Activation of plant immunity by exposure to dinitrogen pentoxide gas generated from air using plasma technology

Daiki Tsukidate1, Keisuke Takashima2, Shota Sasaki2, Shuhei Miyashita1, Toshiro Kaneko2, Hideki Takahashi1, Sugihiro Ando1*

1 Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan, 2 Graduate School of Engineering, Tohoku University, Sendai, Miyagi, Japan

* sugihiro.ando.a2@tohoku.ac.jp

Abstract

Reactive nitrogen species (RNS) play an important role in plant immunity as signaling factors. We previously developed a plasma technology to partially convert air molecules into dinitrogen pentoxide (N$_2$O$_5$), an RNS whose physiological action is poorly understood. To reveal the function of N$_2$O$_5$ gas in plant immunity, Arabidopsis thaliana was exposed to plasma-generated N$_2$O$_5$ gas once (20 s) per day for 3 days, and inoculated with Botrytis cinerea, Pseudomonas syringae pv. tomato DC3000 (Pst), or cucumber mosaic virus strain yellow (CMV(Y)) at 24 h after the final N$_2$O$_5$ gas exposure. Lesion size with B. cinerea infection was significantly ($P < 0.05$) reduced by exposure to N$_2$O$_5$ gas. Propagation of CMV(Y) was suppressed in plants exposed to N$_2$O$_5$ gas compared with plants exposed to the air control. However, proliferation of Pst in the N$_2$O$_5$-gas-exposed plants was almost the same as in the air control plants. These results suggested that N$_2$O$_5$ gas exposure could control plant disease depending on the type of pathogen. Furthermore, changes in gene expression at 24 h after the final N$_2$O$_5$ gas exposure were analyzed by RNA-Seq. Based on the gene ontology analysis, jasmonic acid and ethylene signaling pathways were activated by exposure of Arabidopsis plants to N$_2$O$_5$ gas. A time course experiment with qRT-PCR revealed that the mRNA expression of the transcription factor genes, WRKY25, WRKY26, WRKY33, and genes for tryptophan metabolic enzymes, CYP71A12, CYP71A13, PEN2, and PAD3, was transiently induced by exposure to N$_2$O$_5$ gas once for 20 s peaking at 1–3 h post-exposure. However, the expression of PDF1.2 was enhanced beginning from 6 h after exposure and its high expression was maintained until 24–48 h later. Thus, enhanced tryptophan metabolism leading to the synthesis of antimicrobial substances such as camalexin and antimicrobial peptides might have contributed to the N$_2$O$_5$-gas-induced disease resistance.

Introduction

Developing agricultural systems that minimize environmental impacts remains a major challenge. Excessive use of chemical fertilizers and pesticides include the risks of contaminating
the soil and harming the ecosystem [1]. The concept of Integrated Pest Management (IPM), which effectively combines a variety of control strategies rather than relying solely on chemical pesticides, has become widely accepted in the context of pest control [1]. These efforts can contribute to the achievement of the United Nation’s Sustainable Development Goals (SDGs).

Therefore, it is desirable to develop further technologies to reduce the environmental impacts of agriculture and realize sustainable agricultural systems.

Plasma is a state of matter that can be observed as lightning or auroras in nature and is characterized by electrically charged energetic particles that can form highly reactive states such as radicals. Atmospheric pressure air plasma can be generated using air under atmospheric pressure with low electric power (<100 W) potentially supplied by renewable energy resources. Because of the low resource demands for its generation as well as its ability to generate biologically active reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2], atmospheric pressure air plasma is attracting attention as a potentially sustainable technology in the fields of medicine and agriculture [3, 4].

Typical reactive species produced in atmospheric pressure air plasma include ozone (O$_3$) as a ROS as well as nitric oxide (NO), nitrogen dioxide (NO$_2$), and dinitrogen pentoxide (N$_2$O$_5$) as RNS [5–7]. It is well known that ROS and RNS are important signaling factors in the immune responses of plants. Plants produce ROS and RNS as a defense response when they perceive an infectious stimulus from a pathogen [8, 9]. The generated ROS and RNS function as signaling molecules that contribute to the activation of plant immunity [8, 10]. The functional ROS produced by plant cells include superoxide anion (O$_2^-$), hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (¹O$_2$), among others. O$_2^-$ and H$_2$O$_2$ have been of particular interest in studies of the mechanisms of plant disease defense [11]. Also, RNS such as NO are produced during plant immune responses [12, 13]. Plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are generally known to play important roles in the regulation of plant immunity [14], and many studies have reported that ROS and RNS signals are linked to the activities of these plant hormone signals [15].

Some reports indicate that exogenous application of RNS enhances plant disease resistance. Treatment with NO-releasing compounds appears to suppress tobacco mosaic virus infection in tobacco and rice black-streaked dwarf virus infection in rice through salicylic acid-mediated resistance [16, 17]. Furthermore, exposure of Arabidopsis to NO$_2$ gas enhances basal resistance to Botrytis cinerea and Pseudomonas syringae by activating SA and JA signaling [18]. Exposure to high concentrations of NO$_2$ gas, but not NO gas, induces cell death in A. thaliana [19]. When used to treat plants, NO$_2$ gas is known to convert to nitrate (NO$_3^-$) and nitrite (NO$_2^-$). Thus, NO$_3^-$, NO, and H$_2$O$_2$ might act in a coordinated manner to regulate NO$_2$-induced cell death [19]. Other reports show that NO and NO$_2$ induce protein S-nitrosylation and tyrosine nitration, thereby regulating protein functions such as plant immunity and cell death [9, 20, 21].

Dinitrogen pentoxide is an active nitrogen species whose physiological effects are poorly understood due to difficulties with its synthesis and storage. We have developed a device that uses plasma technology to generate an active species gas containing a high concentration of N$_2$O$_5$ [6]. The gas generated using the plasma technology, which we call “N$_2$O$_5$ gas” in this study, contains a certain amount of O$_3$ and NO$_2$ in addition to its high concentration of N$_2$O$_5$ due to the nature of the matter. However, because the N$_2$O$_5$ gas we generate using this system has unique properties that cannot be created using conventional technology at present, it is meaningful to gain insight into its physiological effects. Although N$_2$O$_5$ readily dissolves in water and results in nitric acid (HNO$_3$) via reactive intermediates [22], the effects on plants of such N$_2$O$_5$-induced chemical reactions are unknown. Moreover, there is currently no knowledge of plant responses after exposure to N$_2$O$_5$ gas. In the present study, we exposed...
Arabidopsis to plasma-generated N$_2$O$_5$ gas and analyzed its effects on disease resistance and post-exposure changes in gene expression after the exposure in order to elucidate the effects of N$_2$O$_5$ gas on plant immune responses.

