Real-Time Detection of Caspase-3-Like Protease Activation in Vivo Using Fluorescence Resonance Energy Transfer during Plant Programmed Cell Death Induced by Ultraviolet C Overexposure[1][C]

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Caspase-like proteases have been demonstrated to be involved in plant programmed cell death (PCD). Here, the time scale of caspase-3-like protease activation was investigated in single living plant cells undergoing PCD induced by ultraviolet C (UV-C) overexposure. The real-time detection of caspase-3-like protease activation was achieved by measuring the degree of fluorescence resonance energy transfer (FRET) within a recombinant substrate containing enhanced cyan fluorescent protein (ECFP) linked by a peptide possessing the caspase-3 cleavage sequence, DEVD, to enhanced yellow fluorescent protein (EYFP; i.e. ECFP-DEVD-EYFP). Microscopic observations demonstrated that the ECFP-DEVD-EYFP fusion protein could be expressed correctly and the FRET from ECFP to EYFP could be found in transfected Arabidopsis (*Arabidopsis thaliana*) protoplasts. At 30 min after exposure to UV-C, caspase-3-like protease activation indicated by the decrease in FRET ratio occurred, taking about 1 h to reach completion in single living protoplasts. Mutation in the DEVD tag or a caspase-3 inhibitor could prevent the changes in FRET ratio induced by UV-C treatment, confirming that the decrease in FRET ratio was due to the cleavage of fusion protein as a result of caspase-3-like protease activation. This activation was further confirmed by in vitro caspase-3 substrate assay and western-blot analysis, showing the occurrence of cleavage in ECFP-DEVD-EYFP protein but not in the protein with a mutant DEVD tag. In summary, these results represent direct evidence for the activation of caspase-3-like protease in UV-C-induced PCD, and the FRET technique is a powerful tool for monitoring key events of PCD in living cells in real time.

Programmed cell death (PCD) is a fundamental genetically controlled process that is responsible for removal of unwanted and potentially dangerous cells; thus, it contributes significantly to both the development and defense of multicellular organisms (Roger and Lam, 1997; Chichkova et al., 2004; Vacca et al., 2004). In animal cells, a central core execution switch for apoptosis, which is a defined type of PCD in the animal kingdom, is the activation of caspases (Cys-containing Asp-specific proteases), which is triggered by the release of cytochrome *c* from the mitochondrial intermembrane space and mediates the cleavage of a variety of apoptotic proteins, ultimately leading to cell demise (Green and Reed, 1998; Los et al., 2001; Shi, 2002). Similarly, accumulating evidence in recent years suggests the existence of caspase-like activity in plants and its functional involvement in various types of plant PCD, although there are many types of plant cell death that do not depend upon caspase-like proteases and do not share aspects of apoptosis (Woltering et al., 2002; Woltering, 2004; Lam, 2005; Sanmartín et al., 2005; Bonneau et al., 2008; He et al., 2008; Reape et al., 2008).

Synthetic fluorogenic substrates and synthetic peptide inhibitors to caspases have been widely used to study caspase-like activity and its functional involvement in plant PCD induced by biotic or abiotic stimuli. Based on the use of the synthetic tetrapeptide fluorogenic substrate to caspase-1 (Ac-YVAD-AMC), caspase-like activity has been demonstrated in extracts from tobacco mosaic virus (TMV)-infected tobacco (*Nicotiana tabacum*) leaves, and this caspase-like activity could be inhibited with caspase-1 (Ac-YVAD-CMK) and caspase-3 (Ac-DEVD-CHO) inhibitors but not by caspase-unrelated protease inhibitors (Lam and del Pozo, 2000). Likewise, it has also been demonstrated that cytosolic extracts from barley (*Hordeum vulgare*) embryonic suspension cells exhibited both caspase-1-like and caspase-3-like activity by the assay using fluorogenic substrates to caspase-1 (Ac-YVAD-AMC) and caspase-3 (Ac-DEVD-AMC; Korthout et al., 2000). Recently, employing caspase-1 inhibitors (Ac-YVAD-CHO, biotin-XVAD-fmk, and biotin-YVAD-chlorome-

[1] This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University (grant no. IRT0829) and the National High Technology Research and Development Program of China (863 Program grant no. 2007AA10Z204).

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.125625

thylketone) and a caspase-1 substrate (Ac-YVAD-MCA), a vacuole-localized protease called vacuolar processing enzyme has been identified as a protease that exhibits mammalian caspase-1 activity and is essential for TMV-induced hypersensitive cell death in tobacco leaves and fumonisin B1-induced cell death in Arabidopsis (*Arabidopsis thaliana*) plants, although it is structurally unrelated to caspases (Hatsugai et al., 2004; Kuroyanagi et al., 2005).

As the central executer of apoptosis, caspase-3 has undoubtedly been the major focus of many studies in animals (Budihardjo et al., 1999). Caspase-3-like activity and its involvement in plant PCD have also attracted intense attention. It has been not only demonstrated that both extracts from barley embryonic suspension cells and TMV-infected tobacco leaves displayed caspase-3-like activity (Korthout et al., 2000; Lam and del Pozo, 2000) but also confirmed that a plant caspase-3-like protease activated during TMV-triggered hypertensive response (HR) of tobacco plants was partially purified and characterized, and a potential genuine plant caspase substrate was shown during this process (Chichkova et al., 2004). In addition, using synthetic fluorogenic caspase-3 substrate, Ac-DEVD-AMC cleavage activity was detected during UV- or heat shock-induced apoptosis of plant cells, and caspase-3 inhibitors were able to suppress these types of cell death (Danon et al., 2004; Vacca et al., 2006). It should also be noted that exogenous (bovine) poly-(ADP-Rib) polymerase (PARP) was found to be cleaved by extracts from fungus-infected cowpea (*Vigna unguiculata*) plants, and this cleavage activity could be inhibited by caspase-3 inhibitor (Ac-DEVD-CHO; D'Silva et al., 1998). All of these experiments suggest the existence of functional caspase-like proteolytic activity and its functional involvement in plant cells undergoing PCD.

Although synthetic fluorogenic substrates and synthetic peptide inhibitors could be used to detect DEVD activity in vivo in plant cells (Korthout et al., 2000; Elbaz et al., 2002; Hatsugai et al., 2004; Kuroyanagi et al., 2005; Bosch and Franklin-Tong, 2007), these experimental results tell us little about the time scale of the activation of caspase-3-like protease at the single cell level. During the past decade, fluorescence resonance energy transfer (FRET) has been proved as a powerful technique to monitor compartmentation and subcellular targeting as well as to visualize protein-protein interactions and protease activity in living cells (Weiss, 2000). FRET is a quantum-mechanical process by which the excitation energy is transferred from a donor fluorochrome to a neighboring acceptor fluorochrome. FRET only occurs when (1) the absorption spectrum of the acceptor chromophore overlaps with the fluorescence emission spectrum of the donor, and (2) the proximity of the two chromophores is less than 10 nm (Gadella et al., 1999; Shah et al., 2001). During the process of staurosporine-induced apoptosis of COS-7 cells, FRET technique has been successfully employed to reveal the time scale of the activation of caspase-3 at the single cell level by measuring the extent of FRET within a recombinant substrate containing cyan fluorescent protein (CFP) linked by a short peptide possessing the caspase-3 cleavage sequence, DEVD, to yellow fluorescent protein (YFP; i.e. CFP-DEVD-YFP; Tyas et al., 2000).

