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INTROGRESSION OF GENETIC MATERIAL FROM *ZEA MAYS* SSP. *MEXICANA* INTO CULTIVATED MAIZE WAS FACILITATED BY TISSUE CULTURE

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Abstract

Zea mays ssp. *mexicana*, a wild relative of cultivated maize (*Z. mays* ssp. *mays*), is a useful gene resource for maize breeding. In this study, two populations were generated by conventional breeding scheme (population I) or tissue culture regime (population II), respectively, to introgress genetic material of *Z. mays* ssp. *mexicana* into maize. Karyotype analysis showed that the arm ratios of 10 pairs of chromosomes in parent maize Ye515 and derivative lines from 2 different populations with 26% and 38% chromosome variation frequencies, respectively. Alien chromatin was detected in the root tip cells of progeny plants through genomic *in situ* hybridization (GISH). There were 3.3 chromosomes carrying alien chromatin on average in population I and 6.5 in population II. The hybridization signals were located mainly at the terminal or subterminal regions of the chromosomes and the sizes were notably variant among lines. Based on those results, it is concluded that the introgression of genetic material from *Z. mays* ssp. *mexicana* into cultivated maize was facilitated by tissue culture, and subsequently some excellent materials for maize breeding were created.

Introduction

A large number of genes from wild relative species are of great value for crops improvement, therefore it is necessary to exploit and make full use of these genetic resources. Teosintes are the closest living wild relatives of cultivated maize in *Maydeae* and considered to be excellent materials for the study of the origin and evolution of maize (Doebley *et al*., 1995; Hilton & Gaut, 1998; Lauter & Deobley, 2002; Vigouroux *et al*., 2002; Wang *et al*., 2005), but till now only a few studies have focused on how to transfer desired genes into domesticated maize for improvement (Wei *et al*., 2003; Tang *et al*., 2005; Wang *et al*., 2008b). Among teosintes, the annual *Z. mays* ssp. *mexicana* belongs to the same species and has the same number of chromosomes as maize. It shows high tillering and ramifying abilities, strong growth vigor, high protein content in the kernel and tolerance/resistance to multiple fungal diseases. Those qualities make it a useful genetic resource for maize improvement. Transferring the desired genes into elite inbred lines to create excellent breeding materials is an interesting strategy to increase yield and quality of maize.

Introgressive hybridization is considered as a successful method to transfer desired genes from the alien species to a chromosome of the cultivated crops with minimal amounts of accompanying foreign chromatin. Larkin *et al*., (1989) and Jain (2001) proposed that tissue culture was a possibly feasible means for the introgression of desirable traits into the genomes of cultivated crops since it frequently induced chromosome breakage and fusion in plant species (Kaeppler *at al*., 2000).

In this study, genes from *Z. mays* ssp. *mexicana* were transferred into maize elite inbred line Ye515 in order to produce excellent inbred lines and the efficiencies of obtaining small-segment introgression lines by conventional methods or the combination of conventional methods with tissue culture were compared. The chromosomes in root tip cells from different introgression lines of both populations were subjected to examine the location, number and size of the introgressed segments by genomic *in situ* hybridization (GISH).

Materials and methods

Plant material and population development: Cultivated parent Ye515 (kind gift from Shandong Denghai Seeds Co., Ltd, China) is an elite inbred line that is widely used in Chinese agriculture. However, it shows some disadvantages such as imperfect kernel quality, high infection of maize stalk rot and maize rough dwarf disease. Wild parent *Z. mays* ssp. *mexicana* was purchased from Shandong Lujiuzhou industrial Co., Ltd.

F_1 hybrids (Ye515 × *Z. mays* ssp. *mexicana*) were crossed with the recurrent parent Ye515 to get BC_1 seeds. Two plants from each of the 60 selected BC_1 lines were self-pollinated to produce BC_1F_2 generation; three ears of each BC_1F_2 line were selected to produce BC_1F_3 individuals by self-pollination, and BC_1F_3 plants were then allowed to self and only one ear was harvested for three successive generations to obtain population I (conventional breeding population). For population II (tissue-culture derived population), immature embryos from ears of self-pollinated BC_1 plants were inoculated to induce embryogenic calli and regenerate plants, and 164 ears of regenerated plants derived from 52 BC_1 lines were self-pollinated to develop BC_1F_3 seeds. Then BC_1F_3 plants were allowed to self and also only one ear was harvested for three consecutive generations to form population II.

