Silencing of DS2 aminoacylase-like genes confirms basal resistance to *Phytophthora infestans* in *Nicotiana benthamiana*

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*Nicotiana benthamiana* is a potential host to several plant pathogens, and immature leaves of *N. benthamiana* are susceptible to *Phytophthora infestans*. In contrast, mature leaves of *N. benthamiana* are weakly susceptible and show basal resistance to *P. infestans*. We screened a gene-silenced mature plant showing high resistance to *P. infestans*, designated as DS2 (Disease suppression 2). The deduced amino acid sequence of cDNA responsible for DS2 encoded a putative aminoacylase. Growth of *P. infestans* decreased in DS2 plants. Trypan blue staining revealed inhibited hyphae growth of *P. infestans* with an increased number of dead cells under the penetration site in DS2 plants. Consistent with growth inhibition of *P. infestans*, defense responses such as reactive oxygen generation and expression of a salicylic acid-dependent *PR-1a* increased markedly in DS2 plants compared with that of control plants. DS2 phenotype was compromised in NahG plants, suggesting DS2 phenotype depends on the salicylic acid signaling pathway. Accelerated defense response was observed in DS2 plants elicited by INF1 elicitor as well as by NbMEK2DD, which is the constitutive active form of NbMEK2, and act as a downstream regulator of INF1 perception. On the other hand, INF1- and NbMEK2DD-induced defense responses were prevented by DS2-overexpressing transgenic tobacco. These results suggest that DS2 negatively regulates plant defense responses against *P. infestans* via NbMEK2 and SA-dependent signaling pathway in *N. benthamiana*.

**Introduction**

Plants have evolved innate immune responses to detect and respond quickly to pathogen infections.1 They use transmembrane pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) at the cell surface. Plants perceive bacterial flagellin, EF-Tu, and fungal chitin oligomers through their cognate receptors FLS2, EFR, and CERK1, respectively.2,3 Plants also recognize avirulent gene products as associated molecular patterns (PAMPs) at the cell surface. Plants respond quickly to pathogen infections.1 They use transmembrane pattern recognition receptors that recognize pathogen- or enzymes involved in biosynthesis of antimicrobial compounds. Other pathogen-induced genes encode proteins with regulatory functions in signal transduction pathways of plant defense responses.2 Knowledge of molecular mechanisms of disease resistance has increased tremendously in recent years. However, little is known about the molecular basis of disease susceptibility.

Our strategy to isolate candidates for genes required for susceptibility (plant disease susceptibility factors) was to screen knock-down plants showing a disease-resistant phenotype. Virus-induced gene silencing (VIGS) is a powerful tool for analyzing gene function. Based on this principle, we performed VIGS screening of genes related to disease susceptibility using *Nicotiana benthamiana* and the potato virus X vector system. We previously isolated a plant that showed high resistance to *Ralstonia solanacearum*, designated as DS1 (Disease suppression 1).10 In this study, we screened a VIGS plant that showed barely noticeable late blight symptoms after inoculation with *Phytophthora infestans*, and designated it as DS2 (Disease suppression 2). We analyzed and discussed the molecular mechanisms of the DS2 phenotype.
Results

DS2 plants showed enhanced resistance against *P. infestans*

We previously reported that DS1 (Disease suppression 1) plants showed high resistance to *Ralstonia solanacearum*, the causal agent of bacterial wilt, which is a serious disease in Solanaceae plants.\(^{10}\) Potato late blight is also an important disease like bacterial wilt disease. *Phytophthora infestans* is the causal agent of late blight in potato, and a potential pathogen to *Nicotiana benthamiana*.\(^{21}\) In this study, we focused on resistance against *P. infestans*. Therefore, we screened a gene-silenced plant showing high resistance to *P. infestans* using high throughput VIGS, and designated the plant as DS2 (Disease suppression 2) (Fig. 1A). Expression of DS2 decreased markedly in the silenced plants compared with that in control plants (Fig. 1B). There was no noticeable morphological difference between control and DS2-silenced plants (Fig. 1C, D). All control leaves were infected and sporulating 6 d after inoculation with *P. infestans*. In contrast, sporulation was barely observed in the DS2 plant leaves (Fig. 1A, E). At 4 d after inoculation with *P. infestans*, an approximately 15-fold reduction in growth of *P. infestans* was observed in DS2 plants compared with control plants (Fig. 1F).
DS2 plants showed accelerated defense responses against *P. infestans*

