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A hypersensitive response-induced ATPase associated with various cellular activities (AAA) protein from tobacco plants

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Abstract

The hypersensitive response (HR) is one of the most critical defense systems in higher plants. In order to understand its molecular basis, we have screened tobacco genes that are transcriptionally activated during the early stage of the HR by the differential display method. Among six genes initially identified, one was found encoding a 57 kDa polypeptide with 497 amino acids not showing significant similarity to any reported proteins except for the AAA domain (ATPase associated with various cellular activities) spanning over 230 amino acids. The bacterially expressed protein exhibited ATP hydrolysis activity, and a green fluorescent protein-fusion protein localized in the cytoplasm of onion epidermis cells. The protein was subsequently designated as NtAAA1 (*Nicotiana tabacum* AAA1). *NtAAA1* transcripts were induced 6 h after HR onset not only by TMV but also by incompatible *Psuedomonas syringae*, indicating that *NtAAA1* is under the control of the *N*-gene with a common role in pathogen responses. Expression of *NtAAA1* was induced by jasmonic acid and ethylene, but not by salicylic acid (SA). It also occurred at a high level in SA-deficient tobacco plants upon TMV infection. When *NtAAA1* was silenced by the RNAi method, accumulation of transcripts for *PR-1a* significantly increased during the HR. Treatments with SA induced higher expression of *PR-1a* and acidic *PR-2* in RNAi transgenic plants than in wild-type counterparts. These results suggest that NtAAA1 mitigates the SA signaling pathway, and therefore that NtAAA1 modulates the pathogen response of the host plants by adjusting the HR to an appropriate level.

Introduction

Plants are equipped with unique and complicated protection systems against microbial pathogens. In addition to constitutive resistance by physical barriers including the cell wall, cuticular and antimicrobial compounds, temporal resistance is induced after pathogen attack. Examples are lignification of cell walls, generation of superoxide species, accumulation of pathogenesis related (PR) proteins and the hypersensitive response (HR). This latter occurs upon pathogen infection, and results in necrotic lesions around attack sites to enclose pathogens, and thus prevention of disease expansion. A series of events in this resistance is activated through recognition of signal molecules derived from the pathogen by plant receptors (Hammond-Kosack and Jones, 1996). Plants have also evolved a system, which is systemically induced upon pathogen attachment to a local area. This is known as systemic acquired resistance, and provides defence against subsequent attacks by a broad spectrum of microbial agents (Ryals *et al.*, 1996).

To explore the molecular basis of the HR, we have screened for genes whose transcripts were specifically induced in tobacco plants in response to the pathogen, tobacco mosaic virus (TMV)(Yoda *et al*., 2002). When tobacco cultivars carrying the *N* gene are attacked by TMV, defense responses including HR are induced (Holmes, 1938). However, this does not occur at 30 °C, and viral multiplication results (Weststeijn, 1981). Transfer to 20 °C activates the *N* gene, and visible necrotic lesions are induced approximately 9 h thereafter. Using this system, in which the HR is synchronously induced by temperature shift, we have identified genes that are involved in the HR and subsequent defense reactions by the differential display method (Yoda *et al*., 2002). Continuing this project, we have further isolated several clones, most of which are known to be stress responsive, but not pathogen associated. Among them, we found a particular gene encoding an ATPase associated with the various cellular activities (AAA) protein.

The AAA super family proteins found in prokaryotes and eukaryotes play important roles in diverse cell functions including protein degradation, formation and maintenance of cell organelles, membrane fusion and vesicle transport (Patel and Latterich, 1998; Lupas and Martin, 2002). They are also critical for the cell cycle, mitochondrial function, peroxisome assembly and proteolysis (Frohlich, 2001). The AAA proteins constitute one group within the P-loop NTPase family, featuring Walker motifs A and B. A distinguished conserved sequence, SRH (second region homology), makes the AAA protein family distinct from other P-loop NTPase proteins (Lupas and Martin, 2002). In mammals, more than hundred genes encoding AAA proteins have been registered, and their functions have been relatively well studied (Iyer *et al*., 2004). In plants also, the number of genes identified which encode AAA proteins has been increasing as exemplified by the over 60 examples found in *Arabidopsis*. However, functional analyses have so far been limited, with the exception of the 26S proteasome (Fu *et al*., 1999), the metalloprotease FtsH (Lindahl *et al*., 1996; Seo *et al*., 2000), NSF acting in vesicle trafficking (Rancour *et al*., 2002), and PEX6 involved in peroxisome biogenesis (Olsen, 1998). In the present study, we describe properties of a novel AAA protein, which is specifically induced during the early phase of the HR, with a role in regulation of the defense system against pathogen infection.

Materials and methods

Plant and bacterial materials

Tobacco plants (*Nicotiana tabacum*, cv Xanthi NC, cv Xanthi NN, cv Samsun nn, NahG transgenic and *NtAAA1*-RNAi lines) were grown in a greenhouse at 23 °C under a 14 h /10 h light/dark photocycle. In the case of RNAi transgenic lines, T_0 and T_1 plants were used after confirmation of stable suppression of *NtAAA1*. About 2 month-old mature leaves were detached and used for subsequent experiments. *Pseudomonas syringae* pv. *glycinea* 801 and *P. syringae* pv. *tabaci* 301075 were obtained from Y. Takigawa (Shizuoka University) and from the Genebank (Ministry of Agriculture, Forestry, Fisheries), respectively. Bacterial cells were grown in King's B medium at 25 °C.

Pathogens and phytohormone treatment

Detached tobacco leaves were mechanically inoculated with or without TMV (10 μg/ml) in 10 mM sodium phosphate buffer (pH 7.0) using carborundum (600 mesh) and incubated at 30 °C under continuous light for 48 h, and then transferred to 20 °C. Detached leaves were also treated with bacteria (2×10^8 cfu/ml) by injection using a syringe without needle and incubated at 22 °C. For exposure to phytohormones, detached leaves were placed in water for 4 h to diminish cutting stress, and treated with 2 mM salicylic acid (SA) dissolved in 0.05% Triton X-100, 50 μM jasmonic acid (JA) methyl ester (MeJA) or 100 μM ethephon to produce ethylene in sealed boxes.

