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Identification and Characterization of Cannabinoids That Induce Cell Death through Mitochondrial Permeability Transition in *Cannabis* Leaf Cells*[S]

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Cannabinoids are secondary metabolites stored in capitate-sessile glands on leaves of *Cannabis sativa*. We discovered that cell death is induced in the leaf tissues exposed to cannabinoid resin secreted from the glands, and identified cannabichromenic acid (CBCA) and Δ^1-tetrahydrocannabinolic acid (THCA) as unique cell death mediators from the resin. These cannabinoids effectively induced cell death in the leaf cells or suspension-cultured cells of *C. sativa*, whereas pretreatment with the mitochondrial permeability transition (MPT) inhibitor cyclosporin A suppressed this cell death response. Examinations using isolated mitochondria demonstrated that CBCA and THCA mediate opening of MPT pores without requiring Ca^{2+} and other cytosolic factors, resulting in high amplitude mitochondrial swelling, release of mitochondrial proteins (cytochrome *c* and nuclease), and irreversible loss of mitochondrial membrane potential. Therefore, CBCA and THCA are considered to cause serious damage to mitochondria through MPT. The mitochondrial damage was also confirmed by a marked decrease of ATP level in cannabinoid-treated suspension cells. These features are in good accord with those of necrotic cell death, whereas DNA degradation was also observed in cannabinoid-mediated cell death. However, the DNA degradation was catalyzed by nuclease(s) released from mitochondria during MPT, indicating that this reaction was not induced via a caspase-dependent apoptotic pathway. Furthermore, the inhibition of the DNA degradation only slightly blocked the cell death induced by cannabinoids. Based on these results, we conclude that CBCA and THCA have the ability to induce necrotic cell death via mitochondrial dysfunction in the leaf cells of *C. sativa*.

Multicellular organisms such as plants and animals possess cell death-inducing systems to eliminate damaged, superfluous, and ectopic cells. In higher plants, these systems participate in a variety of physiologically important events (*e.g.* root cap elimination, somatic embryogenesis, xylogenesis, leaf senescence, and defense against microbial pathogens) (1, 2), and therefore plant cell death has attracted a great deal of attention. To reveal the regulatory mechanism of cell death induction, biochemical and molecular examinations have been extensively carried out to date and have demonstrated that, in many cases, plant cell death can be classified into apoptosis or necrosis and that mitochondria control both types of cell death in plants as well as animals (3–5). For example, plant apoptosis, like animal apoptosis, is caused by release of apoptotic proteins (cytochrome *c* and others) from mitochondria, followed by activation of cell-executing enzymes (caspases and nucleases) (3, 4). On the other hand, a decrease of the ATP level due to serious mitochondrial dysfunction has been shown to induce necrosis in plants and animals (5, 6).

Apoptotic-protein release and mitochondrial dysfunction are often reported to be controlled by mitochondrial membrane permeability, and several systems that regulate this permeability have been discovered in both plants and animals (3–9). Among these, the mitochondrial permeability transition (MPT)[3] is regarded as one of the most important events in some forms of apoptosis and necrosis. Its mechanism has been well investigated particularly in animal cells, demonstrating that the MPT, which is triggered by opening of Ca^{2+}-dependent channels called MPT pores (10, 11), induces loss of mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial swelling, and disruption of the mitochondrial outer membrane, resulting in release of apoptotic proteins and mitochondrial dysfunction (7–9). Although the MPT pores have been believed to consist of voltage-dependent anion channels, adenine nucleotide translocator, and cyclophilin D, recent studies using knockout mice have demonstrated that the adenine nucleotide translocator is not essential for opening of MPT pores (12) and that cyclophilin D is required for inducing Ca^{2+}- and oxidative stress-mediated cell death (13, 14).

Likewise, opening of MPT pores in plants is also considered to be regulated by Ca^{2+}. Indeed, wheat mitochondria (15) and potato tuber mitochondria (16) have been shown to undergo high amplitude swelling through MPT in response to treatment with high concentrations of Ca^{2+}, leading to release of cyto-

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[3] The abbreviations used are: MPT, mitochondrial permeability transition; CBCA, cannabinchromenic acid; CsA, cyclosporine A; 4′,6-diamino-2-phenylindole, 4′,6-diamino-2-phenylindole; FDA, fluorescein diacetate; HPLC, high performance liquid chromatography; $\Delta\Psi m$, mitochondrial membrane potential; PBS, phosphate-buffered saline; THCA, Δ^1-tetrahydrocannabinolic acid; TMRM, tetramethylrhodamine methylester; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end-labeling; Z, benzyloxycarbonyl; FMK, fluoromethyl ketone.

chrome *c*. However, it has remained unknown whether Ca^{2+}-mediated MPT, which causes necrosis in animal cells (13, 14), induces apoptosis or necrosis in plant cells, because the reported MPT was induced in isolated mitochondria and was not examined in wheat cells or potato tuber cells. Furthermore, it has been reported that Ca^{2+} induces little swelling in mitochondria isolated from oats (17) and *Arabidopsis thaliana* (18), suggesting that, in some plants, opening of MPT pores is regulated by molecules other than Ca^{2+}. Thus, the regulatory mechanism of MPT and the characteristics of MPT-mediated cell death in plants are not fully understood. In the present study, we discovered that in *Cannabis sativa* (hemp), two cannabinoids have the ability to act as endogenous cell death regulators by directly mediating the opening of MPT pores, and clarified novel features of this cannabinoid-mediated cell death.

Cannabinoids, which contain alkylresorcinol and monoterpene groups, are unique secondary metabolites found only in *C. sativa* (19). The main cannabinoid of this plant, Δ^1-tetrahydrocannabinolic acid (THCA) is known to be readily converted by non-enzymatic decarboxylation into Δ^1-tetrahydrocannabinol, which has psychoactive and analgesic effects (20). Owing to their interesting chemical and pharmacological properties, numerous studies have been carried out on these cannabinoids, although why this plant produces such unique metabolites remains unknown. Previously, we reported that THCA is biosynthesized from cannabigerolic acid (21, 22) and stored in glandular tissues on *Cannabis* leaves (23). In further studies on cannabinoids, we have discovered that cannabinoid resin secreted from the glands accumulates in regions where cell death occurs and that the major components of this resin, cannabinchromenic acid (CBCA) and THCA, have potent cell death-inducing activity for leaf cells or suspension cells of *C. sativa*. Biological characterization revealed that both cannabinoids cause necrotic cell death through direct induction of MPT independently of Ca^{2+} and H_2O_2. Here we describe the identification and characterization of these novel cell death regulators.

EXPERIMENTAL PROCEDURES

Plant Materials and Reagents—C. sativa (Mexican strain) was cultivated in the herb garden of the Graduate School of Pharmaceutical Sciences, Kyushu University. *Cannabis* cells were cultured in Gamborg B5 medium supplemented with 2.5 μM 2,4-dichlorophenoxyacetic acid at 25 °C (24). *Cannabis* cells used here were confirmed to produce quite low levels of CBCA and THCA (lower than 0.1 μg/g fresh cells in each cannabinoid). Tobacco cells (BY-2) were obtained from RIKEN Bioresource Center (Tsukuba, Japan), and cultured in modified Linsmaier-Skoog medium containing 0.2 mg/liter 2,4-dichlorophenoxyacetic acid at 25 °C (25). Chemical and biochemical reagents were purchased from Sigma-Aldrich, Wako Pure Chemicals (Osaka, Japan), Merck, Promega, Roche Diagnostics, Invitrogen, Yakult (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan). Highly purified cannabinoids were obtained from leaves of *C. sativa* as follows. The dried leaves (200 g) were extracted three times with *n*-hexane (3000 ml), and the combined *n*-hexane extracts were evaporated. The oily residues were resuspended in acetone (200 ml), and the insoluble materials were removed by filtration. Cosmosil 75C18-OPN (Nacalai Tesque, 20 g) was added to the filtrate, and acetone was completely removed by evaporation. The residue was directly loaded onto a column containing Cosmosil 75C18-OPN (2.5 × 25 cm) previously treated with 65% aqueous methanol. After the column was washed with the same solvent (500 ml), CBCA and THCA were simultaneously eluted with 85% methanol (600 ml). The fractions containing both cannabinoids were concentrated to dryness by evaporation and then subjected to silica gel chromatography (3 × 40 cm). After THCA was eluted with *n*-hexane-ethyl acetate (5:1,500 ml), CBCA was eluted with *n*-hexane-ethyl acetate (2:1,600 ml) (26). Solvent was removed by evaporation to afford pure THCA (2.5 g) and CBCA (123 mg).

Fluorescent Microscopy—Fluorescent microscopy was carried out with a Nikon TE2000 fluorescent microscope (Nikon, Tokyo, Japan). Autofluorescence of chlorophyll and cannabinoid-containing glands was imaged using 365-nm excitation light and detected through a 400-nm emission filter. 4′,6-Diamino-2-phenylindole fluorescence was imaged using the same filter setting as used for analyses of the above autofluorescence. The fluorescence of cells stained with fluorescein diacetate (FDA, Sigma-Aldrich) and a terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) method, excited at 450–490 nm light, was collected through a 520 nm filter.

Analyses of Cell Death Induced by Artificial Leakage of Fluorescent Resin—Artificial leakage of fluorescent resin from the glands was induced by exfoliation of cuticles as follows. The leaf veins in the lower surface of young or mature *Cannabis* were pulled up using forceps to exfoliate the cuticle. The leaf samples (1–1.5 mm^2) of which cuticle were removed were floated on Gamborg B-5 medium (1 ml) and incubated at 25 °C. The chlorophyll in the samples was examined by fluorescence microscopy 5 min, 24 h, and 48 h after incubation. Cell death in the samples was evaluated using the cell viability indicator FDA. The leaf samples were incubated with Gamborg B5 medium (1 ml) containing 25 μM FDA at room temperature for 10 min (27), and the FDA intensity in the stained samples was then monitored using a fluorescence microscope.

