} of 0.4. This mixture was utilized to silence the *NbNMNAT* gene, while a combination of pTRV2-GFP and pTRV1 vector was used as a negative control. Two-week-old *N. benthamiana* plants were subjected to gene silencing, and after an additional 2 weeks, the silenced plants were used for subsequent assays. The gene-specific primers (Appendix A) were used for amplification.

2.3. NMN pretreatment

NMN (Shanghai Aladdin Biochemical Technology, China) was dissolved in water at various concentrations, supplemented with 0.01% Silwet L-77. To assess the impact of NMN on plant resistance, each plant was sprayed with 5 mL NMN or water, and the plants were kept in moisture overnight. After 24 h, these treated plants were employed for the subsequent experiments. In the soil drenching inoculation and stem inoculation assay, plants were subjected to four rounds of NMN spraying at 48-h intervals. Following an additional 24 h, these plants were used for the soil-drenching inoculation or stem-inoculation procedure.

2.4. Bacterial inoculation assay

The type III effector HopQ1 is recognized by Roq1 in *N. benthamiana* (Schultink *et al.* 2017), and thus the DC3000 mutant lacking HopQ1 (DC3000 Δ*hopQ1-1*) was employed for the inoculation assay. Additionally, *R. solanacearum* strain CQPS-1, a virulent strain isolated from tobacco, was also employed (Liu *et al.* 2017). The inoculation assay of DC3000 Δ*hopQ1-1* and CQPS-1 was performed according to the previously established protocol (Ouyang *et al.* 2023).

The *R. solanacearum* soil drenching inoculation was carried out as previously described with a minor modification (Gao *et al.* 2013; Cao *et al.* 2022). After NMN pretreatment, 20 tobacco or tomato plants were drenched with *R. solanacearum* strain CQPS-1 or GMI1000 (OD_{600}=0.1) to evaluate the impact of NMN on plant disease resistance. Disease severity was assessed based on the disease index, which gauged wilting symptoms on a scale from 0 to 4 according to the previously described standard (Gao *et al.* 2013). Survival analysis was analyzed based on the disease index, with the disease scoring transformed into binary data with the following criteria: a disease index exceeding 2 was categorized as '1', while others were designated as '0' for each specific time point.

2.5. *Phytophthora parasitica* and *P. capsici* inoculation

Mycelial plugs were aseptically transferred from the original *Phytophthora* plate to a fresh V8 agar medium and cultured for three to 4 days at 28°C. In preparation for inoculation, an inoculation tray was set up by adding 80 mL of sterile water to three layers of filter paper. Subsequently, six leaves, each with 2 cm-long petioles cut from 4-week-old plants, were wrapped in cotton and positioned in the inoculation tray for the impending inoculation. Mycelial plugs of *Phytophthora* were carefully placed on the back of each leaf. To ensure thorough contact, 18 μL sterile water was added to the contact surface between the mycelial plug and the leaf. The inoculation tray was then sealed and covered with plastic, maintaining a consistent temperature for 48 h in darkness. At 2 days post-inoculation (dpi), leaf lesions were observed under a handheld ultraviolet lamp, and the size of each lesion was quantified using ImageJ (http://rsb.info.nih.gov/ij). *Phytophthora parasitica* biomass assay was carried out as previously described (Qiang *et al.* 2021; Wen *et al.* 2021). For *Phytophthora* inoculation in pepper and tomato stems, the procedure closely followed a previously described protocol (Ahmed *et al.* 2000). Stem lesions were assessed at 4 dpi.

2.6. TMV-GFP infection assay

TMV-GFP infection assay was carried out according to Bai *et al.* (2022). The GFP intensity was observed and photographed under UV light, and then quantified using the ImageJ software under the same settings.

2.7. RT-qPCR analysis of defense-related gene expression

RNA extraction was carried out following the Sangon Biotech protocol with the Total RNA Extraction Kit (Sangon Biotech, Shanghai, China). Then, the reverse transcription of RNA samples was performed in a 20 μL reaction using the ABScript II cDNA First-Strand Synthesis Kit (ABclonal Technology, Wuhan, China). *NbEF1α* served as the internal control, and SYBR Green was utilized in qPCR to determine the relative expression levels of defense-related genes. Relative changes in gene transcript levels were quantified using the $2^{-\Delta\Delta Ct}$ method. The primers for RT-qPCR were listed in Appendix A.

2.8. Statistical analysis

Each experiment was performed with three biological

replicates. Mean values are presented along with their standard deviations (SD). Statistical analysis was performed using Student's *t*-test or Duncan's multiple range tests, with the confidence coefficients set at $P<0.05$, $P<0.01$, and $P<0.001$.

