Research Paper

ALY proteins participate in multifaceted Nep1<sub>Mo</sub>-triggered responses in Nicotiana benthamiana and Arabidopsis thaliana

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Abstract

Previously, it was found that Nep1<sub>Mo</sub> (a Nep1-like protein from Magnaporthe oryzae) could trigger a variety of plant responses, including stomatal closure, hypersensitive cell death (HCD), and defence-related gene expression, in Nicotiana benthamiana. In this study, it was found that Nep1<sub>Mo</sub>-induced cell death could be inhibited by the virus-induced gene silencing of NbALY916 in N. benthamiana. NbALY916-silenced plants showed impaired Nep1<sub>Mo</sub>-induced stomatal closure, decreased Nep1<sub>Mo</sub>-induced production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitric oxide (NO) in guard cells, and reduced Nep1<sub>Mo</sub>-induced resistance against Phytophthora nicotianae. It also found that the deletion of AtALY4, an orthologue of NbALY916 in Arabidopsis thaliana, impaired Nep1<sub>Mo</sub>-triggered stomatal closure, HCD, and defence-related gene expression. The compromised stomatal closure observed in the NbALY916-silenced plants and AtALY4 mutants was inhibited by the application of H<sub>2</sub>O<sub>2</sub> and sodium nitroprusside (an NO donor), and both Nep1<sub>Mo</sub> and H<sub>2</sub>O<sub>2</sub> stimulated guard cell NO synthesis. Conversely, NO-induced stomatal closure was found not to require H<sub>2</sub>O<sub>2</sub> synthesis; and NO treatment did not induce H<sub>2</sub>O<sub>2</sub> production in guard cells. Taken together, these results demonstrate that the NbALY916/AtALY4–H<sub>2</sub>O<sub>2</sub>–NO pathway mediates multiple Nep1<sub>Mo</sub>-triggered responses, including stomatal closure, HCD, and defence-related gene expression.

Key words: ALY, disease resistance, hypersensitive response, Nep1, nitric oxide, stomatal closure.

Introduction

Plants have evolved multiple defence mechanisms against phytopathogens, including the hypersensitive response (HR) and stomatal closure (Dangl et al., 1996; Lam et al., 2001; Mur et al., 2008). The HR is considered to be a form of localized hypersensitive cell death (HCD) that results in the formation of necrotic lesions around sites of infection (Hammond-Kosack and Jones, 1996; Greenberg and Yao, 2004). HCD is initiated upon plant–pathogen recognition. In addition to classic gene–gene interactions, the binding of selected pathogen-associated molecular patterns (PAMPs) and elicitors to the receptors can also trigger the HR, leading to PAMP-triggered immunity (PTI) (Nurnberger et al., 2004; Schwessinger and Zipfel, 2008).

Necrosis and the secretion of ethylene-inducing peptide 1 (Nep1) occurs in taxonomically diverse organisms, including bacteria, fungi, and oomycetes (Gijzen and Nürnberger, 2006). In some cases, microbial Nep1-like proteins (NLPs) are considered to be positive virulence factors that accelerate disease and pathogen growth in host plants through disintegration (Amsellem et al., 2002; Pemberton et al., 2005; Ottmann et al.,...
However, NLPs trigger cell death and immune responses in many dicotyledonous plants (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006; Schwessinger and Zipfel, 2008). It is believed that NLPs may associate with the outer surface of the plasma membrane to trigger the HR (Qutob et al., 2006; Schouten et al., 2008), and global gene expression analyses have revealed overlap between the responses to Nep1 and responses to other elicitors that lead to active ethylene and oxygen production (Bae et al., 2006). It was recently demonstrated that the mitogen-activated protein kinase (MAPK) cascade is involved in Nep1Mö (Nep1 from Magnaporthe oryzae)-triggered plant responses, and the MAPK signalling associated with HCD exhibits shared and distinct components with that of stomatal closure (Zhang et al., 2012a). Bioinformatics and functional genetic screens have been used to identify and characterize genes mediated by such elicitor signalling. However, information on the signalling network is still fragmentary, and many important players involved in Nep1-induced plant immunity remain to be discovered.

High-throughput down-regulated expression screening of a plant cDNA library was previously performed using virus-induced gene silencing (VIGS), and several genes that suppress cell death in Nicotiana benthamiana leaves upon elicitor treatment were identified. The involvement of respiratory burst oxidase homologues (RBOH), vacuolar processing enzymes (VPE), G proteins, and MAPKs in elicitor-triggered plant immunity was demonstrated by Potato virus X (PVX)-based VIGS (Zhang et al., 2010, 2012). Here, a cDNA identified in the screen, which encodes an Ally of AML-1 and LEF-1 (ALY) protein that acts as a suppressor of Nep1Mö-mediated immunity in N. benthamiana and Arabidopsis thaliana, is reported. The ALY family is a group of plant RNA-binding proteins. Animals encode only one or two ALY proteins, whereas A. thaliana encodes four ALYs; in comparison, many monocots encode four or more ALYs (Uhrig et al., 2004; Canto et al., 2006). Animal (mouse, Drosophila, and human) and yeast ALY proteins contribute to the export of mRNAs from the nucleus before translation, and join with the exon junction complex of proteins marking mRNAs in the course of splicing (Bruhm et al., 1997; Zhou et al., 2000; Storozhenko et al., 2001). Animal ALY proteins are also known as transcriptional co-activators, and promote the interaction of DNA-binding proteins. However, the function of plant ALYs is unknown.

