Building a Platform for Gene Editing and Transgenic Technologies at CIMMYT

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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

Wheat

- Disease resistance
- Herbicide tolerance
- Heat tolerance
- Grain quality
- Hybrid wheat

Maize

- Disease resistance (MLN)
- Herbicide tolerance
- Heat tolerance
- Grain quality
- Stem-borer resistance

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.

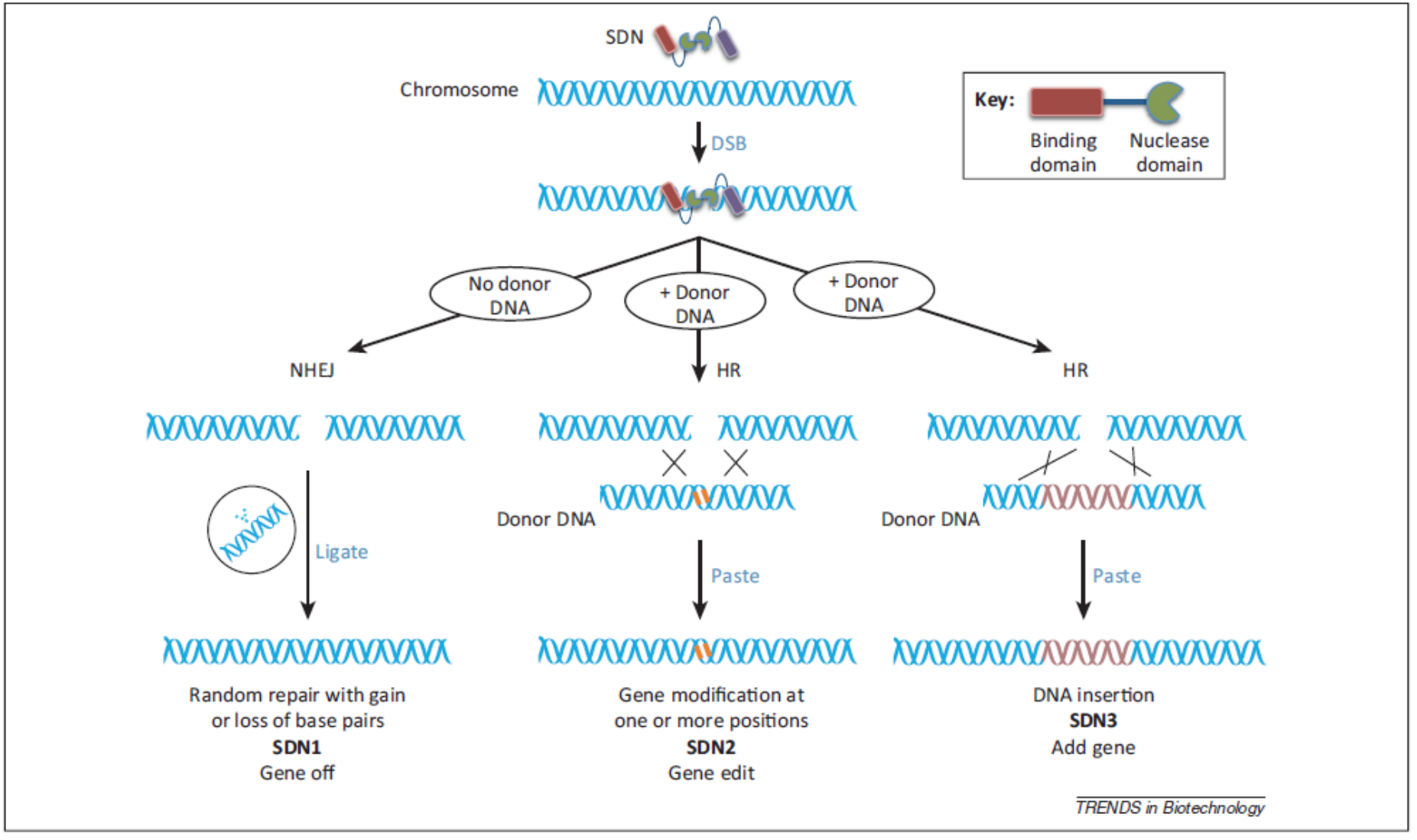


Figure 1. Different site-directed nuclease (SDN) techniques (SDN-1, 2, and 3). An SDN complex is shown at the top in association with the target sequence. The repair can take place via nonhomologous end-joining (NHEJ) or homologous recombination (HR) using the donor DNA. SDN-1 can result in site-specific 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 1 Comparison of SDN-1, -2, and -3 in relation to the legal interpretations (BVL, NGOs, BFN, NTWG, ZKBS, EFSA)

	BVL[1]	ZKBS[2]	NTWG[3]	EFSA[4,5]	NGOs[6]	BFN[7]
SDN-1	Non GMO	Non GMO	Non GMO	Non GMO	GMO	GMO
SDN-2	Non GMO	Non GMO	Non GMO	Non GMO	GMO	GMO
SDN-3	GMO	GMO	GMO	GMO[b]	GMO	GMO
ODM	Non GMO[a]	Non GMO	Non GMO	Non GMO	GMO	GMO
RdDM	n.d	Non GMO	Non GMO	Non GMO	n.d	GMO
Interpretation	Process/product	n.d	n.d	n.d	Process	Process

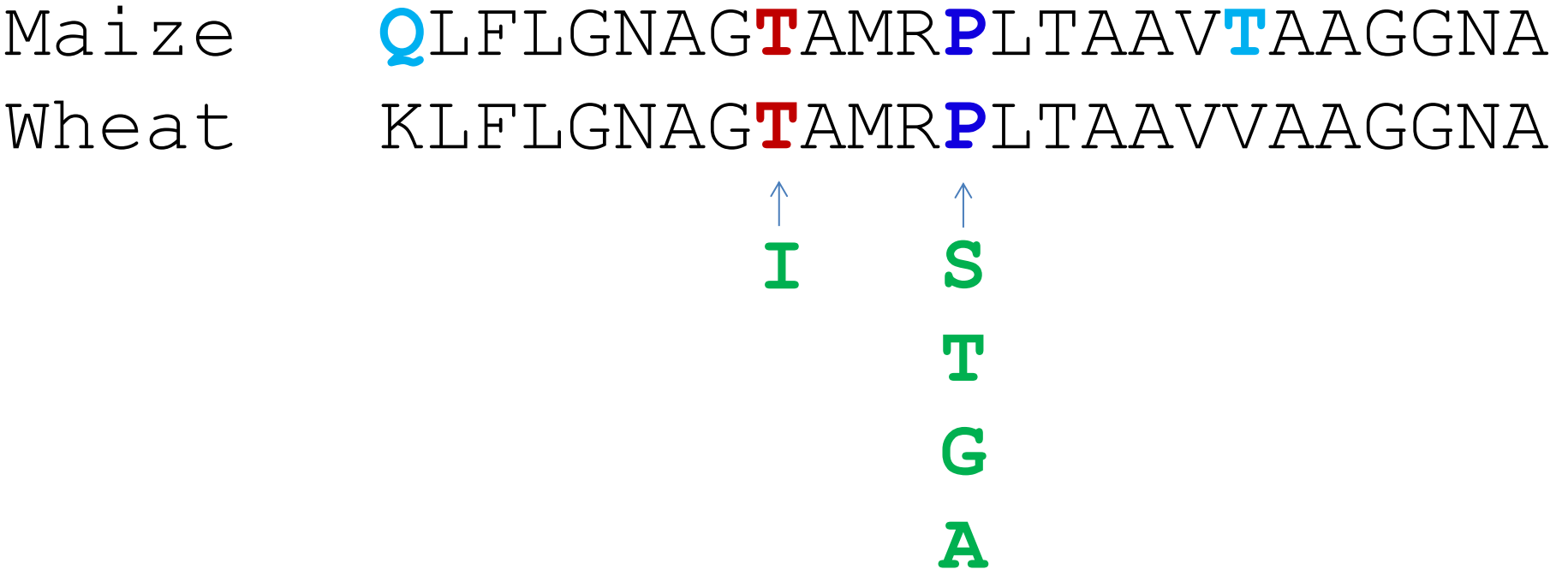
The classification refers to plants generated by using these techniques without stable integration of recombinant DNA

SDN site-directed nucleases, *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

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



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

```
TaALS6DL   MVAITGQVPRRMIGTDAF
TaALS6AL   MVAITGQVPRRMIGTDAF
ZmALS1     MVAITGQVPRRMIGTDAF
ZmALS2     MVAITGQVPRRMIGTDAF
                     ↑
                     S

TaALS6DL   QHLGMVVQWEDRFYKANR
TaALS6AL   QHLGMVVQWEDRFYKANR
ZmALS1     QHLGMVVQLEDRFYKANR
ZmALS2     QHLGMVVQWEDRFYKANR
```

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.

