Mammalian Apoptosis-associated Genes c-myc and p53 in Maize: Homologs and Their Locations

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Summary  The highly conserved proto-oncogene c-myc and tumor suppressor gene p53 in animals has been shown to play a central role in regulating multiple developmental processes including growth, proliferation and differentiation, and are also 2 of the important genes associated with programmed cell death. Using the probes corresponding to the 2 genes derived from human, we detected and localized their DNA homologs in maize (Zea mays L.) at cytogenetic level for the first time utilizing chromosome in situ hybridization. For detection, techniques, 3,3′-diaminobenzidine and fluorescence staining were utilized. Both of them gave the identical results. The signals of the test probe corresponding to c-myc were detected on 4L (the long arm of chromosome 4) and 5L and 1S (the short arm of chromosome 1) simultaneously. And those of p53 were localized on 5S, 1L, 3L and 9L simultaneously. These results further confirmed the existence of the homologs of the 2 genes in maize at DNA level. The improvements of FISH technique using heterologous probes were discussed.

Key Words  c-myc, Homologs, In situ hybridization, p53, Programmed cell death.

The proto-oncogene c-myc and tumor suppressor gene p53 have been highly conserved during evolution and have been shown to play central roles in regulating multiple developmental processes such as proliferation, programmed cell death (PCD), and differentiation of a cell in animals (Packham et al. 1996, Yonish-Rouach 1996). In disease situations in which immortalization of a cell occurs, the expression of various proto-oncogenes and tumor suppressor genes that control PCD appears to be widespread (Raff 1992, Wang et al. 1996). It seems likely that several p53 activities, both transcriptionally dependent and transcriptionally independent, can play a role in mediating PCD in cells suffering DNA damage (Bates and Vousden 1996), and as to c-myc, it plays a dual role opposite to each other in activating PCD and cell proliferation processes (Packham et al. 1996).

Although the relationship between the mechanisms underlying PCD in animals and plants is not known, PCD in plants seems also to be necessary for growth and survival and can occur on a local or large scale. It is now generally accepted that many developmental processes, disease resistance and stress responses in plants are ensured through the operation of PCD (Greenberg 1996, Fukuda 1997, Pennell and Lamb 1997, Heath 1998, Richberg et al. 1998, Vaux and Korsmeyer 1999, Jabs 1999). And there is increasing evidence that plant PCD shares some common morphological and biochemical features with that in animals. More important, caspase-like plant cysteine protease activities have also been found in hypersensitive response (HR) cell death (del Pozo and Lam 1998, Solomon et al. 1999), and plant homologs of PCD suppressor gene Dad-1 have been isolated from Arabidopsis (Gallois et al. 1997), rice (Tanaka et al. 1997) and apple (Dong et al. 1998), suggesting in particular PCD mechanisms may be conserved between animals and plants. Because of these, the search for functional homologs of other genes controlling animal PCD in plants may be warranted and will further resolve the similarities and the differences in cross-king-

Up to present, although only several genes involved in plant PCD have been identified (Greenberg and Guo 1994, Buschges et al. 1997, Dietrich et al. 1997, Gray et al. 1997), however, some genes involved in PCD in animals have been found homologs in plants and some of them (but not all) appear the similar functions (Georgieva et al. 1994, Yang and Klessig 1996, Gallois et al. 1997, Tanaka et al. 1997). In addition to Dad-1, the convincing example, Bel-2 (Dion et al. 1997) and Rb (Xie et al. 1996), for instance, have been detected protein homologs in tobacco and maize plants respectively. These successful researches provide a valuable clue that it is feasible to search for the plant homologous genes associated with PCD using heterologous probes.

C-myc and p53, as another examples, have been detected corresponding protein homologs in maize embryo tissue using specific antibodies and these homologs have been presumed to function similar to that in animals (Georgieva et al. 1994). But the DNA sequences corresponding to these homologs have not been investigated so far. In order to further confirm whether the mechanisms controlling PCD in plants and animals are similar, and to fully understand the role of PCD in plants, it would be necessary to identify, characterize and manipulate the plant genes that control this process (Dangl et al. 1996, Martienssen 1997). For this purpose, we detected the DNA sequences homologous to human c-myc and p53 using the corresponding cDNA probes, and determined their locations on maize chromosomes.

