Doubled Haploid (DH) technology in maize breeding

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Centro Internacional de Mejoramiento de Maíz y Trigo (Spanish)

International Maize and Wheat Improvement Center (English)

Maize and Wheat Science for Improved Livelihoods
Outline of the talk

• Conventional inbred line development vs. DH line development
• Advantages of DH lines in maize breeding
• Technology for production of maize DH lines
• Limitations in maize DH line production
• CIMMYT’s research on DH technology
Hybrid maize breeding

- U.S. Maize yields increased six-fold from 1930-present

- Hybrid varieties played a major role (Duvick, 1999)

- Homozygous inbred lines are essential for hybrid breeding
Traditional inbred line development

- Produced by repeated generations of selfing
- Resulting inbreds are highly homozygous but not 100%

If 1% genome is heterozygous, ~300 genes will be segregating in maize
Inbred line development through DH technology

**Diploid (F1/F2)**
- Chromosome #
- Genotype: Aa, Bb, Cc

**Haploid induction**
- Chromosome #
- Genotype: N

**Chromosomal doubling**
- Chromosome #
- Genotype: 2N

**Doubled haploid**
- Chromosome #
- Genotype: AA, bb, CC

100% homozygous inbred lines can be developed in **two** generations.
Advantage of DH over conventional inbred line development

Efficiency of line development is improved by **reduced time** and **simplified logistics**

- organization of nurseries/inventories
- Seed shipments
- Reduced expenses for selfing and maintenance breeding

**Reduction of costs in line development:** An European breeding company reports about **30% lower costs** when using DH
DH vs. conventional inbred lines in breeding

Use of DH lines results in **increased selection gain**

DH lines in *line per se* and testcross trials exhibit **high heritability**

Results are more reliable and reproducible than materials with various degrees of genetic segregation or levels of inbreeding

Selected DH lines **maintain high yield and outstanding agronomic traits constantly from generation to generation**

Products (Hybrids) to the market in faster time

Perfect compliance with **DUS** (distinctness, uniformity and stability) criteria for **variety protection**

Breeders focus more on germplasm evaluation than generating lines- Line production is separated from breeding
DH lines in target gene fixation

Use of DH lines enables **gametic selection**

Egg cell(n) ➔ Haploid(n) ➔ DH(2n)

Selection at the gamete level is **more efficient** than at the progeny level

**Table: Aa heterozygous selfing**

<table>
<thead>
<tr>
<th>Gametes(DH)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (AA)</td>
<td>50</td>
</tr>
<tr>
<td>a (aa)</td>
<td>50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Progeny</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>25</td>
</tr>
<tr>
<td>Aa</td>
<td>50</td>
</tr>
<tr>
<td>aa</td>
<td>25</td>
</tr>
</tbody>
</table>
DH lines in target gene fixation and genetic studies

Target gene fixation: Sample size required to fix gene combinations

<table>
<thead>
<tr>
<th>Genes/loci</th>
<th>DH($2^n$)</th>
<th>Progeny($4^n$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>1024</td>
</tr>
</tbody>
</table>

Smaller sample sizes could save costs of DNA isolation and genotyping

For marker-trait association studies:
Use of DH lines instead of commonly used F2 plants or F3 families increases the phenotype to genotype linkage facilitate a better estimation of marker/QTL effects
DH in germplasm improvement

DH provides an efficient tool to improve genepool
eliminate unfavorable genes and enrich favorable genes
In haploids, deleterious alleles are unmasked

DH lines from land races and OPVs:
Can be evaluated in replicated trials with high precision (not possible with landraces as such)

Source material for breeding programs (expanding genetic base of germplasm)

Long term conservation of heterotic germplasm without the risk of genetic drift

Allele mining from land races and OPVs
Conclusions

DH technology increases the efficiency of inbred line development and may reduce costs of breeding programs.

Use of DH increase selection gain.

DH when combined with molecular marker technology is very powerful.

DH can be efficiently used for improving landraces and OPVs.
DH technology in maize
In vitro DH line production is not used in maize

- a complex trait and is **highly genotype** dependent

- most genotypes **do not respond**

- expensive and skill required
In maize *in vivo* haploid induction is practiced

Haploids occur **naturally** at a frequency of **0.1%** in maize

**Haploid inducers:**
Specialized maize genetic stocks/lines with **capability to induce haploids at high frequency** (>1%)

**Four major steps in maize DH line production**
1) *In vivo* Haploid induction
2) Haploid identification
3) Chromosomal doubling
4) Selfing putative doubled haploids
Step 1 and Step 2: Haploid induction and identification

Haploids are maternal in origin

Inducer
R1-nj

Donor
r₁

F₁
R₁-nj/r₁

Contaminant or inhibition of R₁-nj

Haploid  Diploid
Step 3: Chromosomal doubling

Haploid Seed Preparation for Chromosome Doubling

Haploid seeds are germinated under controlled conditions (28°C; dark; 3-5 days), after anti-fungal treatment of the seeds. The tip of coleoptile is cut (2 cm) with a razor blade to facilitate penetration of the colchicine solution.
Step3: Chromosomal doubling

Seedlings dip in 0.04% colchicine + 0.5% DMSO for 12 hrs in a tank

How does colchicine work
Colchicine interferes with the cell division at the shoot apical meristem, resulting in exact duplication of the chromosome complement (haploid → doubled haploid).