Materials and methods

Dinitrogen pentoxide gas generation from air

Dinitrogen pentoxide gas, which is highly reactive, not storable under ambient conditions, and not typically available in the gas market, was prepared by an atmospheric pressure plasma device, developed recently in our previous work as shown in S1A Fig [6]. This device, which has electric controls designed for plant exposure experiments, can produce a continuous supply of N$_2$O$_5$ gas from only compressed air via a chemical reaction chain involving N$_2$O$_5$, NO$_2$, and O$_3$. Importantly, to prevent contamination by any chemicals, no chemical compounds were used for the generation of N$_2$O$_5$ gas by the given plasma device. Furthermore, exactly the same conditions without reactive species were created by turning off the generation of air plasmas by the plasma device. Due to the unavoidable generation and decomposition reaction chains involving N$_2$O$_5$ under physiological conditions, N$_2$O$_5$ selectivity is limited up to 10 at a density of approximately 240 ppm, which allows simultaneously high density and high selectivity at room temperature. The minor gas components of O$_3$, NO$_2$, N$_2$O, and HNO$_3$ that we measured that were unavoidably present in the N$_2$O$_5$ gas are summarized in S1C Fig [6]. This N$_2$O$_5$ gas mixture synthesized from air is henceforth described in this paper simply as N$_2$O$_5$ gas.

Plant cultivation and N$_2$O$_5$ gas exposure of plants

Wild-type plants of Arabidopsis thaliana ecotype Columbia (Col-0) and the mutants coi1-1, ein2-1, and npr1-1 in the same background were sown on soilless mix (Metro-Mix 350, San Gro, Canada) and grown for 2 weeks. Each seedling was then transferred to a new pot for further cultivation for 3 weeks in a growth chamber under short day conditions (light 10 h/dark 14 h) at 23˚C. Homozygous coi1-1 plants were screened using a dCAPs marker before transplanting [23]. The N$_2$O$_5$ gas generated by the transportable plasma device was used for exposing plants to N$_2$O$_5$ gas [6]. The plants were incubated for 30 min under a clear cover prior to exposure to N$_2$O$_5$ gas to ensure uniform humidity conditions. Dry air containing N$_2$O$_5$ at a density of approximately 240 ppm was emitted at 2 L/min from the outlet of a polytetrafluoroethylene (PTFE) tube with a 4-mm inner diameter. Each A. thaliana pot was placed 5 cm downstream from the N$_2$O$_5$ gas outlet tube for 20 s once per day with an outer plastic shroud to prevent room air flow disturbances from causing unexpected processes as shown in S1B Fig.

Botrytis cinerea inoculation

Botrytis cinerea isolated from Brassica species (MAFF 237695) was provided from NARO GeneBank [24]. Botrytis Cinerea was cultured on potato dextrose agar (Difco, Detroit, MI) medium at 23˚C for 3 days under dark conditions, and then plates were transferred to incubate under continuous black light (FL10BLB; Toshiba Corp., Tokyo, Japan) for 3 days to induce conidia formation. Conidia were suspended in potato dextrose broth (Difco) using a paint brush and then filtered through four layers of gauze. Conidial suspensions were centrifuged at 400 × g for 5 min and the supernatant was removed. The collected conidia were resuspended in 1/8 diluted potato dextrose broth to a concentration of 2 × 10$^5$ conidia/mL. Five-week-old plants were exposed to N$_2$O$_5$ gas once per day for 3 days, and the plants were inoculated with B. cinerea at 24 h after the final N$_2$O$_5$ gas exposure. A separate set of control plants were sprayed...
with 200 μM methyl jasmonate (MeJA) and incubated for one day under a clear cover for comparison with the effect of the N₂O₅ gas. The conidial suspension (5 μL) was spotted onto a fully expanded leaf and incubated for 2 days while maintaining high humidity. The area of each lesion (mm²) was measured to evaluate disease severity. Trypan blue staining was performed to detect dead cells, as previously described [25]. Statistical analyses were performed using Student’s t-test or the Tukey–Kramer test, depending on the number of experimental groups. Each experiment was performed at least twice and similar results were obtained each time.

**Inoculation with *Pseudomonas syringae pv. tomato* DC3000**

Five-week-old plants were exposed to N₂O₅ gas once per day for 3 days, and then *Pseudomonas syringae pv. tomato* DC3000 (Pst) was inoculated at 24 h after the final gas exposure. King’s B liquid medium supplemented with 50 μg/mL of rifampicin was used for Pst culture at 25˚C for 1 day and the bacterial concentration was adjusted with 10 mM MgCl₂ solution to an OD₆₀₀ value of 0.002. The bacterial suspension was infiltrated into the intercellular spaces of three fully expanded leaves per plant using a syringe. Mock treatment was performed by infiltration with 10 mM MgCl₂ in the absence of bacteria. The inoculated leaves were sampled and ground using a pestle in a tenfold volume of sterile water at 2 days after inoculation. Successive dilutions of the ground leaf tissue were spread onto King’s B solid medium supplemented with rifampicin (50 μg/mL), incubated at 25˚C for 2 days, and the number of Pst colonies formed was counted. In addition, bacterial biomass was assessed by calculating the ratio of bacterial DNA to plant DNA using qPCR. Inoculated leaves were collected at 0, 1, 2 and 3 days after infection and total DNA was extracted using an ISOPLANT II kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer’s protocols. To quantify plant DNA and Pst DNA, RHIP1 and OprF sequences [26], respectively, were amplified by qPCR using TB Green™ Premix Ex Taq™ II (Takara Bio Inc., Shiga, Japan) on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) (S1 Table). Student’s t-test was performed for statistical analysis and no significant differences were found. Each experiment was performed three times with similar results.

**Inoculation with cucumber mosaic virus**

Cucumber mosaic virus strain yellow (CMV(Y)) was inoculated and propagated on *Nicotiana benthamiana* plants and purified as described [27]. Five-week-old *Arabidopsis* plants were exposed to N₂O₅ gas once per day for 3 days, and were inoculated with CMV(Y) at 24 h after the final N₂O₅ gas exposure. Three leaves of the *Arabidopsis* plants were inoculated with CMV (Y) as described [28]. Briefly, mechanical inoculation of the virus was carried out onto leaves sprinkled with carborundum by rubbing the surface lightly with a cotton swab soaked in virus solution. Air exposure was used as control and was also subjected to CMV(Y) inoculation or mock treatment (water). An enzyme-linked immunosorbent assay (ELISA) was performed as described previously to quantify CMV(Y) multiplication [29]. A rabbit antibody against the CMV(Y) coat protein (CP) and alkaline phosphatase-conjugated anti-rabbit IgG (Fc) (Promega, Madison, WI) were used as the primary and secondary antibodies, respectively. The compound p-nitrophenyl phosphate (1 mg/mL) in AP9.5 buffer (10 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM MgCl₂) was used as the substrate for alkaline phosphatase. The absorbance of the resulting phenolate solution was measured at 405 nm. The amount of CP in 0.025 mg total protein was calculated as average absorbance ± standard deviation. Student’s t-test was performed for statistical analysis. Each experiment was performed at least twice with similar results.
Transcriptome analysis by RNA-seq

