Gene Editing to Improve Maize and Wheat Performance at CIMMYT

Kanwarpal S. Dhugga
Genetic Resources Program
International Center for Maize and Wheat Improvement (CIMMYT)
El Batan, Mexico

Symposio de Tecnologias Modernas de Edicion de Genomes IX
Encuentro REDBIO 2016 – Lima, Peru
Average yield increase globally has been \( \sim 40 \text{ kg.ha}^{-1}.\text{yr}^{-1} \) over the last half century and about the same in four blocks each of 14 year.

Total rate of yield gain was calculated as \( \frac{(Y_t-Y_{t-1}) \times 100}{Y_{t-1}} \), where \( Y \) is yield and \( t \) is the year the rate is calculated for. Approximately, half of the rate in any given year could be attributed to genetics and the other half to agronomics. The total rate of annual yield increase was 3.6 % per year in 1961 and 1.2% in year 2015.

Source: USDA, Foreign Agricultural Service, Production, Supply, and Distribution Database.
**Genetic Resources at CIMMYT**

98,220 Wheat Lines Genotyped

41,345 at SAGA:
- 30,500 ICARDA accessions
- 5,300 landraces from Mexicali
- 2,205 wild relatives

56,875 at SAGA & DArT:
- **87% hexaploid** (landraces, elite bread wheat, synthetics and prebreeding)
- **10% tetraploid** (durum, landraces)
- 3% wild relatives
Data Publicly Available – Register and Go

Germinate 3 SeeD Wheat Database

Enter your username and password below to access this site. If you have forgotten your login details please consult the help page.

Username: 
Password: 
Login

Home

This initial version of the SeeD Catalog includes very basic query options. We will substantially expand it over the course of the project, as more data come online.

Data release

All wheat data and attribution information will be made available here, no later than 24 months after concluding the generation of each dataset to give those who generated the data an opportunity to draft scientific publications to add value to the data. We will link the Wheat SeeD Catalogue, with other online resources through web services, where possible.
Reasons for CIMMYT to Develop In-house Transgenic and Gene Editing Capabilities

- Our objective is to extend the benefits of modern technologies to small-holder farmers.
- Licensing of some of these traits from private partners has been difficult.
- Recent technological breakthroughs have opened new avenues to generate agronomically important traits in native state, that is, the product is non-GM.
- CIMMYT possesses state-of-the-art laboratories and expertise to conduct transgenic research and produce novel products that complement conventional breeding.
High Level Strategy

• Maize:
  – In-license de-regulated traits from industry
    • Example: Bt and DroughtGard from Monsanto
  – Transgenics for high-impact traits only, e.g., MLN, Fusarium resistance
  – Evaluate transgenics in Africa, not Mexico
  – Already signed an agreement with DuPont Pioneer on gene editing

• Wheat:
  – Set up high-throughput wheat transformation capability to generate new traits, for example, disease resistance and herbicide tolerance

• Gene Editing – as an alternative to breeding
## Biotech Traits - Prioritization

<table>
<thead>
<tr>
<th>Wheat</th>
<th>Maize</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Disease resistance</td>
<td>• Disease resistance (MLN)</td>
</tr>
<tr>
<td>• Herbicide tolerance</td>
<td>• Herbicide tolerance</td>
</tr>
<tr>
<td>• Heat tolerance</td>
<td>• Heat tolerance</td>
</tr>
<tr>
<td>• Grain quality</td>
<td>• Grain quality</td>
</tr>
<tr>
<td>• Hybrid wheat</td>
<td>• Stem-borer resistance</td>
</tr>
</tbody>
</table>