Here, by employing VIGS, it was found that NbALY916 is involved in the regulation of Nep1Mö-induced stomatal closure, HCD, and pathogen resistance in N. benthamiana. It is also demonstrated that AtALY4, an orthologue of NbALY916 in A. thaliana, plays the same role in Nep1Mö-triggered stomatal closure, HCD, and defence-related gene expression.

**Materials and methods**

Plant materials, elicitors, and treatment protocol

Nicotiana benthamiana and Arabidopsis were grown in a growth chamber under a 16/8h light/dark cycle at 25 °C. The T-DNA insertion line for AtALY4 used in this study was AtALY4 (CS331800) supplied by the Arabidopsis Resource Center (http://www.arabidopsis.org). The PCR primers (P1, GGGCATCAGGAGTTGAAGTT; P2, GGATCCCATGATCCATGTA; and LB1, GCGTGGACCGCTTGCTGCAACT) were used to check the T-DNA insertions. To prepare Nep1Mö, overnight cultures of Escherichia coli BL21 cells carrying pET32b harbouring the Nep1Mö gene (GenBank accession no. MG_08454) were diluted (1:100) in Luria– Bertani medium containing ampicillin (50 mg ml⁻¹) and incubated at 37 °C for 3h. When the OD₆₀₀ of the culture reached 0.6, Nep1Mö secretion into the culture medium was induced via the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside for 6h. Nep1Mö was expressed as a His-tag fusion protein. Protein purification was performed with Ni-nitrilotriacetic acid resin (Qiagen, Valencia, CA, USA), and the purified proteins were dialysed against a phosphate-buffered saline (PBS) buffer (pH 7.4) and stored at 20 °C prior to use. Protein concentration was determined using the Bradford reagent (Qutob et al., 2006), and concentrated stock solution (500 μM) was prepared.

**DNA constructs and seedling infection for virus-induced gene silencing**

To amplify a cDNA encompassing the entire open reading frame of NbALY916, the full-length cDNA for NbALY916 was identified with RACE (rapid amplification of cDNA ends) using a SMART RACE amplification kit (BD Bioscience-Clontech, Palo Alto, CA, USA). VIGS for the NbALY916 gene (GenBank accession no. AM167906) in N. benthamiana was performed using PVX, as previously described by Zhang et al. (2009, 2010). The NbALY916 insert was 450 bp, which was derived from the 3′ terminus of its open reading frame and inserted into the PVX vector in the antisense direction to generate PVX.NbALY916. The construct containing the insert was transformed into Agrobacterium tumefaciens strain GV3101. Bacterial suspensions were applied to the undersides of N. benthamiana leaves using a 1 ml needleless syringe. Plants exhibited mild mosaic symptoms 3 weeks after inoculation. The third or fourth leaf above the inoculated leaf, where silencing was most consistently established, was used for further analyses.

**Diaminobenzidine (DAB) staining**

Following the methods of Thordal-Christensen et al. (1997), leaves were harvested 3h after Nep1Mö treatment and immediately vacuum-infiltrated for 20min with PBS (pH 7.4) containing 0.5% (w/v) DAB. The leaves were placed in light for 10h and then boiled for 20 min in 80% ethanol. Quantitative scoring of hydrogen peroxide (H₂O₂) staining in leaves was analysed using Quantity One software (BIO-RAD, Segrate, Italy).

**RNA isolation, semi-quantitative transcription–PCR (RT-PCR), and quantitative RT–PCR**

The leaf fragments after Nep1Mö inoculation were used for total RNA extraction using the Trizol reagent (TaKaRa, Dalian, China) following the manufacturer’s protocol and then treated with RNase-free DNase (TaKaRa). qRT–PCR was performed on the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the purified proteins were dialysed against a phosphate-buffered saline (PBS) buffer (pH 7.4) and stored at 20 °C prior to use. Protein concentration was determined using the Bradford reagent (Qutob et al., 2006), and concentrated stock solution (500 μM) was prepared.

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**Stomatal aperture measurements**

Stomatal apertures were measured as described by Chen et al. (2004) and Zhang et al. (2009, 2010). Leaves were derived from PVX Nb, NbALY916-silenced plants, Col-0, and AtALY4. Abaxial
(lower) epidermis were peeled off and floated in 5 mM KCl, 50 mM CaCl₂, and 10 mM MES-TRIS (pH 6.15) in light for 3 h to open the stomata fully before experimentation to minimize the effects of other factors in stomatal response, because the mesophyll signals can also significantly influence stomatal behaviour. The epidermal strips then were followed by Nep1Mo (50 nM), sodium nitroprusside [SNP, a nitric oxide (NO) donor, 25 mM], and H₂O₂ (800 μM), respectively, to induce a stomatal response. For cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; an NO scavenger] treatment, cPTIO (400 μM) was applied for 1 h prior to SNP treatment. The maximum diameter of stomata was measured under an optical microscope. At least 50 apertures in each treatment were obtained and the experiments were repeated three times.