Materials and methods

Plant and probes

Maize (Zea mays L.) inbred line Huang Zao 4, which derived from a native cultivar in China, was supplied by Prof. Song Jiancheng (Shandong Agricultural University, Shandong Province, China). Both of the tested cDNA probes corresponding to human c-myc and p53 were cloned in vector pBR322, provided by Prof. W. X. Li (Molecular Virus Laboratory of Wuhan University). The relevant inserted cDNA fragments were 2.2 Kb and 1.1 Kb in size respectively and both of the restriction loci were EcoRI/HindIII. The cDNA inserts were cut out with EcoRI/HindIII, separated by and extracted from low-melting point agarose electrophoresis, then labeled with Bio-11-dUTP following Nick translation protocol offered by the kit (Sino-American Biotechnology Company, China; Gustafson et al. 1990) and subsequently subjected to pass through sepharose CL-6B (Sigma) column for purification.

Chromosome preparation

The root tips of 1–2 mm length from vigorously grown maize plant were treated with saturated α-bromonaphthalene under the temperature 25°C for 2.5 h, and fixed with methanol : gracial acetic acid (3 : 1) for over night. After fully washed with water and a mixture of 2% pectinase and 2% cellulase (SERVA) added in, the treated root tips underwent enzymolysis under the temperature 28°C for around 3 h. Finally, the treated root tips were mounted onto microscopic slide, crushed with a tweezer and flame-dried (Song et al. 1994).

In situ hybridization (ISH)

The method published by Dong and Quick (1995) was adopted with some modifications. The hybridization mix (50 μl) contained 2 μg of probe, 50% deionized formamide (Sigma), 10% (W/V) sodium dextran sulphate (Sigma), 0.2% (W/V) of ssDNA and was denatured at 90°C for 10 min, immediately chilled on ice and placed for 10 min. Hybridization was performed overnight at 37°C in a humid chamber. The slides were then washed in 2×SSC, 0.1×SSC, at 42°C for 5 min each, and subsequently washed in 0.1% of TritonX-100, PBS at room temperature, 5 min each step.
**Detection and visualization**

DAB (3,3′-diaminobenzidine) detection was carried out as the procedure outlined by Gustafson et al. (1990). For microscopy, Olympus BH-2 photomicroscope (Japan) was used.

For fluorescence detection, following washing as above, the slide was loaded with 40 μl 1% (W/V) anti-biotin-FITC-avidin D (diluted in PBS/0.5%BSA) raised in goat (Sigma) and covered carefully with a 22×40 mm clear cover slip without any air bubbles created, and then placed in a humidity chamber with three layer filter papers in the bottom and incubated at 37°C for 60 min in the dark. After rinsed in PBS for 3×5 min in the dark, the slide was conjugated with 40 μl 0.5% (W/V) anti-goat-avidin D antibody (diluted in PBS/0.5%BSA) raised in rabbit (Sigma) instead and covered with a clear cover slip and placed back in the humidity chamber for incubation as above. Cascade amplification was performed for 2 cycles using this immunological reaction and ended with incubation with the goat antibody-FITC. The slide was alerted to keep wet during all these processes. Finally, the slide was rinsed in PBS as above and counterstained with 20 μl 3 μg/ml propidium iodide (PI) containing 10 μg/ml anti-fade reagent p-phenylenediamine dihydrochloride and visualized with an Olympus (BH-2) fluorescence microscope (Japan). Selected interphase nuclei and chromosomes were photographed on ISO 400 color negative film.

Chromosomes were identified by their relative lengths and arm ratios following the standard karyotype (Neuffer et al. 1997). Images gotten from DAB and fluorescence detection systems were processed using the software Photoshop4.0 produced by Adobe Corporation.

**Results**

The improved procedure for chromosome preparation allowed the chromosomes to expose to an optimum degree for hybridization with the labeled probes. On hybridized chromosomes, the discriminated signals showed cardinal red on the Giemsa-counterstained chromosomes and yellow-green on the PI-counterstained ones, round in shape, and were easy to be recognized. The microphotography for the chromosomes with good feature in morphology and clear background and clear signals of division phases was carried out and the relevant data of resultant of the photograph was measured on the screen of transformation device, which was calculated for standard deviation.

