Identification of programmed cell death in situ in individual plant cells in vivo using a chromosome preparation technique

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Abstract

A simple procedure, which combines a chromosome preparation technique with an in situ labelling technique modified from fluorescence in situ hybridization (FISH), has been developed for in situ detection of plant programmed cell death (PCD) at the single-cell level. After exposure of chromosomes and nuclei on slides by enzymolysis, Klenow or TdT was used to incorporate Bio-dUTP or fluorescein-dUTP at sites of DNA breaks. After Klenow-mediated labelling, the signals were amplified by a cascade of antigen–antibody reaction according to the detection system of FISH. This method enables in situ detection of plant PCD in vivo morphologically and biochemically at the chromosome, nuclear and DNA levels without cell culture and histological sectioning. This technique permits labelling of DNA breaks with high sensitivity due to increased chromosome and nucleus exposure to the labelling solutions, as well as due to the immunological amplification of the signals. Moreover, the changes in the cells were easier to be observed because the spatial obstacle of the cell wall and its autofluorescence were eliminated. It is potentially useful for in situ detection of PCD in plant root meristematic cells triggered by various environmental abiotic factors. It is proposed that the root tip is a versatile in vivo system for studying PCD induced by environmental abiotic factors.

Key words: Chromosome preparation, FISH, in situ end labelling, plants, programmed cell death.

Introduction

Programmed cell death (PCD) plays a major role in disease and defence against adverse environments, as well as in cell and tissue homeostasis and specialization, and tissue sculpting (Greenberg, 1996). Biochemically, it seems that chromatin cleavage is the most characteristic feature of PCD (Gavrieli et al., 1992; Tian et al., 1991). In situ detection methods are dependent on the labelling and detection of cleaved fragments. ISEL (in situ end labelling), TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) and ISNT (in situ nick translation) are three methods that can be used to label these DNA breaks in various tissues. The ISEL method uses the Klenow fragment of DNA Polymerase I to incorporate labelled nucleotides into the DNA strand breaks that occur during internucleosomal cleavage, while the TUNEL method uses TdT to end label 3'-OH groups exposed during the cleavage process (Gorczyca et al., 1993).

The TUNEL-positive reaction is considered as a good specific criterion of death by PCD in animals (Kressel and Groscurth, 1994), but some researchers using plant tissues have reported that sample preparation of histological sectioning including fixation, embedding and sectioning, can cause sufficient nicking of nuclear DNA to produce false TUNEL positivity (Wang et al., 1996). Nevertheless, the TUNEL reaction is more specific to PCD when associated with morphological and time-course data, than other death markers such as fluorescein diacetate (FDA) and Evan’s Blue. Additionally, the fixation procedure is simpler when using a protoplast or cell.
culture population and has not been reported to induce false TUNEL positive labelling (Danon et al., 2000).

Morphologically, PCD, known as apoptosis, is generally characterized by a subset of changes such as chromatin and cytoplasm condensation (Vaux, 1993). Little is known about apoptosis in plants, including the morphological changes (Danon et al., 2000). Although some accumulating evidence suggests that some features of plant apoptosis such as nuclear disintegration and chromatin condensation triggered endogenously or environmentally are similar to those in animals (reviewed by Danon et al., 2000; Lam et al., 1999; Vaux and Korsmeyer, 1999), other features such as cytoplasm shrinkage, nuclear periphery and the formation of apoptotic bodies have not been universally identified (Danon and Gallois, 1998; Gunawardena et al., 2001; Stein and Hansen, 1999).

Material for current studies of PCD in animals and plants has been obtained predominantly from two different systems, cell culture for in vitro studies and histological sectioning for in vivo studies (Stein and Hansen, 1999). On tissue sections, PCD changes can only be detected at the tissue level without detailed description in individual cells unless ultramicroscopy is used (Gunawardena et al., 2001), and there exists low sensitivity due to poor penetration without pretreatment. Especially in plants, the cell wall autofluoresces, resulting in high background that increases as a result of the pretreatment process (Wang et al., 1996). It makes changes in the cells undergoing PCD difficult to visualize. In addition, it has been reported that section preparation including fixation, embedding and sectioning, can cause sufficient nicking of nuclear DNA to produce false TUNEL positive nuclei. Therefore, sectioning techniques should be used very cautiously in plants (Wang et al., 1996). Cell culture systems offer the possibility to study apoptosis at the biochemical and molecular levels in a rather homogeneous cell population (Koukalova et al., 1997), and the fixation protocol is simpler when using a protoplast or cell culture population and has not been reported to induce false TUNEL positive labelling (Danon et al., 2000). Most of the current knowledge about the nature of PCD has come from cell culture systems. However, a question arises as to whether PCD observed in vitro also occurs in whole plants (Koukalova et al., 1997). Also, a hallmark of PCD, DNA laddering in cell culture may be caused by mycoplasma endonucleases (Paddenberg et al., 1996). So, in vitro systems might be more biologically relevant than in vitro systems. Therefore, it is necessary to develop more effective techniques for the detection of in vivo plant PCD, both morphologically and biochemically.