Differential display

Differential display was performed as described (Yoshida *et al*., 1994) with a slight modification. First strand cDNA, which was synthesized with oligo(dT) primers and mRNA derived from mock- or TMV-treated leaves, was used for the second strand synthesis and polymerase chain reaction (PCR) with 10-mer arbitrary primers (Operon Technologies, Alameda, CA). The reaction was

carried out with one cycle of 1 min at 94 °C, 30 cycles of 20 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and one cycle of 7 min at 72 °C. Amplified cDNA was fractionated on a 1.5% agarose gel and stained with ethidium bromide. Fractionated cDNA was eluted from gel and subcloned into the pGEM®-T Easy vector (Promega, Madison, WI). To eliminate false positive clones, dot blot hybridization was performed. A 1-μl aliquot of PCR product from the differential display was mixed with 4 μl of 4×SSC, and spotted onto a nylon membrane (Hybond™-N+, Amersham, Buckinghamshire, England). The membrane was then denatured in denaturation buffer (1.5 M NaCl and 0.5 M NaOH), neutralized with neutralization buffer (1.5 M NaCl and 0.5 M Tris-HCl, pH 7.5) and immobilized by UV irradiation. Cloned cDNA fragment was amplified, labeled with AlkPhos Direct (Amersham, Buckinghamshire, England) and used as a probe. The cDNA clone exhibiting hybridization signals was finally selected and further analyzed.

RNA and DNA gel blot analysis

Total RNA was isolated as described (Chomczynski and Sacchi, 1987) with modification. A 20-μg aliquot of total RNA was fractionated on a 1% formaldehyde gel and blotted onto a nylon membrane. After immobilization by UV irradiation, the blot was hybridized with appropriate ^{32}P-labeled probes in 1 mM EDTA, 0.5% SDS, 50 mM Tris-HCl pH 7.5, 1×Denhardt's , 3×SSC, 50% formamide and 10% dextran sulfate at 42 °C for 16 h. The membrane was washed with 0.5 × SSC and 0.1% SDS at 65 °C, and used to expose BAS (Fuji Photo Film, Tokyo, Japan) or X-ray film (Eastman-Kodak, Rochester, NY). Alternatively, probes were labeled with digoxigenin-11-dUTP (Roche Applied Science, Penzberg, Germany) and subjected to hybridization according to the manufacturer's instruction. Genomic DNA was isolated by the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980) with modification. A 20-μg aliquot of genomic DNA was digested with *Hin*dIII or *Bam*HI, fractionated on a 0.8% agarose gel and blotted onto a nylon membrane according to the manufacture's protocol. The blot was hybridized with ^{32}P-labeled probes in 0.5 M Church phosphate buffer, 1 mM EDTA and 7% SDS at 65 °C for 16 h and the membrane was successively washed with 2 × SSC and 0.1% SDS at 65 °C, for exposure of BAS or X-ray film. The cDNA probes were labeled with ^{32}P using a *Bca*BEST™ labeling kit (Takara, Kyoto, Japan).

Isolation of NtAAA1 cDNA and sequencing

To obtain the full-length coding region of *NtAAA1*, DNA segments encoding the N- and C-terminal regions were amplified from a cDNA library constructed in Lambda ZAP®II (Stratagene, La Jolla, CA) by the polymerase chain reaction (PCR). The primer sets used were designed after the vector sequence and a partial sequence of *HRR11 (NtAAA1)* clone: 5′-CTCAG TGTCCCTGACCGATG-3' and 5'-TGCTCCGCC AAACTCATCAG-3' for the 5′ region, and 5′-CT CAGTGTCCATTTAGCAAAAGAGGC-3′ and 5′-GGGAAGGCGTGGAAGCGGGG-3′ for the 3′ region. Nucleotide sequences were determined by the dideoxynucleotide chain termination method on an ABI3100 sequencer (ABI, Sunnuvale, CA).

Enzymatic activity assay

A cDNA encoding the entire NtAAA1 open reading frame was amplified by PCR using a set of forward (5′-GGCCCGGGATGATCAGCACAA TGGGATTGC-3') and reverse (5′-GGGCGGCC GCATTTTGGGTAAAACCATCTAA-3′) primers. The *Sma*I and the *Not*I sites were linked at the 5′ ends of forward and reverse primers, respectively, and the PCR products were introduced into pGEM®-T Easy vector for amplification in *E. coli* DH-5α. The plasmid was digested with *Sma*I and *Not*I, and the fragment was ligated to the corresponding site of pGEX-4T-2 (Amersham, Uppsala, Sweden). *E. coli* BL21 was transformed with the resulting plasmid, and grown at 37 °C in LB medium containing ampicillin (200 μg/ml) to an A_{600} of 0.5. Recombinant proteins were induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. After further growth for 12 h at 18 °C, cells were harvested and resuspended in a 10-fold volume of sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA and 1 mM DTT). Sonicated lysate was mixed with 1% (v/v) of Triton X-100 and recombinant proteins were purified through a glutathione Sepharose 4B resin

(Amersham, Uppsala, Sweden) according to the manufacturer's instructions. The amount of protein was measured by the method of Bradford (1976). Proteins were viewed by SDS-PAGE and Coomassie Blue staining. ATPase activity was determined by the method of Perlin and Spanswick (1981) with modification. A reaction mixture containing 25 μl of 50 mM Tris-HCl (pH 7.5), 3 mM ATP and approximately 10 pmol of recombinant protein or GST protein as a control was incubated at 30 °C for appropriate time periods in the presence or absence of 3 mM $MgCl_2$. The reaction was terminated by placing the samples on ice and addition of 50 μl of developing reagent (0.42% ammonium molybdate in 1 N H_2SO_4:10% ascorbate at 5:1 [v/v] ratio). After incubation at 25 °C for 30 min, the absorbance of the color complex was measured at 820 nm. The amount of released phosphate in the reaction mixture was determined using known concentrations of potassium dihydrogen phosphate as the standard.

Subcellular localization

The coding region of *NtAAA1*, with the stop codon deleted, was amplified by PCR using forward (5′-GGGTCGACATGATCAGCACAATGGGATTGC-3′) and reverse (5′-GGCCATGGAATTTTGGGTAAAACCATCTAA-3′) primers. The *Sal*I and the *Nco*I sites were linked at the 5′ ends of forward and reverse primers, respectively. The PCR products were introduced into pGEM®-T Easy vector as described above. Digested fragments were ligated into the corresponding site of the psGFP(S65T) vector. The Atcys-3A plasmid, which localizes in the cytoplasm (Barroso *et al*., 1995), was used as a positive control. Gold particles coated with plasmids were bombarded into onion epidermal cells according to the manufacturer's instructions (PDS-1000, Bio-Rad Laboratories, Hercules, CA) at a pressure of 1100 psi. After incubation of bombarded onion epidermal cells in the dark for 12 h at 25 °C, GFP was observed by fluorescence microscopy.

