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3-Acetonyl-3-hydroxyoxindole: a new inducer of systemic acquired resistance in plants

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Summary

Systemic acquired resistance (SAR) is an inducible defence mechanism which plays a central role in protecting plants from microbial pathogen attack. Guided by bioassays, a new chemical inducer of SAR was isolated from the extracts of *Strobilanthes cusia* and identified to be 3-acetonyl-3-hydroxyoxindole (AHO), a derivative of isatin. Tobacco plants treated with AHO exhibited enhanced resistance to tobacco mosaic virus (TMV) and to the fungal pathogen *Erysiphe cichoracearum* (powdery mildew), accompanied by increased levels of pathogenesis-related gene 1 (*PR-1*) expression, salicylic acid (SA) accumulation and phenylalanine ammonia-lyase activity. To study the mode of action of AHO, its ability to induce *PR-1* expression and TMV resistance in *nahG* transgenic plants expressing salicylate hydroxylase, which prevents the accumulation of SA, was analysed. AHO treatment did not induce TMV resistance or *PR-1* expression in *nahG* transgenic plants, suggesting that AHO acts upstream of SA in the SAR signalling pathway. In addition, using two-dimensional gel electrophoresis combined with mass spectrometry, five AHO-induced plant proteins were identified which were homologous to the effector proteins with which SA interacts. Our data suggest that AHO may represent a novel class of inducer that stimulates SA-mediated defence responses.

Introduction

Microbial pathogen infections are common in plants, and they have evolved strategies to protect themselves. Localized treatment of plants with specific biotic or abiotic agents can result in the development of enhanced resistance against pathogens in the whole plant. Resistance induced by such treatments is generally characterized by a restriction of pathogen growth and a decrease in disease severity (Hammond-Kosack and Jones, 1996). This form of induced resistance is generally referred to as systemic acquired resistance (SAR) (Ryals *et al*., 1996). SAR is an inducible plant defence response involving a cascade of transcriptional events induced by salicylic acid (SA). Therefore, SAR is characterized by the endogenous accumulation of SA (Yalpani *et al*., 1991) and a concomitant expression of genes encoding pathogenesis-related proteins (Sticher *et al*., 1997). SAR, induced through an SA-mediated signalling pathway, is the best characterized mechanism of induced resistance. Therefore, SA was specifically identified as a signalling molecule during SAR development in plants. In addition, several synthetic chemical SAR inducers, such as *N*-cyanomethyl-2-chloroisonicotinamide, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester and 2,6-dichloroisonicotinic acid, have been reported (Metraux *et al*., 1990; Uknes *et al*., 1992; Friedrich *et al*., 1996; Nakashita *et al*., 2002). These compounds activate the expression of the same set of defence genes and induce resistance against the same spectrum of pathogens as in SA-induced SAR (Kessman *et al*., 1994; Friedrich *et al*., 1996), but they induce SAR by triggering signalling downstream of

SA accumulation. Thus, the identification of new chemicals capable of inducing disease resistance would be useful, not only for elucidating the pathways leading to SAR, but also for developing new plant protection agents.

Guided by bioassays, a compound capable of inducing SAR was isolated from extracts of *Strobilanthes cusia* and identified to be 3-acetonyl-3-hydroxyoxindole (AHO), a derivative of isatin. AHO can induce resistance in tobacco plants against infection with tobacco mosaic virus (TMV) and the fungal pathogen *Erysiphe cichoracearum* (powdery mildew). In this study, it is demonstrated that AHO-induced resistance depends on SA-mediated defences.

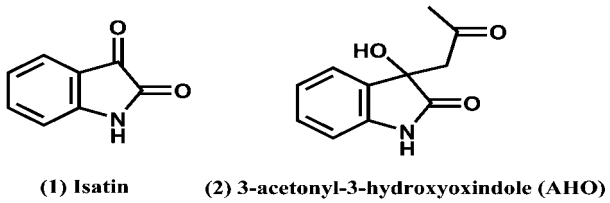
Results

Identification of small-molecule compounds inducing plant SAR

Guided by bioassays, a compound capable of activating SAR was isolated from *S. cusia*. It was identified as AHO, a derivative of isatin (Figure 1).