**NO measurement in guard cells**

NO accumulation was determined using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) according to Ali et al. (2007). Epidermal strips were prepared from control and gene-silenced plants, and incubated in 5 mM KCl, 50 mM CaCl₂, and 10 mM MES-TRIS (pH 6.15) in light for 2 h, followed by incubation in 20 μM DAF-2DA for 1 h in the dark at 30 °C, 65 rpm, and finally rinsed three times with 10 mM TRIS-HCl (pH 7.4) to wash off excess fluorophore. The dye-loaded dark at 30 °C, 65 rpm, epidermal strips then were followed by Nep1Mo, and most reproducible inhibition of cell death triggered since the silencing of #14-4-4 produced the strongest inhibition of cell death triggered by Nep1Mo, the #14-4-4-silenced line was therefore chosen for further study. The DNA sequence of #14-4-4 showed strong similarity to the C-terminal domain of NbALY916 (GenBank accession no. AM167906) from N. benthamiana. To facilitate a more comprehensive comparative analysis of NbALY916, the full-length cDNA for NbALY916 was isolated from N. benthamiana. Three clones of the full-length cDNA, named NbALY916, were sequenced to confirm that the correct gene had been cloned. The predicted protein for NbALY916 is composed of 275 amino acids. The most closely related protein is AtALY4 from A. thaliana (56% amino acid sequence similarity; Fig. 1).

**NO measurement in guard cells**

According to the method described by Pei et al. (2000), reactive oxygen species (ROS) measurement in guard cells was detected by using 2′,7′-dichlorofluorescein diacetate (H₂DCFDA). Epidermal peels were floated in 5 mM KCl, 50 mM CaCl₂, and 10 mM MES-TRIS (pH 6.15) for 2 h under light to induce stomatal opening, followed by incubation with 50 μM H₂DCFDA for 10 min and washing for 20 min with incubation buffer. Images of guard cells were obtained 1 h after Nep1Mo treatment under a fluorescence microscope (excitation wavelength, 484 nm; emission wavelength, 515 nm; Leica DMR, Germany) and analysed using Quantity One software.

**ROS measurement in guard cells**

Disease resistance assay

One half (right side) of a leaf from PVX Nb and NbALY916-silenced N. benthamiana was infiltrated with either PBS (10 nM) or Nep1Mo (50 nM). Three hours later the leaves were collected and transferred to Petri dishes containing sterile water-saturated filter paper. A 9 mm×9 mm hyphal plug of Phytophthora nicotianae was then placed on the surface of the left side of each leaf, which had not been infiltrated with Nep1Mo or PBS. Samples were kept in the dark at 25 °C. Pictures of the lesions were taken at 48 h post-inoculation, leaves then were fixed with 100% ethanol, and homogenized in sterile water. Bacteria were recovered on L-agar medium and induced resistance was defined based on decreases in symptom severity and in planta bacterial numbers (Gerhardt, 1981; Dong et al., 1999).

**Results**

NbALY916 silencing inhibits cell death in response to Nep1Mo

N. benthamiana is the most widely used host for VIGS (Goodin et al., 2008). Purified Nep1Mo inoculated into N. benthamiana leaves can induce cell death (Zhang et al., 2012a). To identify components of the Nep1Mo signalling pathway, a high-throughput in planta down-regulated expression screen of 6000 N. benthamiana cDNAs was performed using a PVX-based VIGS system (Supplementary Fig. S1 at JXB online). Each clone was used to infect two N. benthamiana plants. A common positive control is silencing of the phytoene desaturase (PDS) gene, which results in photobleaching of the silenced regions and is a readily visible phenotype. When photobleaching was apparent, two leaves of candidate plants were infiltrated with Nep1Mo solution. Several cDNAs, including PVX clone #14-4-4, were found to compromise Nep1Mo-triggered cell death upon silencing. Since the silencing of #14-4-4 produced the strongest and most reproducible inhibition of cell death triggered by Nep1Mo, the #14-4-4-silenced line was therefore chosen for further study. The DNA sequence of #14-4-4 showed strong similarity to the C-terminal domain of NbALY916 (GenBank accession no. AM167906) from N. benthamiana. To facilitate a more comprehensive comparative analysis of NbALY916, the full-length cDNA for NbALY916 was isolated from N. benthamiana. Three clones of the full-length cDNA, named NbALY916, were sequenced to confirm that the correct gene had been cloned. The predicted protein for NbALY916 is composed of 275 amino acids. The most closely related protein is AtALY4 from A. thaliana (56% amino acid sequence similarity; Fig. 1).

**NbALY916 silencing and the mutation of AtALY4 reduces cell death and H₂O₂ accumulation following Nep1Mo exposure**

Reduced expression of NbALY916 resulted in a strong phenotype, inhibiting cell death in N. benthamiana leaves after inoculation with Nep1Mo (Fig. 2A). To confirm the suppression of NbALY916 mRNA in the silenced plants, qRT–PCR was performed. A clear reduction in NbALY916 mRNAs was observed in silenced plants compared with control plants infected by the PVX-VIGS vector alone (Fig. 2B; Supplementary Fig. S2 at JXB online). Thomas et al. (2001) reported that at least 23 nucleotides of perfectly matched sequence are necessary to target a gene for silencing (Thomas et al., 2001). BLAST analysis revealed that NbALY916 showed no significant homology with any expressed sequence tags in the N. benthamiana database (http://compbio.dic. harvard.edu/cgi/cgi-bin/tgi). and that it shared 100% identity with stretches of only 10 or fewer nucleotides in NbALY617, NbALY1693, and NbALY615 (three homologues of NbALY916 in N. benthamiana). To test whether the VIGS of NbALY916 could affect the expression of the other NbALY genes, their transcriptional level was examined by
qRT–PCR using primers that specifically annealed to these gene sequences. The results revealed that the expression of these genes was unaffected in the NbALY916-silenced plants (Supplementary Fig. S3). These results indicate a high degree of target specificity using VIGS. It was also observed that the Nep1Mo-triggered HR was not suppressed in NbALY617-, NbALY1693-, and NbALY615-silenced plants. These data demonstrate that the observed inhibition of cell death was due to the specific silencing of NbALY916.