During the past several years, FRET technique has also been widely used to monitor interactions and distances between molecules in living plant cells (Immink et al., 2002; Seidel et al., 2004; Vermeer et al., 2004). In this work, the approach reported by Tyas et al. (2000) was adapted to monitor caspase-3-like protease activation in single living plant cells in real time by transfecting Arabidopsis (ecotype Columbia-0) protoplasts with a constructed recombinant caspase substrate. This recombinant is composed of enhanced (E) CFP as the FRET donor and EYFP as the acceptor, linked by a peptide containing the sequence DEVD, which has been defined as an optimal cleavage sequence for animal caspase-3 (ECFP-DEVD-EYFP; Fig. 1A; Tyas et al., 2000). With careful optimization of transfection efficiency, it is clearly demonstrated that the caspase-3-like activation in single living plant cells could be detected in real time during the process of UV-C-induced plant PCD based on FRET analysis.

RESULTS

Expression and Localization of ECFP-DEVD-EYFP in Protoplasts

To detect caspase-3-like activation in living plant cells during plant PCD in real time, the construct for expression of the ECFP-DEVD-EYFP fusion protein was cloned in plant expression vector under the control of the cauliflower mosaic virus 35S promoter. The linking peptides between ECFP and EYFP contained an optimum sequence for caspase-3 cleavage, as reported previously (Fig. 1A; Tyas et al., 2000). The cleavage of the linker peptide by activated caspase-3-like protease would reduce or abolish the FRET from ECFP to EYFP; thus, the caspase-3-like protease activity could be detected from the degree of FRET.

The correct expression of the chimeric construct in plant cells was first confirmed. After transient transfection of Arabidopsis mesophyll protoplasts, intra-

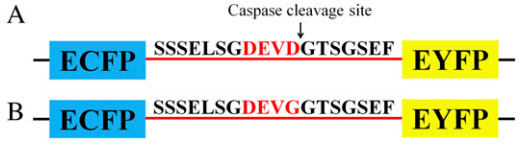

Figure 1. Schematic representations of ECFP-DEVD-EYFP (A) and ECFP-DEVG-EYFP (B) fusion proteins. The fusion proteins contain an 18-amino acid linker possessing the caspase-3 cleavage sequence DEVD and the mutation sequence DEVG, respectively. The arrow indicates the cleavage site by plant caspase-3-like protease. [See online article for color version of this figure.]

cellular distribution of ECFP, EYFP, and chlorophyll fluorescence was visualized by laser confocal scanning microscopy. As can be seen in Figure 2, when the protoplasts were excited with a 458-nm laser, not only ECFP fluorescence but also EYFP fluorescence could be clearly observed in the protoplast transfected with PI-ECFP-DEVD-EYFP but not in the control protoplast without transfection, which showed only the chlorophyll autofluorescence (Fig. 2A). Because EYFP is not excited at 458 nm, the presence of EYFP fluorescence in the transfected protoplasts indicated the occurrence of FRET between ECFP and EYFP (Fig. 2, B and C), which was further confirmed by the higher fluorescence intensity in the spectral range of EYFP emission (520–610 nm) than in that of ECFP emission (470–510 nm; Fig. 3A).

To prove that the fluorescence of ECFP/EYFP specifically indicates the localization of the ECFP-DEVD-EYFP fusion protein expressed in the protoplasts, the spatial distribution of the ECFP-/EYFP-specific signals and chlorophyll autofluorescence was further analyzed. The emission characteristics of ECFP/EYFP fluorescence and chlorophyll autofluorescence along the axis across the protoplast clearly showed that the distribution of ECFP fluorescence at the cytoplasmic compartment matched that of EYFP fluorescence but not that of autofluorescence-specific emission, confirming that ECFP/EYFP fluorescence was emitted by fusion protein and not by chlorophyll (Fig. 3B).

Acceptor Photobleaching FRET Analysis

Next, the FRET acceptor photobleaching experiments were performed to assess the stability and the FRET efficiency of the developed FRET probe. The acceptor EYFP of the FRET probe was photobleached in a selected region of the transfected protoplast using a 100-ms 100% laser intensity photobleaching pulse at 514 nm; simultaneously, the emission spectra from the bleaching area before and after photobleaching were measured. As shown in Figure 4, the ECFP fluorescence intensity increased with the decreasing EYFP fluorescence intensity after bleaching of EYFP when compared with those before bleaching, providing direct evidence for the occurrence of FRET between ECFP and EYFP in our caspase-3 FRET reporter (Fig. 4).

In Vivo Real-Time Detection of Caspase-3-Like Protease Activation by FRET Analysis

It has been reported that caspase-3-like protease activity is involved in different types of plant PCD (Chichkova et al., 2004; Danon et al., 2004; Vacca et al., 2006), although in most cases there is no direct evidence of caspase-3-like protease activation in vivo. For example, UV-C irradiation can induce PCD in Arabidopsis seedlings and protoplasts in the light, and in the process caspase substrate assays from in vitro experiments demonstrated caspase-3-like protease activation in a time-dependent manner during the onset of PCD (Danon et al., 2004; Gao et al., 2008). As described above, the fusion protein ECFP-DEVD-EYFP has been experimentally confirmed to be expressed correctly in the transfected protoplasts, and the FRET phenomena could be detected in this caspase probe. Thus, we use it to detect in vivo caspase-3-like protease activation during the process of plant PCD induced by UV-C exposure by analyzing the changes in FRET.

