Nitric Oxide and Reactive Oxygen Species Do Not Elicit Hypersensitive Cell Death but Induce Apoptosis in the Adjacent Cells During the Defense Response of Oat

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Nitric oxide (NO) acts as a signaling molecule in many cellular responses in plants and animals. Oat plants (*Avena sativa* L.) evoke the hypersensitive response (HR), which shares morphological and biochemical features with mammalian apoptosis, such as DNA laddering and heterochromatin condensation, in response to the avirulent crown rust fungus (*Puccinia coronata* f. sp. *avenae*). We examined the role of NO and reactive oxygen species (ROS) in the initiation of hypersensitive cell death, which is induced by direct contact with the pathogen, and apoptotic cell death in the adjacent cells. Cytofluorimetric analysis using the fluorescent NO probe DAF and the H₂O₂ probe DCF demonstrated that NO and H₂O₂ were generated simultaneously in primary leaves at an early stage of the defense response. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) markedly enhanced H₂O₂ accumulation detected by 3,3-diaminobenzidine staining and DCF, whereas treatment with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) strongly suppressed it. Superoxide dismutase (SOD) increased NO accumulation, suggesting that endogenous NO may modulate the level of H₂O₂ by interacting with O₂⁻ in the HR lesion. Cytological observation showed that administration of cPTIO, SNAP, or SOD had no effect on elicitation of hypersensitive cell death, but clearly reduced heterochromatin condensation in the nearby cells and DNA laddering. These findings indicate that NO and ROS are not essential mediators for the initiation of hypersensitive cell death. However, NO and O₂⁻ but not H₂O₂ are required for the onset of apoptotic cell death in the adjacent cells, where excess NO may exert its anti-apoptotic function by regulating cellular redox state.

Additional keywords: programmed cell death

Recognition of an avirulent pathogen activates disease resistance gene (*R* gene)-dependent innate immune responses in

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plants, referred to as the hypersensitive response (HR) (Dangl and Jones 2001). The reaction involves the transient generation of reactive oxygen species (ROS), such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), through the oxidative burst (Doke 1997). These small signal molecules synergistically amplify the plant defense abilities against particular pathogen attack. ROS exert functions including direct killing of pathogens (Peng and Kuc 1992) and cross-linking of the cell wall (Bradley et al. 1992; Ikegawa et al. 1996), as a regulator for defense-related genes (Chamnonpol et al., 1998; Chen et al., 1995; Jabs et al. 1997; Levine et al. 1994), and for the induction of hypersensitive cell death (Levine et al. 1994). Recent studies have demonstrated that nitric oxide (NO) also plays a key role in the regulation of cellular signaling leading to induction of a battery of defense responses and plant development (Beligni and Lamattina 2000; Clarke et al. 2000; Delledonne et al. 1998, 2001; Durner and Klessig 1999; Wendehenne et al. 2001).

NO plays a variety of physiological and pathophysiological roles, such as regulation of apoptosis, blood vessel relaxation, and inflammation in animal cells (Estevez et al. 1999; Griffith et al. 1984; Ialenti et al. 1992). A number of reports demonstrated that NO had dual effects of cytotoxic and cytoprotective properties in apoptotic signaling pathways (Brune et al. 1998; Mannick et al. 1994; Messmer et al. 1994). NO induces apoptosis in diverse cells such as neuronal cells, thymocytes, peripheral T cells, and myeloid leukemia cells (Brockhaus and Brune 1998; Estevez et al. 1999; Fehsel et al. 1995; Williams et al. 1998). In contrast, NO has been shown to prevent apoptotic cell death in a cell-specific way by up-regulating protective proteins such as heat shock proteins, heme oxygenase-1, and cyclooxygenase-2, or by down-regulating MAP kinase phosphatase which induces apoptosis of endothelial cells in response to tumor necrosis factor (Brune et al. 1998; Rossig et al. 2000). Thus, the role of NO during apoptosis is considered to be ambivalent, and NO positively and negatively regulates the death program.