Plant transformation

For silencing constructs, the pKANNIBAL (Wesley *et al*., 2001) vector designed for producing hairpin RNA with a loop was used. For sense orientation, the coding region of *NtAAA1* cDNA (corresponding to positions 664 to1281) was amplified by PCR using forward (5'-CCCTCGAGAGGGAGAGTAAGGATTTCTATGC-3') and reverse (5′-GGGGTACCTGCAACATCAGCAGGTATGATC-3') primers. For anti-sense orientation, the same region was amplified using forward (5'-CCAAGCTTAGGGAGAGTAAGGATTTCTATGC-3') and reverse (5′-GCATCGATTGCAACATCAGCAGGTATGATC-3′) primers. *Xho*I and *Kpn*I sites were linked to the 5′ends of forward and reverse primers for sense orientation, respectively. The *Hind*III and the *Cla*I sites were linked to the 5' ends of forward and reverse primers for anti-sense orientation, respectively. All PCR products were introduced into the pGEM®-T Easy vector as described above. Digested fragments were ligated to the corresponding site of pKANNIBAL vector, which were introduced into *Agrobacterium tumefaciens* strain LBA4404 cells. Resulting plasmid was digested by *Not*I and ligated to the corresponding site of pART27, which was introduced into *Agrobacterium tumefaciens* strain LBA4404 cells. Tobacco transformation was performed as described previously (Yap *et al*., 2002).

Results

Identification of TMV-responsive cDNAs

Total RNA was isolated from leaves of tobacco carrying the *N*-gene (*N. tabacum*, cv Xanthi NC), which had been treated with either buffer (mock) or TMV. Inoculated samples were maintained at 30 °C for two days, and then transferred to 20 °C to allow plants to initiate the HR. Differentially expressed cDNAs between the two treatments 1 h or 3 h after the temperature shift were screened by the differential display method, and cDNA fragments finally identified were designated as *HRR* (hypersensitive response-related) with serial numbers starting from 8 (see Discussion). Their identity was determined by cloning and sequencing (Table 1), and the expression pattern examined by RNA blot analysis (Figure 1). *HRR8* encodes a 90-amino acid polypeptide, showing a high similarity to 5-epi-aristolochene synthase of *N. tabacum* (Mandujano-Chavez *et al*., 2000). Its transcripts were detected 6 h after the temperature

Table 1. Clones identified by differential display.

Clone	Fragment size (bp)	Best match
HRR7	1050	HSR203J[a] (100% identity)
HRR8[g]	272	5-epi-aristolochene synthase[b] (97% identity)
HRR9[h]	357	lectin-like protein kinase[c] (73% identity)
HRR10[i]	412	amino acid transporter-like protein[d] (51% identity)
HRR11	596	AAA-type ATPase-like protein[e] (53% identity)
HRR12	660	phospho-2-dehydro-3-deoxyheptonate aldolase 1 chloroplast precursor[f] (100% identity)
HRR13	1031	no homology

[a–i]Accession numbers; [a]AF212184; [b]AY313939; [c]AB030083; [d]AB030586; [e]AB010077; [f]M64261; [g]AB196435; [h]AB196436; [i]AB1966437.

shift. *HRR9* showed similarity to lectin-like protein kinase of *Populus nigra* (Nishiguchi *et al*., 2002), and transcripts also accumulated 6 h after temperature shift. *HRR10* encodes a 137-amino acid polypeptide showing a 51% similarity with amino acid transporter-like protein of *A. thaliana*. The transcript level was maximal at 6 h after temperature shift, and decreased markedly by 12 h. *HRR11* encodes a 198-amino acid polypeptide, containing conserved sequences of AAA-type ATPase family proteins, and its transcripts accumulated 3 h after temperature shift reaching a maximum at 12 h. This clone was further characterized in this study as this type of protein has never been reported to be associated with the HR. *HRR12* was found to be identical with phospho-2-dehydro-3-deoxyheptonate aldolase 1 chloroplast precursor of *N. tabacum* (Wang *et al*., 1991), and transcripts were detected 6 h after temperature shift with a gradual decrease to 24 h. Transcripts of *HRR13* showed no significant homology with any reported genes, and were detected only 6 h after temperature shift. In addition to the newly found 6 clones, we isolated a cDNA fragment showing 100% similarity to *HSR203J*. Although we previously identified a small fragment of this clone, designated as *HRR7* (Yoda *et al*., 2002), it was included for characterization in the present study. Its transcripts were quickly induced within 3 h after temperature shift.

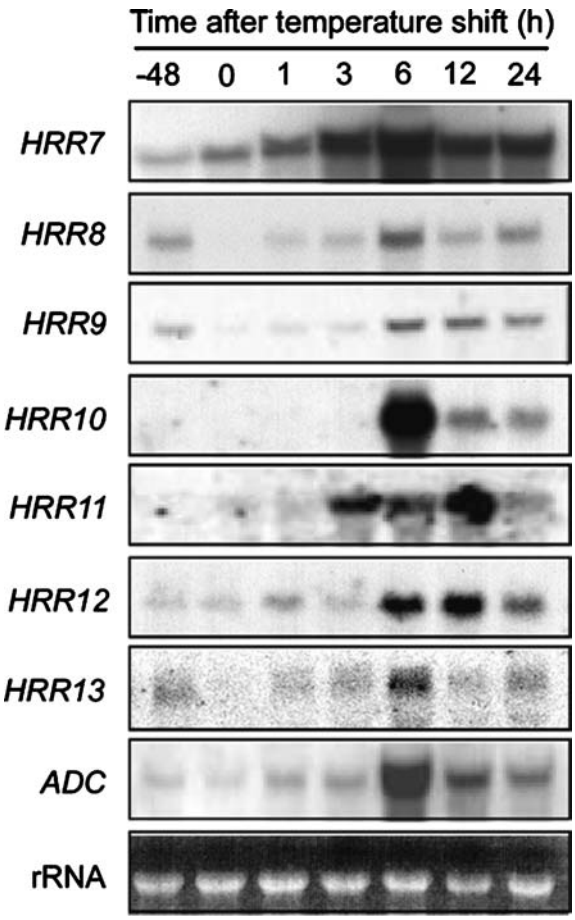

Figure 1. Transcript accumulation profile of TMV-responsive genes. Detached healthy tobacco leaves were inoculated with TMV, incubated at 30 °C for 48 h, and then transferred to 20 °C. Total RNAs were isolated at the indicated time before (−48) and after temperature shift. A 20-μg aliquot of total RNA per lane was fractionated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the indicated radioactively labeled probes (see Table 1). As the positive control for the HR, cDNA encoding arginine decarboxylase (ADC) was used (Yoda *et al*., 2003). As the loading control, rRNA was used (lower panel).