Analyses of Cannabinoids in Cannabis Leaves and Glands—For analysis of gland contents, glands isolated by the following method were used. Mature *Cannabis* leaves (~1 g) were floated on sterile water (20 ml) in Petri dishes so that the lower surfaces of the leaves were exposed to the surface of the water, and sonicated at room temperature for 20 min. The glands separated from the leaves were precipitated by standing the Petri dish at room temperature 30 min. The solvent was carefully removed using a pipette, and the precipitated glands were collected using a CryoLoop™ (Hampton Research). About 100 glands were sonicated with 50 μl of methanol for 10 min and centrifuged at 5000 × *g* for 5 min. A 25-μl aliquot of the supernatant was analyzed by HPLC as described below. For analyses of cannabinoids in leaves, senescent leaves (1.0 g) were homogenized with *n*-hexane (10 ml) and sonicated for 20 min, and insoluble materials were removed by filtration. The filtrate was concentrated to dryness by evaporation, and the residues were dissolved in methanol. The methanol extracts were analyzed by

HPLC as described below or TLC (Silica Gel 60 F_{254}, Merck) using *n*-hexane-ethyl acetate (2:1) as a solvent.

HPLC Conditions—An HPLC system (TOSOH, Japan) composed of a CCPM pump and a UV-8020 absorbance detector equipped with a 0.46- × 15-cm column of Cosmosil 5C18 AR-II (Nacalai Tesque) was used for analyses of CBCA and THCA. Both cannabinoids were eluted with 85% (v/v) acetonitrile containing 50 mM phosphoric acid at a flow rate of 1 ml/min. The eluate was monitored by absorption at 254 nm, and the peak intensities of these cannabinoids were determined with a Chromatocorder 21 (TOSOH). The retention time and concentration of CBCA and THCA were compared with those of authentic samples (21, 26).

Assay for Cell Death Activities of Cannabinoids—Young leaves where cuticles were removed from the lower surface were used to measure the cell death activity of cannabinoids. After the leaf samples (1-1.5 mm^2) were incubated in Gamborg B5 medium (1 ml) containing various concentrations (0, 50, and 100 μM) of CBCA or THCA at 25 °C for 24 h, chlorophyll in each sample was observed by fluorescent microscopy. The same samples were then stained with 25 μM FDA as described above, and cell viability in the sample was evaluated by monitoring the FDA intensity using a fluorescent microscope. For analyses of DNA in the cannabinoid-treated leaves, DNA was extracted from the above leaf samples (10 mg) by the cetyltrimethylammonium bromide method and electrophoresed on a 1.0% agarose gel. The DNA was visualized with 0.5 μg/ml ethidium bromide.

The cell death activities of cannabinoids for suspension cells were measured as follows. *Cannabis* cells or tobacco cells (each 0.1 g) in logarithmic growth phase were incubated with Gamborg B5 medium or LS medium (each 10 ml), respectively, in the presence of CBCA or THCA (each 100 μM) at 25 °C for 3 h. The cells were collected by centrifugation at 1000 × *g* and then stained with FDA as described above. The FDA fluorescence of the cells was visualized by fluorescence microscopy or measured by an MTP-600FE fluorescent microplate reader (Corona, Tokyo, excitation 530 nm, emission 570 nm). DNA degradation in the cells was investigated by the TUNEL assay as described below. For the evaluation of effects of cell death inhibitors, cells (0.1 g) were previously incubated with culture medium (10 ml) containing 25 mM ascorbic acid (Wako Pure Chemicals), 50 μM Z-VAD-FMK (Sigma-Aldrich), 25 μM cyclosporine A (Wako Pure Chemicals), or 25 μM aurintricarboxylic acid (Wako Pure Chemicals) at 25 °C for 1 h, and processed for the cell death assay as described above.

Analyses of the Effects of Cyclosporin A on Cell Death Induced in Cannabis Leaves—Young leaves where cuticles were removed from the lower surface was incubated in Gamborg B5 medium (1 ml) containing 25 μM cyclosporin A at 25 °C for 1 h, and CBCA or THCA (each 100 μM) was then added to the samples. After 24-h incubation, chlorophyll and cell viability in each sample was analyzed as described above.

Concerning the effects of cyclosporin A on senescence naturally occurring in *Cannabis* leaves, mature leaves just before senescence were used. The leaves were cut through their central veins, and each separated piece was incubated in sterile water (10 ml) containing 25 μM cyclosporin A with a 16-h photoperiod at 25 °C for 7 days.

Cannabinoid Synthase Assay—Fresh *Cannabis* leaves (60–70 mg) at various stages were homogenized with 2 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM mercaptoethanol at 4 °C, and the homogenates were centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was used as enzyme solution. The enzyme assay was carried out by a modification of the method described in our previous study (28). The standard mixture solution consisted of 200 μM cannabigerolic acid, 0.1% (w/v) Triton X-100, 100 mM phosphate buffer (pH6.5) in a total volume of 500 μl. The reaction was started by adding of 100 μl of the enzyme solution, and the mixture was incubated at 30 °C for 2 h. After terminating the reaction with 600 μl of methanol, CBCA and THCA in the reaction mixture were analyzed by HPLC as described above.

TUNEL Assay—Suspension cells (0.1 g) treated under various conditions were incubated with 2% (w/v) paraformaldehyde in 10 mM phosphate-buffered saline (PBS, 1.5 ml) for 30 min at room temperature. The fixed cells were washed with PBS (1.5 ml), incubated in permeabilization buffer (1 ml) consisting of 0.1% (w/v) Triton X-100 and 0.1%(w/v) sodium citrate for 2 min in 4 °C, and then washed with PBS (1.5 ml) again. The cells were resuspended in PBS (200 μl), and a 60-μl aliquot was centrifuged at 1000 × *g* for 1 min. After the supernatant was removed, the cells were incubated in reaction mixture (30 μl, *in situ* Cell Death Detection Kit, fluorescein, Roche Diagnostics) at 37 °C for 1 h. The samples were washed with PBS (100 μl), resuspended in PBS (40 μl) containing 0.5 μg/ml 4′,6-diamino-2-phenylindole (Wako Pure Chemicals), and analyzed by fluorescence microscopy as described above. The rate of TUNEL-positive nuclei was calculated by comparing their numbers with those of 4′,6-diamino-2-phenylindole-positive nuclei.

Isolation of Mitochondria—All procedures were carried out at 4 °C. Suspension cells (30 g) were homogenized in a mortar with isolation buffer (pH 7.5, 60 ml) composed of 20 mM HEPES-Tris, 0.3 M sucrose, 5 mM EDTA, 1 mM dithiothreitol, and 0.3% (w/v) bovine serum albumin (5). The homogenates were filtered through 40-μm nylon mesh, and the filtrate was centrifuged at 2500 × *g* for 5 min. The supernatant was filtered through 5-μm nylon mesh, and the filtrate was centrifuged at 28,000 × *g* for 10 min. Further purification of mitochondria from the pellet was carried out using a modified method of Curtis and Wolpert (17). Briefly, the pellet was resuspended in the isolation buffer (500 μl) and layered onto Percoll gradients (20 ml). The gradients were centrifuged at 41,000 × *g* for 30 min. Mitochondria were collected from the bottom layer and washed twice with the isolation buffer (10 ml) by centrifugation at 28,000 × *g* for 10 min. The pellet was used as isolated mitochondria for further examinations.

Analysis of Mitochondrial Swelling—Mitochondria (0.2 mg of mitochondrial protein) isolated from *Cannabis* cells or tobacco cells were resuspended in swelling buffer (pH 7.5, 1 ml) consisting of 10 mM Tris-HCl, 1 mM sodium phosphate, 10 μM EGTA, 25 mM KCl. As soon as CBCA, THCA, Ca^{2+}, or H_2O_2 (final concentrations, 200 μM, 200 μM, 1 mM, or 1 mM, respectively) was added to the mitochondrial solutions, the optical density at 540 nm of the solutions was measured every 4 s using

a U-2001 spectrophotometer (Hitachi, Tokyo, Japan) at room temperature. For analyses using MPT inhibitor or Ca^{2+}-ionophore, mitochondria were incubated with 25 μM CsA Wako Pure Chemicals) for 5 min and then processed for swelling analyses as described above.

Assay for $\Delta\Psi m$—Cannabis cells and tobacco cells (each 0.1 g) were stained by incubation with 2.5 μM tetramethylrhodamine methylester (TMRM) perchlorate (Invitrogen) in Linsmaier-Skoog medium and Gamborg B5 medium (each 10 ml), respectively, at room temperature for 10 min. TMRM staining of isolated mitochondria (0.2 mg of mitochondrial protein) was performed under the same conditions except for the use of the swelling buffer (see "Analysis of Mitochondrial Swelling") instead of culture media. CBCA or THCA (each final concentration, 200 μM) was added to these TMRM-stained samples, and the reaction mixtures were incubated at 25 °C for 10 min. The $\Delta\Psi m$ values in mitochondria were quantitatively evaluated by measuring TMRM fluorescence using a fluorescence microplate reader MTP-600FE (excitation 530 nm, emission 570 nm).

Analyses of Mitochondrial Proteins—Isolated mitochondria (1.0 mg of mitochondrial protein) were incubated with CBCA or THCA (each 200 μM) in 500 μl of the swelling buffer at 25 °C for 40 min. The samples were centrifuged at 21,000 × *g* for 10 min to fractionate supernatants and pellets, and the supernatants were used to detect nuclease and cytochrome *c*, which were released from mitochondria. The nuclease assay was carried out by a modification of the method of Balk *et al.* (29). Aliquots (each 2 μl) of the supernatant were added to substrate buffer (pH 8.0 and 18 μl) consisting of EcoRI-treated pBR322 (0.5 μg) 10 mM HEPES-NaOH, 2 mM NaCl, 2.5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EGTA, 220 mM mannitol, and 68 mM sucrose, and the reaction mixtures were incubated at 30 °C for 2 h. The DNA in each sample was analyzed by agarose gel electrophoresis (1.5% agarose gel). The detection of cytochrome *c* in the same supernatant was carried out by immunoblotting as follows. Aliquots (100 μl) of the supernatants were lyophilized to dryness, and the residues were dissolved in distilled water (10 μl). The samples were subjected to SDS-PAGE (12.5% acrylamide gel) and transferred to a polyvinylidene difluoride membrane, which was probed with the monoclonal anti-cytochrome *c* antibodies (1:500 dilution, Chemicon International). The membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibodies (1:1000 dilution, Wako Pure Chemicals) and the cytochrome *c* was then visualized using 1-chloro-4-naphthol (Sigma-Aldrich). For analyses of proteins (nuclease and cytochrome *c*) retained in mitochondria, the above pellets were used. The pellets were homogenized and sonicated with 500 μl of the mitochondrial isolation buffer (see "Isolation of Mitochondria") containing 0.1% Triton X-100 at 4 °C. After the samples were centrifuged at 21,000 × *g*, the supernatants were used to analyze cytochrome *c* and nuclease as described above.

