High Spontaneous Male-Fertility-Restorer Frequency in a Maize Recurrent Selection Experiment

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Abstract

Using the crosses of the DH lines derived from a single cross hybrid Xianyu 335 as source materials for haploid induction in the recurrent selection scheme, we found that the rate of spontaneous chromosome doubling in the tassels of the haploid plants increased significantly in the second cycle and showed a cumulative genetic effect, and through using DH recurrent selection technique, the doubling rate of the haploid tassels can be doubled. Proved performing continuous recurrent selection in a narrow single cross background, the genetic makeup of the haploids had been enriched for spontaneous doubling restoration. Using hybrids between DH lines from two cycles haploid selection as source materials for haploid induction, the haploid tassel doubling rate had reached 85.15% and haploid ears showed seed set had reached 66.18%, representing a rate increase of 6.99 folds and 9.86 folds of its original source population Xianyu335.The genetic gain of set seeds plant frequency is 29.74% per cycle. Also, because the DH lines had been selected from two cycles of haploids or whole genome gamete selection based on the haploid phenotypes, it was effectively eliminated many deleterious and bad genes, and also cumulatively combined many superior genes for agronomy and plant growth vigor. These new DHs can be directly used as parents for germplasm enhancement and new hybrid selection and breeding.

The discovery of the enrichment for spontaneous chromosome doubling and fertility restoration in of the tassels of the haploid plants tassels that were derived from DH recurrent selection technique and create high spontaneous doubling rate source materials may have showed a significant application value in maize breeding. It shows significant advantages for construct maize two cycle Recurrent selection with high haploid spontaneous doubling rate can to speed up breeding output and cumulative genetic improvements.

Key words Maize; Haploid; Spontaneous doubling; Recurrent selection.

Introduction

Doubled haploid (DH) breeding technology can greatly reduce breeding time, increase breeding efficiency, and is broadly accepted in maize breeding programs around the world. Large seed business uses it as core breeding method and procedures are perfected and developed for purpose to gain advantages in competition in global seed business. . Currently, Many breeding programs have established high throughput method for haploid seed induction and haploid plant production, but still have technical challenges in producing DH plants. ,, Haploid doubling efficiency has become the limiting “bottleneck” for DH breeding method.

In literatures, the haploid doubling rate in different genetic materials ranges from 0% to 10%(Chase.S.S.,1969; Beckert M.,1994; Kato A.,2002). developing a safe, easy and high efficient chromosome doubling technique has been a major research focus in refining DH breeding method (Hui G Q,2012; Liu Z Z,2000; Wen K,2006)., in the past 10 years we used recurrent selection and genetic repairment mechanism, created a high spontaneous doubling rate male tassel haploid population, breeding materials and doubled haploids, expecting this method (patent application number 201610280480.0) can be applied in breeding practice.

1 Materials and Methods

1.1 Materials

Basic Population for haploid Induction: Single cross maize hybrid Xianyu u335(combination code: X1132X) was used for original haploid induction. Its’ female parent PH6WC belongs to Reid group and in most experiments this heterotic group show higher haploid spontaneous rate. Male parent is PH4CV， belongs to Lancaster group, in most experiments this heterotic group show lower haploid spontaneous rate.

Haploid Induction Line: Induction line JiGaoYou3 was selected by Jilin Academy of Agricultural Sciences. Its seeds carry clear and stable purple R-navajo marker, shad large amount of pollen grain, and average induction rate is 10.4%(CAI Zhuo,2007).

1.2 Methods to Obtain Haploids

Xianyu335 used as female and crossed with pollens from JiGaoYou3 for haploid induction. With R-navajo marker, the F1 seeds were screened and the haploid seeds were identified by lacking purple color plumule and having sharp embryo tip and purple crown.

1.3 Field Evaluation

Haploid seeds were planted in the nurseries in Gongzhuling city, Jilin and in Sanya city, Hainan. , plant density was 105,000 plants/ha. The fields were managed intensively. , True haploid plants were mainatained and the false haploid plants with R-navajo marker were remved at early growth stages(CAI Zhuo,2012).

1.4 Methods to Obtain DH Lines

At flowering, , shoots were bagged, the haploid plants that shed pollens were self-pollinated and clearly maked with colored shoot bags. After pollen shed period, plants were counted for shedding pollens and frequency of pollen-shedding was calculated for each population.

Harvest and count haploid ears that set seeds. Use number of ears that set seeds and are confirmed DHs in next planting season to calculate doubling rate.