HRR11 encoding AAA-type protein

The *HRR11* clone was further characterized in detail. The longest cDNA isolated consisted of 1600 bp, in which a 1491-bp open reading frame encoding a polypeptide with 497 amino acids was

found (Figure 2A). Northern blot analysis using this cDNA as the probe showed the size of the corresponding transcript to be approximately 1.5 kb (data not shown), indicating the isolated cDNA to be full length. Southern blot analysis using a 1436-bp fragment as the probe showed two hybridization signals upon digestion by *Hin*dIII, with no recognition site present in the coding region (Figure 2B). Digestion with *BamHI*, which has one recognition site in this region, yielded one major and several minor signals. Although interpretation of such data is generally confounded by the amphidiploid nature of *N. tabacum* and the occurrence of introns, the simple hybridization pattern appears to be consistent with the *HRR11* gene being a single copy gene.

The molecular mass of the predicted polypeptide was 57 kDa, which demonstrated 53% and 47% identity with products of genes from *Arabidopsis* chromosome 5 (accession no. At5g40010) and rice (*Oryza sativa*) chromosome 3 (accession No. AAO72381), respectively. Although the functions of these genes are not known, both are predicted to encode AAA-type ATPase-like proteins based on their characteristic amino acid sequences. Typical AAA proteins have three conserved motifs, Walker motif A, Walker motif B and a second region of homology (SRH) region (Beyer, 1997; Patel and Latterich, 1998) (Figure 3A). Walker motif A, known as the P-loop, binds to phosphate in NTP, and Walker motif B binds to Mg^{2+}. These two motifs are present in common in P-loop NTPases, and are involved in NTP hydrolysis. In contrast, the SRH region is characteristic of the AAA protein family. The HRR11 could be shown to contain sequences showing high similarity to all three motifs (Figure 3A). Although phylogenetic analysis indicated that HRR11 does not belong to the classical AAA protein family, indicating a location far from the major group (Figure 3B), these observations suggested it to indeed be an AAA protein. Consequently, HRR11 was tentatively designated as NtAAA1 (*Nicotiana tabacum* AAA protein 1) in further study (accession no. AB174847).

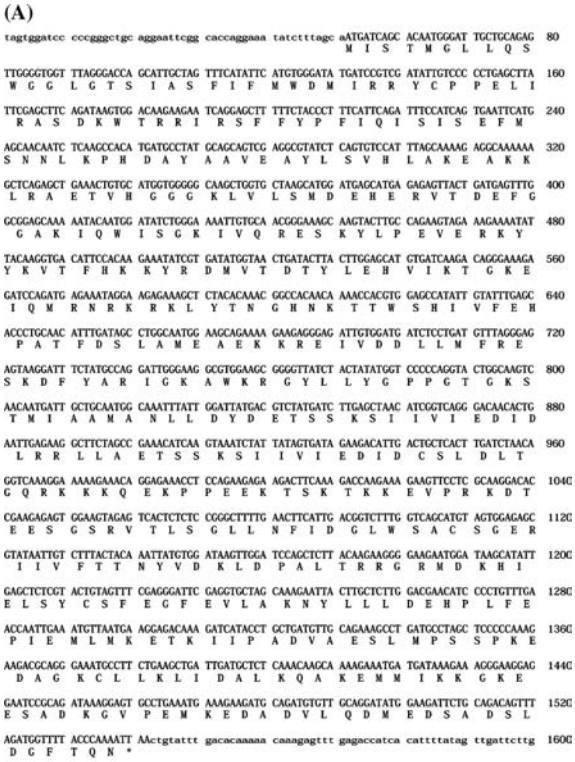

Figure 2. Properties of NtAAA1. (A) Nucleic and deduced amino acid sequences of *NtAAA1* (accession no. AB174847). Deduced amino acids for the ORF are shown under the nucleic acid sequence. Stop codons are indicated by asterisks. (B) Southern-blot analysis of *NtAAA1*. A 20-μg aliquot of genomic DNA from *N. tobacum*, cv Xanthi NN was digested with the indicated restriction enzyme, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the radioactively labeled *Pst*I-cut *NtAAA1* fragment as a probe.

Enzymatic activity

Using energy supplied by their ATPase activity, AAA proteins play critical roles in various cellular functions. To confirm ATP hydrolysis, NtAAA1 was bacterially expressed and ATPase activity assayed. The recombinant protein was produced in

