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Original Paper

Suppression of salicylic acid signaling pathways by an ATPase associated with various cellular activities (AAA) protein in tobacco plants

Mi-Hyun Lee, Hiroshi Sano*

Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Nara 630-0192, Japan
*E-mail: sano@gtc.naist.jp Fax: +81-743-72-5659

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Abstract An ATPase associated with various cellular activities (AAA) protein was previously shown to be involved in pathogen response in tobacco plants and designated as NtAAA1. Transgenic tobacco lines in which *NtAAA1* was suppressed by the RNA-interference (RNAi) were found to exhibit an elevated resistance to *Pseudomonas syringae* infection, suggesting that *NtAAA1* negatively controlled the defense reaction. To identify genes that were regulated by NtAAA1, differential micro-array screening between *NtAAA1*-RNAi and wild type plants was performed. Results brought out 330 affected genes, which were classified into functional categories, including transcriptional regulation, signal transduction, secondary metabolism and others. Notably, 43 genes were stress- and defense-related, among which 10 were phytohormone-related. Subsequent examination revealed that, in RNAi transgenic plants, genes related to salicylic acid were up-regulated, whereas those related to jasmonic acid and ethylene were generally down-regulated. When salicylic acid was exogenously applied to leaves, expression of *PR-1a*, a maker gene of pathogen response, was evidently induced at much higher level in *NtAAA1*-RNAi transgenic lines than in the control. Simultaneous application of jasmonic acid with salicylic acid markedly cancelled the effect of salicylic acid in the control, but not much in *NtAAA1*-RNAi transgenic plants. The present findings suggested that NtAAA1 broadly functions in cellular metabolism, and particularly that, responding to jasmonic acid and/or ethylene signals, it might interfere with salicylic acid signaling. This system maintains the defense response at appropriate levels, so that detrimental necrosis is avoided, and therefore NtAAA1 may be regarded as a molecular switch of the salicylic acid signaling pathway.

Key words: AAA-protein, jasmonic acid, micro-array screening, *Nicotiana tabacum*, *Pseudomonas syringae*, salicylic acid.

Plants have developed the defense system by specifically recognizing pathogens and preparing protecting activities against their attack (Baker et al. 1997). In this procedure, signaling pathways play critical roles, in which signaling molecules, including jasmonic acid (JA), salicylic acid (SA), auxin and ethylene, synergistically or antagonistically function. JA is usually associated with wounding pathways, whereas SA is usually involved in pathogen responses. SA- and JA-dependent pathways are not linear, but constitute a network of cross-talking that establishes stress-responses (Devoto and Turner 2003). In most cases, each pathway antagonistically functions (Doares et al. 1995; Felton et al. 1999, 2000; Niki et al. 1998). However, molecular mechanism for positive and/or negative interactions between SA and JA pathways remains to be determined.

During the search for pathogen-responsive genes in tobacco plants, we identified an ATPase associated with a various cellular activities (AAA) protein (NtAAA1), which suppressed the hypersensitive response (HR) upon tobacco mosaic virus infection (Sugimoto et al. 2004). The AAA proteins generally play important roles in cellular functions, including regulation of cell cycle, gene expression, vesicle-mediated transport, peroxisome assemble, protein degradation by 26S proteaseome (Rechsteiner et al. 1993) and transcriptional activation (Confalonieri and Duguetm 1995). However, NtAAA1 does not structurally belong to any of these classical AAA protein families, and is the first case of AAA protein that is involved in pathogen response. This suggested NtAAA1 to be a novel type of AAA protein (Sugimoto et al. 2004). In the present study, we screened for genes which were under the control of NtAAA1, using *NtAAA1*-RNAi transgenic tobacco plants. Analysis of identified genes indicated that NtAAA1 acts as a negative regulator of the SA signals by mediating the JA

Abbreviations: HR, hypersensitive response; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid.
This article together with supplement materials can be found at http://www.jspcmb.jp/

signals, thereby serving as a molecular switch for SA signaling pathways.

Materials and methods

Plant and bacterial materials

Wild type and RNAi transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi NC) were grown in a greenhouse at 23°C under a 14 h/10 h light/dark photocycle. Leaves from bout 2 month-old mature plants were detached and used for subsequent experiments. *Pseudomonas syringae* pv *glycinea* 801 was obtained from the Genebank of the Ministry of Agriculture, Forestry, Fisheries. Bacterial cells were grown in King's B medium at 25°C as described (King et al. 1954).