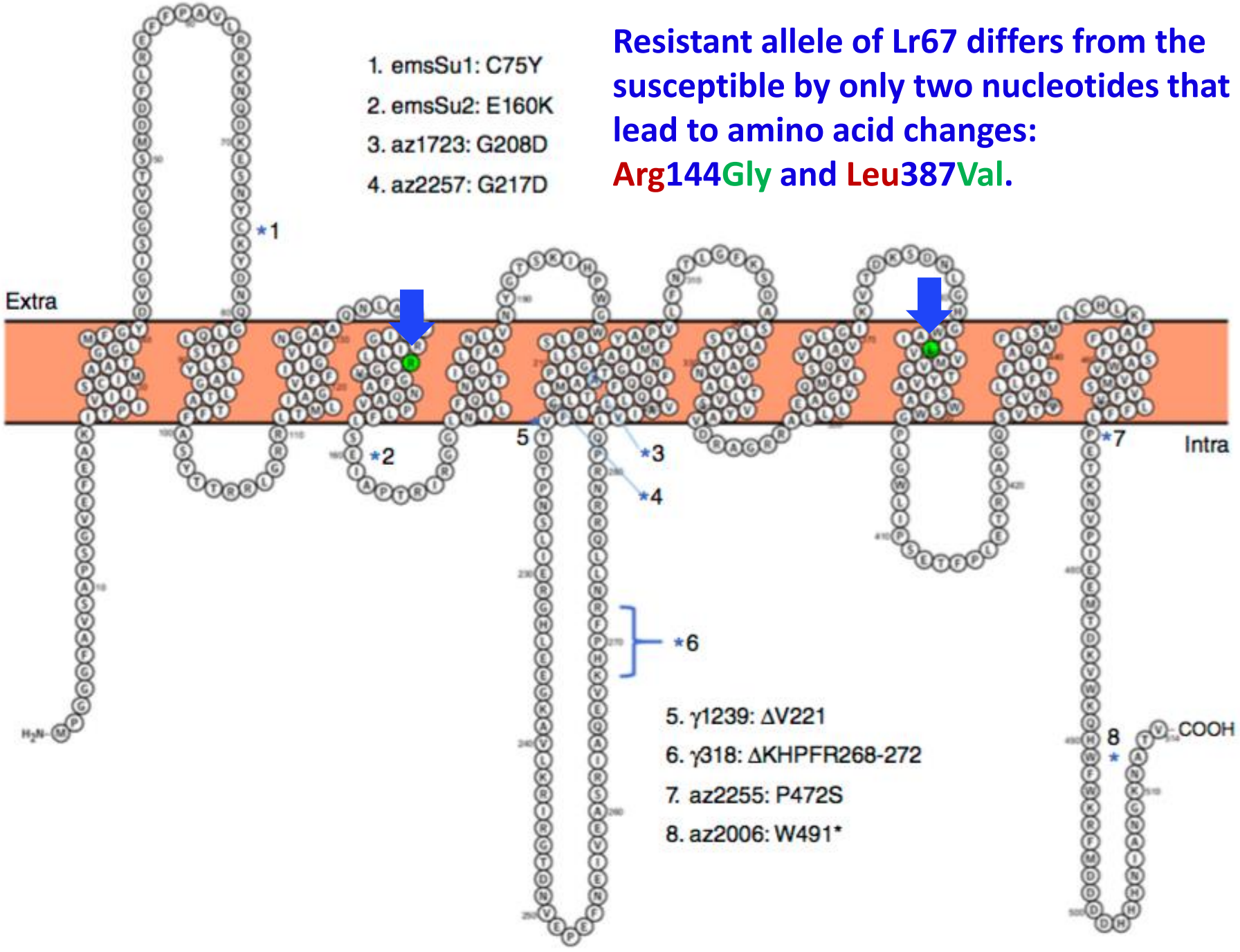
Wheat has multiple gene copies for ALS.

Gene Editing for Herbicide Tolerance in Maize

Edited ALS2 **S**

Unedited ALS2 **P**

Svitashev et al., 2015, Plant Physiol. 169:931
DuPont Pioneer


1. emsSu1: C75Y
2. emsSu2: E160K
3. az1723: G208D
4. az2257: G217D
Resistant allele of Lr67 differs from the susceptible by only two nucleotides that lead to amino acid changes:
Arg144Gly and Leu387Val.
Extra
Intra
*1
*2
*3
*4
5
*6
*7
8
COOH
5. γ1239: ΔV221
6. γ318: ΔKHPFR268-272
7. az2255: P472S
8. az2006: W491*

Candidate Genes for Editing in Wheat and Maize

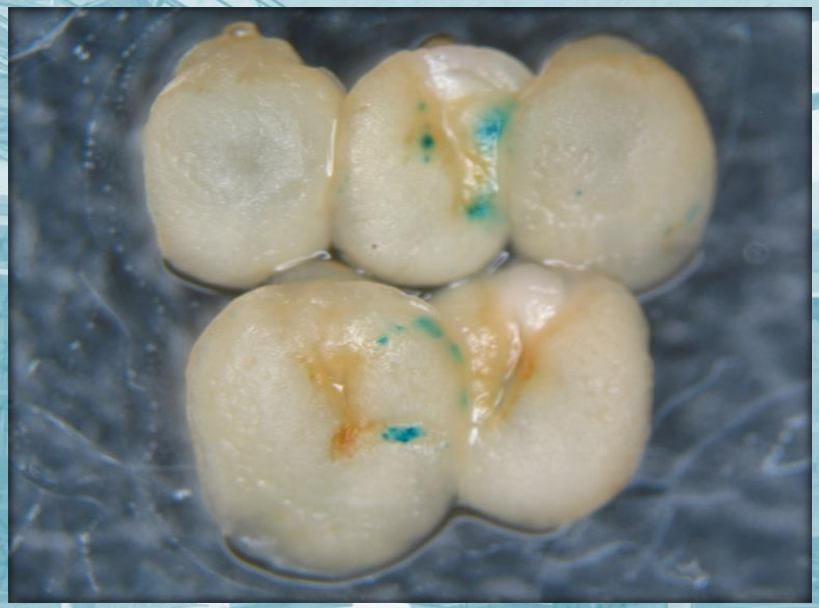
- Disease resistance
  - Wheat: FHB, Lr34, Lr67, MLO
  - Maize: Maize lethal necrosis (MLN), Fusarium
- Herbicide tolerance
  - Wheat: glyphosate, sulfonylureas
  - Maize: sulfonylureas, alternative for glyphosate
- Grain Quality
  - Knockout lysine degrading enzymes
  - Knockout phytate-related enzymes

Line Fielder

Line Baj

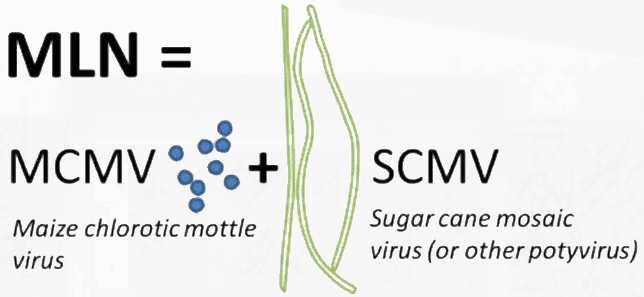
Line Kachu-Saual

Line Navojoa



Maize Lethal Necrosis

MLN is caused by combined action of Maize Chlorotic Mottle Virus (MCMV) and any of the Potyviruses that like cereals, especially Sugarcane Mosaic Virus (SCMV)

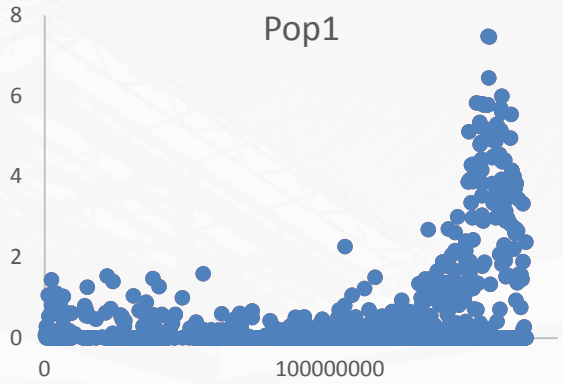


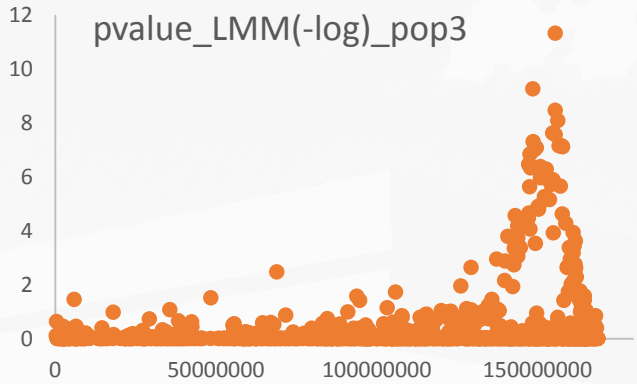
The disease was first reported in Bomet county of Kenya in Sept 2011, and since then in several countries in eastern Africa.