Maize callus induction, subculture and plants regeneration: Ears were removed from plants at 8 through 10 days after pollination and surface-sterilized for 5 minutes in 70% ethanol. Immature embryos (1.1 mm approximately in length) were isolated from the ear by excising kernel crowns and removing the endosperm, and

then transferred onto modified MS medium supplemented with 1 mg/l 2,4-D (Li *et al*., 1990). The scutellar side was exposed while the flat plumule-radicle axis side was in contact with the medium. Callus were induced, subcultured and differentiated according to Li *et al*., (1990). The embryogenic calli were induced to regenerate plants after being cultured for 8 months.

Karyotype analysis and Genomic *in situ* hybridization (GISH): Five lines were randomly selected from each population for karyotype analysis. Qualified chromosome spreads were preprated according to Wang *et al*., (2008b). For C-banding, air-dried slides were incubated in 5% $Ba(OH)_2 \cdot 8H_2O$ for 5 min at 47.5°C, rinsed in distilled water for 3 times, incubated in 2×SSC for 8 min, and then washed thoroughly in distilled water. Slides were stained in diluted Wright's Eosin Methylene Blue Solution for 3-4 h, and rinsed in distilled water. Karyotype classification was performed according to the methods of Levan *et al*., (1964) and Li & Zhang (1996), which satellites were excluded in the total length to calculate arm ratios. The parameters of the karyotypes were based on 10 metaphase cells spreads. Chromosome measurements were made by VIEDO-TEST Karyo 3.1 software.

GISH analysis was performed according to Wang *et al*., (2008b) with 80-fold blocking DNA. The slides were denatured on a heated metal block at 90°C for 2 min and the hybridization was performed overnight at 37°C in a humid chamber. Post-hybridization wash was performed according to Poggio *et al*., (2000). Chromosomes were examined with an Olympus BX51 fluorescence microscope.

Results

Regeneration of plantlets from type II calli: Embryogenic calli were induced from the immature embryos of different BC_1F_2 progenies and maize parent Ye515 on modified MS medium supplemented with 1 mg/l 2, 4-D. The scutellar cells proliferated rapidly in a few days after inoculation, and nodular white or pale-yellow calli were visible in localized regions after14 days in culture. And after 8 months of subculture, the plantlets (Fig. 1b) were generated from the type II calli (Fig. 1a) that were fresh yellow, friable, and fast growing. The regenerated plants were generally similar to maize in morphology and were selfed for BC_1F_3 generation.

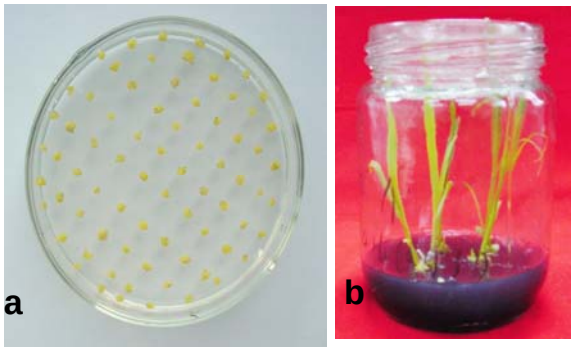

Fig. 1. Embryogenic calli and regenerated plantlets. a Type II embryogenic callus. b Regenerated plantlets on the differentiation medium.

Chromosome counting and karyotype analysis: The chromosome number of plants from the 2 populations was consistent with the euploid number of 20 and no numerical chromosome changes were observed, possibly due to the close affinity between the 2 parents and that the homogenous chromosomes of different parents could conduct normal pairing and separation during meiosis.