1.5 DHs screen and selection, and recombine

The DH lines were planted ear-to-row in field. Based on breeding objectives, pairs of DH-lines with at least two complementary pairs were selected and crossed to produce a series of new F1s. These F1 plants were then crossed with the inducer line to produce haploid seeds and further generate new DH lines through spontaneous chromosome doubling procedure.

The above procedure was repeated to obtain DH lines of DH^2 (DHXDH), DH^3 (DH/DHXDH/DH), DH^n (DH…DHXDH…DH), for next cycle selection: superior DH lines can directly move to breeding process.

1.6 Calculation Methods for Statistical Index:

Pollen Shed Frequency (%) = Haploid Plants producing Pollen/Total Haploid Plants X 100 Set Seeds Plant Frequency (%) = Harvest DH Plants with Seeds/Total Haploid Plants X 100

2. Results and Analysis

2.1 Procedures to Study and to Create High Spontaneous Male-Fertility-Restorer Frequency Maize Population

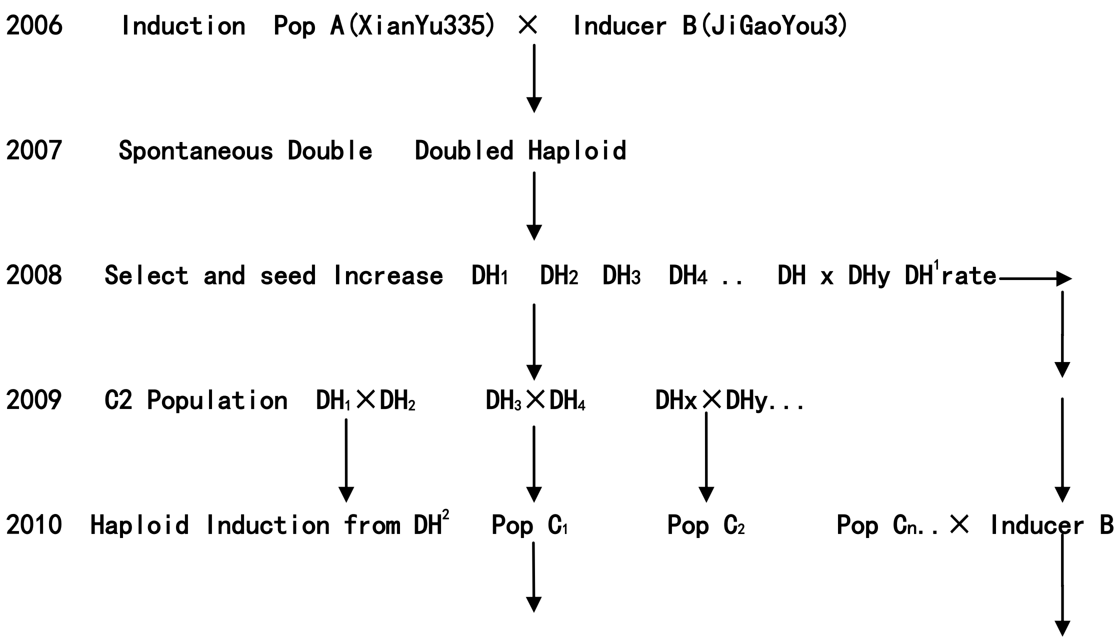
2006-2008 1st Cycle hybrid induction, obtain DH lines