After UV-C exposure and continuous light for 20 min, the EYFP/ECFP FRET in the transfected protoplasts decreased with the decreasing EYFP fluores-

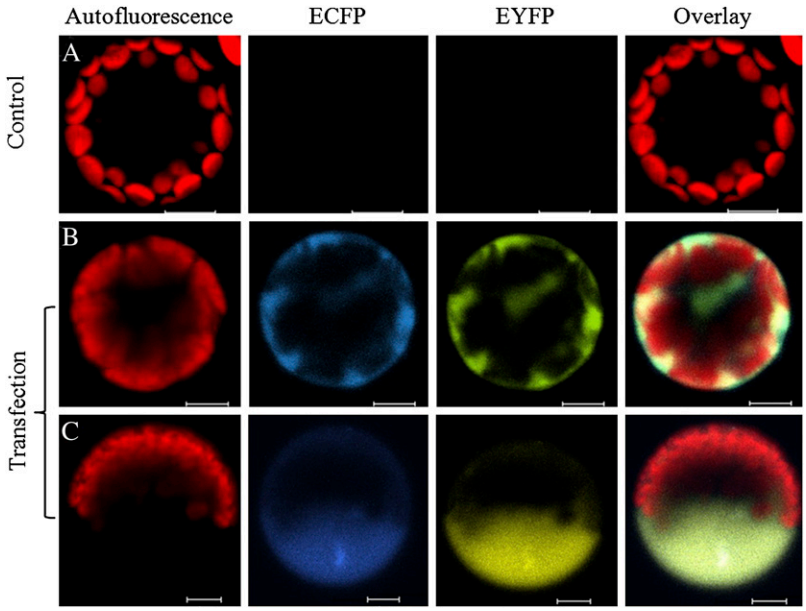

Figure 2. Expression of the chimeric fusion protein ECFP-DEVD-EYFP in protoplasts. Control protoplast without transfection and the transfected protoplasts were excited using a 458-nm laser; the emission fluorescence was divided into chlorophyll autofluorescence (red), ECFP (blue), and EYFP (yellow). Representative images from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars = 5 μm. [See online article for color version of this figure.]

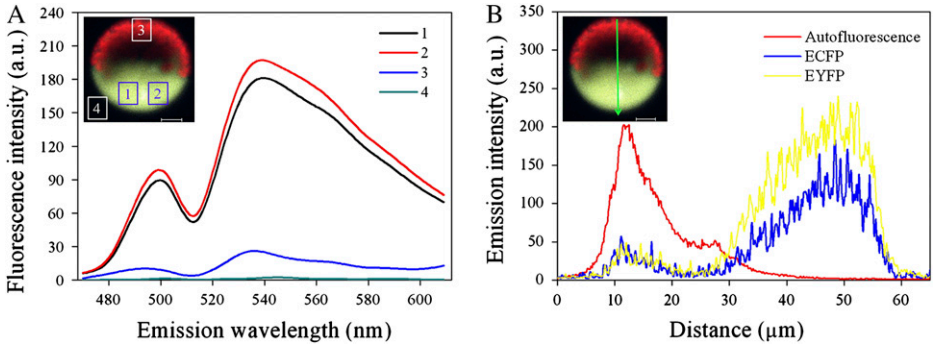

Figure 3. Spectral characteristics and distribution profile of the fluorescence in transfected protoplasts (excitation at 458 nm). A, The emission spectra were recorded between 470 and 600 nm from different areas of the transfected protoplasts. B, The fluorescence emission intensity of CFP at 495 nm (blue curve), YFP at 545 nm (yellow curve), and the chlorophyll autofluorescence at 685 nm (red curve) along the arrow represents their distribution profile inside the transfected protoplast. Representative images and data from one experiment are presented. Repetition of experiments led to results very similar to those shown. a.u., Arbitrary units. Bars = 5 μm. [See online article for color version of this figure.]

cence intensity when compared with those without UV-C exposure (Fig. 5A), suggesting the occurrence of the cleavage of the fusion protein ECFP-DEVD-EYFP. The temporal profile of EYFP/ECFP emission ratio measured in a single cell showed that, after UV-C exposure and continuous light for 20 min, the EYFP/ECFP ratio remained almost unchanged within the first 10 min, then it started to decrease gradually (Fig. 5D, red arrow) and reached a minimum at 90 min (Fig. 5D, black arrow), indicating that the caspase-3-like protease was activated and the fusion protein ECFP-DEVD-EYFP was cleaved in a time-dependent manner in the transfected protoplasts after UV-C treatment. It should be noted that during the whole investigation period, the protoplast morphology remained intact (Fig. 5, differential interference contrast images); therefore, the decrease of EYFP fluorescence and FRET ratio should be due to the caspase-3-like protease activation and the subsequent cleavage of ECFP-DEVD-EYFP during UV-induced PCD, not to the rupture of protoplasts. In contrast, if the transfected protoplasts were kept in continuous light for 20 min without UV-C treatment, the EYFP fluorescence and the EYFP/ECFP FRET ratio underwent only a slight decrease during the whole investigation period (Fig. 5A), indicating that there was neither change in FRET nor photobleaching during the process.

Mutation in the DEVD Tag Prevents the Changes in FRET Ratio

To exclude the background fluorescence interference during the process of plant PCD induced by UV-C exposure, we constructed a plasmid with a site mutation in the DEVD tag by substituting a Gly residue for the critical Asp residue in the caspase-3 cleavage site to prevent the cleavage of the recombinant caspase substrate by the caspase-3-like protease (i.e. ECFP-DEVG-EYFP; Fig. 1B). The results demonstrated that, even after UV-C exposure and continuous light for 20 min, the fluorescence of EYFP and ECFP in the protoplasts, which were transfected with the mutant plasmid and expressed correctly in the ECFP-DEVG-EYFP fusion protein, did not change like that in the same transfected protoplasts without UV-C exposure (Fig. 6A; data not shown). The temporal profile of the EYFP/ECFP emission ratio and the fluorescence of EYFP and ECFP measured in the single cell transfected with the plasmids with the DEVG tag showed that, regardless of UV-C exposure or not, the EYFP/ECFP

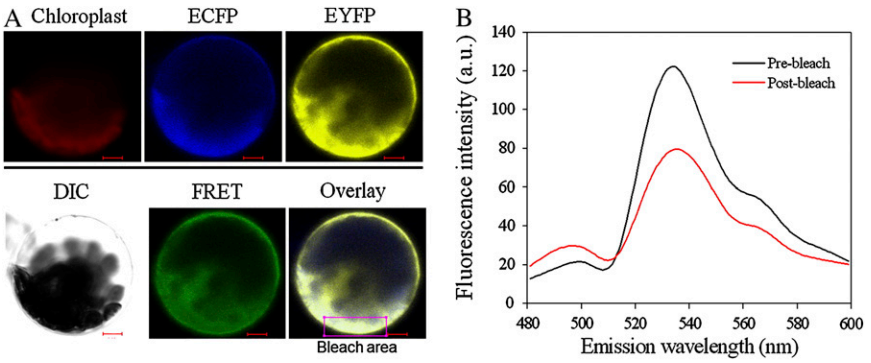

Figure 4. Acceptor photobleaching analysis of protoplasts expressing ECFP-DEVD-EYFP. A, EYFP was selectively bleached by repeated scanning of the indicated cell area at a high laser power of 514 nm. B, The emission spectrum from the indicated bleach area was recorded before and after photobleaching. Representative images and data from one experiment are presented. Repetition of experiments led to results very similar to those shown. a.u., Arbitrary units; DIC, differential interference contrast. Bar = 5 μm. [See online article for color version of this figure.]

