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Chromosome G-banding in Situ Hybridization of RFLP Marker umc58 Linked with the Gene hm1 Dictating *Helminthosporium carbonum* Susceptibility1 in Maize*

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Abstract **The technique of simultaneous G-banding and in situ hybridization has been developed in plants for the first time. Using this technique, RFLP marker umc58 closely linked with the hm1 gene dictating *Helminthosporium carbonum* susceptibility1 was localized onto 1L3 (chromosome 1, long arm, the third band from the centromere to the end of the arm), 5L5 and 9L5. The results demonstrated that umc58 was a triplicated sequence. It was deduced that umc58 probably was in a duplicated region that includes a part of *Helminthosporium carbonum* susceptibility genes (hm1 and hm2), as the hybridization sites of umc58 in chromosomes 1 and 9 were those at which the genes localize. The techniques of simultaneous G-banding and ISH in plants are discussed.**

Key words **In situ hybridization, chromosome G-banding, maize, RFLP markers**

In humans, G- or R- banding and in situ hybridization (ISH) have been successfully combined into a single procedure. In plants studies of the combination of C- or N-banding and ISH have been reported[1], but not of G-banding and ISH. Chromosome G-banding is more difficult in plants than in mammals. After G-banding was successfully developed in mammals, plant G-banding had not been broken through for more than twenty years. The G-banding technique was first introduced into plants by Drewry[2], in *Pinus resinosa*. Since then many studies on plant G-banding were reported. However, the technique of plant G-banding was still unreliable and ideal G-banded karyotypes could not be obtained easily. Recently an available G-banding procedure, by an improved ASG method, has been developed in plants[3]. Using this procedure, clear G-banded karyotypes of many plant speices have been obtained. Therefore, it has created favorable conditions for developing the technique of combination of G-banding and ISH in plants.

The sensitivity of ISH techniques for humans is now down to 0. 25 kb[4]. Many single copy genes have been located onto human chromosomes. ISH of single or low copy sequence DNA has lagged behind in plants compared with that in humans. It was not until 1986 that Ambros et al[5]. Reported the mapping of single copy DNA in *Crepis capillaris*. Since then some ISH research on single copy or low copy DNA sequences has been reported in plants including maize, pea, barley, rye alfalfa, rice, wheat and *Arabidopsis thaliana*. However, in these studies, most of the targeted DNA sequences were more than 5 kb, only a few studies of mapping of small DNA sequences around

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1 kb in size have reported[6~8].

In this study, one of the purpose was to develop a method for simultaneous G-banding and ISH in maize with the RFLP marker umc58 closely linked with the gene hm1, *Helminthosporium carbonum* susceptibility1 (a disease lesion on leaves)[9]. The disease caused by *Helminthosporium carbonum* is an epidemic in some maize production countries including China. It makes production and quality of maize reduce and causes great danmage in agriculture. To locate the *Helminthosporium carbonum* susceptibility gene is meaningful for maize breeding enhancing the resistance to *Helminthosporium carbonum*, because based on its position the structural reformation of the gene and separation of its dominant allele, *Helminthosporium carbonum* resistance gene, Hm1[9], which could be applied to gene transfer or other genetic research, can be performed. Therefore, another important purpose of this study was to localize this gene onto the specific chromosome region, using chromosome G-banding ISH of the RFLP marker umc58 closely linked with it.

1 Materials and methods

Maize inbred line Huang Zao 4, which derived from a native cultivar in China, was used as the tested plant materials; seeds were provided by Professor Song Jiancheng, Shandong Agricultural University, Shandong Province, P. R. China. The RFLP marker, umc58, is close to hm1 gene dictating *Helminthosporium carbonum* susceptibility1 (a disease lesion on leaves) near the centromere of linkage group 1 in the classical genetic map[9]. The RFLP probe was obtained from the RFLP laboratory and probe bank of the US Department of Agriculture, Agricultural Research Service, and Department of Agronomy, University of Missouri, Columbia, USA. The probes umc58 is 920 bp in size and it is present in plasmid pUC19. The improved ASG method developed by Song *et al* (1994)[3] was basically adopted for chromosome G-banding with the following modifications. After fixation in methanol acetic acid the root tips were washed in deionized water for 15 min and then treated in 5 mol/L HCl for 30 min at room temperature. The materials were digested in 1% cellulase (Shanghai Institute of Biochemistry, Chinese Academy of Sciences) and 1% pectinase (SERVA Feinbiochemica, Heidelberg) at 30℃ for 90 min and 3 ~ 4 root tips were squashed with forceps in 2 ~ 3 drops of the fixative on a slide and frame dried. Subsequently, the slides were not used for Giemsa staining instead of ISH.