One pair of satellites was found on the short arm of chromosome 6 of all the examined lines and the parent Ye515. Ten pairs of chromosomes in the examined lines were apportioned to either metacentric or submetacentric chromosomes (m or sm) according to their arm raitos. All of the chromosomes were generally euchromatic, with some terminal or subterminal C-bands, but there were some obvious differences in C-banding patterns and arm ratios and so on. For example, in line I-12 (population I) and II-96 (population II) (Table 1 and Fig. 2), C-banding patterns were obviously different from that of Ye515. Ye515 (Fig. 2a & b) (18 m + 2 sm) possessed metacentric chromosomes (m) except chromosome 6. In line I-12 (Fig. 2c & d) (10 m + 10 sm), chromosome 4, 7, 8 and 9 exhibited a much increased arm ratio and changed into sm from m with the arm ratio of chromosome 4 significantly different from that of Ye515. While for line II-96 (Fig. 2e & f) (12 m + 8 sm) obtained *via* tissue culture, the arm ratios of chromosome 7, 8, 9 increased from m to sm and the ratio of chromosome 3 decreased greatly. In addition, the karyotypes revealed much more C-banding polymorphisms or possible rearrangements in different lines. The polymorphism mainly resulted from the presence / absence of particular bands and variation was also observed for band size and staining intensity. The statistic analysis confirmed the significant differences of arm ratio between derivative lines and the parent ($p<0.05$).

Some chromosomal structure aberrations such as deletions, duplications and translocations could result in the variation of chromosome arm ratio. Therefore, the arm ratios and karyotypic formula of the derivative lines were analyzed (Table 1). The variations were obvious within and between the populations. Among 5 lines for population I, totally 13 (26%) chromosomes were significantly ($p<0.05$) different from those of Ye515, while 19 (38%) ones for population II. With respect to arm ratios, the chromosomes in population II showed larger portion of alteration compared with those of population I.

Alien chromosome segments in the cells from different populations: GISH analysis was conducted using *Z. mays* ssp. *mexicana* genomic DNA as a probe and Ye515 genomic DNA as a blocker. The chromosomes in maize showed red fluorescence while those in *Z. mays* ssp. *mexicana* with yellow-orange or green-yellow fluorescence along all of their length or on the nucleus (Fig. 3a), suggesting that the identification of alien chromatin was reliable and convincing. The loci and numbers of hybridization signals varied among different lines, indicating different introgression events involved. The average number of chromosomes with hybridization signal was 3.3 in population I. Alien chromosome segments were detected in 6 of 10 lines ranging from 8

for line I-93 to 4 for line I-10 or line I-11 (Table 2, Fig. 3b & c). For population II derived from tissue culture scheme, alien chromosome segments were present in all the analyzed lines, with averaged 6.5 chromosomes per line carrying hybridization signals. For example, there were 7 signals on the chromosomes in line II-90 (Fig. 3d, Table 2). The number of chromosomes involved in heterogenic chromatin translocation or insertion in population II was significantly higher than that in population I ($p<0.05$).

The results above suggested that the chromatin of *Z. mays* ssp. *mexicana* were effectively introgressed into maize genome and the process of tissue culture substantially promoted the introgression of small alien segments into maize genome.

Table 1. Karyotypic formula and arm ratios of mitotic chromosomes in derivative lines from hybrids of Ye515 and *Z. mays* ssp. *Mexicana*.

