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Effects of scavengers for active oxygen species on cell death by cryptogein

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Abstract

The hypersensitive reaction is a type of programmed cell death in plants. Cryptogein is a proteinaceous elicitor secreted from *Phythophthora cryptogea*. In one current model, active oxygen species (AOS) trigger programmed cell death in plants. In this study, we examined a variety of AOS scavengers to elucidate the function of AOS in the death program. Most of these AOS scavengers, including tiron, a scavenger for superoxide radical, catalase for hydrogen peroxide, and hydroquinone, sodium ascorbate and propyl gallate for free radicals, almost completely removed extracellular AOS. However, none of the reagents completely blocked the cell death process. Other reagents, such as histidine and dimethylfuran, scavengers for singlet oxygen, and diphenyleneiodonium chloride, an inhibitor of NADPH oxidase, showed significant toxicity in BY-2 cells. These results indicate that AOS produced in the extracellular space do not play a role in hypersensitive cell death.

Keywords: *Nicotiana tabacum*; *Solanaceae*; AOS; BY-2; Cryptogein; Fluorescein diacetate; Hypersensitive response; *Phythophthora cryptogea*

1. Introduction

The hypersensitive response (HR) is a universal phenomenon found in plant cells that helps to protect against attacks from incompatible pathogens (Greenberg et al., 1994). This reaction is mediated by an interaction between specific receptors and pathogen-derived signal molecules called elicitors (Ebel, 1998; Nespoulous et al., 1992). The final stage in HR involves the rapid appearance of necrotic lesions around the point of invasion by the pathogen.

Cryptogein is a proteinaceous elicitor secreted by *Phythophthora cryptogea* (O'Donohue et al., 1995; Ricci et al., 1989). This elicitor induces a remarkable and reproducible cell death in tobacco plants. A rapid and substantial calcium influx (Tavemier et al., 1995), alkalization of the extracellular medium (Blein et al., 1991; Lebrun-Garcia et al., 1999; Mathieu et al., 1996), and the transient production of active oxygen species (AOS) (Rusterucci et al., 1996) have all been observed following cryptogein treatment.

AOS are known to be key molecules in the execution of hypersensitive cell death. In one current model, AOS are produced outside of cells, and then returned inside to initiate the death program. Several reports support this hypothesis. For example, the direct addition of H_2O_2 to cultured tobacco BY-2 cells (Houot et al., 2001) or *Arabidopsis* cells (Desikan et al., 1998) induced their death. However, since results to the contrary have also

Abbreviations: AOS, active oxygen species; BY-2, *Nicotiana tabacum* L. cv. Bright Yellow 2; FDA, fluorescein diacetate; HR, hypersensitive response.

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been reported (Sasabe et al., 2000), further research is needed on the involvement of AOS in HR.

There are several kinds of AOS, including superoxide radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical. We used various scavengers for AOS (Rusterucci et al., 1996) that were specific for each of these species. Tiron is a scavenger of superoxide radical (Miller and Macdowall, 1975), catalase is a scavenger for hydrogen peroxide (Chance and Oshino, 1971), and dimethylfuran (Noguchi et al., 1977; Takayama et al., 1977) and histidine quench singlet oxygen (Hartman et al., 1990). Hydroquinone (O'Brien, 1991), propyl gallate (Haseloff et al., 1990) and sodium ascorbate (Bielski and Cabelli, 1991) are effective against free radicals. We also examined another type of reagent, diphenyleneiodonium chloride (DPI), which inhibits NADPH oxidase (Dorey et al., 1999; Pugin et al., 1997), a key enzyme that generates superoxide radicals in plant cells and readily converts the radicals into hydrogen peroxide (Pugin et al., 1997).

In this report, we describe the effects of AOS scavengers on cell death. Five of the eight scavengers, i.e., tiron, catalase, hydroquinone, sodium ascorbate and propyl gallate, were effective for removing AOS from the medium, however the rate at which cell death was induced was not affected. The remaining three compounds, histidine, dimethylfuran and DPI, acted as potent poisons to kill tobacco cells even when no cryptogein was added to the medium.

