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Detection of DNA ladder during cotyledon senescence in cotton

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Abstract

The asynchronous programmed cell death (PCD) in different regions of the cotton cotyledon was studied during senescence. We showed that changes in chlorophyll contents do not occur at the same time in different parts of the cotyledon. By using light microscopy (LM) and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end *in situ* labelling (TUNEL) methods, the symptoms of cell death were detected in cotyledon margins earlier than in the central part. DNA ladders were detected by gel electrophoresis in senescent cotyledon margins and in the centre respectively, but not in the whole cotyledons.

Additional key words: chlorophyll content, *Gossypium hirsutum*, programmed cell death, DNA fragmentation.

Introduction

Leaf senescence is a genetically regulated developmental programme: sequential events at the morphological, physiological and molecular levels are orchestrated and specific signatures of its stages can be identified (Lim *et al.* 2003). Full understanding of leaf senescence requires analysis of this process in different regions of leaves of different age (Kołodziejek *et al.* 2006).

Visible yellowing, which correlates with drop of chlorophyll content, change of cell structure and degradation of DNA occur during leaf senescence. One key event in programmed cell death (PCD) is DNA degradation, because the degradation of the genome is considered to be a means by which the cell death program is made irreversible and facilitates the disassembly of the nucleus (Ito and Fukuda 2002). Indeed, DNA laddering phenomenon is a very specific character of PCD in animals and plants (Danon *et al.* 2000, Zhang *et al.* 2006). DNA laddering is one of the easiest PCD markers to detect and is used in numerous systems (Wagstaff *et al.* 2003).

Internucleosomal DNA fragmentation, specific for PCD, is one of the earliest symptoms of leaf senescence (Leśniewska *et al.* 2000, Simeonova *et al.* 2000). Horii and Marubashi (2005) showed evidence of PCD (DNA laddering by agarose gel electrophoresis and DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays) for juvenile and young leaves of *Nicotiana tabacum*. However, other reported that PCD-associated cleavage is restricted exclusively to the final brown stage of leaf development in cucumber (Delorme *et al.* 2000). DNA degradation and the activation of proteases are also common to most plant PCD systems, where they have been studied; however, breakdown of DNA into nucleosomal units (DNA laddering) is not observed in all systems (Rogers 2005). Lee and Chen (2002) found no indication of DNA laddering in senescing rice leaves. Whereas, in all of these studies, the whole leaves from the seedlings have been used (Leśniewska *et al.* 2000, Simeonova *et al.* 2000, Horii and Marubashi 2005, Delorme *et al.* 2000, Lee and Chen 2002).

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Abbreviations: CTAB - cetyltrimethyl-ammonium bromide; PCD - programmed cell death; TE - tracheary element; TUNEL - terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end *in situ* labeling.
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Until now, the different studies interested in PCD during leaf senescence focused especially on the whole organ, and comparison of cell death of different leaf region to a lesser extent. Most of the data concerning PCD have been performed on experimental systems such as tobacco or *Arabidopsis* cell suspensions, and little information is available about other species at the organ level, even less the report about how to improve the detection of DNA ladder. Consequently, it is valuable to understand PCD at the tissue level (such as leaves) or the whole plant level (Fan and Xing 2004). Therefore, the aim of this work was studied the asynchronous cell death in cotton cotyledon of different regions. Consequently, explore the method of DNA ladder detection.

Materials and methods

Plants and growth conditions: Cotton (*Gossypium hirsutum* L. cv. Shannong NO.6) seeds were imbibed overnight in cold running tap water prior to planting in *Vermiculite*, and grown in the growth chamber at temperature of 30 ± 3 °C, relative humidity 50 %, and 12-h photoperiod with an irradiance of 400 μmol m^{-2} s^{-1}. Seedlings were sub-irrigated with water as required. Cotyledon age was measured in days after leaf emergence (DALE). In order to get the cotyledons of different age, seeds were sown every three days. Cotyledons were harvested simultaneously when the first batch cotyledons age reached 30 DALE.