Seedlings in an iron tank with colchicine
Step 3: Chromosomal doubling

Recovery of the treated seedlings in the greenhouse
Step 4: Selfing putative doubled haploids
Thanks to Dr. Shiwan Ryu and his team from Maize Research Institute GangwonARES for translation into Korean language

http://knowledgecenterblog.cimmyt.org/
DH technology adoption in Korea

Performance of tropicalized haploid inducers in Korea

First set of DH lines produced in Korea

Source: Dr. Shiwan Ryu and colleagues from Maize Research Institute, Gangwon ARES
Haploid induction
Maternal haploid inducer lines

- Stock 6 (Coe, 1959)
- KMS and ZMS (Tyrnov and Zavalishina 1984)
- KEMS (Shatskaya et al., 1994)
- MHI (Eder and Chalyk, 2002)
- RWS, RWK (Röber et al., 2005)
- UH400
- PK6 (Barret et al., 2008)
- HZI1 (Zhang et al., 2008)
- CAUHOI (Li et al., 2009)
- PHI (Rotarenco et al., 2010)
- HIL1, HIL2, HIL3 (Sarakr, 2011)
- TAILs (Prigge et al., 2011)

Genetic mechanism of haploid induction is not yet clearly understood.
First generation tropically adapted inducer lines (TAILs)

TAILs have haploid induction rate 5.5-8%

There is further scope to improve TAILs with respect to HIR and agronomy.
Major QTL conditioning high haploid induction rate

Three independent studies pointed to a **QTL on chromosome 1** for conditioning high HIR

Deimling et al, 1997
Barret et al, 2008
Prigge et al, 2012

Named **qHIR1**

QTL region fine mapped to 11.6 cM (Barret et al, 2013)

Two markers flanking **qHIR1** (umc1917 and bnlg1811) are used for validation in tropical populations
Validation of \textit{qHIR1} in tropical populations

- for high HIR, \textit{qHIR1} appears necessary
- \textit{qHIR1} alone does not guarantee high HIR

<table>
<thead>
<tr>
<th>Haploid Induction Rate(%)</th>
<th>% families with \textit{qHIR1} (N=57)</th>
<th>% families without \textit{qHIR1} (N=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 3</td>
<td>54.39</td>
<td>93.75</td>
</tr>
<tr>
<td>3.1 to 8</td>
<td>28.07</td>
<td>6.25</td>
</tr>
<tr>
<td>&gt;8</td>
<td>17.54</td>
<td>0.00</td>
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</table>

P value=\textless .0001

P value=0.0034

P value=0.01
Second generation haploid inducers

36 potential candidates with >6% HIR were identified

<table>
<thead>
<tr>
<th>Inducer</th>
<th>AF13B</th>
<th>MZ14B</th>
<th>AF15A</th>
<th>MZ15A</th>
<th>average</th>
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</thead>
<tbody>
<tr>
<td>2nd Gen TAILs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM2GT007</td>
<td>14.32</td>
<td>9.61</td>
<td>8.41</td>
<td>10.73</td>
<td>10.8</td>
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<tr>
<td>CM2GT009</td>
<td>6.67</td>
<td>12.49</td>
<td>12.98</td>
<td>13.12</td>
<td>11.3</td>
</tr>
<tr>
<td>1st Gen TAILs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAIL 8</td>
<td>9.49</td>
<td>-</td>
<td>6.77</td>
<td>8.84</td>
<td>8.4</td>
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<td>TAIL 9</td>
<td>6.10</td>
<td>-</td>
<td>10.00</td>
<td>10.77</td>
<td>9.0</td>
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<td>Temperate inducers</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>UH400</td>
<td>-</td>
<td>-</td>
<td>6.06</td>
<td>9.75</td>
<td>7.9</td>
</tr>
<tr>
<td>RWS</td>
<td>-</td>
<td>-</td>
<td>9.99</td>
<td>10.98</td>
<td>10.5</td>
</tr>
</tbody>
</table>

AF = Aguafria, Mexico; Mz = Metztitlan, Mexico  A = Winter; B = Summer

Two best candidate inducer lines will be released in 2017
Agronomic performance of 2nd generation inducer candidates

Temperate inducer

2nd generation tropical inducer (006)