Recent analyses of NO signaling in plants indicate that NO-activated signaling pathways in mammals are also functional in plants. NO stimulates the activation of defense-related genes such as phenylalanine ammonia lyase and PR-1 genes in tobacco and *Arabidopsis* cells (Delledonne et al. 1998; Durner et al. 1998; Klessig et al. 2000). It has been demonstrated that NO cooperates with ROS to promote host cell death in soybean suspension cells (Delledonne et al. 2001). Furthermore, NO has been shown to induce programmed cell death (PCD) via cGMP-dependent signaling pathways in *Arabidopsis* cells (Clarke et al. 2000). In contrast, methylviologen-induced oxidative damage

could be negated by NO treatment (Beligni and Lamattina 1999). In summary, NO may regulate the cellular redox state, gene expression, and modification of target proteins as reported in mammals (Bolwell 1999; Durner and Klessig 1999; Van Camp et al. 1998). Although the ability of NO to function as a signaling molecule and to affect signaling pathways may redirect apoptotic signaling into anti-apoptotic signaling and vice versa, the versatility of NO-signaling mechanisms has not been well defined in plants.

Cell death induced by a certain pathogen often exhibits the morphological and biochemical features of mammalian apop-

tosis, such as heterochromatin condensation and DNA laddering (Dangl et al. 1996; Gilchrist 1998; Greenberg 1997; Heath 1998a; Lam et al. 1999; Pennell and Lamb 1997; Tada et al. 2001; Yao et al. 2001). We already have shown that hypersensitive cell death in oat (*Avena sativa* L.) caused by an avirulent crown rust (*Puccinia coronata* f. sp. *avenae*) race shares similar characteristics of mammalian apoptosis (Tada et al. 2001). Heterochromatin condensation and DNA laddering also were evident in leaves treated with biotic or abiotic elicitors, indicating that apoptotic cell death is associated with the disease resistance response in oat (Tada et al. 2001; Yao et al. 2001). Nuclear apoptosis induced by the host-selective toxin victorin C, which is produced by *Cochliobolus victoriae*, was reduced by treatment with the ROS scavengers superoxide dismutase or *N*-acetyl-cysteine, suggesting that ROS act as an upstream messenger for apoptotic cell death (Yao et al. 2002). Considering that NO also is a mediator for PCD in plants, the apoptotic response of oat plants most likely is regulated by NO.

Until now, there was not enough cytological evidence to determine whether NO modulates ROS accumulation in the HR lesion. Furthermore, because the HR lesion includes HR cell death of cells in direct contact with the pathogen and the surrounding dead cells, it must be elucidated whether NO and ROS affect both hypersensitive cell death and the death of the adjacent cells. Here we demonstrate that in oat leaves, NO and O_2^- may not be essential mediators for the initiation of hypersensitive cell death induced by the crown rust fungus, although they are important regulators for apoptotic cell death in the adjacent cells. We also demonstrate that endogenous NO plays an important role in the modulation of H_2O_2 accumulation during the defense response.

RESULTS

The crown rust fungus induces apoptotic cell death and the generation of NO and ROS in resistant oat leaves.

Primary leaves of oat cv. Shokan 1 were inoculated with the crown rust fungus, *P. coronata* f. sp. *avenae*, and analyzed for apoptotic cell death after various time intervals by monitoring DNA laddering. We previously have demonstrated that the nucleosomal cleavage of DNA and heterochromatin condensation can be used as reliable markers for apoptotic cell death, and that the intensity of the laddering reflects the number of dead cells (Tada et al. 2001; Yao et al. 2001). In the incompatible interaction of Shokan 1 and race 226, hypersensitive cell death occurs in the mesophyll cells by 36 h and hyphal growth is concomitantly retarded (Mayama et al. 1982). Although the avirulent pathogen clearly induced DNA fragmentation from 36 h after inoculation and the intensity increased in a time-dependent manner (Fig. 1A), obvious DNA laddering was not observed in the compatible interaction (race 203-Shokan 1) during the period tested.

The kinetics of NO and H_2O_2 accumulation induced by the fungus was determined by monitoring the fluorescence of NO and H_2O_2 probes 4,5-diaminofluorescein (DAF) and 2',7'-dichlorofluorescein (DCF), respectively. The lower epidermal cells of the rust-infected leaves were peeled off 12 h after inoculation, and the leaf segments were floated on DAF or DCF solution by the indicated time. The rapid and synchronous increases in NO and H_2O_2 accumulation occurred between 18 and 36 h after inoculation (Fig. 1B and C). The fluorescence intensity of DAF and DCF reached 2.4-fold and 1.8-fold higher than that of control treatments, respectively, and the generation of NO and H_2O_2 declined between 36 and 60 h.