Pathogen and phytohormone treatments

P. syringae pv. *glycinea* 801 were grown to the density approximately 0.5 at 600 nm. Detached healthy leaves from wild type or *NtAAA1*-RNAi transgenic plants were subjected to bacterial injection using a syringe without needle, and incubated at 25°C for appropriate time periods up to 24 h. Leaf discs were collected immediately after infection (0 h), or after 24 h, homogenized in 10 mM $MgSO_4$, and crude extract was used as bacterial solution after appropriate dilution by plating onto KB agar. After incubation at 25°C for 1 day, number of colonies was visually determined. In this article, the hypersensitive response is defined as the resistance gene product-dependent programmed cell death around the site of pathogen infection (Morel and Dangle 1997). For treatments with phytohormones, healthy leaves were cut into 1.5-cm^2 pieces and placed in water for 4 h to diminish cutting stress. Resulting leaf pieces were submerged in a solution containing 0.5 mM salicylic acid dissolved in 0.05 % Triton X-100, or exposed to 50 μM jasmonic acid methyl ester in a sealed box. Samples were harvested at indicated time periods and used for further assay.

Detection of hydrogen peroxide and cell death

Cell viability was estimated by staining with an alcoholic lactophenol Trypan Blue mixture (30 ml ethanol, 10 g phenol, 10 ml water, 10 ml glycerol, 10 ml of 10.8 M lactic acid, and 10 mg of Trypan Blue). Samples were placed in a boiling water bath for 3 min, left at room temperature for 1 h, then transferred to a chloral hydrate solution (2.5 g ml^{-1}), and boiled for 20 min for destaining. Hydrogen peroxide was visualized using 3,3′-diaminobenzidine as the substrate (Thordal-Christensen et al. 1997). Leaves were excised and floated on solution containing 1 mg ml^{-1} DAB for 5 to 8 h at 25°C. The treatment was terminated by immersion of the leaves in boiling ethanol (95%) for 10 min. After cooling, leaves were retained in ethanol and photographed.

Micorarray Analysis

Detached leaves from wild type and NtAAA1-RNAi lines (R15-3, R15-8 and R15-9) were treated *P. syringae* pv. *glycinea* 801, and incubated at 25°C for 9 h. Total RNA was isolated as described (Chomczynski and Sacchi 1987), and mRNA was prepared from 100 μg total RNA using a Gen Elutie mRNA miniprep kit (Sigma, St Lois, MO). The probe cDNA was synthesized and labeled with a Label Star Array kit (Qiagen, Helden, Germany). Independent experiments was performed using two duplicate slides with reverse labeling. cDNA differentially labeled with cy3 or cy5 was dissolved in 60 μl of hybridization buffer (Amersham Life Science) and 120 μl of formamide. The probe was denatured at 95°C for 2 min and incubated at room temperature for 10 min. Printing slide procedures were preformed as described (Katoh et al. 2003). The slide was then exposed to UV crosslinker (ultraviolet crosslinker, Amersham Life Science). Careful handing of slide surfaces before hybridization as well as thorough steps ensured a minimum of dust. Microarray hybridization was performed by an automated slide processor (Amersham Biosciences) and then scanned by FLA-8000 version 1.0 Fuji Film, Tokyo). For data analysis, spot intensities from scanned slides were quantified by using Array vision version 6.0 (Amersham Biosciences). Grids were predefined and manually adjusted to ensure optimal spot recognition, discarding spots with dust or locally high background. Spots were individually quantified by using array vision. Gene expression data were normalized, and induced and repressed genes in *NtAAA1*-RNAi plants were selected based on values greater than 1.5 times of the wild type control.

RNA blot analysis

A 20-μg aliquot of total RNA isolated as described (Chomczynski and Sacchi 1987) was fractionated on a 1% formaldehyde gel and blotted onto a nylon membrane. After immobilization by UV irradiation, blots were hybridized with appropriate ^{32}P-labeled probes in a solution containing 1 mM EDTA, 0.5% SDS, 50 mM Tris-HCl pH 7.5, 1×Denhardt's solution, 3×SSC, 50% formamide and 10% dextran sulfate at 42°C for 16 h. The membrane was washed with 0.5×SSC containing 0.1% SDS at 65°C, and used to expose BAS (Fuji Photo Film, Tokyo, Japan) or x-ray film (Eastman-Kodak, Rochester, NY). The probes were radioactively labeled using [α-^{32}P]dCTP using a *Bca*BEST^{TM} labeling kit (Takara, Kyoto, Japan).