The root tip of various plant species is generally one of the most sensitive tissues to various environmental impacts (Katsuhara and Kawasaki, 1996), and has previously been used for studying PCD induced by external abiotic factors (Katsuhara, 1997; Stein and Hansen, 1999). Roots have been also used for studying PCD during aerenchyma formation (reviewed by Drew et al., 2000) and during sloughing of root cap cells (Wang et al., 1996). In this work, a method was developed, which is based on a chromosome preparation and in situ labelling (ISEL and TUNEL) techniques. After exposure of chromosomes and nuclei on slides by enzymolysis, Klenow or TdT was used to incorporate Bio-dUTP or fluorescein-dUTP at the sites of DNA breaks. After Klenow-mediated labelling, the signal was amplified by a cascade of antigen–antibody reactions according to the detection system of fluorescence in situ hybridization (FISH). It enables detection of PCD in situ at the chromosome, nuclear and DNA levels without cell culture and histological sectioning.

Materials and methods

Plant material, germination and cell death induction

Seedlings were grown and cell death was induced by the methods described previously (Katsuhara, 1997) with some modification. Sterilized seeds of maize (Ze a mays L.) ‘Yidan 6’ were soaked in double-distilled (dd) H2O for about 1 d with aeration. Germinated seeds were transplanted into a nutrient solution containing 4 mM KNO3, 1 mM NaH2PO4, 1 mM MgSO4, 1 mM CaCl2, and 1 mg l−1 Fe-citrate (pH 6.0 with NaOH) at 28 °C with a 13/11 h light/dark cycle. When roots were approximately 2 cm long, the younger plants were subcultured into 500 mM NaCl dissolved in a replaced nutrient solution for varying time periods (Katsuhara, 1997). This concentration (500 mM NaCl) was chosen by a dose-effect experiment and was appropriate for analysis of the levels of cell death as below. A parallel culture was grown without subcultivation at 28 °C as a positive control.

Sample preparation for in situ end labelling

The apical 1–2 mm of the roots was excised from the treated seedlings and positive controls at the end of each incubation period, and fixed in 2% paraformaldehyde for 2 h at room temperature with gentle rotation. Then the root caps were removed. Roots of this length have not initiated physiological death processes (Katsuhara, 1997), and include a mass of mitotic cells. After washing with water to remove the fixative, the root tips were incubated in 2% pepsin and 2% cellulase (SERVA corp., New York, USA), in 6.8 mM of CaCl2, 11 mM of KH2PO4, 0.6 M of mannitol, and 4 g l−1 polyvinyl-pyrrolidone (PVP), pH 5.8, at 28 °C for approximately 3 h. After gentle rinsing with ddH2O, 2 or 3 softened tips were subsequently squashed with fixative onto a clean cool slide, and spread with a tweezer. The slide was then air-dried (Song and Gustafson, 1995).

In situ end labelling with Klenow

In order to analyse the distribution of DNA breaks on metaphase chromosomes, the large fragment of DNA polymerase I (Klenow) was used instead of the holoenzyme of DNA polymerase I. The latter can extend DNA strands from DNA
nicks and is not appropriate for analysis of the sites of DNA nicks on metaphase chromosomes. While the former can localize the DNA breaks because it only incorporates dNTP at DNA nicks.

An ISEL protocol modified from the probe labelling procedure of FISH (Song and Gustafson, 1995) was carried out on the prepared samples. Samples were dehydrated in a graded ice-cold ethanol series (70%, 95%, 100%) before labelling with the labelling mixture, which contained 30 µl of dNTP (equal amount of dATP, dCTP and dGTP, 30 µM of each), 10 µl of Bio-11-dUTP (30 µM), 10 µl of 10 x Buffer of Klenow, 2 µl of Klenow (Sigma, Missouri, USA) (5 U µl⁻¹) and 48 µl of ddH₂O. The slides were covered with clear cover slips, bubbles were removed, and the slides were then incubated at 15 °C in a humid chamber for 2 h. The labelling reaction was stopped by washing in 0.5 M of EDTA solution (in ddH₂O).

