Forward and reverse genetics for functional genomics and breeding

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**Food Security:** “A situation that exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life”. FAO, 2002

- ~1 in 8 people in the world suffer from chronic undernourishment
- Pressures are expected to increase (climate, population, etc.)
- Many approaches for increasing food security, including plant breeding
Variation within a species is a major resource for the crop breeder.
Mutations are changes in the DNA sequence of a cell's genome caused by radiation, viruses, transposons, mutagenic chemicals, or errors that occur during meiosis or DNA replication.

Mutations are naturally occurring, or can be induced.

Induced mutations broaden diversity.
Inducing mutations in plants

Lewis John Stadler: Genetic effect of X-rays in maize, wheat, and barley (1920s)

**Mutation Breeding:** The process of treating plant cells with mutagens to facilitate crop breeding

- Seed, pollen, cell culture, plant tissues
- Chemical or Physical (ionizing radiation) mutagenesis
- Orders of magnitude faster than natural mutation
- First released variety: “Vorsteland” Tobacco with improved quality in 1934 in Indonesia

Image under public domain, National Institutes of Health
Registered Mutant Crop Varieties

Total: 3218
Plant Species: 224

Jankowicz-Cieslak & Till, 2015 in press

http://mvgs.iaea.org/
Examples of mutant varieties

VIETNAM

- Mutant varieties since the 1980s (>55 total)

- Economic impact of over 2 billion USD

- Mutant rice growing in 30% of Mekong delta (salinity tolerant)

- Award winning quality

- Rice, soybean, maize, ornamentals

- Over 8 million farmers have profited (income increase by 30%)

- 2014 FAO/IAEA Breeding Award

Data from: Agricultural Genetics Institute (VAAS), Department of Agriculture and rural development, Institute of Agricultural Science for Southern Vietnam
Examples of mutant varieties

PERU

• Barley & Amaranth
• High Andes (>3500 m)
• Six-fold yield increase
• Adding >6 million USD annually
• 2014 FAO/IAEA Breeding Award

Photo courtesy Dr. Luz Gomez Pando
Examples of mutant varieties

INDIA

- Bhabha Atomic Research Centre (Mumbai)
- 42 crop varieties (groundnut, mustard, soybean, mung bean, pigeon pea, urdbean, cowpea)
- Groundnut varieties cover over 2,970,000 ha.
- 2014 FAO/IAEA Breeding Award
Mutation Breeding Attributes

• tested, proven, robust
• ubiquitously applicable
• no IP, not regulated
• cost effective
• increasing local economies
THE PLANT BREEDING AND GENETICS LABORATORY

- Training (>30/year)
- Services (Mutagenesis)
- Adaptive R&D/Tech Transfer

  - In direct support of TC projects
  - “upstream” efforts to develop, evaluate and test new approaches (CRPs)
  - “Smart” methods for developing countries
    (Low-cost, non-toxic, do-it-yourself)
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**THE PLANT BREEDING AND GENETICS LABORATORY**

**Services (Mutagenesis)**

- International treaty on plant genetic resources for food and agriculture & SMTA
- Dosage optimization
- Seed & veg. prop
- ~ 40 requests/year

![Pie chart showing distribution of service requests by country](chart.png)

*Mirta Matijevic*
Adaptive R&D/Tech Transfer

- In direct support of TC projects
- “upstream” efforts to develop, evaluate and test new approaches (CRPs)
- methods for labs with limited infrastructure (Low-cost, non-toxic, do-it-yourself)
Mutation discovery & optimization

Traditional “forward” genetics

- Mutagenesis
- Phenotyping
- Varietal release
- Clone gene causing trait

Reverse genetics

- Mutagenesis
- Find mutations in genes
- Phenotypic validation
TILLING
Targeting Induced Local Lesions IN Genomes
McCallum, et. al., 2000

Mutagenesis

Mutation discovery

Sequencing

seed bank

PCR

heat, cool
Advantages of TILLING

• Yields a spectrum of point alleles and small indels
• Mutations are irreversible
• Efficient for small genes
• Can focus on region of interest
• Applicable to almost any organism
• Only phenotype selected plants (10s versus 1000s)
• Non-transgenic
# Examples of TILLING Projects