**Initial Focus:** Disease resistance and herbicide tolerance in maize and wheat.

**Medium Term:** Heat tolerance when external funds become available.

**Longer term:** Photosynthetic efficiency, epigenetics with external funding.
Figure 1. Different site-directed nuclease (SDN) techniques (SDN-1, 2, and 3). An SDN complex is shown at the top in association with the target sequence. The repair can take place via nonhomologous end-joining (NHEJ) or homologous recombination (HR) using the donor DNA. SDN-1 can result in site-specific random mutations by NHEJ. In SDN-2, a homologous donor DNA is used to induce specific nucleotide sequence changes by HR. In SDN-3 DNA is integrated in the plant genome via HR.
<table>
<thead>
<tr>
<th></th>
<th>BVL(^1)</th>
<th>ZKBS(^2)</th>
<th>NTWG(^3)</th>
<th>EFSA(^4,5)</th>
<th>NGOs(^6)</th>
<th>BFN(^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDN-1</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>GMO</td>
<td>GMO</td>
</tr>
<tr>
<td>SDN-2</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>GMO</td>
<td>GMO</td>
</tr>
<tr>
<td>SDN-3</td>
<td>GMO</td>
<td>GMO</td>
<td>GMO</td>
<td>GMO(^b)</td>
<td>GMO</td>
<td>GMO</td>
</tr>
<tr>
<td>ODM</td>
<td>Non GMO(^a)</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>GMO</td>
<td>GMO</td>
</tr>
<tr>
<td>RdDM</td>
<td>n.d</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>Non GMO</td>
<td>n.d</td>
<td>GMO</td>
</tr>
</tbody>
</table>

The classification refers to plants generated by using these techniques without stable integration of recombinant DNA.

SDN site-directed nuclease, ODM oligonucleotide-directed mutagenesis, RdDM RNA-dependent DNA methylation, n.d no opinion given, GMO genetically modified organism, BVL German Federal Agency for Consumer Protection and Food Safety, ZKBS Zentrale Komission für biologische Sicherheit, NTWG New technology working group, EFSA European Food Safety Authority. 1 BVL 2015d, 2 ZKBS 2012, 3 Lusser et al. 2011, 4 EFSA 2012, 5 EFSA GMO unit 2015, 6 Krämer 2015, 7 Spranger 2015

\(^a\) Serial steps should be considered separately

\(^b\) Due to the known target site of the transgene lesser amounts of event-specific data might be necessary for the risk assessment
Gene Editing for Herbicide Tolerance in Maize

Edited ALS2  Unedited ALS2
S            P

Svitashev et al., 2015, Plant Physiol. 169:931
DuPont Pioneer
Resistant allele of Lr67 differs from the susceptible by only two nucleotides that lead to amino acid changes: 
\text{Arg}144\text{Gly} \text{ and } \text{Leu}387\text{Val}. 
Resistance is recessive as hets are as susceptible as the susceptible ones. Likely a suppressor, the mutant form of which is not released from the regulatory site by the viral signal, not allowing the defense genes to be turned on.
Transformation and Regeneration of Fielder and Navojoa

Carlos Slim Laboratories - April 5, 2016

Fielder

Navojoa
Candidate Genes for Editing in Wheat and Maize

- **Disease resistance**
  - Wheat: FHB, Lr34, Lr67, MLO
  - Maize: Maize lethal necrosis (MLN), Fusarium

- **Herbicide tolerance**
  - Wheat: glyphosate, sulfonylureas
  - Maize: sulfonylureas, alternative for glyphosate

- **Grain Quality**
  - Knockout lysine degrading enzymes
  - Knockout phytate-related enzymes
The Gene Editing Platform at CIMMYT

Testing for altered function

Screening for alterations

Molecular biology

Transformation and regeneration

Testing for altered function

Screening for alterations

Molecular biology

Transformation and regeneration
EPSPS Gene Needs to be Edited at Only Two Nucleotides to Make it Glyphosate-tolerant

Maize  QLFLGNAGTAMRPPLTAAVTAAGGNA
Wheat  KLFLGNAGTAMRPPLTAAVVAAGGNA

↑  ↑
IST

G
A
Gene Editing for Sulfonylurea and Glyphosate Tolerance

Can’t resist glyphosate or SU

Gene editing

Can resist glyphosate or SU
Can resist glyphosate and SU

Transgenic approach: Introduce a bacterial gene resistant to herbicide
Gene editing: Change a single or a couple of nucleotides. No foreign DNA in the product.
### Selected Regions of Acetolactate Synthase Protein