The DAB and fluorescence detection systems gave the identical results. The tested probe c-myc was hybridized onto 4L (the long arm of chromosome 4), 5L and 1S (the short arm of chromosome 1), and the probe p53 onto 5S, 1L, 8L and 9L under microscope. For c-myc, 243 mitotic phases at metaphase, early metaphase or prophase by DAB detection and 40 by fluorescence detection were observed in 3 independent repeated experiments, and for p53, 290 by DAB detection and 62 by fluorescence detection were observed. The arm ratio of all the chromosomes on which the signal appeared and the percentage distance from the signal to centromere were measured. The calculation of the averages of these 2 parameters for each detected chromosome was presented as Table 1. It may be seen that the detection rate of fluorescence was much higher than that of DAB. To DAB detection, the hybridization signals infrequently appeared simultaneously on a pair of homologous chromosomes or on sister chromatids, and less simultaneous occurrence of two or more hybridization signals was found in one cell, all of which only made up to 8% of the total phases (Fig. 1 A–C, F, H). As to FISH, however, it resulted in displaying much more mitotic phases with the signal dots simultaneously occurred on a pair of homologous chromosomes or on sister chromatids, or two or more signals simultaneously occurred on different chromosomes. It made up to 25% of the total phases on which signals presented (Figs. 2, 3). The detection rate of hybridization signals at interphase in both detection systems was much higher than that at the metaphase. To FISH detection, it was 54.2% and 66.7% for c-myc and p53 respectively.
Our results of ISH supported that the homologs of c-myc and p53 of human were present in maize at DNA level besides at protein and mRNA levels proved by the study of Georgieva et al. (1994). As to the detected sites, the result obtained from DAB system coincided with that from FISH, which corroborated each other. It has been proved and deduced that the homologs of the 2 genes should be also present in other plants and lower eucaryotes (Georgieva et al. 1994).

It has been showed that duplicated sequences in maize genome are arranged on chromosomes following the modes of 5-1-9, 3-8-6 and 10-2-7, which means chromosome 1 shares similarity with both chromosomes 5 and 9, etc. (Dowty and Helentjaris 1992). Helentjaris (1996) proposed further that the long arm of chromosome 4 (4L) shared similarity with 5L and 1L shared similarity with 5S. The results of our experiment essentially just followed these patterns and indicated the distribution of multiple loci of homologous sequence of c-myc and p53 in maize.

Undoubtedly, it is more difficult to do localization for chromosome ISH by using heterologous gene. Especially, when the gene or cDNA from animals are used as probe to search for homologous sequence in plants, it is hard to achieve a satisfied experimental result without the probes with enough length and high conservation, and the support of perfect technology in this field. Analogous researches have been done with other cancer suppressor genes or cDNAs, but we have not obtained satisfied results (data not shown). On the other hand, to low or single- or low-copy genes, it was reported that the detection rates for individual sites were 10-20% when the sizes of probes were around 1 kb, and it could rise to 90% by nonisotopic labeling if the sizes were over 3.5 kb (Jiang and Gill 1994). However, in this study, the detection rates of the tested two cDNA probes were much lower than 90% by FISH for most detected sites. Even to the same probe, the detection rates on different detected chromosomes obviously differed from each other. It may be explained that homologous sequences on different chromosomes have undergone variation at varying levels and brought about different homology at different levels during its evolution route. Perhaps the loci of higher detection rates represented the genes homologous to c-myc and p53, yet those of lower de-

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Table 1. Signal locations and arm ratios averaged for the chromosomes detected by the tested probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Chromosome arm</th>
<th>DAB detection</th>
<th>FISH detection</th>
<th>Ratio of long to short arm</th>
<th>Percentage distance from centromere to signal site (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of detection</td>
<td>Detection rate (%)</td>
<td>No. of detection</td>
<td>Detection rate (%)</td>
</tr>
<tr>
<td>c-myc</td>
<td>5L</td>
<td>102</td>
<td>42.0</td>
<td>18</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td>4L</td>
<td>87</td>
<td>35.8</td>
<td>20</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>1S</td>
<td>71</td>
<td>29.2</td>
<td>11</td>
<td>27.5</td>
</tr>
<tr>
<td>p53</td>
<td>5S</td>
<td>96</td>
<td>33.1</td>
<td>35</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>1L</td>
<td>55</td>
<td>19.0</td>
<td>21</td>
<td>33.9</td>
</tr>
<tr>
<td></td>
<td>3L</td>
<td>21</td>
<td>7.2</td>
<td>9</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>9L</td>
<td>11</td>
<td>3.8</td>
<td>4</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Standard deviation.

Table 2. Combination of FISH signals for the tested probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>No. of cells observed</th>
<th>No. of cells detected</th>
<th>Detected rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>40</td>
<td>32</td>
<td>80.0</td>
</tr>
<tr>
<td>p53</td>
<td>62</td>
<td>44</td>
<td>71.0</td>
</tr>
</tbody>
</table>

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Discussion

Our results of ISH supported that the homologs of c-myc and p53 of human were present in maize at DNA level besides at protein and mRNA levels proved by the study of Georgieva et al. (1994). As to the detected sites, the result obtained from DAB system coincided with that from FISH, which corroborated each other. It has been proved and deduced that the homologs of the 2 genes should be also present in other plants and lower eucaryotes (Georgieva et al. 1994).