Biotin labeling of the probe included two steps. The first, 5 μL plasmid DNA (2×10^{-7} g/μL) was added to a 5 μL mixture of 1 μL 10 NTB, 1.5 μL DNase I (2×10^{-12} g/μL) and 2.5 μL sterile deionized water and the mixture was incubated at 37℃ for 15 min. In the second step, to the mixture was added 4 μL 10 NTB, 2 μL dNTP (mixture of 0.5 mmol/L dATP, dGTP and dCTP), 2 μL 0.5 mmol/L bio-11-dUTP, 30 μL sterile deionized water, and 2 μL DNA polymeraseI, and the reaction was incubated at 14℃ for 12~16 h. Labeling was terminated by adding 2 μL 0.5 mmol/L EDTA. All of the labeling reagents came from a kit supplied by Beijing Medical University. Labeled probe was separated from unincorporated nucleotides by passing the reaction through a Sepharose column. Incorporation of bio-11-dUTP was evaluated by means of dot blots following the streptavidin alkaline phosphatase detection system.

The technique for ISH was that used by Gustafson and Dillé (1992)[7]. So was the technique for detection of hybridized probes except that the preparations were stained in 2% Giemsa solution for 20~30 min instead of 1 min after DAB staining in order to get both hybridization signals and clear G-banding patterns simultaneously. After detection and photography the slides were destained in 1/15 mol/L phosphate buffer, pH6.8, for removing the G-bands and showing hybridization spots only on the chromosomes.

2 Results and discussion

Fig. 1 presents G-banded chromosomes of the mitotic cells (Fig. 1-a, b) and the band destained single chromosomes (Fig. 1-c) labeled by probe umc58 (Fig. 1-a,b). Both hybridization signals and G-bands are apparent on G-banded chromosomes. Even though the G-banding patterns are not as clear as those obtained by routine G-banding pro-

cedures, the bands could be recognized and distinguished for most chromosomes including the labeled ones. The hybridization spots still keep and show clearly on the band destained chromosomes (Fig. 1-c).

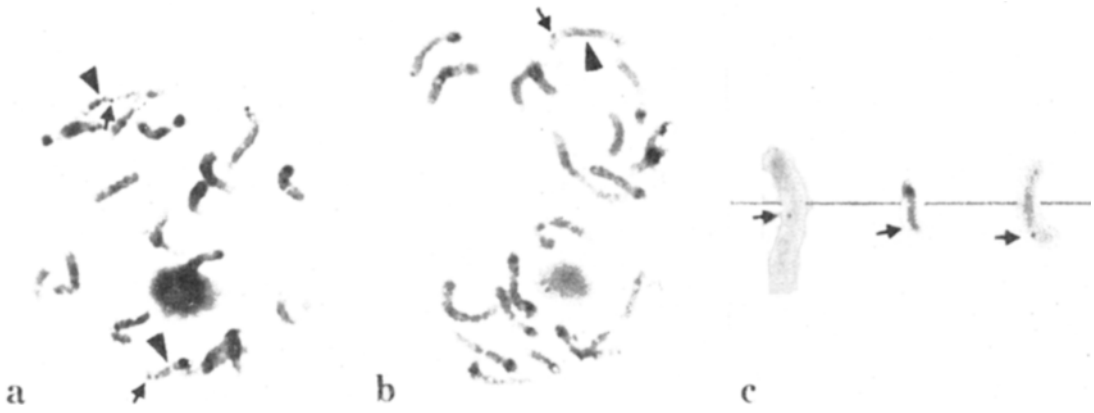

Fig. 1 The mitotic chromosomes showing hybridization sites of the tested probe

The arrow denotes the hybridization site and the arrow head denotes the centromere in all the figures except Fig. 1c in which the position of the centromeres is showed by a lateral line. (a) Late prophase showing two sites with probe umc58 at 1L3 and 9L5 in G-banded chromosomes. (b) Late prophase showing a site with probe umc58 at 5L5 in G-banded chromosomes. (c) The single band destained chromosomes showing the hybridization sites corresponding to those at figures a and b, from left to right, probe umc58 at 1L, 9L and 5L in proper order