The FISH detection system (Dong and Quick, 1995) was adopted with modifications. The cover slips were removed and the samples were washed in PBS (pH 7.4) for 5 min three times. The slides were then covered with 40 µl of 1% (w/v) goat anti-biotin-FITC (Sigma, diluted in PBS) and subsequently incubated with 40 µl of 0.5% (w/v) rabbit anti-goat-biotin antibody (diluted in PBS 1% BSA), then, the incubation process was repeated twice, at 37 °C for 30 min each with washing in PBS for 3 x 5 min between each step in this process.

**TUNEL assay**

Samples were dehydrated in a graded ethanol series as above before labelling. In situ nick end labelling of nuclear DNA fragmentation was performed in a humid chamber for 1 h in the dark at 37 °C with a commercially available TUNEL kit (fluorescein, in situ Cell Death Detection Kit, Boehringer Mannheim, Mannheim, Germany), at a dilution of 1:2 in reaction buffer. The TUNEL reaction incorporated fluorescein-dUTP at DNA breaks.

For each ISEL and TUNEL labelling sample, both negative and positive controls were arranged. For negative controls, salt-stressed samples were labelled in parallel as above except for the absence of Klenow or TdT enzyme. For positive controls, samples without subcultivation (without salt stress) were treated with 5 U µl⁻¹ DNase I for 10 min at 37 °C before labelling as above (Gavrieli et al., 1992).

After being labelled by either ISEL or TUNEL, the slides were rinsed in PBS for 3 x 5 min and counterstained with 3 µg ml⁻¹ propidium iodide (PI) containing 10 µg ml⁻¹ anti-fade agent, p-phenylenediamine dihydrochloride, for fluorescence microscopy (Olympus BX60, Japan). Computer images were captured with a SenSys 1401E digital camera.

At any given time point, the percentage of cells labelled with Klenow minus those labelled with TdT represented the percentage of necrotic cells, since Klenow labels both apoptotic and necrotic cells simultaneously, yet TdT only labels apoptotic cells (Kressel and Groscurth, 1994).

**Image processing and densitometric analysis**

The surface plots and the values of FITC intensity of chromosomes or nuclei were determined with the software ‘Scion Image’ (Release beta 3b, Scion Corporation, NIH, USA). The values of FITC intensity were indicated by the pixel intensity values (PIVs) of FITC (Fath et al., 2001).

**DNA extraction and gel electrophoresis**

Maize root tissues exposed to different time-courses of salt treatments were frozen in liquid nitrogen and ground to fine powder. DNA was isolated by means of phenol–chloroform extraction (Doyle and Doyle, 1990). Extracted DNAs were treated with 25 µg ml⁻¹ DNase-free RNase A (Sigma, Missouri, USA) for 1 h at 37 °C, before electrophoresis on a 1% agarose gel. After electrophoresis, the gel was stained with 0.5 µg ml⁻¹ ethidium bromide for 30 min, destained in 1 mM MgSO₄ for 1 h, and observed and photographed on a UV light box (Stein and Hansen, 1999).

**Results**

On the NaCl-treated samples labelled by Klenow, the FITC fluorescence intensity indicative of the degree of DNA cleavage (Fig. 1 A–F, G–L, M–R; Fig. 2) and the percentage of FITC-labelled cells (Fig. 3) increased dramatically as the treatment continued. At 0 h, all of the chromosomes and nuclei only fluoresced with PI (red) without visible FITC signal (Fig. 1A, G, W; Fig. 3). NaCl treatment for 1.5 h showed about 30% FITC-stained cells (Fig. 3), and the mean FITC relative intensity (PIV) of the FITC-distributed regions of nuclei and chromosomes was about 200 and 120, respectively (Fig. 2). By 3.5 h of the treatment, the fraction of FITC-labelled cells increased to over 70% (Fig. 3). By 5.5 h, almost all cells showed a high degree of DNA cleavage indicated by their bright FITC fluorescence (Fig. 1X), and the PIV of each cell was over 240 (Fig. 2). The signals exhibited were so strong that the whole chromosomes and nuclei were covered with bright FITC (Fig. 1E, F, K, L). The high intensity of FITC indicated the condensation of chromatin in the nuclei (Stein and Hansen, 1999).