Induction of resistance to a broad range of diseases in tobacco by AHO

A virus disease (caused by TMV) and powdery mildew disease (caused by *E. cichoracearum*) were chosen as test diseases, because their development mechanisms are markedly different from each other. AHO was applied via foliar spraying to 7-week-old *Nicotiana tabacum* cv. Xanthi-nc plants and TMV inoculation was performed 6 h later. Although many necrotic lesions appeared on the leaves of control tobacco plants treated with 0.05% dimethyl sulphoxide (DMSO), sparse lesions were seen on the leaves of AHO-treated plants (Figure 2a). Furthermore, the size of the lesions decreased gradually with increasing concentration of AHO (Figure 2b). The TMV lesion size on 500 nM AHO-treated plants was 0.42 ± 0.15 mm. This was 85% less than the lesion size on 0.05% DMSO-treated tobacco plants (2.82 ± 0.23 mm).



(1) Isatin **(2) 3-acetonyl-3-hydroxyoxindole (AHO)**

Figure 1 Structures of isatin (1) and 3-acetonyl-3-hydroxyoxindole (AHO) (2).

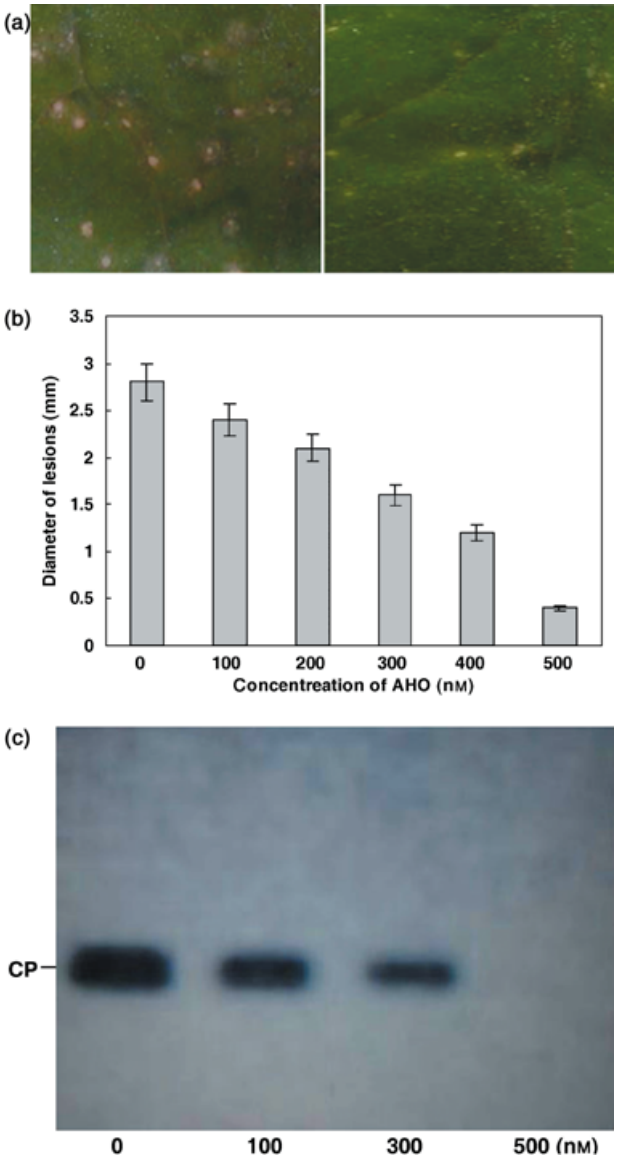


Figure 2 3-Acetonyl-3-hydroxyoxindole (AHO) induced virus resistance in tobacco. *Nicotiana tabacum* cv. Xanthi-nc (NN genotype) plants were pretreated with 0.05% dimethyl sulphoxide (DMSO) (control) or a solution containing AHO 6 h prior to inoculation with tobacco mosaic virus (TMV). (a) AHO enhanced resistance against TMV. Necrotic lesions developed on leaves were photographed 5 days after inoculation with TMV. Left panel: control plant. Right panel: 500 nM AHO-treated plant. (b) Average size of TMV lesions. The diameters of 50 randomly selected lesions were measured under a microscope 5 days after TMV inoculation. Each treatment with the indicated concentration of AHO was performed with five plants, and three leaves of each plant were inoculated with TMV. Values are shown as means ± standard deviation. The experiment was repeated three times with similar results. (c) Total protein extracted from leaves treated with 0, 100, 300 or 500 nM of AHO was subjected to protein gel blot analysis probed with anti-TMV antibody. CP, TMV coat protein.