Fig. 1. Avirulent crown rust induces DNA laddering and the generation of NO and H_2O_2 in oat leaves. **A**, Genomic DNA was extracted from cv. Shokan 1 leaves inoculated with uredospores of *Puccinia coronata* f. sp. *avenae*, race 226 (AV: avirulent) or 203 (V: virulent) and electrophoresed on a 2% (wt/vol) agarose gel. The size markers are shown to the left. **B** and **C**, Primary leaves of resistant cv. Shokan 1 (closed square) and susceptible cv. CW-491-4 (closed circle) were inoculated with race 226. **B**, NO was measured by monitoring the fluorescence of 4,5-diaminofluorescein (DAF). **C**, Reactive oxygen species was measured by monitoring the fluorescence of 2',7'-dichlorofluorescein (DCF). Points and bars represent the mean value and standard deviation of three independent experiments.

Cytochemical localization of H₂O₂ during the resistance response.

3,3'-diaminobenzidine (DAB) staining was performed in the inoculated leaves to determine where H₂O₂ accumulates and whether changes in the intracellular level of H₂O₂ detected by DCF correlate with the staining. In the incompatible combination, although H₂O₂ accumulation was not observed at 12 h after inoculation (Fig. 2A), DAB deposits were unambiguously detected at 36 h postinoculation (Fig. 2B), and continuous expansion of DAB deposits was observed during the time course (Fig. 2C). In contrast, distinct H₂O₂ accumulation was not detected in leaves infected with the virulent fungus (Fig. 2D to F). To further determine the precise localization of H₂O₂ accumulation in the infected tissue, we observed cross-sections of oat leaves 60 h after inoculation. DAB deposits were observed in the hypersensitively collapsed cells that exhibited autofluorescence under UV irradiation (Fig. 2G and H), but not in the compatible interaction (Fig. 2I and J), indicating that strong DAB precipitation is associated with the occurrence of hypersensitive cell death.

NO modulates ROS accumulation in the HR lesion.

Delledonne and associates (2001) demonstrated that, in soybean suspension cultures, NO worked synergistically with ROS to promote cell death. If NO coordinately exerts its physiological functions with ROS in the HR lesion, NO would interact with ROS and affect ROS accumulation at infection sites. To determine whether the reaction of NO with ROS is involved in the defense response, we evaluated the effect of superoxide dismutase (SOD) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) on the accumulation of NO and H₂O₂, respectively. Because the highest rate of NO and H₂O₂ accumulation occurred from 18 to 20 h (Fig. 1B and C), and no DAB deposits were observed at 12 h postinoculation (Fig. 2A), pharmacological agents were applied 12 h after inoculation. The administration of SOD (200 µg/ml) significantly enhanced NO accumulation induced by the avirulent fungus (race 226 plus water) (Fig. 3A), and the NO scavenger cPTIO (500 µM) also increased the intensity of DCF fluorescence (H₂O₂) at 48 h after inoculation (Fig. 3B). This indicates that NO may directly interact with O₂⁻ and regulate the level of H₂O₂. Furthermore, we carried out cytological analysis

Fig. 2. Cytological detection of H₂O₂ and the hypersensitively dead cells. Primary leaves of oat cv. Shokan 1 were inoculated with avirulent race 226 (AV: avirulent) or virulent race 203 (V: virulent), and H₂O₂ accumulation was detected by 3,3'-diaminobenzidine (DAB) staining. **A to F**, Time course of changes in the intensity of DAB deposits observed by bright field, showing the accumulation of H₂O₂ at the infection sites (arrows). **A to C**, Race 226; **D to F**, race 203. Note that all DAB deposits were observed at mesophyll cells under appressoria, which are located along the vein. Bars = 100 µm. **G to J**, Cross-sections of rust-infected leaves at 60 h after inoculation. **G** and **I**, Bright-field observation of the leaf sections inoculated with race 226 (AV) or race 203 (V), respectively, showing H₂O₂ accumulation in the hypersensitively collapsed cells (arrows). **H** and **J**, Same sections as in **G** and **I**, respectively, but visualized by fluorescence microscopy. Note that the dead cells contain autofluorescent compounds (arrows). Bars = 50 µm.