3. Results

3.1. NMN enhances plant resistance against multiple phytopathogens in tobacco

In recent studies, it has been demonstrated that application of 300 μmol L^{-1} NMN induces enhanced disease resistance against pathogenic fungi in barley, wheat, and Arabidopsis (Miwa *et al.* 2017; Sidiq *et al.* 2021). To extend these findings to pathogenic bacteria and oomycetes, we conducted experiments using 4-week-old tobacco leaves. Leaves were inoculated with *P. parasitica* after pretreatment with either water (control) or 300 μmol L^{-1} NMN. The lesion diameters in NMN-pretreated leaves decreased significantly compared to the water control (Fig. 1-A and B), implying that NMN pretreatment enhances tobacco resistance against *P. parasitica*. Similarly, the bacterial colonization of *P. syringae* strain DC3000 Δ*hopQ1-1* and *R. solanacearum* strain CQPS-1 in 4-week-old tobacco leaves pretreated with NMN were less than those treated with water (Fig. 1-C), indicating that NMN also confers resistance against pathogenic bacteria. Additional soil-drenching inoculation assays demonstrated that NMN-pretreated tobacco plants exhibited a significantly lower disease index and higher survival rate upon CQPS-1 infection (Fig. 1-D and E). This result substantiates the positive impact of NMN on tobacco resistance to bacteria. The resistance observed in the aboveground parts of root-infected plants suggest that NMN may stimulate systemic acquired resistance (SAR) in tobacco plants. Besides, upon TMV-GFP infection, NMN pretreatment significantly suppressed the GFP fluorescence intensity in tobacco compared to the water control (Fig. 1-F and G), indicating that NMN pretreatment promotes plant resistance to TMV. *GFP* relative expression confirmed less TMV population after NMN pretreatment (Fig. 1-H). Taken together, these findings point towards NMN's capacity to confer broad-spectrum resistance in tobacco plants against multiple phytopathogens, including not only fungi, but also bacteria, oomycetes and viruses.

3.2. NMN at various concentrations activates plant resistance against *P. parasitica* in tobacco

To determine the effective concentration range of NMN, we investigated its impact on disease resistance of tobacco leaves across concentrations ranging from 0 to 600 μmol L^{-1}. A total of 24 hours post-NMN pretreatment, tobacco leaves were inoculated with *P. parasitica*, and the lesion diameters were observed at 2 dpi. Results showed that