Table 1
Effects of AOS scavengers on AOS production and death progression activity induced by cryptogein

	Concentration	AOS production (%)[a]		Death progression (%)[b]	
None	–	100	(2.53)[d]	100	(9.91)[d]
Tiron	10 mM	1.43	(1.17)	135	(5.1)
Catalase	84 μg/ml	1.63	(1.28)	111	(6.17)
Hydroquinone	0.1 mM	2.23	(1.18)	95.9	(3.90)
Sodium ascorbate	1 mM	1.62	(1.16)	90.1	(13.0)
Propyl gallate	0.1 mM	6.39	(1.50)	83.3	(18.2)
Histidine	10 mM	82.5	(1.07)	76.9	(5.30)
Dirnethylfuran	87 mM	5.95	(1.35)	–[e]	
DPI[c]	100 μM	1.37	(0.68)	8.84	(1.11)

[a] AOS production was determined from the quenching rate of pyranine fluorescence. Values are expressed relative to the control. AOS production (%) = Quenching rate of pyranine fluorescence with scavenger/without scavenger × 100.

[b] Rate of death progression was estimated from cell viability at 6 h after treatment. Cell viability was verified by spectrofluorimetric analysis using FDA. Death progression (%) = (Viability at 6 h with scavenger/without scavenger) × 100.

[c] Diphenyleneiodonium chloride.

[d] Standard error, calculated from three independent experiments.

[e] It was impossible to determine the viability due to the toxicity of dimethylfuran.

2. Results and discussion

2.1. Effects of AOS scavengers

Tobacco cells produce considerable amounts of AOS during HR, including superoxide radical, hydrogen peroxide, singlet oxygen, and hydroxyl radicals. In this study, we investigated which species were involved in HR, and used specific AOS scavengers to quench the radicals. The quantity of AOS was determined from the quenching rate of pyranine fluorescence (Lecourieux-Ouaked et al., 2000). In this assay system, pyranine is a substrate for peroxidase localized at the tobacco cell wall. Thus, the AOS referred to here represents H_2O_2 in a narrow sense. The relative rate of AOS production was calculated from the quenching rate of pyranine fluorescence. The quenching rate of tobacco cells seen with 500 nM of cryptogein was defined as 100% AOS production. The effects of AOS scavengers were compared to the quenching rate of pyranine fluorescence obtained from tobacco cells treated with each scavenger. The results are summarized in Table 1.

The addition of 0.1 mM tiron, which is a scavenger for superoxide radical (Fig. 1(a)), resulted in a 70% reduction in AOS production. Moreover, when the concentration of tiron was increased to 10 mM, AOS production was dramatically reduced to only 1.43% of the original rate. Catalase is a scavenger of hydrogen peroxide. When catalase was added at 84 μg/ml, only 1.63% of H_2O_2 remained (Fig. 1(c)). Hydroquinone, propyl gallate and sodium ascorbate are effective against free radicals. Upon the addition of hydroquinone (Fig. 1(e)) and propyl gallate at 0.1 mM (Fig. 1(g)), the production of AOS was reduced to 2.23% and 6.39%, respectively. Although sodium ascorbate did not have any effect at 0.1 mM, the production of AOS was reduced to 1.62% at 1 mM (Fig. 1(i)). Sodium ascorbate is a bioactive reagent, and thus ascorbate was probably degraded by some biological reactions. In this experiment, a lower concentration of ascorbate was not effective.

Dimethylfuran and histidine, which are scavengers for the hydroxyl radical, had different effects on AOS quenching (Fig. 2(a) and (c)). With 87 mM dimethylfuran, the rate of AOS production was lowered to 5.95%, while that with 10 mM histidine remained at 82.5%. In addition to these scavengers, we used another reagent, diphenyleneiodonium chloride, which inhibits NADPH oxidase (Fig. 2(e)) (Dorey et al., 1999; Pugin et al., 1997). NADPH oxidase is a key enzyme that generates superoxide radicals in plant cells, which are readily converted into hydrogen peroxide. When DPI was added at a concentration of 100 μM, AOS production was reduced to 1.37%.