As described above, NbALY916 is closely related to an A. thaliana gene, AtALY4. To determine whether mutations in the AtALY4 gene affect Nep1Mo-induced cell death, homozygous lines containing T-DNA insertions in this gene were obtained from the Salk Institute Genomic Analysis Laboratory collection by PCR screen. The T-DNA in the AtALY4 mutant is inserted into the fifth exon of AtALY4. In the allele, wild-type AtALY4 transcripts were did not detected by qRT–PCR (Fig. 2C; Supplementary S2 at JXB online). As expected, Col-0 seedlings showed obvious cell death after treatment with Nep1Mo. Conversely, the AtALY4 mutant showed hyposensitivity to Nep1Mo by displaying no cell death (Fig. 2D).

To test whether the alteration in Nep1Mo-induced cell death was associated with H2O2 accumulation, the H2O2 levels were quantified in vector control and NbALY916-silenced plants. H2O2 production in response to Nep1Mo was much lower in NbALY916-silenced plants (75% reduction over control treatment; Fig. 3A). Light staining was also observed on AtALY4 mutant leaves following Nep1Mo treatment (Fig. 3B). N. benthamiana rbohA and rbohB and A. thaliana rbohD and rbohF, which encode plant NADPH oxidases, are responsible for generating the H2O2 involved in HRs and plant resistance (Torres et al., 2002; Yoshioka et al., 2003; Sagi and Fluhr, 2006). The expression level of rboh genes (NbrbohA and NbrbohB, and AtrbohD and AtrbohF) was
ALYs in Nep1\textsubscript{Mo}\-triggered plant immunity

Fig. 3. In situ detection of hydrogen peroxide using DAB staining on leaves of NbALY916-silenced N. benthamiana and the Arabidopsis AtALY4 mutant in response to Nep1\textsubscript{Mo}. (A) Nicotiana benthamiana leaves silenced for NbALY916 were compared with PVX-only leaves 6h after infiltration of PBS (10 mM) or Nep1\textsubscript{Mo} (50 mM). Elicitation with the elicitor was conducted on plants by infiltrating an equivalent elicitor solution of 25 μl. Quantitative scoring of staining in leaves of the control and gene-silenced plants with Nep1\textsubscript{Mo} treatment. The analysis was repeated for three sets of independently silenced plants in each experiment; the values shown were the means ± SD of duplicate assays. The experiment was repeated twice with similar results. (B) DAB staining of ROS accumulation in the Arabidopsis AtALY4 mutant leaves 6h after inoculation with Nep1\textsubscript{Mo}. (C) NrbohA and NrbohB were analysed by qRT–PCR and normalized to NbEF1α expression. (D) AtrbohD and AtrbohF were analysed by qRT–PCR and normalized to ATEF1α expression. Each measurement is an average of three replicates; Experiments were repeated twice with similar results.

decreased significantly in NbALY916-silenced and AtALY4 mutant lines compared with control plants (Fig. 3C, D; Supplementary Fig. S4 at JXB online). These data demonstrate that ALYs regulate H\textsubscript{2}O\textsubscript{2} accumulation in response to Nep1\textsubscript{Mo}. These results suggest that the compromised cell death observed in NbALY916-silenced plants and the AtALY4 mutant may be associated with a Nep1\textsubscript{Mo}\-induced decrease in H\textsubscript{2}O\textsubscript{2}.

NbALY916 silencing and the mutation of AtALY4 impairs Nep1\textsubscript{Mo}\-activated stomatal closure

Recent studies have shown that stomata play an active role in the innate immune system (Melotto et al., 2006, 2008). It has been reported that elicitors trigger RBOH-, VPE-, and G protein-dependent stomatal closure in N. benthamiana leaves (Zhang et al., 2009, 2010, 2012a), but whether ALY proteins contribute to elicitor-induced stomatal closure remains unclear. The stomatal response of NbALY916-silenced plants exposed to Nep1\textsubscript{Mo} was examined. It was found that NbALY916-silenced plants failed to close their stomata in response to Nep1\textsubscript{Mo}, whereas the stomata of control plants showed normal closure responses (Fig. 4A). These results suggest that NbALY916 silencing in plants affects the response to Nep1\textsubscript{Mo} or affects a general step in stomatal closure that is common to multiple stomatal closure pathways. The AtALY4 mutant was also tested for Nep1\textsubscript{Mo}\-induced stomatal closure. AtALY4 mutant plants were defective in Nep1\textsubscript{Mo}\-induced stomatal closure (Fig. 4B). Nep1\textsubscript{Mo}\-induced stomatal aperture analyses were consequently performed on the NbALY916-silenced plants and AtALY4 mutants. It was found that the NbALY916-silenced plants and AtALY4 mutant plants showed a markedly reduced response to Nep1\textsubscript{Mo}. Therefore, N. benthamiana NbALY916 and A. thaliana AtALY4 are involved in the Nep1\textsubscript{Mo} signalling pathways leading to stomatal closure.