Results

NtAAA1-silenced transgenic plants

In order to identify genes that are controlled by NtAAA1, we programmed the differential micro-array screening. To this end, transgenic tobacco plants were used, in which *NtAAA1* expression was suppressed, so that comparison of transcript population with the wild type control was readily performed. Five such lines were initially constructed (Sugimoto et al. 2004), and one was subjected to pathogen challenge to confirm its involvement in defense response. Leaves of wild-type or *NtAAA1*-RNAi transgenic line R15 plants were inoculated with *P. syringae* pv *glycinea*, which is one of the incompatible pathogens of tobacco, and lesion development was periodically examined up to 24 h (Figure 1A). In wild type control leaves, lesions were distinct by 24 h after infection, while in transgenic R15 leaves, no clear lesions were detectable up to 24 h. To see

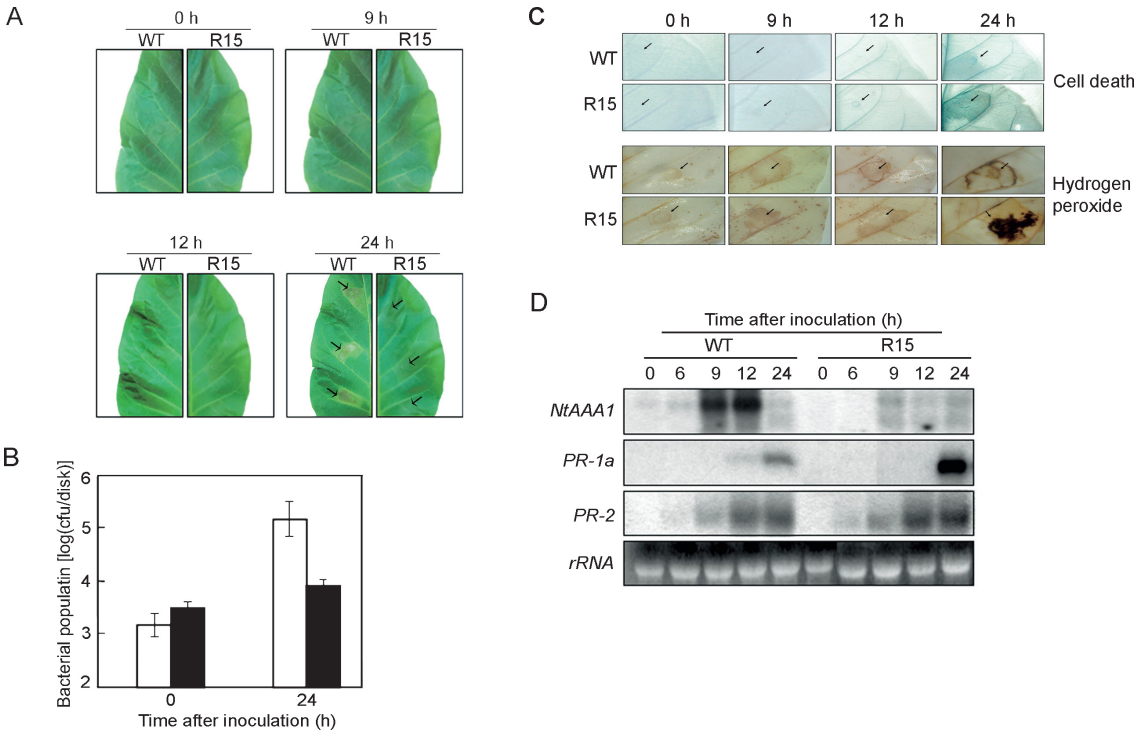

Figure 1. Analysis of *NtAAA1*-RNAi transgenic tobacco plants. (A) Pathogen response. Necrotic lesion development was observed at indicated time period after infiltration of *P. syringae* pv *glycine* into leaves from wild-type (WT) or RNAi transgenic line R15 (R15) plants. Infiltrated positions are indicated by arrows. (B) Quantification of *P. syringae* pv *glycine* cells propagated in wild type (open bar) or RNAi transgenic line R15 (closed bar) plants. Leaf discs were collected at 0 and 24 h after infection, and numbers of propagated bacterium were estimated as described in the text. Values are from triplicate measurements with standard deviation and expressed in logarithmic scale. (C) Cell death and hydrogen peroxide production. After leaf samples from wild type (WT) or transgenic R15 (R15) were inoculated with *P. syringae* pv. *glycine*, dead cells were detected by trypan blue (upper panel), and hydrogen peroxide was detected by 3,3′-diaminobenzidine staining at indicated time period. Arrows indicate the position of infiltration of chemicals. (D) Accumulation profile of transcripts for defense related genes. Detached healthy leaves of wild-type (WT) and RNAi transgenic line (R15) were inoculated with *P. syringae* pv *glycinea* and incubated for indicated time period, when total RNA was extracted and subjected to RNA blot hybridization with probes for *NtAAA1*, *PR-1a* (X06361) and acidic *PR-2* (M5944). As the equal loading control, rRNA was used.