Measurement of Cellular ATP—The ATP level in *Cannabis* cells was determined as described by Casolo *et al.* (5). *Cannabis* cells (0.1 g) were incubated with 100 μM CBCA or THCA in Gamborg B5 medium (10 ml) at 25 °C for 2 h, collected by centrifugation at 1000 × *g* for 5 min, and heated in 50 mM Tris-HCl (pH 7.5, 2.5 ml) containing 0.05% (w/v) Triton X-100 and 5 mM EDTA at 95 °C for 2 min. After centrifugation at 1000 × *g* for 5 min, the supernatants were used for the determination of total cellular ATP. Aliquots (200 μl) of the supernatants were treated with an ATP bioluminescence detection kit (ENLITEN® rLuciferase/Luciferic Reagent, Promega) according to the manufacturer's instructions, and the resulting fluorescence was assayed in a luminometer (Luminometer Model 9501, Berthold).

Isolation of Nuclei from Cannabis Cells—*Cannabis* nuclei were isolated from protoplasts of *Cannabis* cells. *Cannabis* cells (10 g) were incubated in digestion solution (100 ml) consisting of 0.4 M mannitol, 88 mM sucrose, 1% (w/v) Cellulase Onozuka R-10 (Yakult, Tokyo), 0.2% (w/v) Macerozyme R-10 (Yakult, Tokyo), 0.1% (w/v) pectolyase Y-23 (Kikkoman, Tokyo) at 30 °C for 4 h with gentle agitation. The protoplasts were collected by filtration through 40-μm nylon mesh and washed with 0.5 M mannitol (50 ml). The protoplasts were resuspended in buffer (pH 7.5 and 100 ml) consisting of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.02% Triton X-100, 10 mM β-mercaptoethanol, incubated at 4 °C for 10 min with gentle agitation, and filtered through 40- and then 15-μm nylon mesh. All further procedures for isolation of nuclei were performed as described by Balk *et al.* (29).

DNA Degradation in Nuclei by Mitochondrial Nuclease—The mitochondria isolated from *Cannabis* cells (1.0 g) were incubated with the swelling buffer (1 ml) in the presence of CBCA or THCA (each 200 μM) at 25 °C for 40 min to release mitochondrial nuclease. The reaction mixture was centrifuged at 21,000 × *g* for 15 min, and $MgCl_2$ (final concentration, 2 mM) was added to the supernatant. The nuclei prepared from *Cannabis* cells (each 1 g) as described above were added to the supernatants, and incubated at 30 °C for 2 h. After the reaction mixtures were centrifuged at 21,000 × *g* for 15 min, DNA was extracted by the cetyltrimethylammonium bromide method and analyzed by agarose gel electrophoresis (1.0% agarose gel). For experiments on inhibition of DNA degradation, the supernatant supplemented with aurintricarboxylic acid (final concentration, 25 μM) and $MgCl_2$ (final concentration, 2 mM) were used as enzyme solution.

RESULTS

Direct Visualization of Fluorescent Resin Causing Cell Death in Cannabis Leaves—In the present study, we discovered that fluorescence microscopy can directly visualize resin, which is assumed to induce cell death in *Cannabis* leaves. As shown in Fig. 1*A*, numerous capsules (40–60 μm in diameter) exhibiting light bluish fluorescence under UV irradiation were observed on the lower surface of *Cannabis* leaves. Such fluorescence capsules on hemp leaves have not been reported, but we identified them as capitate-sessile glands, based on their morphological features (sizes and shapes) (30, 31). In intact leaves at the young (*L1* and *L2*) or mature (*L3* and *L4*) stage, the light bluish fluorescence was localized only within these glands (Fig. 1*A*), whereas in young or mature leaves having lesions, the fluorescence, which seemed to leak from the glands, accumulated in the damaged tissues (Fig. 1*B*). Furthermore, such leakage of fluorescence was observed in senescent tissues of more aged

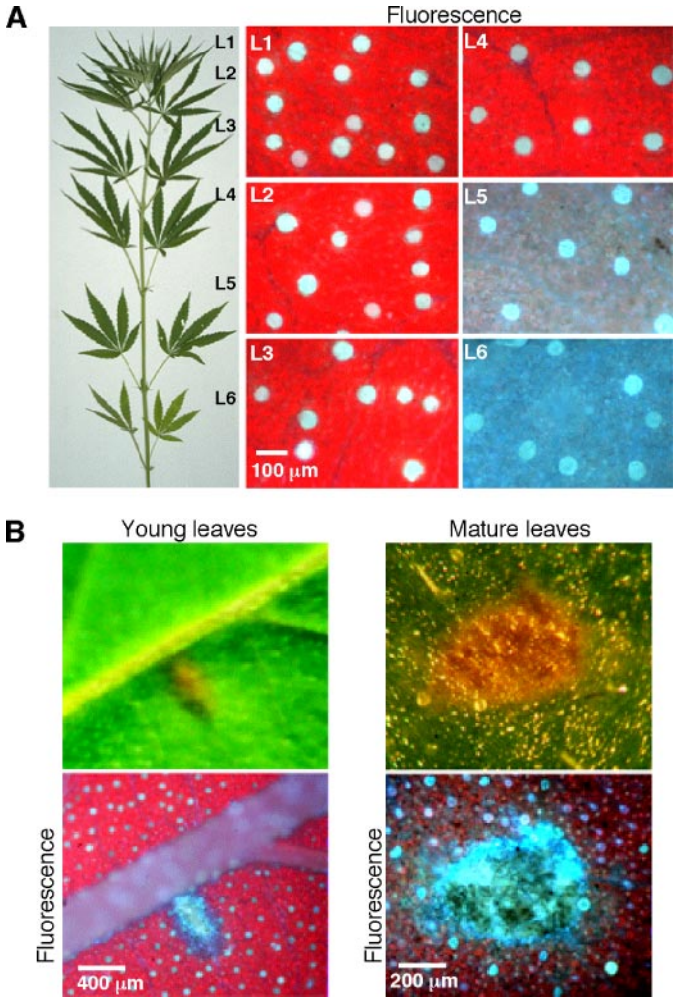

FIGURE 1. **Direct visualization of fluorescent glands on *Cannabis* leaves.** *A*, fluorescent glands on *Cannabis* leaves. Leaves at various stages were collected from 7-week-old *C. sativa* (*left panel*), and their lower surfaces were observed by fluorescence microscopy (*right panels*). *B*, distribution of fluorescent resin in *Cannabis* leaves having lesions. The same regions of the upper surface and lower surface of the damaged area were analyzed by light microscopy (*upper panel*) and by fluorescence microscopy (*lower panel*). Young leaves (*right panels*) and mature leaves (*left panels*) were collected from plants at the stages L2 and L3, respectively.

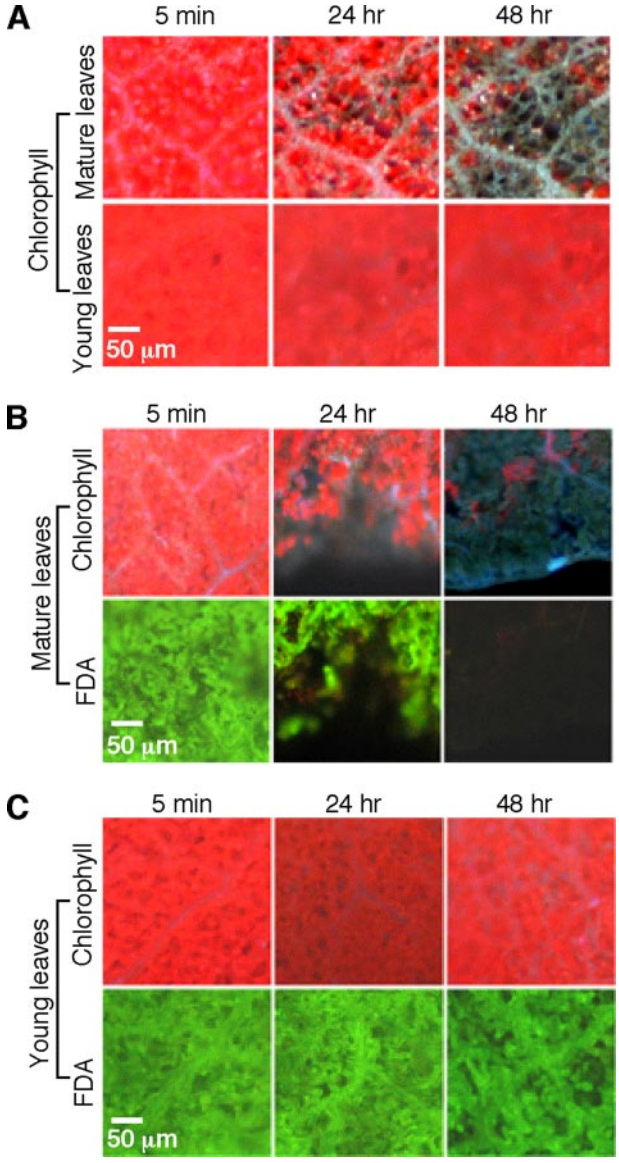

FIGURE 2. **Cell death induced by artificial leakage of fluorescent resin.** *A*, chlorophyll degradation by artificially induced leakage of fluorescent resin. Cuticles were exfoliated from the lower surfaces of mature leaves or young leaves, and the leaf samples were incubated as described under "Experimental Procedures." Fluorescent resin and chlorophyll in the samples were examined by fluorescent microscopy after incubation for the times indicated in the figure after incubation. *B* and *C*, change of cell viability and chlorophyll by artificially induced leakage of fluorescent resin. Mature leaves (*B*) or young leaves (*C*) were incubated for 5 min, 24 h, or 48 h after removal of their cuticles. Chlorophyll in each sample was investigated by fluorescent microscopy. The same sample was then stained with FDA, and its fluorescence was visualized by fluorescent microscopy.

leaves (*L5* and *L6* in Fig. 1*A*). Because nuclear DNA in the above damaged tissues and senescent leaves underwent extensive degradation (supplemental Fig. S1*A*), cell death was considered to occur in the tissues exposed to the fluorescent resin.