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Fig. 1. DAB detection for the tested probes. Small arrows in A-D indicated the signal locations of probe c-myc and in E-H indicated the signal locations of probe p53 on the mitotic metaphase chromosomes of maize. The tailless arrows showed centromeres. Bar=0.5 μm. A) Signals were detected simultaneously on a pair of 4L, B) Simultaneously on a pair of 5L, C) Simultaneously on 5L and a pair of 4L, D) On 1S, E) Signal was detected on 1L, F) Simultaneously on two separated 3L, G) On 5S, H) Simultaneously on 1L and 9L.
Fig. 2. FISH for the tested probes. Small arrows in A-D indicated the signal locations of probe c-myc and in E-H indicated the signal locations of probe p53 on the mitotic metaphase chromosomes of maize. The tailless arrows showed centromeres. Bar=0.5 μm. A) Signals were detected simultaneously on sister chromatids of a pair of 4L, B) Simultaneously on 4L and 1S, C) Simultaneously on sister chromatids of 1S, D) On 5L. E) Signals were detected simultaneously on sister chromatids of 1L, 9L and 5S, F) Simultaneously on sister chromatids of a pair of 5S, G) Simultaneously on a pair of 3L, H) Simultaneously on 9L and a pair of 1L.
Fig. 3. FISH karyotypes for the tested probes. A) Chromosome identification for the detected sites of probe c-myc, B) Chromosome identification for the detected sites of probe p53, C) Karyotypes prepared from A and B according to the ideogram of standard karyotype of maize chromosomes. The upper arrange was for c-myc and the bottom one was for p53 respectively. D) Ideogram of the chromosomal locations of the tested probes on the standard karyotype. The detected sites were indicated with percentage distances on the right of each chromosome. The percentage distances were averaged for DAB and fluorescence detection systems. The number of each chromosome corresponded to that in C.
tection rates only represented the DNA fragments homologous to them to certain extent (Ren et al. 1997a, b).

Due to that the experimental operation directly affected the result of detection and the detection rate, in addition to the factors affecting the detection rate mentioned as above, we strictly performed every step of the processes of ISH and detection. We have made a lot of investigation and explorative works on chromosome preparation, Giemsa and PI counterstaining as well as photography. And a perfect experiment process and operation experience of laboratory has been summed up. First of all, we obtained a number of available mitotic phases with clear background, disjunction and nice feature of individual chromosomes with appropriate length, which related positively to hybridization effect. By comparison, we found that the cellulase produced by SERVA corporation brought up the most satisfied effects in enzymolysis, and revealed that the detection rate of the probe of short DNA fragment was raised by means of appropriate multiple cascade amplification and of prolonging the time of antigen-antibody coupling. Besides these, we found that PI counterstaining might conceal the hybridization signal.

At present, the technique FISH has been one of the more advanced techniques used in plant molecular cytogenetics (Jiang et al. 1996, Palotie et al. 1996). FISH is generally much superior to DAB with high sensitivity and resolution, strong contrast, large volume (bi- or multi-color) and simply process in gene localization in situ and can bring about much higher detection rate than DAB detection, indicating a nice prospect for development and application (Palotie et al. 1996). For example, Fiber-FISH, molecular combing and quantitative DNA fine mapping techniques have been developed based on FISH (Raap 1998, de Jong et al. 1999). Our experimental results also indicated that the detection of FISH was much higher than that of DAB in general. The more and more reports on detection of single-copy gene by FISH in plant have been issued (Dong and Quick 1995, Bi et al. 1997, Li et al. 1998). But the ultimate shortcoming of FISH is that the fluorescence signal easily quenches, difficult to be caught, even the signal may quench before its being observed, and this is one of the essential factors causing lower rate of detection (Li et al. 1998). In our procedure, besides above-mentioned improvements and appropriate anti-quenching agent being applied, multiple cascade amplification of signal was also applied for matching the length of the sequence of probe.

The ubiquitous occurrence and necessity of PCD for plant development and defense suggest that the underlying mechanism of regulation and execution of this process merit further examination (Crute and Pink 1996, Gan and Amasino 1997). Using both molecular and genetic approaches for searching and mapping the PCD-associated genes and further identification and characterization of them to decipher the mechanism will provide a foundation for further molecular analysis of gene regulation during PCD in plants. Although we cannot conclude according to our results that the homologs of c-myc and p53 in maize also play a predicted role in plant PCD, we obtained the primary result for this purpose. We are now investigating the temporal and spatial expression patterns of the homologs of these genes using in situ RNA hybridization and in situ immunohistochemical methods and will further study whether these homologs are surely associated with PCD during maize embryogenesis.

Acknowledgments

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References