It was noted that the FITC signals were inclined to appear on the same sites of the sister chromatids of each chromosome (Fig. 1B), and were first apparent in the terminal regions of the chromosomes (Fig. 1, C) and lastly in the centromere regions. This implied that, at first, large segments were produced and then they were further cleaved into smaller segments. By comparison, agarose gel electrophoresis of DNA extracted from treated roots showed that there was an increase in DNA fragmentation during the treatment period, forming a laddering pattern (Fig. 4). The regular distribution of FITC signals and the steady increase in FITC intensity indicate that the cleavage of nuclear DNA was under regulation during the cell death process.

Morphologically, at 0 h, the chromosomes and nuclei were intact (Fig. 1A, G, W). However, as the treatment progressed, both chromosomes and nuclei showed an increase in the degree of disorganization, which was accompanied by the increase in the degree of DNA cleavage. Between 1.5 h and 4.5 h of treatment, the staining in many of the nuclei had a crescent shape pattern (Fig. 11, J). In longer incubations, this crescent...
Fig. 1. Identification of maize cell death in individual cells by using Klenow-mediated in situ end labelling. The maize roots were treated with 500 mM NaCl. The prepared chromosomes and nuclei from the treated root tips were labelled with Bio-11-dUTP and detected with goat anti-biotin-FITC. Bars in (L), (V) and (X) represent 5 μm. (A–F) Chromosomes at metaphase (1155×). (A) At 0 h, the chromosomes show no FITC signal, and are intact and regular in shape. (B) 0.5 h of treatment, the chromosomes are intact and regular in shape yet, with slight FITC signals on the same sites of the sister chromatins of each chromosome. (C) 1.5 h of treatment, morphological changes are not obvious, but strong FITC in the terminal regions is observed. (D) 2.5 h of treatment. Intensive FITC was displayed on each chromosome, but only slight deformation is found. (E) 4.5 h of treatment, the chromosomes are further deformed, and the high intensity of FITC indicates that they are condensed. (F) 6.5 h of stress, showing further condensation in morphology. (G–L) Interphase nuclei (1155×). (G) At 0 h, the nucleus is stained red with PI, and is intact and round in shape. (H) 0.5 h of treatment. The nucleus is intact and round in shape except for slight FITC in local regions. (I) 2.5 h of treatment. The nucleus is deformed and locally condensed. (J) 4.5 h of treatment. Periphery of condensed nuclear content is observed. (K, L) 6.5 h of treatment. Degraded nuclei (without nucleoli) are further condensed and fragmented. (M–R) The surface FITC intensity plots of the Klenow-labelled nuclei of the left row. The surface FITC intensity plots were obtained using the software 'Scion Image', as described in the Materials and methods. (S–V) Controls (1155×). (S, T) Negative controls. Samples treated with 500 mM NaCl for 4.5 h, were labelled as above, except that the Klenow enzyme was omitted. No FITC was detected in the chromosomes and nuclei. (U, V) Positive controls. Samples were cultured without salt treatment. Prior to labelling, cells were pretreated with 5 U μl⁻¹ DNase I for 10 min at 37 °C. Both of the chromosomes and nucleus are labelled, but they are intact in shape (no morphological change). (W, X) Labelled samples were observed under a lower magnification (460×). (W) At 0 h. Cells are stained with PI, red in colour and intact in shape. (X) 5.5 h of treatment. The nuclei are labelled with FITC, and are deformed (with nucleoli) or degraded (without nucleoli).
expanded till the whole nucleus was stained, and eventually became condensed and fragmented (Fig. 1K, L). By 1.5 h of treatment, the chromosomes and nuclei had no visible morphological changes, but the DNA was obviously cleaved (Fig. 1C, h). It can be concluded that the DNA breaks occurred prior to changes in cell morphology. By 2.5 h, both chromosomes and nuclei were condensed and disorganized as well as a high degree of cleavage of DNA (Fig. 1D, I). Degradation of the nucleoli became visible by 4.5 h. After this stage, condensation and fragmentation of chromosomes and nuclei were observed (Fig. 1E, F, J–L). A disintegrating nucleus with nucleoli is said to have deformed, while one without nucleoli is said to have degraded (Katsuhara and Kawasaki, 1996). In conclusion, cleavage of DNA occurred before the shrinkage and fragmentation of chromosomes, followed by nuclear deformation and fragmentation, and then the degradation of its nucleolus occurred.