We next investigated whether artificially induced leakage of the substance with light bluish fluorescence causes these cell death responses. Although resin composed of various secondary metabolites is known to be stored in glandular trichomes of plants (32), methods to induce it to leak into host leaf tissues have not been established. We found that exfoliation of the cuticle from the lower surface of the mature leaves removed the glands but also quickly induced leakage of fluorescent resin into the leaf tissues (Fig. 2, *A* and *B*). The disappearance of red fluorescence, which reflected chlorophyll degradation, was initiated within 24 h after inducing fluorescence leakage and thereafter expanded to a wide region (Fig. 2*A*). When the viability of these leaf tissues was investigated using FDA (27), green fluorescence characteristic of living cells extensively decreased with chlorophyll degradation (Fig. 2*B*), confirming that cell death was induced in the chlorophyll-lacking tissues. On the other hand, similar treatment of young leaves, which leaked little fluorescence, caused neither chlorophyll degradation (Fig. 2, *A* and *C*) nor cell death within 48 h (Fig. 2*C*). These results suggest that secretion of the fluorescent resin from the glands into leaf tissues leads to cell death in *Cannabis* leaves.

Identification and Characterization of Cell Death Regulators in Glandular Trichomes—Because the above results indicated the presence of cell death-mediating factors in the fluorescent resin, we attempted their identification by chemical and phar-

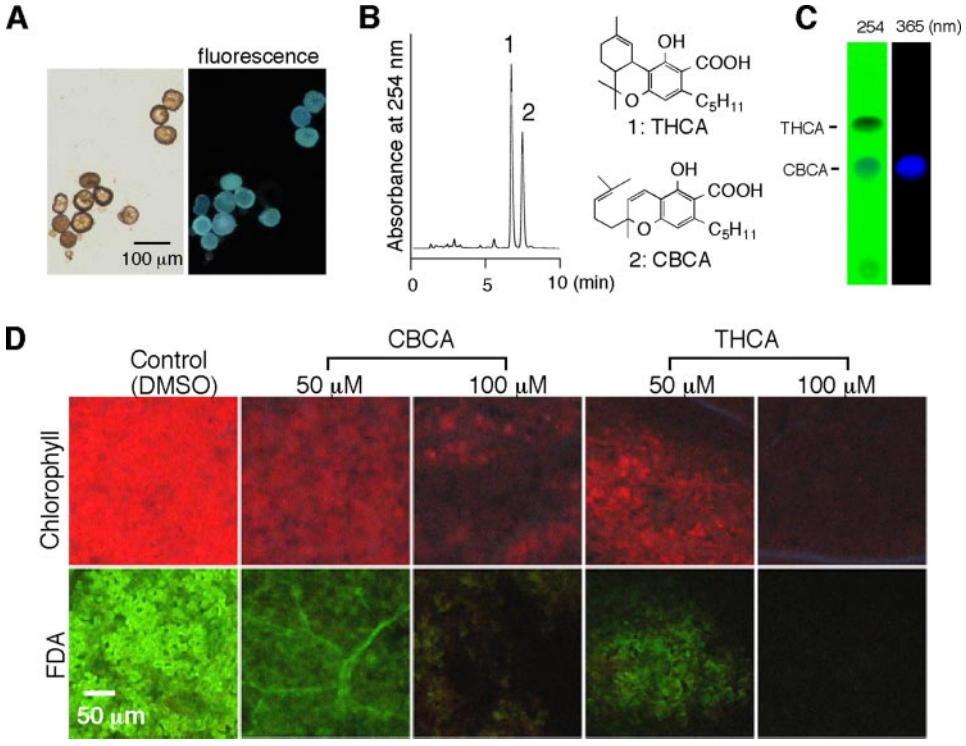

FIGURE 3. **Identification of cell death regulators in the glands.** *A*, glands isolated from *Cannabis* leaves. The glands were isolated by sonication of mature *Cannabis* leaves and then visualized by fluorescent microscopy. *B*, HPLC of gland extracts. Methanol extracts of the isolated glands were analyzed by reverse-phase HPLC. *C*, TLC of extracts from senescent *Cannabis* leaves. Extracts prepared by *n*-hexane treatment of senescent leaves were loaded onto silica gel TLC. After TLC was developed using *n*-hexane-ethyl acetate (2:1), the spots were visualized under UV irradiation. *D*, cell death activity of cannabinoids for *Cannabis* leaves. After young leaves from which the cuticles had been removed from the lower surface were incubated with various concentrations of cannabinoids for 24 h, chlorophyll in the samples was analyzed by fluorescent microscopy. Thereafter, the same samples were stained with FDA and cell viability was analyzed by fluorescent microscopy.

macological analyses. As a first step, the glands were isolated using our newly developed method (Fig. 3*A*) and then analyzed by HPLC. As shown in Fig. 3*B*, two major compounds were eluted, and were identified as THCA and CBCA by comparison of their retention times with those of authentic samples (21, 26). Because only CBCA potently exhibited light bluish fluorescence upon TLC of leaf extracts (Fig. 3*C*), we concluded that the fluorescence leakage found in lesions (Fig. 1*B*) and senescent leaves (*L5* and *L6* in Fig. 1*A*) represented CBCA secretion. In contrast, owing to its low fluorescence, it was impossible to confirm THCA leakage by fluorescence microscopy. However, HPLC demonstrated that THCA (0.953 mg/g tissues) as well as CBCA (0.196 mg/g tissues) accumulated in the tissues of mature leaves (leaves corresponding to the *upper panel* shown in Fig. 2*A*) in which fluorescence leakage was artificially induced by cuticle removal, indicating that THCA is secreted with CBCA.

Thus, CBCA and THCA were regarded as candidates for cell death mediators, and therefore we examined whether they can induce cell death in *Cannabis* leaves. To accelerate the uptake of cannabinoids into leaf cells and to avoid influence arising from endogenous cannabinoids, we used the young leaves (leaves corresponding to the *lower panel* shown in Fig. 2*A*) from which cuticles and glands had been removed. Incubation with higher than 100 μM (0.035 mg/ml) CBCA or THCA effectively caused cell death responses such as chlorophyll degradation (Fig. 3*D*), disappearance of FDA fluorescence (Fig. 3*D*), and DNA degradation (supplemental Fig.S1*B*). Because *Cannabis* glands store, together with these cannabinoids, other minor secondary metabolites (Fig. 3*B*), we prepared a fraction containing only these minor metabolites by collecting effluent from an HPLC column and analyzed the cell death activity of this fraction. However, neither chlorophyll degradation nor a decrease of cell viability was found to be induced by this fraction (data not shown). Based on this evidence, we concluded that the cell death regulators present in *Cannabis* glands are CBCA and THCA. Judging from the contents of CBCA (0.39 mg/g fresh leaves) and THCA (5.5 mg/g fresh leaves) in mature leaves, *C. sativa* is considered to produce sufficient amounts of CBCA and THCA to expose all the leaf cells to higher than 0.035 mg/ml of both cannabinoids. Therefore, we concluded that secretion of both cannabinoids can induce cell death throughout *Cannabis* leaves.

We also compared the cell death activity of these cannabinoids with that of small molecules, which are known to induce cell death in plants (27, 33, 34), and found that treatment with the high concentrations of H_2O_2 (1 mM), (−)-catechin (300 μM) and camptothecin (500 μM) did not cause cell death within 48 h (data not shown). Thus, both cannabinoids caused cell death in *Cannabis* leaves much more effectively than these known cell death regulators.

Concerning the production of CBCA and THCA in *Cannabis* leaves, we previously demonstrated that these cannabinoids constitutively occur in *Cannabis* leaves at various stages (35) and are biosynthesized from cannabigerolic acid by CBCA synthase and THCA synthase (21, 26). Because little is known about the leaf stages in which their biosynthetic systems are activated, we measured the activities of these cannabinoid-synthesizing enzymes in the leaves at various stages. The young leaves showed much higher activities than other leaves, and both activities markedly decreased after leaf maturation (supplemental Fig. S2), indicating that CBCA and THCA are mostly biosynthesized in young leaves during the developmental stage.

Features of Cell Death Catalyzed by Cannabinoids—To precisely characterize the mechanism of cell death mediated by cannabinoids, suspension-cultured cells derived from *Cannabis* leaves were also used. We first examined whether both cannabinoids induce cell death in *Cannabis* cells as well as *Cannabis* leaves. *Cannabis* cells were incubated with various concentrations of CBCA or THCA and then stained with FDA. The cell death-inducing effects of these cannabinoids were