<table>
<thead>
<tr>
<th></th>
<th>TaALS6DL</th>
<th>MVAITGQVPRRMIGTDAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TaALS6AL</td>
<td>MVAITGQVPRRMIGTDAF</td>
</tr>
<tr>
<td></td>
<td>ZmALS1</td>
<td>MVAITGQVPRRMIGTDAF</td>
</tr>
<tr>
<td></td>
<td>ZmALS2</td>
<td>MVAITGQVPRRMIGTDAF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>TaALS6DL</th>
<th>QHLGMVVQWEEDRFYKANR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TaALS6AL</td>
<td>QHLGMVVQWEEDRFYKANR</td>
</tr>
<tr>
<td></td>
<td>ZmALS1</td>
<td>QHLGMVVQLEEDRFYKANR</td>
</tr>
<tr>
<td></td>
<td>ZmALS2</td>
<td>QHLGMVVQWEEDRFYKANR</td>
</tr>
</tbody>
</table>

Ten amino acids can be substituted in the ALS enzyme, conferring resistance against sulfonylurea and imidazolinone herbicides without affecting the activity. Two examples are shown where the alteration of a proline (P) to serine or a few other residues and that of tryptophan (W) to leucine (L) makes the enzyme resistant to this class of herbicides.

Wheat has multiple gene copies for ALS.
Genomic Copies of Acetolactate Synthase in Wheat
Genomic Copies of EPSPS: Maize 1, Wheat 7

Maize has only one copy, making it difficult to modify it without affecting plant performance. Solutions...

Wheat has seven gene copies; it should be possible to edit one or more of these to confer glyphosate tolerance
Fig. 2  Number of genes reported edited by CRISPR/Cas9 to date, by plant species. Publications reporting these were gathered using PubMed and searching the terms “crispr” and “plant”. “Tobacco” includes *Nicotiana tabacum* and *Nicotiana benthamiana*.
Gene Editing

• The technology has been around for several decades but was difficult to use, only for the resource-rich outfits.
• A recent advancement, clustered regularly-interspersed short palindromic repeat (CRISPR)-Cas9 system, has revolutionized gene editing.
• Initial successes already achieved in maize, rice, soybean, tomato, and wheat.
• We will employ CRISPR-Cas9 to edit genes in wheat and maize, the latter in collaboration with DuPont Pioneer.
Fig. 3: Rate of Gain (%) Extrapolated From Year 2000 to 2030
Red Marker Equals 1%

\[ y = -0.018x + 37.9 \]
\[ R^2 = 1 \]
Recent Transgenic Research Projects at CIMMYT

Water Efficient Maize for Africa (WEMA)
- **Funding:** Bill & Melinda Gates Foundation and Howard G. Buffett Foundation.
- **Partners:** AATF, Monsanto, Kenya’s KALRO, Mozambique’s IIAM, South Africa’s ARC, Tanzania’s COSTECH, Uganda’s NARO.
- **Expected outputs:** Transgenic drought tolerant and Bt insect resistant hybrids.

Improved Maize for African Soils (IMAS)
- **Funding:** Bill & Melinda Gates Foundation and USAID
- **Partners:** DuPont-Pioneer; KALRO; ARC-South Africa.
- **Expected outputs:** Native trait alleles to enhance yield under N stress; transgenic maize varieties with increased yield under N stress.

Development of Abiotic Stress Tolerant Crops by DREB Genes
- **Funding:** Min. Agric. Forestry and Fisheries (MAFF), Japan.
- **Partners:** JIRCAS, RIKEN PSC, CIMMYT, IRRI, CIAT.
- **Expected outputs:** Identify useful regulatory genes for drought tolerance; contribute to sustainable food production.
Deliverables

• **Year 1**
  – Laboratory is equipped and fully functional
  – High throughput wheat transformation established
  – Gene editing initiated in wheat for disease resistance
  – Gene editing in maize undertaken in collaboration with a private partner for the same traits

• **Year 2**
  – Gene editing expanded to multiple targets, including heat tolerance and increased grain lysine
  – Gene editing initiated for agronomic traits other than heat tolerance

• **Year 3**
  – First products ready for testing in the greenhouse and possibly field
  – Gene editing expanded to possibly herbicide tolerance

• **Year 4**
  – Ramp up gene editing platform for whole-genome targeting
  – Test edited genes for performance in the field

• **Year 5**
  – Screen the events from whole-genome targeting for sequence alteration
  – Develop a seed resource database with known variants and make available to other scientists
  – Screen for traits of interest
Transcriptional regulation
Alberts et al., Molecular Biology of the Cell

Metabolic regulation

Changes made at the transcriptional, translational, or enzymatic levels must eventually reflect in the form of CHO and storage protein.