Parameters of cotyledon senescence: Chlorophyll content, fresh mass and dry mass were estimated in the margins or in the central parts of the cotyledon blades without midrib. Fresh mass and dry mass of the whole cotyledon were determined as well. Samples were examined at different ages starting at 12 DALE when the cotyledon had just reached its maximum area.

Chlorophyll was extracted from 500 mg cotyledon samples in 10.0 cm^3 80 % acetone for 3 h in the darkness and was determined spectrophotometrically (*UV2201*, *Shimadzu*, Tokyo, Japan) at 652 nm using the method of Arnon (1949).

Paraffin sections: Pieces of fresh tissue (5 × 4 mm^2) at various stages were cut from the centre and cotyledon margin (Fig 2) with a razor blade and fixed immediately in Carnoy's Fluid (acetic acid: ethanol; 3:1, by vol.) for 12 h. The samples were dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin, and then the embedded tissues were sectioned using a rotary microtome to be 8 μm thick and mount on glass slides. Tissue sections were deparaffinized with xylene, and rehydrated through a graded series of ethanol washes. Sections (8 μm) were stained with Safranin/Fast Green, then observed and photographed under a light microscope (*Olympus BX-51*, Tokyo, Japan).

TUNEL assay: Tissue sections prepared for light microscopy were de-waxed with xylene, and rehydrated through a graded series of ethanol washes. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end *in situ* labelling procedure was performed according to the manufacturer's instruction (*Roche*, Basel, Switzerland). The sections were observed with blue fluorescence and photographed with an *Olympus BH-51* fluorescence microscope.

Isolation of nuclear DNA and DNA laddering analysis: Total DNA was isolated using the cetyltri-methyl-ammonium bromide (CTAB) method as described previously (Tanaka *et al.* 2001). Cotton cotyledons were frozen in liquid nitrogen immediately after being collected and ground to a fine power with a mortar and pestle. A 0.5-cm^3 aliquot of 2 % CTAB DNA extraction buffer (2 %; m/v CTAB, 1.4 M NaCl, 0.2 % v/v 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added to each ground sample. The supernatant was extracted twice with chloroform/isoamyl alcohol (24/1) after being incubated at 60 - 65 °C for 30 - 60 min. Afterwards, the supernatant was treated with cold isopropanol and RNase in succession. To analysis DNA laddering, samples (6 μg per lane) were run on a 2 % agarose gel at constant 70 V (*Bio-Rad*, Hercules, USA). The gel was stained with ethidium bromide for visualization and photographed by *Gel Documentation System* (*BioSens SC750*, Shanghai, China).

Statistical evaluation: All experiments were repeated four times; in the case of each experiment measurements were replicated 3 times. Similar results and identical trends were obtained in all experiments. The data reported here are from four times experiments with 12 replications. Data were evaluated using program *Sigma Plot 2000* (USA). The data are reported in figures and tables as means with standard errors (SE).

Results

The cotyledon reached maximum expansion by 12 d after emergence. Cotyledons of 12 to 18 DALE are green. The cotyledon yellowing started from its margin at 21 DALE. The yellowing then progresses inwards. After 30 DALE,

the cotyledons were yellowish completely and margins turned brown.

The water content of intact cotyledon was maintained steadily high between days 12 and 30, but the onset of dry mass could be detected on day 18 (Fig. 1). On the other hand, water content of both cotyledon margin and centre remained nearly constant during the course of the study. As the water and dry mass lost, the water content of cotyledon margin and the central part was fairly stable; the ratio was about 1. Water content in each part of the cotyledons from 12 DALE through 30 was, in fact, the most stable factor measured throughout this study. As such it provided a stable base for monitoring trends in other factors under test.