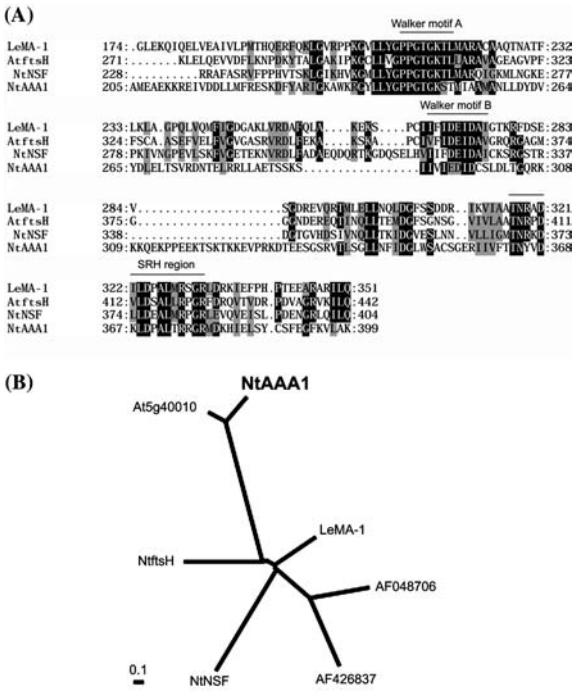

Figure 3. Amino acid sequence comparison of AAA domains. (A) Multiple alignment of NtAAA1 with the 26S proteasome S6a subunit homologue from tomato (*L. esculentum*) (LeMA-1; accession No. X74426), the ftsH homologue from *A. thaliana* (AtftsH; accession No. X99808) and the NSF homologue from *N. tabacum* (NtNSF; accession No. D86506) was performed with the ClustalW program. Structural motifs including Walker motif A, Walker motif B and SRH region are underlined. Identical and similar amino acids in at least three out of four AAA proteins are framed in black and gray boxes, respectively. (B) Phylogenetic tree showing the relationship between NtAAA1 and other AAA proteins. Amino acid sequences were aligned using the ClustalW program and the tree was constructed with Tree View software. Sequences were from above the three AAA proteins and three functionally unknown AAA proteins (accession Nos. At5g40010, AF426837, AF048706).

E. coli as a glutathione *S*-transferase (GST) fusion protein, which was purified through a glutathione Sepharose 4B column (Figure 4A). Immunoblot analysis using an anti-GST antibody showed the purified recombinant protein to have an approximate molecular mass of 65 kDa, being close to that calculated from deduced amino acid sequence. When subjected to ATPase assay, the recombinant protein exhibited distinct activity, in marked contrast to the GST protein itself showing no detectable hydrolysis (data not shown) (Figure 4B). ATP hydrolysis was evident in the absence of Mg^{2+}, but greatly enhanced in its presence, suggesting that NtAAA1 is an Mg^{2+}-dependent ATPase.

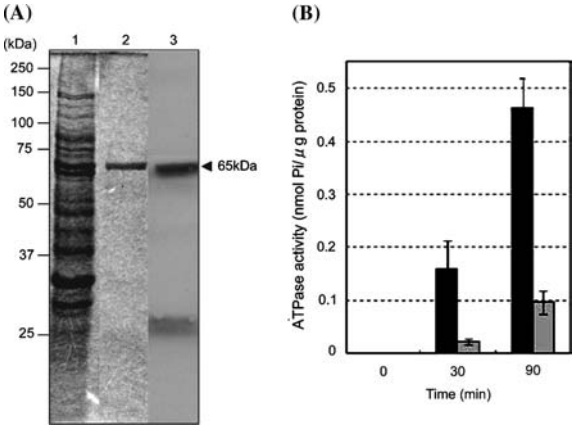

Figure 4. Expression and enzymatic activity of recombinant NtAAA1 protein. (A) SDS-PAGE profile. Recombinant NtAAA1 protein was induced by treating *E. coli* with isopropylβ-D-thiogalactopyranoside, and total cellular extracts (lane1) and proteins purified through glutathione-Sepharose column (lane2) were fractionated by 10% SDS-PAGE and stained with Coomassie brilliant blue. Proteins were subjected to immunoblot analysis with antibodies raised against glutathione *S*-transferase (GST; lane3). The arrowhead indicates purified recombinant protein. (B) Enzymatic activity. ATP-hydrolyzing activity of recombinant NtAAA1 protein was estimated in the presence (black bar) or absence (gray bar) of 3 mM magnesium sulfate. As the control, GST protein alone was used, which showed no activity (not shown). Values represent the mean of triplicates and standard deviations are shown by bars.

Cellular localization

The cellular localization of NtAAA1 was examined. A reporter gene encoding GFP was fused to *NtAAA1*, which was driven by the cauliflower mosaic virus (CaMV) 35S promoter, and subjected to transient assay. As controls, constructs containing GFP alone or GFP fused with *Atcys3A* encoding *O*-acetylserine-(thiol)lyase (Barroso *et al*., 1995), localized in the cytoplasm were used. When the control GFP was introduced into onion epidermis cells by biolistic bombardment, fluorescence signals were observed throughout the cell with particularly strong intensity in cytoplasm and nuclei (Figure 5A, B). The fusion construct of CaMV35S:: NtAAA1-GFP showed GFP signals throughout the cell (Figure 5C, D). The positive control containing CaMV35S::Atcys3A-GFP also showed ubiquitous signals (Figure 5E, F). Since the signal pattern of NtAAA1-GFP was the same as that of Atcys3A-GFP, it was concluded that

NtAAA1 is localized in cytoplasm, consistent with a lack of any distinguishing cellular localization sequence in the protein (Figure 2A).

Expression profile during the defense response

N. tabacum cv Xanthi NN possesses a resistant *N*-gene, which confers HR upon TMV infection. This was visibly evidenced by formation of necrotic lesions 9 h after temperature shift (data not shown). *NtAAA1* transcripts were found to accumulate 6 h after temperature shift, and gradually declined thereafter (Figure 6A upper panel). In contrast to cv Xanthi NN, *N. tabacum* cv Samsun nn does not carry the *N* gene, and is highly susceptible to TMV infection (data not shown). When this plant was treated with TMV in the same manner as Xanthi NN, *NtAAA1* transcripts were not induced (Figure 6A lower panel). In order to examine whether *NtAAA1* is causatively associated with HR, similar experiments were carried out with bacterial pathogens. *Psuedomonas syringae* pv. *glycinea* is an incompatible pathogen, causing severe necrosis on leaves 24 h after infection (data not shown). Transcripts of *NtAAA1* were clearly accumulated 9 h after its inoculation (Figure 6B upper panel). However, upon inoculation with a compatible pathogen, *P. syringae* pv. *tabaci*, transcripts were not induced (Figure 6B lower panel). These results suggest that *NtTAAA1* functions in incompatible pathogen–plant interactions.