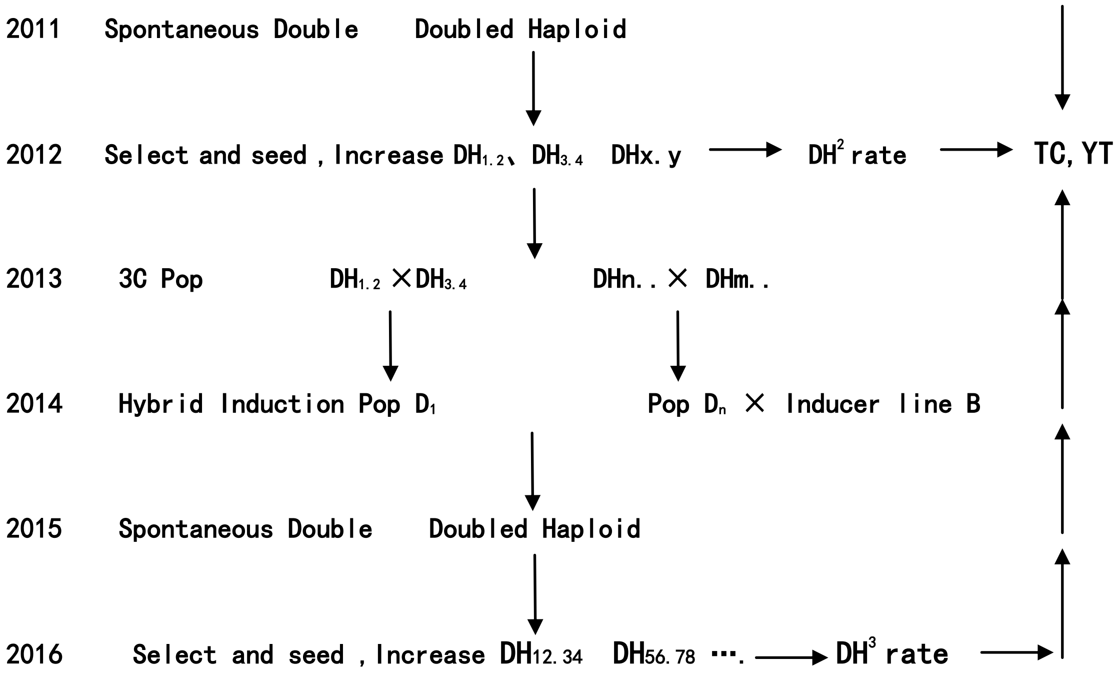
2009-2012 2nd Cycle DH^2 lines from recombined population

2013-2015 3rd Cycle DH^3 lines from recombined population,

Please see details in Figure 1

Fig. 1 Diagram of high spontaneous doubled frequency maize population creation procedures.





2.2 Discover the Genetic Repair Ability and Additive Effects of Haploid Male Tassels in their Spontaneous Doubling Rate

In the process of continuous recurrent selection over 10 years, we observed an increase in the number of male tassels shed pollen grains of the haploids, the number of anthers, the size of anthers, and the amount of pollen grains in the haploid populations in the higher cycles of recurrent selection. The increase in these traits appeared to be affected by many genes with minor effects, and show a typical continuous normal distribution.

In 2006, the initial haploid population induced from xianyu335 had the spontaneous doubling rate for haploid male tassels of 6.71% . In 2009, use crosses between selected DH sister lines as source induced materials, the spontaneous doubling rate of the 2011 haploids increased to 17.63%. Among those, the haploids derived from 07xian335-58-2 X 07xian335-128 cross had a spontaneous doubling rate 31.26%.

2.3 Evidence for the Genetic Repair Ability and Additive Effects of Haploid Male Tassels in their Spontaneous Doubling Rate

In 2014 and 2015, we induced haploid seeds from two DH-line crosses 07xian335-18 X 09xianyu335-18 and 06xian335-18 X 06xianyu335-9 - The spontaneous doubling rate and plants shedding pollen of the haploids increased to 49.96% and 39.94% respectively. Seed setting by self-pollinating these haploid increased to 35.56% and 27.04%. These results indicate that through recurrent selection the late cycle generated DH materials did showed genetic repair ability and additive effects to increase its spontaneous doubling capabilities.

2.4 Create Breeding Materials with High Spontaneous Doubling Rate of Haploid Male Tassels

Starting from 2013 , we were selecting those DH(DH^2) lines that were obtained from last crop with superior overall agronomic traits, and traits that could compensating each other to recombine to become source population materials for the 3rd cycle induction. In 2015, we obtained new spontaneous DH (DH^3) lines. It is clearly shown in Table 1 that the spontaneous pollen shedding ratio and harvest ratio again clearly elevated. The average pollen shedding of the haploids reached to 85.15%, and the average seed set ratio increased to 66.18%. Also, the size of anthers, number of shedding anthers, and amount of shed pollen grains had significantly increased, and not necessary need to very careful collecting pollens like the first cycle. Clearly, by using recurrent selection for genetic repairing ability, it can efficiently increase the frequency of haploid male tassel doubling, shedding pollen and setting seeds.

Most recombined populations from DH lines had shown doubled of their spontaneous doubling rate of their original population in this experiment. The spontaneous doubling rate of recombined populations from DH^2 lines could reach as high as 90%, and 65% could be selfed and produce seeds. Also, after two cycle whole genome gamete selection, plants which carried most harmful and deleterious genes had been eliminated and useful genes had been effectively accumulated. Either the haploid or the DH plants which carried more useful genes had shown better agronomic traits and growth vigor. Among those, some hybrids that were derived from recombined DH^2 lines had been entered yield testing experiment in Jilin province.