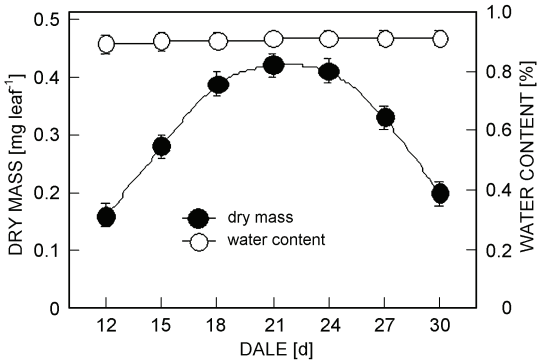

Fig. 1. Changes associated with age in water content and dry mass in intact cotyledons of cotton. Means ± SE, n = 12.

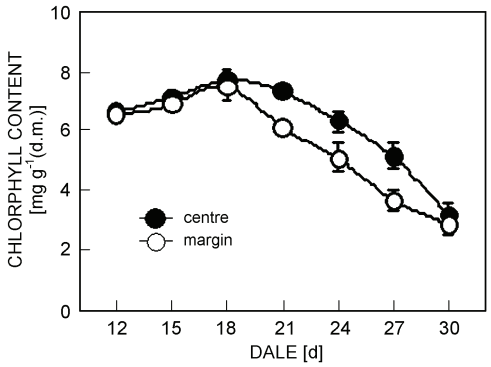

Fig. 2. Comparisons of chlorophyll content changes between margin and centre in cotton cotyledons. Means ± SE, n = 12.

There was no significant difference in the chlorophyll contents between cotyledon margin and centre from 12 to 18 DALE. After 18 DALE, chlorophyll content declined faster in the margin than in the central part. However, after 30 DALE, there were no differences between cotyledon margin and central part (date not shown).

Cell structure in different regions between 12 and 18 DALE was similar. The palisade mesophyll could be distinguished as a layer of closely packed, columnar cells beneath the upper epidermis (Fig. 3*A*). Notable differences between margin and central part were observed after 24 DALE. In margin sections cell-to-cell adhesion decreased (Fig. 3*B*), and with large intercellular spaces appeared among them, but these changes in central sections were not so remarkable (Fig. 3*C*). On 30 DALE, distortion, disintegration of cell could be detected at margin sections (Fig. 3*D*); however, intact cell with nuclei could be detected around the midrib vascular