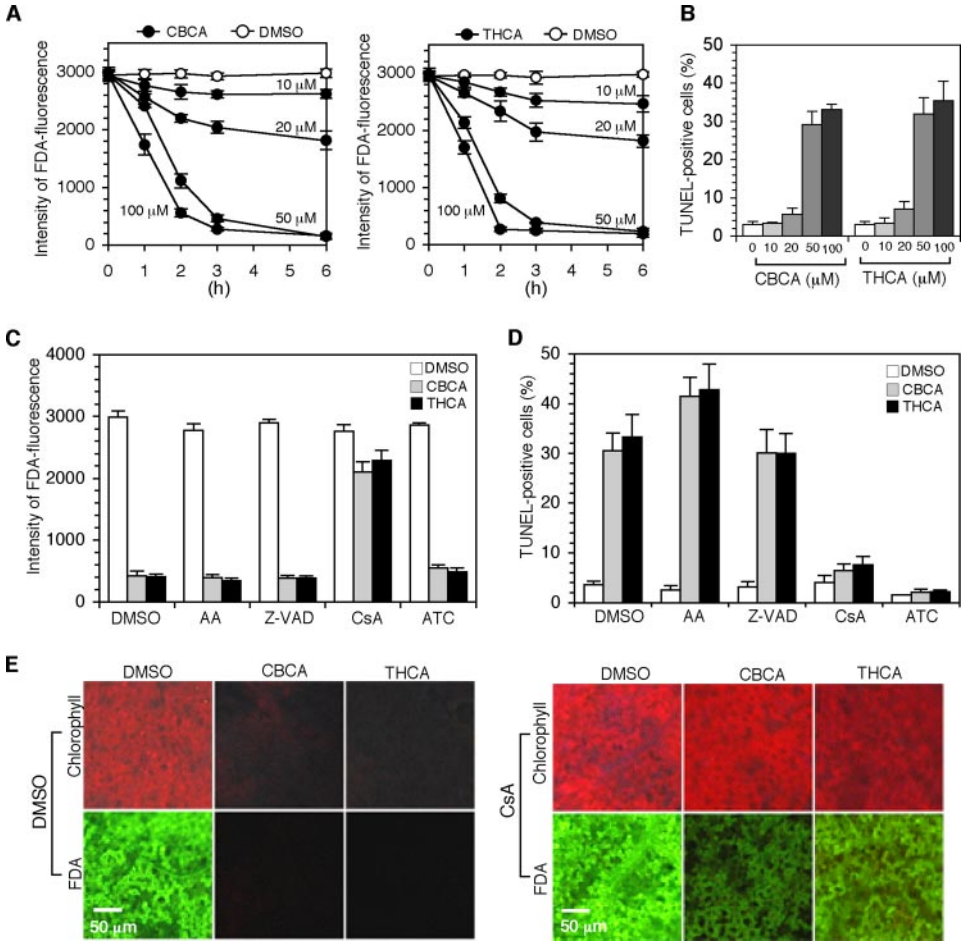

FIGURE 4. **Features of cannabinoid-mediated cell death.** *A*, FDA assay of *Cannabis* cells. *Cannabis* cells were incubated with various concentrations of CBCA or THCA. The cells were harvested after incubation for the times indicated in the figures and stained with FDA. The FDA fluorescence intensity was quantified using a fluorescent microplate reader. The data are means of five replicate assays. *B*, TUNEL assay of *Cannabis* cells. *Cannabis cells* were incubated with various concentrations of CBCA or THCA for 3 h and stained with a TUNEL assay kit. The rates of TUNEL-positive cells were calculated as described under "Experimental Procedures." The data are means of three replicate assays. *C* and *D*, effects of cell death inhibitors on cell death catalyzed by cannabinoids in *Cannabis* cells. *Cannabis* cells were incubated with 25 mM ascorbic acid (*AA*), 50 μM Z-VAD-FMK (*VAD*), 25 μM cyclosporin A (*CsA*), or 25 μM aurintricarboxylic acid (*ATC*) for 1 h. Thereafter, 50 μM CBCA or 50 μM THCA was added to the cultures. After incubation for 3 h, the cells were stained with FDA or a TUNEL assay kit. The FDA fluorescence intensity (*C*) and the rates of TUNEL-positive cells (*D*) were determined as described above. The data are means of five replicate assays. *E*, effects of CsA on cell death induced by cannabinoids in *Cannabis* leaves. Young *Cannabis* leaves from which the cuticles had been removed from the lower surface were treated with Me_2SO (*DMSO*, *left panels*) or 25 μM CsA (*right panels*) for 1 h. After the leaves were incubated with 100 μM CBCA or 100 μM THCA for 24 h, chlorophyll in each sample was analyzed by fluorescence microscopy. The same sample was stained with FDA, and its cell viability was analyzed by fluorescent microscopy.

evaluated by measuring the fluorescence intensity of the FDA-stained cells. As shown in Fig. 4*A*, treatment with higher than 50 μM CBCA or THCA caused a marked decrease of cell viability in most cells within 3 h. In contrast, lower concentrations (10 μM and 20 μM) of each cannabinoid slowly decreased the viability of *Cannabis* cells until 6 h after treatment (Fig. 4*A*), and the cell viability was only slightly reduced by a further 24-h incubation (data not shown). Moreover, we investigated DNA damage in cannabinoid-treated cells by the TUNEL assay. We found that DNA degradation was induced by treatment with higher than 50 μM CBCA or THCA (Fig. 4*B*) and that the number of TUNEL-positive nuclei reached the maximum level (>30% of total nuclei) 3 h after incubation. In contrast, 10 μM or 20 μM cannabinoids only slightly increased the population of TUNEL-positive nuclei (Fig. 4*B*). Thus, we confirmed that treatment of *Cannabis* cells with higher than 50 μM CBCA or THCA quickly caused cell death responses, which were similar to those induced by cannabinoid treatment of young *Cannabis* leaves from which the cuticles had been removed.

We next treated *Cannabis* cells with these cannabinoids (50 μM each) in the presence of cell death inhibitors and attempted to clarify the cell death mechanism by investigating the effects of these inhibitors on cell viability reduction and DNA degradation. Pretreatment with ascorbic acid or the pan-caspase inhibitor Z-VAD-FMK failed to inhibit the decrease of cell viability (Fig. 4*C*) and DNA fragmentation (Fig. 4*D*) caused by cannabinoids, suggesting that cannabinoids induce cell death in H_2O_2- and caspase-independent manners. Cyclosporin A (CsA) clearly suppressed these cell death responses in cannabinoid-treated cells. Because CsA inhibits the MPT in plants as well as animals (10, 16, 36), CBCA and THCA were considered to cause cell death through MPT.

We examined whether cell death in *Cannabis* leaves is also blocked by CsA treatment. When young *Cannabis* leaves from which the cuticles had been removed were incubated with cannabinoids in the absence or presence of CsA (Fig. 4*E*), this MPT inhibitor delayed the chlorophyll degradation and cell viability reduction (*right panel* in Fig. 4*E*). These results suggested that CBCA and THCA mediate cell death though MPT in *Cannabis* leaves.

Mitochondrial Swelling and ΔΨm Reduction by Cannabinoids—Like animal MPT, plant MPT is also considered to be triggered by opening of MPT pores, leading to mitochondrial swelling and loss of mitochondrial membrane potential (ΔΨm) (16). Therefore, we examined whether CBCA and THCA induce such responses in *Cannabis* mitochondria. As a first step, mitochondria were isolated from *Cannabis* cells, and their light scattering (absorbance at 540 nm) was then monitored to evaluate mitochondrial swelling. We found that a decrease in the absorbance, which is characteristic of mitochondrial swelling, was quickly initiated by addition of >100 μM of each cannabinoid and reached the maximum level at 5 min of treatment (Fig. 5, *A* and *B*). Up to 200 μM of each cannabinoid dose-dependently decreased the absorbance at 5 min of incubation, and the value of $\Delta A_{540\,nm}$ (~0.25 *A* at 5 min) implied that high

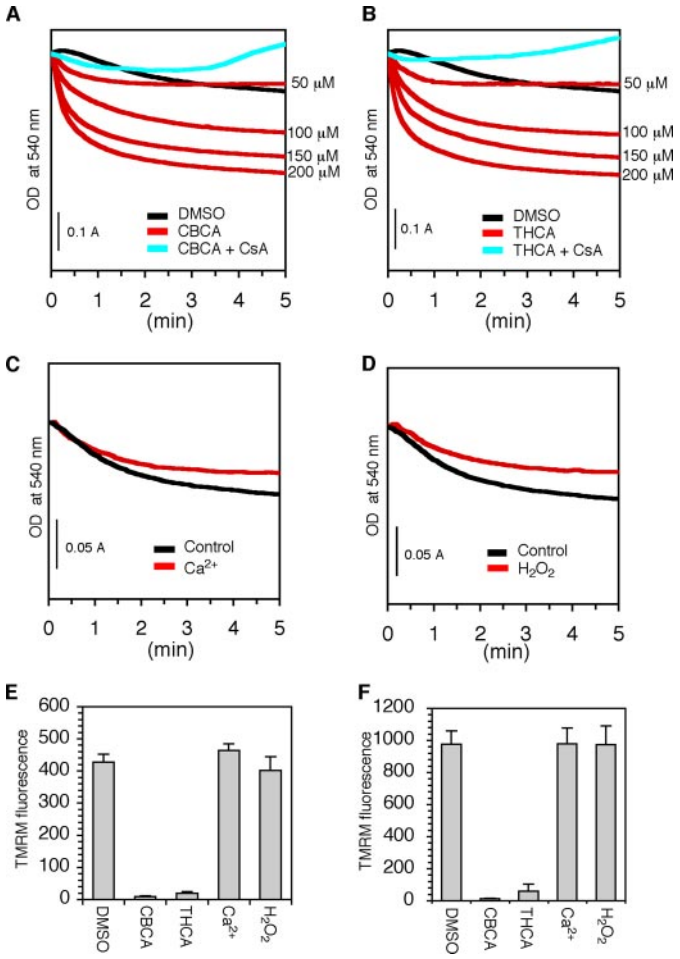

FIGURE 5. **MPT induced by cannabinoids.** *A–D*, mitochondrial swelling induced by cannabinoids. After CBCA (*A*), THCA (*B*), 1 mM Ca^{2+} (*C*), or 1 mM H_2O_2 (*D*) was added to isolated mitochondria, and their light scattering was monitored at $A_{540\ nm}$. The *numbers* on the *right* of *panels A* and *B* indicate the concentrations of cannabinoids used for the swelling assay. For experiments using MPT inhibitor (*A* and *B*), isolated mitochondria previously treated with 25 μM CsA for 5 min were incubated with 200 μM CBCA or 200 μM THCA. The data are means of five replicate assays. *E* and *F*, ΔΨm reduction by cannabinoids. The isolated mitochondria (*E*) and *Cannabis* cells (*F*) were stained with 2.5 μM TMRM, and then incubated with 200 μM CBCA, 200 μM THCA, 1 mM Ca^{2+}, or 1 mM H_2O_2 for 10 min. The intensity of TMRM fluorescence was measured using a fluorescence microplate reader. The data are means of five replicate assays.

amplitude mitochondrial swelling was induced by treatment with each cannabinoid at 200 μM (Fig. 5, *A* and *B*). These swelling effects were suppressed by CsA pretreatment (Fig. 5, *A* and *B*), suggesting that cannabinoid-mediated swelling is regulated by the MPT pores.