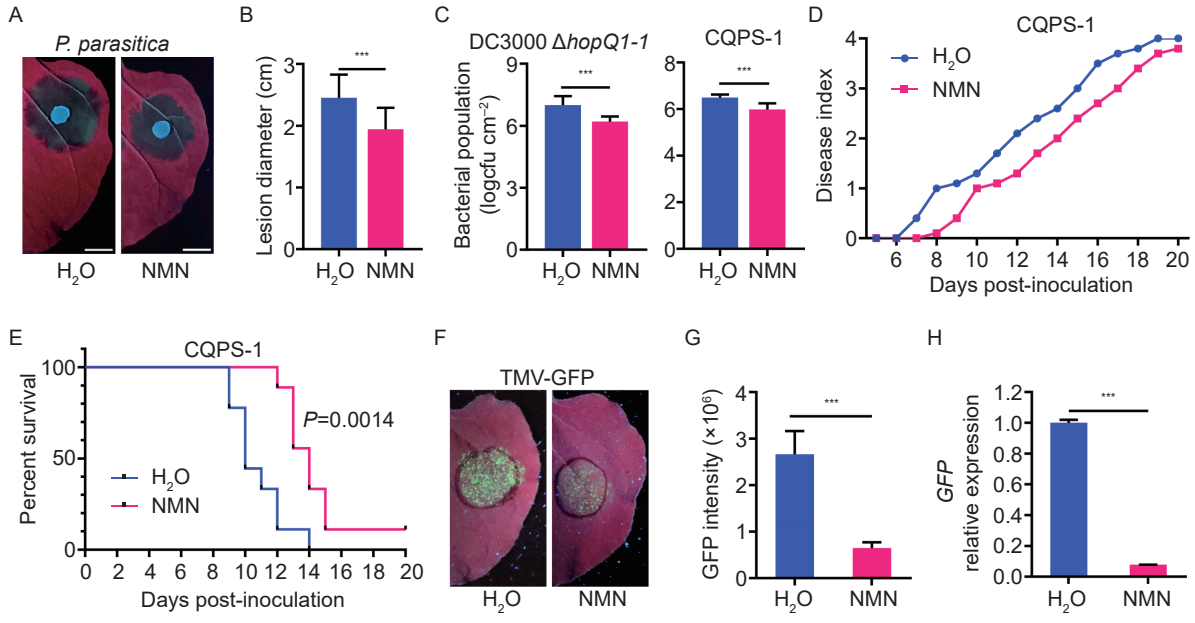


Fig. 1 Nicotinamide mononucleotide (NMN) enhances plant resistance against multiple phytopathogens in tobacco. A, effect of NMN pretreatment on tobacco resistance to *Phytophthora parasitica*. The 300 μmol L^{-1} NMN-pretreated leaves were inoculated with *P. parasitica* at 24 hours post-NMN treatment. Water was used as control. Phenotypes of *P. parasitica*-inoculated leaves were observed at 2 days post-inoculation (dpi). Bar: 10 mm. B, quantification of lesion size in A. C, bacterial population in leaves of NMN-pretreated tobacco plants. After treatment with 300 μmol L^{-1} NMN, the treated leaves were inoculated with DC3000 Δ*hopQ1-1* or CQPS-1. Bacterial colonization was measured at 2 dpi. D, disease index of tobacco plants pretreated with water or NMN after soil-drenching inoculation of CQPS-1. Twenty plants per treatment were used for one biological replicate. E, survival analysis of the data in D. Statistical analysis was performed using a Log-rank (Mantel_x0002_Cox) test and the corresponding *P*-value is shown. F, effect of NMN pretreatment on tobacco resistance to tobacco mosaic virus (TMV). The 300 μmol L^{-1} NMN-pretreated leaves were infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying the TMV-GFP vector at 24 hours post-NMN treatment. Phenotypes of *P. parasitica*-inoculated leaves were observed at 60 hours post-inoculation (hpi) under UV light. G, quantification of GFP intensity in F. H, *GFP* relative expression at 48 hpi measured by RT-qPCR. Data are shown as the mean±SD (B and G, $n=12$; C, $n=6$; H, $n=3$). *** indicates significant differences at $P<0.001$.

leaves pretreated with NMN ranging from 50 to 600 μmol L^{-1} exhibited significantly smaller lesions and less *P. parasitica* biomass compared to the water control, with the most pronounced effect observed at 75 μmol L^{-1} (Fig. 2). This result demonstrates that NMN concentrations between 50 and 600 μmol L^{-1} are effective in improving plant disease resistance, with the optimal application concentration identified as 50–75 μmol L^{-1}, considering both effectiveness and cost. Here, we selected 75 μmol L^{-1} NMN for the following experiments. We inoculated tobacco leaves with *P. parasitica* at different time points (1, 4, 7, 10, or 13 days) after pretreatment with 75 μmol L^{-1} NMN and found that the enhanced plant resistance effect of NMN pretreatment persisted for at least ten days (Appendix B). Given the more significant effect within the initial 7 days, pretreating plants 1 week in advance may be deemed suitable for practical field applications.

3.3. NMN induces plant resistance in tomato and pepper

Phytophthora parasitica and *P. capsici* are prevalent plant pathogens known to inflict severe diseases in tobacco, tomato, pepper, and various other crops, often leading to crop failure (Blaker and Hewitt 1987; Granke *et al.* 2012; Meng *et al.* 2014). In this study, we extended our evaluation of NMN's impact on crop resistance by employing the *P. capsici*-pepper and *P. parasitica*-tomato pathosystems. Four-week-old tomato and pepper plants were subjected to a spray of 75 μmol L^{-1} NMN. After 24 h, the leaves of pepper and tomato plants were inoculated with *P. capsici* and *P. parasitica*, respectively. Results showed that the lesion diameters of NMN-pretreated pepper leaves (Fig. 3-A and B) and tomato leaves (Fig. 3-C and D) were significantly reduced compared to the water control, indicating that NMN pretreatment enhances the disease resistance of pepper and tomato plants. The milder disease symptoms and the shorter stem lesions in stem-infected pepper and tomato plants pretreated with NMN further affirm the positive effect of NMN in inducing immunity (Fig. 3-E–H). Furthermore, we investigated the impact of NMN on crop disease resistance using the *R. solanacearum* GMI1000-tomato pathosystem (Peeters *et al.* 2013). Tomato plants pretreated with NMN exhibited a compromised disease index and higher percent survival compared with the water control (Fig. 3-G–I), suggesting increased resistance of NMN-pretreated tomato plants against GMI1000. These findings demonstrate NMN's potential to confer resistance against multiple phytopathogens, suggesting broad applicability in enhancing crop immunity.