Effects of signal molecules

To identify signaling pathways by which *NtAAA1* is controlled, effects of phytohormones on its

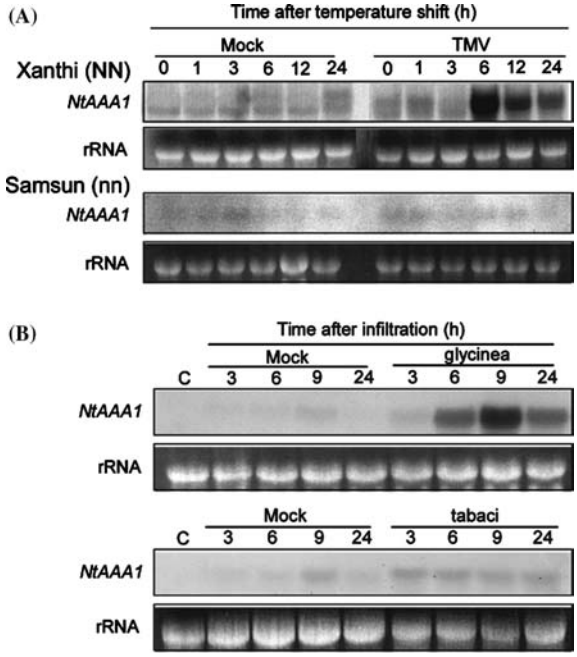

Figure 6. Induction of *NtAAA1* transcripts upon pathogen inoculation. (A) Accumulation of *NtAAA1* transcripts in tobacco plants treated with TMV. Detached healthy leaves from *N. tabacum*, cv Xanthi NN and Sumsun nn were treated with 10 mM sodium phosphate buffer alone (Mock), or containing TMV (10 μg/ml) (TMV), incubated at 30 °C for 48 h, and then transfered to 20 °C. Total RNA was isolated at the indicated time points, and 20 μg aliquots per lane were fractionated with agarose gel electrophoresis, transferred to a nylon membrane and subjected to RNA blot hybridization with the radioactively labeled *NtAAA1* probe. (B) Detached healthy leaves were treated with 10 mM magnesium sulfate alone (Mock), or containing *P. syringae* pv. *glycinea* (glycinea) or pv. *tabaci* (tabaci) (2×10^8 cfu/ml) using a syringe without a needle and incubated at 25 °C. Total RNA was isolated at the indicated time points after infiltration and subjected to RNA blot hybridization as described above and ethidium bromide stained. As the loading control, rRNA was used (lower panel).

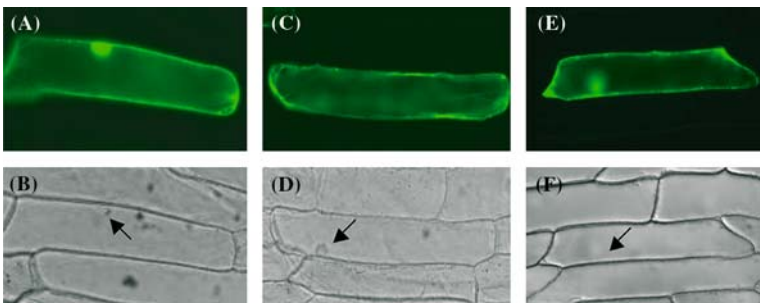

Figure 5. Cellular localization of NtAAA1. Onion epidermal cells were transformed with plasmids expressing green fluorescent protein (GFP; A, B), NtAAA1::GFP (C, D) or Atcys3A::GFP fusion protein (E, F). Green fluorescence under UV illumination (A, C, E) or corresponding differential interference contrast images (B, D, F) are shown at the same magnification. Arrow indicates the position of nucleus.

transcript accumulation were examined. When tobacco leaves were treated with SA, *NtAAA1* transcripts were not induced (Figure 7A). Since *PR1a* transcripts were correctly induced 6 h after treatment, *NtAAA1* expression appears to be SA-independent. This was confirmed with NahG transgenic tobacco plants, in which accumulation of endogenous SA is diminished, by rapid induction of *NtAAA1* transcripts (Figure 7B). In contrast, treatments with JA and ethephon (ethylene) induced *NtAAA1* transcripts within 1 h and 3 h after treatment, respectively (Figure 7C, D). The time course of induction was comparable with that of the respective control genes encoding ornithine decarboxylase (*ODC*) for JA (Yamaguchi *et al*., 2003) and chitin-binding protein (*CBP20*) for ethylene (Ponstein *et al*., 1994) (Figure 7C, D). These observations suggest that activation of *NtAAA1* is mediated through a pathway in which JA and ethylene, but not SA, are involved.

Analysis of RNAi transgenic plants

Transgenic tobacco plants were generated, in which the endogenous *NtAAA1* gene was silenced by the RNAi method (Wesley *et al*., 2001). Among fifteen kanamycin-resistant shoots initially obtained, two (#8 and #15) showed almost no induction of *NtAAA1* transcripts in leaves upon

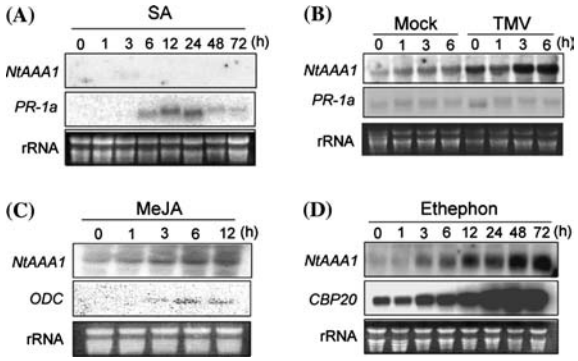

Figure 7. Effects of SA and MeJA. Detached healthy leaves of *N. tabacum*, cv Xanthi NN were left for 4 h for acclimatization to the initial wound stress, then treated with 2 mM SA (A), 50 μM MeJA (C) or 100 μM ethephon (D). Detached healthy leaves of NahG transgenic tobacco (B) were treated with phosphate buffer without (Mock) or with TMV (TMV), incubated at 30 °C for 48 h, then transfered to 20 °C. Total RNA was isolated at the indicated time points after the temperature shift, and 20 μg of total RNA per lane was fractionated and hybridized with the indicated cDNA probe. As the loading control, rRNA was used (lower panel).

TMV-infection 6 h after temperature shift (Figure 8A), indicating efficient suppression by RNAi. That TMV equally propagated in the samples before temperature shift was confirmed by hybridization, showing equal accumulation of coat protein transcripts (Figure 8A). In order to determine whether or not silenced *NtAAA1* influenced the defense reaction, transcripts for HSR203J, PR-1a and acidic PR-2, all participating in HR, were examined by RNA blot hybridization, followed by densitometric estimation of signal intensity (Figure 8A). The results showed that their accumulation was generally higher in transgenic lines than in wild type counterparts. For example, line #15 accumulated transcripts for *HSR203J* and acidic *PR-2* at two- and three-fold, respectively, the level in the wild-type control. *PR-1a* transcripts were 14-fold higher than in the control. These genes were not expressed in the absence of virus challenge, or HR (Figure 8B). In contrast, transcripts for PR-5 (osmotin), basic PR-3 and thionin were not affected during the HR (data not shown). A similar accumulation profile was evident with line #8 (Figure 8A).

