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DNA Endonuclease- and Active Oxygen-Associated Degradation of Chloroplast DNA in Response to Paraquat-Induced Oxidative Stress

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Activity of chloroplast-localized DNA endonuclease was observed in detached tobacco leaves that had been treated with paraquat and light. The DNA endonuclease was able to cleave the chloroplast, plasmid, and single-stranded DNA, as estimated on an agarose gel. Activity was sensitive to two endonuclease inhibitors: aurintricarboxylic acid and $ZnSO_4$. The time course for activity showed a peak 4 h after the stress treatment. These results suggest that this enzyme plays a specific physiological role during oxidative stress. Probable roles for this enzyme are also discussed.

Keywords: DNA endonuclease, oxidative stress, paraquat

Chloroplasts enable plants to grow autotrophically by photosynthesizing in the presence of light. The light-initiated electron transfer within the thylakoid membrane is primarily used to generate ATP and NADPH for carrying out CO_2 fixation in the Calvin cycle. Under various environmental stresses that perturb photosynthetic electron flow, such as high light or drought, the chloroplasts frequently undergo oxidative stress during which superoxide anion radicals ($\cdot O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH$\cdot$) (collectively known as reactive oxygen species, ROS) are generated.

Of these ROS, the hydroxyl radicals are particularly potent, reacting with most macromolecules such as DNA or membrane lipids (Badway and Karnovsky, 1980; Slater, 1984). As a protective means against ROS, chloroplasts have evolved with antioxidizing, nonenzymatic agents and enzymes such as Cu/Zn superoxide dismutase (SOD), ascorbate peroxidase, and glutathione reductase (Foyer and Halliwell, 1976; Knox and Dodge, 1985; Smith et al., 1989; Bowler et al., 1992). Among the ROS scavengers in chloroplasts, Cu/Zn SOD appears to be a primary defensive enzyme by which superoxide anion radicals are converted into hydrogen peroxide and water. The well-controlled activity of the Cu/Zn SOD is critical to inhibiting production of hydroxyl radicals because the uncontrolled presence of superoxide anion radicals and hydrogen peroxides would favor the production of hydroxyl radicals (Tepperman and Dunsmuir, 1990).

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Oxidative stresses exceeding the cell's ROS-scavenging capacity accelerate cellular damage, with DNA being one of the major targets (Bowler et al., 1992). The wide variety of oxidative damage to DNA may include modifications to the bases as well as cleavage of the sugar phosphate backbone (Demple and Harrison, 1994). Most of the damage is corrected by a repair system, but damage that escapes repair can be mutagenic and/or inhibitory to DNA synthesis or replication (Demple and Harrison, 1994). Because plants are vulnerable to various kinds of environmental disturbances, which prompt extensive oxidative stresses, it is expected that their nuclear and organellar genomes will be damaged frequently (Britt, 1996). For example, chloroplast genomes can be damaged when plants are exposed to a photoinhibitory stress that discourages normal photosynthetic electron flow. In a study of ozone-treated peas and beans, the 8-hydroxyguanine contents increased eight-fold in the chloroplast genome, indicative of oxidative damage (Floyd et al., 1989). Hydroxyl radicals were suspected as the major factor for the damage. Because of technical difficulties in monitoring DNA damage in vivo, the defined kinetics for damage occurring under oxidative stresses are not well understood, particularly in plants.

DNA endonuclease is involved in several vital cellular functions, including DNA repair and programmed cell death. Various types of endonucleases participate in this repair (Demple and Harrison, 1994). In principle, they are involved in excising the DNA region that carries the damaged base(s). Several reports have demonstrated induced activity of repair-related endonuclease in response to UV damage in

plants (Strickland et al., 1991; Britt, 1996). It is also well known that DNA endonuclease participates in the initial event of cell death in both mammals (Ueda and Shah, 1992; Ueda et al., 1995) and plants. The endonuclease usually cleaves the nuclear genome in a very controlled manner to produce either DNA fragments, i.e., the markers for apoptotic cell death, or else random DNA degradation, which frequently occurs in a necrotic type of cell death. For a few DNA endonucleases, activities have been detected during the hypersensitive response-associated cell death in TMV-infected tobacco plants (Mittler and Lam, 1995).