2.2. Effect of AOS scavengers on death progression

The induction of cell death is known to be closely associated with the production of AOS (Rusterucci

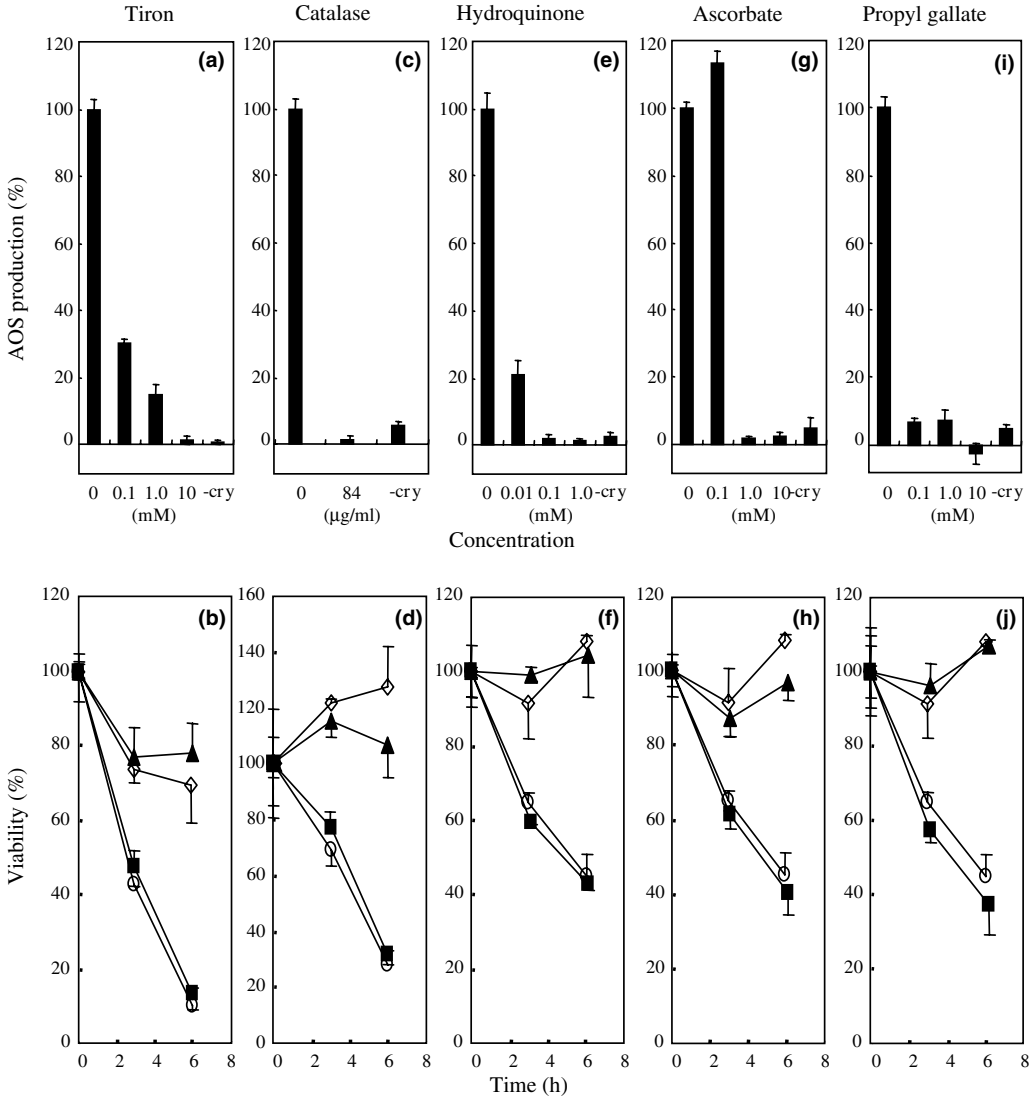

Fig. 1. Effects of AOS scavengers that are harmless to tobacco BY-2 cells. Upper panels (a), (c), (e), (g) and (i) show relative AOS production. When cryptogein was added to tobacco cells at 500 nM, the rate of AOS production was defined as 100%. After various concentrations of scavenger were added with cryptogein, extracellular AOS was measured based on the quenching rate of pyranine fluorescence. Lower panels (b), (d), (f), (h) and (j) show the time course of cellular viability measured by the FDA method, (○), cryptogein at 500 nM; (◇), without cryptogein; (▲), scavenger only; (■), scavenger and cryptogein. Each point represents the average of at least three experiments. The bar indicates the standard error. The concentrations of scavengers in the analysis were fixed as follows: tiron 10 mM, catalase 84 μg/ml, hydroquinone 0.1 mM, propyl gallate 0.1 mM, and sodium ascorbate 1 mM.