Grain Yield

85% CHO
11% Protein
2% Oil
2% Ash

\[
C_{Ei}^{Ji} = \frac{\partial J / J}{\partial E / E}
\]
Possible Limiting Steps in Grain Yield Formation

• Photosynthate production
• Transient storage (leaf starch)
• Medium term storage (stem reserves)
• Transport (photosynthate and stem reserves)
• Utilization (in the developing grain)
**Fig. 7:** Coupled Assay for Glucose & Fructose Using MTT as a Color Reagent

- **Blank**
- **Glucose -6-phosphate dehydrogenase + Hexokinase**
- **Phosphoglucose isomerase**

**Fig. 8:** Glucose and Fructose Standards

- Glucose
- Fructose
- Glu + Fru

Absorbance ($A_{600}$) vs. Sugar (ug)

- $y = 0.34x + 0.0063$
  - $R^2 = 0.99$
- $y = 0.17x + 0.0005$
  - $R^2 = 0.99$
- $y = 0.16x - 0.0185$
  - $R^2 = 0.99$
Fig. 9: Sugar Composition of the Mid-section of Maize Ear Leaf

The section (inset) was separated into three longitudinal planes of approximately equal
Our objective is to make the fruits of useful technologies accessible to small-holder farmers
Systems Biology

Fig. 5: Biological complexity is minimal at metabolite level.

This schematic is drawn for humans. Wheat has ~100K genes so its proteome would be ~300K. Metabolites would still be ~5K.

Horgan and Kenry (2011)
Obstet Gynaec 13;189-195
Effect of Weeds on Wheat Yield

- Favorable conditions for competitive weed species
- Unfavorable conditions &/or weakly competitive weed species
CRISPR-Cas9-mediated Resistance Against Powdery Mildew in Wheat

Figure 2 Loss of TaMLO function confers resistance of bread wheat to powdery mildew disease. (a) Percentage of microcolonies formed from the total number of germinated spores of 

Blumeria graminis f. sp. tritici (Bgt) inoculated on the leaves of wild-type (WT) and various tamlo mutants. At least 2,000 germinated spores per genotype per experiment were examined 72 h after inoculation with virulent Bgt isolate E09. Values are the mean ± s.d. of four independent experiments. **P < 0.01 (t-test). (b) Micrographs of microcolony formation of Bgt on the surfaces of leaves of the indicated genotypes 3 d postinoculation. Powdery mildew spores and colonies were stained with Coomassie blue. Scale bars, 200 μm. (c) Macroscopic infection phenotypes of representative leaves of WT and the indicated mlo mutants 7 d after inoculation of detached leaves with Bgt. Scale bar, 1 cm. (d) Disease symptoms of wild-type (WT) and tamlo-aabbdd mutant plants. The photograph was taken 7 d after inoculation in planta. Scale bars, 2 cm.
Fig. 3. Effects of the CRISPR/Cas9-induced mutations in ripening fruits. A) Appearance of mutant fruits harvested five days after the breaker stage. B) Expression analysis of RIN in mutant plants. Fruits of the mutants were harvested five days after the breaker stage and total RNAs and nuclear proteins were prepared from an identical fruit. RIN mRNA was detected by Northern blotting analysis (middle panel) and the RIN protein was detected by Western blotting analysis with the RIN-antibodies, the recognition region of which is indicated in Fig. 1 (bottom panel). Deduced molecular sizes of mutant proteins are >16.0-kDa for the proteins with the Guide 1-induced mutations and >23.3-kDa for the proteins with the Guide 2-induced mutations. The wild-type RIN protein was detected at a larger molecular weight than the deduced weight (28.8-kDa), which is a specific property of RIN as reported previously [26].
Mutations Introduced in a Rice Gene Family With a Single Probe

Off-target editing can overcome the homeoallelic complexity of wheat.