Five-week-old plants were exposed to N₂O₅ gas for 20 s once per day for 3 days, and fully expanded leaves were sampled at 24 h after the final exposure to N₂O₅ gas (hereafter, N₂O₅-gas-exposed). Plants exposed to air served as the control experiment (hereafter, Air-control). Total RNA was extracted from leaves using the TRIzol method [30]. Macrogen Japan on the Illumina platform was used for RNA-seq analysis in order to obtain 151-bp paired-end sequences. Fifty-one M reads were obtained for the Air-control, and 46 M reads for the N₂O₅-gas-exposed sample were obtained. Differentially expressed gene analysis was performed between the Air-control and N₂O₅-gas-exposed samples. Fold changes (fc) in transcript abundances were calculated using exactTest in the edgeR package [31] for each sequence pair comparison. Significant results are indicated with |fc| ≥ 2 at an exactTest raw p-value < 0.05. Functional category enrichment was defined by implementing the Gene Ontology (GO) tool online (http://geneontology.org/). The data for RNA-Seq have been deposited in the DDBJ Sequence Read Archive (DRA) (https://www.ddbj.nig.ac.jp/dra/index-e.html) and are accessible through DRR Run accession numbers: DRR345814 and DRR345815.

Gene expression analysis by qRT-PCR

Total RNA was extracted from individual seedlings using the TRIzol method [30]. Reverse transcription and subsequent PCR were performed using PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio Inc.). The relative abundances of mRNA transcripts for each gene of interest were determined by qRT-PCR using TB Green® Premix Ex Taq™ II (Takara Bio Inc.) and a 7300 Real-Time PCR system (Applied Biosystems). Transcript abundance was calculated and represented as fold difference relative to the abundance of ACTIN2 transcripts. The average and standard deviation of values of three independent seedlings were then calculated. Student’s t-test, Dunnett’s test, or Tukey–Kramer test were performed for statistical analysis depending on the number and character of experimental groups. Each experiment was performed at least twice with similar results. Primers used in this study are listed in S1 Table.

Statistical analysis

All data were subjected to analysis of variance and various post-hoc tests using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Determination of N₂O₅ gas exposure conditions for Arabidopsis thaliana

To determine the N₂O₅ gas exposure conditions that would not be harmful for plant growth, A. thaliana plants were exposed to N₂O₅ gas by placing them 5 cm downstream from the gas outlet tube for 0 s, 10 s, 20 s, 30 s, 40 s, 60 s, 2 min, or 5 min. Immediately after the N₂O₅ gas exposure for 300 s, leaves turned brown and died by 24 h after exposure (Fig 1). Clear injury was observed with N₂O₅ gas exposure of 40 s at 6 h post-exposure (Fig 1). However, no apparent change in leaves was observed with up to 30 s of N₂O₅ gas exposure even at 4 days after exposure (Fig 1). We have also confirmed that N₂O₅ gas exposure for 20 s repeated once per day for 3 days did not cause apparent injury to plants. Therefore, exposure to N₂O₅ gas for 20 s was chosen as the experimental treatment for the following analyses.

Effects of the N₂O₅ gas exposure on disease resistance

Air-exposed and N₂O₅-gas-exposed plants were inoculated with B. cinerea. The lesions were smaller on the N₂O₅-gas-exposed plants than on the control plants at 2 days post-inoculation.
Fig 1. Observation of plant damage after exposure of *Arabidopsis* plants to N$_2$O$_5$ gas. The N$_2$O$_5$ gas exposure time for each plant is shown in the lower left corner. Observations were made before, immediately after, 6 h after, 24 h after, and 4 days after N$_2$O$_5$ gas exposure. Magnified images at 4 days after some of the N$_2$O$_5$ gas exposure times (0 s, 30 s, 40 s, and 5 min) are shown in the lower right corner. Red arrowheads indicate a leaf that turned brown immediately after the N$_2$O$_5$ gas exposure. Yellow arrowheads indicated the location of leaves that were damaged by the N$_2$O$_5$ gas. Scale bar is 2 cm.

https://doi.org/10.1371/journal.pone.0269863.g001
To evaluate changes in global gene expression caused by exposure to $N_2O_5$ gas, RNA-Seq analysis was performed using $N_2O_5$-gas-exposed and air-exposed leaf samples at 24 h following the third exposure to $N_2O_5$ gas. The transcripts of 828 genes had increased in abundance by more than twofold after exposure to $N_2O_5$ gas (S2 Table), and the transcript abundances of 871 genes had decreased by less than half (S3 Table). The expression of several selected genes was checked by qRT-PCR and similar results were obtained (S2 Fig). Gene ontology term analysis of the genes exhibiting increased transcript abundance suggested that JA- and ET-dependent signaling pathways and disease resistance including systemic acquired resistance are activated by exposure to $N_2O_5$ gas (S3A Fig). Although GO term analysis showed that responses to SA and abscisic acid were suppressed by exposure to $N_2O_5$ gas (S3B Fig), the expression of $PR1$, a marker gene for responses involving SA, was induced (S2 Table).

Among the genes exhibiting increased transcript abundance, we chose to further analyze changes in expression over time after $N_2O_5$ gas exposure of the defense-related genes $PAD3$ and $PDF1.2$ and transcription factors (TFs) $WRKY26$ and $ORA59$, which may be involved in JA and ET responses. *Arabidopsis* plants were exposed to air (control) or $N_2O_5$ gas once for 20 s, and shoots of each individual plant were collected as independent samples for qRT-PCR at 1, 3, 6, 12, 24, and 48 h after exposure. Expression of $PAD3$ and $WRKY26$ transcripts was transiently induced within 3 h after $N_2O_5$ gas exposure (Fig 5). Protein encoded by $PAD3$ gene is a cytochrome P450 involved in the biosynthesis of the antimicrobial compound camalexin from tryptophan (S6A Fig). Similarly, expression of transcripts of $CYP71A12$, $CYP71A13$, $PEN2$, and $NIT2$, which are involved in tryptophan metabolism (S6A Fig), was also induced (Fig 5 and S4 Fig), suggesting that tryptophan metabolism, including synthesis of camalexin and indole-glucosinolate derivatives, is enhanced by exposure of plants $N_2O_5$ gas. It has also been reported that the functions of $WRKY26$ are redundant with those of the homologous $WRKY25$ and $WRKY33$ [32]. We also confirmed that the gene expression of $WRKY25$ and $WRKY33$ showed similar changes in gene expression as $WRKY26$ after $N_2O_5$ gas exposure (Fig 5). These TFs might coordinate regulation of the response to treatment of plants with $N_2O_5$ gas.
Fig 2. Induction of Botrytis cinerea resistance in Arabidopsis plants by N$_2$O$_5$ gas exposure. (A) Arabidopsis plants exposed to N$_2$O$_5$ gas were inoculated with B. cinerea. Scale bar is 1 cm. (B) Areas of the lesions at 2 days after B. cinerea inoculation were measured and the mean (± standard deviation) is shown. Different letters indicate significant differences according to the Tukey–Kramer test (n = 11–12, P < 0.05).