Although Ca^{2+} is indispensable for opening of MPT pores in animal mitochondria (10, 11), surprisingly, the above swelling of isolated *Cannabis* mitochondria occurred even in the absence of Ca^{2+}. Moreover, it became evident that Ca^{2+} does not stimulate opening of *Cannabis* MPT pores in isolated *Cannabis* mitochondria, because addition of 1 mM Ca^{2+} decreased rather than increased their light-scattering change (Fig. 5*C*). This effect may be related to the inhibitory effect of Ca^{2+} through an external Me^{2+} binding site (37), as we have found that *Cannabis* mitochondria do not take up Ca^{2+} after treatment with high concentrations of Ca^{2+} even in the presence of A23187 (data not shown). Together with Ca^{2+}, H_2O_2 is also assumed to mediate opening of MPT pores in plant cells (36), although apparent swelling was not induced by treatment with 1 mM H_2O_2 (Fig. 5). These results indicated that CBCA and THCA regulate the opening of MPT pores independently of Ca^{2+} and H_2O_2.

We also examined the effects of CBCA and THCA on ΔΨm using the specific ΔΨm indicator TMRM. After isolated mitochondria previously stained with TMRM were incubated with CBCA or THCA, the intensity of the TMRM fluorescence was measured. As shown in Fig. 5*E*, these cannabinoids quickly caused a marked decrease of the fluorescence, which indicates ΔΨm reduction, whereas Ca^{2+} and H_2O_2 did not decrease ΔΨm after >1 h of treatment. Furthermore, the same experiments using *Cannabis* cells yielded results similar to those obtained using isolated mitochondria (Fig. 5*F*). This evidence demonstrated that, like mitochondrial swelling, ΔΨm reduction was also induced by cannabinoids in a Ca^{2+}- and H_2O_2-independent manner.

Release of Mitochondrial Proteins and Induction of Mitochondrial Dysfunction by Cannabinoids—High amplitude swelling of animal mitochondria causes release of mitochondrial proteins (*e.g.* cytochrome *c*) through collapse of the mitochondrial outer membrane (12, 13, 15, 16, 38, 39). To examine whether CBCA and THCA induce such collapse, we analyzed proteins released from *Cannabis* mitochondria treated with these agents. We investigated the release of nuclease as well as cytochrome *c*, because nuclease, which can hydrolyze plant nuclear DNA and plasmid DNA, has recently been confirmed to be present in the mitochondrial intermembrane space in plants (29). The isolated mitochondria were incubated with CBCA or THCA, and the reaction mixtures were fractionated into supernatants and pellets by centrifugation. Western blotting using anti-cytochrome *c* antibodies showed a higher level of cytochrome *c* in the supernatants from the isolated mitochondria treated with CBCA and THCA, whereas nuclease assays also demonstrated a clear increase of the nuclease activity in the same supernatants (*left panels* of Fig. 6*A*), thus indicating the release of mitochondrial proteins into the supernatant. Similar assays for the above pellets demonstrated that cannabinoid treatment of the isolated mitochondria significantly decreased the levels of cytochrome *c* and nuclease in the pellets (*left panels* of Fig. 6*B*), which is consistent with their release from the isolated mitochondria. Similar results were obtained from experiments using *Cannabis* cells. We first treated *Cannabis* cells with cannabinoids, isolated mitochondria from the cells and analyzed cytochrome *c* and nuclease in the isolated mitochondria. As shown in Fig. 6*C* (*left panel*), we confirmed that cannabinoid treatment of *Cannabis* cells reduced the levels of both proteins in the isolated mitochondria. Taken together, these results suggest that cannabinoid-mediated MPT induces release of mitochondrial proteins, and this suggestion was also supported by evidence that CsA clearly inhibited their release (*right panels* of Fig. 6, *A–C*).

Accordingly, we concluded that both cannabinoids cause rupture of the mitochondrial outer membrane through MPT-mediated swelling, resulting in the release of mitochondrial proteins. Such membrane rupture is known to promote mitochondrial dysfunction (14), leading to a decrease of the cellular

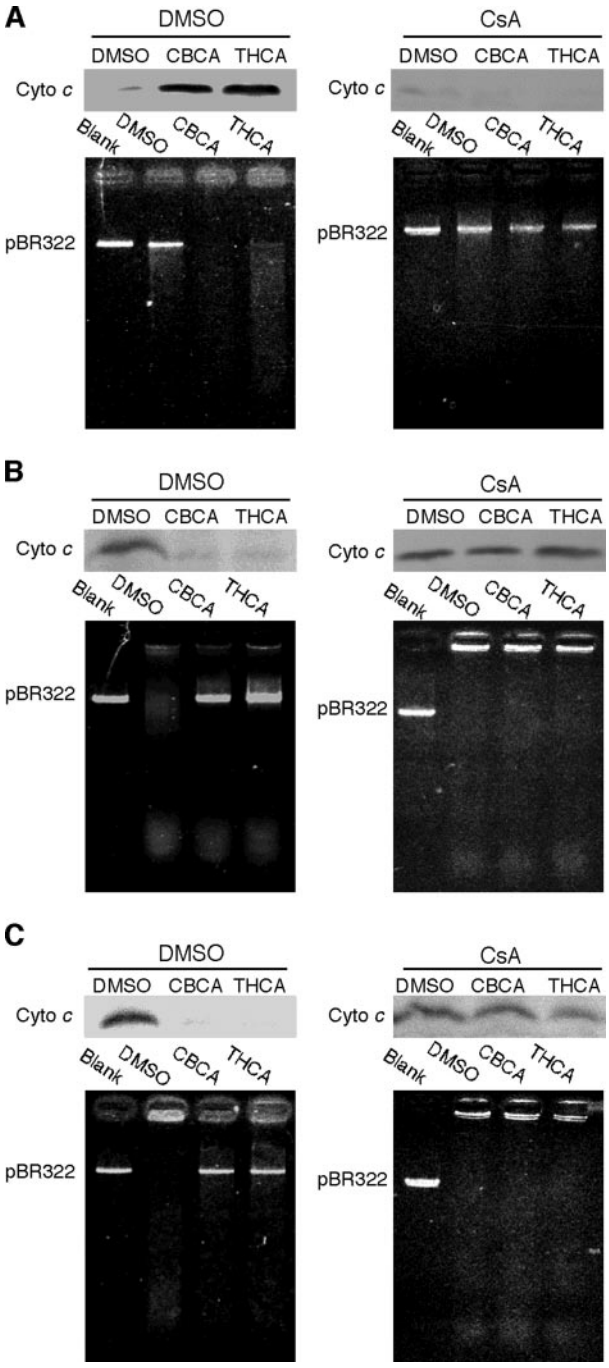

FIGURE 6. **Release of mitochondrial proteins in response to cannabinoids.** *A*, proteins released from isolated mitochondria in response to cannabinoids. Isolated mitochondria previously treated with Me_2SO (*DMSO*, *left panels*) or 25 μM CsA (*right panels*) for 5 min were incubated with 200 μM CBCA or 200 μM THCA for 40 min. The reaction mixtures were fractionated into supernatant and pellet by centrifugation. An aliquot (11 μg of protein) of the supernatant was analyzed by SDS-PAGE (12.5% acrylamide gel) and processed for immunoblotting using anti-cytochrome *c* (*upper panels*). The nuclease activity in the same supernatant was detected using EcoRI-cleaved pBR322 as a substrate (*lower panels*). *B*, proteins in isolated mitochondria after cannabinoid treatment. Mitochondrial proteins in the above pellets were extracted as described under "Experimental Procedures." Cytochrome *c* (*upper panels*) and nuclease (*lower panels*) in the extracts were analyzed as described above. *C*, proteins in mitochondria isolated from cannabinoid-treated cells. *Cannabis* cells were incubated with DMSO (*left panels*) or 25 μM CsA (*right panels*) for 1 h and then with 100 μM CBCA or 100 μM THCA for 3 h. After mitochondria were isolated from the cells, proteins in the isolated mitochondria were extracted. Cytochrome *c* (*upper panels*) and nuclease (*lower panels*) in the extracts were analyzed as described above.

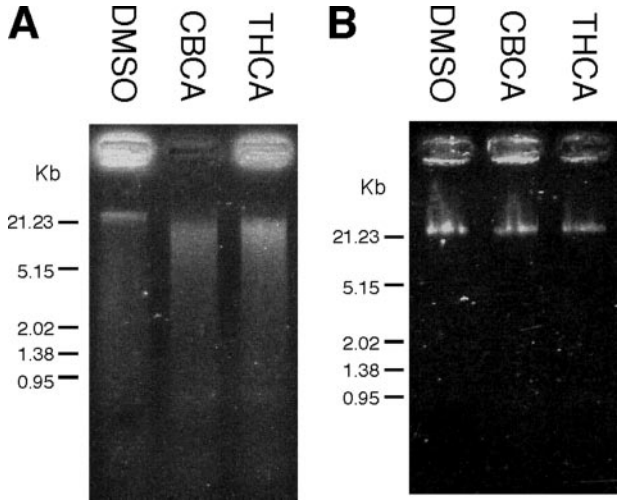

FIGURE 7. **Degradation of nuclear DNA by mitochondrial nuclease.** The supernatant prepared as described in the legend of Fig. 6*A* was used as nuclease solution. Nuclei isolated from *Cannabis* cells were incubated with the supernatant in the absence (*A*) or the presence (25 μM, *B*) of aurintricarboxylic acid for 2 h. The nuclei were collected by centrifugation, and nuclear DNA was extracted using the cetyltrimethylammonium bromide method and analyzed by agarose gel electrophoresis (1.0%). The labels (*DMSO*, *CBCA*, and *THCA*) of each lane indicate that the sample was incubated with supernatant prepared by treatment of isolated mitochondria with Me_2SO (DMSO), CBCA, or THCA, respectively.

ATP level. Indeed, we also confirmed that cannabinoid treatment decreases the cellular ATP level: when *Cannabis* cells were incubated with CBCA and THCA for 2 h, the ATP levels in the cells markedly decreased (supplemental Fig. S3). Thus, it was evident that the features of cannabinoid-induced cell death are consistent with those of necrotic cell death (5, 6).