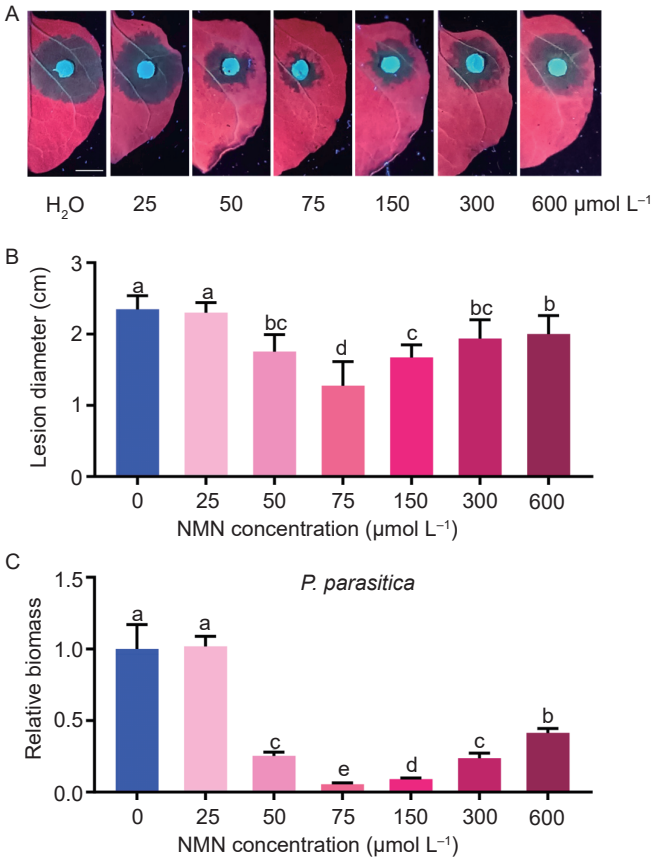


Fig. 2 The effective concentration range of nicotinamide mononucleotide (NMN). The 300 μmol L^{-1} NMN-pretreated leaves were inoculated with *Phytophthora parasitica* at 24 hours post-NMN treatment. Water was used as control. A, phenotypes of *P. parasitica*-inoculated leaves at 2 days post-inoculation (dpi). Bar: 10 mm. B, quantification of lesion size in A. C, *P. parasitica* biomass was quantitated by RT-qPCR at 48 hours post-inoculation (hpi). *Nicotiana benthamiana NbEF1α* and *P. parasitica UBC* were used to determine *P. parasitica* biomass. Data are shown as the mean±SD (B, n=12; C, n=3). The same letters indicate no significant difference at P<0.05.

3.4. NMN induces the expression of PTI- and SA-related genes

To explore the mechanisms underlying the stimulation of plant resistance by NMN, we detected the expression of key marker genes associated with PTI, SA, and JA pathways in tobacco leaves pretreated with NMN. The PTI marker gene, *NbCYP71D20* (Heese *et al.* 2007), the SA marker gene, *NbPR1a* (Dean *et al.* 2005), and the JA marker gene, *NbPDF1.2* (Zhu *et al.* 2014), were selected for analysis. At 12 h post-NMN treatment, the relative expression levels of *NbCYP71D20* and *NbPR1a* in NMN-treated plants were significantly elevated (Fig. 4-A). Notably, the expression of *NbPR1a* continued to increase at 24 h, while that of *NbCYP71D20* decreased compared to the 12 h time point. These results indicate that NMN induces both PTI and SA-mediated immunity in tobacco plants. When tobacco plants pretreated with NMN were subsequently inoculated with DC3000 Δ*hopQ1-1*, the expression levels of *NbCYP70D20* and *NbPR1a* at 24 h post-NMN treatment were extremely higher than in the control (Fig. 4-B), further supporting the conclusion that NMN improves plant disease resistance through the induction of PTI and SA signaling, particularly the latter. In contrast, the expression of the JA marker gene *NbPDF1.2* did not exhibit a significant response to NMN treatment, irrespective of pathogen infection. This result suggests that NMN-induced immunity may not involve the JA signaling pathway.

3.5. NMN-induced plant resistance is at least partially independent of its conversion to NAD

NMN is a precursor of NAD (Yamamoto *et al.* 2014;