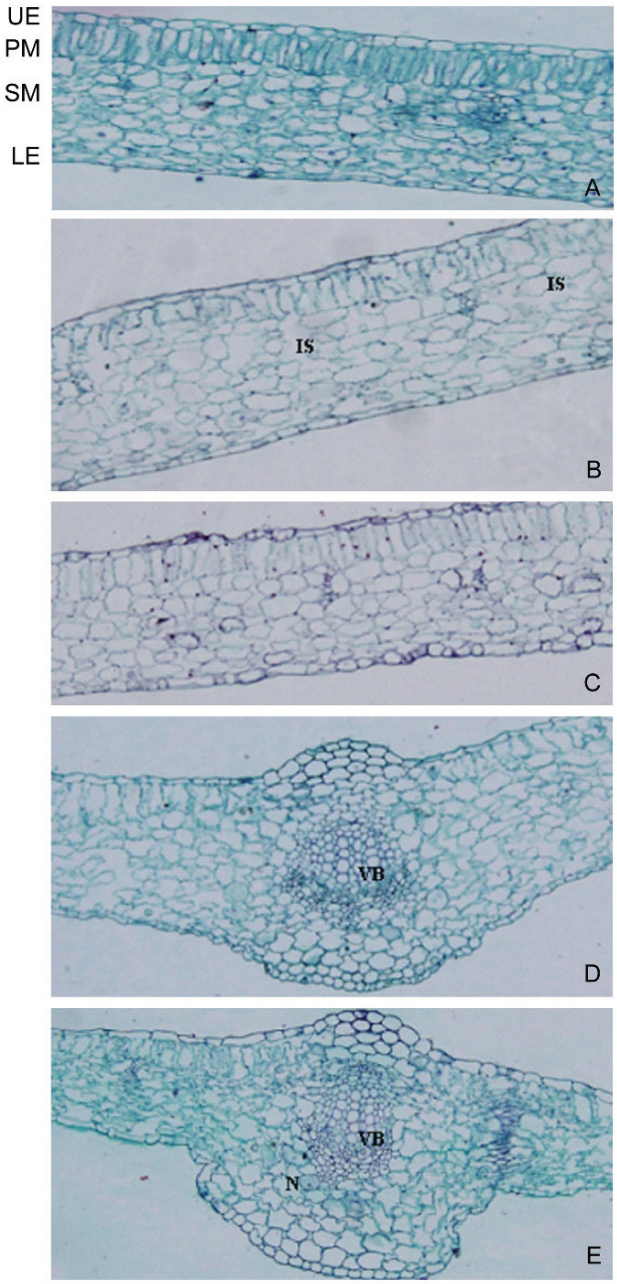

Fig. 3. Light microscopic analysis of cotyledons. Transverse sections of cotyledons sampled in 12 DALE (*A*), 24 DALE (*B* - margin, *C* - centre), and 30 DALE (*E* - midrib); magnifications 10×. IS - intercellular spaces, LE - lower epidermis, N - nucleus, PM - palisade mesophyll, SM - spongy mesophyll, UE - upper epidermis, VB - cells of the vascular bundle.

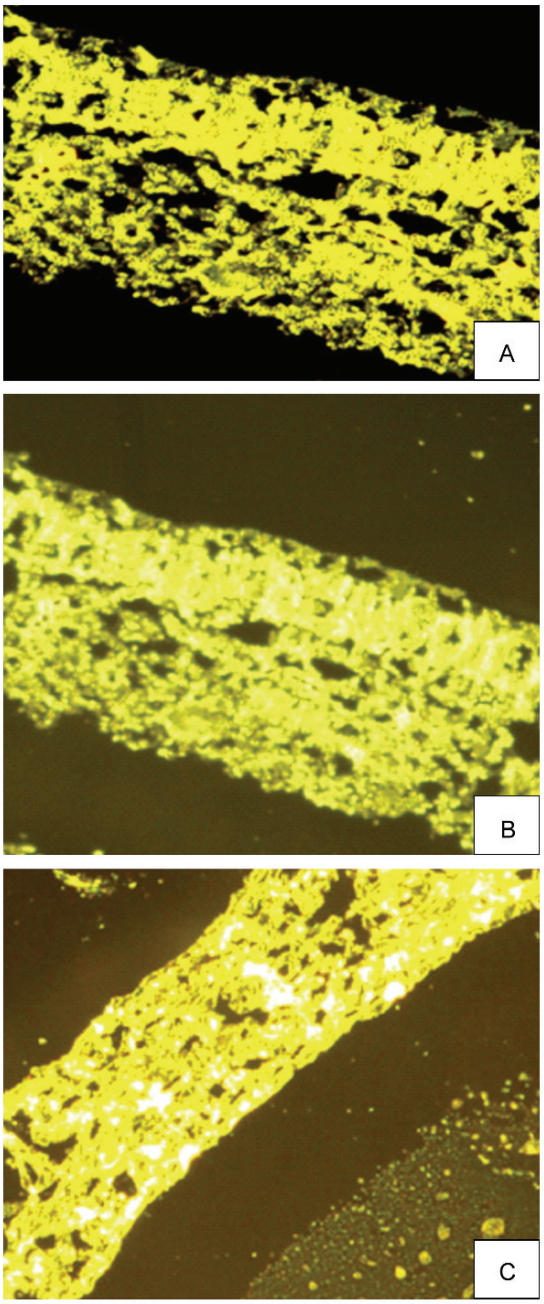

Fig. 4. Cytological detection of DNA cleavage in cotyledon nuclei by the TUNEL procedure. Transverse sections of cotyledons center (*A*) and margin (*B*) sampled in 21 DALE. The positive control (cleavage of DNA by DNase I according to the manufacturer's instructions of the kit) used cotyledon sampled in 12 DALE (*C*). Note that the TUNEL signals (*white dot*) appeared initially in the cells of cotyledon margin sampled in 21 DALE.

bundle in shed cotyledon (Fig. 3*E*). The gradual reduction of number of mesophyll cells with nucleus were also detected during senescence (Fig. 3).

