Synthesizing double haploid hexaploid wheat populations based on a spontaneous alloploidization process

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Abstract

Doubled haploid (DH) populations are useful to scientists and breeders in both crop improvement and basic research. Current methods of producing DHs usually need in vitro culture for extracting haploids and chemical treatment for chromosome doubling. This report describes a simple method for synthesizing DHs (SynDH) especially for allopolyploid species by utilizing meiotic restitution genes. The method involves three steps: hybridization to induce recombination, interspecific hybridization to extract haploids, and spontaneous chromosome doubling by selfing the interspecific F1s. DHs produced in this way contain recombinant chromosomes in the genome(s) of interest in a homogeneous background. No special equipment or treatments are involved in the DH production and it can be easily applied in any breeding and/or genetic program.

Keywords: Allopolyploid; Doubled haploid; Unreduced gametes

1. Introduction

Double haploids (DHs) are plants produced from haploid sporophytes by chromosome doubling. DHs have the advantage that all genes are homozygous. Hence, they are excellent materials for both basic research and practical breeding. Production of haploids and chromosome doubling are two key steps in DH production. Methods that have been used for DH production in common wheat generally involve chemical treatment for chromosome doubling. The different ways of producing haploids include (see reviews by Devaux and Pickering, 2005; Forster et al., 2007): 1) utilization of androgenesis through anther or isolated microspore culture, 2) wide hybridization followed by elimination of chromosomes from male parents, and 3) gynogenesis by female gametophytes (usually unfertilized egg cells) culture. However, these methods have common weaknesses derived from the processes of in vitro culture or artificial chromosome doubling. The production of haploids by in vitro culture is a highly technical procedure that is labor-intensive, time-consuming and costly. Haploid production may be restricted by species- or genotype-dependency and low frequencies of embryogenesis, segregation distortion, and gametoclonal and somaclonal variation occurring at the time of induction and in vitro regeneration. The use of colchicine or other chemical agents for chromosome doubling is cumbersome and may cause abnormalities to the plants (Niemiebowicz-Szczytty, 1997).

Many important crops like durum wheat, common wheat, cotton, oat, canola, coffee, oilseed rape and tobacco are allopolyploids, derived from hybridization of two or more distinct species with related (homoeologous) genomes followed by chromosome doubling. For example, common wheat (Triticum aestivum L., 2n = 6x = 42, AABBDD) is one of the most

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remarkable allopolyploids. It was formed by a two-step spontaneous allopolyploidization process (Kihara, 1944; McFadden and Sears, 1944). During the first step diploid Aegilops speltoides (Tausch) Gren. \((2n = 2x = 14, SS)\), or a closely related species, was pollinated by diploid \(T. urartu\) Tumanian ex Gandilyan \((2n = 2x = 14, AA)\), resulting in tetraploid \(T. turgidum\) L. \((2n = 4x = 28, AABB)\). Domesticated \(T. turgidum\) was then pollinated by diploid \(Ae. tauschii\) (Coss.) Schmalh \((2n = 2x = 14, DD)\), resulting in common wheat after chromosome doubling. Unreduced gametes that were produced due to meiotic restitution in the interspecific or intergeneric hybrids played an important role in these polyploidization events (see reviews by Harlan and de Wet, 1975; Ramanna and Jacobsen, 2003; Jenczewski and Alix, 2004).

Previously, we hypothesized that meiotic restriction could be utilized to produce DH production (Zhang et al., 2007). We have tested this hypothesis by applying meiotic restriction to production of synthetic DH populations. In this communication, we report our test results and demonstrate the efficacy of this method with molecular markers.

2. Materials and methods

2.1. Plant materials

Plant materials used in this study included \(T. turgidum\) ssp. \(dura\)m cultivar Langdon (abbreviated as LDN) from USA, \(T. turgidum\) ssp. \(turgidum\) landraces Yuanzhuimai (AS2255) and Ailanmai (AS313) from southwestern of China, \(Ae. tauschii\) ssp. \(tauschii\) accession AS60 from Middle East. LDN (Zhang et al., 2008a, 2010), AS2255 and AS313 (Zhang et al., 2007, 2010) have genes for the formation of unreduced gametes in their hybrids with \(Ae. tauschii\) accession AS60.

2.2. Production of hybrids

The emasculation and pollination were done as described by Liu et al. (1999). AS2255 and AS313 were both crossed with \(T. turgidum\) LDN. Their \(F_1\) hybrids were then pollinated by \(Ae. tauschii\) AS60. No embryo rescue technique or hormone treatment was applied for embryo rescue. Hybrid seeds were germinated in Petri dishes and the seedlings were transplanted 10 cm apart in the experimental field with 60 cm between rows. Randomly selected spikes were sampled from the \(F_1\) plants for cytological observation. Spikes that were selected for seed production were bagged before anthesis to prevent cross contamination.

