N-Cyanomethyl-2-chloroisonicotinamide Induces Systemic Acquired Resistance in Arabidopsis without Salicylic Acid Accumulation


1Plant Functions Laboratory and 2Microbial Toxicology Laboratory, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
3Laboratory for Growth Regulation, Plant Science Center, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
4Graduate School of Science and Engineering, Saitama University, 255 Shimookubo, Saitama-shi, Saitama 338-8570, Japan
5Biological Research Laboratories, Nissan Chemical Industries Ltd., 1470 Shiraoka, Minamisaitama, Saitama 349-0294, Japan
6Department of Applied Biological Chemistry, Tamagawa University, Machida, Tokyo 194-8610, Japan

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Systemic acquired resistance (SAR) is a potent innate immunity system in plants that is induced through the salicylic acid-mediated pathway. N-cyanomethyl-2-chloroisonicotinamide (NCI) is able to induce a broad range of disease resistance in tobacco and rice and induces SAR marker gene expression without SA accumulation in tobacco. To clarify the detailed mode of action of NCI, we analyzed its ability to induce defense gene expression and resistance in Arabidopsis mutants that are defective in various defense signaling pathways. Wild-type Arabidopsis treated with NCI exhibited increased expression of several pathogenesis-related genes and enhanced resistance to the bacterial pathogen, Pseudomonas syringae pv. tomato DC3000. NCI induced disease resistance and PR gene expression in NahG transgenic plants, but not in the npr1 mutant. NCI could induce PR gene expression in the ein2-1, ein3-1, and jin1-1 mutants. Thus, NCI activates SAR, independently from ethylene and jasmonic acid, by stimulating the site between SA and NPR1.

Key words: systemic acquired resistance; Arabidopsis; salicylic acid; PR protein; Pseudomonas syringae

Systemic acquired resistance (SAR) is an inducible defense mechanism and plays an important role in defending the plants from attack by pathogens. SAR is induced after a hypersensitive response, including tissue necrosis, caused by pathogens such as viruses, bacteria and fungi and is effective against a broad spectrum of pathogens. SAR in tobacco and Arabidopsis thaliana has been well-characterized and a set of pathogenesis-related (PR) genes has been identified as SAR marker genes. To investigate the mechanism for SAR in plants, the chemical probes capable of inducing or inhibiting the development of SAR would also be very useful, and several chemicals have been reported as plant activators which induce SAR in plants. For example, the exogenous application of salicylic acid (SA), which is indigenously produced and functions in the development of SAR, results in the induction of resistance against pathogens and the expression of a set of SAR genes. In addition, some synthetic compounds exhibit several essential criteria of SAR inducers: they induce a broad range of disease resistance; their effects are not due to their antibiotic activities; and they induce an SAR molecular marker, PR gene expression, in plants. 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) induce SAR by stimulating the same site or downstream of SA accumulation in the signal transduction for SAR development (Fig. 1). In contrast, probenazole (PBZ) and its derivative, benzothiazole (BIT), induce SAR accompanying SA

1 To whom correspondence should be addressed. Fax: +81-48-462-4959; E-mail: nakashi@postman.riken.go.jp
* Present address: Polymer Chemistry Laboratory, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
** Present address: Laboratory for Remediation Research, Plant Science Center, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

Abbreviations: BIT, benzothiazole; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester; INA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; NCI, N-cyanomethyl-2-chloroisonicotinamide; PBZ, probenazole; Psi, Pseudomonas syringae pv. tomato DC3000; SA, salicylic acid; SAR, systemic acquired resistance
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![Fig. 1. Structures of Chemicals Capable of Inducing Disease Resistance.](image)

NCl, N-cyanomethyl-2-chloroisonicotinamide; BTH, benzo(1,2,3)thiadiazole-7-carboxylic acid S-methyl ester; BIT, benzothiazole; SA, salicylic acid.

accumulation by stimulating the SAR signaling pathway upstream of SA (Fig. 1).\(^{10,11}\) Despite extensive studies with these chemicals over years, however, many parts of the signaling pathway still remain to be clarified; for example, the targets of these chemicals have not been determined so far.