The Mechanism of DNA Degradation Induced by Cannabinoids—In many cases, DNA degradation in apoptotic cells is catalyzed by nuclease, which is activated by caspases in the presence of ATP (3, 4, 6–8), whereas the DNA degradation catalyzed by CBCA and THCA occurred in the presence of a pan-caspase inhibitor (Fig. 4*D*) and at a lower ATP level. We measured caspase activities in cannabinoid-treated cells using substrates for caspase 1 and caspase 3, whose activities are detected in many plants (34, 40), but little increase of the activities was detected (data not shown). This evidence suggests that both cannabinoids induce DNA degradation though a mechanism other than the caspase-dependent pathway.

We hypothesized that the above DNA degradation is catalyzed by nuclease released from mitochondria through MPT, because a previous study showed that plant mitochondrial nuclease has the ability to catalyze DNA degradation in isolated nuclei (29). To test this hypothesis, we characterized the properties of the nuclease in *Cannabis* mitochondria. When the isolated *Cannabis* nuclei were incubated in nuclease solution prepared by treatment of isolated *Cannabis* mitochondria with cannabinoids, we found that DNA in the isolated nuclei underwent degradation (Fig. 7*A*). Therefore, a mitochondrial nuclease released by MPT was considered to catalyze DNA degradation in cannabinoid-treated cells. Because the degradation of DNA by mitochondrial nuclease was strongly inhibited by aurintricarboxylic acid (Fig. 7*B*), the effect of this inhibitor on cannabinoid-mediated cell death was investigated. The inhibi-

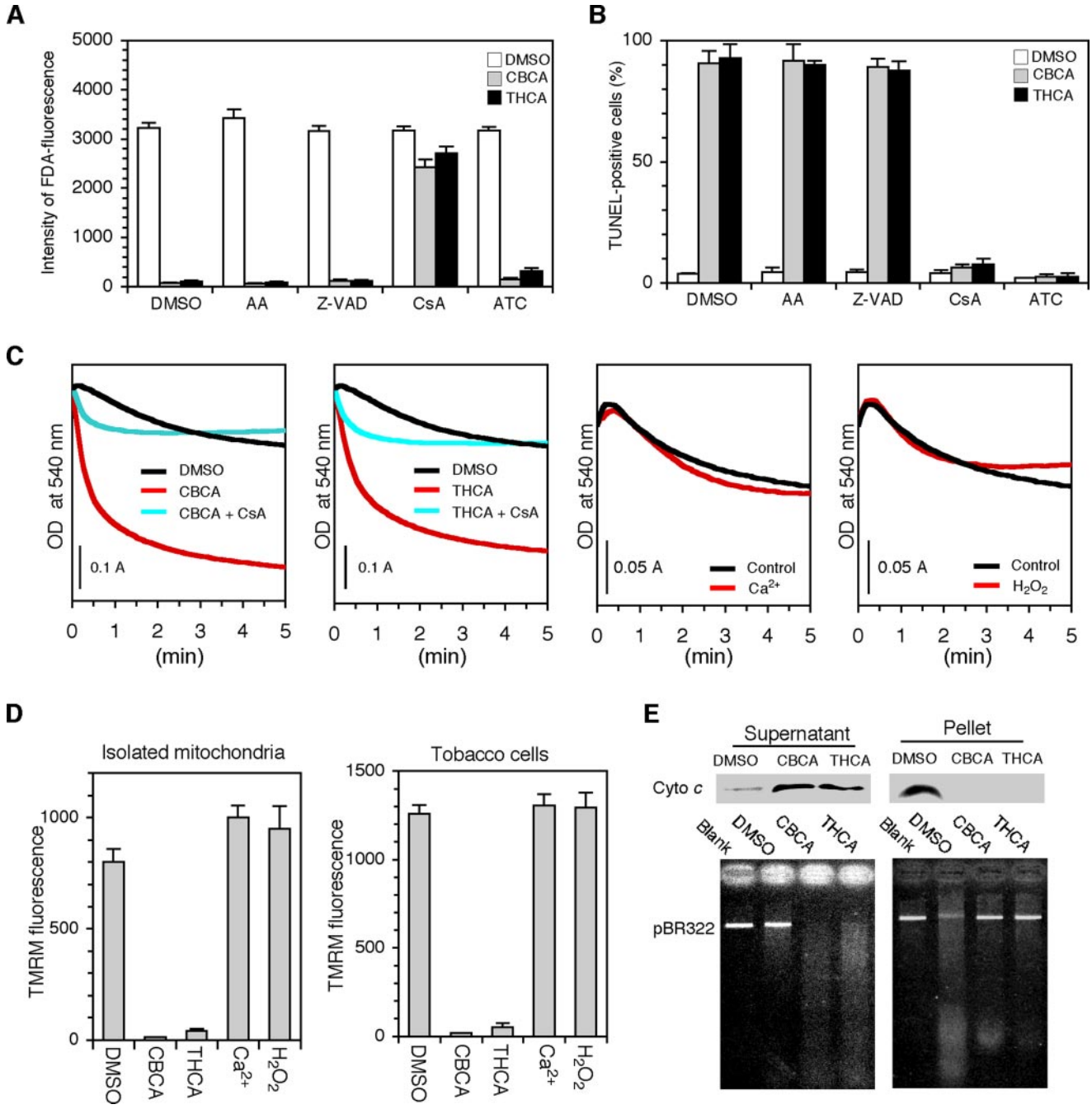

FIGURE 8. **Features of cell death mediated by cannabinoids in tobacco cells.** *A* and *B*, effects of cell death inhibitors on cell death catalyzed by cannabinoids in tobacco cells. Tobacco cells were incubated with 25 mM ascorbic acid (*AA*), 50 μM Z-VAD-FMK (*VAD*), 25 μM cyclosporin A (*CsA*), or 25 μM aurintricarboxylic acid (*ATC*) for 1 h. After 100 μM CBCA or 100 μM THCA was added to the cultures, the cell viability (*A*) and DNA degradation (*B*) in the samples were analyzed as described under "Experimental Procedures." *C*, mitochondrial swelling induced by cannabinoids. Mitochondria isolated from tobacco cells were incubated with 200 μM CBCA, 200 μM THCA, 1 mM Ca^{2+}, or 1 mM H_2O_2, and their light scattering was monitored at optical density at 540 nm. For experiments using CsA, the isolated mitochondria were treated as described in the legend of Fig. 5 (*A* and *B*). The data are means of five replicate assays. *D*, $\Delta\Psi$m reduction by cannabinoids. Isolated mitochondria and tobacco cells were treated as described under "Experimental Procedures," and the intensity of TMRM fluorescence was quantified using a fluorescence plate reader. *E*, release of proteins from the isolated mitochondria by cannabinoid. The isolated tobacco mitochondria were treated and fractionated into supernatant and pellet as described in the legend of Fig. 6*A*. Cytochrome *c* and nuclease in the samples were analyzed by immunoblotting and nuclease assay, respectively, as described under "Experimental Procedures."

tor suppressed the increase of TUNEL-positive nuclei (Fig. 4*D*) but only slightly increased the viability of cannabinoid-treated cells (Fig. 4*C*), suggesting that DNA degradation contributes less than mitochondrial dysfunction to the induction of cell death by cannabinoids.

The Effects of Cannabinoids on Cell Death of Tobacco Cells—We investigated whether CBCA and THCA cause cell death in tobacco cells (BY-2 cells), which have often been used as model plant cells. We confirmed that incubation with higher than 20 μM CBCA or THCA caused a marked reduction of cell viability within 3 h (supplemental Fig. S4*A*). In addition, TUNEL assays demonstrated that DNA degradation was induced in most cells (~90% of total nuclei) after 3 h of treatment with the higher concentrations (50 μM or 100 μM) of each cannabinoid and that

incubation with 20 μM cannabinoids also increased the population (>50% of total nuclei) of TUNEL-positive nuclei (supplemental Fig. S4*B*). These results indicate that CBCA and THCA induced cell viability reduction and DNA degradation more effectively in tobacco cells than in *Cannabis* cells.

We also examined the effects of various cell death mediators on cannabinoid-mediated cell responses (cell viability reduction and DNA degradation). These responses were clearly inhibited by CsA but not by ascorbic acid or pan-caspase inhibitor (Fig. 8, *A* and *B*), indicating that cannabinoid treatment of tobacco cells also induced cell death through MPT. We confirmed that tobacco mitochondria underwent Ca^{2+}-independent opening of MPT pores in response to cannabinoid treatment, resulting in high amplitude mitochondrial swelling (Fig. 8*C*) and loss of $\Delta\Psi$m (Fig. 8*D*). Furthermore, this swelling induced release of cytochrome *c* and nuclease from the isolated tobacco mitochondria (Fig. 8*E*). Taken together, these results indicate that CBCA and THCA induce necrotic cell death through mitochondrial dysfunction in tobacco and that the properties of MPT are conserved in *Cannabis* cells and tobacco cells.

DISCUSSION

In the present study, we discovered that cannabinoid resin accumulates in leaf tissues where cell death occurs, and identified cell death-inducing cannabinoids (CBCA and THCA) from the resin by assays using *Cannabis* leaves where cuticles were removed. Judging from their amounts in *Cannabis* leaves and their cell death-inducing activity, the cannabinoid resin is considered to have the ability to regulate cell death in *Cannabis* leaves.

In addition, analysis of the distribution of cannabinoid resin demonstrated that this plant accumulates these cannabinoids in leaf cells through a unique system to induce cell death. Generally, cell death-mediating molecules are maintained at quite low or undetectable levels in intact cells, and death stimuli and/or death signals lead to their accumulation at higher levels by activating their synthetic systems. In contrast, despite their highly toxic properties, large amounts of CBCA and THCA are biosynthesized in young leaves and constitutively accumulate in the glands on leaves at various stages. *C. sativa* is considered to protect leaves from the toxicity of cannabinoids by storing them in the glands and to regulate cell death by secreting them from the glands. To our knowledge, organisms storing large amounts of cell death regulators for later use have not been reported to date. Glandular tissues in higher plants, which have been investigated for over 300 years, are known to contribute to attracting pollinators and protecting host plants against herbivores by secreting secondary metabolites (32), but it has never been reported that glandular tissues in other plants act as "death capsules" like the *Cannabis* glands described here.