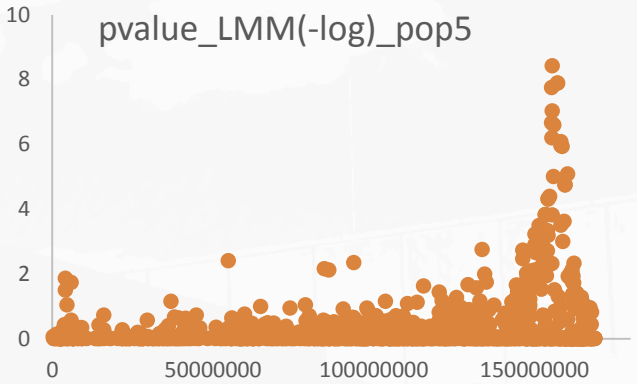
Resistance Against MLN From an Exotic Genetic Resource

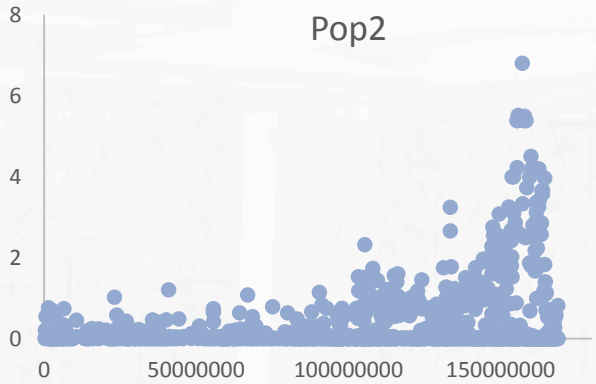
CML395 KS-23-6 CML444

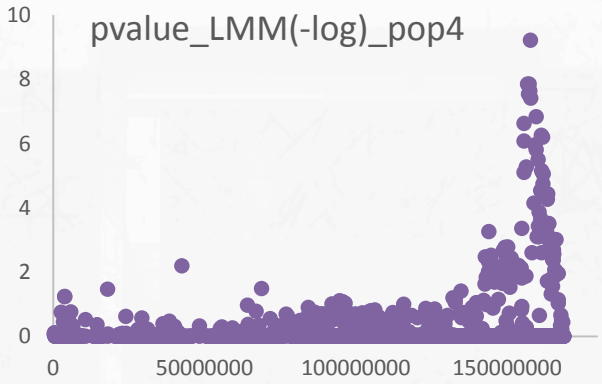
MLN Maps to Chr. 6 in All Six Subpopulations Derived From Crosses of KS523-5 or KS23-6 with CML Lines