et al., 1996). Therefore, we determined the concentrations of scavengers that completely quenched AOS. We also studied the effects of AOS scavengers on death progression. Cells were harvested every 3 h, and viability was measured by the FDA method (Amano et al., 2003). Previously, we found that 500 nM of cryptogein was sufficient to kill tobacco cells (Amano et al., 2003). Under such conditions, cell viability decreased at least 50% within 6 h, which is consistent with the results in this study. The effects of various scavengers were compared under these conditions to assess whether the cells could be rescued by treatment with the scavengers.

Before we evaluated the effects of the scavengers, we first examined the effects of the scavengers on FDA fluorescence in the absence of cells in control experiments. The addition of scavengers showed a sharp peak in fluorescence just after addition, but this peak rapidly decreased within 10 s, and then remained at a constant base level (data not shown). This indicates that the scavengers used had no effect on FDA fluorescence.

To evaluate the effects of the scavengers, we initially examined the effect of tiron at up to 10 mM on the viability of tobacco cells (Fig. 1(b)). When 10 mM tiron was added to cultured cells, cell viability deceased to 77.9% (Fig. 1(b), ◇), while without tiron, viability was

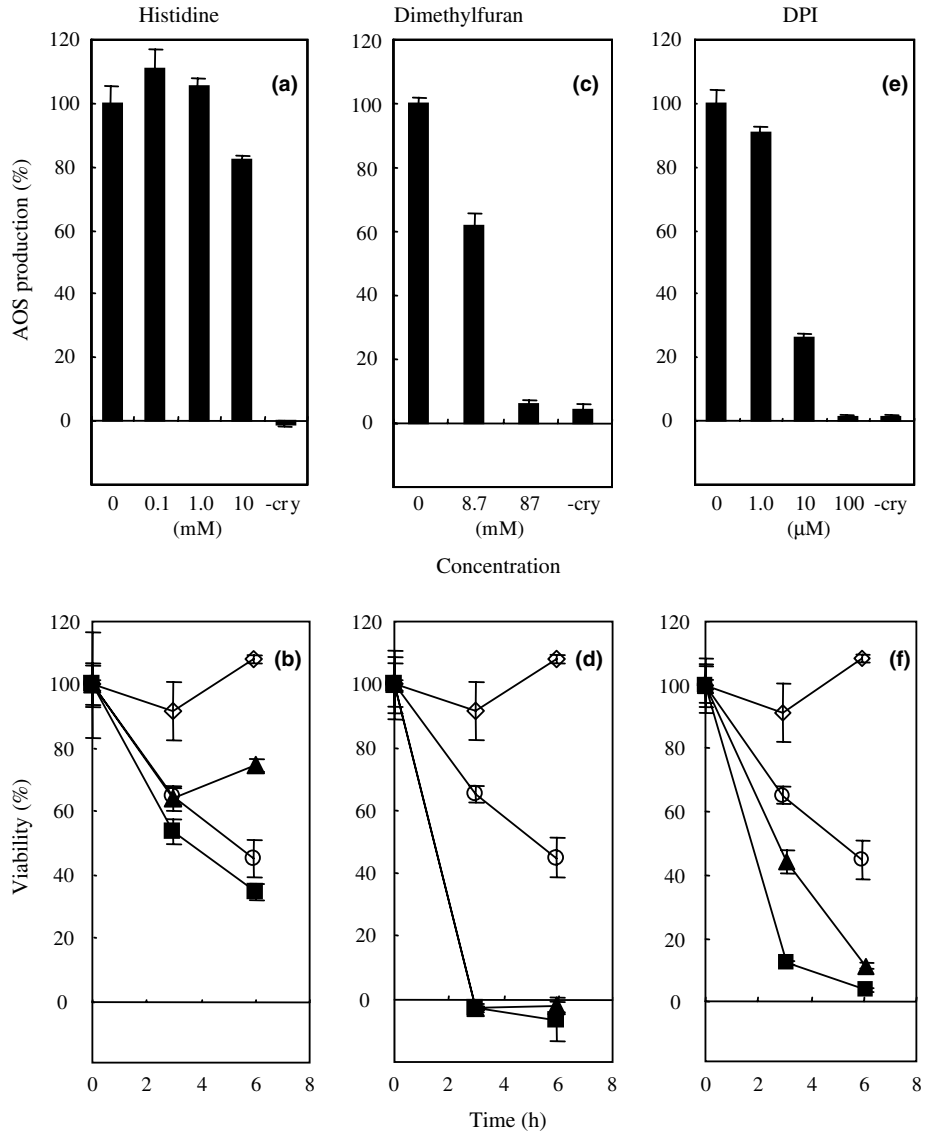

Fig. 2. Effects of AOS scavengers that are toxic to BY-2 cells. Upper panels (a), (c) and (e) show relative AOS production. Lower panels (b), (d) and (f) show the time course of cell viability, (○), cryptogein at 500 nM; (◇), without cryptogein; (▲), scavenger only; (■), scavenger and cryptogein. Each point represents the average of at least three experiments. The bar indicates the standard error. The concentrations of histidine, dimethylfuran and diphenyleneiodonium chloride (DPI) were set at 10 mM, 87 mM and 100 μM, respectively. For details, see the legend for Fig. 1.