Crossability was expressed as the percentage of the number of the \(F_1\) seeds obtained to the number of florets pollinated. The selfed seedset was calculated as the percentage of the number of the seeds obtained to the number of florets.

2.3. Cytological observations

Somatic chromosomes were determined on squashed root tip preparations as reported by Zhang et al. (2007). Root tips (1–3 cm long) were cut from germinating seeds and pretreated in ice water at 0–4 °C for 20–24 h, then fixed in Carnoy’s fixative I (3 parts 95% ethanol to 1 part glacial acetic acid) overnight. Feulgen staining was applied to the fixed root tips to visualize chromosomes with an Olympus BX-51 microscope coupled with a Photometric SenSys CCD camera.

2.4. SSR analysis

For each DH line and parents, total genomic DNA was isolated from young fresh leaves collected from five plants using the 2XCTAB method (Zhang et al., 2004). Since about 20% of the \(F_2\) plants were aneuploids a bulked leaf sample from five plants greatly reduced the impact of chromosome loss on SSR genotyping. A total of 327 SSR primers on A or B genome chromosomes were selected according to Röder et al. (1998) and Somers et al. (2004). PCR amplifications were done in 20 μL mixtures containing 1 unit ExTaq polymerase (TaKaRa, Dalian, China), 1 × ExTaq buffer (Mg²⁺ free), 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 200 nmol/L of each primer, and 50–100 ng template DNA at 94 °C for a 5 min initial denaturation, followed by 38 cycles of 30 s at 94 °C, 30 s at either 50, 55 or 60 °C (depending on the individual SSR), 30 s at 72 °C and a final extension at 72 °C for 5 min. PCR was performed in a MJ Research thermocycler (Watertown MA, USA). The amplified fragments were separated by electrophoresis in 6% polyacrylamide denaturing gels and visualized with silver-staining method (Zhang et al., 2004).

2.5. Identification of high molecular weight glutenin subunits (HMW-GSs)

The procedure described by Wan et al. (2000) was followed to isolate HMW-GSs from wheat grains and to separate them by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

2.6. Chi-square analysis

Chi-squared analyses were performed with SPSS software version 11.0 (SPSS Inc., Chicago) to assess the goodness-of-fit of SSR and HMW-GS markers between observed and expected segregation ratios in the DH population.

3. Results

Two tetraploid \(F_1\) hybrids, LDN × AS2255 and LDN × AS313, were made. The tetraploid \(F_2\)s were then pollinated by \(Ae. tauschii\) AS60 to form triploid \(F_1\) hybrids. The triploid \(F_1\) hybrids were made with no difficulty. The crossabilities were 9.17% for LDN/AS313//AS60 and 7.90% for LDN/AS2255//AS60. Over 50% of \(F_1\) seeds germinated without embryo rescue or special treatment. In total, 118 and 83 \(F_2\) hybrid plants were obtained from LDN/AS313//AS60 and LDN/AS2255//AS60, respectively. These triploid \(F_1\) hybrid plants were cytologically confirmed. They grew vigorously and had tough tenacious brown glumes, which were obviously
inherited from *Ae. tauschii*. All were fertile although variable in selfed seedset (Table 1). After selfing of F1 plants, 118 and 83 F2 lines were obtained from the LDN/AS313//AS60 and LDN/AS2255//AS60 crosses, respectively. To confirm somatic chromosome numbers, 89 F2 plants derived from 10 random LDN/AS313//AS60 F1 plants, were subjected to somatic chromosome counts. Among them, 71 (79.8%) were euhexaploid DHs with 42 chromosomes. Spontaneous chromosome doubling obviously occurred in F1 plants via the union of functional unreduced gametes. However, 20.2% of the F2 plants were aneuploids with 2n = 40, 41 and 43. Such aneuploids were presumably derived from gametes with missing or additional chromosomes.

Of the 327 SSR primer sets, 50 revealed clear polymorphism between *T. turgidum* AS313 and LDN and *Ae. tauschii* AS60 and each chromosome of the A and B genomes was represented by at least one SSR marker. These 50 polymorphic markers were used to genotype 117 of the 118 LDN/AS313//AS60 F2 lines (the exception involved a bad DNA sample). The results revealed that the 117 DH lines all have either the LDN allele or the AS313 allele for every SSR marker. These results proved that they were all homozygous. Forty three (86%) of the 50 SSR markers segregated in ratios of 1 (LDN allele): 1 (AS313 allele), agreeing with the expected segregation in a usual DH population. These results validated the usefulness of this DH population in further genetic analysis. Segregation for seven SSR markers did not fit the expected 1:1. Of which, four were on a closely linked region of chromosome 6B while others were located on three chromosomes. Segregation distortion was not caused by meiotic restitution alleles since all the 118 F1 haploid hybrid plants from LDN/AS313//AS60 produced F2 doubled haploid plants. Segregation distortion (a deviation from the expected Mendelian segregation ratio) is a common biological phenomenon and occurs in a number of species, which may be caused by segregation-distortion loci (SDL) (Lyttle, 1991; Lorieux et al., 1995).