to determine an *in vivo* effect of NO on H₂O₂ accumulation detected by DAB staining. Spots of localized DAB deposition were formed in water-control leaves (Fig. 3C), and a strong, red-dish-brown color of DAB (arrowhead) and the surrounding H₂O₂ accumulation were detected by microscopy (Fig. 3D). These responses were observed only at infection sites (data not shown). cPTIO (500 μ M) significantly increased H₂O₂ accumulation in the spreading area around cells that exhibited dense deposition of DAB (Fig. 3C and E); however, it had no obvious effect on H₂O₂ accumulation in mock-inoculated leaves (data not shown). Although addition of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 200 μ M) failed to suppress strong DAB deposition at infection sites, clearly abrogated the accumulation of H₂O₂ in the surrounding area (Fig. 3C and F). These results indicate that endogenous NO may play an important role in the modulation of cellular redox state, negatively regulating H₂O₂ accumulation elicited by the pathogen infection.

Effect of NO and ROS on the induction of hypersensitive cell death.

To study the effect of NO and ROS on the initiation of the HR, we performed a statistical analysis of the frequency of autofluorescence, which is a marker of a hypersensitively dead cell (Fig. 2H). Cell autofluorescence was observed only around infection sites (Fig. 4A), and treatment with cPTIO, SNAP, or SOD at 12 h after inoculation had no effect on the intensity of autofluorescence. Hypersensitive cell death at 48 h after inoculation accounted for 52% of total appressoria formed on stomata (Fig. 4B, water). Treatment with SOD,

Fig. 3. Interaction of NO with reactive oxygen species (ROS) in the hypersensitive response lesion. Primary leaves of oat cv. Shokan 1 were inoculated with avirulent race 226. At 12 h postinoculation, leaf segments were treated with pharmacological agents described below for 36 h. Samples were treated with **A**, superoxide dismutase (SOD; 200 μ g/ml) or **B**, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO; 500 μ M), and NO and ROS were measured by 4,5-diaminofluorescein (DAF) and 2',7'-dichlorofluorescein (DCF), respectively. Mock samples were not treated with the pathogen. The results are means $\pm$ standard deviation of three independent experiments. **C** to **F**, Samples were stained with DAB to visualize H₂O₂ accumulation. **C**, Localized DAB deposition in the infected leaves treated with water, cPTIO (500 μ M), or *S*-nitroso-*N*-acetylpenicillamine (SNAP; 200 μ M). **D** to **F**, Microscopic observation of DAB deposition in the same samples shown in **C**. **D**, Water; **E**, cPTIO (500 μ M); **F**, SNAP (200 μ M). Arrowheads show examples of dense deposition of DAB (D through F). Bars (D through F) = 100 μ m.

cPTIO, or SNAP could not reduce or increase cell death at any concentration tested (Fig. 4B), indicating that NO and ROS may not function as essential mediators for the initiation of hypersensitive cell death in oat leaves. Then we performed cytological analysis of the same samples to confirm the occurrence of hypersensitive cell death. Leaf cross-sections stained with toluidine blue showed that hypersensitive cell death in the water control was associated with the less-stained and

Fig. 4. Effect of NO and reactive oxygen species (ROS) on the induction of hypersensitive cell death. Primary leaves of oat cv. Shokan 1 were inoculated with avirulent race 226. After 12 h, leaf segments were treated with the indicated reagents for 36 h. **A**, Bright-field observation (upper). Arrowheads show examples of appressoria on stomata. Same sections were visualized by fluorescence microscopy showing bright autofluorescence, a marker of hypersensitive response (HR) (bottom). Bars = 50 μ m. **B**, Statistical analysis of the frequency of HR per appressoria formed on the stomata. At least 3000 appressoria were counted in each treatment. The results are means $\pm$ standard deviation of three independent experiments. **C** to **F**, Cross-sections of the treated leaf. Samples were fixed, embedded, sectioned, and stained with toluidine blue for light microscopy. **C**, Water; **D**, superoxide dismutase (SOD; 200 μ g/ml); **E**, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO; 500 μ M); **F**, *S*-nitroso-*N*-acetylpenicillamine (SNAP; 200 μ M). Arrowheads show examples of hypersensitive cell death (C through F). Bars (C through F) = 50 μ m.

degraded chloroplasts (Fig. 4C, arrowhead). Although cell death was observed in SOD-, cPTIO-, or SNAP-treated leaves showing abnormal shape of the cells, nuclei, and chloroplasts, the cellular components retained at least part of their integrity in the dead cells (Fig. 4D to F). These data suggested that NO and ROS may play a role in the destruction of the infected cells.