N-cyanomethyl-2-chloroisonicotinamide (NCI) (Fig. 1) has been reported to induce resistance against rice blast disease.\(^{12}\) We have recently demonstrated that NCI induces SAR in tobacco by triggering the signaling pathway at the same level as or downstream of SA, as do both BTH and INA.\(^{13}\) Various types of SAR-deficient Arabidopsis mutants were identified and used to investigate the mechanism for SAR. To identify the exact mode of action of NCI, we examined its ability to enhance resistance in Arabidopsis. We show here that NCI induces SAR in Arabidopsis by stimulating the SA/NPR1-mediated defense-signaling pathway downstream of SA.

Materials and Methods

Plant materials. Arabidopsis thaliana was grown in sterilized potting soil (Kureha, Japan) in pots (5 x 5 x 5 cm\(^3\)) inside a growth chamber under a 16:8 h light:dark regimen at 22°C with 60% humidity.

RNA extraction and northern blot analysis. The plants were treated with various concentrations of NCI, 2 mM BIT, 0.5 mM BTH, or water by foliar spraying, and the leaves were harvested at various times after the application. Total RNA was extracted from frozen leaf samples of these plants by using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer’s instructions.

DNA fragments of the coding regions for the PR-1, PR-2, and PR-5 genes\(^{14}\) were amplified by the polymerase chain reaction (PCR) from cDNA prepared from SA-treated Arabidopsis. The primers used for the PR-1 gene were TGTCTCTACACT- TCTTATTCTCTA and GAGTTACGCAAAC- CACCTAGTAT; those for the PR-2 gene were ATCTCCCTTGCTGTAATCTTAC and TCTCATAGTGTGTCCTTATT; and those for the PR-5 gene were TGTTACTCATGTCGCCAC- TTTTGA and TCAATTCAAGGCGAGAG- ACAAAC. The PCR products were cloned into plasmid pCR 2.1 (Invitrogen Co. Ltd.) and the nucleotide sequences of these were confirmed.\(^{15}\)\(^{16}\) P-labeled cDNA probes were synthesized by random priming of these fragments of the PR-1, PR-2, and PR-5 genes.

Total RNA samples were subjected to 1.2% agarose-1.1% formaldehyde gel electrophoresis and then transferred to a nylon membrane (Hybond N\(^{\oplus}\), Amersham). After the transfer, RNA was cross-linked to the membrane by using a UV linker (GS Gene Linker, Bio-Rad). Prehybridization and hybridization were performed at 68°C for 1 h or longer and for 8 h or longer, respectively. The membrane was washed twice with 2 X SSC containing 0.1% SDS for 30 min at 68°C and then washed twice with 0.1 X SSC containing 0.1% SDS for 15 min at 68°C. Detection was performed with a BAS2500 image analyzer (Fujifilm).

Bacterial infection. For the Pseudomonas syringae pv. tomato DC3000 (Pst) infection assay, the plants were pretreated by soil drenching with 0.2 or 0.5 mg/pot of NCI or by foliar spraying with 1 mM NCI. The control plants were pretreated with water. Five days after the pretreatment, Pst was inoculated by dipping the plant in the bacterial solution (2 x 10\(^6\) colony-forming units per ml) or by infiltration of the bacterial solution (1 x 10\(^6\) colony-forming units per ml) in the leaves of the foliar-treated plants. After incubation for 1 to 4 days at 22°C, the leaves were harvested from the inoculated plants. At each time point, three leaves were combined and homogenized in 10 mM MgCl\(_2\); the homogenate was placed on nutrient broth agar containing rifampicin (50 \(\mu g/ml\)) at appropriate dilutions. After incubation at 28°C, the number of rifampicin-resistant bacterial colonies was counted. The experiment was repeated three times.