In addition to SSR markers we also genotyped the 118 LDN/AS313//AS60 DH lines for HMW-GS composition. In LDN, both the x- and y-type subunit alleles at the *Glu-1A* are silent and the 6 + 8 pair occur at Glu-1B (Xu et al., 2004). *T. turgidum* AS313 has a composition of 23 + 18 (Yang et al., 2000). Our results indicated that, among the 118 LDN/AS313//AS60 DH lines, 55 showed Langdon allele with 6 + 8 and 63 showed AS313 allele with 23 + 18, clearly a 1:1 ratio of alleles.

**4. Discussion**

Doubled haploidy provides the fastest route to homozygosity. DHs have been widely used in plant breeding and basic research (Jauhar et al., 2009). Taking wheat as an example, we demonstrate here a procedure for producing DH populations by a synthesis method based on allopolyploidization. This method takes the advantage of meiotic restitution genes and has three steps (Fig. 1). The first cross brings two genotypes of a species of interest together and creates an opportunity for them to recombine normally. A following interspecific hybridization between these intraspecific F1 and an alien parent will capture the recombinant gametes in a haploid condition. Spontaneous chromosome doubling via unreduced gametes of these haploids will then generate double haploid sporocytes with the recombinant chromosomes in a homozygous condition and in a uniform background of the second species. The interspecific hybridization step is necessary because meiotic restitution genes are functional only in haploid plants (Zhang et al., 2007; Wang et al., 2010). The alien species can be one that has additional valuable traits to

### Table 1

Selfed seedset of triploid F1s between *Triticum turgidum* F1s and *Aegilops tauschii*.

<table>
<thead>
<tr>
<th>Hybrid combination</th>
<th>No. of triploid F1 plants</th>
<th>No. of florets</th>
<th>No. of F1 seeds</th>
<th>Seed per plant</th>
<th>Seedset (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDN/AS2255//AS60</td>
<td>83</td>
<td>42678</td>
<td>11595</td>
<td>139.70 (15–575)</td>
<td>27.17 (3.10–56.65)</td>
</tr>
<tr>
<td>LDN/AS313//AS60</td>
<td>118</td>
<td>59054</td>
<td>19817</td>
<td>167.94 (15–702)</td>
<td>33.56 (5.52–71.48)</td>
</tr>
</tbody>
</table>

* Value ranges among plants are given in parentheses.
transfer from, such as *Ae. tauschii*, or one that induces alien chromosome elimination in interspecific hybrids, such as maize. Obviously, two key factors determine the success of the synthesis method: meiotic restitution and interspecific hybridization. A large interspecific F1 hybrids is necessary for an adequate recovery of recombinants. In the current example this was a relatively easy step since we were able to get a mean crossability of about 8% for two interspecific crosses, all in the absence of hormone treatment or embryo rescue. In a previous larger scale experiment, 372 wide hybridization combinations were made by crossing 196 *T. turgidum* lines belonging to seven subspecies with 13 *Ae. tauschii* accessions. Again, without embryo rescue and hormone treatment, 3713 F1 seeds were obtained from 66220 pollinated florets with a mean crossability of 5.61% (Zhang et al., 2008b).

Obviously the availability of genotypes with meiotic restitution genes is the key pre-requirement for DH production. In the present study, both *T. turgidum* parents Langdon and AS313 had meiotic restitution genes although they have genetic differences in ability to produce unreduced gametes. A strong allele in LDN (Matsuoka and Nasuda, 2004; Xu and Joppa, 1992, 1995, 2000; Matsuoka and Nasuda, 2004; Cai and Xu, 2007; Zhang et al., 2007, 2010) may be related to the variation in the fertility of the *T. turgidum × Ae. tauschii* triploid F1 plants, which was the result of production of functional unreduced gametes (Fukuda and Sakamoto, 1992; Xu and Dong, 1992; Xu and Joppa, 1995, 2000; Matsuoka and Nasuda, 2004; Cai and Xu, 2007; Zhang et al., 2007). Meiotic restitution seems to be a common phenomenon in plants since more than half of the known plant species have resulted from polyploidization for which meiotic restitution is the believed driving force (Stebins, 1971; Wendel, 2000). Meiotic restitutions that lead to unreduced gametes have been observed in many crops (Peloquin et al., 1989; Bingham et al., 1994; Zhang et al., 2007). A recent study indicated that 102 of 115 *T. turgidum × Ae. tauschii* F1 hybrid combinations, involving 76 genotypes of seven *T. turgidum* subspecies and 24 *Ae. tauschii* accessions, produced unreduced gametes (Zhang et al., 2010). Therefore, identification of lines containing meiotic restitution genes should be relatively easy. However, we should consider the probability that sometimes only one of two *T. turgidum* wheats used in SynDH has meiotic restriction gene. The potential loss of gametes that do not have the meiotic restriction gene in DH production could be a pitfall of this method if the meiotic restriction gene is involved in the first hybridization but not the second hybridization. This could happen because the meiotic restriction gene is segregated during the meiosis in the F1 plants and 50% of the gametes theoretically would not have this gene and, as a result, not be able to produce DHs by the second hybridization since haploid plants without meiotic restriction gene are sterile. In such case, segregation distortion will occur in the chromosomal region flanking meiotic restriction gene.