https://doi.org/10.1371/journal.pone.0269863.g002
Fig 3. Response of N$_2$O$_5$-gas-exposed Arabidopsis plants to infection with *Pseudomonas syringae* pv. *tomato* DC3000. (A) Lesion formation was observed at 3 days after *Pst* inoculation. Red arrows indicate inoculated leaves.
Meanwhile, ORA59 showed biphasic induction of expression at around 3 h and 24 h after N\textsubscript{2}O\textsubscript{5} gas exposure (Fig 5). The expression of PDF1.2, which encodes an antimicrobial peptide, gradually increased from 3 h after N\textsubscript{2}O\textsubscript{5} gas exposure and remained at a high level until at least 48 h later (Fig 5). In contrast, the expression of VSP2, a gene specifically inducible by JA, was slightly responsive to N\textsubscript{2}O\textsubscript{5} gas exposure with a significant but low level of induction at 12 h after N\textsubscript{2}O\textsubscript{5} gas exposure (S4 Fig). In addition, although the expression of PR1 was induced at 12 to 24 h after N\textsubscript{2}O\textsubscript{5} gas exposure, it decreased to its basal level after 48 h (S4 Fig).

Responses to N\textsubscript{2}O\textsubscript{5} gas in Arabidopsis mutants deficient in phytohormone signaling pathways

To determine whether the observed responses to N\textsubscript{2}O\textsubscript{5} gas exposure are mediated by the JA and ET signaling pathways, we exposed mutant plants for each of these phytohormone

![Graph showing accumulation of virus coat protein (A\textsubscript{405})](https://doi.org/10.1371/journal.pone.0269863.g004)

**Fig 4.** Induction of cucumber mosaic virus resistance in Arabidopsis plants by N\textsubscript{2}O\textsubscript{5} gas exposure. Arabidopsis plants exposed to N\textsubscript{2}O\textsubscript{5} gas were inoculated with CMV(Y). Two days after inoculation, the inoculated leaves were harvested and subjected to enzyme-linked immunosorbent assay using an antibody against the CMV CP. Asterisks denote significant differences (Student’s t-test, n = 6, \(P < 0.05\)).

https://doi.org/10.1371/journal.pone.0269863.g004
Stimulation of plant immunity by N2O5 gas

Relative expression to ACT2 gene

Time after N2O5 gas exposure (h)

Air  N2O5
signaling pathways to \( N_2O_5 \) gas and analyzed their responses. \textit{Arabidopsis} plants were exposed to air (control) or \( N_2O_5 \) gas for 20 s, and shoots of each individual plant were harvested at 2 and 24 h later for qRT-PCR. The induction of WRKY33, WRKY26, PEN2, and PAD3 expression was reduced in \textit{coi1-1}, a JA signaling mutant at 2 h after \( N_2O_5 \) gas exposure, whereas expression of these genes was similar to the wild type in \textit{ein2-1}, an ET signaling mutant (Fig 6 and S5 Fig). The transcript abundance of \textit{ORA59} was relatively lower in plants carrying either mutation especially \textit{ein2-1}, at 2 h after \( N_2O_5 \) gas exposure. The transcript abundance of \textit{ORA59} was significantly lower only in \textit{coi1-1} at 24 h after exposure to \( N_2O_5 \) gas. The contribution of ET and JA to the regulation of \textit{ORA59} expression seems to differ between 2 h and 24 h after \( N_2O_5 \) gas exposure (Fig 6). Induction of the expression of \textit{PDF1.2} was greatly attenuated in both the \textit{coi1-1} and \textit{ein2-1} mutants, especially at 24 h after \( N_2O_5 \) gas exposure (Fig 6). These results indicated that both JA and ET signaling have important roles in the activation of gene expression by \( N_2O_5 \) gas.

In addition, we analyzed the induction of disease resistance by exposure to \( N_2O_5 \) gas in \textit{coi1-1} and \textit{ein2-1} mutants. The SA signaling mutant \textit{npr1-1} was also used in these experiments. Wild-type, \textit{coi1-1}, \textit{ein2-1}, and \textit{npr1-1} plants were exposed to \( N_2O_5 \) gas under the same conditions as in Fig 2. The sizes of lesions caused by \textit{B. cinerea} infection were reduced with exposure to \( N_2O_5 \) gas as compared with the Air-control in \textit{ein2-1} and \textit{npr1-1} but were not significantly different in \textit{coi1-1} as shown in Fig 7A. These results indicated that JA signaling has a major role in the enhancement of \textit{B. cinerea} resistance by \( N_2O_5 \) gas. In contrast, the induction of CMV resistance by exposure to \( N_2O_5 \) gas was compromised in \textit{ein2-1} and \textit{npr1-1}, but was maintained in \textit{coi1-1} (Fig 7B). In particular, the effect of the \( N_2O_5 \) gas tended to be weak in \textit{ein2-1}, suggesting that ET may be a major factor in CMV resistance induced by \( N_2O_5 \) gas.

**Discussion**

In this study, we showed that exposure of \textit{Arabidopsis thaliana} to \( N_2O_5 \) gas produced from air-derived plasma can activate plant immunity mainly through JA and ET signaling. Specifically, we showed that exposure of plants to \( N_2O_5 \) gas enhanced \textit{B. cinerea} and CMV resistance but not \textit{Pst} resistance. However, it is important to note that although \( N_2O_5 \) gas is composed mainly of highly concentrated \( N_2O_5 \), it also contains a certain amount of the other active species such as \( O_3 \) and \( NO_2 \) (S1C Fig). Methods for exogenously exposing plants to ROS or RNS in a gaseous state for disease control have been reported using \( O_3 \) and \( NO_2 \) [18, 33]. Increased production of SA, ET, and JA has been reported upon \( O_3 \) exposure in \textit{A. thaliana} [34–36]. Ethylene is thought to be involved in \( O_3 \) sensitivity because \( O_3 \)-induced damage is reduced in ethylene-deficient mutants. However, ET production is suppressed and damage is reduced in MeJA-treated plants after \( O_3 \) exposure, suggesting that JA acts antagonistically with ET [34]. With regard to SA, the accumulation of SA after \( O_3 \) exposure was high in the ET-overproducing mutant \textit{eto1}, whereas ET production was low in the SA-deficient mutant, suggesting that ET and SA work cooperatively after \( O_3 \) exposure [35]. Ozone exposure also effectively inhibits the propagation of several plant viruses such as tobacco mosaic virus and soybean mosaic virus [37, 38]. In the present study, the \( N_2O_5 \) gas exposure shown to enhance CMV resistance was dependent mainly on ET signaling pathways according to our analysis using phytohormone signaling mutants (Fig 7B). Ethylene signaling is also partially involved in the CMV resistance...
Fig 6. Expression of defense-related genes in coi1-1 and ein2-1 mutant Arabidopsis plants after exposure to N₂O₅ gas. The accumulation of mRNA transcripts of defense-related genes, including WRKY33, PAD3, ORA59, and PDF1.2 was analyzed in wild-type, coi1-1, and ein2-1 Arabidopsis plants. Different letters denote significant differences among treatments (Tukey–Kramer test, n = 3, P < 0.05). ACT2, ACTIN2.