Line	Karyotypic formula (2n)	Arm ratio of chromosome									
		1	2	3	4	5	6[a]	7	8	9	10
Ye515	18m + 2sm	1.15 ± 0.09	1.24 ± 0.21	1.62 ± 0.37	1.35 ± 0.20	1.49 ± 0.22	2.04 ± 0.15	1.58 ± 0.12	1.47 ± 0.39	1.38 ± 0.30	1.19 ± 0.20
I-01	12m + 8sm	1.14 ± 0.14	1.26 ± 0.23	1.71 ± 0.31	1.38 ± 0.12	1.17 ± 0.16	2.43 ± 0.23	1.98 ± 0.19*	3.23 ± 0.31*	1.59 ± 0.20	1.31 ± 0.06
I-12	10m + 10sm	1.19 ± 0.16	1.27 ± 0.30	1.38 ± 0.26	1.95 ± 0.17*	1.26 ± 0.01	2.17 ± 0.21	2.04 ± 0.49	1.82 ± 0.06	1.86 ± 0.30	1.48 ± 0.04
I-14	14m + 6sm	1.22 ± 0.06	1.68 ± 0.17	1.66 ± 0.14	1.27 ± 0.10	1.12 ± 0.08*	2.32 ± 0.17	2.68 ± 0.15*	2.43 ± 0.08*	1.38 ± 0.18	1.66 ± 0.31
I-22	12m + 8sm	1.29 ± 0.14	2.16 ± 0.20*	1.45 ± 0.10	1.41 ± 0.10	1.42 ± 0.24	2.02 ± 0.49	2.36 ± 0.43*	1.77 ± 0.34	1.52 ± 0.27	1.54 ± 0.27
I-38	10m + 10sm	1.32 ± 0.05*	1.93 ± 0.33*	1.74 ± 0.45	1.48 ± 0.17	1.25 ± 0.22	2.31 ± 0.10*	2.26 ± 0.45	2.41 ± 0.20*	1.41 ± 0.08	1.61 ± 0.18*
II-09	10m + 10sm	1.17 ± 0.08	1.58 ± 0.13*	1.53 ± 0.06	1.98 ± 0.13*	1.53 ± 0.39	2.24 ± 0.21	2.09 ± 0.27*	2.15 ± 0.48*	1.85 ± 0.06*	1.16 ± 0.10
II-25	8m + 12sm	1.84 ± 0.21*	1.76 ± 0.16*	1.55 ± 0.25	1.26 ± 0.12	2.53 ± 0.39*	1.81 ± 0.12*	1.69 ± 0.33	1.71 ± 0.23	1.98 ± 0.19*	1.66 ± 0.46
II-65	12m + 8sm	1.10 ± 0.03	1.31 ± 0.11	1.70 ± 0.14	1.29 ± 0.07	1.46 ± 0.37	2.20 ± 0.30	1.44 ± 0.24	2.49 ± 0.56*	1.81 ± 0.49	1.23 ± 0.18
II-90	12m + 8sm	1.22 ± 0.12	1.64 ± 0.07*	1.59 ± 0.07	1.30 ± 0.03	1.11 ± 0.07*	2.22 ± 0.26	2.18 ± 0.34*	1.81 ± 0.34	1.21 ± 0.21	1.72 ± 0.17*
II-96	12m + 8sm	1.20 ± 0.10	1.42 ± 0.01	1.20 ± 0.05*	1.45 ± 0.38	1.42 ± 0.21	2.23 ± 0.36	2.16 ± 0.31*	2.15 ± 0.07*	1.88 ± 0.13*	1.33 ± 0.21

[a]= Satellite chromosome; *= Significant at 0.05 level

Distribution of hybridization signals on different chromosomes: There existed different introgression frequencies for the 10 chromosomes within and between populations. As shown in Fig. 4, no hybridization signals were detected on chromosome 1 and 10 in population I, while the other 8 chromosomes carried strong signals with the frequencies varied from 10% to 60% (10% for chromosome 4 and 9, and 60% for chromosome 6). In population II, introgressed segments existed on all of the chromosomes excluding chromosome 10, with rather higher frequencies (above 50% for 5 chromosomes) than population I except chromosome 6 (Fig. 4). In both populations, high frequencies of signals were detected on chromosome 5 (above 50%), revealing that recombination hot spot might exist there.

The distribution of the introgressed segments on the chromosomes was not random. Most signals located at the terminal or subterminal region of the chromosomes (Fig. 3). These findings concurred with the data reported by Tian *et al*., (2006) and Chetelat & Meglic (2000) in rice and tomato, respectively.