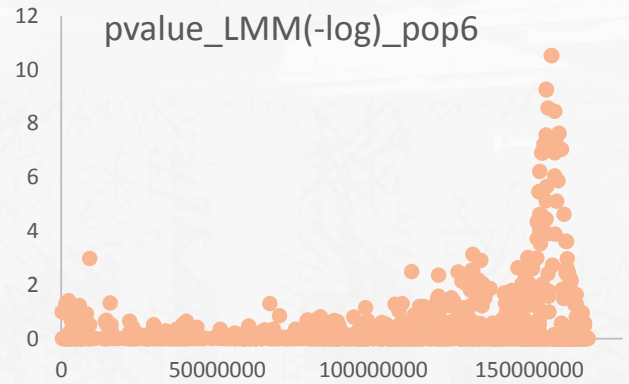


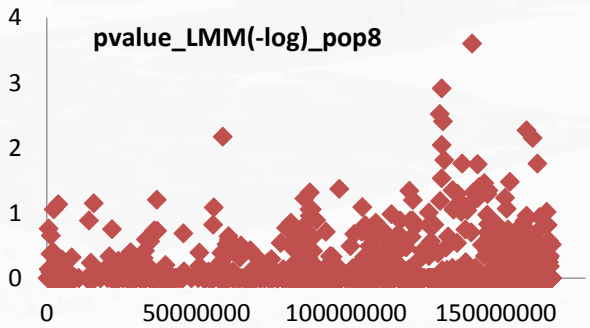


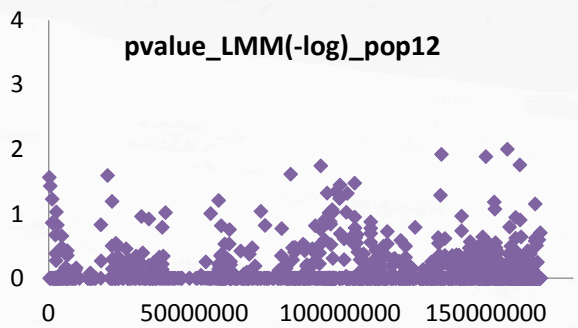








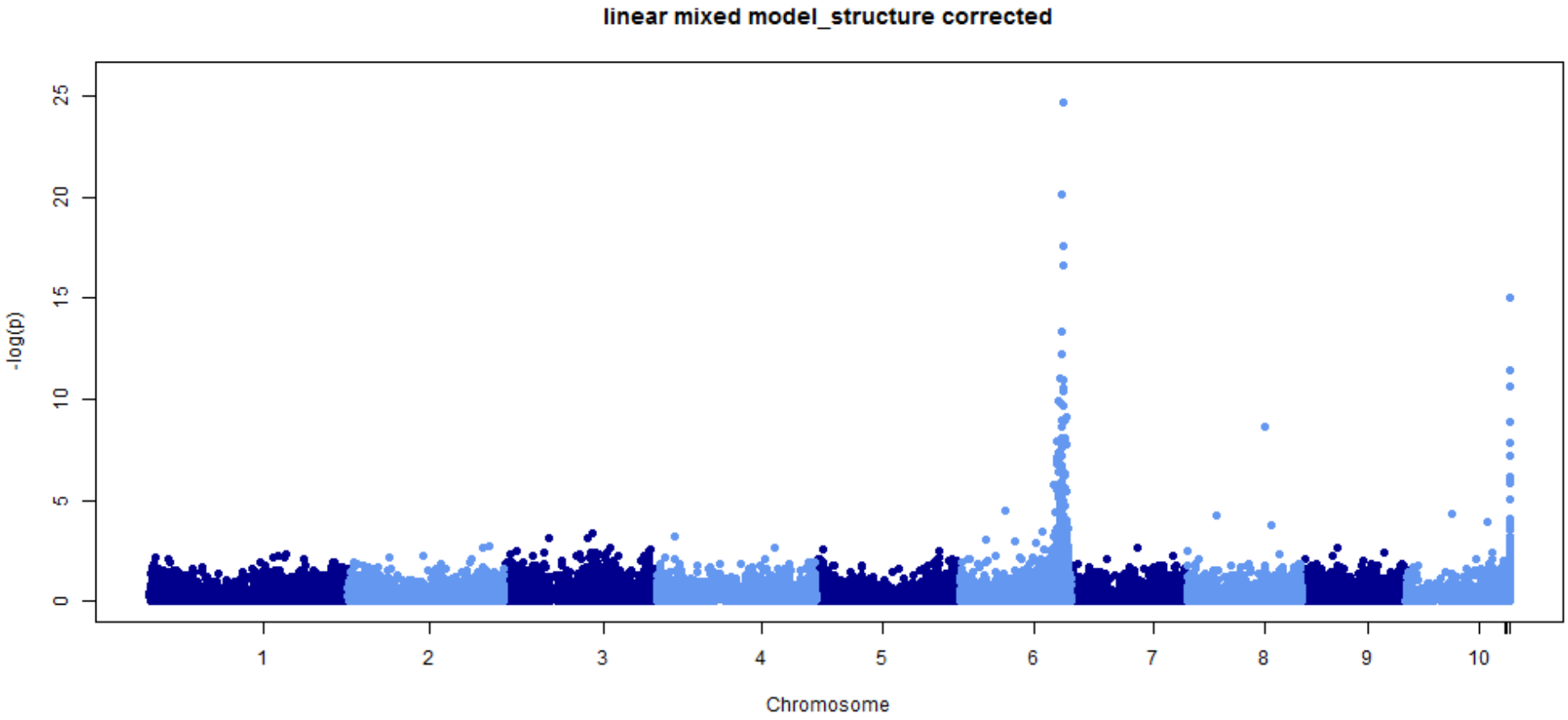




Mike Olsen

MLN Resistance in Maize

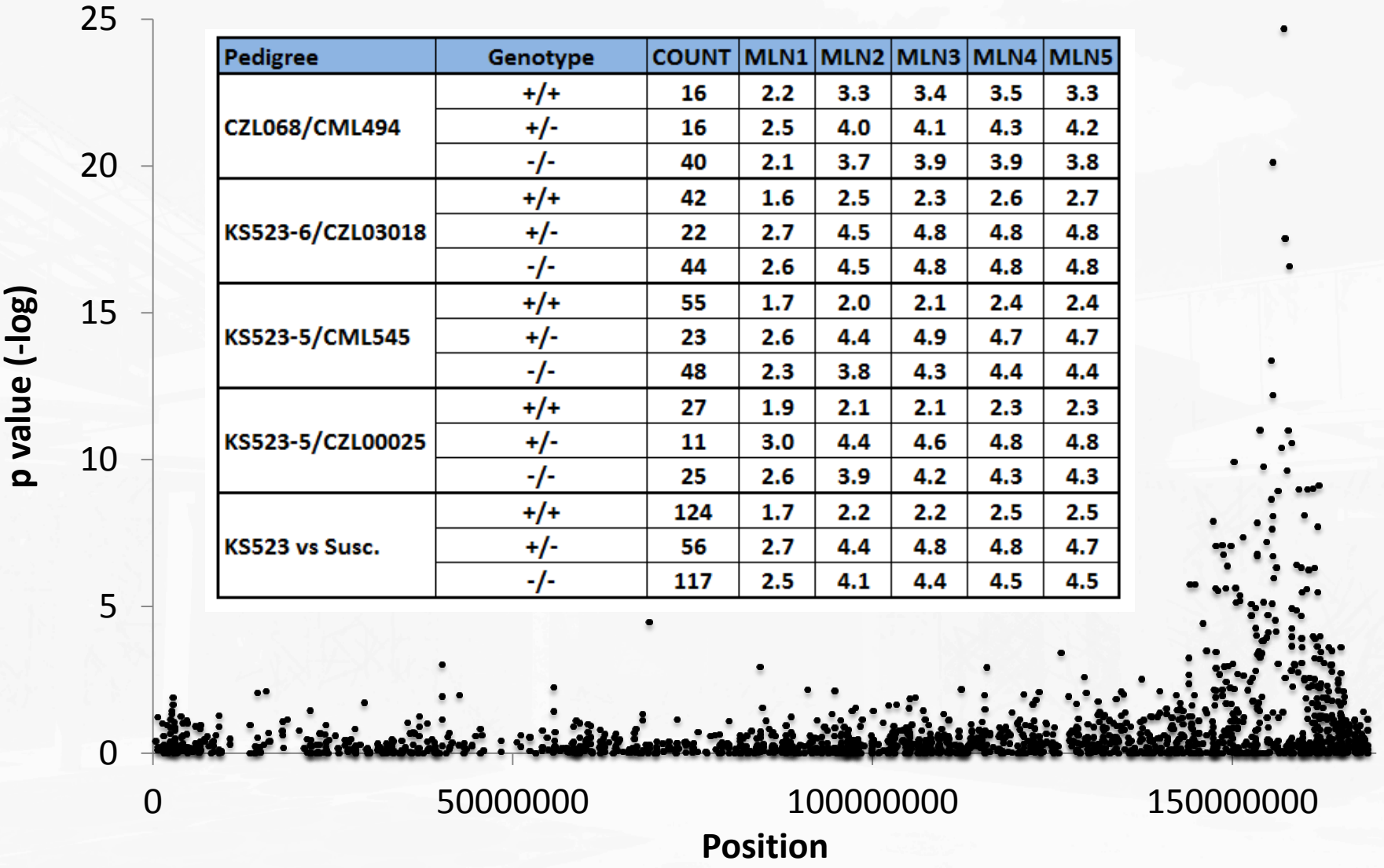
Kenya



Association mapping of MLN in three populations under field conditions.

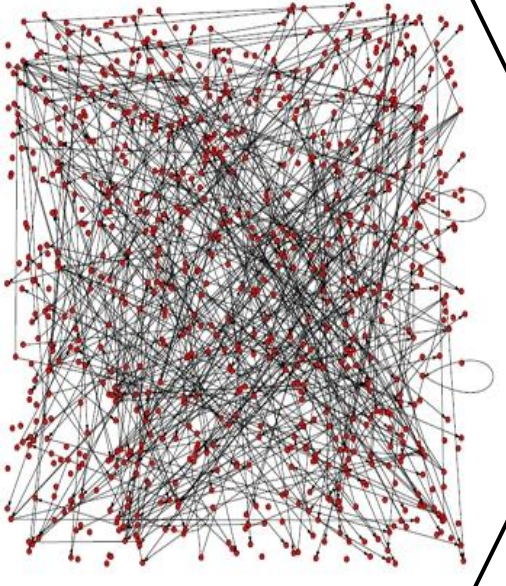
MLN Maps to Chr. 6 and Consistently Reduces Score by 2 Points

Pedigree	Genotype	COUNT	MLN1	MLN2	MLN3	MLN4	MLN5
CZL068/CML494	+/+	16	2.2	3.3	3.4	3.5	3.3
	+/-	16	2.5	4.0	4.1	4.3	4.2
	-/-	40	2.1	3.7	3.9	3.9	3.8
KS523-6/CZL03018	+/+	42	1.6	2.5	2.3	2.6	2.7
	+/-	22	2.7	4.5	4.8	4.8	4.8
	-/-	44	2.6	4.5	4.8	4.8	4.8
KS523-5/CML545	+/+	55	1.7	2.0	2.1	2.4	2.4
	+/-	23	2.6	4.4	4.9	4.7	4.7
	-/-	48	2.3	3.8	4.3	4.4	4.4
KS523-5/CZL00025	+/+	27	1.9	2.1	2.1	2.3	2.3
	+/-	11	3.0	4.4	4.6	4.8	4.8
	-/-	25	2.6	3.9	4.2	4.3	4.3
KS523 vs Susc.	+/+	124	1.7	2.2	2.2	2.5	2.5
	+/-	56	2.7	4.4	4.8	4.8	4.7
	-/-	117	2.5	4.1	4.4	4.5	4.5

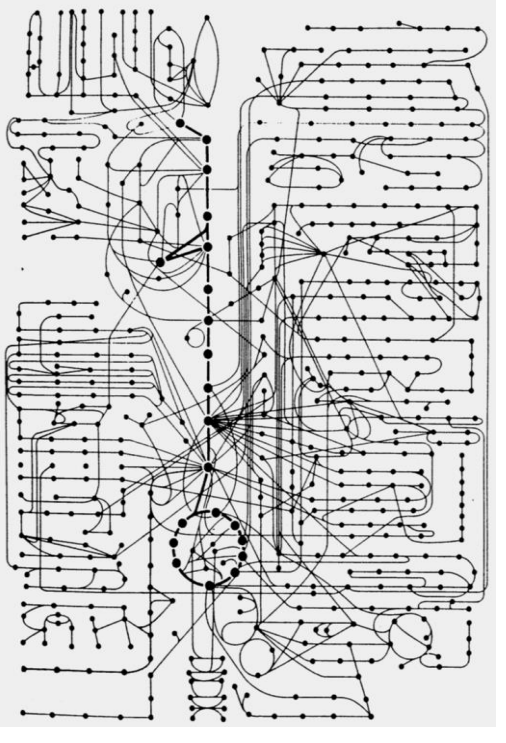


Resistance is recessive as hets are as susceptible as the susceptible ones. Likely a suppressor, the mutant form of which is not released from the regulatory site by the viral signal, not allowing the defense genes to be turned on.

Fig. 4: Genome to Grain Yield



Transcriptional regulation



Metabolic regulation

Alberts et al., Molecular Biology of the Cell

Grain Yield

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

$$C_{Ei}^{Ji} = \frac{\partial J / J}{\partial E / E}$$

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

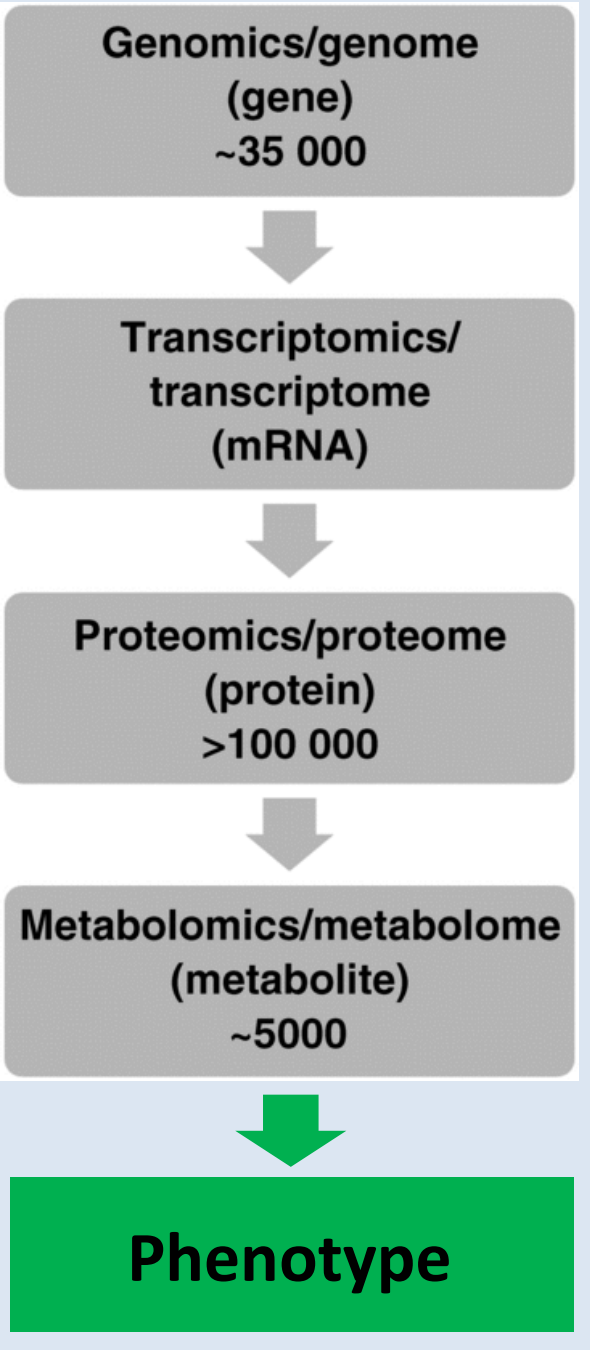


Fig. 5: Biological complexity is minimal at metabolite level.