The time course of transcript accumulation was then examined in leaves of T_1 plants infected by bacterial pathogen, *P. syringae* pv. *glycinea*. Transcripts of *NtAAA1* were induced 6 h after infection in wild-type plants, but not in the transgenic line, #15 (Figure 9A), being consistent with the case of TMV infection (Figure 8A). Transcripts for *PR-1a* began to accumulate 12 h after infection in both wild-type and transgenic plants, but the level was more than 2-fold higher in the latter (Figure 9A). A similar profile was also observed 6 h after infection for *PR-2* transcripts, although the acceleration was not so clear as that in *PR-1a* (Figure 9A). Since *PR-1a* strongly responds to SA, effect of exogenously applied SA was examined. In the wild-type plant, its transcripts began to accumulate 6 h after SA treatment, reached the maximal level 12 h and then declined almost to the basal level 24 h later (Figure 9B). In the transgenic line, the induction profile of transcripts appeared to be the same as in the control, but accumulation continued up to 24 h without showing decline as seen in the control (Figure 9B). The *PR-2* showed a similar profile with that of *PR-1a* (Figure 9B). These results indicated that suppression of *NtAAA1* results in acceleration of HR-induced transcript accumulation,

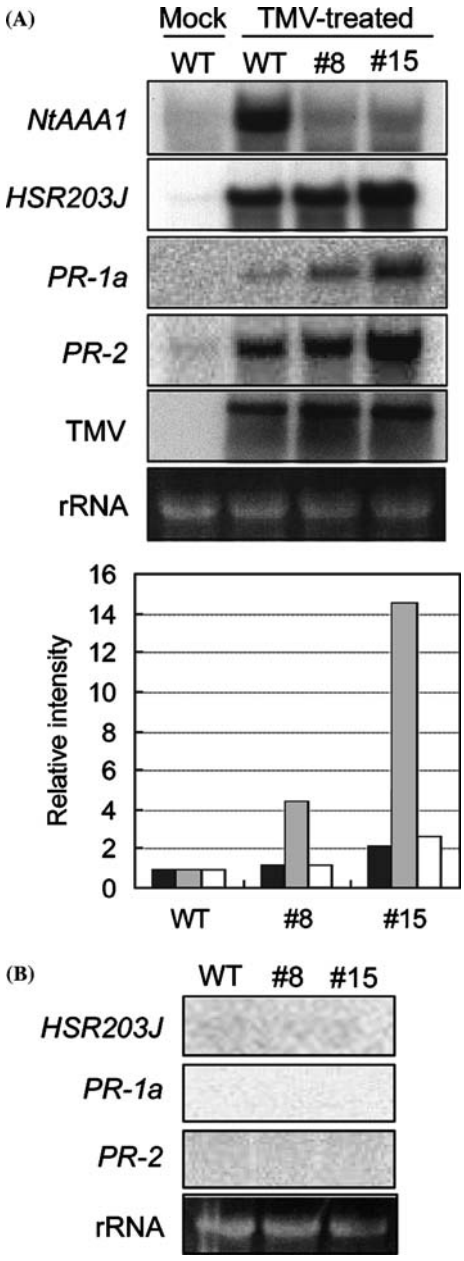

Figure 8. Analysis of *NtAAA1*-silenced tobacco plants. (A) Accumulation profile of transcripts of defense related genes upon 6h after HR onset. Detached healthy leaves of wild-type (WT) and T_0 of *NtAAA1*-RNAi transgenic plant lines #8 and #15 (#8, #15) were inoculated with TMV as described in the legend for Figure 6. Mock-treated wild-type (WT) was used as a negative control (Mock). A 10-μg aliquot of total RNA per lane was fractionated and hybridized with radioactively labeled probes for *NtAAA1*, *HSR203J* (X77136), *PR-1a* (Matsuoka *et al*., 1987) or acidic *PR-2* (M59443). Hybridization patterns are shown in the upper panel, and signals are densitometrically quantified (lower panel). The intensity for *HSR203J* (closed bar), *PR-1a* (shaded bar) or acidic *PR-2* (open bar) in wild-type was taken as one, and their relative intensity in transgenic lines was plotted. (B) Accumulation profile of transcripts of defense related genes under non-induction conditions. Total RNA was isolated from healthy leaves of wild-type (WT) or *NtAAA1*-RNAi transgenic plant lines #8 and #15 (#8, #15), and subjected to RNA blot hybridization as described above. As the loading control, rRNA was used (lower panel).

which is mediated through SA. The morphology of these transgenic plants was apparently normal, and upon infection by TMV or bacterial pathogen *P. syringae* pv. *glycinea,* visible necrotic lesions were formed to the similar extent as in wild-type controls under our experimental conditions (data not shown).

Discussion

Using the differential display method, we isolated six TMV-responsive cDNAs from TMV-infected leaves. Since we have previously identified seven TMV-responsible genes by a similar method, and designated these as *HRR* (hypersensitive response related) with serial numbers from 1 through 7 (Yoda *et al*., 2002), we referred to the present genes as *HRR* with serial numbers 8 through 13. Characteristics of the encoded proteins were as follows. HRR8 showed a high similarity to 5-epi-aristrochene synthethase, which is one of the key enzymes acting in sesquiterpene phytoalexin biosynthesis and is induced by a fungal elicitor and MeJA (Mandujano-Chávez *et al*., 2000). HRR9 resembled poplar lectin-like protein kinase, which responds to wounding (Nishiguchi *et al*., 2002). HRR10 showed similarity to amino acid transporters, some of which are reported to be induced by salt stress (Ueda *et al*., 2001). HRR11 appeared to belong to the AAA type ATPase family, whose function in plants is not known. Consequently it was designated as NtAAA1, and its properties were further analyzed in the present work. HRR12 was found to be 100% identical with tobacco phospho-2-dehydro-3-deoxyheptonate aldolase 1, one of the enzymes involved in shikimate pathway. Its potato homolog responds to wounding (Dyer *et al*., 1989). HRR13 is a novel protein, whose function is currently not clear. Although the majority of these proteins have been shown to be