Our long-term objective is to determine whether these physiological roles of DNA endonucleases, studied primarily with the nuclear genome, are also operative in chloroplasts. As an initial step, we concentrated on understanding how ctDNA is degraded in response to oxidative stress. To impose this type of stress, we applied the herbicide paraquat, also known as methyl viologen (MV), to leaf discs in combination with intervening light. In chloroplasts under illumination, MV is known to transfer electrons from the Photosystem into dioxygen, thus imposing an artificial oxidative stress. We also investigated whether the two means were present by which ctDNA is degraded, i.e., ROS- and endonuclease-associated.

MATERIALS AND METHODS

Plant Material and Growth Condition

Tobacco (*Nicotiana tabacum* L. cv. *Xanthi*) plants were grown at 25°C in a Phytotron growth chamber (Shin Young Inc., Korea) with a light intensity of 90 μmol $s^{-1}m^{-2}$ and a 15/9 h (light/dark) photoperiod. The experiments involved the fourth to sixth leaves from the top of each plant, approximately eight weeks after sowing.

MV Treatment

MV treatment was performed essentially according to the method of Gupta et al. (1993). Leaf discs, 1.5 cm^2 in size, were initially incubated for 16 h in the dark at 25°C in a floating solution that contained 0.4 M sorbitol plus different concentrations of MV to allow for absorption of the plant cells. The discs were then illuminated for 2 h with a light intensity of 100 μmol $s^{-1}m^{-2}$. They were further incubated for various time periods (1-10 h) in the dark at 25°C. For the experiments with the inhibitors, o-phenanthroline and aurintricarboxylic acid (ATC), the leaf discs were preincubated with different inhibitor concentrations for 2 h prior to commencement of the light treatment. For testing the inhibition of protein syntheses, leaf discs were incubated with 30 μmol cycloheximide for 3 h prior to the light treatment.

Isolation of Chloroplasts and Chloroplast DNA

Leaves were homogenized in an ice-cold homogenization buffer containing 50 mM HEPS, 0.33 M sorbitol, and 0.1% BSA (pH 7.5). The crude homogenates were filtered through eight layers of cheesecloth, and the filtrates were centrifuged at 2,500*g* for 2 min at 4°C. After being resuspended in the homogenization buffer, the pellets were layered on top of a sucrose gradient (1.50, 1.00, 0.75 M) and centrifuged at 1,000*g* for 30 min at 4°C. The chloroplast band at the 1-M density was collected and centrifuged at 2,000*g* for 7 min at 4°C. The pellets were then washed twice with a washing buffer containing 50 mM HEPES and 0.33 M sorbitol (pH 7.5), then resuspended in a 0.6-M sucrose solution. Chloroplast extracts were prepared by freezing (–20°C, 1 h) and thawing the suspension. ctDNA was isolated essentially according to the cetyltrimethylammonium bromide method. To estimate the degradation, the ctDNA that was isolated from leaves in the different MV treatments was subjected to 1% agarose gel electrophoresis.

Assay of DNA Endonuclease Activity

Activity of endonucleases was assayed by estimating the extent of degradation of the substrate DNA. The reaction mixture comprised 10-15 *u*L substrate DNA and 10 *u*L of the chloroplast extracts in a 30-*u*L solution containing 50 mM MES/NaOH (pH 5.6) and 3 mM $MgCl_2$. This reaction was carried out for 1 h at 37°C, and the resulting substrate DNA, after DNA extraction, was subjected to 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Degradation of cpDNA by MV Treatment

MV stress in combination with a 2-h light treatment (at a photon flux of 100 μmol $s^{-1}m^{-2}$) was imposed in such a way that chloroplasts became the major target. When fresh tobacco leaf discs were incubated with MV for 16 h at 25°C in the dark prior