Probe umc58 hybridized onto the third band near the centromere of the long arm in chromosome 1 (Fig. 1-a, and Fig. 2, Table 1), the fifth band in the interstitial region of the long arm in chromosome 5 (Fig. 1-b and Fig. 2, Table 1), and the fifth band in the terminal region of the long arm in chromosome 9 (Fig. 1-a, and Fig. 2, Table 1). For brevity, we adopted 1L3, 5L5 and 9L5 to stand for the hybridization sites of the probe, respectively. For the symbols, the first numeral is the number of the chromosome; L stands for long arm; the terminal numeral is the band order from centromere to the end of the arm. A total of 370 late prophase or early metaphase cells were analyzed and 83 labeled cells were detected (22.43%). Most of the labeled cells had signals on one member of the homologues, only three cells had signals on two members of chromosome 1 and two cells on two members of chromosome 9. Two cells had signals on both 1L3 and 9L5. One of them showed in Fig. 1-a. In the 83 labeled cells, 44 had signals on 1L3, 15 on 5L5, and 26 on 9L5 (Table 1). All the signals were found on one chromatid of a chromosome in each labeled cell except in two cells which showed hybridization site on each chromatid of a chromosome 1. The average arm ratios of labeled chromosomes 1, 5 and 9 were 1.24 ± 0.03, 1.06 ± 0.04 and 2.10 ± 0.07, respectively (Table 1).

Table 1 Hybridization locations and arm ratios for the chromosomes in the maize genome

Probe	In situ location	No. of detections	Ratio of long to short arm
umc58	1L3	44	1.24 ± 0.03*
	5L5	15	1.06 ± 0.04
	9L5	26	2.10 ± 0.07

* Standard deviation

Probe umc58 showed 3 hybridization sites, at triplicated sequences. It has been demonstrated that many RFLP markers were duplicated at least once somewhere else in the maize genome[10]. That maize is a complex allotetraploid was supported in the work of several different maize geneticists[11]. Some studies[10] demonstrated that the duplicated sequences of maize RFLP markers probably were relevant to properties of the allotetraploid. The marker umc58 belongs to triplicated sequences. This hints that maize originated not only from polyploidization but also from chromosomal duplication. The detection ratio of umc58 hybridization site on 1L3 was higher than that on 5L5 and 9L6, especially on 5L5. It means that the umc58 sequence probably is only partially homologous with those on chromosomes 5 and 9. It is sure that the higher the homologous degree, the higher the ratio

hybridized by a given probe, e.g., probe umc58 among the duplicated sequences. Therefore, we think the variations of duplicated sequences in

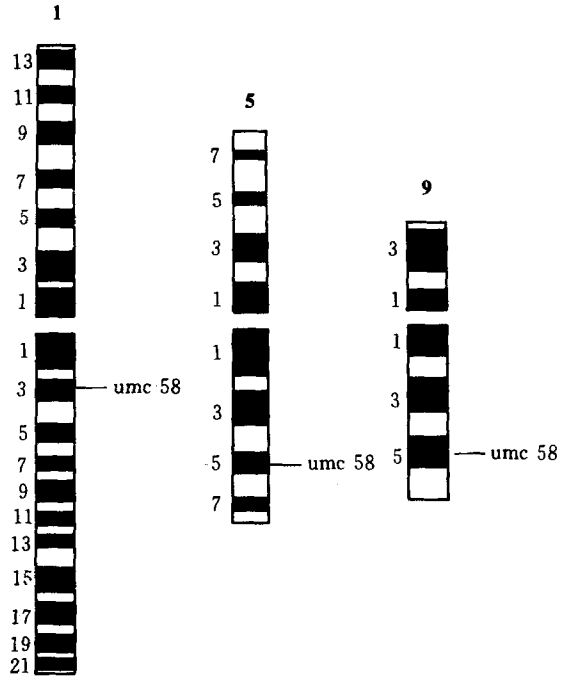

Fig. 2 G-banded idiogram of chromosomes 1, 5 and 9 showing hybridization sites with probe umc 58 at 1L3, 5L5 and 9L5 respectively. The top numeral is the number of the chromosome

evolutionary processes may be analyzed according to the differences in their detection ratios to a certain degree at least.