NbALY916 silencing and the mutation of AtALY4 decreases Nep1\textsubscript{Mo}\-mediated NO production in guard cells

NO functions as a signal in plant disease resistance (Delledonne et al., 1998; Desikan et al., 2002). It was previously shown that NO plays a critical role in Nep1\textsubscript{Mo}\-induced stomatal closure (Zhang et al., 2012b). The NbALY916-silenced plants and AtALY4 mutants were insensitive to Nep1\textsubscript{Mo}\-induced stomatal closure (Fig. 4). To determine further the cellular role of ALYs in the regulation of stomatal closure, NO production was compared in NbALY916-silenced and control plants using the NO-specific fluorescent dye DAF-2DA. Without Nep1\textsubscript{Mo} treatment, the guard cells of the control and NbALY916-silenced plants exhibited similar basal staining for NO. The treatment of epidermal strips with Nep1\textsubscript{Mo} induced a rapid increase in NO levels, as indicated by the change in fluorescence intensity compared with control (PBS) treatment. An ~90% increase in NO occurred in the guard cells of control plants following Nep1\textsubscript{Mo} treatment. However, no increase in NO was observed in the guard cells of NbALY916-silenced plants after Nep1\textsubscript{Mo} treatment (Fig. 5A). Similarly, a decrease in Nep1\textsubscript{Mo}\-induced NO was observed in the AtALY4 mutant (Fig. 5B). The expression levels of nitrate reductase (N. benthamiana NR, A. thaliana NIA) also decreased sharply in the NbALY916-silenced and AtALY4 mutant lines compared with control plants (Fig. 5C, D; Supplementary Fig. S4 at JXB online). This suggests that NR may be the main resource of NO production in Nep1\textsubscript{Mo}.
signalling. Taken together, these results indicate that ALY proteins are required for Nep1$_{Mo}$-mediated NO production in guard cells.

**NO acts downstream of ALY and H$_2$O$_2$**

In addition to NO, H$_2$O$_2$ is also involved in elicitor-induced stomatal closure (Srivastava et al., 2009). To investigate the possible interaction between ALY, H$_2$O$_2$, and NO in Nep1$_{Mo}$-induced stomatal closure, the effects of an NO scavenger (cPTIO) on Nep1$_{Mo}$-, H$_2$O$_2$-, and NO-induced stomatal closure were assessed in NbALY916-silenced plants or AtALY4 mutant lines. Nep1$_{Mo}$-induced stomatal closure was greatly reduced in the presence of cPTIO, in agreement with previous reports (Zhang et al., 2012b). Here, cPTIO inhibition of SNP-induced stomatal closure was observed, similar to the inhibition of Nep1$_{Mo}$-induced stomatal closure ($P<0.001$), indicating a requirement for NO in Nep1$_{Mo}$-induced stomatal closure.
closure and that NO does not require H$_2$O$_2$ generation to initiate stomatal closure.

The role of NO in H$_2$O$_2$-induced stomatal closure was further examined by monitoring NO synthesis in response to applied H$_2$O$_2$. Epidermal fragments were loaded with DAF-2DA to test for changes in NO-induced fluorescence. A significant increase in NO-induced guard cell fluorescence was observed in H$_2$O$_2$-treated epidermal fragments compared with control tissue (P<0.001), demonstrating H$_2$O$_2$-mediated NO production in guard cells (Fig. 6B). Importantly, SNP-induced NO synthesis was abolished by co-incubation with cPTIO, correlating these data with those from the stomatal bioassays (P<0.001; Fig. 6A). Moreover, NO-induced fluorescence was not significantly different between Nep$_{1Mo}^+$ and H$_2$O$_2$-treated epidermal fragments (P>0.05).

The data in Fig. 6A indicate that H$_2$O$_2$ is not required for NO-induced stomatal closure. However, He et al. (2013) reported that in Vicia faba guard cells, exogenous NO, applied in the form of the NO donor SNP, did induce H$_2$O$_2$ production. Consequently, the effects of SNP on H$_2$O$_2$ generation in A. thaliana guard cells were analysed using the fluorescent probe H$_2$DCFDA (Fig. 6C). SNP did induce guard cell H$_2$DCF fluorescence, as did Nep$_{1Mo}$ treatment, which has been previously shown to mediate H$_2$O$_2$ production (Pei et al., 2000; Desikan et al., 2002). However, H$_2$DCFDA is not specific for H$_2$O$_2$ and it also reacts with NO (Hempe et al., 1999). To determine whether the effects of SNP treatment on