Table 1 Spontaneous male Fertility Restorer Situation in Recombinant Population

Generations	Basis Population	Haploid Plants	PSF[a] （%）	Harvest Ears	SSPF[b] （%）
DH1 Line	XY335	6801	11.04	457	6.71
DH2 Line	07XY335-18×09XY335-18	1229	49.96	437	35.56
	06XY335-18×06XY335-9	1302	39.94	352	27.04
	07XY335-58×07XY335-57	1566		373	23.82
	07XY335-58×07XY335-34	980		159	16.22
	07XY335-58×07XY335-128	998		312	31.26
	07XY335-151×07XY335-128	398		96	24.12
	07XY335-98×07XY335-1	420		46	10.95
	07XY335-98×07XY335-58	432		31	7.18
	07XY335-98×07XY335-171	376		22	5.85
	07XY335-171×07XY335-10	121		10	8.26
	07XY335-10×07XY335-1	566		53	9.36
	07XY335-10×07XY335-58	982		117	11.91
	Mean		44.95		17.63
DH3 Line	(X1132X-1/06XY 335-18）-4×(X1132X-1/06XY335-18）-3	982	91.24	713	72.61
	(X1132X-1/06XY 335-18）-4×(X1132X-1/06XY335-18）-2	699	83.98	485	69.38
	(X1132X-1/06XY335-18）-5×(X1132X-1/06XY335-18）-2	883	74.97	505	57.19
	(X1132X-1/06XY335-18）-2×(X1132X-1/06XY335-18）-3	913	85.1	679	74.37
	(X1132X-1/06XY335-18)-4×(X1132X-1/06XY335-18）-6	889	88.75	487	54.78
	(X1132X-1/06XY335-18）-2×(X1132X-1/06XY335-18）-1	975	86.87	670	68.72
	Mean		85.15		66.18

a PSF: Pollen Shed Frequency = Haploid Plants Shed Pollen/Total Haploid Plants X 100%

b SSPF: Set Seeds Plant Frequency = Harvest DH Plants with Seeds/Total Haploid Plants X 100%

2.5 Based on spontaneous doubling method to establish a doubled recurrent haploid breeding technic system

Based on the evidence that maize doubled recurrent selection method is the foundation for modern commercial breeding, also the recurrent selection repairing ability to increase spontaneous doubling rate of the haploids, it is possible to combine both to construct a haploid double recurrent selection breeding system, based on spontaneous doubling rate increase. Also it is possible to use recombined DH lines as source materials in recurrent selection to improve female population (stiff-stalk group) and male population (non-stiff stalk group). This n cycle DH recombination intra population improvement can be used to construct two set core germplasm groups. And at different breeding stages, based on trait improvement at different breeding stage, it is possible to introgress new useful genes to generate new DH lines. We also can use hybrid cross, and backcross method to integrate high spontaneous doubling rate from DH to core germplasm or major inbreds, to create high spontaneous rate DH lines in different heterotic group for practical breeding application.

Fig.2 Selfed ears of DH_0 and DH_1 after C3 Selection
Up is selfed ears from DH_0, down is selfed ears from DH_1.

Fig.3 Plants of DH_0 and DH_1 after C3 selection
Left is haploid(or DH_0) plants, right is DH_1 lines.

3. **Discussion and Conclusion:**

Based on meiosis and double fertilization theories in higher plants, maize haploid plants are unable to perform normal meiotic division, therefore, unable to produce normal pollen and egg cells to perform normal fertilization and set seeds. But in our research we found that most haploid plants could form viable haploid eggs and occasionally few haploid male tassels could shed viable pollen grains. Under favorable growing condition, haploids derived from populations with favorable genetic makeup showed higher percentage pollen shedding plants and large amount of pollens. These results indicate that maize haploid genome contains genes for self-repair and automatic adjustment ability to recover its diploid genome through mis-division and to produce normal haploid gametes. The favorable growing condition can help haploid plants to perform more mis-division to generate more or larger chimeric diploid tissues to yield normal haploid gametes. Therefore, the limiting factor or bottleneck for a haploid plant to become a DH plant is determined by complete or partial recovering of haploid cell diploidization and yield normal haploid pollen grains and eggs(Chalyk S.T.,1994; Kleiber D,2012; Duan M,2012).