CBCA and THCA showed novel features in cell death induction as well as the process of their accumulation. Interestingly, the induction of cell death by these cannabinoids was not suppressed by pretreatment with the H_2O_2-scavenging agent ascorbic acid. H_2O_2 is known to be involved in many cases of plant cell death as an important signaling molecule and to mediate cell death by increasing Ca^{2+} influx and by inducing various cell death regulators (33). However, the findings of this study demonstrate that both cannabinoids induce cell death independently of these H_2O_2-regulated cell death systems. The MPT inhibitor CsA clearly blocked cannabinoid-mediated cell death in the suspension-cultured cells or leaf cells of *C. sativa*, indicating that this cell death is significantly regulated by MPT. Although the mechanism of MPT in plants is not yet fully understood, because of the lack of precise evidence, our examinations using isolated mitochondria provided important evidence about the characteristics of cannabinoids as MPT mediators and about the mechanism of regulation of *Cannabis* MPT.

One notable feature of CBCA and THCA is that both of these cannabinoids (each at 200 μM) directly mediated MPT in isolated mitochondria without requiring Ca^{2+}, leading to high amplitude mitochondrial swelling and $\Delta\Psi$m reduction. The system that regulates MPT in a Ca^{2+}-independent manner has not been identified in plants to date. In contrast, Ca^{2+}-mediated MPT has been reported in the mitochondria of several plants: swelling is induced in wheat mitochondria and potato tuber mitochondria by treatment with high concentrations (2.5–5.0 mM) of Ca^{2+} in the presence of inorganic phosphate (15, 16). However, we found that the properties of the swelling described in those studies are different from those of cannabinoid-treated mitochondrial swelling. For example, the addition of Ca^{2+} to wheat mitochondria and potato tuber mitochondria first induces shrinkage, and swelling starts 2–4 min after treatment (15, 16). On the other hand, CBCA and THCA quickly caused high amplitude swelling in *Cannabis* mitochondria without inducing such a shrinking phase. Interestingly, cannabinoid-induced swelling resembles cyclophilin D-dependent swelling of mouse mitochondria, which is induced without a shrinking phase by treatment with 50–250 μM Ca^{2+} (13, 14). In addition, CsA, which is known to block the opening of animal MPT pores by inhibiting the function of cyclophilin D, strongly suppresses mitochondrial swelling mediated by cannabinoids, indicating that cyclophilin D is essential for cannabinoid-mediated opening of *Cannabis* MPT pores. In contrast, wheat mitochondrial swelling is not inhibited by CsA, whereas dithiothreitol is required for inhibition of Ca^{2+}-mediated swelling of potato tuber mitochondria by CsA. Thus, the features of cannabinoid-driven MPT in *Cannabis* mitochondria are similar to those of the above cyclophilin D-dependent MPT in mouse mitochondria (13, 14).

However, in contrast to animal mitochondria, *Cannabis* mitochondria underwent MPT-dependent swelling even in the absence of added Ca^{2+}. In addition, examinations using a cell-free system showed that other cytosolic factors are not required for the opening of cyclophilin D-dependent MPT pores by THCA and CBCA. Based on this evidence, we hypothesized that some component of *Cannabis* MPT pores possesses sites that control their opening upon binding of cannabinoids or cyclophilin D. Although at present we could not identify such sites in *Cannabis* MPT pores owing to a lack of structural and biochemical information on the components of MPT in animals as well as plants, it may be possible to use CBCA as a probe to identify the component having the cannabinoid-binding site by taking advantage of its fluorescent property.

Recently, cyclophilin D-dependent MPT in mouse mitochondria was shown to induce mitochondrial dysfunction through $\Delta\Psi$m reduction and through collapse of the mitochondrial outer membrane, which was confirmed by the release of cytochrome *c* upon mitochondrial swelling, resulting in necrotic cell death (13). In agreement with this study, we confirmed that cyclophilin D-dependent MPT mediated by cannabinoids induces mitochondrial-protein release and $\Delta\Psi$m reduction, leading to mitochondrial damage in *Cannabis* cells as well as isolated mitochondria. This mitochondrial damage was also confirmed by a decrease of the ATP level in cannabinoid-treated cells. Furthermore, CBCA and THCA induce cell death in *Cannabis* cells independently of caspases, suggesting that this cell death is not induced through a caspase-dependent apoptotic pathway. Accordingly, we concluded that CBCA and THCA induce necrotic cell death based on mitochondrial dysfunction in *Cannabis* cells.

Dysfunction in isolated mitochondria could be induced by treatment with 200 μM (0.07 mg/ml) CBCA or THCA. Taken together with the amounts of CBCA (0.39 mg/g fresh leaves) and THCA (5.5 mg/g fresh leaves) in mature *Cannabis* leaves, these results indicate that *Cannabis* leaves produce sufficient amounts of these cannabinoids to induce mitochondrial dysfunction in whole leaves. Endogenous secondary metabolites acting as necrosis-inducing factors have not been identified in other plants to date.

Interestingly, treatment of *Cannabis* cells with cannabinoids induced DNA degradation characteristic of apoptotic cells, together with the necrotic features. DNA degradation in apoptotic cells of plants is often mediated by caspase-dependent nuclease, whereas we demonstrated that cannabinoid-induced DNA degradation is catalyzed by nuclease released from mitochondria during MPT. Similar DNA degradation is found in animal cells, where endonuclease G released from mitochondria mediates degradation of nuclear DNA (41, 42). Although such mitochondrial nuclease has also been identified in *A. thaliana* (29), the mechanism of the release of this nuclease from mitochondria has not been clarified. Therefore, these cannabinoids are the first endogenous regulators shown to induce the release of nuclease from plant mitochondria. To clarify the role of nuclease release in cannabinoid-mediated cell death, we inhibited this nuclease using aurintricarboxylic acid and found that the decrease of cell viability was only slightly suppressed, suggesting that the DNA degradation by mitochondrial nuclease contributes little to cell death. Indeed, >60% of cannabinoid-treated cells were negative in the TUNEL assay, but most of the cells had a marked decrease of viability. Moreover, mitochondrial dysfunction in mouse cells has been reported to effectively induce cell death without inducing DNA degradation (13, 14). Hence, DNA degradation induced by cannabinoids can be regarded as a side effect of mitochondrial dysfunction.

Like *Cannabis* cells, tobacco cells also immediately suffered mitochondrial dysfunction in a Ca^{2+}-independent manner upon treatment with CBCA and THCA, resulting in necrotic cell death. In addition, examinations using cell death inhibitors indicated that the features of MPT in tobacco cells and *Cannabis* cells are similar to each other. These results indicate that the properties of MPT pores are conserved in the cells of both plants, whereas production of cannabinoids has not been confirmed in tobacco or other plants. Higher plants produce numerous secondary metabolites, and it is possible that some of these metabolites may act as cannabinoid homologues that mediate opening of MPT pores.

Although there have been few studies about the induction of necrosis through MPT in tobacco cells, apoptosis in tobacco cells has been extensively characterized (36, 43, 44). Therefore, we attempted to compare the features of necrosis and apoptosis in tobacco cells. Apoptosis in tobacco cells has been shown to involve the induction of DNA degradation, which is inhibited by a caspase inhibitor (43). Although cannabinoid treatment of tobacco cells also induced DNA degradation, the caspase inhibitor Z-VAD-FMK did not inhibit this degradation. Moreover, an increase of caspase activity was not observed during the cell death induced by cannabinoids, indicating that caspases do not participate in cannabinoid-mediated DNA degradation. Our study suggested that nuclease released from tobacco mitochondria catalyzes this degradation. Furthermore, we found that cannabinoid treatment induced cell death in tobacco cells more rapidly than apoptotic stress did. For example, 8-h incubation with H_2O_2 (36) or 24-h incubation with benzyladenosine (44) have been shown to induce apoptosis in 32% of total tobacco protoplasts or in most of tobacco cells, respectively, whereas we showed that cannabinoids induce necrotic cell death in most of both tobacco cells and *Cannabis* cells within 3 h. Such a rapid induction of necrosis can be explained by the evidence that cannabinoids directly mediate opening of MPT pores, which immediately induces mitochondrial dysfunction. In contrast, apoptosis significantly depends on caspases, and it has been shown to take 6-15 h to activate caspase-3-like protease in tobacco cells (45). Indeed, we also confirmed that caspase 3-like activity in tobacco cells started to increase at 6 h after H_2O_2 treatment and that H_2O_2-induced apoptosis was inhibited by a caspase-3 inhibitor.[4] Therefore, much more time may be required for inducing cell death through the caspase-dependent apoptotic pathway than the cannabinoid-mediated necrotic pathway. To conduct similar comparison in *Cannabis* cells, we also attempted to induce apoptosis, but could not induce this type of cell death using various reagents, including H_2O_2 and camptothecin (data not shown).

In conclusion, we confirmed that CBCA and THCA have the ability to very rapidly cause necrotic cell death through serious mitochondrial dysfunction without requiring other cell death-inducing molecules, including H_2O_2 and Ca^{2+}. These cannabinoids are well known secondary metabolites found only in *C. sativa* (19), although why this plant produces these novel metabolites remains unknown. The properties of these cannabinoids suggest the possibility that they participate in physiologically important events in *C. sativa*. For example, we assume that CBCA and THCA may play an important role in the defense systems of *Cannabis* leaves against pathogens, because necrotic cell death in higher plants is known to confine pathogens to infected sites (3). Indeed, secretion of cannabinoid resin

[4] S. Morimoto, Y. Tanaka, K. Sasaki, H. Tanaka, T. Fukamizu, Y. Shoyama, Y. Shoyama, and F. Taura, unpublished data.

was induced in most of cell death lesions of *Cannabis* leaves. Moreover, similar cannabinoid secretion was also observed in the senescent leaves. This secretion is considered to occur after the initiation of senescence, because in *Cannabis* leaves at the senescence-initiating stage, chlorophyll degradation characteristic of senescence is often found in the regions, which are not exposed to cannabinoid resin (supplemental Fig. S5). Accordingly, CBCA and THCA may also contribute to the promotion of cell death, which is usually induced at the final step of the leaf senescence process. To unequivocally prove these hypotheses about the physiological roles of cannabinoids, more precise experimental evidence will be essential.

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