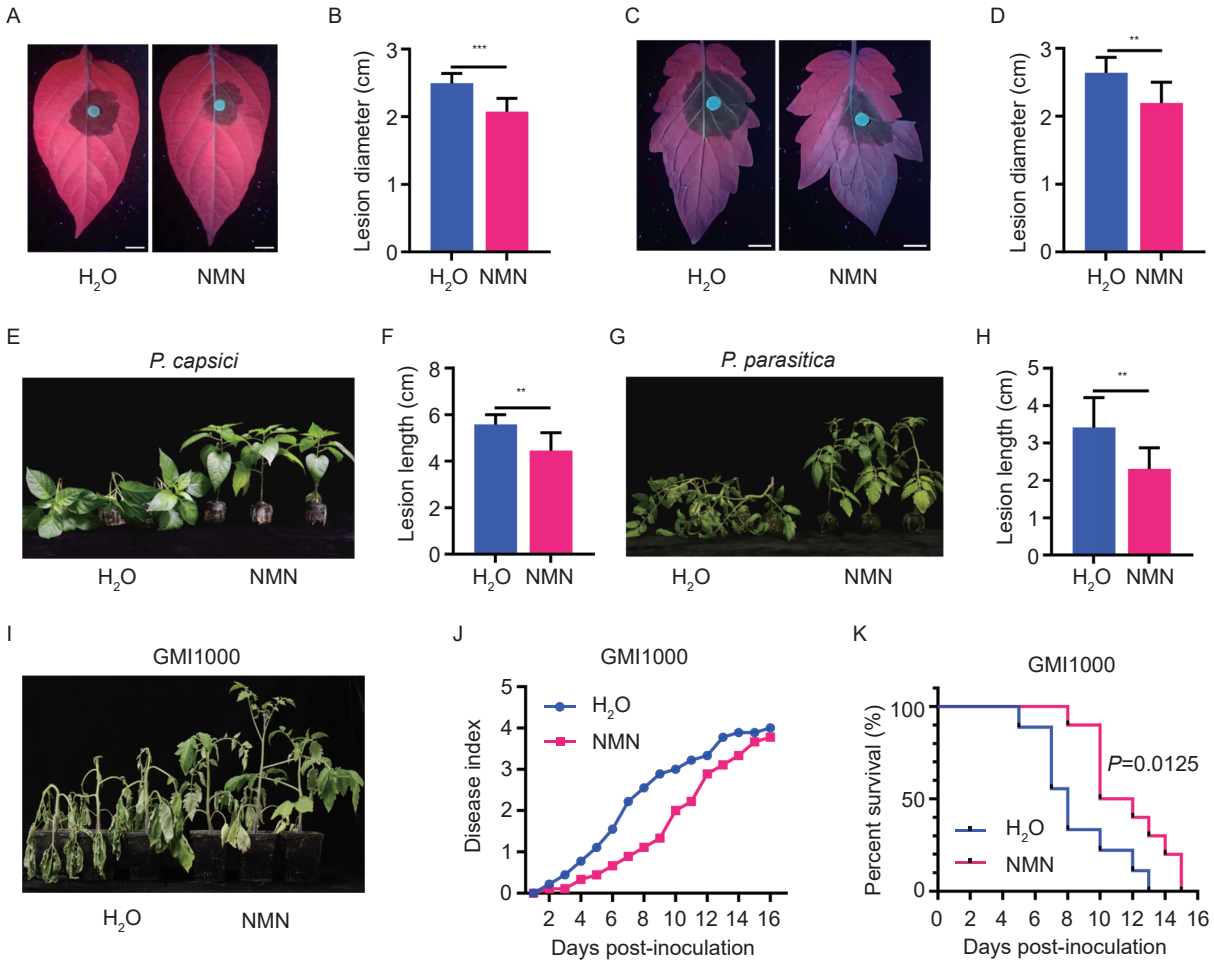


Fig. 3 Nicotinamide mononucleotide (NMN) enhances disease resistance in tomato and pepper plants. A, NMN enhances resistance of pepper leaves to *Phytophthora capsici*. The 75 μmol L^{-1} NMN-pretreated pepper leaves were inoculated with *P. capsici* at 24 hours post-NMN treatment. Phenotypes of *P. capsici*-inoculated leaves were observed at 2 days post-inoculation (dpi). B, quantification of lesion size in A. C, NMN enhances resistance of tomato leaves to *P. parasitica*. The 75 μmol L^{-1} NMN-pretreated tomato leaves were inoculated with *P. parasitica*. Phenotypes of *P. parasitica*-inoculated leaves were observed at 2 dpi. D, quantification of lesion size in C. E, NMN enhances resistance of pepper plants to *P. capsici*. The 75 μmol L^{-1} NMN-pretreated pepper plants were stem-inoculated with *P. capsici* at 24 hours post-NMN treatment. Phenotypes of *P. capsici*-inoculated plants were observed at 4 dpi. F, quantification of lesion length in stems in E. G, NMN enhances resistance of tomato plants to *P. parasitica*. The 75 μmol L^{-1} NMN-pretreated tomato plants were stem-inoculated with *P. parasitica* at 24 hours post-NMN treatment. Phenotypes of *P. parasitica*-inoculated plants were observed at 4 dpi. The red lines on stems indicate the origin and the end of the lesions. H, quantification of lesion length in stems in G. I–K, soil-drenching inoculation of GMI1000 in tomato plants treated with water or 75 μmol L^{-1} NMN. I, plant phenotypes at 14 dpi. J, disease index of tomato plants pretreated with water or NMN after soil-drenching inoculation of GMI1000. Twenty plants per treatment were used for one biological replicate. K, survival analysis of the data in J. Statistical analysis was performed using a Log-rank (Mantel_x0002_Cox) test and the corresponding *P*-value is shown. Bar: 10 mm. Data are shown as the mean±SD (B and D, n=12; F and H, n=8). ** and *** indicate significant differences at P<0.01 and P<0.001, respectively.