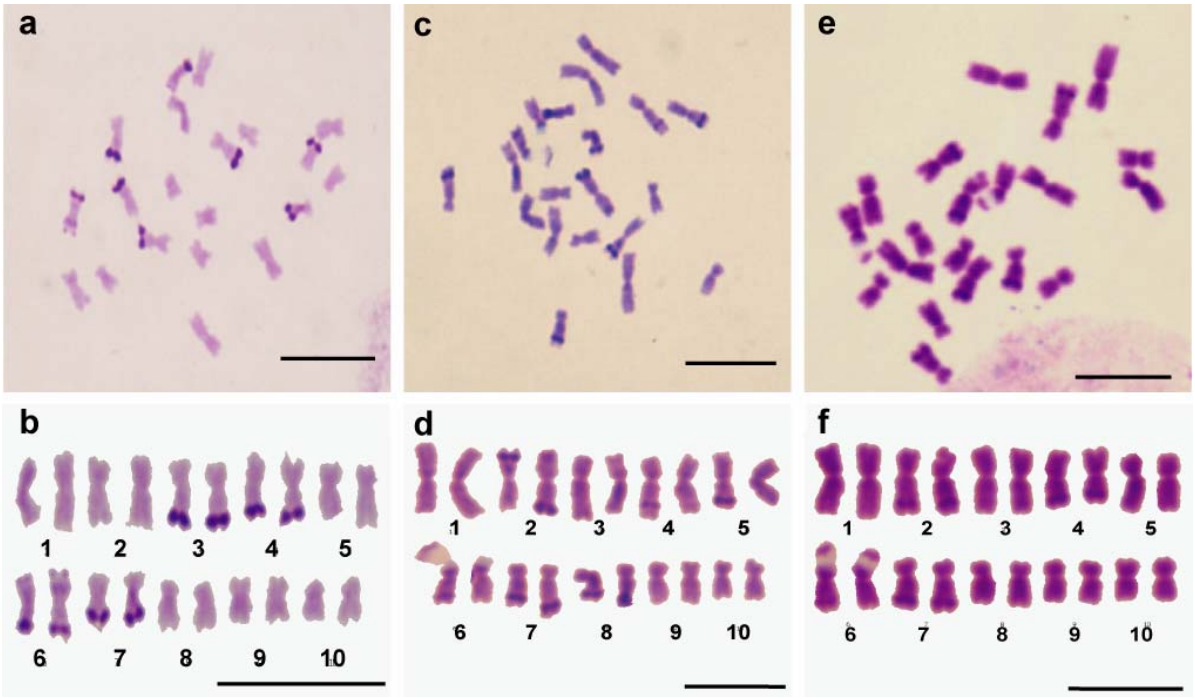

Fig. 2. The C-banding karyotypes of the chromosomes of parent maize Ye515 and its derivative lines. a, c, e,: the metaphase chromosomes of Ye515, line I-12 (population I) and line II-96 (population II) orderly; b, d, f: the karyotype of Ye515, line I-12, and line II-96. Scale bars represent 10 μm.

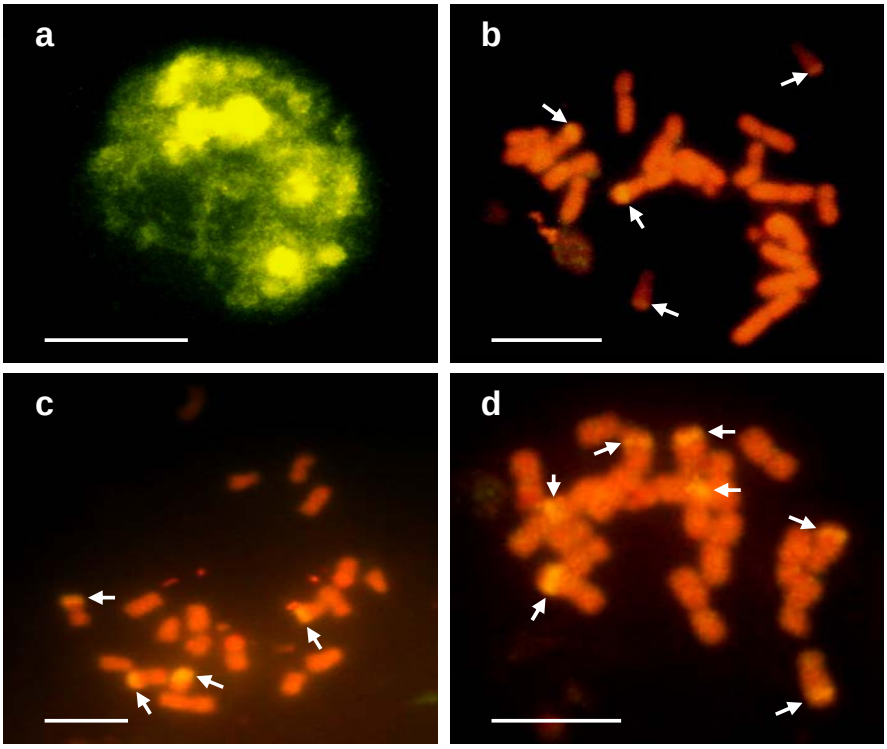

Fig. 3. Genomic *in situ* hybridization (GISH) on the mitotic cells of root tips from wild parent *Z. mays* ssp. *mexicana* and the progenies lines I-10, I-11, II-90 using genomic DNA from *Z. mays* ssp. *mexicana* as probe and maize genomic DNA as a blocker. Sites of hybridization show yellow-orange or green-yellow fluorescence with FITC, while non-hybridized sites show red fluorescence with propidium-iodide counterstain. a: A mitotic interphase cell of *Z. mays* ssp. *mexicana* shows strong hybridization signal on the whole nucleus. b: Chromosomes of line I-10, showing four chromosomes carrying alien chromatin, marked with arrows c: GISH pattern of line I-11, showing four hybridization signals on metaphase chromosomes marked with arrows. d: GISH pattern of line II-90, showing seven hybridization signals on chromosomes, marked with arrows.