Effect of NO and ROS on the induction of apoptotic cell death in the adjacent cells.

To examine whether NO and ROS are critical regulators of cell death in cells adjacent to the hypersensitively dead cells, cytological observation of leaf cross-sections 60 h after inoculation was carried out. In the water control, the cytoplasm of the adjacent dead cells was stained heavily with toluidine blue, and an abnormal distribution of the chloroplasts with a spherical morphology and stronger staining of nuclei were observed (Fig. 5A, open arrowheads). Treatment with SOD or cPTIO at 12 h after inoculation clearly suppressed the morphological changes detected in the water control (Fig. 5B and C). Al-

though the NO donor SNAP induced partial cellular damage in the nearby cells, it effectively reduced the staining in nuclei, indicating that they were morphologically normal (Fig. 5D).

We performed electron microscopic analysis to further confirm the roles of NO and ROS in apoptotic cell death. To focus on the effect of pharmacological agents on the adjacent cells, treatment with SOD, cPTIO, or SNAP was done at 18 h after inoculation, just before the occurrence of the HR. Cells adjacent to the hypersensitively collapsed cells displayed a localized condensation of heterochromatin in the water control (Fig. 5F). Addition of SOD, cPTIO, or SNAP effectively suppressed heterochromatin condensation, and the other organelles remained structurally intact at 60 h after inoculation (Fig. 5G to I).

To examine whether NO and ROS affect another prominent feature of apoptotic cell death, we tested the effect of NO and ROS on the induction of DNA laddering. Treatment with SOD (100 μ g/ml) effectively suppressed DNA laddering induced by the avirulent fungus (Fig. 6). Similar to the results of heterochromatin condensation shown in Figure 5, the presence of

Fig. 5. Effect of NO and reactive oxygen species (ROS) on the induction of apoptotic cell death in the adjacent cells. Primary leaves of oat cv. Shokan 1 were inoculated with avirulent race 226. **A to D**, After 12 h, leaf segments were treated with the indicated reagents for 48 h and the samples were fixed, embedded, sectioned, and stained with toluidine blue for light microscopy. Hypersensitively dead cells and the adjacent dead cells are represented by closed and open arrowheads, respectively. **E to I**, For electron microscopy, leaf segments 18 h after inoculation were treated with the same reagents for 42 h. Open arrowheads show localized condensation of heterochromatin in the cells adjacent to the hypersensitively dead cells. **A and F**, Water control; **B and G**, superoxide dismutase (SOD; 200 μ g/ml); **C and H**, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO; 500 μ M); **D and I**, S-nitroso-N-acetylpenicillamine (SNAP; 200 μ M). **E**, Mock samples were not treated with the pathogen. N, nucleus; Ch, chloroplast; Hc, heterochromatin. Bars (**A** through **D**) = 20 μ m. Bars (**E** through **I**) = 2 μ m.

SNAP (100 and 200 μM) or cPTIO (200 and 500 μM) strikingly reduced the nucleosomal cleavage of DNA (Fig. 6).

DISCUSSION

A growing body of evidence suggests that plants employ NO to regulate a variety of processes, such as maturation, senescence (Leshem et al. 1998), seed germination (Beligni and Lamattina 2000), drought stress (Mata and Lamattina 2001), and defense responses (Delledonne et al. 1998; Hausladen and Stamler 1998; Wendehenne et al. 2001). Delledonne and associates (1998, 2001) clearly demonstrated that NO and ROS produced during a pathogen-induced HR cooperate to activate cell death. They proposed that the reaction of NO with H_2O_2 is required to elicit cell death, whereas scavenging NO with O_2^- or scavenging O_2^- with NO leads to the formation of a powerful oxidant, peroxynitrite (ONOO^-), which is not an essential signal for cell death in soybean cells (Delledonne et al. 2001). In contrast, urate, a natural scavenger of ONOO^- , has been shown to compromise cell death induced by the avirulent *Pseudomonas syringae* pv. *phaseolicola* in *Arabidopsis* leaves (Alamillo and García-Olmedo 2001), suggesting the involvement of ONOO^- in PCD. Taken together, it is strongly suggested that the coordinate levels of NO, O_2^- , and H_2O_2 are required for plant immune responses.