Extraction and analysis of SA. Plants were treated by soil drenching with NCI (0.5 mg/pot), BIT (0.5 mg/pot) or water, or by foliar spraying with 1 mM NCI. At 1-5 days post treatment leaves were harvested from the treated plants, and the SA and SAG levels were measured as previously described.\(^{11,14}\)

Results

NCl induces SAR in Arabidopsis

In Arabidopsis, SAR induced by either the chemical treatment or pathogen infection is associated with enhanced resistance to a variety of pathogens and with the expression of SAR marker genes such as PR-1, PR-2 (β,1-3-glucanase) and PR-5 (thiamatin-like).\(^{7,8,15}\) To determine whether NCI acts as a SAR activator in Arabidopsis, we analyzed its ability to
enhance disease resistance and induce PR gene expression in plants from the Colombia (Col-0) ecotype.

The ability of NCI to enhance resistance to infection by the virulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Pst), was then assessed. The growth of Pst was reduced in the Col-0 plants treated with NCI (0.2 or 0.5 mg/pot) by soil drenching (Fig. 2A). By 4 days post inoculation, these plants contained more than 10-fold lower titers of bacteria than were detected in the water-treated control leaves (Fig. 2A). These concentrations of NCI did not cause any phytotoxicity such as inhibition of growth or bleaching of leaves. In addition, NCI at concentrations of up to 4 mM did not affect the rate of Pst growth in the liquid culture (data not shown). The enhancement of disease resistance in *Arabidopsis* by NCI was also observed when it was treated by foliar spraying (Fig. 2B). Thus, foliar treatment was used for subsequent analysis of NCI’s ability to induce PR gene expression in *Arabidopsis*.

An RNA gel blot analysis indicated that the transcripts for PR-1, PR-2 and PR-5 accumulated in the leaves treated with 1 mM NCI by foliar spraying (Fig. 3A). While NCI was somewhat less effective than BIT or BTH at inducing PR-1 expression, NCI was more effective than BIT at inducing PR-2 and PR-5 expression. In contrast, no transcript for PR-1 and very small amounts of transcripts for PR-2 and PR-5 were detected in water-treated leaves of the control plants. The kinetics of mRNA accumulation for PR-1 were monitored over a time course in the leaves sprayed with 0.5 mM NCI. An increased level of transcripts was detected by 12 hours post treatment (Fig. 3B). The transcripts for PR-1 had reached a maximum by level 72 hours post treatment (Fig. 3B) and remained constant for at least another 5 days (data not shown). A similar pattern of expression for PR-1, PR-2 and PR-5 was observed in the NCI-treated leaves of plants from the ecotypes, Wassilewskija (Ws-0) and Nössen (Nö-0) (data not shown). NCI also induced PR-1 mRNA accumulation in a dose-dependent manner when it was sprayed onto the leaves at concentration ranging from 0.1 to 2.5 mM (Fig. 3C). The ability of NCI to induce SAR marker gene expression and enhance disease resistance in the absence of antibacterial activity confirms that NCI can activate SAR in *Arabidopsis*.

NCI induces SAR without SA accumulation

Biologically induced SAR accompanies PR gene expression and SA accumulation even in healthy leaves. To examine whether NCI induces SA accumulation in *Arabidopsis*, the levels of free SA and total SA (free SA and salicylic acid glucoside; SAG) were measured in NCI- and water-treated *Arabidopsis* over a 5-day time course. BIT was used as a control compound that induce SA accumulation in this plant.\(^1\) The levels of free and total SA detected in the NCI-treated plants were not significantly different from those observed in the water-treated controls at all the times sampled (Fig. 4). In contrast, the levels of free and total SA in the BIT-treated plants were approximately 7- and 5-fold greater, respectively, than those detected in the control plants 5 days post treatment (Fig. 4). This result indicates that NCI does

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**Fig. 2.** Induction of Pathogen Resistance by NCI in *Arabidopsis.*