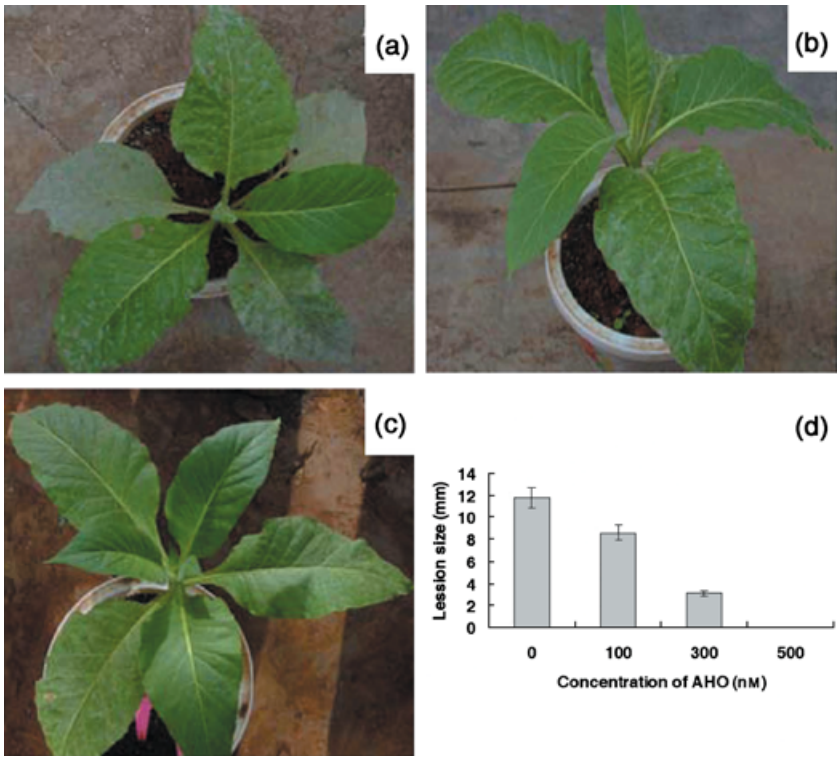


Figure 3 Effect of 3-acetonyl-3-hydroxyoxindole (AHO) on powdery mildew in tobacco. *Nicotiana tabacum* cv. Xanthi-nc (NN genotype) plants were treated with AHO (0, 100, 300 or 500 nM) 6 h prior to inoculation with *Erysiphe cichoracearum*. The conidia of *E. cichoracearum* were powdered on to the leaves of treated plants and cultured in a glasshouse at 25 °C for 5 days. (a) Lesion phenotypes of plants treated with 0.05% dimethyl sulphoxide (DMSO) (control plants). (b) Lesion phenotypes of plants treated with 300 nM of AHO. (c) Lesion phenotypes of plants treated with 500 nM of AHO. (d) Average lesion sizes of powdery mildew disease. Bars indicate the standard errors obtained from three measurements. Student's *t*-test ($P < 0.05$) was used for statistical analysis of the results.

TMV coat protein (CP), as a molecular marker of TMV propagation, was not detected in 500 nM AHO-treated plants (Figure 2c). These results demonstrate the inhibitory effect of AHO against TMV infection.

The treatment of tobacco plants with AHO also greatly reduced the disease symptoms caused by the virulent fungal pathogen *E. cichoracearum* (powdery mildew). *E. cichoracearum*-infected *N. tabacum* cv. Xanthi-nc leaves of untreated control plants developed typical disease symptoms (Figure 3a). Treatment with 300 nM of AHO greatly reduced the severity of the symptoms (Figure 3b). Plants treated with 500 nM of AHO were nearly symptomless (Figure 3c). AHO-induced resistance to the progression of this disease was also dose dependent, as indicated by the lesion sizes (Figure 3d).

Taken together, these results indicate that AHO can induce SAR-like disease resistance to different pathogens in tobacco plants.