In general, disease resistance to pathogens correlates to enhanced defense responses. Therefore, we first examined cell death, the most characterized defense response, in control and DS2 plants inoculated with *P. infestans*. Trypan blue staining showed that there were no detectable dead plant cells in control plants 36 h after inoculation, and *P. infestans* hyphae developed extensively in the intercellular spaces of mesophyll cells. In DS2 plants, blue-stained epidermal cells were clearly observed under and around the penetration site of *P. infestans*, whereas hyphal growth was barely detected (Fig. 1G). These data suggest that hyphal growth of *P. infestans* was inhibited in DS2 plants with an increased number of dead cells. Because cell death responses are usually accompanied by ROS production and defense-related gene expression, we evaluated ROS accumulation and PR-gene level in control and DS2 plants in response to *P. infestans* infection. At 24 h after inoculation with *P. infestans*, a high level of ROS production was observed in DS2 plants compared with control plants (Fig. 1H). Moreover, expression of salicylic acid (SA)-responsive *PR-1a* increased significantly in DS2 plant leaves 24 h after inoculation (Fig. 1I).

**DS2 encodes aminoacylase-like gene**

The deduced amino acid sequence of full-length DS2 shows 97% and 86% identity with its ortholog in *N. tabacum* (NtACY1) and *Solanum tuberosum* (StACY), and 85%, 72%, 70%, and 67% identity with uncharacterized protein from *Solanum lycopersicum* (SlUP), putative aminoacylase 1 from *Ricinus communis* (RcACY1P), peptidase M20 family protein from *Arabidopsis thaliana* (AtM20FP), and aminoacylase 1 from *Medicago truncatula* (MtACY1) (Fig. 2A), respectively. The residues required for ACY activity, His\(^80\), Asp\(^113\), Glu\(^148\), Glu\(^175\), and His\(^373\) were highly conserved in DS2 (Fig. 2).\(^{12}\) These results suggest that DS2 might act as an ACY1 in *N. benthamiana*.

**SA signaling plays crucial role in DS2 phenotype**

DS2 phenotype was accompanied by enhanced expression of *PR-1a* during *P. infestans* infection. To determine whether the enhanced resistance in DS2 plants is caused by SA signaling or not, we used the *N. benthamiana*-expressing SA-degrading enzyme NahG that suppresses SA signaling. We created a VIGS plant based on NahG (NahG:DS2) plants without noticeable growth phenotypes (Fig. 3A, B, C). Both NahG control and NahG:DS2 plants were infected with *P. infestans* (Fig. 3D, E). At 4 d after inoculation with *P. infestans*, an increase in the biomass of *P. infestans* was observed in NahG:DS2 plants similar to NahG control (Fig. 3F). Furthermore, no significant differences were observed in defense responses to *P. infestans*, such as cell death, ROS production, and *PR-1a* expression, between NahG control and NahG:DS2 plants (Fig. 3G, H, I). These results suggest that SA signaling pathways are required for DS2 phenotype.

**DS2 regulates PTI signaling**

It has been shown that INF1 is a major secretory protein of *P. infestans*, and acts as a PAMP, inducing PAMP-triggered immunity (PTI) in *Nicotiana* spp.\(^{13,14}\). To determine whether DS2 phenotype was related to PTI, control, and DS2 plant leaves were
inoculated with *Agrobacterium* carrying the INF1. We observed necrotic lesions 2 d after inoculation with *Agrobacterium* carrying INF1 in *DS2*-silenced plants, whereas no lesions were visible in control plants (Fig. 4A). At 3 d after inoculation, INF1-triggered necrotic lesions also appeared in control plants (data not shown). To investigate a possible role of DS2 in PTI, we further analyzed INF1-triggered responses in control and *DS2*-silenced plants. Compared with control plants, *DS2* plants showed accelerated cell death in response to INF1 (Fig. 4B, C). Furthermore, INF1-elicited ROS production also increased in *DS2* plants compared with that in control plants (Fig. 4D).

**Relationship of DS2 to MAP kinase signaling**

Recent studies have revealed that mitogen-activated protein kinase (MAPK) cascades have a crucial role in PTI. In *N. benthamiana*, *NbMEK2* positively regulates production of ROS and cell death elicited by INF1 protein. To further elucidate the role of *DS2* in PAMP signaling after INF1 perception, *NbMEK2DD*, a constitutive active form of *NbMEK2*, was transiently expressed in control and *DS2* plant leaves. Consistent with the enhanced responses to INF1, *DS2* plants showed acceleration of *NbMEK2DD*-induced necrotic lesions, cell death, and ROS production (Fig. 4E, F, G, H), compared with control plants.