The TUNEL reaction was developed specifically to detect in situ DNA fragmentation occurring in apoptotic processes and has frequently been employed in animals (Barrett et al., 2001; Gavrieli et al., 1992; Gorczyca et al., 1993; Kressel and Groscurth, 1994) and higher plants (Jones et al., 2001; Weir, 2001). The results obtained by Klenow-mediated ISEL were compared with those of TUNEL. TUNEL resulted in fewer cells fluorescing with FITC at any given time point (Fig. 3; Fig. 5B). 1.5 h of treatment resulted in about 30% of the cells being TUNEL-positive, and the rise in the percentage of FITC-stained cells continued till 4.5 h of treatment. A longer induction decreased the number of TUNEL-stained cells (Fig. 3), and increased the FITC intensity in most of them, while the number of Klenow-stained cells increased with induction time till 6.5 h of treatment (Fig. 3). This suggested that salt stress induced necrosis rather than PCD after 4.5 h. The necrotic cells with...
disintegrated morphology were not labelled using the TUNEL reaction, but were labelled using the Klenow-mediated in situ labelling. At a given time point, the number of necrotic cells could be determined, which was the difference between the numbers of Klenow-labelled cells and those of TdT-labelled cells.

In order to check the results of in situ labelling mediated by Klenow and TdT, both negative and positive controls were arranged for each test sample. The negative controls were the roots treated and labelled in parallel with the test samples, except that Klenow or TdT enzyme was omitted. All cells of the negative controls only fluoresced with PI (red) without visible FITC signal (Fig. 1S, T), and no FITC-labelled cell was observed, either in ISEL or TUNEL (Fig. 5A). The positive controls, which were the roots cultured without salt stress, were pretreated with DNase I before labelling in parallel with test samples. Both of the TdT- and Klenow-mediated labelling assays showed that pretreatment with DNase I caused an intensive staining of all chromosomes and nuclei (Fig. 1U, V).

Discussion

Salt stress, especially at a higher concentration of NaCl, is toxic to plant cells (Katsuhara, 1997). The present results show that, at the chromosome, nuclear and DNA levels, the toxicity of NaCl in maize roots is associated with the main features characteristic of animal PCD: DNA fragmentation (TUNEL-positive and DNA laddering pattern), chromatin condensation and fragmentation. Nevertheless, PCD induced by salt stress in maize was not absolutely identical to that in animals. Apoptotic bodies were not observed. As the data reported previously, in most plant PCD, and even in some animal PCD, no apoptotic bodies were detected either. Apoptotic bodies may not be functionally necessary for plants due to the absence of possible phagocytosis by adjacent cells in the presence of a cell wall (Danon et al., 2000). PCD has often been observed without the formation of apoptotic bodies.

As in animals, DNA fragmentation, and chromatin condensation and fragmentation have been well documented in a variety of plant PCD systems (reviewed by Danon et al., 2000). However, the distribution of DNA breaks on chromosomes and morphological changes of chromosomes have not been reported to date. Interestingly, in this study, FITC signals tended to appear on the same sites in sister chromatids (Fig. 1B), suggesting that they probably represented some specific endonuclease sites, since DNA could only be cut specifically by endonucleases in vivo. Another phenomenon was that FITC signals were first apparent in the terminal regions of the chromosomes (Fig. 1C) and last in the centromere regions (Fig. 1D). This is most likely to be because, the distal regions were the most decondensed parts of cereal chromosomes and contained a large number of actively transcribed genes which are highly active during the cell cycle of mitotically active cells. Therefore, such regions were more susceptible to endonucleases. Heterochromatin regions such as the centromere region, however, were presumably less amenable for access of endonucleases, since they were condensed during the cell cycle (Pedersen et al., 1997). The third phenomenon observed was that condensation and disorganization of nuclei occurred earlier than that of metaphase chromosomes, and FITC intensity of TUNEL-positive interphase nuclei also increased more rapidly than that of TUNEL-positive metaphase chromosomes (Figs 1, 2). It is likely that the chromatin in interphase nuclei was much more decondensed than that of metaphase chromosomes, and was more accessible to endonucleases. In the positive controls, intensive DNA fragmentation (intensive FITC signals) did not result in disorganization of chromosomes and nuclei (Fig. 1U, V). This indicates that DNA is dispensable for keeping the morphology of chromosomes and nuclei. Therefore, the disorganization
of chromosomes and nuclei in the PCD-occurring cells should result from protease activity-involved hydrolysis of chromosomal and nuclear skeleton proteins. Hence, nuclease and protease activities, two biochemical features of PCD, are implied in the PCD process.