We investigated whether NO modulates the accumulation of ROS in the HR lesions. The avirulent rust fungus induces a strong, localized precipitation of DAB and the surrounding H_2O_2 accumulation in the infection sites (Fig. 3D). When NO is scavenged by the NO scavenger cPTIO, DAB deposition markedly expands in the spreading area (Fig. 3E), indicating that cPTIO abolishes the reaction of NO with O_2^- , and then H_2O_2 may accumulate by the SOD-catalyzed or spontaneous dismutation of O_2^- to H_2O_2 . Conversely, an excess level of NO generated by the NO donor SNAP strikingly reduces H_2O_2

accumulation in the surrounding cells (Fig. 3F), presumably generating ONOO^- by the reaction between NO and O_2^- . These findings strongly suggest that intracellular NO plays an important role in the regulation of H_2O_2 accumulation, and that at least NO, O_2^- , H_2O_2 , and ONOO^- may be co-generated in the HR lesions.

We demonstrated that cPTIO and SOD do not suppress the initiation of hypersensitive cell death (Figs. 4 and 5), whereas they effectively reduce the loss of organelle integrity. These data indicate that NO and O_2^- may not trigger hypersensitive cell death but, rather, play an important role in the destruction of the infected cells. The HR in cowpea plants elicited by the cowpea rust fungus also is not affected by treatment with SOD, catalase, or horseradish peroxidase, suggesting that oxidative stress is not involved in the initiation of the HR (Heath 1998b). These results contradict previous studies suggesting that the oxidative burst is the primal trigger of the HR (Grant et al. 2000; Jabs et al. 1996; Levine et al. 1994). Torres and associates (2002) clearly have shown, using reverse genetics, that *Arabidopsis* *gp91^{phox}* homologues *AtrbohD* and *AtrbohF* are responsible for ROS generation and cell death, respectively. Interestingly, HR cell death is enhanced in the *atrbohF* and *atrbohD/F* double mutants after inoculation with the obligate biotroph *Peronospora parasitica* isolate Emco5, although H_2O_2 accumulation is abrogated in the *atrbohD/F* double mutant. Therefore, it may be assumed that a particular pathogen, such as an obligate parasite, might cause HR in the directly infected cells independent of the oxidative burst.

Unlike hypersensitive cell death, pharmacological studies using cPTIO and SOD demonstrated that NO and O_2^- may be key players in stimulating apoptotic DNA laddering and heterochromatin condensation in the adjacent cells (Figs. 5 and 6). These results also suggest that NO or O_2^- alone may not be cytotoxic, because cPTIO and SOD would increase O_2^- and NO (Fig. 3A and B), respectively, by inhibiting the reaction between NO and O_2^- . Although addition of SOD enhances NO accumulation (Fig. 3A) and induces overwhelming H_2O_2 accumulation in the HR lesions (data not shown), the cytotoxic effect of NO/ H_2O_2 , as reported in soybean cells (Delledonne et al. 2001), does not occur. These data may imply that ONOO^- is a critical intermediate of cell death in oat leaves, but additional NO from SNAP reduces apoptotic cell death (Figs. 5D and I and 6) and only causes partial damage to the nearby cells (Fig. 5D). These discrepant results may be explained by the effects of NO on O_2^- or ONOO^- . The activity of alcohol dehydrogenase, which is a target for cellular oxidants, is inactivated by low equimolar concentrations of NO and O_2^- , or a ONOO^- -generating agent SIN-1 (Daiber et al. 2002). Interestingly, an excess level of NO abolishes the significant oxidations induced by SIN-1 or co-treatment with NO and O_2^- , suggesting that NO acts as an antioxidant interacting with ONOO^- to form nitrogen dioxide (Daiber et al. 2002; Wink and Mitchell 1998). Thus, in this study, administration of SNAP may lead to the formation of ONOO^- in the infected leaves, and then suppress its cytotoxic functions by excess NO. Therefore, it is likely that an optimal rate of NO/ O_2^- induced by appropriate levels of exogenous NO or O_2^- would enhance cell death. Our findings support the idea that NO can exert pro-apoptotic and anti-apoptotic functions depending on its concentration (Brune et al. 1998; Wink and Mitchell 1998) during defense responses.