![Fig. 6. NO generation is required for H$_2$O$_2$-induced stomatal closure. (A) Wild-type Arabidopsis and AtALY4 mutant leaves were treated with Nep$_{1Mo}$, H$_2$O$_2$, or SNP in the absence or presence of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO). Stomatal apertures were measured 3 h after treatment. The data are displayed as estimated means and associated 95% confidence intervals (CIs). (B) Epidermal fragments were incubated with DAF-2-DA in MES-KCl buffer. NO synthesis was monitored in controls and 3 h after treatment with Nep$_{1Mo}$, H$_2$O$_2$, or SNP, in the absence or presence of cPTIO. Data are displayed as estimated mean pixel intensities and associated 95% CIs. (C) Quantitative analysis of in vivo H$_2$O$_2$ generation monitored using H$_2$DCF fluorescence as shown in D. (D) Epidermal fragments were incubated with (H$_2$DCFDA) in MES-KCl buffer. Hydrogen peroxide synthesis was monitored in controls and 3 h after treatment with Nep$_{1Mo}$, H$_2$O$_2$, or SNP, in the absence or in the presence of cPTIO.](https://example.com/figure6.png)
H$_2$DCF fluorescence were merely attributable to the reaction of NO with H$_2$DCF, a number of experimental treatments were performed (Fig. 6D). Most importantly, treatment with cPTIO greatly reduced H$_2$DCF fluorescence in response to SNP. The reaction with cPTIO is specific to NO and not H$_2$O$_2$; therefore, these data indicate that the fluorescence observed in the SNP-treated guard cells was due to exogenously applied NO and not H$_2$O$_2$, and NO does not induce H$_2$O$_2$ production in N. benthamiana or A. thaliana. Together, these data demonstrate unequivocally that NO functioned downstream of H$_2$O$_2$ and was involved in ALY-mediated stomatal closure triggered by Nep1$_{Mo}$.

NbALY916 participates in the Nep1$_{Mo}$-triggered resistance of N. benthamiana to P. nicotianae

Elicitors can induce systemic resistance in plants (Garcia-Brugger et al., 2006; Yang et al., 2009). Here it was tested whether the silencing of NbALY916 had effects on Nep1$_{Mo}$-triggered disease resistance against P. nicotianae. All of the NbALY916-silenced leaves were infiltrated with Nep1$_{Mo}$ at one spot, and 4 h later the leaf surfaces opposite the Nep1$_{Mo}$-infiltrated sides were inoculated with 2 mm×2 mm mycelial plugs of P. nicotianae. Systemic resistance was assessed 48 h after P. nicotianae inoculation by comparing and measuring the sizes of the lesions. Typical water-soaked Phytophthora lesions appeared within 24 h post-inoculation (hpi). Expanding disease lesions around the inoculated spots were observed in the silenced plants at 48 hpi. However, Nep1$_{Mo}$ treatment significantly inhibited the expansion of lesions in PVX control-inoculated leaves of N. benthamiana (Fig. 7). These findings indicate that NbALY916 is required for Nep1$_{Mo}$-induced disease resistance to P. nicotianae.

AtALY4 is involved in Nep1$_{Mo}$-triggered resistance towards Pst DC3000

To determine whether AtALY4 has an effect on Pst DC3000-induced disease symptoms upon Nep1$_{Mo}$ treatment in A. thaliana, leaves from AtALY4 mutant plants and Col-0 were infiltrated with Nep1$_{Mo}$ at one spot, then wild-type and AtALY4 mutant plants were infiltrated (10$^6$ cfu ml$^{-1}$) with Pst DC3000 using a syringe. As expected, Col-0 showed water-soaked necrotic lesions accompanied by chlorosis, while the AtALY4 mutant plants exhibited accelerated necrotic lesions without visible chlorosis upon PBS treatment. Interestingly, when the growth of Pst DC3000 was monitored at 0, 1, 2, and 4 d post-infiltration, the bacterial population on the AtALY4 mutant was significantly increased compared with that on the inoculated control plants in response to Nep1$_{Mo}$ (Fig. 8). These results suggest that AtALY4 significantly contributed to Nep1$_{Mo}$-induced plant resistance against Pst DC3000 in A. thaliana, at least for the length of time that bacterial growth was monitored.

NbALY916-silenced and AtALY4 mutant plants show altered defence-related gene expression

Differences in the accumulation of H$_2$O$_2$ and NO were detected between the NbALY916-silenced plants and control plants. Distinct levels existed in the AtALY4 mutant and wild type as well, suggesting that the NbALY916-silenced plants and AtALY4 mutant exhibit altered defence-related gene expression. To address this possibility, the kinetics of the expression of selected genes following Nep1$_{Mo}$ infiltration were examined by qRT–PCR not only in NbALY916-silenced and PVX control plants, but also in AtALY4 mutant and wild-type plants (Fig. 9; Supplementary Fig. S4 at JXB online). The jasmonic acid signalling gene LOX encodes a lipoxigenase (Delker et al., 2006), while ERF1 is involved in ethylene signalling (Yang et al., 1997; Alonso et al., 1999; Ding et al., 2002). The expression of these genes showed no significant difference in NbALY916-silenced plants and control plants treated with PBS. After treatment with Nep1$_{Mo}$, the expression levels of ERF1 and LOX3 were up-regulated by 9- and 1.8-fold compared with their previous expression levels, respectively. However, the Nep1$_{Mo}$-mediated expression of ERF1 and LOX3 was down-regulated by 7- and 5-fold, respectively, in the NbALY916-silenced plants compared with control plants. Such suppression of the Nep1$_{Mo}$-mediated