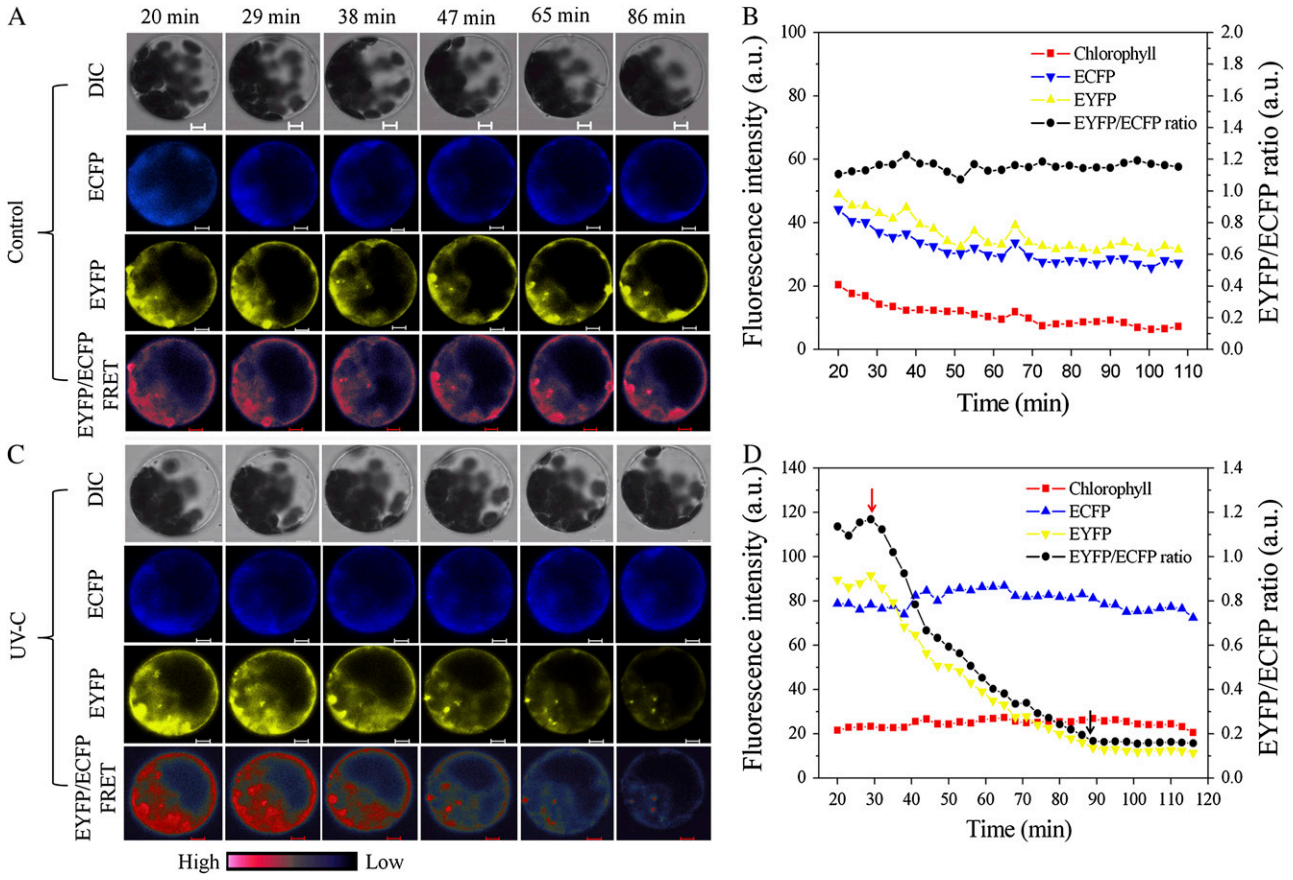

Figure 5. Dynamics of caspase-3-like activation in response to UV-C treatment. A and B, The transfected protoplasts without UV-C treatment in light for 20 min were excited by a 458-nm laser. ECFP, EYFP, and EYFP/ECFP FRET images were recorded, and the fluorescence intensity of ECFP, EYFP, and chlorophyll as well as the EYFP/ECFP ratio were measured during a time lapse. C and D, After treatment with 15 kJ m^{-2} UV-C and constant light for 20 min, the transfected protoplasts were excited by a 458-nm laser. ECFP, EYFP, and EYFP/ECFP FRET images were recorded, and the fluorescence intensity of ECFP, EYFP, and chlorophyll as well as the EYFP/ECFP ratio were measured during a time lapse. The red arrow and the black arrow indicate the start and end of the decrease of EYFP/ECFP ratio, respectively. Representative images and data from one experiment are presented. Repetition of experiments led to results very similar to those shown. a.u., Arbitrary units; DIC, differential interference contrast. Bars = 5 μm.

ratio and the fluorescence of EYFP and ECFP both remained almost unchanged during the whole investigation period (Fig. 6B; data not shown), indicating that the decreases in the fluorescence of EYFP and the EYFP/ECFP ratio in the protoplasts transfected with the nonmutation plasmid were not due to background fluorescence interference (Fig. 5). Chlorophyll fluorescence is also presented to show whether the cells were in out-of-focus appearance (Fig. 5).

A Caspase-3 Inhibitor Inhibits the Changes in FRET Ratio

To further confirm that the changes in EYFP/ECFP FRET and the loss of YFP signal correlate with caspase-3-like activity, an assay using the mammalian caspase-3 inhibitor Ac-DEVD-CHO was performed. The protoplasts were preincubated for 1 h with Ac-DEVD-CHO (100 μM) before UV-C exposure. Inhibitor analysis showed that the decrease in the fluorescence of YFP and the EYFP/ECFP FRET ratio were totally inhibited by Ac-DEVD-CHO (Fig. 7A). In the presence of Ac-DEVD-CHO, the fluorescence of YFP and the EYFP/ECFP FRET ratio both remained constant during the whole investigation period (Fig. 7B), confirming that the changes in EYFP/ECFP FRET and the loss of EYFP signal correlated with the caspase-3-like activity.