Nadeeshani *et al.* 2022). Previous studies indicated that both NMN and NAD levels increased following NMN treatment (Miwa *et al.* 2017). NAD also plays a role in enhancing plant immunity through the SA signaling pathway (Wang *et al.* 2017). However, the exact mechanism by which NMN treatment exerts its effects, whether through increased NAD biosynthesis, remains unclear. In this study, we silenced the gene encoding NMNAT, the key enzyme responsible for converting NMN into NAD in tobacco (Fig. 5-A). RT-qPCR analysis confirmed efficient silencing of *NMNAT* (Fig. 5-B). Subsequently, *NMNAT*-silenced and *GFP*-silenced plants were sprayed with NMN and then inoculated with DC3000 Δ*hopQ1-1* after 24 h of NMN treatment. Results showed a significant increase in bacterial colonization of DC3000 Δ*hopQ1-1* in *NMNAT*-silenced plants compared to *GFP*-silenced plants (Fig. 5-C), implying the involvement of NMNAT in plant defense response. When compared to the water control, the bacterial colonization in the leaves of *GFP*-silenced or *NMNAT*-silenced plants treated with NMN was significantly reduced by 7.0 and 5.2%, respectively (Fig. 5-C). These results indicate that *NMNAT* silencing slightly, albeit not significantly, suppresses the effect of NMN. Additionally, the promotive effect of NMN on *PR1a* expression was not significantly affected in *NMNAT*-silenced plants compared to *GFP*-silenced plants (Fig. 5-D). These results suggest that NMN-induced plant immunity may not completely depend on its conversion to NAD.

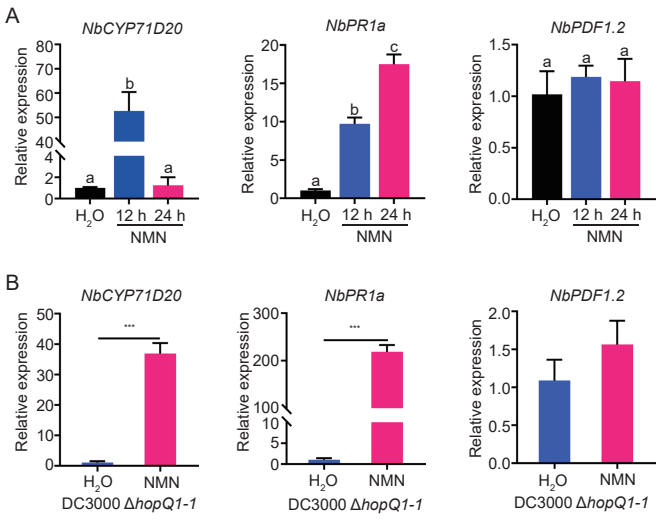


Fig. 4 Nicotinamide mononucleotide (NMN) upregulates the expression of defense-related genes. A, relative expression of *NbCYP71D20*, *NbPR1a*, and *NbPDF1.2* in tobacco leaves treated with NMN for 12 and 24 hours. B, 75 μmol L^{-1} NMN-pretreated tobacco leaves were inoculated with DC3000 Δ*hopQ1-1* at 24 hours post-NMN treatment. The relative expression of *NbCYP71D20*, *NbPR1a*, and *NbPDF1.2* in leaves at 24 hours post-inoculation. Data are shown as the mean±SD (n=3). Different letters indicate significant differences at P<0.01, and *** indicate significant differences at P<0.001.

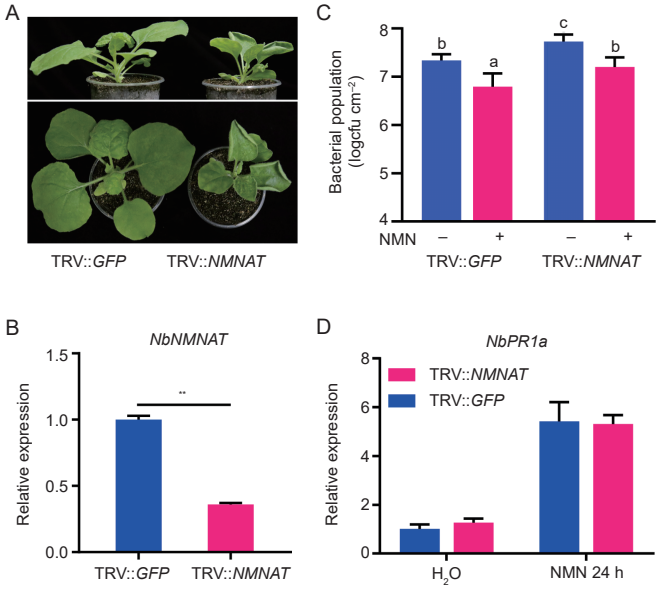


Fig. 5 Effect of NMNAT silencing on nicotinamide mononucleotide (NMN)-induced plant resistance against DC3000. A, phenotype of 5-week-old *TRV::GFP* and *TRV::NMNAT* tobacco plants. B, relative expression level of *NbNMNAT* in *TRV::GFP* and *TRV::NMNAT* plants. C, the 75 μmol L^{-1} NMN-pretreated leaves of *TRV::GFP* and *TRV::NMNAT* were inoculated with DC3000 Δ*hopQ1-1* at 24 hours post-NMN treatment. Bacterial colonization was measured at 2 days post-inoculation (dpi). Data are shown as the mean±SD (n=6). D, relative expression level of *PR1a* in *TRV::GFP* and *TRV::NMNAT* plants at 24 hours post-NMN treatment. Data are shown as the mean±SD (n=3). Different letters indicate significant difference at P<0.05. ** indicates significant differences at P<0.01.