Plants were treated with chemicals 5 days prior to the inoculation of *Pseudomonas syringae* pv. *tomato* DC3000. Leaves were collected 0, 1, 2 and 4 days post inoculation and homogenized in 10 mM MgCl₂. The number of colony-forming units (CFU) was estimated by growth on nutrient broth agar plates after suitable dilution. Each value is shown by the average with standard deviation. (A) Effect of a soil-drenching treatment with NCI. Plants were treated with H₂O or with 0.2 or 0.5 mg/pot of NCI by soil drenching. ▲, H₂O; ○, 0.2 mg/pot of NCI; ●, 0.5 mg/pot of NCI. (B) Effect of a foliar treatment with NCI. Plants were treated with H₂O or with 1 mM NCI by foliar spraying. Leaves were collected 3 days post inoculation.
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Fig. 3. Induction of SAR Marker Gene Expression by NCI.
Each lane was loaded with 4 μg of total RNA. rRNA was used as an internal control for gel loading and transfer. (A) RNA gel blot analysis of acidic PR-1, PR-2 and PR-5 gene expression in the leaves of wild-type plants treated with H2O, 2 mM BIT, 0.5 mM BTH, or 1 mM NCI. Leaves were collected 2 days after the treatment. (B) Wild-type plants were sprayed with 0.5 mM NCI, and the leaves were harvested at the indicated times. (C) RNA gel blot analysis of acidic PR-1 gene expression in leaves treated with H2O or NCI (0.1, 0.5 or 2.5 mM) via foliar spraying. Leaves were collected 2 days after the treatment.

Fig. 4. Accumulation of Free and Total Salicylic Acid in Wild-type Plants Treated with NCI.
Leaves were harvested at the indicated times after treatment with chemicals, and the free and total SA (free SA + SAG) levels were quantified by HPLC. •, H2O; ▲, soil-drenching treatment by 0.5 mg/pot of BIT; ◯, soil-drenching treatment by 0.5 mg/pot of NCI; ○, foliar treatment by 1 mM NCI.

The NCI-treated NahG transgenic plants, PR-1 expression and enhanced resistance to Pst were detected (Fig. 5AB), indicating that the induction of PR gene expression and SAR by NCI is independent of SA.

Genetic analyses have indicated that a key component of the SA-mediated signaling pathway leading to SAR is encoded by the NPR1 gene.\textsuperscript{16-18} Therefore, we tested whether mutations in NPR1 would affect NCI’s ability to induce SAR. No PR-1 gene expression was detected in the BIT-, BTH- or NCI-treated plants containing either the npri-1 mutation (Fig. 5A) or the allelic mutation, npri-5 (Nö-0 ecotype, data not shown). The plants containing these mutations also failed to develop enhanced resistance to Pst following the NCI treatment (Fig. 5B). Thus, NCI requires a functional NPR1 gene to induce SAR in Arabidopsis.

To assess the role of the other defense-related hormones, ethylene and JA, in the NCI-induced activation of SAR, PR expression by NCI was examined in ethylene-insensitive mutants ein2-1 and etr1-1 and in JA-insensitive mutant jar1-1.\textsuperscript{19-21} Nearly wild-type levels of the PR-1 transcripts were detected in the NCI-treated jar1-1, ein2-1 and etr1-1 mutants (Fig. 5C). Thus, NCI appears to activate PR gene expression, and presumably SAR, via a pathway that is independent of ethylene or JA.

Discussion

We have demonstrated that NCI acts as a chemical inducer of SAR in Arabidopsis. NCI induces PR gene expression and enhances resistance to the bacterial pathogen Pst, but it does not exhibit antibacterial activity. Analysis of various Arabidopsis signaling mutants further revealed that NCI does not require ethylene or JA to induce SAR. NCI activates the SA-mediated defense pathway because it induced

\textit{Role of the defense-related hormones and NPR1 in the induction of SAR by NCI}

Whether the defense-related hormone SA played a role in the NCI-induced activation of SAR was determined by using NahG transgenic Arabidopsis that is unable to accumulate SA due to the expression of salicylate hydroxylase, an SA-degrading enzyme.\textsuperscript{4} In