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mutagen</th>
<th>Spectrum</th>
<th>Density (per kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>EMS</td>
<td>&gt;99% GC-AT</td>
<td>1/200</td>
</tr>
<tr>
<td>Barley</td>
<td>EMS</td>
<td>~70% GC-AT</td>
<td>1/140 1/1000</td>
</tr>
<tr>
<td>Maize</td>
<td>EMS</td>
<td>&gt;99% GC-AT</td>
<td>1/500</td>
</tr>
<tr>
<td>Pea</td>
<td>EMS</td>
<td>&gt;99% GC-AT</td>
<td>~1/700</td>
</tr>
<tr>
<td>Rice</td>
<td>EMS, Az-MNU, Gamma</td>
<td>Mixture</td>
<td>~1/130 – 1/6000</td>
</tr>
<tr>
<td>Sorghum</td>
<td>EMS</td>
<td>&gt;90%GC-AT</td>
<td>~1/500</td>
</tr>
<tr>
<td>Soybean</td>
<td>EMS, MNU</td>
<td>75-90% GC-AT</td>
<td>1/140 – 1/500</td>
</tr>
<tr>
<td>Tomato</td>
<td>EMS</td>
<td>~90% GC-AT</td>
<td>1/300-1/700</td>
</tr>
<tr>
<td>Wheat</td>
<td>EMS</td>
<td>&gt;99% GC-AT</td>
<td>1/20-1/40</td>
</tr>
</tbody>
</table>

- **Individual projects**
- **Public services**

* Many different groups
Mutation density and polyploidy

• Density and spectrum allow estimation of ideal population size to recover desired alleles

Data from chemical mutagenesis

## Examples of TILLING for trait improvement

<table>
<thead>
<tr>
<th>Crop</th>
<th>Trait</th>
<th>Target genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>Plant height</td>
<td>gibberellin 3β-hydrolase</td>
<td>Tirques et al, 2007</td>
</tr>
<tr>
<td>Potato</td>
<td>Improved Starch</td>
<td>GBSSI</td>
<td>Muth et al, 2008</td>
</tr>
<tr>
<td>Tomato</td>
<td>Fruit color</td>
<td>Phytoene synthase 1</td>
<td>Gady et al, 2011</td>
</tr>
</tbody>
</table>
Why so few vegetatively propagated food crop varieties?

Banana & Cassava:
Staples for 1 billion
Primarily veg prop.
Years of breeding support
In total only 5 released mutant varieties (14% of population, only 0.16% of database)
Tissue mutagenesis & TILLING for banana

- **High density of induced point mutations (1/57kb)**
- **Rapid dissolution of chimeric sectors**

Joanna Jankowicz-Cieslak, Dec 2012
Most mutant varieties by forward genetics

- Many crops only self-fertilized
- Almost no information on types of mutations and genes involved in traits
- It is very difficult to identify gene/mutation in large genome crops
- Knowledge of mutation and gene for functional genomics, markers, etc.
Example of TC project support: The spread of wheat stem rust race Ug99

- First report of virulence for Sr31 in 1999 (Pretorius et al. 2000 Plant Dis. 84:203)
- Stem rust did not appear in Uganda during the following years
- Presence in Kenya reported in 2002 although Kenyan data indicate that it may have existed since 1999
- Detected in Ethiopia in 2003
- Most leading cultivars and breeding lines became susceptible to Ug99
An interregional approach for enhancing resistance to stem rust

• IAEA interregional project started in 2009, through the department of Technical Cooperation (INT/5/150)
• 21 countries and 4 international institutions (CIMMYT, FAO, IAEA, ICARDA) participate in this project
• University of Eldoret, Kenya, used as the screening hot spot
• Goal: to generate novel genetic variation among wheat and barley varieties using mutagenesis to improve resistance
An example from Kenyan materials

- Mutation induction was done using gamma rays from Cobalt source \((60\text{co})\) in Seibersdorf Laboratories Vienna, Austria

- Plants grown and tested in Kenya

- Training provided at Seibersdorf

- Two new varieties released in 2014

Ego Amos
What next? Lab supporting cloning mutated genes causative for resistance

Cloning by sequencing

1. Seed from Kenya

2. Genotyping by Sequencing

3. Disease resistance validation – Canada, Australia

4. Exome capture for mutation discovery

Mapping (~5 years)

Ug99 partners: Jorge Dubcovsky, Ksenia Krasileva, UC Davis, Hermann Buerstmayr, BOKU, Austria, Miriam Kinyua, Amos Ego, U. Eldoret, Kenya, Tom Fetch, Agri-food Canada, Rob Park, U. Sydney
Exome is a fraction of the genome
Reducing sequencing loads- (CRP D24012)

Genomic library (no selection)

RESCAN (restriction site)

Exome capture (hybridization)

Figure: Isabelle Henry and Luca Comai, UC Davis Genome Center
Pilot exome capture for discovery of rare mutations (sorghum and cassava)

15 plants from 2 Sudanese genotypes selected for sequencing

17 selected for sequencing (1 genotype)
Recovery of indels in mutagenized material