reduced to 69.2% of the initial levels (Fig. 1(b), ▲). Thus, tiron had no significant effect on cellular viability. To normalize the effects of the scavengers, we calculated the relative viability with and without cryptogein after 6 h. Using this approach, the death progression activity with tiron was calculated to be 135%, which is the percentage of viability with tiron to that without tiron. This result indicates that tiron could not stop HR to produce cell death, even though AOS were strongly quenched under these conditions (Fig. 1(b)). With catalase (84 μg/ml, final concentration), death progression (111%) was similar to that in the control treatment with cryptogein (Fig. 1(d)). In addition, 0.1 mM hydroquinone (Fig. 1(f)), 0.1 mM propyl gallate (Fig. 1(h)), and 1 mM sodium ascorbate (Fig. 1(j)), which is effective against a wide range of free radicals, were also examined to determine whether they could counteract death progression. In each case, the activity of cryptogein was not reduced by the scavenger.

When living cells were used, it was impossible to obtain consistent results regarding the death induction rate. The rates shown in Fig. 1(b), (d), (f), (h) and (j) are not consistent, likely because different batches of cells were used. However, for the purposes of this study, it was most important to compare the results with and without scavengers. Thus, an identical batch of cells was used in at least one scavenger experiment (subpanel).

We also found that 10 mM histidine (Fig. 2(b)), 87 mM dimethylfuran (Fig. 2(d)) and 100 μM DPI

(Fig. 2(f)) were toxic to the cells. When 10 mM histidine was added to the cells, viability at 3 h was 64.2%, and that at 6 h was 74.5%, and these values were 91.4% and 108%, respectively, in the control experiment. Next, treatment with 87 mM dimethylfuran reduced the viability to −2.86% at 3 h and −6.48% at 6 h after the addition of cryptogein. This reagent may not only harm cells but also may impair the conversion of FDA.

DPI also acted as a poison to kill cells. Viability with 100 μM DPI alone decreased to 44.1% at 3 h, and finally reached 11.4% at 6 h. The toxicity of this reagent is considered to be due to its active site on the tobacco cell. Usually, it inhibits the essential enzyme NADPH oxidase (Dorey et al., 1999; Pugin et al., 1997), which plays a key role in the production of AOS. Specifically, this enzyme is localized at the cell membrane, and its active sites are oriented toward the outside of the cell. This can explain why the death progression activity upon treatment with DPI was remarkably reduced to 8.84% of the original rate (Fig. 2(f)).