**Overexpression of DS2 exhibited INF1- or NbMEK2DD-elicited defense responses**
INF1- or NbMEK2DD-elicited responses were accelerated in DS2 plants, suggesting that DS2 might act as a negative regulator of these responses. To test this hypothesis, we created DS2-overexpressing transgenic tobaccos (DS2-OX#2, OX#9) (Fig. 5A, B, C). We observed necrotic lesions caused by INF1 or NbMEK2DD in GFP-OX plants, whereas only weak lesions were visible in both DS2-OX#2 and DS2-OX#9 plants at least 2 d after inoculation (Fig. 5D). Both INF1 and NbMEK2DD expression induced cell death in GFP-OX leaves, whereas DS2-overexpressing plants showed markedly suppressed cell death (Fig. 5E, F, G).

**Discussion**

Potato late blight’s impact on humankind is rivaled by few other plant diseases. Phytophthora infestans is the causal agent of late blight in potato and has continued to wreak havoc on potato fields throughout the world. Late blight remains the most destructive disease, resulting in annual losses of potatoes. Therefore, development of an effective method of controlling the disease is important for plant pathologists.\(^6\) P. infestans is a potent pathogen of N. benthamiana.\(^11\) Therefore, we searched for a P. infestans-resistant plant by high-throughput screening with VIGS, and isolated a DS2 plant. Growth of P. infestans was dramatically suppressed in DS plants. Microscopic analysis showed inhibited hyphae growth of P. infestans. We also observed cell death induction around P. infestans-penetrating areas (Fig. 1).

These observations suggested that induction of HR-like defense in the DS2 plant against P. infestans.

Previous reports showed the relationship between SA and defense responses against P. infestans. Hyphal wall elictors of P. infestans activate a 51-kD SA-inducible protein kinase-like (SIPK-like) MAPK in potato tubers.\(^17\) In addition, resistance against P. infestans required NbrbohB, which was induced by SA.\(^18\) SYNTAXIN-RELATED 1 (StSYR1) silencing confirmed resistance against P. infestans with correlation with the constitutive accumulation of salicylic acid and PR-1 transcripts.\(^19\) Transgenic NahG plants showed a drastic enhancement of pathogen growth in potato plants by depletion of SA, indicating SA is an important compound required for basal defense of potato against P. infestans.\(^20\) In addition, virus-induced gene silencing of NbICS1, the genes for SA biosynthesis, reduced resistance of mature N. benthamiana against P. infestans indicating the requirement of SA for the resistance of mature N. benthamiana.\(^21\) Present data showed that the appearance of the DS2 phenotype might be SA-dependent signaling, since DS2 plants showed overexpression of SA-dependent PR-1a against P. infestans infection, and DS2 phenotype was compromised in NahG plants (Fig. 1, 3). Plants activate distinct defense responses depending on the lifestyle of the attacking pathogens.\(^22\) In these responses, SA plays an important signaling role, and induces defense against biotrophic pathogens that feed and reproduce on live host cells. The oomycete P. infestans is a near-obligate (a potential biotroph) pathogen.\(^23\) Therefore, dependency of DS2 phenotype on SA is in good agreement with the lifestyle of P. infestans.
Plants commonly use two distinct classes of immune responses, including PTI and ETI. DS2 plants showed accelerated defense response, HR and ROS, induced by INF1. Additionally, the defense responses induced by NbMEK2\(^{DD}\), which act as a signaling component downstream regulator of INF1 perception,\(^{18}\) were also enhanced in DS2 plants. In addition, INF1- and NbMEK2\(^{DD}\)-induced defense responses were prevented by overexpression of DS2. Therefore, DS2 may act in INF1 signaling downstream of NbMEK2. In contrast, no differences between control and DS2 plants were observed in the cell death caused by the interaction between Avr3a and its cognate R3a (data not shown). These results suggest that DS2 negatively regulates plant defense responses triggered by INF1, but is not related to defense triggered by Avr3a-R3a interaction. Further analysis is needed to not only clarify the role of DS2 in signaling other different PAMPs and effectors, but also different signaling mechanisms between INF1 (PAMP) and Avr3a-R3a (effector). DS2 might be a useful tool for the analysis.