Pathogenesis-related gene 1 (*PR-1*) expression associated with AHO-induced resistance

PR-1 is a useful molecular marker for SAR. Therefore, its expression in AHO-treated plants was examined. Northern blot analysis indicated that the application of AHO to tobacco leaves triggered *PR-1* expression. The increase in the expression level of *PR-1* was proportional to the AHO concentration

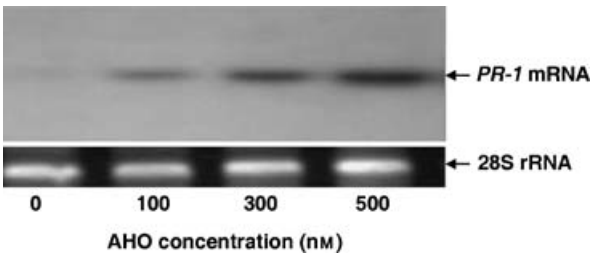


Figure 4 Induction of pathogenesis-related gene 1 (*PR-1*) expression in tobacco by 3-acetonyl-3-hydroxyoxindole (AHO). Kinetics of *PR-1* mRNA accumulation in *Nicotiana tabacum* cv. Xanthi-nc (NN genotype) leaves in response to treatment with different concentrations (0, 100, 300 or 500 nM) of AHO. Total RNA (20 μg) extracted from leaves for each treatment was fractionated on a formaldehyde-containing 1.2% agarose gel, blotted on to a nylon membrane and hybridized with ^{32}P-labelled probes derived from the cDNA clones of tobacco acidic *PR-1*. Equal loading of RNAs was indicated by ethidium bromide staining of 28S rRNA.

administered. In contrast, treatment of leaves with 0.05% DMSO (control) failed to induce *PR-1* expression (Figure 4). These results demonstrate that AHO is effective in inducing SAR.

SA level and phenylalanine ammonia-lyase (PAL) activity in AHO-treated plants

To examine whether AHO-mediated activation of the defence responses was SA dependent, the levels of SA were

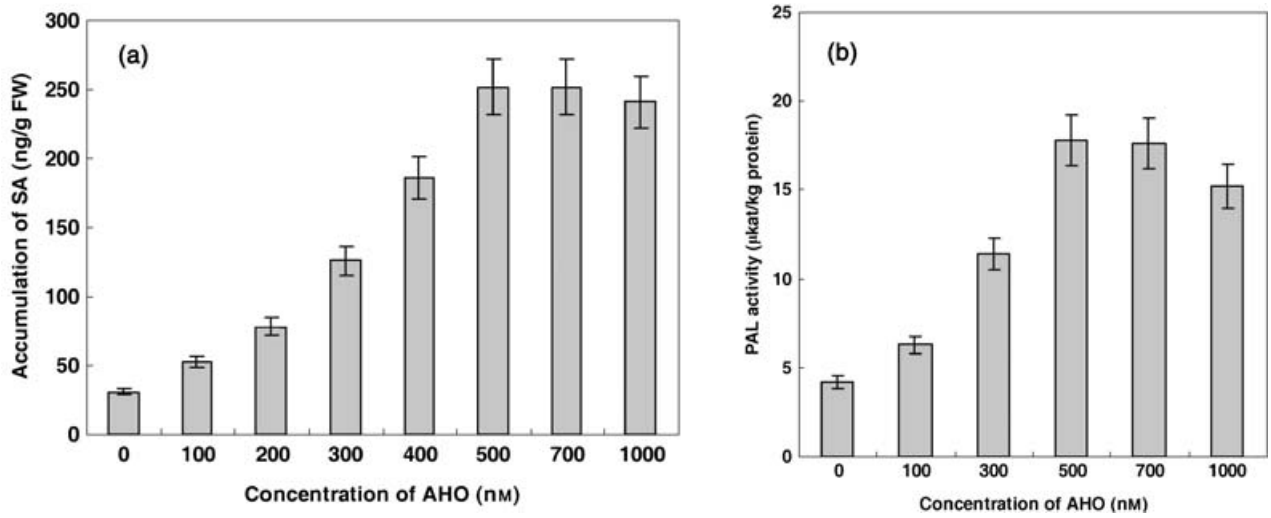


Figure 5 Enhancement of salicylic acid (SA) accumulation and phenylalanine ammonia-lyase (PAL) activity in tobacco plants treated with 3-acetonyl-3-hydroxyoxindole (AHO). *Nicotiana tabacum* cv. Xanthi-nc (NN genotype) plants were sprayed with 0.05% dimethyl sulphoxide (DMSO) (control) or different concentrations of AHO. Leaves were harvested 6 h after spraying and used for quantification of the SA level and PAL activity. (a) AHO treatment induces SA accumulation. Total SA levels (free SA plus conjugated forms) are expressed as ng/g leaf fresh weight (FW). (b) PAL activities in plants treated with AHO. Values shown are the means ± standard deviation from three measurements (each with triplicate samples).