Western Blotting Confirms the Occurrence of the Cleavage of EYFP-DEVD-ECFP

To confirm further that the cleavage between EYFP and ECFP indeed occurs, immunoblot analysis with the polyclonal antibody against GFP was performed in extracts from the protoplasts transfected with constructed recombinant caspase substrates with

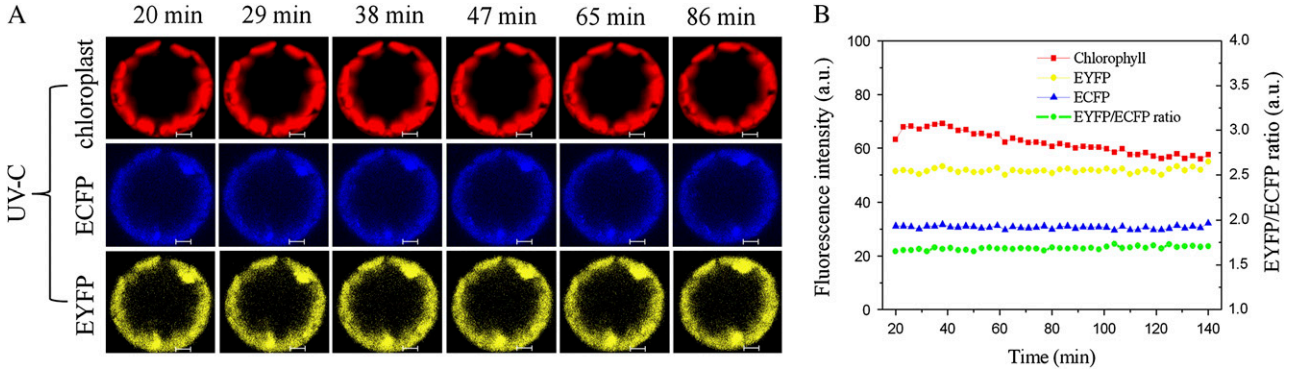

Figure 6. Changes in the fluorescence of ECFP and EYFP as well as the EYFP/ECFP FRET ratio in protoplasts transfected with the plasmid containing ECFP-DEVG-EYFP in response to UV-C treatment. A and B, After treatment with 15 kJ m^{-2} UV-C and constant light for 20 min, the transfected protoplasts were excited by a 458-nm laser. Chloroplasts, ECFP, and EYFP were recorded, and the fluorescence intensity of ECFP, EYFP, and chlorophyll as well as the EYFP/ECFP ratio were measured during a time lapse. Representative images and data from one experiment are presented. Repetition of experiments led to results very similar to those shown. a.u., Arbitrary units. Bars = 5 μm.

and without mutation. The results demonstrated that, after UV-C exposure and continuous light for 2 h, the cleavage of the ECFP-DEVD-EYFP fusion protein could be clearly observed in the transfected protoplasts but not in the control protoplasts without UV-C irradiation (Fig. 8A). It should be mentioned that there was no obvious cleavage in the transfected protoplasts after UV-C exposure and continuous light for 1 h (Fig. 8A). The cleavage of the ECFP-DEVD-EYFP fusion protein was completely blocked when the critical Asp at the fourth residue of the cleavage motif was substituted with a Gly residue (ECFP-DEVG-EYFP; Fig. 8B). It should be noted that transfection of protoplasts with the constructed recombinant caspase substrates has no apparent effect on basal caspase-3-like activity and cell viability (data not shown).

The Time Course of Cleavage Correlates with the Activation of Caspase-3-Like Protease

As shown in Figure 5, the changes in the EYFP/ECFP FRET ratio indicated that the time course of cleavage appears to fit the data of Danon et al. (2004), who showed that caspase-3-like activity was detected at 30 min after UV-C irradiation and reached a peak at 1 h. Due to the fact that the seedlings used to make protoplasts were grown in different conditions and the UV-C treatment protocol used was different when compared with Danon et al. (2004), the caspase-3-like

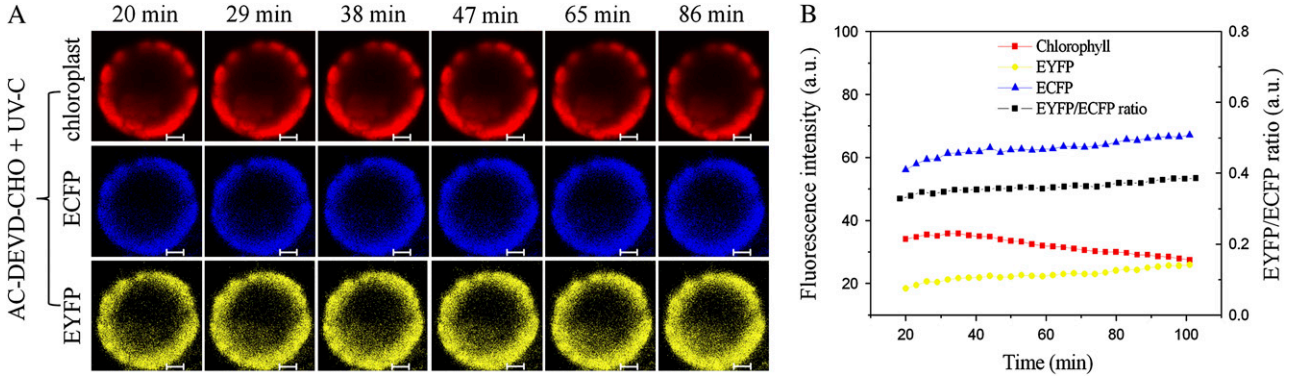

Figure 7. Changes in the fluorescence of ECFP and EYFP as well as the EYFP/ECFP FRET ratio in transfected protoplasts preincubated with the caspase-3 inhibitor Ac-DEVD-CHO in response to UV-C treatment. A and B, The transfected protoplasts were preincubated with the caspase-3 inhibitor Ac-DEVD-CHO (100 μM), exposed to 15 kJ m^{-2} UV-C and kept in light for 20 min, and then excited by a 458-nm laser. Chloroplasts, ECFP, and EYFP were recorded, and the fluorescence intensity of ECFP, EYFP, and chlorophyll as well as the EYFP/ECFP ratio were measured during a time lapse. Representative images and data from one experiment are presented. Repetition of experiments led to results very similar to those shown. a.u., Arbitrary units. Bars = 5 μm.

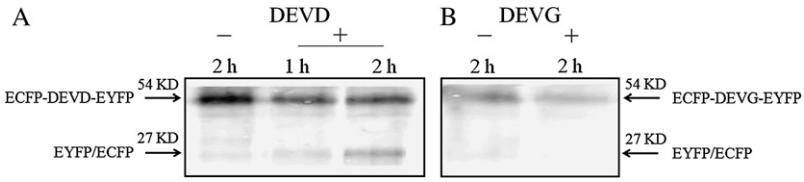

Figure 8. Western-blot evidence that the ECFP-DEVD-EYFP fusion protein was cleaved in response to UV-C treatment. Protoplasts transfected with ECFP-DEVD-EYFP (A) or ECFP-DEVG-EYFP (B) were irradiated with (+) or without (−) 15 kJ m^{-2} UV-C and then kept in the light for different times. The extent of cleavage of ECFP-DEVD-ECFP or ECFP-DEVG-EYFP was detected by western blotting with anti-GFP antibody.

activity was further measured in protoplast extracts using the fluorogenic substrate to see whether the consistency of the time course of cleavage with the data of Danon et al. (2004) was a false positive. The extracts from protoplasts irradiated with UV-C and then kept in the light for different times were used to test caspase-3-like activity using the caspase-3 substrate Ac-DEVD-pNA (200 μM). The results demonstrated that the induction of caspase-3-like activity was detected in the protoplasts at 30 min in the light after UV-C irradiation, reached a peak at 1 h, and subsequently decreased with the increase of the exposure time (Fig. 9), in agreement with the data of Danon et al. (2004).