https://doi.org/10.1371/journal.pone.0269863.g006

Fig 7. Resistance induced by N₂O₅-gas against Botrytis cinerea and CMV in Arabidopsis phytohormone signaling mutants. (A) The areas of the lesions at 2 days after B. cinerea inoculation were measured and the mean (± standard deviation) is shown. Asterisks denote significant differences (Student’s t-test, n = 9, P < 0.05). (B) Two days after CMV inoculation, the inoculated leaves were harvested and subjected to ELISA. Asterisks denote significant differences (Student’s t-test, n = 6, P < 0.05). n.s.: not significant.

https://doi.org/10.1371/journal.pone.0269863.g007
conferred by RCY1, a disease-resistance protein in A. thaliana [39]. The CMV resistance induced by N₂O₅ gas and that conferred by RCY1 might employ a common ET-mediated mechanism. Meanwhile, although activation of SA responses was not detected during the GO term analysis of our RNA-Seq results, transient activation of PR1 expression was confirmed (S3B and S4 Figs and S2 Table). These results imply that SA signaling may also be partially activated. The fact that there was no significant enhancement of CMV resistance by N₂O₅ gas exposure in the npr1-1 mutant supports this possibility (Fig 7B). Because a cooperative function of ET and SA has also been reported for O₃ [35], it is possible that O₃ contained in the N₂O₅ gas partially contributes to the CMV resistance induced by the N₂O₅ gas. Otherwise, N₂O₅ has a strong oxidative effect much like that of O₃, so an unknown mechanism might be operating due to an oxidative effect of N₂O₅. Further analysis is needed to understand the mechanistic details of the enhancement of CMV resistance by N₂O₅ gas.

Botrytis cinerea resistance is regulated by a complex network of SA, ET, and JA signals [40]. However, JA and ET play major roles in resistance to necrotrophic pathogens including B. cinerea [41]. An increase in SA content has been observed immediately after exposure in the case of exposure to NO₂ gas [18]. Interestingly, gene expression related to the synthesis and metabolism of JA is also activated after exposure to NO₂ gas. Also, a reduction in the amount of active JA and accumulation of its metabolites is observed [18]. Exposure to NO₂ gas enhances B. cinerea resistance, but this effect is impaired in both SA-deficient NahG plants and JA biosynthetic mutants, suggesting that NO₂-induced resistance enhancement involves not only SA accumulation but also activation of JA metabolism [18]. In contrast, our results suggest that N₂O₅ gas enhances resistance to B. cinerea mainly by activating JA signaling (Figs 2 and 7A). Gene expression analysis suggests that JA and ET signals are activated in a coordinated manner by exposure to N₂O₅ gas (S3A Fig). Analysis using phytohormone signaling mutants also supports that JA and ET signals are activated by exposure to N₂O₅ gas (Fig 6). However, GO term analysis of our RNA-Seq results suggests that SA signaling was either not activated or was somewhat suppressed by the exposure of plants to N₂O₅ gas (S3 Fig), although transient induction of PR1 gene was observed (S4 Fig). These findings suggest that different regulatory mechanisms likely operate in the B. cinerea resistance induced by NO₂ gas or N₂O₅ gas.

In general, SA plays a central role in resistance to Pst, while JA and ET act antagonistically [42, 43]. However, JA is reported to play a cooperative role with SA in the induction of Pst resistance by oligogalacturonides and chitosan oligosaccharides [44, 45]. In the present study, we did not observe any enhancement of Pst resistance by the N₂O₅ gas (Fig 3), which might have been because SA signaling was not predominantly activated compared with JA (S3 Fig). Because NO₂-gas exposure enhances Pst resistance via SA signaling [18], the differential effects of N₂O₅ gas and NO₂ gas on disease resistance can be confirmed again.

A key transcription factor, WRKY33, controls the expression of genes regulating resistance to B. cinerea via ET/IA signaling and camalexin biosynthesis such as CYP71A12, CYP71A13, and PAD3 [46, 47]. The transient increase in the abundance of WRKY33 transcripts by exposure N₂O₅ gas suggests activation of WRKY33-mediated B. cinerea resistance (Fig 5). Because the N₂O₅ gas induced the expression of CYP71A12, CYP71A13, PAD3, and PEN2 (Fig 5), it is likely that secondary metabolites derived from tryptophan such as camalexin, indole-glucosinolate derivatives, and indole-carboxylic acid (S6A Fig) are involved in the N₂O₅-gas-induced B. cinerea resistance. Furthermore, WRKY25 and WRKY26, which are functionally redundant with WRKY33 [32], are thought to regulate these metabolic systems in a coordinated manner (Fig 5). However, it has been reported that the induction of B. cinerea resistance by NO₂ is PAD3-dependent but not accompanied by an increase in camalexin content [18]. Further analysis is needed to determine which metabolites, including camalexin, contribute to the enhancement of B. cinerea resistance by N₂O₅ gas. Meanwhile, although exposure to N₂O₅ gas...
strongly induced the expression of ORA59 and PDF1.2 (Fig 5), the induction of VSP2 was relatively weak (S4 Fig). The expression of ORA59, which encodes a TF, is regulated by both ET and JA and controls the expression of PDF1.2, which is involved in disease resistance (S6B Fig). However, VSP2 is thought to function in wounding response and insect resistance under the control of the TF MYC2 in a JA-specific manner (S6B Fig) [14, 48]. It is possible that ET signaling becomes more dominant in the relationship between ORA59 and MYC2 during exposure to N₂O₅ gas.

Interestingly, the response of WRKY33 transcript to exposure to N₂O₅ gas is highly similar to the responses to treatments with damage-associated molecular patterns (DAMPs) such as HMGB3 and Pep1 [49]. In plants and animals, DAMPs released from cells due to injury induce immune responses [50, 51]. Therefore, exposure to N₂O₅ gas causes slight cellular damage (Fig 1), which might result in the release of DAMPs into the apoplast of plant tissues. Proteinaceous DAMPs such as HMGB1 and HSPs are known to play an important role in the inflammatory response in animal cells [52]. Pattern recognition receptors (PRR, e.g., Toll-like receptor 4) recognize DAMPs released from damaged cells, and thereby transmit the damage stimulus to surrounding cells [52]. Furthermore, post-translational modifications of DAMPs are known to alter their functions [53]. A recent report indicates that proteinaceous DAMPs, in which tyrosine residues have been modified by nitration, activate the PRR more strongly than do unmodified DAMPs in HeLa cells [54]. Dinitrogen pentoxide is a powerful oxidizing and nitrating agent, and is an important agent widely used in the nitration and S-nitrosylation of organic compounds, as for the production of nitrotyrosine when tyrosine is treated with the N₂O₅ gas [6]. Therefore, exposure to the N₂O₅ gas might contribute not only to the release of DAMPs, but also to the modification of DAMPs by nitration or S-nitrosylation to influence their function in plant immunity. The involvement of DAMPs in the enhancement of disease resistance by N₂O₅ gas will be elucidated in future analyses.