2.3. Signal transduction pathways for cell death

The HR pathway leading to cell death is composed of a vast number of signal transduction cascades. Among these responses, external alkalization, AOS production and death initiation are indispensable for programmed cell death in plants. However, little is known about how these responses contribute to or interact with the death reaction. Three reactions act sequentially toward the death reaction. Notably, the AOS production signal has been shown to be a main factor in death progression (Houot et al., 2001; Rusterucci et al., 1996).

In this study, we focused on the interaction of AOS production and death initiation. As mentioned above, the signal cascade for initiating cell death should be triggered by AOS produced outside of plant cells, and the AOS are then returned into the cells to initiate the program. If this is the case, we expected that AOS scavengers would effectively stop the cell death process. However, none of the scavengers prevented cell death, although all nearly completely removed AOS (Table 1). These results indicate that the induction of cell death was independent of the production of AOS. In this study, we showed that extracellular AOS was not involved in the mechanisms that initiate cell death.

3. Experimental

3.1. Chemicals

Tiron, histidine, mannose, hydroquinone, sodium ascorbate, and diphenyleneiodonium chloride were dissolved in distilled water at a concentration of 0.1 M. Propyl gallate was dissolved in methanol and adjusted with distilled water to 0.1 M (final methanol concentration was 20%, v/v). Catalase (84 μg protein/ml) and dimethylfuran (8.7 M) were used for measurements. All stock solutions were kept in the dark at 4 °C.

3.2. Plant materials

BY-2 tobacco cells were cultured in Murashige-Skoog medium containing 3% (w/v) sucrose, 0.2 mg/l 2,4-dichlorophenoxyacetic acid, 200 mg/l KH_2PO_4, 10 mg/l myo-inositol and 1 mg/l thiamine-HCl at 25 °C with rotary shaking at 130 rpm. The cells were used after 4 d culturing.

3.3. Expression and purification of cryptogein

The *Picha pastoris* strain GS115 bearing the plasmid pLEP3 was used for cryptogein production. Expression of cryptogein was performed according to O'Donohue et al., 1996. Cryptogein was dissolved in distilled water and adjusted to the required concentration using ε_{277} as 8306 M^{-1} cm^{-1} (O'Donohue et al., 1995).

3.4. AOS measurements

AOS production was measured primarily by detecting H_2O_2 from the quenching rate of pyranine fluorescence ($\lambda_{excitation}$ = 405 nm; $\lambda_{emission}$ = 512 nm), since the AOS are randomized and change form. The rate of AOS production was measured according to Lecourieux-Ouaked et al. (2000). Before the start of the measurements, AOS scavenger was added to the cuvette until the desired final concentration was reached.

Before the start of the reaction, pyranine and BY-2 cells were added to the cuvette, and the scavenger was then added at 10 s. The cells were successively stabilized for 300 s. Cryptogein was added at 300 s. AOS production rate was calculated from the slope between 400 and 520 s. In Fig. 2, the scavenger was replaced by water as a control experiment.

3.5. Viability measurements

A quick and accurate method for calculating the viability of plant cells was previously reported (Amano et al., 2003). The measurement was performed with a spectrophotofluorimeter (HITACHI F-2500, Japan) equipped with a stirrer. First, 2 ml of PBS (2.7 mM KCl, 137 mM NaCl, 1.8 mM KH_2PO_4, and 4.0 mM Na_2HPO_4) and 5.0×10^{-4}% (w/v) PBS (2.7 mM KCl, 137 mM NaCl, 1.8 mM KH_2PO_4, and 4.0 mM Na_2HPO_4) and 5.0×10^{-4}% (w/v) PDA were placed in a quartz cuvette, and tobacco cells were mixed in this cuvette to determine the viability. Next, the rate of fluorescence was recorded over 40 s using excitation

and emission wavelengths of 493 and 510 nm, respectively. The slope of the increase in fluorescence (from 25 to 35 s) was calculated for each cellular suspension. Cell viability is correlated with the rate of PDA conversion.

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