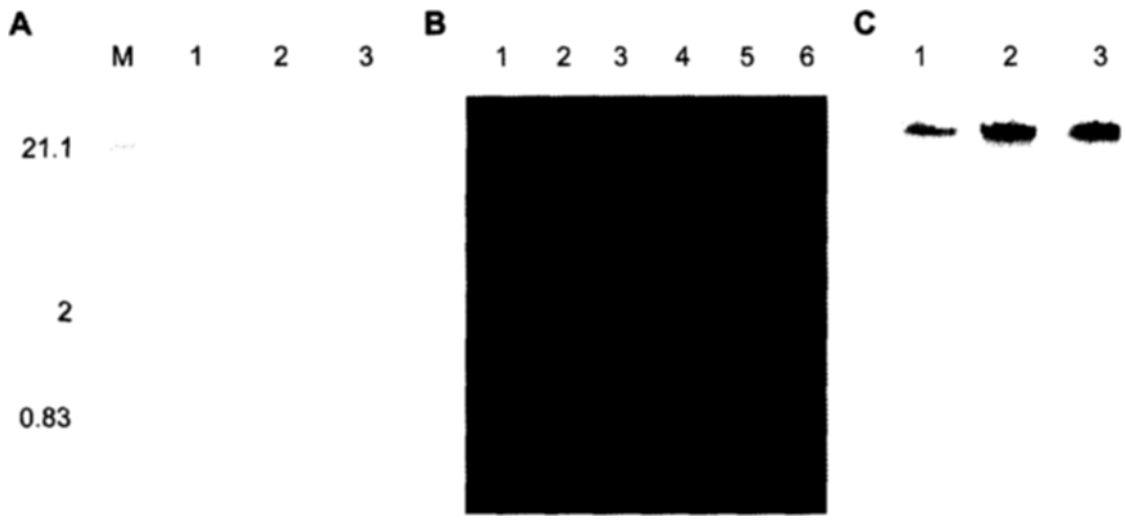

Figure 1. Degradation of chloroplast DNA (ctDNA) isolated from MV-stressed leaves. The DNA was subjected to 1% agarose gel electrophoresis. (**A**) Degradation with different MV concentrations. Lane 1, ctDNA from untreated leaves; Lane 2, treated with 1 *u*M MV; Lane 3, treated with 5 *u*M MV. (**B**) Degradation at 2.5 *u*M MV for the designated time periods after light treatments (as described in "Material and Methods"). Lane 1, 4 h, without MV; Lane 2, 4 h, without light treatment; Lane 3, 1 h; Lane 4, 2 h; Lane 5, 4 h; Lane 6, 8 h. (**C**) Degradation of cpDNA (20 *u*g) by inhibitors. Leaves were treated with inhibitors for 2 h prior to light treatment, followed by 8 h further incubation (2.5 *u*M MV). Lane 1, treated with o-phenanthroline (100 *u*M); Lane 2, control, no inhibitor; Lane 3, treated with ATC (100 *u*M).

to light treatment, the plant cells were allowed to be absorbed by MV. During this light treatment, we expected MV to initiate the production of ROS, resulting in oxidative stress in the chloroplasts.

In the course of further incubation (25°C, dark) for designated time periods following light treatment, ROS should have reacted with the cellular macromolecules in the chloroplast, such as DNA or membrane lipids. Degradation of the ctDNA in MV-treated leaves, therefore, was estimated by the extent of the DNA smears on the agarose gel electrophoresis (Fig. 1). The degree of degradation was positively correlated with the concentration of MV treated (0, 1, or 5 μm; Fig. 1A). When MV was omitted under otherwise identical stress conditions (i.e., 8 h of incubation after the light treatment), the degradation was negligible (Fig. 1B). Similarly, there was no sign of degradation when only the light treatment was omitted (Fig. 1B). As the length of the incubation period following light treatment increased, with the MV concentration fixed at 2.5 μm, the level of degradation also increased (Fig. 1B). These results indicated that MV treatment, with intervening light, was an appropriate experimental system for studying the degradation of ctDNA under MV-induced oxidative stress.