In conclusion, we have shown that N₂O₅ gas has potential for development as a new technology for plant disease control. N₂O₅ is converted to nitric acid by reacting with water, and it can be used by plants as a source of nitrogen. Therefore, treatment with N₂O₅ gas would be almost free from risks of environmental pollution. In addition because the amount of electricity required for production of N₂O₅ gas is relatively low [6], control of plant diseases using N₂O₅ gas could contribute as a low-cost and environmentally friendly technology to the establishment of a sustainable agricultural system. Furthermore, the device used in this study can selectively supply O₃ or NO/NO₂ by mode switching [6]. Since the present study was performed under laboratory conditions using A. thaliana, validation under field conditions using crops is a subject for future work. However, this type of approach might be useful for efficiently controlling plant diseases by exposing crop species to the appropriate active gas composition for the type of disease. Recently, Kumar and co-workers reported that glycine betaine and Arbuscular mycorrhizal fungi treatment reduces chromium toxicity via reduction of oxidative stress [55–57]. Combining such treatments with N₂O₅ gas exposure may allow for the development of more effective and harmless disease control methods.

**Supporting information**

S1 Fig. Atmospheric-pressure plasma device for the generation of N₂O₅ gas. The atmospheric-pressure plasma device was developed at the Graduate School of Engineering, Tohoku University [6]. (A) Device installation status. (B) Photographs showing exposure of Arabidopsis thaliana plants to N₂O₅ gas. The area in the box in (A). (C) Typical densities of reactive species in the gas generated by the plasma device.

(PDF)
S2 Fig. Analysis of N\textsubscript{2}O\textsubscript{5}-gas-inducible gene expression in \textit{Arabidopsis thaliana} plants by qRT-PCR. To confirm the results of RNA-Seq, five genes with elevated transcript expression after exposure to \textsubscript{N}2\textsubscript{O}3 gas were chosen for further analysis of their relative transcript abundances by qRT-PCR. \textit{Arabidopsis} plants were exposed to air (control) or \textsubscript{N}2\textsubscript{O}5 gas for 20 s once a day for 3 days. Shoots of each individual plant were collected as independent samples at 24 h after the third exposure. Total RNA was extracted and subjected to analysis of the relative mRNA transcript abundances of defense-related genes, including PDF1.2, PDF1.4, ORA59, WRKY26, and PR4. Data were normalized to ACTIN2 mRNA transcript abundance. Asterisks denote significant differences relative to the air control (Student’s \textit{t}-test, \(n = 3\), \(P < 0.05\)). 

\textit{ACT2}, \textit{ACTIN2}. The fold change (\textsubscript{N}2\textsubscript{O}5/Air) calculated from qRT-PCR was compared to the fold change obtained from RNA-seq.

S3 Fig. Gene Ontology term analysis of genes with increased or decreased transcript abundance after exposure to \textsubscript{N}2\textsubscript{O}5 gas. Genes with increased or decreased transcript abundance after exposure to \textsubscript{N}2\textsubscript{O}3 gas from the RNA-Seq results were subjected to Gene Ontology term analysis. Enrichment of functional categories was defined implementing Gene Ontology tool online (http://geneontology.org/). Fold enrichment of genes exhibiting increased (A) or decreased (B) transcript abundance after exposure to \textsubscript{N}2\textsubscript{O}5 gas is shown.

S4 Fig. Changes in the expression of genes related to plant disease defense responses over time after exposure to \textsubscript{N}2\textsubscript{O}5 gas. The gene expression of \textit{CYP71A12}, \textit{NIT2}, \textit{VSP2}, and \textit{PRI} was analyzed by qRT-PCR as in Fig 5.

S5 Fig. Expression of plant disease defense-related genes in \textit{coi1-1} and \textit{ein2-1} mutants after exposure to the \textsubscript{N}2\textsubscript{O}5 gas. The gene expression of \textit{WRKY26} and \textit{PEN2} was analyzed by qRT-PCR as in Fig 6.

S6 Fig. Model of tryptophan metabolism pathway and crosstalk between JA and ET signaling in \textit{Arabidopsis thaliana}. (A) Model of tryptophan metabolism pathway. 4MI3G, 4-methoxy indolyl-3-methyl glucosinolate. (B) Model of JA and ET signaling crosstalk. Arrows indicate positive effects. Negative interaction of ORA59 and MYC2 is known. The genes analyzed in this study are shown in red letters.

S1 Table. Primers used in this study.

S2 Table. Genes whose transcript abundance increased more than twofold after exposure to \textsubscript{N}2\textsubscript{O}5 gas compared to air control in RNA-seq analysis.

S3 Table. Genes whose transcript abundance decreased less than half after exposure to \textsubscript{N}2\textsubscript{O}5 gas compared to air control in RNA-seq analysis.

Acknowledgments

We would like to thank the NARO Genebank for supplying \textit{Botrytis cinerea}. 
Author Contributions
Conceptualization: Hideki Takahashi, Sugihiro Ando.
Data curation: Daiki Tsukidate, Shota Sasaki, Shuhei Miyashita, Sugihiro Ando.
Formal analysis: Daiki Tsukidate, Shuhei Miyashita, Sugihiro Ando.
Funding acquisition: Toshiro Kaneko, Hideki Takahashi, Sugihiro Ando.
Investigation: Daiki Tsukidate, Keisuke Takashima, Sugihiro Ando.
Methodology: Keisuke Takashima, Shota Sasaki, Sugihiro Ando.
Project administration: Toshiro Kaneko, Hideki Takahashi, Sugihiro Ando.
Resources: Keisuke Takashima, Shota Sasaki, Toshiro Kaneko, Sugihiro Ando.
Supervision: Toshiro Kaneko, Hideki Takahashi, Sugihiro Ando.
Validation: Daiki Tsukidate, Shota Sasaki, Sugihiro Ando.
Visualization: Daiki Tsukidate, Shota Sasaki, Sugihiro Ando.
Writing – original draft: Sugihiro Ando.
Writing – review & editing: Daiki Tsukidate, Keisuke Takashima, Shota Sasaki, Shuhei Miyashita, Toshiro Kaneko, Hideki Takahashi, Sugihiro Ando.