In plant-pathogen interactions, the rate of NO or ROS generation may be different even among adjacent cells, and the difference leads to the establishment of distinct signal networks in individual cells. Alteration of the NO/ROS ratios may induce a variant set of defense responses. Actually, pharmacological manipulation of NO and ROS accumulation suppresses cell death in the HR lesions (Figs. 5 and 6), and our preliminary results

Fig. 6. Effect of NO and reactive oxygen species (ROS) on the induction of DNA laddering. Primary leaves of oat cv. Shokan 1 were inoculated with avirulent race 226. Leaf segments 18 h after inoculation were treated with water, superoxide dismutase (SOD), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), or *S*-nitroso-*N*-acetylpenicillamine (SNAP). After 24 h of incubation, genomic DNA was extracted and separated on a 2% (wt/vol) agarose gel. The size markers are shown to the left.

indicate that NO modulates the expression of defense-related genes *PAL* and *PR-10* (data not shown) as reported elsewhere (Delledonne et al. 1998; Durner et al. 1998; Klessig et al. 2000). A putative role of NO in plant immune responses may be to limit the expanding defense reaction and cell death, as reported for the *Arabidopsis LSD 1* gene that responds to O_2^- and negatively regulates cell death (Dietrich et al. 1997). A gradation of NO generated at the infection site might form a discrete unit for defense response as an HR lesion.

MATERIALS AND METHODS

Plant materials.

Oat (*Avena sativa* L.) cvs. Shokan 1 and CW-491-4 were used in this study. Shokan 1 is highly resistant to race 226 and susceptible to race 203 of the crown rust fungus of oat, *Puccinia coronata* f. sp. *avenae*. CW-491-4 carries the *Pc38* gene, which confers an adverse interaction with the two races (Mayama et al. 1982). Seed were dipped in water for 12 h, sown on vermiculite, and grown in a growth chamber at 20°C under illumination for 16 h daily with 10,000 lux fluorescent light. Seven-day-old primary leaves were used for the present study.

Pathogens.

Uredospores of the oat rust fungus, 226 and 203, were produced on susceptible host cvs. CW-491-4 and Shokan 1, respectively. Uredospores (10 mg) mixed with 90 mg of talc were sprayed onto both surfaces of the primary leaves. Inoculated seedlings were incubated in a humid chamber in the dark for 12 h and then transferred to the conditions described above. Uredospores were collected and stored at 4°C until use.

Chemical treatment.

SOD and SNAP were obtained from Nacalai Tesque (Kyoto, Japan). cPTIO was purchased from Tocris Cookson (Ellisville, MO). SOD was used at concentrations of 50, 100, and 200 µg/ml. SNAP was used as an NO donor in concentrations of 100 and 200 µM. cPTIO was used as an NO scavenger at concentrations of 100, 200, and 500 µM. The lower epidermis of rust-infected oat leaves was peeled off at the indicated time, and 5-cm segments were taken from 1 to 6 cm from the leaf tip. The segments were floated on 3 ml of testing solutions, which contained chemical agents, such that the exposed mesophyll surface was in contact with the solution, and kept at 20°C under continuous light for the indicated time. To determine the effect of pharmacological agents on hypersensitive cell death and the death of the adjacent cells, treatments were done 12 h after inoculation and incubated for 36 or 48 h, because the fungus begins to enter leaf tissue through stomata 8 to 12 h after inoculation (Tani et al. 1978). The fungus-invaded mesophyll cells displayed a hypersensitive cell death from 20 to 24 h followed by the death of the surrounding cells.

Detection of ROS and NO accumulation.