For most genotypes, the haploid spontaneous doubling rate is very low. The genetic effect is weak, and the environmental influence is variable, it is therefore defined as multiple gene control genetic traits. Shatskaya et. al. believe that the spontaneous doubling rate variation among different genotypes is a genetically controlled trait(Shatskaya O A,1994). Penghao Wu et.al. found 8 genetic loci responsible for high spontaneous doubling rate in a mapping population and classified their functions including additive, dominant, partial dominant and over dominant effects(Wu PH,2014). Our study showed that through repeated recombination of DH genomes from each breeding cycle we could be continuous improve related traits of spontaneous doubling, including anther traits, anther extrusions, and amount of pollen sheds among plants. The effects were minor and expressed a typical continuous distribution curve, evidently it was controlled by multiple genes with minor genetic effect.

Materials used for this research is single cross hybrid PH6WC×PH4CV with relative narrower genetic background and with limited gene pairs that controlled repairing. We observed a significant increase of haploid spontaneous doubling rate by repeated DH recombination recurrent selection and possibly an accumulation of favorable genes from each breeding cycle. The pollen shed rate among haploid plants could reach as high as 91.24% and seed set plants as high as 66.18%, clearly shown an additive effect. Just going through one cycle recombination, the spontaneous doubling rate for good materials was doubled and superior doubling materials could be obtained from recombination among new generated DH lines.

Even the effect of genes controlling spontaneous doubling time and rate is minor, it is possible to eliminate those inert alleles or representing lethal genes with no doubling effect through repeated DH recombination and selection. Therefore the time and frequency of chromosome doubling were significantly moved forward and increased and the size of chimeric normal pollen grains became large enough to spontaneous shed its normal pollen grains. In most cases, an anther with chimeric tissue consisting small portion of doubled cells that are embedded or surrounded by degenerated germ tissue would never be able to open and shed and should be treated as non-functional anther. Therefore, anthers with very late spontaneous doubling event may not produce large enough size normal pollen grain sector to make it open and shed pollen naturally. That is the reason why haploid tassels are often unable to shed pollen, because of their very late occurring doubling events. On the other hand, every normal egg can be fertilized. Therefore, the rate of female side doubling is always higher than male side. It is an artifact or biased estimate. The DH recurrent selection method can effectively accumulate additive gene effects for spontaneous doubling rate and move doubling time earlier to form large functional normal pollen sectors, and to eliminate inert useless alleles. Favorable growing conditions has double assured its occurrence. Therefore, the spontaneous doubling rate is significantly increased.

Based on single cross narrow genetic background source materials, we used DH recombined recurrent selection method to create high haploid spontaneous doubling materials, we belive that chromosome doubling of maize haploid plants can be achieved through genetic repairing in addition to spontaneous doubling, chemical doubling, and Physical doubling methods. The genetic repairing method is significantly effective and application prospective for future. There are very little reports in this area, except relative researches from Zabirova and Geiger, but no new progress (Zabirova E R,1993; Geiger,2011). In China, no reports are in this area. The genetic repairing mechanism is not clear. It is quite similar to transposable elements which are shown changes in timing and in frequency. The high spontaneous doubling rate could be very similar to changes of transposition in timing and in frequency. It is *possible* to make doubling events occur at earlier growth and more frequently, thus forms more large chimeric sectors in many anthers that filled with normal pollen grains, and those anthers could be opened and released naturally. It is therefore to suspect that the genetic repairing mechanism could be due to improvement in time and in frequency of doubling events. Further test is required to confirm our suspect.

In practical breeding application, maize DH recurrent selection repairing method and construction of high spontaneous haploid doubling materials has bright future prospective. Combined with doubled recurrent selection breeding procedures, it is possible to build a DH recombined doubled recurrent selection network to enhance the frequency of spontaneous doubling rate. It is also possible to improve haploid doubling rate of two heterotic core groups for DH production and for making single cross hybrids. This high efficiency spontaneous haploid doubling technic network can be used to replace the complicate laboratory or factorial doubling technic process. Valeriu's haploid recurrent selection experiment proved that the yield level and agronomic traits of the improved population had been significantly improved(Rotarenco Valeriu,2012). It is also possible to use high frequency doubling DHs as donor and backcross to transfer high frequency genes to inbreds or core germplasm of two compensate recipient heterotic groups to yield isogenic inbreds with high spontaneous haploid doubling rate, and to constitute core breeding germplasm to make commercial single cross hybrids. The finding from this study should be improve the breeding efficiency and practical application, and move breeding methodology to a new platform.

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