References
1. Lykogianni M, Bempelou E, Karamaouna F, Aliferis KA. Do pesticides promote or hinder sustainability in agriculture? The challenge of sustainable use of pesticides in modern agriculture. Sci Total Environ. 2021; 795: 148625. https://doi.org/10.1016/j.scitotenv.2021.148625 PMID: 34247073
2. Tendero C, Tixier C, Tristant P, Desmaison J, Leprince P. Atmospheric pressure plasmas: A review. Spectrochim Acta—Part B At Spectrosc. 2006; 61: 2–30. https://doi.org/10.1016/j.sab.2005.10.003
3. Kaneko T, Kato H, Yamada H, Yamamoto M, Yoshida T, Attri P, et al. Functional nitrogen science based on plasma processing: quantum devices, photocatalysts and activation of plant defense and immune systems. Jpn J Appl Phys. 2022; 61: SA0805. https://doi.org/10.35848/1347-4065/ac25dc
4. Adhikari B, Pangomm K, Veerama M, Mitra S, Park G. Plant disease control by non-thermal atmospheric-pressure plasma. Front Plant Sci. 2020; 11: Article 77. https://doi.org/10.3389/fpls.2020.00077 PMID: 32117403
5. Takashima K, Kaneko T. Ozone and dinitrogen monoxide production in atmospheric pressure air dielectric barrier discharge plasma effluent generated by nanosecond pulse superimposed alternating current voltage. Plasma Sources Sci Technol. 2017; 26: 065018. https://doi.org/10.1088/1361-6595/aa7082
6. Sasaki S, Takashima K, Kaneko T. Portable plasma device for electric N2O5 production from air. Ind Eng Chem Res. 2021; 60: 798–801. https://doi.org/10.1021/acs.iecr.0c04915
7. Misra NN, Pankaj SK, Segat A, Ishikawa K. Cold plasma interactions with enzymes in foods and model systems. Trends Food Sci Technol. 2016; 55: 39–47. https://doi.org/10.1016/j.tifs.2016.07.001
8. Baxter A, Mittler R, Suzuki N. ROS as key players in plant stress signalling. J Exp Bot. 2014; 65: 1229–1240. https://doi.org/10.1093/jxb/ert375 PMID: 24253197
9. Yu M, Lamattina L, Spoel SH, Loake GJ. Nitric oxide function in plant biology: A redox cue in deconvolution. New Phytol. 2014; 202: 1142–1156. https://doi.org/10.1111/nph.12739 PMID: 24611485
10. Bellin D, Asai S, Deledonne M, Yoshioka H. Nitric oxide as a mediator for defense responses. Mol Plant-Microbe Interact. 2013; 26: 271–277. https://doi.org/10.1094/MPMI-09-12-0214-CR PMID: 23151172
11. Apel K, Hirt H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology. 2004. pp. 373–399. https://doi.org/10.1146/annurev.arplant.55.031903.141701 PMID: 15377225
12. Gaupels F, Kuruthukulangarakola GT, Durner J. Upstream and downstream signals of nitric oxide in pathogen defence. Curr Opin Plant Biol. 2011; 14: 707–714. https://doi.org/10.1016/j.pbi.2011.07.005 PMID: 21816662
13. Mur LAJ, Hebelstrup KH, Gupta KJ. Striking a balance: does nitrate uptake and metabolism regulate both NO generation and scavenging? Front Plant Sci. 2013; 4: Article 288. https://doi.org/10.3389/fpls.2013.00288 PMID: 23908662

14. Pieterse CMJ, Van Der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM. Hormonal modulation of plant immunity. Annu Rev Cell Dev Biol. 2012; 28: 489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055 PMID: 22559264

15. Wendehenne D, Gao Q ming, Kachroo A, Kachroo P. Free radical-mediated systemic immunity in plants. Curr Opin Plant Biol. 2014; 20: 127–134. https://doi.org/10.1016/j.pbi.2014.05.012 PMID: 24929297

16. Lu R, Liu Z, Shao Y, Su J, Li X, Sun F, et al. Nitric oxide enhances rice resistance to Rice black-streaked dwarf virus infection. Rice. 2020; 13: 20. https://doi.org/10.1186/s12284-020-00382-8 PMID: 32291541

17. Song F, Goodman RM. Activity of nitric oxide is dependent on, but is partially required for function of, salicylic acid in the signaling pathway in tobacco systemic acquired resistance. Mol Plant-Microbe Interact. 2001; 14: 1458–1462. https://doi.org/10.1094/MPMI.2001.14.12.1458 PMID: 11768542

18. Mayer D, Mithöfer A, Glawischnig E, Georgii E, Ghirardo A, Kanawati B, et al. Short-term exposure to nitrogen dioxide provides basal pathogen resistance. Plant Physiol. 2018; 178: 468–487. https://doi.org/10.1104/pp.18.00704 PMID: 30076223

19. Kasten D, Mithöfer A, Glawischnig E, Georgii E, Ghirardo A, Kanawati B, et al. Short-term exposure to nitrogen dioxide provides basal pathogen resistance. Plant Physiol. 2018; 178: 468–487. https://doi.org/10.1104/pp.18.00704 PMID: 30076223

20. Mur LAJ, Prats E, Pierre S, Hall MA, Hebelstrup KH. Integrating nitric oxide into salicylic acid and jasmonic acid/ethylene plant defense pathways. Front Plant Sci. 2013; 4: Article 215. https://doi.org/10.3389/fpls.2013.00215 PMID: 23811890

21. Jain P, Bhatla SC. Molecular mechanisms accompanying nitric oxide signalling through tyrosine nitration and S-nitrosylation of proteins in plants. Functional Plant Biology. 2018. pp. 70–82. https://doi.org/10.1017/FP16279 PMID: 32291022

22. Gailib M, Limmer DT. Reactive uptake of NO by atmospheric aerosol is dominated by interfacial processes. Science (80-). 2021; 371: 371–925. https://doi.org/10.1126/science.abc7716 PMID: 33632842

23. Reeves PH, Ellis CM, Ploense SE, Wu MF, Yadav V, Tholl D, et al. A regulatory network for coordinated flower maturation. PLoS Genet. 2012; 8: e1002506. https://doi.org/10.1371/journal.pgen.1002506 PMID: 22346763

24. Narusaka M, Yao N, Iuchi A, Iuchi S, Shiraishi T, Narusaka Y. Identification of Arabidopsis accession with resistance to Botrytis cinerea by natural variation analysis, and characterization of the resistance response. Plant Biotechnol. 2013; 30: 89–95. https://doi.org/10.5511/plantbiotechnology.12.1226a

25. Ando S, Obinata A, Takahashi H. WRKY70 interacting with RCY1 disease resistance protein is required for resistance to Cucumber mosaic virus in Arabidopsis thaliana. Physiol Mol Plant Pathol. 2014; 85: 8–14. https://doi.org/10.1016/j.pmpp.2013.11.001

26. Ross A, Somssich IE. A DNA-based real-time PCR assay for robust growth quantification of the bacterial pathogen Pseudomonas syringae on Arabidopsis thaliana. Plant Methods. 2016; 12: 48. https://doi.org/10.1186/s13007-016-0149-z PMID: 27895701

27. Takahashi H, Ebara Y. Severe chlorotic spot symptoms in cucumber mosaic virus strain Y-infected tobaccos are induced by a combination of the virus coat protein gene and two host recessive genes. Mol plant-microbe Interact. 1993; 6: 182–189. https://doi.org/10.1094/pmpp-6-182 PMID: 8471793

28. Takahashi H, Goto N, Ebara Y. Hypersensitive response in cucumber mosaic virus-inoculated Arabidopsis thaliana. Plant J. 1994; 6: 369–377. https://doi.org/10.1046/j.1365-313X.1994.00603069.x