measured in AHO- and 0.05% DMSO-treated tobacco plants. The administration of AHO to tobacco leaves resulted in a significant increase in SA levels. However, leaves treated with 0.05% DMSO (control) failed to show elevated SA levels. The increase in SA level was proportional to the AHO concentration. AHO elevated the SA level moderately at a concentration of 100 nM, and the maximum SA values were observed after treatment with 500–700 nM of AHO (Figure 5a). Compared with the average basal level of SA in control plants, which was 34 ng/g fresh weight, the SA level increased 15-fold in tobacco plants treated with 500 nM of AHO. The increase in endogenous SA levels is sufficient to induce plant SAR (Verberne *et al*., 1999).

Plants most probably synthesize SA from *trans*-cinnamic acid, the product derived from phenylalanine by the activity of PAL, although SA can also be synthesized from chorismic acid derived from the shikimic acid pathway, especially during a resistance response (Coquoz *et al*., 1998; Wildermuth *et al*., 2001). The activation of PAL is triggered by a range of biotic and abiotic stresses, and PAL is a key regulator of the phenylpropanoid pathway which yields a variety of phenolics with structural and defence-related functions (Lois *et al*., 1989; Bate *et al*., 1994; Mauch-Mani and Slusarenko, 1996). Thus, it was examined whether AHO promoted PAL activity in plants. As expected, spraying tobacco leaves with different concentrations of AHO resulted in an increase in PAL activity (Figure 5b). Furthermore, the increase in PAL activity preceded the increase in SA level in AHO-treated plants (data not shown). These results indicate that AHO stimulates SA biosynthesis.

Table 1 3-Acetonyl-3-hydroxyoxindole (AHO) did not induce resistance to tobacco mosaic virus (TMV) infection in *nahG* tobacco plants

Tobacco plant	Inducer	Average lesion size (mm)*
Xanthi-nc	0.05% DMSO	1.34 ± 0.21
Xanthi-nc	500 nM AHO	0.56 ± 0.26
nahG Xanthi-nc	0.05% DMSO	2.28 ± 0.32
nahG Xanthi-nc	500 nM AHO	2.27 ± 0.26

DMSO, dimethyl sulphoxide.

*Diameters of TMV-induced lesions on AHO- or DMSO-treated leaves were measured 7 days after inoculation. For each treatment, 50 randomly selected lesions were measured. Values are means ± standard deviation.

AHO does not induce disease resistance in *nahG* transgenic plants

To further determine whether AHO requires SA to induce disease resistance, the effect of AHO on *nahG* transgenic tobacco plants, which are unable to accumulate SA, was examined. In *nahG* transgenic tobacco plants, AHO had no inhibitory effect on TMV infection, as judged by the sizes of the necrotic lesions (Table 1). Furthermore, the treatment of *nahG* plants with AHO did not induce the expression of *PR-1* (Figure 6). These results confirm that AHO is effective in inducing disease resistance in an SA-dependent manner.

Furthermore, AHO-induced SAR also operated in other plant species: *Arabidopsis* plants displayed a similar AHO-