Systems Biology

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

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. 6

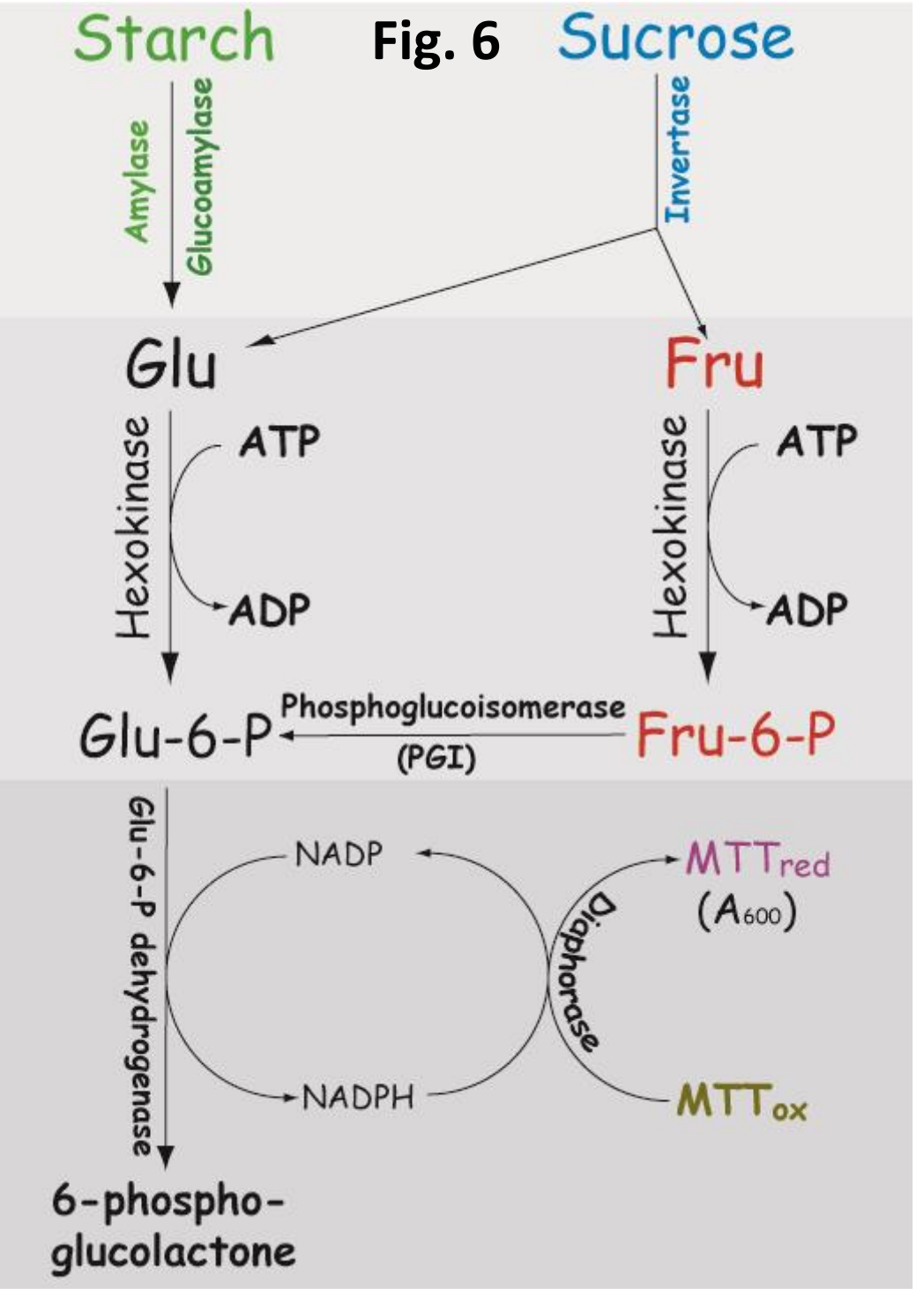

Starch
Sucrose
Amylase
Glucoamylase
Invertase
Glu
Fru
Hexokinase
ATP
ADP
Hexokinase
ATP
ADP
Glu-6-P
Phosphoglucoisomerase
(PGI)
Fru-6-P
Glu-6-P dehydrogenase
NADP
NADPH
Diaphorase
MTTred
(A600)
MTTox
6-phospho-
glucolactone