Superscaffold 12, ~ 575 kb
Heterozygous deletion
ABU genotype, 75b_1 and 75b_2

Chromosome 1, ~ 400 kb
Heterozygous deletion
ABU genotype
75b_1 and 75b_2

25 kb bin analysis

Collaborators: Isabelle Henry and Luca Comai, UC Davis Genome Center
## Preliminary summary of sorghum indel analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Sample(s)</th>
<th>Chr/Scaffold</th>
<th>Start</th>
<th>End</th>
<th>Length (kb)</th>
<th>Type</th>
<th>In/del</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>75b_1 &amp; 75b_2</td>
<td>Chromosome1</td>
<td>39125000</td>
<td>39525000</td>
<td>400</td>
<td>Het</td>
<td>Deletion</td>
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<tr>
<td>B</td>
<td>75b_1 &amp; 75b_2</td>
<td>chromosome 1</td>
<td>44825001</td>
<td>45525000</td>
<td>699.999</td>
<td>?</td>
<td>Insertion</td>
</tr>
<tr>
<td>C</td>
<td>75b_1 &amp; 75b_2</td>
<td>super12</td>
<td>3725001</td>
<td>4300000</td>
<td>574.999</td>
<td>Het</td>
<td>Deletion</td>
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<tr>
<td>D</td>
<td>300a_2</td>
<td>Chromosome 2</td>
<td>42950001</td>
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<td>E</td>
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<td>Deletion</td>
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<td>Het</td>
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<tr>
<td>G</td>
<td>75_b_1</td>
<td>chromosome_8</td>
<td>32800001</td>
<td>32910000</td>
<td>109.999</td>
<td>?</td>
<td>Insertion</td>
</tr>
</tbody>
</table>

- Both insertions and deletions recovered
- Indels found in 4/5 analysed mutants in one Sorghum line (But not the other)
- Smaller bin analysis suggests some false negatives
- More individuals would help with detection
Transferring technologies to developing countries

Issues to consider for molecular biology:

• Varying infrastructure

• Toxic waste storage and disposal

• Lack of constant power makes -80°C freezers problematic

• Can be difficult to get liquid nitrogen

• Kits for sample analysis are expensive, and you need to practice to get good at molecular methods

• Many applications require a high quality genomic DNA from leaf tissue
Low-cost methods suitable for most labs

- High quality DNA at 1/10th the cost
- No toxic chemicals
- Protocols and kits for MSs
- > 80 scientists from >30 MSs trained since 2013

Adapted method for Tef (Ethiopia) & Sugarbeet (Poland)
Low-cost methods suitable for most labs

1st National Workshop on TILLING in Crop Plants – University of Hyderabad, India
April 13-24 – Prof. Ramesh Sharma

Genomic DNA
Activity of extracted nucleases
Technology transfer - Low-cost methods for Mauritius

- Low cost Ecotilling to characterize local germplasm
- Low cost methods for DNA extraction:
  Banana, barley, cassava, wheat, rice, tomato, breadfruit, chilli, sorghum, mango, litchi, peach, Ceylan olive, coffee, roses, anthurium, jackfruit, chrysanthemum, etc.

EcoTILLING in Cochliobolus sativus – Mohammed Jouhar - SYRIA
Some other collaborative projects

- **Tomato TILLING by Sequencing (Univ. Hyderabad)**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Position</th>
<th>Variant Type</th>
<th>Call</th>
<th>Frequency</th>
<th>Depth</th>
<th>Genome</th>
<th>Unique in pools</th>
<th>Comments</th>
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</thead>
<tbody>
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<td>c5</td>
<td>727</td>
<td>SNP</td>
<td>T-&gt;[T/A]</td>
<td>0.02</td>
<td>33647</td>
<td>sharm001</td>
<td>727</td>
<td>non-EMS three pools</td>
</tr>
<tr>
<td>d4</td>
<td>727</td>
<td>SNP</td>
<td>T-&gt;[T/A]</td>
<td>0.02</td>
<td>6719</td>
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<tr>
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<td>2181</td>
<td>EMS type three pools</td>
</tr>
</tbody>
</table>
Some other collaborative projects

- NIRS, Proteomics, Metabolomics of mutant rice (BOKU, U. Vienna)
- Citrus greening disease & TILLING (University of Florida)
- Grass pea TILLING (JIC, RevGenUK)
- Banana disease Tropical Race 4 resistance (TBRI, Bioversity, Stellenbosch Univ, DuRoi)
- Epigenomics of adaptation in barley (GMI, Vienna)
Summary

• Global factors such as population growth and climate change have a negative impact on food security

• Nucleotide variation is the major source of variability for the plant breeder

• Mutations can be induced in organisms at a much faster rate than occur naturally

• Mutation breeding has been widely successful in developed and developing countries, but more needs to be done

• Improving traditional approaches and innovative methods
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THANK YOU!
Seedling and adult plant phenotyping

- Seedling infection types
- Adult plant infection response

0 0; ; 1 2- 2+ 3 3+ 4

-----Resistant-----

---Susceptible---

*13 new resistant lines identified

Peterson et al., 1972
Natural (Spontaneous) mutation

Rate is about 1 mutation every billion base pairs

In humans that means that we have about 6 mutations in each of our cells

However, most mutations are functionally silent & only those mutations in gametes (egg & sperm/pollen) are passed onto offspring…

Plant breeders use natural mutations that have accumulated over many thousands of years

But, slow rate of spontaneous mutation is a major limitation