3.6. Plants sense NMN independently on LecRK-I.8

Previous studies demonstrated that LecRK-I.8 acts as the receptor of NAD (Wang *et al.* 2017). *lecrk-I.8* mutants treated with NAD concentrations below 400 μmol L^{-1} exhibited no discernible difference from those treated with water (Wang *et al.* 2017). Here, we investigated the impact of NMN on *lecrk-I.8* mutants. The results of bacterial colonization and leaf phenotype showed that, in comparison to the water control, both Col-0 and *lecrk-I.8* treated with NMN displayed enhanced resistance against DC3000 (Fig. 6). The similarity in the responses of Col-0 and *lecrk-I.8* to NMN suggest that LecRK-I.8 may not significantly affect the plant's response to NMN. This finding underscores the presence of alternative pathways, independent of NAD signaling, that mediate NMN-induced immunity.

4. Discussion

NMN, renowned for its role as a precursor to NAD and its potential therapeutic applications, has gained significantly attention in the medical field. Because of its involvement in cellular energy metabolism and its potential anti-aging properties (Hou *et al.* 2018; Okabe *et al.* 2019), NMN has become a focal point of research in pharmaceutical and anti-aging studies. However, despite its prominence in medicine, the utilization of NMN in the field of plant science has received relatively limited attention. While previous studies have indicated its efficacy in enhancing plant resistance against pathogenic fungi in specific plant species (Miwa *et al.* 2017; Sidiq *et al.* 2021), comprehensive investigations into its applications across diverse plant-pathogen interactions remain scarce. Our study contributes to this gap by demonstrating that NMN can enhance plant resistance in tobacco against various pathogens, including DC3000 Δ*hopQ1-1*, CQPS-1, *P. parasitica*, and TMV (Fig. 1). Furthermore, NMN boosted the resistance of tomato plants against *P. parasitica* and GMI1000, and induced pepper resistance against *P. capsici* (Fig. 3). These findings indicate that NMN confers broad-spectrum resistance in plants, making it a promising candidate for practical application in the field.

The effective concentration of plant immunity inducers is a key factor in determining application costs, directly influencing their suitability for use in agricultural production. Previous studies showed that, among the tested concentrations (30, 300, and 3,000 μmol L^{-1}), 300 μmol L^{-1} NMN significantly alleviated disease symptoms of Arabidopsis leaves upon pathogen infection (Miwa *et al.* 2017). In the present study, a more detailed concentration range for NMN (25–600 μmol L^{-1}) was investigated, and all concentrations except 25 μmol L^{-1} proved effective in enhancing plant disease resistance in our experimental systems (Fig. 2). We noticed that the defense-promoting effect of NMN is dosage-dependent within a certain range. Specifically, within the range of 0–75 μmol L^{-1}, we observed a better defense enhancement effect in *N. benthamiana* with the increase of NMN concentration. However, concentrations exceeding 75 μmol L^{-1} of NMN may lead to side effects

in *N. benthamiana*. A similar phenomenon was observed when NMN was applied to Arabidopsis to promote resistance to *Fusarium graminearum* (Miwa *et al.* 2017). Taking cost considerations into account, we recommend application concentrations of 50–150 μmol L^{-1}, particularly 75 μmol L^{-1}, which showed the most pronounced effect. The lower effective concentration of NMN further suggest its substantial application potential. It is important to note that the optimal concentration of NMN may vary depending on the type of phytopathogen and plant species (Miwa *et al.* 2017). Therefore, further research is essential to verify the optimal concentration of NMN in different plant species before its practical application in the field. The defense-promoting effect of NMN persisted for at least 10 days, indicating that pretreating plants can effectively protect them from pathogen infection. The loss of this effect over a specific time period avoids overactivation of plant immunity, ensuring that the crop growth and yield will not be significantly impacted.

Recent research showed that, similar to NAD, NMN promotes SA signaling and inhibits JA signaling in Arabidopsis (Miwa *et al.* 2017; Wang *et al.* 2017). Consistent with these findings, our results showed that NMN significantly induced SA-mediated immunity in tobacco, as indicated by the markedly enhanced transcription of the SA marker gene, *NbPR1a* (Dean *et al.* 2005) (Fig. 4), thereby confirming the induction activity of NMN on SA signaling. Additionally, the PTI marker gene, *CYP71D20* (Heese *et al.* 2007), exhibited a substantial upregulation in NMN-pretreated leaves upon pathogen infection, suggesting that NMN also induces PTI. However, NMN did not influence the transcription of the JA marker gene, *PDF1.2* (Zhu *et al.* 2014) (Fig. 4), indicating that the mechanism of NMN-induced plant immunity in tobacco may not be completely the same as that in Arabidopsis. These results collectively demonstrate that NMN enhances plant defense against pathogens through inducing PTI and SA signaling pathways, providing valuable insights for further in-depth study.