DISCUSSION

A vast number of studies have prompted the suggestion that true caspase-like activity was present in plants and was crucially involved in the implementation of various types of plant PCD occurring during HR, developmental cell death of the weaker shoots, and various abiotic stresses, although, at present, no obvious homolog for an animal caspase gene has yet been identified in the available Arabidopsis genome, which does have a metacaspase family that is distantly related to the caspase family (Lam et al., 2001; Madeo et al., 2002; Belenghi et al., 2004; Chichkova et al., 2004; Danon et al., 2004; Zuppini et al., 2004; Vacca et al., 2006; He et al., 2008). For example, the extracts prepared from tobacco plants undergoing TMV-induced HR displayed Ac-YVAD-AMC cleavage activity (del Pozo and Lam, 1998), and specific inhibitors of animal caspase-1 and -3 (Ac-YVAD-cmk and Ac-DEVD-CHO, respectively) could not only attenuate bacteria- and TMV-induced HR in tobacco leaves (del Pozo and Lam, 1998) but also reduce the death of tobacco cells induced by isopentenyladenosine (Mlejnek and Prochazka, 2002) and inhibit the cell death during menadione-induced apoptosis in tobacco protoplasts (Sun et al., 1999). Recently, the assays performed in incompatible pollen using commercially available fluorescent substrates demonstrated that the caspase-3-like protease is activated remarkably rapidly during self-incompatibility-induced PCD in *Papaver* pollen (Bosch and Franklin-Tong, 2007). Also in that study, the temporal and spatial activation of caspase-like enzymes was demonstrated in living cells (Bosch and Franklin-Tong, 2007). It is possible to detect DEVD activity and to follow the activation of caspase-like proteases in vivo using fluorescent caspase substrates and synthetic caspase inhibitors (Korthout et al., 2000; Elbaz et al., 2002; Hatsugai et al., 2004; Kuroyanagi et al., 2005; Bosch and Franklin-Tong, 2007); however, this tells us little about the characteristics of the activation of caspase-like proteases in specific tissues. Therefore, it is intriguing to develop new strategies for real-time monitoring of the key events of PCD in specific tissues or cells.

In many animal cell apoptosis pathways, activation of the effector caspases is considered to be the final step. Among the spectrum of various caspases, caspase-3 is believed to be the major executioner to induce the cleavage of the PARP, DNA fragmentation, chromatin condensation, and final death program in animal cells (Cohen, 1997; Thornberry and Lazebnik, 1998; Budihardjo et al., 1999). In plants, there are two different types of PARP, and Arabidopsis PARP-1 shows high homology to human PARP-1, including a conserved caspase-3 recognition site (DSVD-N; Woltering et al., 2002). The PARP has been used as a

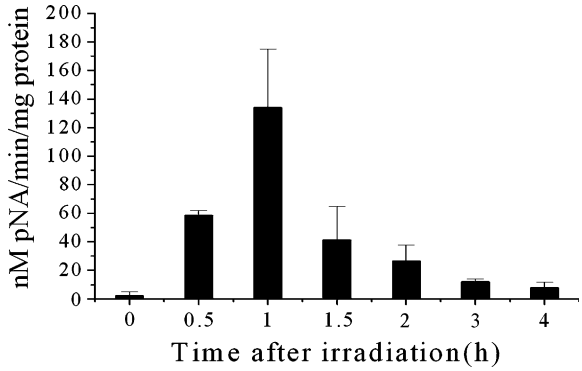

Figure 9. Caspase-3-like activity tested in an in vitro assay using the caspase-3 substrate. The extracts from protoplasts irradiated with UV-C and kept in the light for 0.5, 1, 1.5, 2, 3, or 4 h were used to test caspase-3-like activity. The samples were incubated with the caspase-3 substrate Ac-DEVD-pNA (200 μM) in assay buffer containing phenylmethylsulfonyl fluoride and EDTA. Error bars indicate SD values for five replicates.

substrate to study proteolytic activity in plant cells undergoing PCD. For example, exogenous (bovine) PARP has been found to be endoproteolytically cleaved by extracts from fungus-infected cowpea plants that were developing a HR but not by extracts from noninfected leaves. This cleavage activity was inhibited by caspase-3 inhibitor (Ac-DEVD-CHO) but not by caspase-1 inhibitor (Ac-YVAD-CHO; D'Silva et al., 1998). Moreover, it has also been found that the cleavage of endogenous (plant) PARP occurred during menadione-induced PCD in tobacco protoplasts, and this was inhibited by caspase-3 inhibitor (Ac-DEVD-CHO; Sun et al., 1999). In addition, it has been demonstrated that the degradation of plant PARP during PCD was dependent on the release of cytochrome *c* into the cytosol (Amor et al., 1998; Sun et al., 1999). These experiments suggest the existence of caspase-3-like activity and the presence of a caspase-3-like activating pathway during plant PCD (Amor et al., 1998; D'Silva et al., 1998; Sun et al., 1999; Woltering et al., 2002).

Because caspase-3 activation is a landmark event in apoptosis, the detection of caspase-3 activation and the measurement of caspase-3-like activity have been widely used as a definitive way of detecting PCD in animals and plants, respectively. Although caspase-3 activation could be studied in animals by western blotting using anti-caspase-3 antibody, and caspase-3-like activity could also be measured in plants using caspase-3 activity detection kits, these techniques are time consuming and cannot be used to dig out the more specific details of the caspase-3-like activity in real time and at the single cell level (Belenghi et al., 2004; Chichkova et al., 2004; Danon et al., 2004; Zuppini et al., 2004). As a noninvasive and stable technique for the spatiotemporal monitoring of living cell protein-protein interactions, FRET has been demonstrated to be superior over other protein interaction reporter assays and become a powerful tool for studying the cellular events (Immink et al., 2002; Seidel et al., 2004; Vermeer et al., 2004). Using the FRET technique to study MADS box transcription factor interactions, it was found that, in addition to the FLORAL BINDING PROTEIN11 (FBP11) heterodimers, homodimers of FBP2, FBP5, and FBP9 were also formed in living plant cells, which could not be detected by the yeast two-hybrid systems (Immink et al., 2002). The findings obtained in staurosporine-induced COS-7 cell apoptosis by FRET imaging confirmed that once caspase-3 activation is initiated, it moves to completion within 5 min or less. Individual cells initiate their response at quite different times; therefore, when this is averaged out over the cell population, the apparently "slow" increases in caspase-3 activity would be observed, which could not be measured by western blotting or other approaches (Tyas et al., 2000).