These results show that DNA breakage occurred prior to the visible morphological changes in both nuclei and chromosomes during this PCD process. This is partly consistent with the pattern of DNA fragmentation in plant apoptosis: DNA fragmentation occurs in morphologically normal nuclei (round in shape), in deformed nuclei and in fragmented nuclei (Danon et al., 2000). It indicates that this pattern may be a universal marker of nuclear change during plant PCD. By contrast, it has been demonstrated that in some animal PCD, the DNA fragmentation occurred after the formation of separate apoptotic bodies or after final cell lysis (Collins et al., 1997). One possible explanation for the difference might be that, in these PCD processes, the related endonucleases were activated earlier than the related proteinases. Another possibility might be the differences between animal and plant genomes. The maize genome has a large number of unexpressed sequences, which are less important than functional genes. Therefore, it is possible that these unexpressed sequences are unstable and more easily degraded.

Previous studies (Katsuhara, 1997; Ryerson and Heath, 1996; Wang et al., 1996; S-B Ning, L Wang, Y-C Song, unpublished data) have shown that there are several serious defects such as low efficiency and false positives using the sectioning technique for in situ detection of PCD in plants. The approach developed in this study overcame these limitations and displayed several advantages as follows. (i) It allows in situ distinction between individual cells in vivo, both morphological and biochemical. Moreover, the change order of chromosomes and nuclei during PCD can be identified. In these results, the order is: cleavage of DNA occurred before the shrinkage and fragmentation of chromosomes, followed by nuclear deformation and fragmentation, and then the degradation of its nucleolus occurred. There is increasing evidence that as well as in animals, a large number of physical or chemical factors can induce PCD in plants besides the convincing examples which have been well described in in vitro-cultured tissues or suspension-cultured cells induced by UV irradiation (Danon and Gallois, 1998; Mitsuhashi et al., 1999), cold stress (Koukalova et al., 1997), KCN (Ryerson and Heath, 1996), heat shock (Balk et al., 1999), and mannose (Stein and Hansen, 1999), and using root meristematic cells induced by salt stress (Katsuhara, 1997) and by mannose (Stein and Hansen, 1999). (ii) Enables quantitation of DNA breakage in a single PCD-occurring cell using appropriate image process software, as well as quantitation of PCD process in a cell population. In particular, it allows analysis of DNA breaks on chromosomes. (iii) High sensitivity due to increased chromosome and nucleus exposure to the labelling solutions. In addition, the signals can be magnified by a cascade of antigen–antibody after labeling with Klenow. It has been reported TUNEL was over 10-fold more sensitive compared to ISNT assay in animals (Gorzyczka et al., 1993). But in these experiments, with immunological cascade amplification of signals, it was found that Klenow could even result in equivalent efficiency to TdT. (iv) Simple and direct investigation of cell death. The autofluorescence of the cell wall as a spatial obstacle is eliminated. In addition, the protoplast spreading protocol described by Danon and Gallois (Danon and Gallois, 1998) was simplified; polylysine was not used because it was found to be unnecessary.

Overall, the method presented here allows the analysis of sensitivity to DNA breakage in chromosomes and nuclei, as well as the observation of morphological changes in cells undergoing PCD, and is available for in situ analysis of PCD resulting from various adverse environments in plants in vivo, in situ. DNA damage in plant cells without PCD morphology is also amenable to this technique. Nevertheless, this method is more readily applicable for in situ detection of PCD in meristematic tissues induced by physical or chemical factors such as cold or salt stress rather than biotic or physiological ones. The understanding of PCD in plants is only at its initial stage. The main reason is a lack of suitable test materials, which can provide large amounts of PCD-occurring cells and synchronous cell populations (Zhao et al., 1999). Plant roots include a mass of mitotic cells and are very readily accessible. The meristematic cells are some of the most sensitive to environmental impacts (Katsuhara and Kawasaki, 1996), and PCD can be induced in a large population of cells (Stein and Hansen, 1999). It is proposed that the root tips of various plant species are a versatile in vivo induction system of PCD under environmental factors, and the method presented in this paper is a useful tool to study PCD in vivo.

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