1st generation tropical inducer

2nd generation tropical inducer (006)
Second generation inducer hybrids

2nd generation TAIL hybrid

1st generation TAIL hybrid

2014 winter cycle at Agua Fria, Mexico

2nd generation TAIL hybrids are best suited for isolation nurseries
Influence of source germplasm on haploid induction rate

171 CIMMYT Maize Lines

236 Drought Tolerant Maize Association (DTMA) panel members

Genome wide association analysis to identify background genetic factors influencing HIR

(Chaikam and Nair– for publication)
R1-cn marker is most commonly used for haploid identification

All haploid inducers are equipped with R1-cn marker system

R1-cn marker expression depends on source germplasm
Inhibition of \(R1-nj\) marker expression

<table>
<thead>
<tr>
<th>Type of germplasm</th>
<th>N</th>
<th>Full expression (%)</th>
<th>Segregating for expression (%)</th>
<th>Complete inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>Inbred lines</td>
<td>896</td>
<td>49.33</td>
<td>21.43</td>
<td>29.24</td>
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<tr>
<td>Elite crosses</td>
<td>157</td>
<td>56.05</td>
<td><strong>40.13</strong></td>
<td>3.82</td>
</tr>
<tr>
<td>Landraces</td>
<td>155</td>
<td>3.9</td>
<td><strong>69.48</strong></td>
<td>27.27</td>
</tr>
</tbody>
</table>

Using \(R1-nj\) marker for haploid identification is not possible/efficient in all tropical germplasm

Chaikam et al, 2015
Molecular markers for prediction of color marker inhibition

C1-I ins (indel) developed from putative functional polymorphism

<table>
<thead>
<tr>
<th>inhibition</th>
<th>no inhibition</th>
<th>C1-I SNP identified by GWAS of color inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-I ins + / C1-I SNP +</td>
<td>162</td>
<td>97</td>
</tr>
<tr>
<td>TP</td>
<td></td>
<td>259</td>
</tr>
<tr>
<td>C1-I ins -/ C1-I SNP -</td>
<td>25</td>
<td>438</td>
</tr>
<tr>
<td>FN</td>
<td></td>
<td>463</td>
</tr>
<tr>
<td>Total inhibition</td>
<td></td>
<td>722</td>
</tr>
<tr>
<td>Total no inhibition</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 87% of all the lines with inhibition are predicted with the marker combination
- Use of these markers can save resources in haploid induction

Chaikam et al, 2015
Development and Validation of Red Root Marker-Based Haploid Inducers in Maize

Vijay Chaikaon, Leocadio Martinez, Albrecht E. Melchinger, Wolfgang Schipprack, and Prasanna M. Boddupalli*

ABSTRACT
One of the critical limitations for the in vivo production of doubled haploid (DH) lines in maize (Zea mays L.) is the inability to effectively identify haploids in a significant proportion of induction crosses due to the possibility of complete or partial inhibition of the currently used RT-ν (Navajo) color marker. In this study, we demonstrate that the RT-ν marker could result in a high proportion of false positives among the haploids identified, besides being ineffective in germplasm with natural anthocyanin expression in pericarp tissue. To address these limitations, we developed haploid inducer lines with triple anthocyanin color markers, including the expression of anthocyanin coloration in the seedling roots and leaf sheaths, in addition to the Navajo marker on the seed. Although these inducers show acceptable haploid induction rates ranging from 6.8 to 10.2%, they exhibited relatively poor agronomic performance compared with tropicalized haploid inducers within tropical environments. The addition of the red root marker more accurately identified haploids among the germinating seedlings, including four tropical inbred lines and eight breeding populations that showed complete inhibition of RT-ν. We also demonstrate that the red root marker can be used for haploid identification in germplasm with natural anthocyanin expression in the pericarp. A survey of 548 tropical inbreds and 244 landraces showed that anthocyanin accumulation in the roots of germinating seedlings is very rare compared with anthocyanin accumulation in the seed and leaf sheath tissues. As a result, the red root marker can serve as a highly complementary marker to RT-ν to enable effective identification of haploids within a wide range of tropical maize germplasm.

The efficient production of inbred lines through the doubled haploid (DH) technology provides significant economic advantages to crop breeding programs (Darwall, 2010). In maize breeding, use of DH lines enhances genetic gains and improves breeding efficiency via simplification of the logistics and cost reduction for line development and maintenance (Schmidt, 2003; Melchinger et al., 2005; Lee and Tracy, 2009). The DH technology in maize essentially involves in vivo induction using a haploid inducer stock, identification of haploids, doubling the haploid chromosome complement, and the production of seed from fertile DH plants (Prasanna et al., 2012; Prigge and Melchinger, 2012).