Fig. 7: Coupled Assay for Glucose & Fructose Using MTT as a Color Reagent

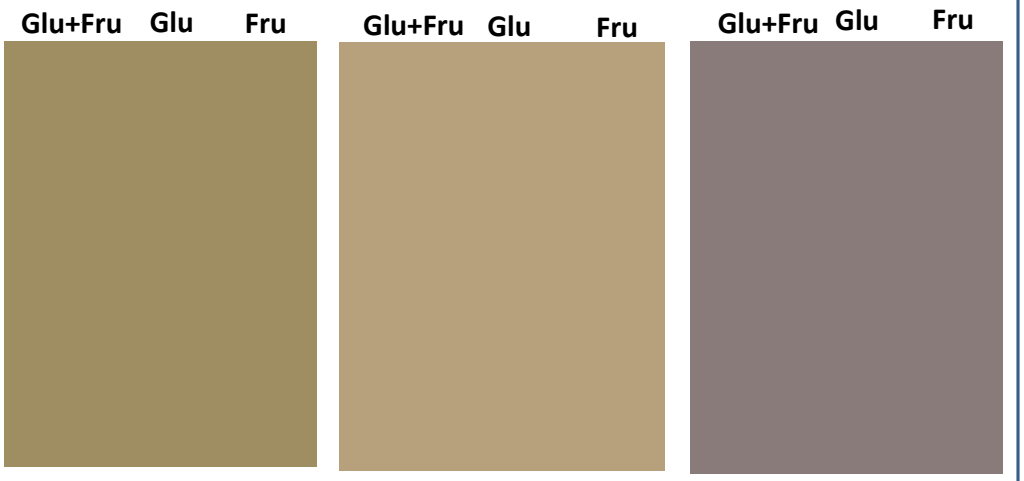


Fig. 8: Glucose and Fructose Standards

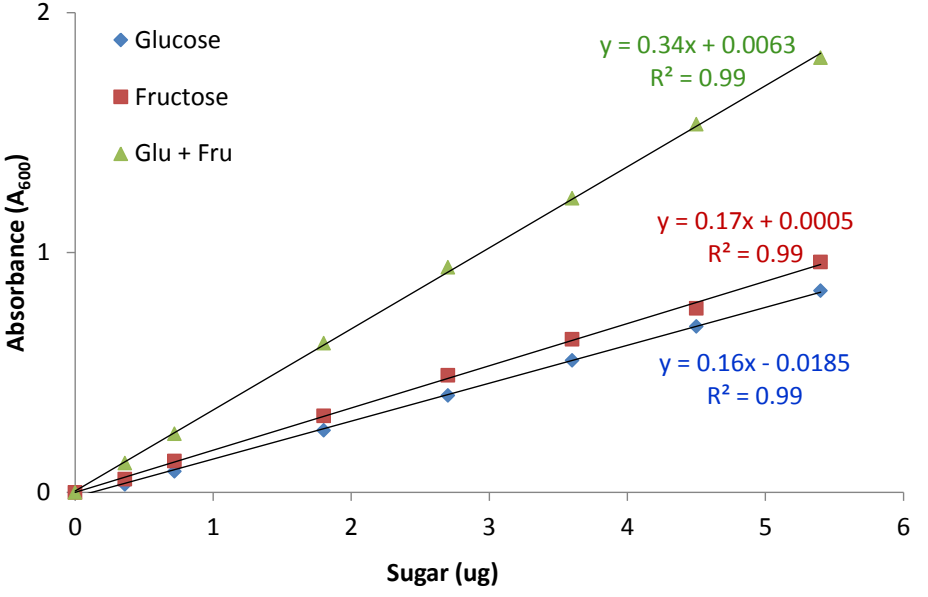
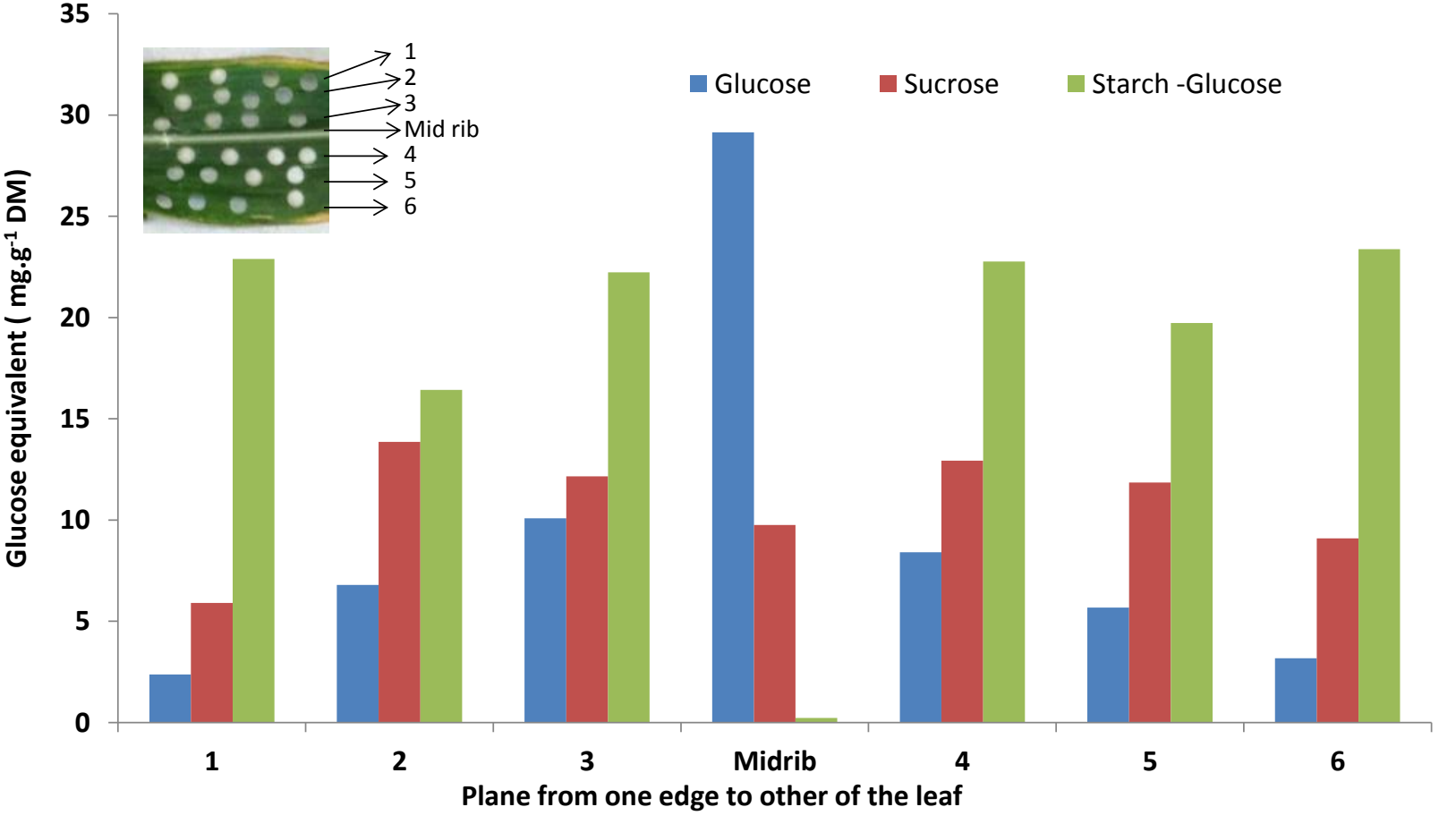


Fig. 9: Sugar Composition of the Mid-section of Maize Ear Leaf

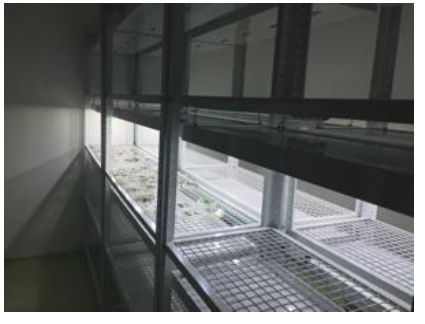
The section (inset) was separated into three longitudinal planes of approximately equal