![Fig. 7. NbALY916-silenced plants display enhanced sensitivity to P. nicotianae. (A) For the control and gene-silenced plants, fully expanded leaves collected 4 h after Nep1$_{Mo}$ treatment (10 µl, black circle) were inoculated with a 2 mm×2 mm hyphal plug on the left leaf surfaces opposite the Nep1$_{Mo}$-infiltrated sides. Then, the leaves were placed in Petri dishes containing filter paper saturated with sterilized distilled water and kept under a 16 h day/8 h night regime at 25 °C. Pictures of the lesions were taken at 48 h post-inoculation and the lesion diameter (red circle) was measured. (B) Resistance evaluation based on diameter of lesion spots. Inhibition rate=(diameter of necrosis with PBS treatment–diameter of necrosis with Nep1$_{Mo}$ treatment)/diameter of control. Data are means ±SE from eight experiments.](image-url)
expression of ERF1 and LOX3 was also detected in the AtALY4 mutant compared with the wild type. These results reveal that the silencing of NbALY916 in N. benthamiana and the mutation of AtALY4 both influence the expression of defence-related genes.

Discussion

The observation that Nep1Mo can induce cell death in N. benthamiana and the ability to conduct VIGS in N. benthamiana provided an excellent strategy for identifying plant genes that play a role in Nep1Mo signalling. Here, it was found that a loss of the N. benthamiana gene NbALY916 and its orthologue (AtALY4) in A. thaliana resulted in the inhibition of Nep1Mo-mediated cell death and stomatal closure. The inoculation of NbALY916-silenced N. benthamiana with P. nicotianae induced accelerated necrotic lesions upon Nep1Mo treatment. Similar to NbALY916-silenced N. benthamiana, inoculation with Pst DC3000 of the AtALY4 mutant line induced accelerated necrotic lesions in response to Nep1Mo. Moreover, it was found that NO acts downstream of ALY and H2O2 to participate in Nep1Mo signalling. Based on the phenotypes observed, a model was developed that involves ALY, H2O2, and NO in Nep1Mo-mediated immunity (Fig. 10).

NbALY916 silencing and the mutation of AtALY4 suppresses Nep1Mo-triggered HR and stomatal closure

ALY proteins, which belong to a highly conserved polypeptide nuclear localization signal protein family, exist in plants, yeast, Drosophila, nematodes, and mammals. A previous study found that ALY proteins play an important role in the activation of transcription, pre-mRNA splicing, and mRNA export in mammals (Zhou et al., 2000; Gatfield and Izaurralde, 2002). However, no subsequent reports were found regarding the function of ALY proteins in plants. Unlike previous strategies, VIGS was employed to investigate the role of NbALY916 in Nep1Mo-mediated HR. The results indicate that the silencing of NbALY916 and mutation of its orthologue in A. thaliana, AtALY4, compromised Nep1Mo-mediated HR,

![Fig. 8.](image)

**Fig. 8.** Nep1Mo induced a significant increase in number and size of Pst DC3000 lesions on AtALY4 mutant lines. Resistance evaluation based on diameter of Pst DC3000 lesion spots.

![Fig. 9.](image)

**Fig. 9.** Expression analysis of ERF and LOX in response to Nep1Mo (50 nM). (A) At 6 h after treatment with or without Nep1Mo (50 nM), leaf samples were harvested from the inoculation site on the lower and the upper leaves; N. benthamiana EF1α expression is used to normalize the expression value in each sample, and relative expression values were determined against buffer or PVX-infected plants using the comparative Ct method \(2^{-\Delta\Delta Ct}\). (B) Transcript levels of ERF and LOX in the wild-type and the Arabidopsis AtALY4 mutant 6 h after inoculation with Nep1Mo. Bars represent the mean (three biological replicates) ±SD.
confirming a positive role for ALYs in cell death regulation. ALY-mediated cell death caused by Nep1$_{Mo}$ was associated with the rapid generation of H$_2$O$_2$, displaying a similarity to pathogen-induced HRs. Most importantly, an ALY loss-of-function study demonstrated that ALY is involved in the regulation of HRs caused by the M. oryzae elicitor Nep1$_{Mo}$. These results indicate that ALYs may be a convergence point for Nep1$_{Mo}$ signal transduction pathways.

The present results show that the silencing of NbALY916 and mutation of AtALY4 compromised Nep1$_{Mo}$-induced stomatal closure and was accompanied by less NO accumulation in guard cells (Fig. 4 and 5), which suggests that ALYs control Nep1$_{Mo}$-mediated stomatal closure. NO is a key mediator of abscisic acid (ABA)-induced stomatal closure in peas (Neill et al., 2002), V. faba (Garcia-Mata and Lamattina, 2002, 2003), and A. thaliana (Bright et al., 2006). These data indicate that ALY proteins mediate Nep1$_{Mo}$-triggered stomatal closure via NO signalling. An increasing number of studies in plants have confirmed the importance of stomata in plant immunity, and stomatal closure has been observed as a result of PTI. The results showed that Nep1$_{Mo}$-induced resistance against P. nicotianae was also compromised in NbALY916-silenced plants (Fig. 7). This suggests that NbALY916 is involved in the regulation of plant immunity, controlling stomatal apertures and inhibiting the entry of pathogens into plant leaves during infection.