whether or not such a low level of lesion formation was correlated with resistance activity, bacterial numbers around infected areas were directly counted. In wild type leaves, the bacterium linearly increased up to 2.1×10^5 within 24 h whereas in R15 leaves, the increase was low, reaching only 1×10^4 at 24 h point, being 1/20 that of the control (Figure 1B). This suggested that R15 plants acquired higher level of pathogen resistance than wild type control. When cell viability was monitored by staining with trypan blue, dead cells were detectable in both wild type and R15 plants 24 h after inoculation, but at much higher level in the latter (Figure 1C, upper panel). Production of hydrogen peroxide was also higher in R15 than the control plants (Figure 1C, lower panel). Since quick cell death and hydrogen peroxide production are considered to be representative features of hypersensitive response (Morel and Dangle 1997; Lamb and Dixon 1997), these results indicated that the HR was accelerated in R15 plants, and that such an acceleration was apparently due to suppression of *NtAAA1*, as seen from scarce accumulation of its transcripts during infection up to 24 h (Figure 1D, the first panel). Concomitantly, expression of marker genes for HR, *PR-1a* and acidic *PR-2*, was higher in R15 than in the control (Figure 1D, second and third panels). Densitometric estimation showed that transcripts for *PR-1a* and acidic *PR-2* were 5-fold and 1.7-fold higher than the control at 24 h, respectively (data not shown). These results were consistent with our previous observation that suppression of *NtAAA1* accelerated expression of HR-related genes (Sugimoto et al. 2004), and convinced us to proceed to the micro-array analyses.

Identification of differentially expressed genes

The micro-array analysis was performed using cDNA probes prepared from healthy leaves of wild type and transgenic (line R15) plants. Leaf samples were inoculated with *P. syringae* pv *glycinea*, and harvested 9 h later, when expression of *NtAAA1* was the highest in the wild type plants (Figure 1C). A total of 330 genes was found to be differentially expressed in RNAi transgenic plants; 91 genes were up-regulated and 239 were down-regulated (Supplement Table 1 in the attached file). Among up-regulated 91 genes, 82 were