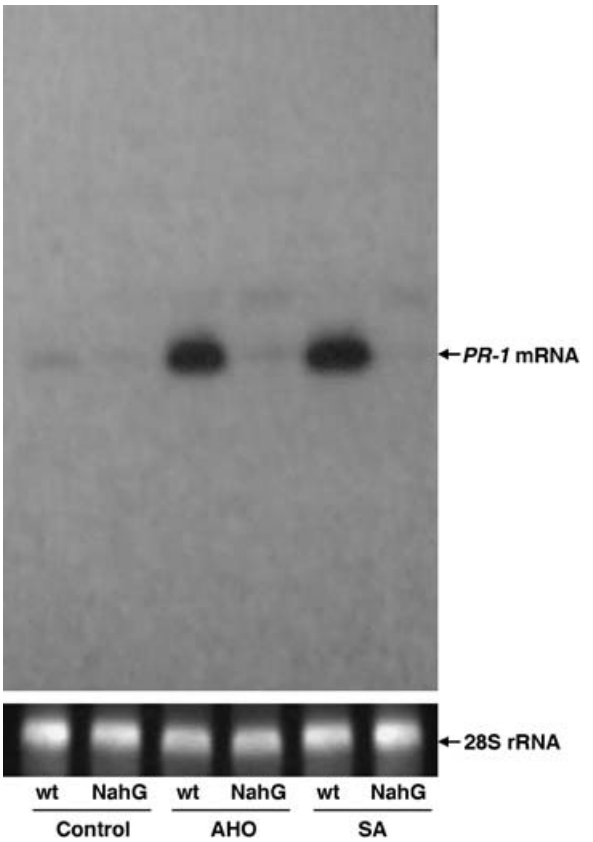


Figure 6 3-Acetonyl-3-hydroxyoxindole (AHO) did not induce pathogenesis-related gene 1 (*PR-1*) expression in *nahG* tobacco plants. *PR-1* mRNA accumulation in wild-type (wt) and *nahG* tobacco plants in response to treatment with salicylic acid (SA) (300 μM) or AHO (500 nM). The treatment of leaves was as described in 'Experimental procedures'. Leaf samples were taken 6 h after treatment for the extraction of total RNA. Northern blot hybridization for *PR-1* mRNA was essentially as described in the legend of Figure 4. Ethidium bromide-stained 28S rRNA is shown as a loading control.

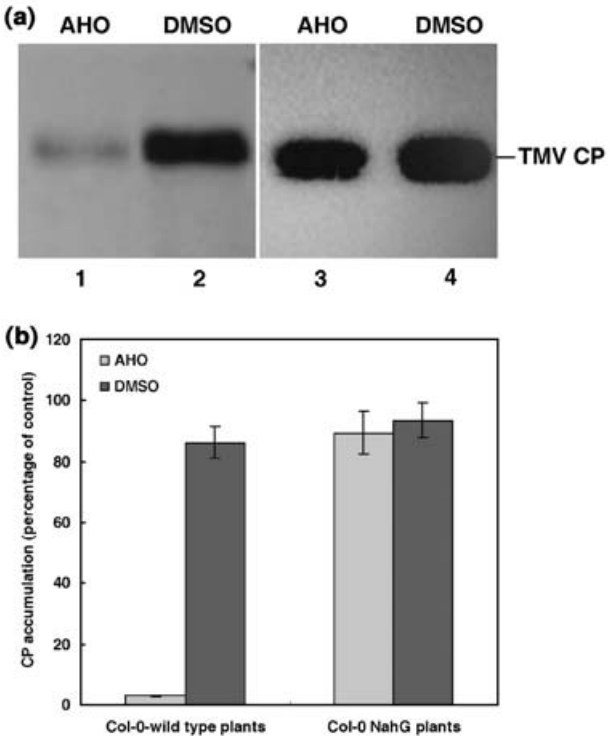


Figure 7 Activity of 3-acetonyl-3-hydroxyoxindole (AHO) against tobacco mosaic virus (TMV) infection in Col-0 wild-type plants and Col-0 *nahG* plants. (a) TMV coat protein (CP) accumulation in Col-0 wild-type plants (lanes 1 and 2) and Col-0 *nahG* plants (lanes 3 and 4) treated with 500 nM AHO or 0.05% dimethyl sulphoxide (DMSO), as determined by Western blotting (described in the legend of Figure 2). (b) TMV CP accumulation in Col-0 wild-type plants and Col-0 *nahG* plants treated with 500 nM AHO or 0.05% DMSO, as assayed by enzyme-linked immunosorbent assay (ELISA). All data represent the average values of three independent measurements with standard deviations.

induced disease resistance to that found in tobacco plants. After inoculation with TMV, wild-type Col-0 plants pretreated with 500 nM AHO were nearly symptomless, in contrast with the severe disease symptoms that developed in wild-type Col-0 plants mock-treated with 0.05% DMSO (data not shown). The quantification of TMV titres, as represented by the amount of TMV CP determined by protein gel blotting, revealed an approximately 80-fold lower level of TMV in AHO-treated wild-type Col-0 plants compared with that in DMSO-treated plants (Figure 7a, lanes 1 and 2). By contrast, TMV consistently multiplied to a high level in both AHO- and DMSO-treated *nahG* plants (Figure 7a, lanes 3 and 4). There was no significant difference in TMV CP levels between AHO- and DMSO-treated *nahG* Col-0 plants (Figure 7b). These results are consistent with the hypothesis that AHO is a plant SAR inducer, and that the activity of AHO is fully dependent on the function of SA in the SAR signalling pathway. Furthermore, using two-dimensional gel electrophoresis, combined with mass spectrometry, some of the AHO-induced proteins were identified as important components acting upstream of SA in the signal transduction pathway(s) leading to SAR (Supplementary material).