The TUNEL assay was a good method to identify nuclei that were undergoing DNA fragmentation (Dangl *et al.* 2000). Before 18 DALE, there was no positive labelling in cotyledon cells (data not shown). Striking differences were observed in sections of leaves from 21 DALE, positive labelling could be detected in the margin sections (Fig. 4*B*), but not in the central ones (Fig. 4*A*).

We monitored the integrity of DNA by electrophoresis in a 2 % agarose gel (Fig. 5). Results indicated that DNA extracted from both cotyledon margin and centre before 21 DALE did not show DNA laddering or smearing, but an unresolved high molecular mass band of more than 10 kb in length (Fig. 5*A*). DNA fragmentation with preferential oligonucleosomal cleavage, event characteristic of PCD, was found in the advanced stage of cotyledon senescence. Cotyledon margin at 24 DALE showed a laddering pattern (consisting of multiples of approximately 180 bp in length) (Fig. 5*A*), however, central part of cotyledons detected the laddering pattern in the 27 DALE, but not in the 24 DALE (Fig. 5*A*).

Isolation of nuclear DNA and DNA laddering analysis were also performed in the whole cotyledon. DNA extracted from intact cotyledons detected an unresolved high molecular mass band before 21 DALE (data not shown). At 24 DALE, extensive DNA smearing was detected, which indicate degradation of DNA. Increasing smearing was observed at 27 and 30 DALE (Fig. 5*B*). However, there was no obvious "laddering" of DNA degradation of the genomic DNA into internucleosomal fragments of ~180 bp.

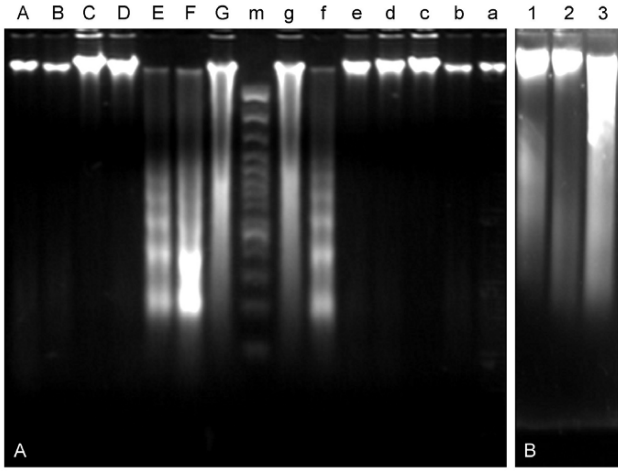

Fig. 5. DNA cleavage in developing cotyledons. *A* - Agarose gel analysis of DNA extracted from cotyledon margin and center. *Lanes A - G*: DNA extracted from cotyledon margin of 12, 15, 18, 21, 24, 27 and 30 DALE, respectively, *lane m*: molecular mass markers, stained with ethidium bromide, and separated electrophoretically. *B* - Agarose gel analysis of DNA extracted from intact cotyledon. *Lanes 1 - 3*: genomic DNA isolated from developing cotyledons taken at 24, 27 and 30 DALE, respectively. Extensive DNA smearing was present, indicating the degradation of DNA must relate to the asynchronous PCD in naturally senescing cotyledons.