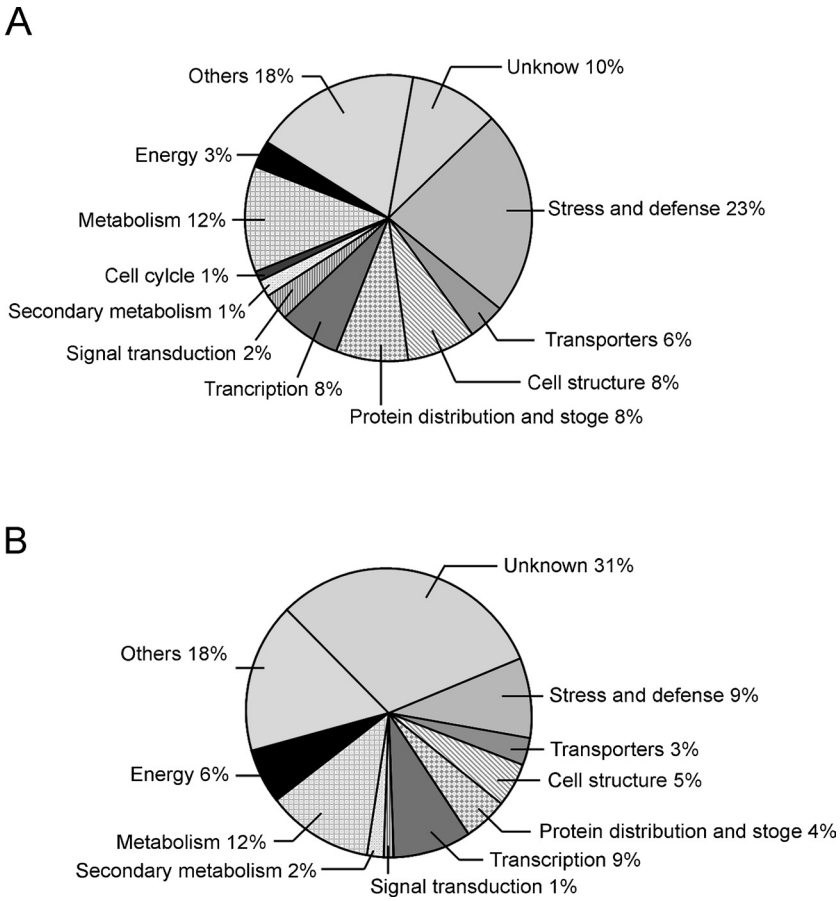

Figure 2. Identification of differentially expressed genes. Healthy leaves from wild type or transgenic *NtAAA1*-RNAi line R15 plants were inoculated with *P. syringae* pv *glycinea* and incubated for 9 h. Total RNA was extracted and reverse-transcribed to yield probe cDNAs for microarray hybridization. Functional classification of 231 up-regulated (A) and 91 down-regulated (B) genes in the transgenic line in comparison with the control is shown with the ratio to the total gene numbers in percentage.

Table 1. Selection of genes responding to signaling molecules.

Clone ID	BLAST hit	Putative function[a]	Ratio[b]	*p*-value[c]
Salicylic acid				
L-095_g04	M97359	Sar8.2b protein	3.22	0.020215
I-105_b01	AAA57551	Salicylic acid binding catalase	1.81	0.001289
R-101_h08	AAD17207	ADP-ribosylation factor, ARF	1.64	0.043165
R-112_f03	NP_916283	Putative Regulatory protein NPR1	1.58	0.018590
Jamonic acid				
I-031_e03	AAN05433	Phospholipase D delta isoform 1a	1.81	0.093642
R-038_a11	AAP83135	Lipoxygenase	0.28	0.001932
I-088_E03	BAA95697	Thionin like protein	0.19	0.039900
Etylene				
R-057_C09	AB012857	ACC oxidase	1.59	0.001023
Chitinase III	Z11563	Acidic chitianse III	0.10	0.002717
L-077_h02	AAA91063	Ethylene-inducible protein	0.05	0.048167

[a] Putative functions are based on sequence homology to known or predicted genes. [b] Ratio of relative expression intensity in *NtAAA1*-RNAi plants to that in wild type control plants after infection with *P. syringae* pv *glycine*. [c] Estimated by the test of significance at $p<0.05$.

assigned to encode proteins with known function; for example, 21 were involved in stress response and defense (23%), 11 in metabolism (12%), 7 in cell structure (8%), and 7 in protein distribution and storage (8%) (Figure 2A). Among 239 down-regulated genes, 76 encoded proteins of unknown function (31%). The remaining included 29 genes encoding proteins involved in metabolism (12%), 21 in transcription (9%), and 22 in stress and defense (9%). Notably, defense-related genes appeared to be one of the major gene groups affected by NtAAA1. Among such genes, 10 were found to possibly participate in hormonal response (Table 1). Four genes

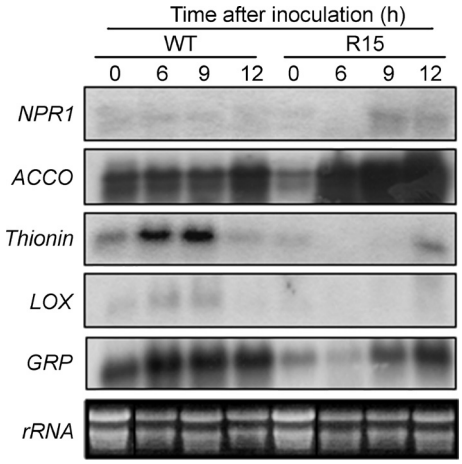

Figure 3. RNA blot hybridization. Healthy leaves from wild type (WT) or transgenic *NtAAA1*-RNAi line R15 (R15) plants were inoculated with *P. syringae* pv. *glycinea*, incubated for indicated time period and total RNA was extracted, which was fractionated on agarose gel and blotted onto nylon membrane. Hybridization was performed with indicated probe cDNAs prepared after microarray results. Probes were, *NPR1* (nonexpresser of PR gene 1, NP_916283), *ACCO* (aminocyclopropane carboxylic acid oxidase, AB012857), *Thionin* (BAA95697), *LOX* (lipoxygenase, AAP83135), *GRP* (glycine rich protein, M37152). As the equal loading control, rRNA was used.