29. Ando S, Jaskiewicz M, Mochizuki S, Koseki S, Miyashita S, Takahashi H, et al. Priming for enhanced ARGONAUTE2 activation accompanies induced resistance to cucumber mosaic virus in Arabidopsis thaliana. Mol Plant Pathol. 2021; 22: 19–30. https://doi.org/10.1111/mp.13005 PMID: 33073913

30. Chromczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques. 1993; 15: 532–537. PMID: 7692896

31. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016; 11: 1650–1667. https://doi.org/10.1038/nprot.2016.095 PMID: 27560171

32. Li S, Fu Q, Chen L, Huang W, Yu D. Arabidopsis thaliana WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance. Planta. 2011; 233: 1237–1252. https://doi.org/10.1007/s00042-011-1375-2 PMID: 21336597

33. Wohlgemuth H, Mittelstrass K, Kschieschan S, Bender J, Weigel HJ, Overmyer K, et al. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant, Cell Environ. 2002; 25: 717–726. https://doi.org/10.1046/j.1365-3040.2002.00859.x
Rao M V., Koch JR, Davis KR. Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant J. 2002; 32: 447–456. https://doi.org/10.1046/j.1365-313x.2002.01434.x PMID: 12445117

Kangasjärvi J, Jaspers P, Kolivist H. Signalling and cell death in ozone-exposed plants. Plant, Cell Environ. 2005; 28: 1021–1036. https://doi.org/10.1111/j.1365-3040.2005.01325.x

Bilgin DD, Aldea M, O’Neill BF, Benitez M, Li M, Clough SJ, et al. Elevated ozone alters soybean-virus interaction. Mol Plant-Microbe Interact. 2008; 21: 1297–1308. https://doi.org/10.1094/MPMI-21-10-1297 PMID: 18785825

Yalpani N, Nyedey AJ, León J, Raskin I. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta. 1994; 193: 372–376. https://doi.org/10.1007/BF00201815

Takahashi H, Miller J, Nozaki Y, Sukamoto, Takeda M, Shah J, et al. RCY1, an Arabidopsis thaliana RPP8/HRT family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism. Plant J. 2002; 32: 655–667. https://doi.org/10.1046/j.1365-313x.2002.01453.x PMID: 12472683

AbuQamar S, Moustafa K, Tran LSP. Mechanisms and strategies of plant defense against Pseudomonas syringae pv. tomato DC3000 in Arabidopsis thaliana by activating both salicylic acid–and jasmonic acid–mediated pathways. Mol Plant-Microbe Interact. 2018; 31: 1271–1279. https://doi.org/10.1094/MPMI-03-18-0071-R PMID: 29869942

Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol. 2005; 43: 205–227. https://doi.org/10.1146/annurev.phyto.43.040204.135923 PMID: 16078883

Nomura K, Melotto M, He SY. Suppression of host defense in compatible plant-Pseudomonas syringae interactions. Curr Opin Plant Biol. 2005; 8: 361–368. https://doi.org/10.1016/j.pbi.2005.05.005 PMID: 15936244

Gimenez-Ibanez S, Rathjen JP. The case for the defense: plants versus Pseudomonas syringae. Microbes Infect. 2010; 12: 428–437. https://doi.org/10.1016/j.micinf.2010.03.002 PMID: 20214999

Howlader P, Bose SK, Jia X, Zhang C, Wang W, Yin H. Oligogalacturonides induce resistance in Arabidopsis thaliana by triggering salicylic acid and jasmonic acid pathways against Pst DC3000. Int J Biol Macromol. 2020; 164: 4054–4064. https://doi.org/10.1016/j.ijbiomac.2020.09.026 PMID: 32910959

Jia X, Zeng H, Wang W, Zhang F, Yin H. Chitosan oligosaccharide induces resistance to Pseudomonas syringae pv. tomato DC3000 in Arabidopsis thaliana by activating both salicylic acid–and jasmonic acid–mediated pathways. Mol Plant-Microbe Interact. 2018; 31: 1271–1279. https://doi.org/10.1094/MPMI-03-18-0071-R PMID: 29869942

Birkenbihl RP, Diezel C, Somssich IE. Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. Plant Physiol. 2012; 159: 266–285. https://doi.org/10.1104/pp.111.192641 PMID: 22392279

Liu S, Kracher B, Ziegler J, Birkenbihl RP, Somssich IE. Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards Botrytis cinerea 2100. Elife. 2015; 4: e07295. https://doi.org/10.7554/eLife.07295 PMID: 26076231

Yang J, Duan G, Li C, Liu L, Han G, Zhang Y, et al. The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. Front Plant Sci. 2019; 10: Article 1349. https://doi.org/10.3389/fpls.2019.01349 PMID: 31681397

Choi HW, Manohar M, Manosalva P, Tian M, Moreau M, Klessig DF. Activation of plant innate immunity by extracellular High Mobility Group Box 3 and its inhibition by salicylic acid. PLoS Pathog. 2016; 12: e1005518. https://doi.org/10.1371/journal.ppat.1005518 PMID: 27007252

Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, et al. The grateful dead: Damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immuno Rev. 2007; 220: 60–81. https://doi.org/10.1111/j.1600-065X.2007.00579.x PMID: 17979840

Hou S, Liu Z, Shen H, Wu D. Damage-associated molecular pattern-triggered immunity in plants. Front Plant Sci. 2019; 10: Article 646. https://doi.org/10.3389/fpls.2019.00646 PMID: 31191574

Vénéreau E, Ceriotti C, Bianchi ME. DAMPs from cell death to new life. Front Immunol. 2015; 6: Article 422. https://doi.org/10.3389/fimmu.2015.00422 PMID: 26347745

Lim SY, Rafty MJ, Geczy CL. Oxidative modifications of DAMPs suppress inflammation: The case for S100A8 and S100A9. Antioxidants Redox Signal. 2011; 15: 2235–2248. https://doi.org/10.1089/ars.2010.3641 PMID: 20919339
54. Ziegler K, Kunert AT, Reinmuth-Selzle K, Leifke AL, Widera D, Weller MG, et al. Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress. Redox Biol. 2020; 37: 101581. https://doi.org/10.1016/j.redox.2020.101581 PMID: 32739154

55. Kumar P. Stress amelioration response of glycine betaine and Arbuscular mycorrhizal fungi in sorghum under Cr toxicity. PLoS One. 2021; 16: e0253878. https://doi.org/10.1371/journal.pone.0253878 PMID: 34283857

56. Kumar P, Tokas J, Singal HR. Amelioration of Chromium VI Toxicity in Sorghum (Sorghum bicolor L.) using Glycine Betaine. Sci Rep. 2019; 9: 16020. https://doi.org/10.1038/s41598-019-52479-w PMID: 31690803

57. Kumar P. Soil applied glycine betaine with Arbuscular mycorrhizal fungi reduces chromium uptake and ameliorates chromium toxicity by suppressing the oxidative stress in three genetically different Sorghum (Sorghum bicolor L.) cultivars. BMC Plant Biol. 2021; 21: 336. https://doi.org/10.1186/s12870-021-03113-3 PMID: 34261429