Table 2. Number of chromosomes with alien segments in different population*.

Population I		Population II	
Line	**CIBAC**	**Line**	**CIBAC**
I-03	5	II-04	6
I-9	6	II-05	8
I-10	4	II-25	8
I-11	4	II-27	6
I-12	6	II-60	6
I-14	0	II-89	8
I-15	0	II-88	4
I-93	8	II-90	7
I-94	0	II-92	8
I-100	0	II-96	4
Mean	**3.3 ± 3.06[b]**		**6.5 ± 1.58[a]**

*= There are 10 lines in each population for the analysis of chromosomes
CIBAC= chromosomes introgressed by alien chromatin
[ab]=The means (± SE) from them show significant difference (p<0.05)

Location and size of hybridization signals in different populations: Three sizes of signal (S, M, L) were classified according to the ratio between hybridization signal area and the chromosome where it was located. The ratio for S was less than 20%, M from 20 to 30% and L larger than 30%. A total of 99 hybridization signals were classified for 2 populations based on the data obtained with VIDEO TEST software. The distribution of signal sizes was notably different between them (Fig. 5). The frequency of M type was 54.5%, L type 27.3%, and S type 18.2% in population I while that of M type was 33.3%, L type 24.3% and S type 42.4% in population II. From these results, we concluded that population II, the self-pollinated progeny of BC_1F_3 *via* tissue culture scheme was better for obtaining small segment introgressants through crossing with wild species. Among introgressants, the linkage drag effect of alien chromosome segment would be much reduced if the small size of the alien segments were introgressed. Small segment translocations carrying desirable genes were the ultimate aim of alien introgression.

Discussion

Introgression hybridization has been successfully applied in wheat, rice, coffee and potato for academic research or crop improvement. (Lshermes *et al*., 2000; Ko *et al*., 2002; Multani *et al*., 2003; Kazi *et al*., 2007; Kazi *et al*., 2008). The narrow germplasm base of maize would reduce potentials for long-term gains in productivity and increase possible susceptibility to new pathotypes of disease-causing pests. Alleles from teosintes for domestication genes and landraces for improvement genes could be introduced into maize breeding programs to reintroduce variation into maize breeding programs. It is specifically these genes that need to be added to maize breeding from exotic sources to broaden maize genetic base. Alternatively, transgenic alteration of the expression patterns of selected genes can be tested for desired effects on the relevant agronomic traits (Yamasaki *et al*., 2005). A bright practical prospect can be predicted in some derivative lines in this work (Wang *et al*., 2008a).

It is reported that *Z. mays* ssp. *mexicana* is able to be hybridized readily with maize (Wilkes 1977) and its genome is homologous enough to produce normal chromosomes pairing during the meiosis of hybrid. Meanwhile it has the second higher value for heterozygosity and gene diversity among teosintes (Fukunaga *et al*., 2005). So it is valuable to make full use of *mexicana* for maize improvement.

In this study, it was demonstrated that the chromatin of *Z. mays* ssp. *mexicana* could be successfully introgressed into maize. The introgression frequencies were significantly different between the 2 populations. Population II, obtained *via* tissue culture, had the significantly higher introgression frequency (6.5 chromosomes with alien chromatin per line) than population I obtained *via* conventional breeding method. For the karyotypes of the two populations, great changes of the arm ratio of chromosomes and the increased proportion of sm chromosomes in derivative lines were also observed, possibly because sm chromosomes of some derivative lines might be inherited from their wild parent. Shenoy *et al*., (1983) reported that chromosome 5, 7, 8, 9 of *Z. mays* ssp. *mexicana* were sm, differring from Ye515. McClintock (1984) hypothesized that when a plant suffered a shock, its genome may change greatly in response to the challenge and be reprogrammed, even over successive plant generations. Under the tissue culture environment, the resetting of genome may not follow the same orderly sequence that occurs under natural conditions, which result in more alterations of chromosomes. As a result, the chromosome abnormalities, such as translocations, inversions, deficiencies and duplications would be enhanced.