We found that DS2 encoded a gene containing conserved amino acid residues responsible for ACY activity (Fig. 2). A homology search of DS2 indicated ACY-like genes are conserved in several dicot plant species, including potato and tomato. Therefore, DS2-like ACY might function in potato plants during natural interaction between potato and \(P.\) infestans. Aminoaoylase I (EC 3.5.1.14) is a homodimeric zinc binding metalloenzyme located in the cytosol, and it catalyzes the hydrolysis of N-linked acyl groups in L-amino acids, including the N-acetylated derivatives of serine, glutamic acid, alanine, methionine, glycine, leucine, and valine.\(^{24}\) In mammal cells, the biological functions of ACY have been well documented mainly in N-acetylated amino acid metabolisms and its relationship to cell death. ACY3 efficiently deacetylates and detoxifies 4-hydroxy-2-nonenal and acrolein, which are highly reactive neurotoxic products of lipid peroxidation.\(^{25}\) ACY3 deacetylates halogenated mercapturic acids, which are a cytotoxic agent in mice.\(^{26}\) In contrast, transfection
of ACY1 inhibited cell proliferation and increased apoptosis in renal cell carcinoma cell lines.\textsuperscript{27} The ACYs are also shown to relate to intracellular catabolism of N-acetylated proteins that are generally assumed by the ubiquitin system. This proteolytic system is involved in fundamental biological functions such as the cell cycle, apoptosis, metabolism, signal transduction, immune response, and protein quality control.\textsuperscript{28} Plant immune responses, including resistance gene-dependent HR, are also related to protein degradation via the ubiquitin system.\textsuperscript{29} In this experiment, we observed acceleration of cell death by inoculation with \textit{P. infestans} and \textit{Agrobacterium}-expressing INF1- and NbMEK2\textsuperscript{\textregistered} in DS2 plants (Fig. 1, 4). Therefore, we believe that DS2 may negatively regulate cell death and suppression of immune responses, resulting in establishing disease susceptibility. As far as we know, this is the first report showing the possible function of ACY in plants, especially as a disease susceptible factor. Furthermore, confirmation is needed of the functional analysis of DS2 and the functional link between DS2, SA, and suppression of plant immune responses (disease susceptibility).

**Material and Methods**

**Plant growth conditions and pathogen inoculation**

\textit{Nicotiana benthamiana}, transgenic \textit{N. benthamiana} expressing NahG, and \textit{N. tabacum} were grown as previously described.\textsuperscript{30} \textit{Phytophthora infestans} race 1.2.3.4 was maintained on susceptible potato (\textit{Solanum tuberosum}) tubers.\textsuperscript{31} Immature leaves of \textit{N. benthamiana} are susceptible, whereas mature leaves of \textit{N. benthamiana} are weakly susceptible and show basal resistance to \textit{P. infestans} race 1.2.3.4.\textsuperscript{31} A suspension of the zoospores was prepared as described by Yoshioka et al. \textit{Nicotiana benthamiana} leaves were inoculated with a 0.3 ml drop of \textit{P. infestans} zoospores (2 x 10\textsuperscript{5} zoospores/ml) and covered with lens paper to disperse the zoospores. The inoculated leaves were kept at high humidity at 25 °C for 1 d after inoculation, and were then moved to a plant growth condition as described above. Disease development of late blight was evaluated daily using the following scoring index for each sample as shown previously.\textsuperscript{13} Data are based on 3 independent biological replicates.

**RNA and DNA isolation, and cDNA synthesis**

Total RNA and DNA were isolated from \textit{N. benthamiana} leaves with a NucleoSpin RNA plant kit in combination with an RNA/DNA Buffer Set (Macherey-Nagel, Düren, Germany) according to the manufacturer's manual. Reverse transcription was performed with 1 µg total RNA using a PrimeScript RT reagent Kit (Takara Shuzo, Shiga, Japan).

**Isolation of full-length cDNA**

PCR amplification was performed with the primers DS2S (CATATGAGTTTTTGGCGGCCTTAG) and DS2A (GGA TCCCTCAATGATGATGATGATGATGCAATTTCTTCCC TCGG). Cycling parameters were as follows: 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The full-length cDNA was cloned into pGEMT-Easy (Promega), creating pGEM-DS2.

**Sequencing**

Sequencing analysis was performed using M4 (GTTTTCCCAGTCAGACGAC) and RV (CAGGAAACAGATATGACGAC) primers with the reagents for the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and Applied Biosystems 3100 Avant Automated Sequencer (Applied Biosystems) according to the manufacturer's instructions. The sequence analysis was performed using DNASIS (version 3.6; Hitachi) and the BLAST network service from the National Center for Biotechnology Information.