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\begin{itemize}
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\item PR2
\item PR5
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\item PR1
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\item 0 6 12 24 48 72 h
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\item H2O 0.1 0.5 2.5 mM
\item PR1
\item rRNA
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Fig. 5. Induction of SAR by NCI in the Defense-signaling-deficient Mutants.
(A) Induction of PR-1 gene expression in NahG transgenic and npr1 plants. Plants were treated with H2O, 2 mM BIT, 0.5 mM BTH, or 0.5 mM NCI by spraying. All leaf samples were collected 2 days post treatment. Each lane was loaded with 4 μg of total RNA. rRNA was used as an internal control for gel loading and transfer. (B) NCI-induced resistance in NahG transgenic and npr1 plants. Plants were treated by soil drenching with H2O, 0.5 mg/pot of BIT, or 0.5 mg/pot of NCI 5 days prior to inoculation with Pst. The growth of Pst in the leaf tissue was evaluated 2 days (NahG) or 3 days (npr1-5) after the inoculation as described in the Materials and Methods section. Each value is shown as the average with standard deviation. (C) Induction of PR-1 gene expression in the signaling mutants, ein2-1, etr1-1 and jar1-1. Plants were treated with H2O, 2 mM BIT, or 0.5 or 2.5 mM NCI by foliar spraying. All leaf samples were collected 2 days post treatment. Each lane was loaded with 5 μg of total RNA. rRNA was used as an internal control for gel loading and transfer (data not shown).

the same responses as SA and required a functional NPR1 gene; however it does not require SA to induce SAR. These data suggest that NCI activates defense response via the SA/NPR1 signaling pathway by triggering it at the same level as or downstream of SA.

Studies on NCI's activity in tobacco have demonstrated that it induces SAR in tobacco by triggering the signaling pathway at the same level as or downstream of SA, as do BTH and INA.7) However, the requirement of NPR1, a key regulator of SAR in Arabidopsis, and the roles of other phytohormones in NCI-induced SAR have not been determined. Studies using Arabidopsis, as shown here, revealed that NCI, similarly to BTH and INA, acts between SA and NPR1 in the SAR signaling pathway independently from ethylene or JA (Fig. 6).7) However, no information about the target proteins of these compounds has yet been obtained. It has recently been reported that NPR1 functions not only in the nucleus but also in the cytosol which is likely regulated through phosphorylation of the NPR1 protein by unknown proteins.22) NCI, in addition to BTH and INA, are expected to be useful for investigating the regulation mechanism of the NPR1 protein.

Three chemicals, NCI, BTH and INA, stimulate the same or close points along the SAR signaling pathway. It has been reported that BTH was a much more potent SAR inducer than other substances including INA and exhibited PR-1 gene induction from a concentration of 120 μM.7) An NCI concentration as low as 100 μM induced PR-1 gene expression as shown here, suggesting that the activities for PR-1 gene induction by BTH and NCI were almost same in Arabidopsis. This is also supported by the fact that NCI and BTH exhibited similar levels of disease resistance induction activities against rice blast and bacterial blight diseases, as reported previously.13)
In the previous report, the rise in free SA levels in the BIT-treated Arabidopsis plants was little, whereas total SA levels increased.\textsuperscript{13} However, the results shown in Fig. 4 indicate that the BIT treatment induced an increase in both the free and total SA levels in Arabidopsis. On the other hand, it also induced an increase in both levels in tobacco plants.\textsuperscript{14} An analysis using NahG transgenic Arabidopsis and tobacco plants indicated the requirement of SA for the induction of SAR by BIT. Thus, we conclude that BIT, and presumably PBZ, are able to increase both SA levels in Arabidopsis and assume that this difference was due to experimental influence of the culture conditions or treatment of chemicals.

We have previously demonstrated that NCI induces SAR in tobacco, which is effective against viral, bacterial and fungal pathogens.\textsuperscript{13} NCI is hardly incorporated from the tobacco leaf surface,\textsuperscript{13} however, the foliar treatment of Arabidopsis with NCI induced SAR as shown here. This suggests that the effect of chemicals by a foliar treatment is different between plant species. In addition to tobacco and Arabidopsis, treatment of NCI is also effective in rice against the bacterial and fungal pathogens.\textsuperscript{12,13} Thus, NCI would presumably be effective in many types of plant species and also against various kinds of pathogens. Extensive studies on SAR have been conducted by many research groups, mainly by characterizing various SAR-related Arabidopsis mutants; however, many aspects still remain to be clarified, not only about the SAR signaling pathway in Arabidopsis but also about the SAR mechanism for other agriculturally important crop plants. Thus, chemicals effective on various plants, like NCI, can be used as a potent tool to investigate the defense-response mechanism of higher plants.

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