were involved in the salicylic acid pathway, including *NPR1* (NP_916283), *ARF* (AAD17207), *Sar 8.2b* (M97359) and a gene for SA binding catalase (AAA57551). All were up-regulated in the transgenic line. Three were related to jasmonic acid response, *PLD* being up-regulated, and *LOX* and *thionin* down-regulated. The remaining 3 were involved in ethylene response; *ACCO* was up-regulated and genes for chitinase and ethylene-inducible protein were down-regulated (Table 1). For confirmation of these micro-array results, 5 genes representing different expression patterns were subjected to RNA gel-blot analysis using RNA samples isolated from pathogen-treated leaves (Figure 3). Transcripts for *NPR1* and *ACCO* accumulated to a higher level in the transgenic line than in the wild type control, while those for *thionin*, *LOX* and *GRP* showed almost no accumulation. Results were consistent with micro-array data, which were thus proven to be reliable for further analyses.

Modulation of SA signaling pathway

The above-described results suggested that NtAAA1 may play an important role in modulation of SA and JA pathways. Since suppression of NtAAA1 appeared to result in acceleration of SA pathway and in suppression of JA and ethylene pathways, effects of exogenously applied SA, alone and in combination with JA on expression of marker genes were examined (Figure 4). Experimentally, healthy leaves from wild type or *NtAAA1*-RNAi transgenic plants were detached and treated with SA for up to 48 h, and accumulation of *PR-1a* transcripts were profiled by the RNA blot hybridization. Results showed that, although transcripts were equally induced in both transgenic and control plants, the amount was over 3-fold higher in the former than in the latter (Figure 4A). When JA was simultaneously applied with SA, *PR-1a* induction was suppressed in both plants, but to a less extent in the transgenic line in comparison with the control (Figure 4B). This was particularly distinct 48 h after treatment, when the transcript level was nearly the same as that by SA alone, indicating the failure of JA to repress SA signals. These results suggested that NtAAA1 can

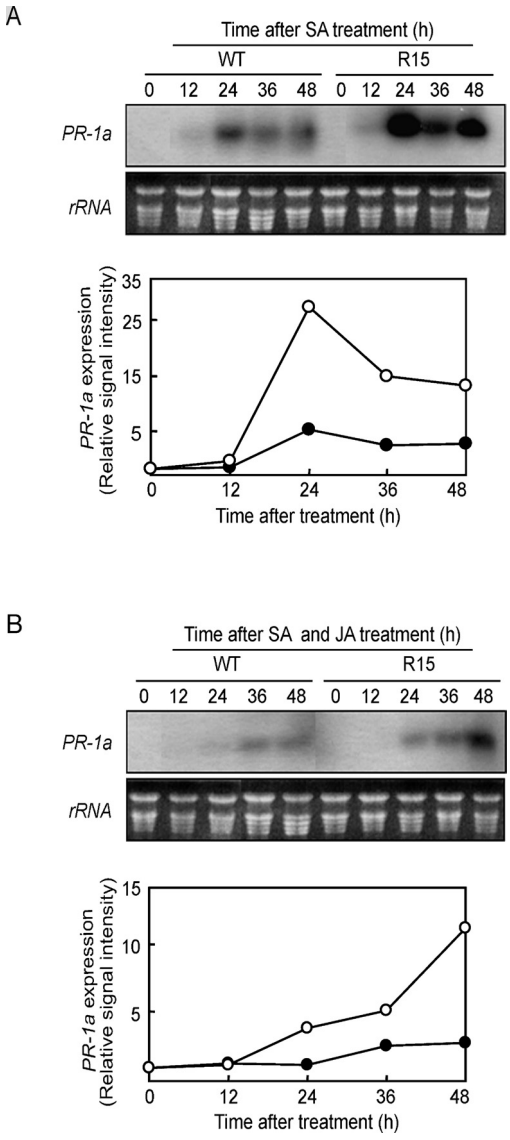

Figure 4. Effects of exogenously applied signaling molecules. Leaves of wild type (WT) and transgenic *NtAAA1*-RNAi (R15) plants were cut into 1.5-cm^2 and floated on buffer solution containing 500 μM SA (A), or 500 μM SA and 50 μM JA methyl ester (B) and sampled at indicated time periods. Total RNA was isolated and subjected to RNA blot hybridization with the *PR-1a* probe (upper panel). Signals are densitometrically quantified (lower panel). As the equal loading control, rRNA was used.