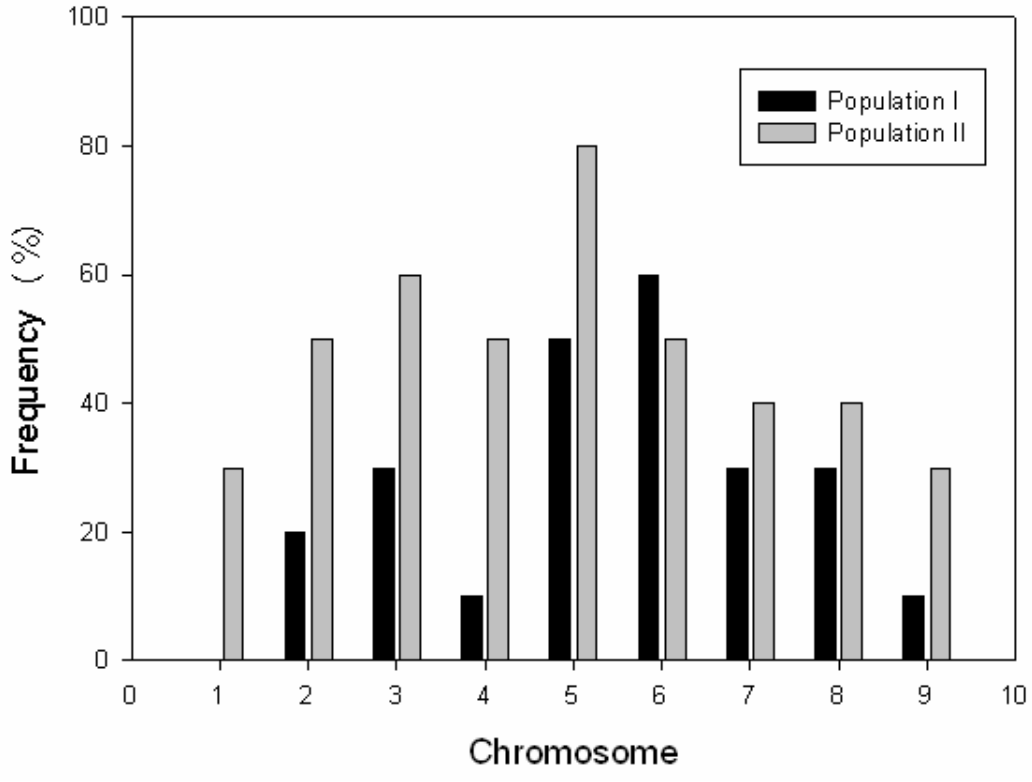

Fig. 4. Distribution of hybridization signals on different chromosomes of both populations. Population I was generated using conventional breeding program; Population II was generated using a scheme involving tissue culture.