In vivo haploid induction is achieved by crossing the source germplasm with pollen from maternal haploid inducers that have the capability to induce haploid embryos. Inducers with 6 to 15% haploid induction rates are now available in both temperate (Röber et al., 2005; Prigge et al., 2012b) and tropical genetic backgrounds (Prigge et al., 2012a). Haploids can be distinguished from diploids based on phenotypic markers or differences in plant characteristics at the adult stage (Xu et al., 2013; Weber, 2014; Wu et al., 2014). However, identification of haploids at the seed or early seedling stage is important for large scale production of DH lines.
Use of red root marker when $R1-nj$ marker is inhibited

Red root inducer × Source germplasm

Diploids (F1)
Red root
Haploids
White root

Complete inhibition of $R1-nj$

When $R1-nj$ is completely inhibited, red root marker can help to identify haploids from diploids
Other applications of red root marker

*R1-nj* marker based classification leads to significant proportion of false positives

Redroot marker helps for haploid identification when expression of *R1-nj* is masked

Purple corn crossed with inducer
High oil marker

**High oil trait**

- Embryo phenotype
- Dominantly inherited

Haploid inducers need to be equipped with high oil trait

**High oil inducer** × **source germplasm**

- Diploids (F1)
  - High oil
- Haploids
  - Low oil

**Advantage:**
Can be automated
Chromosomal doubling method - seedling dip

0.04% colchicine + 0.5% DMSO for 12 hrs in a tank

High seedling mortality (~30-60%)
Lower efficiency of doubling
Chromosomal doubling methods - Root dip

Colchicine 0.75% + 0.5% DMSO for 5 hours

Advantages: Less labor requirement
Low seedling mortality
Higher efficiency in doubling
Alternative doubling agents

Colchicine is very toxic to humans

Colchicine Alternatives for Chromosome Doubling in Maize Haploids for Doubled-Haploid Production

Albrecht E. Melchinger,* Willem S. Molenaar, Vilson Mirdita, and Wolfgang Schipprack

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitosis inhibiting agent</th>
<th>No. of seedlings treated</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amiprophos-methyl</td>
<td>Pronamid</td>
<td></td>
</tr>
<tr>
<td>E4T1</td>
<td>20</td>
<td>-</td>
<td>284</td>
</tr>
<tr>
<td>E4T2</td>
<td>20</td>
<td>0.3</td>
<td>322</td>
</tr>
<tr>
<td>E4T3</td>
<td>20</td>
<td>0.7</td>
<td>277</td>
</tr>
<tr>
<td>E4T4</td>
<td>20</td>
<td>1.0</td>
<td>350</td>
</tr>
<tr>
<td>E4T5</td>
<td>20</td>
<td>1.5</td>
<td>270</td>
</tr>
<tr>
<td>E4T6</td>
<td>20</td>
<td>2.0</td>
<td>341</td>
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<td>E4T7</td>
<td>20</td>
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<td>286</td>
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<td>E4T8</td>
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<td>E4T9</td>
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<tr>
<td>E4T10</td>
<td>Colchicine control</td>
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<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>RR</th>
<th>OSR</th>
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<tbody>
<tr>
<td>E4T1</td>
<td>83.8ab†</td>
<td>4.6c</td>
<td>3.9d</td>
</tr>
<tr>
<td>E4T2</td>
<td>87.9a</td>
<td>6.4c</td>
<td>5.6cd</td>
</tr>
<tr>
<td>E4T3</td>
<td>84.1a</td>
<td>10.3bc</td>
<td>8.7c</td>
</tr>
<tr>
<td>E4T4</td>
<td>81.7b</td>
<td>10.1bc</td>
<td>8.3c</td>
</tr>
<tr>
<td>E4T5</td>
<td>70.7c</td>
<td>12.6b</td>
<td>8.9b</td>
</tr>
<tr>
<td>E4T6</td>
<td>73.6c</td>
<td>12.0b</td>
<td>8.8c</td>
</tr>
<tr>
<td>E4T7</td>
<td>52.5d</td>
<td>28.7a</td>
<td>14.0b</td>
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<td>E4T8</td>
<td>46.5d</td>
<td>34.7a</td>
<td>16.1a</td>
</tr>
<tr>
<td>E4T9</td>
<td>81.6b</td>
<td>3.8c</td>
<td>3.1d</td>
</tr>
<tr>
<td>E4T10</td>
<td>75.8bc</td>
<td>29.2a</td>
<td>22.1a</td>
</tr>
</tbody>
</table>

† Values followed by the same letter are not significantly different at the 0.05 probability level.

Seedling dip in Amiprophos methyl 20mg/L + Pronamid 4mg/L for 8 hours

Less efficient than colchicine

Pure herbicide formulations are very expensive
Influence of source germplasm on male fertility of haploids

171 CIMMYT Maize Lines analyzed for spontaneous male fertility

Genome wide association analysis to identify genetic factors influencing male fertility in haploids
Thank you for your interest!