In ISH studies of rice RFLP markers it was indicated that considerable variation could, and did, exist between genetic and physical maps[7,8,12]. Heslop-Harrison (1991)[12] thought there was often little correlation between the separation distances of markers on the genetic and physical map. However, in this study, the umc58 probe was located near centromere of the long arm in linkage group 1 in the classical linkage map[9], according to our results of physical mapping it was also located near the centromere of the long arm in chromosome1, besides the other sites to which they hybridized. It demonstrates that the maize physical map is basically consistent with the genetic map for umc58 and the centromere of chromosomes 1. At present, the physical location of most genes or markers is still unknown. We suggest that different genes and species should not be the same for the relationship between the genetic and physical map. Although there are variations between these two types of map, the correlation between them will be displayed for more and more genes or markers as their physical mapping is developed.

Helminthosporium carbonum susceptibility genes of maize include hm1 and hm2. In the genetic map, hm1 is located near the centromere of the long arm of linkage group 1, and hm2 in the interstitial region of the long arm of linkage group 9[9]. It is interesting that the hybridization site of umc58 is shown to be physically located just in the corresponding chromosome arms and even in their corresponding regions of the arms of these two chromosomes besides at 5L5. The gene hm2, like hm1, was masked by Hm1[9]. The genes hm1 and hm2 are probably homologous or at least partly similar. Therefore, it can also be deduced that umc58 is a part of hm2 too and where umc58 is physically located in chromosome 9, the hm2 gene is located. Probe umc58 showed hybridization sites on 5L5 besides 1L3 and 9L6. Whether chromosome 5 also has *Helminthosporium carbonum* susceptibility gene as chromosomes 1 and 9 remains to be studied.

It has been shown that the activities for proteinase and DNase in both cellulase and pectinase which we used could not only digest cell wall and middle lamella, but also could act on cytoplasm and nuclear matrix, and induce chromosome G-band differentiation[3]. Therefore, when chromosomes are prepared, the chromosome G-bands can be simultaneously differentiated under a proper cellulase and pectinase treatment. In this study, treating in 1% cellulase and 1% pectinase at 30℃ for 1 h followed by 5 mol/L HCl treatment at room temperature for 30 min was chosen as the optimum condition for preparation of both protoplast chromosomes and G-banding. This condition was so mild that the detection rate of ISH was higher (22.43%) than that observed in routine ISH. In addition, this procedure is very simple and has the same steps as routine ISH. However, it is very important that the Giemsa staining should be carefully kept to an optimum degree so that the chromosome G-bands show blue or purple blue. Thus, the G-bands can be easily distinguished from the labeled spots, which are red. Heavy staining makes the chromosome G-bands red purple so that it will be difficult to distinguish them from the la-

beled spots in color, while insufficient staining can not show clear G-bands.

The reason that development of the ISH technique lagged behind in plants compared with that in humans is manifold. It was suggested that the major factor contributing to slow progress should be the presence of cell walls. We think, besides the obstacle of the cell walls, all the difficulties of the G-banding technique are important factors which restrict development of the chromosome ISH in plants. C- or N-banding can only show a limited number of bands and most of the chromosomal regions can not be marked by C- and N-bands. Therefore, it is more difficult to exactly map most genes or genetic markers onto specific regions of chromosomes by ISH in plants. Although a percent distance from centromere to detection site was calculated and analyzed in some mapping studies[7,8] in order to eliminate the problems associated with variation in chromosome condensation, the mapping error can not be avoided because the different chromosomal regions are not the same in condensation degree. In recent years, a human cytogenetic map can be constructed in detail and region specific DNA fragments can be isolated for any part of the human genome by microdissection of human chromosomes. One of the important factors is that a technique combining G-banding and ISH has been developed. It is sure that in plants, construction of fine cytogenetic maps and isolation of region specific DNA fragments will be developed rapidly as the techniques combining G-banding and ISH are improved and applied.

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