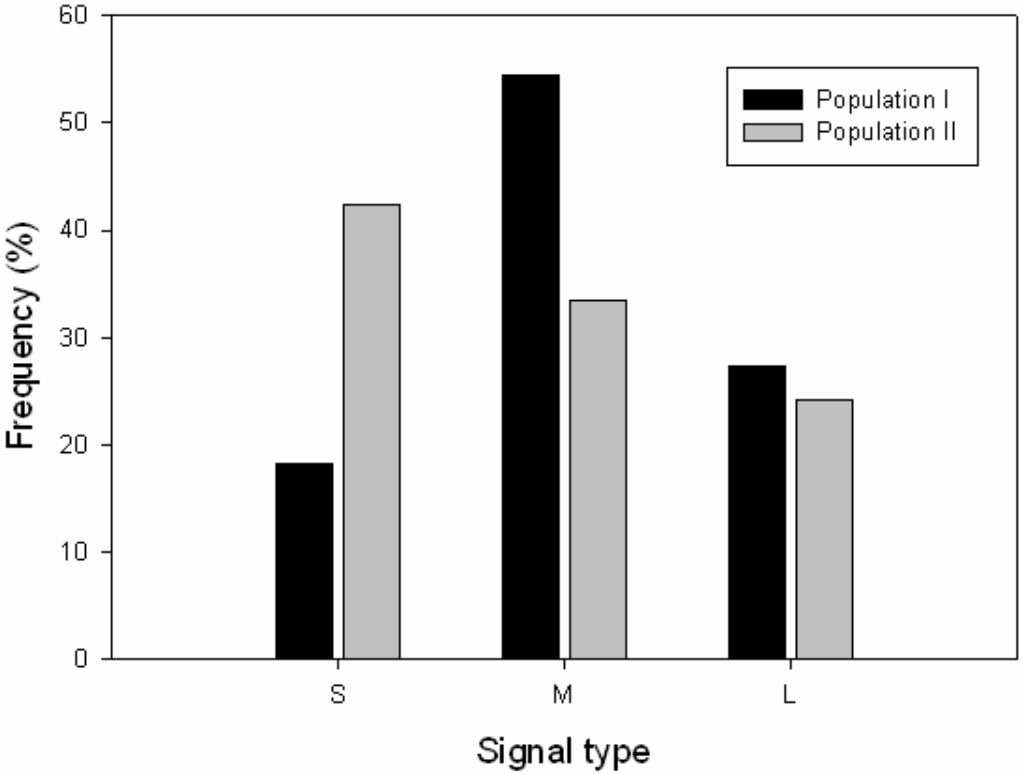

Fig. 5. Frequency of different types of hybridization signals on chromosomes of the two populations. Population I was generated using conventional breeding program; Population II was generated using a scheme involving tissue culture.

GISH, a powerful technique for the detection of alien genetic material transfer in introgressive hybridization, can visualize the size of alien chromatin, and follow the alien chromosomes or chromosome segments in the subsequent backcrosses and selection procedure. In this study, the presence of hybridization signal on chromosome 10 in all examined lines was not observed, and the possible explanations include (1) homologous pair of chromosome 10 of the hybrid was very similar, so the result of crossover between the homologous chromosomes could not be detected by *in situ* hybridization or they had little homology which rendered crossover that was rare to occur, and (2) the gamete or zygote bearing exotic chromatin in chromosome 10 was of low vitality. Meanwhile, the highest average introgression frequency was observed in chromosome 5, followed by chromosome 6. Maybe the recombination hotspots existed on them and/or the homologous chromosomes of them hand the remarkable differences. This phenomenon was reported by Wei *et al*., (2003), who analyzed the introgressed segments in lines from *Zea mays* × *Zea diploperennis* by GISH. Their results showed that the majority of signals were localized on the long arms of chromosomes 2, 3, 6 and 8. In this study, chromosome 5 and 6 possessed more alien introgression segments detected by GISH, whereas chromosome 2, 7 and 8 showed higher variant frequencies by karyotype analysis. Such difference suggested that the karyotype changes are produced not only from alien chromatin introgression, but also somaclonal variation and genome restructure in hybrid cells.

Linkage drag produces negative effects on the utilization of wild relatives during crossbreeding. Small exotic introgressed segments carrying desirable genes are the ultimate aim of introgression hybridization. During plant tissue culture, the introgression of alien chromatin is promoted (Li *et al*., 2000; Molnár-Láng *et al*., 2000; Lewis 2005). A plenty of variants produced by plant tissue culture have been reported (Phillips *et al*., 1994; Kaeppler *et al*., 2000). Cytogenetic abnormalities including ploidy and aneuploidy changes and chromosome rearrangements have been found among regenerants from tissue culture. Late-replicating heterochromatic regions may replicate even later in the tissue culture，which leads to the breakage of hromosomes. If the breakage occurred simultaneously in non-homologous chromosomes, translocation or reciprocal interchanges happened. Tissue culture is considered to be an effective way to get translocation lines (Hohmann *et al*., 1996; Li *et al*., 2000; Lewis 2005). An increased transferring rate of potato virus Y (PVY) resistance gene from a *Nicotiana africana* addition chromosome to tobacco using method involving *in vitro* culture was observed, compared to a scheme using conventional backcrossiong (Lewis 2005). In this study, population II was obtained *via* tissue culture pathway, and the lines from which manifesting traits of wild parent and the number of chromosomes with alien segments were much higher than those of population lobtained through conventional breeding. The results proved that tissue culture promoted the introgression of alien chromatin to maize genome.

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