This contribution primarily establishes a method that is capable of in vivo, real-time detection of caspase-3-like protease activation during UV-C-induced plant PCD based on the principle of FRET analysis. Microscopic observations and the analysis of emission spectra and acceptor photobleaching first demonstrated that the constructed caspase-3 substrate could be expressed correctly and the FRET from ECFP to EYFP could be found in transfected Arabidopsis protoplasts (Figs. 2–4). Cleavage of the ECFP-DEVD-EYFP fusion protein and activation of caspase-3-like protease were indicated by the decreases in the fluorescence intensity of EYFP and in the EYFP/ECFP emission ratio, which reflected a decrease in FRET from ECFP to EYFP. Clearly, cleavage of the ECFP-DEVD-EYFP fusion protein occurred at 30 min after UV-C irradiation when compared with the transfected protoplasts without UV-C exposure (Fig. 5, control). The data from Figure 5 also demonstrate that once caspase-3-like protease activation is initiated, it moves to completion within 60 min, which is not consistent with the results obtained in COS-7 cells, in which caspase-3 activation from initiation to completion needs only 5 min or less (Tyas et al., 2000). This discrepancy may result mainly from the intrinsic difference in the regulation of the PCD machinery between plants and animals. Alternatively, it may be due to the different methods used for induction of PCD.

UV-C stress has been shown to induce plant PCD mediated by caspase-like activity, and the DEVDase (caspase-3-like protease) activity could be detected at 30 min after UV-C irradiation (Danon et al., 2004). A recent study has further shown the important role of reactive oxygen species and mitochondria in participating in UV-C-induced Arabidopsis protoplast PCD (Gao et al., 2008). Therefore, UV-C is a very convenient trigger to induce PCD in plants and protoplasts in a reproducible manner and can be used to as a strength stimulus to study intrinsic plant PCD pathways. In this study, it was found that after UV-C exposure, the EYFP/ECFP FRET ratio remained at its baseline for 30 min and then decreased gradually (Fig. 5), suggesting that the caspase-3 probe was cleaved at this stage and the caspase-3-like protease was activated. The decrease in the EYFP/ECFP emission ratio induced by UV-C exposure was completely blocked when the critical Asp at the fourth residue of the cleavage motif was substituted with a Gly (Fig. 6), indicating that the decrease in the EYFP/ECFP emission ratio was due to the cleavage of EYFP-DEVD-ECFP fusion protein but not to background fluorescence interference. The results of experiments using the caspase-3 inhibitor showed no decrease in the EYFP/ECFP emission ratio induced by UV-C exposure (Fig. 7), confirming that the observed decrease in the EYFP/ECFP emission ratio was attributed to the activity of caspase-3-like protease. The activation of caspase-3-like protease induced by UV-C exposure was further strengthened by western blotting, showing that the cleavage of the EYFP/ECFP protein indeed occurs in the EYFP-DEVD-ECFP fusion protein but not in the EYFP-DEVG-ECFP fusion protein (Fig. 8). Interestingly, when compared with results obtained by western blotting and the data obtained by FRET imaging (Figs. 5 and 8A), it is found

that at the single cell level the initiation and completion of caspase-3-like protease activation appeared to be more rapid than at the cell population level. This may be due to the fact that individual cells initiate their responses at quite different times (data not shown; Tyas et al., 2000). Actually, the in vitro measurement of DEVD activity using the synthetic fluorogenic substrates to caspase-3 demonstrated that the timing of the activation of caspase-3-like protease was in good agreement with the findings obtained by Danon et al. (2004; Fig. 9). These experiments demonstrate that the changes in FRET from ECFP to EYFP could in vivo monitor the degree of ECFP-DEVD-EYFP cleavage and the activation of caspase-3-like protease at the single cell level. It should be pointed out that the in vitro detection of the cleavage of the EYFP-DEVD-ECFP fusion protein by human caspase-3 would further strengthen these findings.

In summary, a FRET probe has been successfully constructed and used to detect caspase-3-like protease activation in Arabidopsis protoplasts undergoing PCD. These preliminary experimental results represent direct in vivo evidence for the activation of caspase-3-like protease during UV-C-induced Arabidopsis PCD and demonstrate the availability of the FRET probe in detecting caspase-3-like protease activity in real time at the single cell level. The FRET probe presented here would allow study of the activation of caspase-3-like protease and the key events of PCD in specific tissues or cells using tissue-specific or cell type-specific promoters and transgenic plants. As a powerful tool for studying protein-protein interactions and protease activity, FRET will be attractive and useful in visualizing the signaling pathway underlying plant PCD in living cells and unraveling the essential components of PCD that are highly conserved in eukaryotes. It should be noted that, at present, we do not know whether the EYFP/ECFP construct could enter the nucleus to detect the activity of caspase-3-like protease, which has been suggested to be present inside the nucleus (Elbaz et al., 2002; Chichkova et al., 2004). Further investigation is needed to address this question and to confirm the stability and reliability of caspase-3 probe when extensively used in plants.

MATERIALS AND METHODS

Plant Material

Plants of Arabidopsis (*Arabidopsis thaliana* Columbia-0) were grown in soil culture in a plant growth chamber (Conviron model E7/2) with a 16-h-light photoperiod (120 μmol photons m^{-2} s^{-1}) and a relative humidity of 80%/85% (light/dark) at 22°C/20°C (light/dark) for 3 to 4 weeks.

Plasmid Constructs

ECFP and EYFP were amplified by PCR under the templates of pECFP and pEYFP (Clontech Laboratories), respectively. ECFP was amplified using the following primers: forward, 5′-TCGACGAATTCGACGAGATGTCTAGAGAGAAGGACCTGAAGGTGAGCAAGGGCGAGG-3′; reverse, 5′-TTTGGGGTACCATCGACCTCATCTCCGCTGAGCTCGGACGAGGACTTGTACAGCTCGTCCAT-3′. EYFP was amplified using the following primers: forward, 5′-TTTGGGGTACCAGCGGAAGCGAATTCGTGAGCAAGGGCGAGGAG-3′; reverse, 5′-TTTCGCTCGAGGGATCCGCGGCCGCTTACTTGTACAGCTCGTCCAT-3′. The PCR products amplified from ECFP and EYFP were digested with *Eco*RI-*Kpn*I and *Kpn*I-*Bam*HI, respectively, and then gel purified and three-way ligated into the sites of the clone vector pUC18 to yield the pUC-ECFP-DEVD-EYFP plasmid. There was an *Xba*I restrictive site behind the *Eco*RI site of the ECFP PCR product. The fragment ECFP-DEVD-EYFP was digested with *Xba*I-*Bam*HI from pUC-ECFP-DEVD-EYFP plasmid and cloned in the expression vector PIB1 to yield the PI-ECFP-DEVD-EYFP plasmid under the control of the cauliflower mosaic virus 35S promoter as C-terminal fusions after excision of the GFP gene from the vector. This method of construction introduces an 18-amino acid linker peptide (SSSELSGDEVDGTSGSEF), which joins the C terminus of ECFP to the N terminus of EYFP (Fig. 1A). To yield the ECFP-DEVG-EYFP construct, the critical Asp residue in the caspase-3 cleavage site was substituted with a Gly residue by PCR using the forward primer of ECFP and the mutagenic primer (5′-TTTGGGGTACCACCGACCTCATCTCCGCTGAGCTCGGACGAGGACTTGTACAGCTCGTCCAT-3′; Fig. 1B). The complete sequence integrity of all constructs was confirmed by DNA sequencing.