attenuate the effect of SA, and that such an attenuation might be activated by the JA pathway.

Discussion

This paper documents that transgenic *NtAAA1*-RNAi tobacco plants showed an elevated resistance to pathogenic bacterium, *P. syringae* pv *glyciena*, in comparison with the wild type counterparts, and that such a resistance might be mediated through acceleration of salicylic acid signaling pathways. *Pseudomonas syringae* is one of the major gram-negative plant pathogenic bacteria, which induce hypersensitive cell death in non-host plants (Taguchi et al. 2003). Inducible defense responses in non-host plants comprise the synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, and translation products from pathogenesis-related genes as well as the localized reinforcement of the plant cell wall and hypersensitive programmed cell death (Mysore and Ryu 2004; Nümberger and Brunner 2002; Thordal-Christensen 2003). Our present results are consistent with this view, and clearly indicate that observed resistance was attributable to hypersensitive cell death through accelerated HR. In previous studies, we reported that, upon pathogen infection, *NtAAA1*-RNAi plants exhibited visible necrotic lesions to the same extent as the wild type plants (Sugimoto et al. 2004), but actual resistance activity was not directly estimated. In the present study, the same plants showed less lesions and high resistance, being apparently contradictory to the previous report. This inconsistence may be due to different experimental conditions, such as initial numbers of inoculated bacteria, age of leaf samples, humidity and temperature of cultivation, and incubation period, all of which are critical factors for effective induction of HR. A high level of resistance without clear lesion formation as observed here has occasionally been observed in non-host HR (van Wees and Glazebrook 2003; Takeuchi et al. 2003). This is perhaps due to quick programmed cell death occurring at limited cells, thereby preventing the spread of visible tissue death as observed in ordinary HR.

Intensive survey has suggested that SA- and JA-signaling pathways are antagonistic (Doares et al. 1995; Felton et al. 1999; Niki et al. 1998). For example, application of SA to tobacco leaves suppressed expression of genes encoding lipoxygenase-2, an enzyme for JA biosynthetic (Spoel et al. 2003) and thionin, an anti-fungal basic PR protein which typically responds to JA (Niki et al. 1998). The opposite is also true, as JA suppressed expression of genes for acidic PR proteins (Niki et al. 1998). However, the molecular mechanism of such antagonistic actions has not been established. It is generally considered that SA interferes with JA synthesis, and vice versa, resulting in shut-down of signaling pathway of the counterpart (Spoel et al. 2003; Laudert and Weiler 2002; Doherty et al. 1988). Another idea is that whether SA or JA predominates depends on the concentration of each *in planta* (Mur et al. 2006). The present finding proposes an additional idea that, responding to JA signals, NtAAA1 directly suppresses SA signals.

The underlying mechanism is currently not clear, but two features must be separately considered; transcriptional level and protein interaction level. Since AAA proteins are supposed to be functional upon interaction with other proteins (Lupas and Martin 2002), suppression of SA signals by NtAAA1 might be mediated through protein-protein interaction. In this context, it is conceivable that NtAAA1 is first activated by a JA-induced protein, and then interacts with SA-related protein(s), which in turn switch off the SA signaling. If it is the case, NtAAA1 might serve as a stand-by receptor of the JA signal. This is consistent with our previous observation that *NtAAA1* transcripts were evidently induced only by ethylene but not by SA or JA (Sugimoto et al. 2004), indicating production of NtAAA1 itself to be independent on SA and JA. This hypothesis also accounts for the decreased expression of JA- and ethylene-responsive genes in *NtAAA1*-RNAi plants upon infection, as uncontrolled excess SA signals resulting in suppression and/or imbalance of JA/ethylene signaling pathways. Overall, it is tempting to speculate that NtAAA1 functions as a kind of molecular switch to shut down SA pathways.

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