We then examined how ROS and DNA endonuclease were involved in the stress-response degradation of ctDNA. To confirm the role of ROS, we added o-phenanthroline to the incubation medium for 2 h before the light treatment. This compound inhibits the iron-mediated production of hydroxyl radicals (Hallick et al., 1977; Starke and Farber, 1985; Kvietys et al., 1989), a prime cause of oxidative stress-induced DNA damage. The control, without the inhibitor, represented the degradation with 2.5 μm MV and 8-h incubation following the light treatment.

As shown in Figure 1C, o-phemanthroline significantly inhibited degradation compared with the non-treated control. This indicated that ROS was actively participating. However, a significant portion of the ctDNA remained undegraded. This incomplete inhibition was consistently observed (data not shown), even when the concentration of inhibitor increased 10- or 100-fold, far higher concentrations than those normally used in other systems (Arnoma et al., 1989). Similarly, the endonuclease-specific inhibitor ATC (see Schraufstatter et al., 1988), was used for determining whether DNA endonuclease also participated in the degradation. As with o-phenanthroline, significant inhibition was observed in the ATC-treated sample, but ATC could not completely block degradation (Fig. 1C.). These results strongly suggested that the MV-caused degradation of ctDNA occurred via both ROS and endonuclease.

Identification of a Chloroplast-Localized DNA Endonuclease

To confirm the participation of DNA endonuclease in the MV-associated degradation of ctDNA, we incubated several substrate DNAs with chloroplast extracts prepared from the MV-treated leaves, using various periods of incubation following light treatment (Fig. 2). When the cpDNA prepared from unstressed leaves was incubated as substrate DNA with extracts from the 2.5-μm MV-treated leaves, activity was not significant for extracts with only 1 or 2 h of incubation, but it was sharply enhanced for extracts with 4 h of incubation. This was indicated by the significant increase in low-molecular-weight DNAs (Fig. 2A). Activity was again less for extracts that were incubated longer than 4 h. This transient increase at the 4-h mark was consistently observed in numerous independent experiments under identical stress conditions (data not shown).

To further demonstrate that degradation was mediated by endonuclease, a supercoiled circular plasmid (pBluscript DNA) was incubated with our 4- or 8-h-incubated chloroplast extracts. The activity of endonuclease was confirmed by observing the conversion of the supercoiled DNA to linear DNA (Fig. 2B). Single-stranded M13mp9 DNA was also degraded (data not shown), which demonstrated the ability of endonulease to degrade both double- and single-stranded DNA.

To substantiate the involvement of DNA endonuclease in response to MV, the endonuclease inhibitors, ATC and $ZnSO_4$, were added to the reaction mixture that contained substrate ctDNA from unstressed leaves as well as chloroplast extracts from

Figure 3. Dependency of endonuclease activity on aurintricarboxylic acid (ATC). Chloroplast extracts prepared from leaves treated with 2.5 *u*M MV with 4 h of incubation after light treatment were then incubated with substrate ctDNA in the presence of ATC (100 *u*M). Lane 1, 100 *u*M ATC; Lane 2, 100 *u*M fuchsin acid, a non-functional structural analogue of ATC; Lane 3, 100 *u*M $ZnSO_4$; Lane 4, control, no inhibitor; Lane 5, heated chloroplast extracts (90°C, 3 min).