Isolation and Transient Transfection of Mesophyll Protoplasts

The isolation and transient transfection of protoplasts from Arabidopsis plants (21–27 d old) were performed at room temperature, according to a modified procedure as described previously (He et al., 2006; Zhang and Xing, 2008). Healthy leaves were sliced with a razor blade into small leaf strips (0.5–1 mm), which were vacuum infiltrated with enzyme solution (1%–1.5% [w/v] cellulase R10 [Yakult Honsha], 0.2%–0.4% [w/v] macerozyme R10 [Yakult Honsha], 0.4 M mannitol, 20 mM MES, pH 5.7, 20 mM KCl, 10 mM $CaCl_2$, and 0.1% [w/v] bovine serum albumin) for 10 to 20 min and then incubated in the dark at room temperature for 3 h without shaking. Protoplasts were isolated by filtration through 75-μm nylon mesh sieves and collected by centrifugation at 100*g* for 3 min. The purified protoplasts were washed three times with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glc, and 1.5 mM MES-KOH, pH 5.6) and finally suspended in W5 solution. Protoplasts were counted in a hematocytometer. Before transfection, the protoplasts in W5 solution were bathed in ice for 30 min, and the supernatant was removed as much as possible without touching the protoplast pellet. The protoplasts were resuspended in mannitol-$MgCl_2$ solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES pH 5.7) at room temperature, and the concentration was adjusted to about 2×10^5 protoplasts mL^{-1} with the mannitol-$MgCl_2$ solution. For transfection, 100 μL of protoplasts in mannitol-$MgCl_2$ solution was incubated with 10 to 20 μg of plasmid DNA and 120 μL of polyethylene glycol (PEG) solution (40% [w/v] PEG-4000, 0.2 M mannitol, and 0.1 M $CaCl_2$). After incubation for 5 to 30 min at room temperature, 0.5 mL of W5 solution was added to stop the transfection. After centrifugation for removing PEG solution, the protoplasts were collected and resuspended in 250 μL of W5 solution (24-well plates). Transfected protoplasts were left in the dark at room temperature for 8 to 20 h to allow the expression of ECFP-DEVD-EYFP or ECFP-DEVG-EYFP fusion protein.

UV-C Irradiation of Protoplasts

Cell suspension cultures of transfected protoplasts were placed on 24-well plates and incubated with or without caspase-3 inhibitor (Ac-DEVD-CHO [100 μM]; Calbiochem) for 1 h in the dark at room temperature. After incubation, the cell cultures were irradiated using a 254-nm UV lamp (EF-180C; Spectroline). The UV-C energy delivered in each experiment was measured by a laser power/energy meter (LPE-1A; Physcience). After UV-C treatment, the protoplasts were placed in white light (90–100 μmol photons m^{-2} s^{-1}) at room temperature for the indicated time periods.

Confocal Microscopy, FRET, and Acceptor Photobleaching Analysis

A Zeiss Observer Z1 epifluorescence motorized microscope coupled to a Zeiss LSM 510 META system was used. The system was controlled using LSM software (version 4.2). For spectral imaging, the HFT 458/514 beam splitter was used in combination with the 32-channel spectral detector and optical

grating system to collect lambda stacks consisting of a series of x-y images at emission wavelengths (464–604 nm) separated by 10-nm steps. Photobleaching of the acceptor of FRET was performed using the Zeiss LSM 510 META laser scanning confocal microscope. For FRET detection, the 458-nm laser line from an argon ion laser was used for ECFP excitation. A Zeiss Plan-Neofluar 40× (numerical aperture = 1.3) oil-immersion objective lens was used. Images were acquired through three channels. Two band-pass (BP) filters (BP 465–510 nm and BP 520–550 nm) were used for ECFP and EYFP, respectively, and the chlorophyll autofluorescence was collected with a 650- to 700-nm BP filter. The 514-nm laser line from an argon ion laser was used for photobleaching the acceptor of ECFP-DEVD-ECFP. All of the quantitative analysis of the fluorescence images was performed using Zeiss Rel4.2 image-processing software.

Protein Extraction, Western Blotting, and Caspase-3-Like Activity Assays

Protoplasts (2×10^5 protoplast mL^{-1}) expressing ECFP-DEVD-EYFP or ECFP-DEVG-EYFP fusion protein were placed on 24-well plates and irradiated with UV-C of 15 kJ m^{-2}. Then, the protoplasts were kept in white light (90–100 μmol quanta m^{-2} s^{-1}). At the indicated time points, protoplasts were harvested and kept at −80°C until they were to be analyzed. For protein extraction, the treated protoplasts were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 15 mM NaCl, 1% Triton X-100, and 100 mg mL^{-1} phenylmethylsulfonyl fluoride) and incubated on ice with gentle shaking on a level shaker for 30 min. Samples were centrifuged for 5 min at 12,000*g* and 4°C, and the supernatants were transferred to new 1.5-mL tubes. Protein concentrations were determined by the method of Bradford (1976). The samples were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The resulting membrane was blocked with 5% skim milk and incubated with the polyclonal antibody against GFP (Beyotime) followed by IRDye 800 secondary antibody (Rockland Immunochemicals). Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR). Caspase-3-like activity was measured by determining the cleavage of the fluorogenic caspase-3 substrate Ac-DEVD-pNA using supernatant prepared from cell lysates. Reactions were adjusted to 100 μL in assay buffer (20% glycerol, 0.1% Triton, 10 mM EDTA, 3 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 50 mM sodium acetate, pH 7.4) and incubated with Ac-DEVD-pNA (200 μM final concentration) at room temperature for 2 h. The extent of Ac-DEVD-pNA cleavage was measured as the change in A_{405} because of the release of free fluorescent pNA.

ACKNOWLEDGMENTS

We are grateful to Professor Hongqing Li (School of Life Science, South China Normal University) for providing the plasmid PIB1. We thank Mrs. Yonghong Tang for her help using the laser scanning confocal microscope in our research group. We thank Dr. Feng Wen, Xichao Wang, Xianwang Wang, Liujing Lai, and Lei Liu at our institute for their help with revision of the manuscript. We thank four anonymous reviewers for their constructive comments on the manuscript.

Received June 30, 2008; accepted June 11, 2009; published June 17, 2009.

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