Discussion

In this study, it has been demonstrated that AHO induces resistance to a broad range of diseases in plants. In tobacco, AHO enhances resistance against the viral pathogen TMV and the fungal pathogen *E. cichoracearum*. During resistance induction, AHO itself triggers PAL activity, elevates the SA level and induces *PR-1* gene expression in tobacco plants. Several reports have suggested that PAL is a key regulatory enzyme in the biosynthesis of SA and the establishment of SAR (Mauch-Mani and Slusarenko, 1996). Tobacco plants in

which PAL activity has been epigenetically suppressed are unable to express SAR (Howles *et al*., 1996). It was observed in this study that the PAL activity in 500 nM AHO-treated plants showed a 4.5-fold increase with respect to that in untreated plants (Figure 5b). The increase in PAL activity paralleled the increase in the level of SA accumulation. Furthermore, the increase in PAL activity preceded the increase in SA level in AHO-treated plants (data not shown). These results suggest the possibility that the initial signal from AHO treatment leads to the activation of PAL, which is responsible for the production of *trans*-cinnamic acid, an indirect precursor of SA, and demonstrate that AHO is an effective activator of induced plant SAR by triggering the SA biosynthesis pathway. Furthermore, the use of *nahG* transgenic tobacco and *Arabidopsis* plants revealed that AHO induces SAR by triggering signalling upstream of SA accumulation. Using two-dimensional gel electrophoresis, combined with mass spectrometry, it was found that certain AHO-induced proteins were important components in the SA signal transduction pathway(s) leading to SAR (Supplementary material). Together, these results demonstrate that AHO is an effective inducer of plant SAR acting via the SA-mediated signal transduction pathway. These findings may enhance the understanding of the pathway(s) leading to disease resistance and provide new opportunities for the control of microbial diseases in plants.

In addition, this study provides the first demonstration that an indole-type compound (AHO) can act as an inducer of plant SAR. AHO has the potential to be developed into a novel type of plant protection agent that works by inducing the plant's inherent disease resistance mechanisms.

Experimental procedures

Plant materials

Seeds of the *nahG* transgenic tobacco and *Arabidopsis* lines were obtained from the Arabidopsis Biological Resource Center of Ohio State University (Columbus, OH, USA). *N. tabacum* cv. Xanthi-nc (NN genotype) and its *nahG* transgenic plants were grown at 25 °C in a growth room programmed for a 14-h light cycle for 7–8 weeks before being used in the experiments.

TMV infection

TMV (strain U1) was used in this work and purified from infected *N. tabacum* K326 leaves harvested 7 days after inoculation. Fully expanded leaves of 7–8-week-old *N. tabacum* cv. Xanthi-nc (NN) plants were mechanically inoculated with either 5 μg/mL TMV solution in 20 mM phosphate buffer (pH 7.0) or buffer only (mock-inoculated). After inoculation, the plants were maintained at 25 °C in a growth chamber. *Arabidopsis* plants were grown as described previously (Penninckx *et al*., 1996). Inoculation of *Arabidopsis* plants with TMV was performed on 4-week-old, soil-grown plants.

Assays for AHO-induced disease resistance in tobacco plants

Seven-week-old *N. tabacum* cv. Xanthi-nc (NN) plants were pretreated with various concentrations (0, 100, 300 or 500 nM) of AHO by foliar spraying. In the TMV infection assay, three leaves of pretreated tobacco plants were mechanically inoculated with TMV 6 h after pretreatment, followed by incubation at 25 °C. The number and size of the lesions were measured 5 days after TMV inoculation under a light microscope. For the *E. cichoracearum* infection assay, *N. tabacum* cv. Xanthi-nc (NN) plants were inoculated with the fungal pathogen 6 h after pretreatment by transferring conidia (asexual spores) directly from previously infected plants via direct leaf-to-leaf contact. They were incubated at 25 °C with 60–70% humidity and a 16-h/8-h light/dark cycle. The plants were incubated for 5 days and the degree of disease symptoms was evaluated.