The Gene Editing Platform at CIMMYT

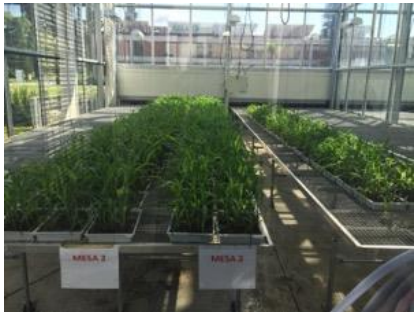
Molecular biology

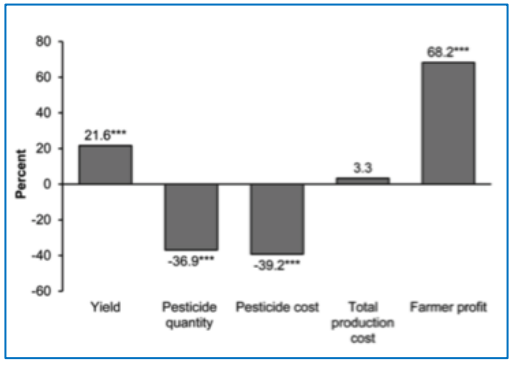
Transformation and regeneration



Screening for alterations

Testing for altered function

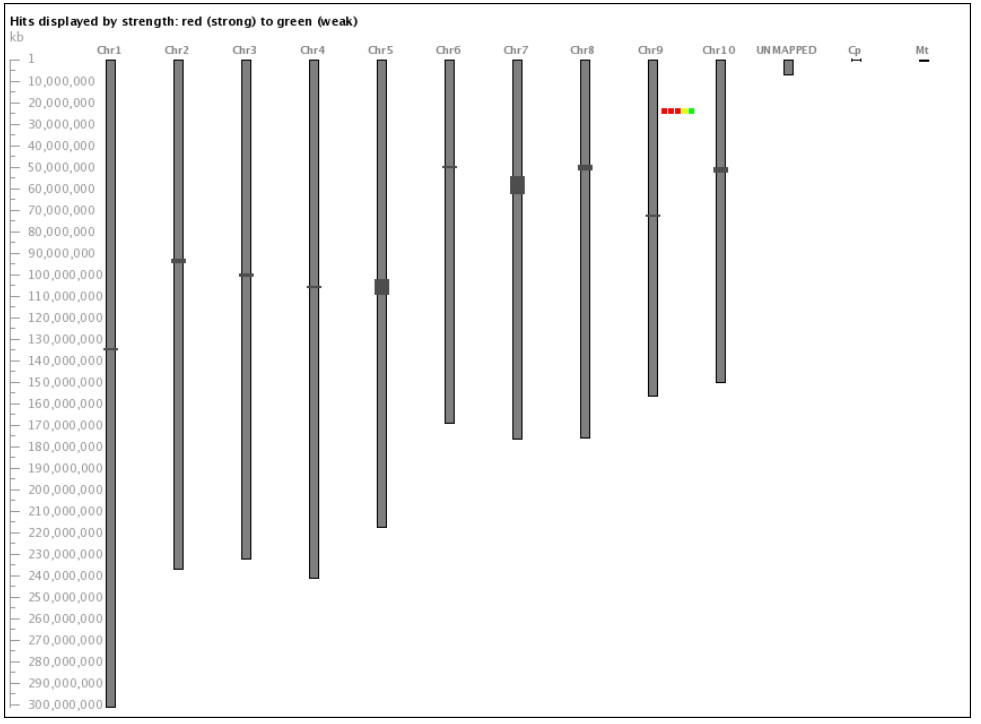




Traits for Gene Alteration

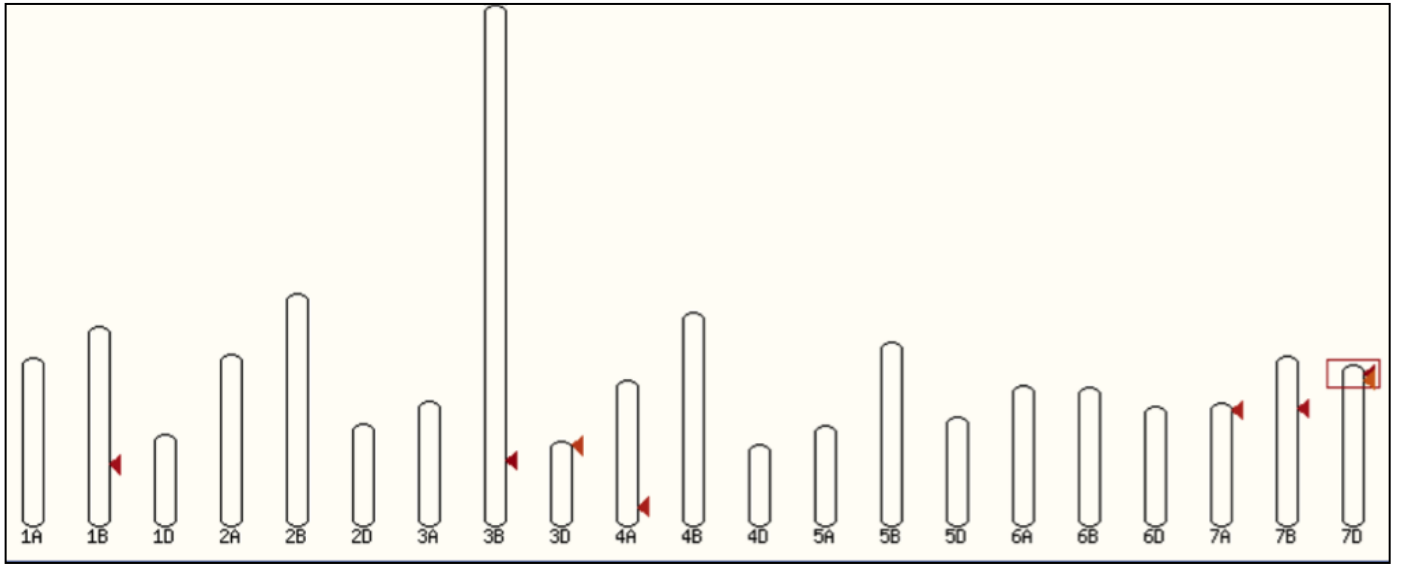
- **Maize**
  - MLN resistance (priority: high).
  - Striga resistance
    - Strigolactone synthase (priority: low).
    - ALS resistance (priority: high).
  - Increase provitamin A by down-regulating CCD genes (priority: low).
  - Gamma-zein knockout (priority: low).
  - Fusarium resistance – transgenic. Proof-of-principle.
- **Wheat**
  - Rust resistance (Lr34 and Lr67) (priority: high).
  - Phytate down-regulation (priority: medium).

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



Plant Cell Rep (2016) 35:1417–1427

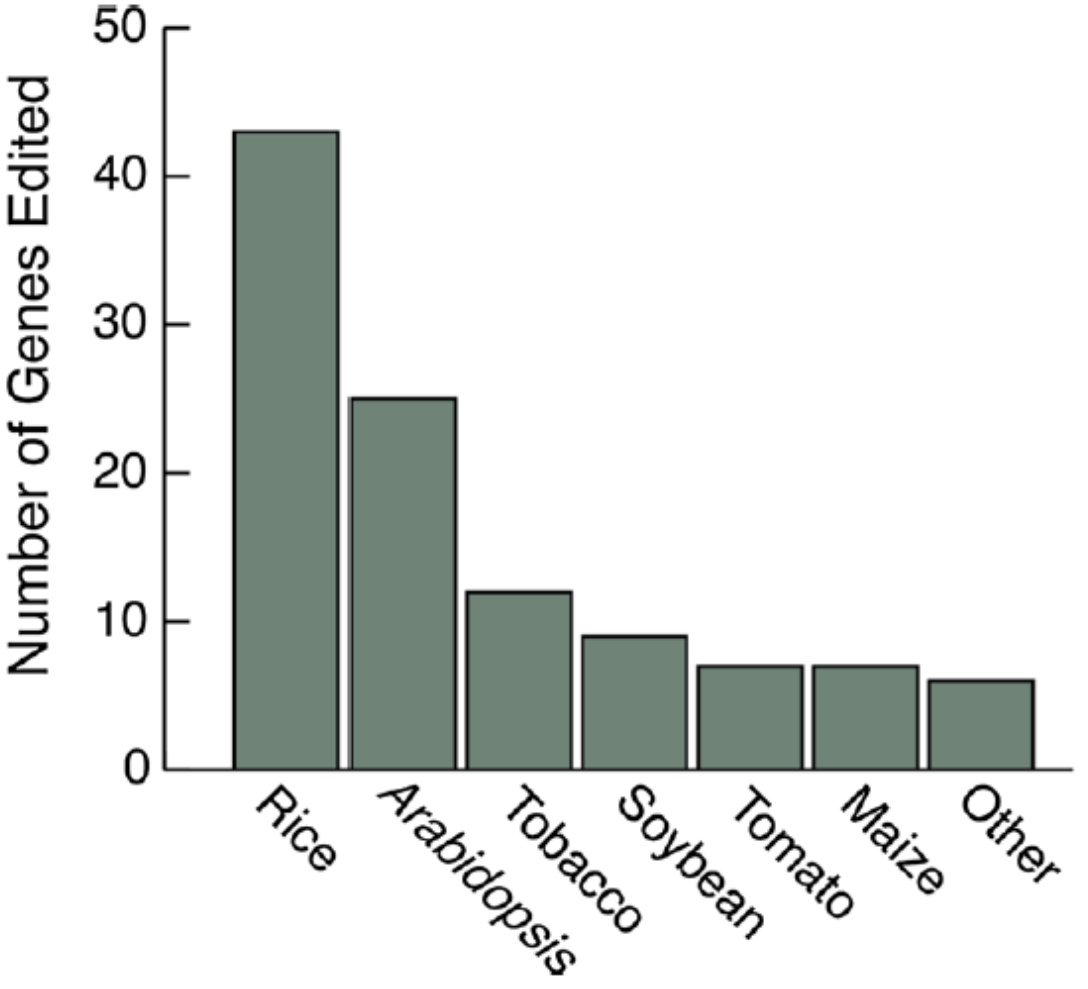
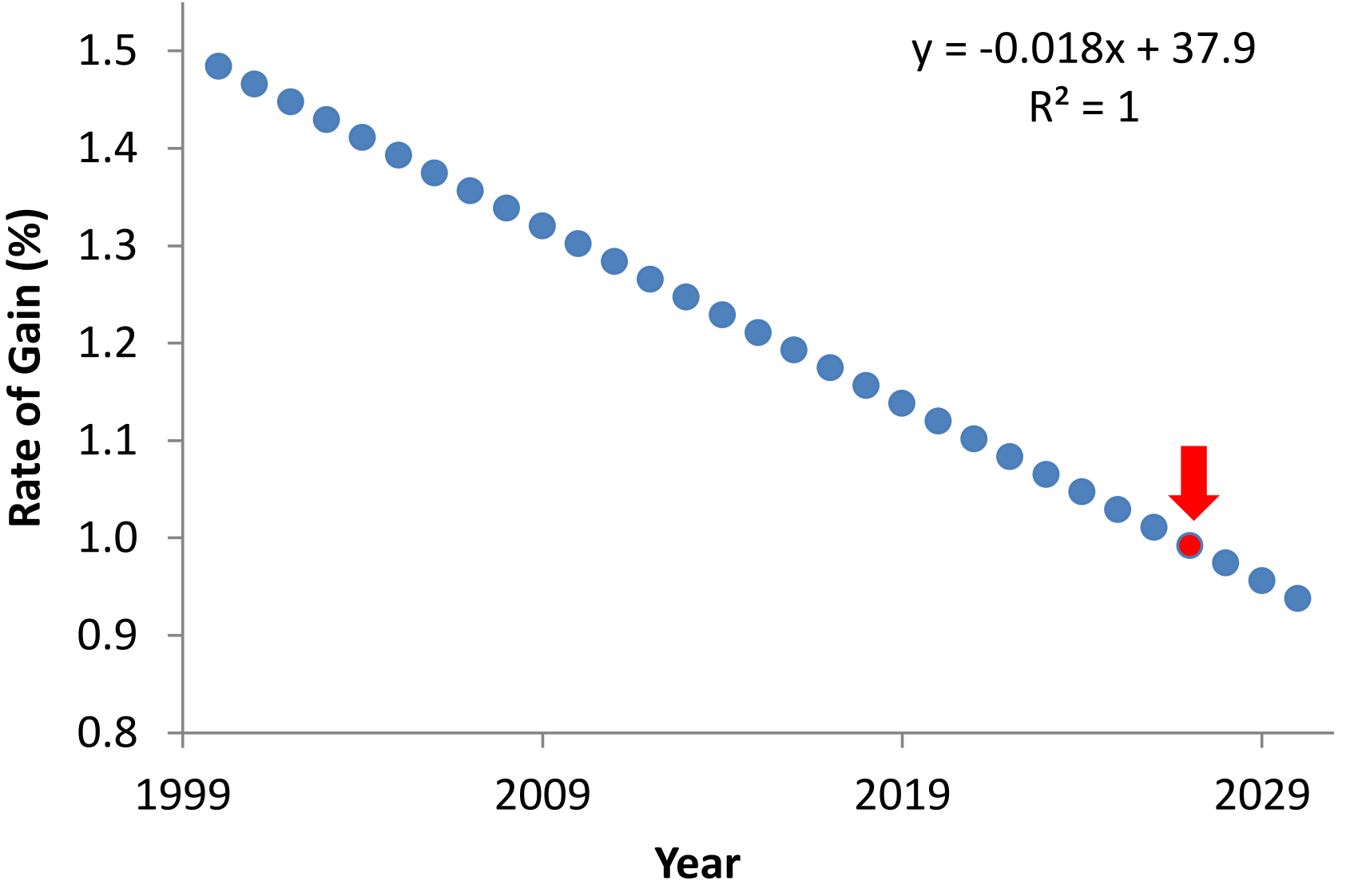