NO acts downstream of ALY-mediated H$_2$O$_2$ generation in response to Nep1$_{Mo}$

The requirement for NO and H$_2$O$_2$ in elicitor-mediated stomatal closure has been shown previously (Zhang et al., 2009). However, NO and H$_2$O$_2$ synthesis were thought to occur in parallel, until Lum et al. (2002) demonstrated that exogenous H$_2$O$_2$ induced the rapid production of NO in Phaseolus aureus guard cells. A report provided pharmacological evidence indicating that endogenous H$_2$O$_2$-mediated NO generation plays an important role in UV-B-induced stomatal closure in V. faba (He et al., 2013). In the present study, NO synthesis in response to H$_2$O$_2$ in N. benthamiana and A. thaliana guard cells was demonstrated. Importantly, this process was correlated with Nep1$_{Mo}$-induced stomatal closure. Using a pharmacological approach, it has been shown that NO synthesis is required for H$_2$O$_2$-induced stomatal closure. In contrast to the findings of He et al. (2013), the data in the present study demonstrate that NO does not induce H$_2$O$_2$ synthesis as required for stomatal closure. Moreover, the data show unequivocally that NO does not induce H$_2$O$_2$ synthesis in guard cells. Rather, the H$_2$DCF fluorescence monitored in the presence of SNP was actually attributable to the reaction of the dye with NO, not H$_2$O$_2$. However, He et al. (2013) did report that SNP induced H$_2$DCF fluorescence and stomatal closure, so it is possible that guard cell responses differ between N. benthamiana, A. thaliana, and V. faba.

Further evaluation of this response and the potential source of NO induced by exogenous H$_2$O$_2$ revealed that NO production and stomatal closure could both be attributed to NR activity. The expression level of NR in NbALY916-silenced plants and AtALY4 mutants in Nep1$_{Mo}$-induced NO synthesis and stomatal closure was significant and indicates a role for NR activity in these responses (Fig. 5C, D). Previous studies have reported that H$_2$O$_2$-induced NO generation in the guard cells of V. faba was related to NR activity. The analysis of NR–NO activity provides unequivocal evidence that A. thaliana NR has the capacity to generate NO from nitrite (Bright et al., 2006). Guard cells of the double mutant nia1, nia2 do not generate NO in response to H$_2$O$_2$, indicating that NR is responsible for the production of NO in guard cells in response to exogenous H$_2$O$_2$. Importantly, it has been demonstrated that nia1, nia2 plants do generate H$_2$O$_2$ in response to ABA, providing further evidence that NR acts downstream of ABA-mediated H$_2$O$_2$ generation to produce NO and to induce stomatal closure. It has been suggested that NR-mediated NO generation may act downstream of ALY-mediated H$_2$O$_2$ generation in Nep1$_{Mo}$ signalling.

 NbALY916 silencing and the mutation of AtALY4 suppresses Nep1$_{Mo}$-induced pathogen resistance

NbALY916-silenced plants and AtALY4 mutants exhibited increased susceptibility to pathogen invasion. The data presented suggest that ALYs function as a positive regulator of broad spectrum resistance during pathogen invasion. The silencing of NbALY916 in N. benthamiana conferred decreased resistance to P. nicotianae after Nep1$_{Mo}$ treatment. Moreover, upon Nep1$_{Mo}$ application, NbALY916-silenced N. benthamiana showed a decrease in LOX and ERF expression compared with control plants, suggesting that NbALY916 is critically involved in Nep1$_{Mo}$-triggered defence responses. AtALY4 plants homozygous for the T-DNA insertion were susceptible to infection by Pst DC3000 upon Nep1$_{Mo}$ treatment. As expected, decreased LOX and ERF expression was observed in the AtALY4 mutant relative to wild-type plants during Pst infection. Interestingly, this suggests that AtALY4 functions in the A. thaliana defence
response to *Pst* infection in a manner similar to that of *N. benthamiana* upon *P. nicotianae* infection. The decreased level of salicylic acid (SA)-resistant (*LOX*) and ethylene-responsive (*ERF*) gene expression in *N. benthamiana* and *A. thaliana* supports the notion that ALYs are involved in SA and ethylene-mediated signalling pathways during pathogen infection. Analyses of *NbALY916*-silenced and AtALY4 mutant plants revealed that plant disease resistance is conferred by ALY gene expression during pathogen infection.

In conclusion, a VIGS-based forward genetic screen was developed for the identification of new targets involved in Nep1Mo signalling. Although the original intention of the study was to identify genes involved in Nep1Mo-induced HR, a gene, *NbALY916*, that when silenced inhibited cell death and stomatal closure upon Nep1Mo application was identified. Although the precise role of *NbALY916* in the Nep1Mo signalling pathway is debatable and requires confirmation, the results present a new role for ALYs in *P. nicotianae* and bacterial disease development.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1 Screening of *Nicotiana benthamiana* gene coding hypersensitive cell death induced by Nep1Mo by virus-induced gene silencing.

Fig. S2 Expression analysis of *NbALY916* and AtALY4 in *NbALY916*-silenced plants and the AtALY4 mutant by qRT–PCR.

Fig. S3 Local induction of hypersensitivity responses on PVX-, PVX-NbALY615-, PVX-NbALY617-, and PVX-NbALY693-infected *N. benthamiana* leaves in response to Nep1Mo.

Fig. S4 Expression analysis of genes associated with redox control in *NbALY916*-silenced plants and the AtALY4 mutant by qRT–PCR.

Table S1. Gene-specific primers for qRT–PCR.

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