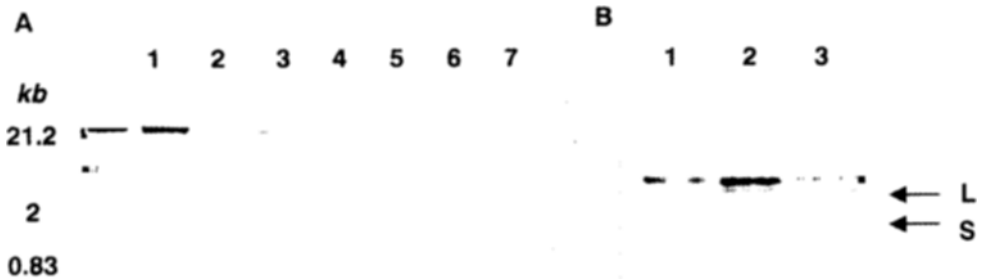

Figure 2. Endonuclease activity in MV-treated leaves. Chloroplast extracts (10 *u*g), prepared from leaves MV-treated for the designated time periods after light treatment, were incubated with substrate DNA, ctDNA (15 μg) isolated from MV-untreated leaves (A) or pBluscript SK plasmid (15 *u*g) (B). Supercoiled and linear DNAs are indicated by arrows, as described in "Material and Methods". Lane 1, substrate ctDNA incubated for 1 h without chloroplast extracts; Lanes 2-7, incubated with chloroplast extracts prepared from leaves incubated for 1, 2, 4, 6, 8, or 10 h following light treatment, respectively. (B), as described in "Materials and Methods." (A) Lane 1, substrate ctDNA incubated for 1h without chloroplast extracts; Lanes 2-7, incubated with chloroplast extracts prepared from leaves incubated for 1, 2, 4, 6, 8, or 10 h following the light treatment, respectively. (B) Lane 1, substrate pBluscript DNA was incubated for 1 h without chloroplast extracts; Lane 2, with chloroplast extracts from 4-h incubated leaves; Lane 3, from 8-h incubated leaves. Supercoiled (S) and linear (L) DNAs are indicated by arrows.

leaves with 4 h of incubation. ATC and $ZnSO_4$ almost completely inhibited the activity of endonuclease (Fig. 3), whereas fuchsin acid, a non-functional structural analogue of ATC, could not prevent degradation. Likewise, the reaction mixture containing boiled chloroplast extracts did not cause degradation either. These results clearly indicated that the ATC- and Zn^{++}-sensitive endonucleases were involved in the degradation.

Our study demonstrated that a chloroplast-localized endonuclease was induced and that it participated in the degradation of ctDNA in response to MV treatment. This endonuclease was able to cleave the supercoiled circular plasmid and single-stranded DNA, which suggested that it initially generated a single-stranded nick and, in turn, a double-stranded break to produce linear DNA. These characteristics can be directly applicable to the chloroplast genome, a 155-kb, supercoiled circular DNA in tobacco.

We speculate that this chloroplast-localized endonuclease play's an active role in the early stage of a plants adaptive response to MV. The primary function of this endonuclease might be to excise a particular DNA region that contains a harmful oxidative modification. Numerous reporters have inferred that nuclease activity, which could be involved in excision repair of the chloroplast genome, was induced in response to various DNA-damaging agents (Cerutti et al., 1993), UV-irradiation (Murphy et al., 1993; Yajima et al., 1995), or oxidative damage (Cerutti et al., 1993; Eide et al., 1996; Ramotar and Demple, 1996) in plants. However, direct evidence is still lacking for involvement of an endonuclease in the repair during oxidative stresses.

A nuclear-encoded plastid protein, homologous to the *Escherichia coli* Rec A protein, has been induced in response to several DNA-damaging agents in *Arabidopsis* and pea (*P. sativum*). This suggests that the protein plays a role in DNA repair. We have no evidence for that. However, the transient induction of endonuclease activity at the 4-h incubation mark following light treatment prompted us to assume that the stressed cells were actively resisting MV by repairing their damaged DNA at this early stage of oxidative damage. Once the damage exceeds the resistance, however, the cells would no longer require active endonuclease activity. This possibility was supported by the finding that endonuclease activity again decreased after the 4-h peak (Fig. 2A).

The endonuclease may also play a role in accelerating the degradation of cpDNA in order to effectively destroy it. That would, therefore, prevent possible dissemination of the chloroplast genome to the other organelles. We might speculate that the endonuclease is involved in programmed organellar death, if there is one specific to chloroplasts. Because no histone and nucleosome-equivalent genome structure exists, the fragmentation of DNA, which is frequently observed in apoptotic cell death, would not be expected, even if endonuclease participated in DNA cleavage during organelle death.

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