Discussion

Reports on leaf senescence are limited mainly to the whole organ in the past. Until now, the different studies interested in leaf senescence focused especially on *Arabidopsis*, and other annual species to a lesser extent (Shen *et al.* 2006). In this report, we present cell death at different stages of development and during senescence of cotyledon. We found that different in chlorophyll content in different regions of cotyledon during aging. Although the dry mass and water decreased after 21 DALE, the water content of each cotyledon region remained constant. Programmed cell death of margin region occurs earlier than in the central part. Moreover, DNA ladders were detected in senescent cotyledon margins and in the centre, respectively.

Programmed cell death (PCD) is a genetically defined process associated with common morphological and biochemical changes, which is critical for normal development, maintenance of tissue homeostasis, and for defence responses in plants (Coupe *et al*. 2004). The loss of dry mass and water, the stability of water content of each region indicate translocation of leaf metabolites maintenance of tissue homeostasis. The differential cell death from cotyledon margin to centre that occurs during senescence is associated, at least in part, with efficient recycling of nutrients that are released during senescence. Therefore, the question arises as to whether there are differential expressions of genes along the length of leaf during senescence, and how the molecular mechanisms involved in regulating asynchronous cell death in leaf.

Nuclear DNA fragmentation often occurs with PCD and can be detected by the banding pattern of total DNA seen after agarose gel electrophoresis (Xu and Roossinck 2000). Detection of DNA laddering can be hampered in many experiment systems, e.g. leaf perforations formation (Gunawardena *et al.* 2003, Gunawardena *et al.* 2005), tracheary elements development (TEs) (Mittler and Lam, 1995, Groover *et al.* 1997), barley aleurone protoplasts undergoing PCD (Fath *et al.* 1999) and rice starchy endosperm development (Li *et al.* 2004). However, in many experimental systems such as cell cultures, DNA ladder can be detected easier. Probably, there exit variations among different experimental systems. Moreover, detection of the DNA ladder may be difficult for technical reasons (Groover *et al.* 1997, McCabe *et al.* 1997, Gao and Showalter 1999).

Lu *et al.* (2004) reported that there exists a two-stage DNA fragmentation process during apoptosis in the cell free extract from *Xenopus* eggs, and there is an over-lap within the two different stages. In the first stage the chromatin DNA is cut into large molecular weight fragment (Wyllie *et al.* 1980, Oberhammer *et al.* 1993) and in the second stage the chromatin DNA is cut into oligo-nucleosomal DNA ladders. Finally, the fragmented DNA is digested completely by specific endonucleases (Gunawardena *et al.* 2003). In this study, by using the whole cotyledon, only DNA smearing is detected. Nevertheless, asynchronous DNA ladder is detected in both cotyledon margin and centre. We showed that these changes in DNA degradation do not proceed at the same time in different parts of cotton cotyledons. Taking these facts into consideration, the cells are not synchronized in intact tissues during the development of cotyledon senescence, only a fraction of the cells are undergoing the nuclear DNA fragmentation process at the same time, insufficient synchrony of cells undergoing PCD bring us some difficulties to detect DNA ladder.

TUNEL assays are used for *in situ* detection of DNA fragmentation (Mittler and Shulaev 2003). The TUNEL *in situ* assay has been reported to give variable results due to fixation methods, reaction times, reagent concentrations, *etc*. False positives can occur as a result of, for example, DNase activity in necrotic cells or as a result of different washing procedures during the assay. A combination of electrophoresis of isolated DNA and the TUNEL assay was a good method to identify nuclei that were undergoing DNA fragmentation (Gunawardena *et al.* 2003). The average cost of the kit is expensive, but it could extract various DNA fragment at the advantage of avoiding toxic reagents. Accordingly, in the course of this study, TUNEL assay was used as an assistant method to detect DNA fragment.

In conclusion, DNA ladder detection depends on having a substantial population of cells undergoing PCD synchronously. In order to detect DNA ladder during PCD, morphological methods and TUNEL assay should be utilized firstly to analyze each region of the cotyledon at different points in the senescence progression, which can provide the most valuable information on the cell death process. Further study will shed light on the molecular mechanisms involved in regulating asynchronous cell death in leaf.

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