Approximately 20% of F2 plants were aneuploids in our study. However, this should not be a big problem to genetic analysis or breeding because most aneuploids are cytologically unstable and should revert to the euploid state within a few generations. Other types of aneuploids are weak and morphologically distinctive and can easily be eliminated from the population.

One might argue that the existence of an unwanted alien genome could be troublesome to future genetic analysis. This should not be the case because the alien genome is not involved in genetic recombination with the genomes of interest and will be homogenous in all the DH derivatives of a particular cross. For example, if we are interested in analyzing QTLs in the A and B genomes of *T. turgidum*, we can use tetraploid wheat to make the first hybrids and *Ae. tauschii* as the alien parent in the second cross. In the resulting DHs, tetraploidization-hexaploid DHs, only A and B chromosomes should be involved in genetic recombination (Fig. 1A). Similarity, if the purpose is to analyze a gene/QTL of the D genome, two *Ae. tauschii* lines are used in the first cross and a *T. turgidum* line becomes the alien parent in the second cross. The resultant DHs, diploidization-hexaploid DHs, will then have a recombinant D genome under a background of non-recombinant A and B genomes (Fig. 1B). However, we should keep caution that the non-recombinant genome may complicate phenotyping of the synthesized DH population in some degree since it may also contribute to the final phenotype of the DH plants. In an extreme case, such as the existence of an epigenetic effect controlled by the non-recombinant genome, the phenotypic variations controlled by the recombinant genomes could be largely overshadowed. This complicity by the non-recombinant genome can be minimized by skillful phenotyping in most cases.

It is not surprising that the majority of accessions of an ancestral species were not involved in speciation of the polyploid crop and thus their unique genes are not present in the crop (Reif et al., 2005; Warburton et al., 2006). These wild genetic resources are treasures for crop improvement. However, utilization of this treasure is a great challenge. Not only do we need to spend much effort to transfer a gene of interest into the crop, but also we have to face the fact that many economically important traits are quantitatively inherited and thus controlled by multiple genes or quantitative trait loci (QTLs). Multiple genes/QTLs then need to be transferred and evaluated simultaneously, hence aggravating the difficulty. Also to utilize quantitative traits in an ancestral species, wild hybridization is necessary, and is usually followed by making derivatives such as amphiploids or backcross offspring. The quantitative trait loci (QTLs) of interest are then identified by analyzing recombinant inbred lines (RILs) or double haploids (DHs) generated from the hybrid derivatives. Making RILs is time-consuming. On the contrary, DHs have the advantage over RILs because they can be generated much faster. DHs are a very useful tool for QTL mapping, particularly when the effect of the QTLs is small (Devaux and Pickering, 2005). As demonstrated for wheat in this study, the synthesis method combines these two time-consuming, labor-intensive steps into one if one of the parents in the first cross is an accession of the related ancestral species that carries the trait of interest.
The DH lines we produced are actually synthetic common wheat lines and any potentially useful variation can be incorporated into breeding programs. Allopolyploids like common wheat have multiple genomes. RILs and DHs of such a species that are created via another culture or corn hybridization have recombination equally distributed among the genomes, making the analysis complex. With the synthesis method, genetic recombination can be restricted to one or two genomes, making genetic analysis simpler. For instance, the diploidization-hexaploid DHs made in this study contain recombinant D chromosomes while all the A and B chromosomes are homogenous across the whole population (Fig. 1B). Similar tetraploidization-hexaploid DHs can be made for recombinant A or B chromosomes as well (Fig. 1A).

In conclusion, we present a novel method for DH production in an allopolyploid species without in vitro culture or artificial chromosome doubling. The requirements for the system are an inducer line with gene(s) for meiotic restitution and an alien species that can be crossed without embryo rescue. The method can be an efficient and accurate tool for genetic analysis while unlocking the gene bank in a related species.

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