In both animals and plants, NMN promotes the *in vivo* synthesis of NAD (Miwa *et al.* 2017; Wang *et al.* 2017; Li *et al.* 2023). Both exogenous NAD and NMN could induce the transcription of the *PR1a* gene (Miwa *et al.* 2017; Wang *et al.* 2017). It is likely that NMN functions by promoting NAD synthesis. However, in wheat, despite the significant upregulation of NMN concentration and antimicrobial compounds, the NAD content remains unchanged at 7 days post-NMN treatment (Sidiq *et al.* 2021). Whether NMN induces plant immunity solely dependent on NAD remains unclear. NMNAT, the key enzyme converts NMN to NAD, exhibits a strong defense ability against pathogens upon overexpression in plants (Miwa *et al.* 2017). Consistently, here, when we silenced *NMNAT*, the plant's resistance was reduced. Interestingly, in *NMNAT*-silenced plants, NMN still induced immunity in tobacco plants and the transcription of *PR1a* (Fig. 5). This suggests that NMN-enhanced plant immunity is not entirely dependent on NAD synthesis.

The earlier findings indicated that plants can perceive exogenous NAD through LecRK-I.8 (Wang *et al.* 2017). The plant's response to exogenous NAD was weakened, and the lower concentration of NAD failed to induce plant defense response and *PR1* transcription in the *lecrk-I.8* mutant. NAD is synthesized through NMN and ATP (Nadeeshani *et al.* 2022), and ATP does not bind to LecRK-I.8 (Wang *et al.* 2017). As the precursor of NAD, NMN shares structural similarities with NAD. It remains possible that LecRK-I.8 can bind to NMN. However, our results demonstrated that even at low concentrations, exogenous NMN still effectively induced plant immunity in *lecrk-I.8* (Fig. 6). These results indicate that plant perception of NMN may not depend on LecRK-I.8, further suggesting the alternative signaling pathway of NMN independent of NAD. It holds promise for the future research to delve deeper into how NMN is perceived in plants.

5. Conclusion

In this study, we explored the significance of NMN in eliciting plant immunity against pathogens. The application of NMN on tobacco demonstrated a notable reduction in *Phytophthora* disease symptoms in tobacco leaves, evident 10 days post-treatment. The observed broad-spectrum resistance extended beyond tobacco, encompassing other

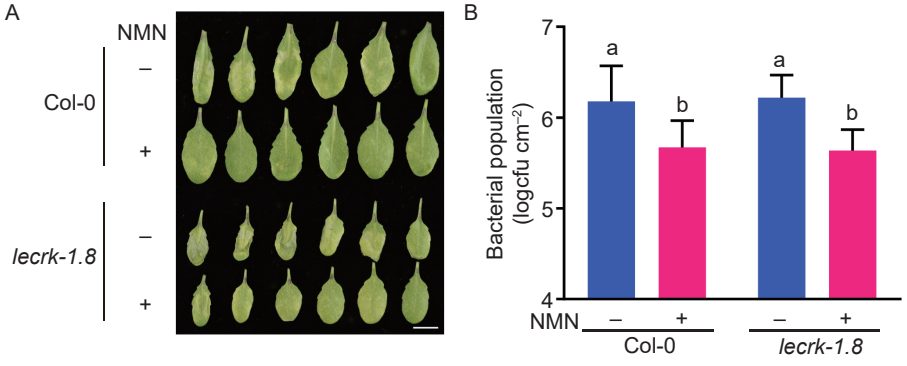


Fig. 6 Effect of nicotinamide mononucleotide (NMN) treatment on *lecrk-I.8* mutants. Water or 300 μmol L^{-1} NMN was sprayed onto the leaves of wild-type Arabidopsis (Col-0) and *lecrk-I.8* mutants, incubating for 24 hours. The treated leaves were then inoculated with DC3000. A, leaf phenotypes at 2 days post-inoculation (dpi). Bar: 10 mm. B, quantification of bacterial colonization in A. Data are shown as the mean±SD (n=6). Different letters indicate significant difference at P<0.05.

crops. Subsequent investigations implied that NMN-induced plant immunity is not entirely dependent on NAD. Our results not only certify that NMN confers broad-spectrum resistance in plants but also establish a groundwork for unraveling the mechanisms underlying NMN-induced plant immunity.

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Declaration of competing interest

The authors declare that they have no conflict interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used Grammarly and Wordvice AI to improve the readability, grammar, and language clarity of the manuscript. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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