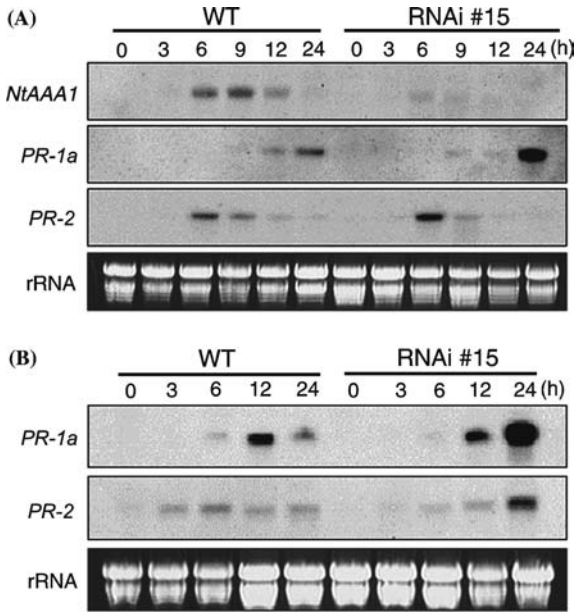

Figure 9. Time course analyses of pathogen responsive transcripts and effects of SA. (A) Induction profiles of HR-related genes. Healthy leaves of wild-type (WT) and T_1 of *NtAAA1*-RNAi transgenic (#15) plants were inoculated with bacterial pathogen, *P. syringae* pv. *glycinea,* and sampled at indicated time periods. A 10-μg aliquot of total RNA per lane was fractionated and hybridized with DIG-labeled probes for *NtAAA1*, *PR-1a* or *PR-2*. (B) Effects of SA on transcript accumulation. Healthy leaves of wild-type (WT) and T_1 of RNAi transgenic (#15) plants were cut into 2-cm^2 pieces and floated on a buffer containing 2 mM SA and sampled at indicated time periods. A 10-μg aliquot of total RNA per lane was fractionated and hybridized with DIG-labeled probes for *PR-1a* or *PR-2*. As the loading control, rRNA was used (lower panel).

responsive to various stresses, this is the first report, to our knowledge, showing them to be associated with the HR.

NtAAA1, which comprises 497 amino acids, contains the AAA conserved region with approximately 230 amino acid residues. Outside this AAA conserved region, however, the deduced protein contained no sequences resembling those in other major AAA proteins, nor distinct regions suggestive of particular functions. Nevertheless the conserved AAA motif implied that this protein hydrolyzes ATP as do other AAA proteins, and subsequent assay using recombinant protein indeed indicated it to possess Mg^{2+}-dependent ATPase activity. Coupled with ATP hydrolysis, AAA proteins generally change their conformation, and rearrange their interactions with macromolecular substrates (Vale, 2000). For example, the N-terminal domain of the AAA conserved region in yeast Yme1, a subunit of *i*-AAA protease in mitochondria, has been reported to play an important role in recognition of unfolded protein substrate, thus functioning like a chaperone (Leonhard *et al*., 1999). It is tempting to speculate that the AAA module in NtAAA1 plays a similar role in protein-protein interactions.

Transcripts of *NtAAA1* were readily induced by TMV infection in a resistant tobacco cultivar carrying the *N* gene. This was in marked contrast to the case with a susceptible tobacco cultivar without the *N*-gene, indicating *NtAAA1* to be under its control. Such an induction of *NtAAA1* in incompatible plant-pathogen interactions appeared to be general as evidenced by the interaction between tobacco and the bacterial pathogen, *P. syringae* pv. *glycinea*. In both cases, *NtAAA1* transcripts began to accumulate prior to formation of HR lesions, and declined when expression of PR proteins was initiated. This suggests NtAAA1 to specifically function in the early stage of the HR. An involvement of AAA proteins in plant-pathogen interaction was earlier indicated by a study demonstrating that transcripts of *DS9*, which encodes a tobacco homolog of chloroplast metalloprotease FtsH, constitutively accumulated in healthy leaves, dramatically decreased by inoculation with TMV (Seo *et al*., 2000). On analysis of transgenic tobacco lines both overexpressing and suppressing *DS9*, an inverse relationship was found between its expression and lesion formation. Thus FtsH may affect lesion formation through chloroplast homeostasis (Seo *et al*., 2000). Another example is the finding of a protein possessing an AAA conserved sequence, isolated by a yeast two-hybrid system using TMV replicase domain as the bait (Abbink *et al*., 2002). Our current results together with these reports suggest that AAA proteins may be generally involved in plant–pathogen interactions.

The mode of NtAAA1 induction appears to be complex. *In planta* assays using RNAi suppression lines showed that, after temperature shift, the amount of TMV is the same in both transgenic and control wild-type plants. This indicated that virus equally propagated in the absence of the activated *N*-gene in both plants. Once the *N*-gene was activated, transcripts of defense-related genes were equally induced in both plants, but their level was much higher in transgenic lines than in the

control. Since *NtAAA1* was expressed in the control, but not in transgenic lines at this stage, the accelerated transcript accumulation in the latter was clearly due to the absence of NtAAA1. This implies that NtAAA1 functions in mitigation of the HR.

The molecular basis for such a regulation remains to be clarified in detail, but responses to SA and JA of *NtAAA1* itself and genes involved in HR in RNAi transgenic lines are very suggestive. We found that induction of *NtAAA1* transcripts was dependent on JA and ethylene but not on SA. Indeed, *NtAAA1* was transcriptionally activated more rapidly in SA-deficient plants than in the wild type controls, suggesting an association of *NtAAA1* with genes controlled via interaction between SA- and JA-signaling pathways, which antagonistically or synergistically affect each other (Felton *et al*., 2000). This was partially confirmed by a finding that expression of *PR-1a* and acidic *PR-2*, dependent on SA, was enhanced in *NtAAA1* silenced plants. In contrast, expression of basic *PR-2* and *thionin*, both known to be JA responsive, was apparently not affected (unpublished observation). These results indicate that suppression of *NtAAA1* might cause an amplification of SA signaling. It was therefore conceivable that *NtAAA1* usually acts as a negative regulator of the SA signaling pathway, thereby alleviating detrimental excess HR.

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