An IX70 (Olympus, Tokyo) fluorescent microscope equipped with a MicroMax 12-bit CCD camera system (Princeton Instruments, Inc., Trenton, NJ) and an optical filter changer (Lambda 10-2; Sutter Instruments, Novato, CA) were used. A xenon lamp was coupled with a monochromator (Polychrome II; TILL Photonics, Planegg, Germany) to provide the excitation light source. ROS generated from oat leaves were measured by monitoring the fluorescence of DCF (Molecular Probes, Eugene, OR). NO accumulation was measured by the fluorescence of DAF (Daiichi Kagaku, Tokyo). The epidermis was peeled from leaves 12 h after inoculation, and five 1-cm segments of Shokan 1 and CW-491-4 leaves were incubated

with the testing solution containing 100 µM DCF or 100 µM DAF by the indicated times (16, 18, 24, 36, 48, and 60 h). For control experiments, we also measured the accumulation of NO and H_2O_2 induced by talc treatment (mock). The testing solution was observed using 488 and 515 nm with excitation at 520 and 495 nm emission wavelengths, respectively. Images from the microscope were acquired during a 200-ms exposure time, and the fluorescence intensity values were analyzed by MetaMorph software (Universal Imaging Co., Downingtown, PA, U.S.A.). All fluorescence intensity values ranged between 0 and 4,095, and experimental points represented the mean $\pm$ standard deviation of three replicates of the same experiment.

DNA extraction and analysis.

Oat leaves (0.3 g) were frozen in liquid nitrogen and ground into a fine powder. Each sample was incubated for 30 min at 65°C in 600 µl of 2% (wt/vol) cetyltrimethylammonium bromide (CTAB) solution (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 2% [wt/vol] cetyltrimethyl ammonium bromide), then mixed with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol). After gently shaking for 5 min, the mixture was centrifuged for 15 min at $10,000 \times g$. Total DNA was precipitated by the addition of 800 µl of 1% (wt/vol) CTAB solution (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% [wt/vol] cetyltrimethyl ammonium bromide) to the supernatant. After centrifugation for 10 min at $8,000 \times g$, the pellet was dissolved in 500 µl of 1 M CsCl and subjected to alcohol precipitation with a twofold volume of 100% ethanol. DNA was recovered by centrifugation for 10 min at $10,000 \times g$, followed by washing with 70% ethanol, and dissolved in 500 µl of Tris-EDTA buffer containing RNase at 40 µg/ml. After incubation at 37°C for 30 min, phenol extraction and ethanol precipitation were performed to recover DNA. To detect DNA laddering, samples were run on a 2% (wt/vol) agarose gel and stained with ethidium bromide at 0.5 µg/ml.

Histochemical detection of hydrogen peroxide.

For histochemical detection of H_2O_2 using 3,3-diaminobenzidine-HCl (Thordal-Christensen et al. 1997), primary leaves inoculated with rust race 226 or 203 were sampled at the indicated time. The inoculated leaves were excised and placed in an aqueous solution containing 3,3-diaminobenzidine-HCl (Nacalai, Kyoto, Japan) at 1 mg/ml, and incubated in Petri dishes for 4 h at 20°C in the dark.

Analysis of the HR frequency.

The treated leaves were collected and washed for 3 days in alcoholic lactophenol (70% ethanol, 7% phenol, 7% lactic acid, 7% glycerin) with several changes of wash solution. Cytological response of the specimens was observed under bright and fluorescent fields of a fluorescence microscope (Olympus, Tokyo). A total of 3,000 appressoria were counted for each treatment.

Light and electron microscopic analysis.

The treated tissues were cut into approximately 1- to 2-mm² pieces. The samples then were prefixed in 2.5% (vol/vol) glutaraldehyde and 2% (vol/vol) paraformaldehyde with 1:15 M phosphate-buffered saline (PBS) (pH 7.2). After three washes with PBS buffer, the samples were postfixed with 1% (wt/vol) osmium tetroxide in PBS for 1 h and then dehydrated. Leaf segments were embedded in Quetol 812 resin. For light microscopic analysis, samples were cut using a Sorvall-microtome to obtain semi-thin sections (2 µm) and mounted on glass slides and stained with 1% toluidine blue. For electron microscopic observation, samples were embedded in Epon 812 resin

(Nisshin EM, Tokyo), and blocks were cut with a Histo diamond knife (DIATOME, Bienne, Switzerland) on a Reichert-Nissei Ultracut (Leica AG, Vienna, Austria) to obtain ultrathin sections (90 nm), which then were collected on copper grids (200 mesh). Sections were examined and photographed using a Hitachi-7100 (Hitachi, Tokyo) transmission electron microscope at an accelerating voltage of 75 kV.

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