**Quantitative real-time PCR**

Expression levels of defense-related genes and the growth of \textit{P. infestans} were determined by quantitative real-time PCR as described by Maimbo et al.\textsuperscript{32} and Asai et al.,\textsuperscript{33} respectively. Quantitative PCR was performed in a 20 µl reaction mixture containing 1 µl cDNA or DNA, and 10 pM respective primers using SYBR GreenER qPCR Reagent System (Invitrogen) on an Applied Biosystems 7300 real-time PCR instrument. The cycling parameters were the same for all primers: initial 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Primers used in this experiment were as follows: DS2rtpF (CCTTCTCCGGCCACCTTAGA) and DS2rtpR (GAGAACCCTGGGCGTAGA) for N\textit{bac}, PR1rtpF (GGTCAACAGCGGAAACC) and PR1rtpR (GCCCTAGCAGCGCTACTG) for \textit{PR-1}, FboxrtpF (GCCACTACAACAGGCTGACTAC and FboxrtpR (ACCTGGGAGCATTGGGAGCTTAT) for F-Box, L23rtpF (AAGGATGCCGTGAAGAGATG) and L23rtpR (GCATCGTAGTCAGAGTCAAC) for L23, EF1rtpF (CCTCAAGAAGGTTGGATACAC) and EF1rtpR (TTATGCTCAACATCGGTTGTC for N\textit{hEF-1} gene, and PirtpF (TGCACTGTTGAGGCCGGAAG) and PirtpR (GCCCGAGGCCCAATTCA) for biomass of \textit{P. infestans}. Melting curve runs were performed at the end of each PCR reaction to verify the specificity of primers by the presence of a single product. The relative amount of \textit{P. infestans} genes was calculated using N\textit{hEF-1} as standard.\textsuperscript{32} The normalization factor of plant gene expression was calculated as a geometric mean of F-box and L23 for each sample as shown previously.\textsuperscript{34} Data are based on 3 independent biological replicates.

**Virus-induced gene silencing**

A 264 bp cDNA fragment of DS2 was amplified with primers DS2-\textit{PVX}S (AAATAGCCGTGATCTGCTAGCAGA AA) and DS2-\textit{PVX}A (AAATAGCCGTGATCTGCTAGCAGA AA). This cDNA fragment was subcloned into the TA cloning site of pGEM-T-Easy, creating pGEM-PVX::DS2. The pGEM-PVX::DS2 plasmid was digested with PstI and Sall, and ligated into the PVX vector p\textit{PVX}201 digested with Sce83871 and Sall.\textsuperscript{35} The construct containing this insert in the antisense orientation was designated as p\textit{PVX}::DS2. The plasmids, p\textit{PVX}201 (empty control) and p\textit{PPVX}::DS2, were transformed into \textit{A. tumefaciens} strain GV3101, and inoculated into \textit{N. benthamiana} leaves as described previously,\textsuperscript{13} and we used approximately 30-d-old silencing plants, which showed basal resistance (weakly susceptible) to \textit{P. infestans}.\textsuperscript{18}
Trypan blue staining
Trypan blue staining was performed as described previously with minor modifications. Inoculated leaves were boiled for 2 min in trypan blue staining solution (60 ml ethanol, 10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 10 ml distilled water, 67 mg trypan blue). The leaves were decolorized in 2.5 g ml\(^{-1}\) chloral hydrate for at least 1 h. Stained leaves were observed using a fluorescence microscope (FSX100; Olympus).

Cell death quantification
Cell death was quantified by ion conductivity as described previously. For ion leakage measurements, 6 leaf discs (8 mm in diameter) were collected from each plant and floated on 1 ml distilled water for 4 h at room temperature with gentle shaking. Conductivity of the water was measured using a twin conductivity meter (Twin Cond D-173; Horiba).

ROS measurement
ROS measurements were conducted as described by Kobayashi et al. The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence. The L-012 probe (0.5 mM in 10 mM MOPS-KOH, pH 7.4) was infiltrated into \(N.\) benthamiana leaves using a needleless syringe. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera (LAS-4000 mini), and quantified with Multi Gauge ver. 3.0 software (Fujifilm, Tokyo, Japan).

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration)

Agrobacterium tumefaciens strain GV3101 carrying GUS, INF1, R3a, AVR3a, NbMEK2 were prepared as described previously. These constructs were transiently expressed in \(N.\) benthamiana as described previously.

Creation of transgenic tobacco plants
The pGEM-DS2 was digested with BamHI and SacI (Takara Bio), and ligated into the pRI101 vector (Clontech, Tokyo, Japan) digested with the same enzymes. The construct containing the insert was designated as pRI::DS2. Tobacco plants (\(N.\) tabacum cv Samsun NN) aseptically grown from seed for approximately 1 mo were transformed with pRI::DS2 via the \(A.\) tumefaciens-mediated leaf disc procedure and selected using 5 µg ml\(^{-1}\) kanamycin as the selection reagent. Genomic PCR was applied to confirm successful transformation with pRI::DS2 using primers based on CaMV35S promoter (35S-S) and NOS terminator (NOS-A) sequences and tobacco \(NbEF-\alpha\) primers as described previously.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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