Purification of AHO from *S. cusia* guided by anti-TMV assays

Fresh *S. cusia* plants were collected in Guangdong Province, China (a voucher specimen was identified by Professor Xiao-yi Wei, South China Botanical Garden, Chinese Academy of Sciences, and deposited at the South China Institute of Botany, Chinese Academy of Sciences). The dried plant material (25 kg) was extracted with methanol (containing a small quantity of acetone). The extract was successively partitioned into petroleum ether, ethyl acetate, $CHCl_3$, CH_3OH and aqueous solution portions. Each fraction was evaporated to remove the solvents, and the remaining residue was dissolved in 0.05% DMSO to a concentration of 100 mg/mL. Aliquots of the solution were sprayed on to *N. tabacum* Xanthi-nc (NN) plant leaves. Plants sprayed with 0.05% DMSO served as controls. Six hours after spraying, the plants were inoculated with TMV. The plants were then maintained in a growth chamber at 25 °C with a 16-h/8-h photoperiod for 5 days, and the propagation level of TMV was examined by enzyme-linked immunosorbent assay (ELISA), as described previously (Bendahmane *et al*., 1997). The fractions with anti-TMV activity were combined, evaporated and subjected to chromatography on a C18 cartridge column (Waters, Milford, MA, USA). The column was eluted with increasing concentrations of CH_3OH in water, starting with 10% (v/v). The anti-TMV activity could be detected in a fraction eluted with 80% CH_3OH (v/v), which was further separated on a reversed-phase high-performance liquid chromatography (HPLC) column (Li Chrospher 100 RP-18, 5 μm particle size, 4 mm × 25 cm; Hewlett-Packard, Palo Alto, CA, USA). The column was eluted with mobile phase A [water–methanol, 1 : 4 (v/v)] at a flow rate of 1 mL/min, monitored at 254 nm. Starting at 0.1 min after injection, 30 fractions (each of 5 mL) were collected. The anti-TMV activity was found in fractions with retention times of 11.2–16.5 min. The fractions were further separated on another HPLC column (RP-18, 5 μm particle size, 4 mm × 25 cm; Hewlett-Packard) by elution with mobile phase B [water–acetonitrile, 4 : 6 (v/v)]. A fraction showing anti-TMV activity was eluted at the retention time of 16.2 min to yield 17 mg of racemic AHO.

Quantification of SA and PAL enzymatic activity

Nicotiana tabacum cv. Xanthi-nc (NN) plants were sprayed with various concentrations (0, 100, 200, 300, 400, 500, 700 or 1000 nM) of AHO dissolved in 0.05% DMSO. Plants sprayed with 0.05% DMSO were used as controls. Leaves were harvested 6 h after spraying for the determination of PAL activity and SA accumulation level following the methods described in Gurr *et al*. (1992) and Seo *et al*. (1995), respectively.

RNA blot analysis

Northern blot hybridization was performed essentially following the standard protocols described in Tang *et al*. (1999). Total RNA was extracted from tobacco plant leaves treated with different concentrations (0, 100, 300 or 500 nM) of AHO using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (20 μg) was fractionated on a formaldehyde-containing 1.2% agarose gel, blotted on to nylon membranes and hybridized with ^{32}P-labelled probes derived from cDNA clones of the tobacco acidic *PR-1* gene (Zhao *et al*., 2003).

TMV CP assay

Tobacco and *Arabidopsis* plants were sprayed with various concentrations (0, 100, 300 or 500 nM) of AHO or 0.05% DMSO (control). After 6 h, the pretreated plants were inoculated with TMV. Leaves were harvested 3 days after inoculation, and the accumulation levels of TMV CP were determined by Western blotting (Cruz *et al*., 1996).

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of protein changes in plants treated with 3-acetonyl-3-hydroxyoxindole (AHO).

Table S1 Identified proteins associated with 3-acetonyl-3-hydroxyoxindole (AHO)-induced plant systemic acquired resistance (SAR)

Appendix S1: Effect of exogenous application of 3-acetonyl-3-hydroxyoxindole (AHO) on protein changes in tobacco leaves.

Appendix S2: Preparation of protein samples for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

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