Fig. 2 Number of genes reported edited by CRISPR/Cas9 to date, by plant species. Publications reporting these were gathered using PubMed and searching the terms "crispr" and "plant". "Tobacco" includes *Nicotiana tabacum* and *Nicotiana benthamiana*

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%



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

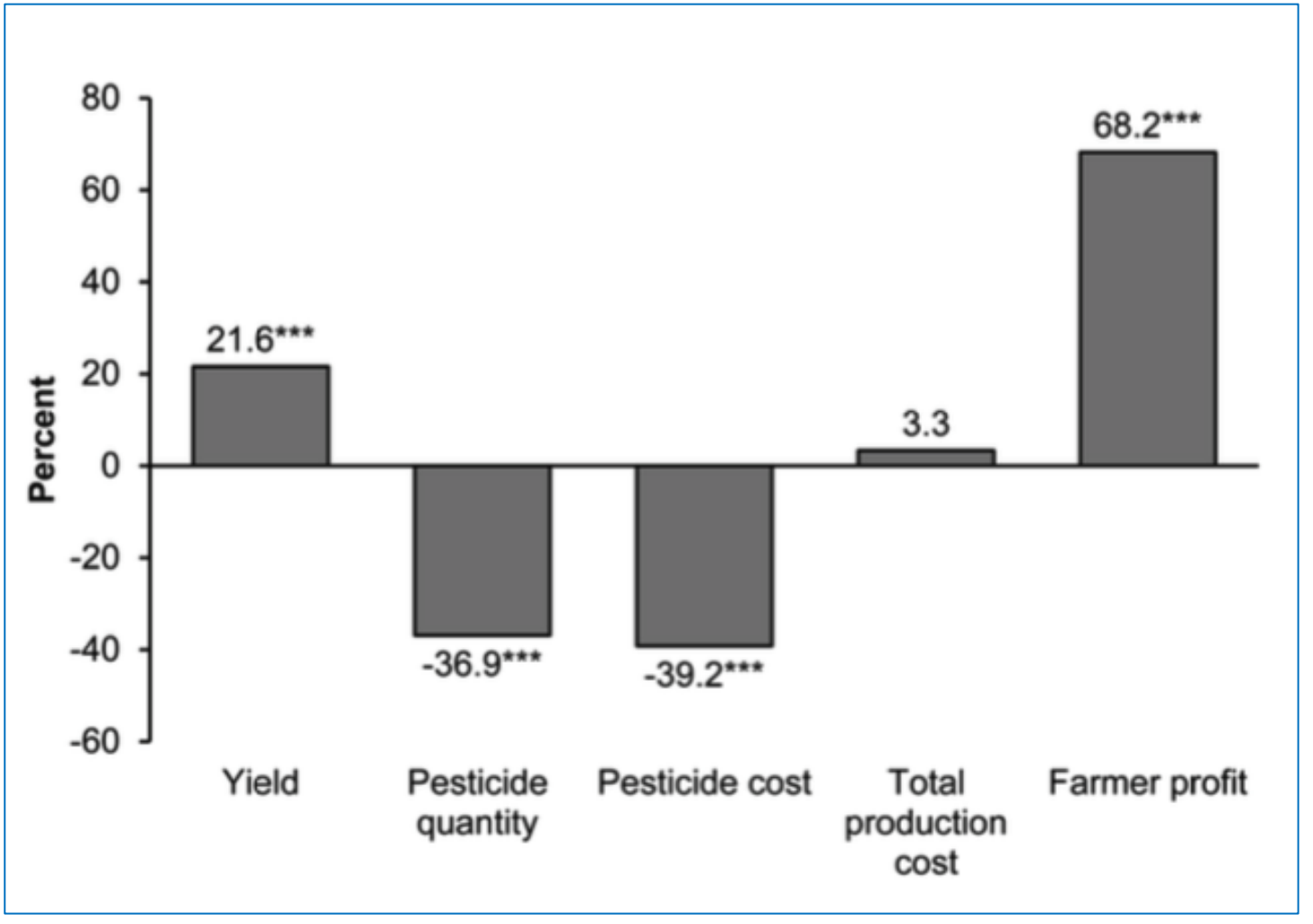
- **Funding**: Min. Agric. Forestry and Fisheries (MAFF), Japan.
- **Partners**: JIRCAS, RIKEN PSC, CIMMYT, IRRI, CIAT.
- **Expected outputs**: Identify useful regulatory genes for drought tolerance; contribute to sustainable food production.

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

Impact of Adoption of GM Crops

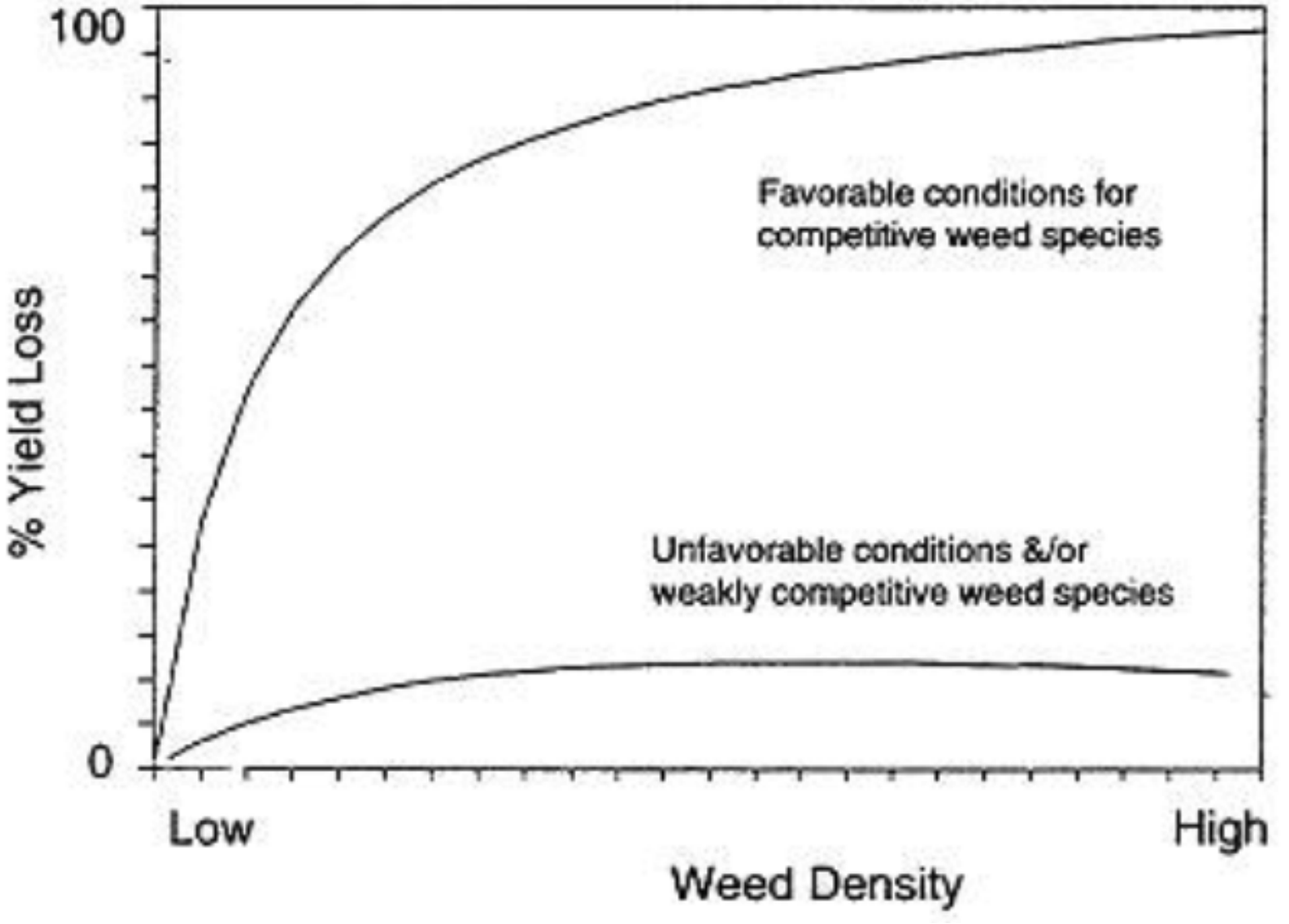
A Meta Analysis of 147 Studies



Klumper and Qaim, 2014, PLoS ONE

Our objective is to make the fruits of useful technologies accessible to small-holder farmers

Effect of Weeds on Wheat Yield



Alberta Agric. Forest.

CRISPR-Cas9-mediated Resistance Against Powdery Mildew in Wheat

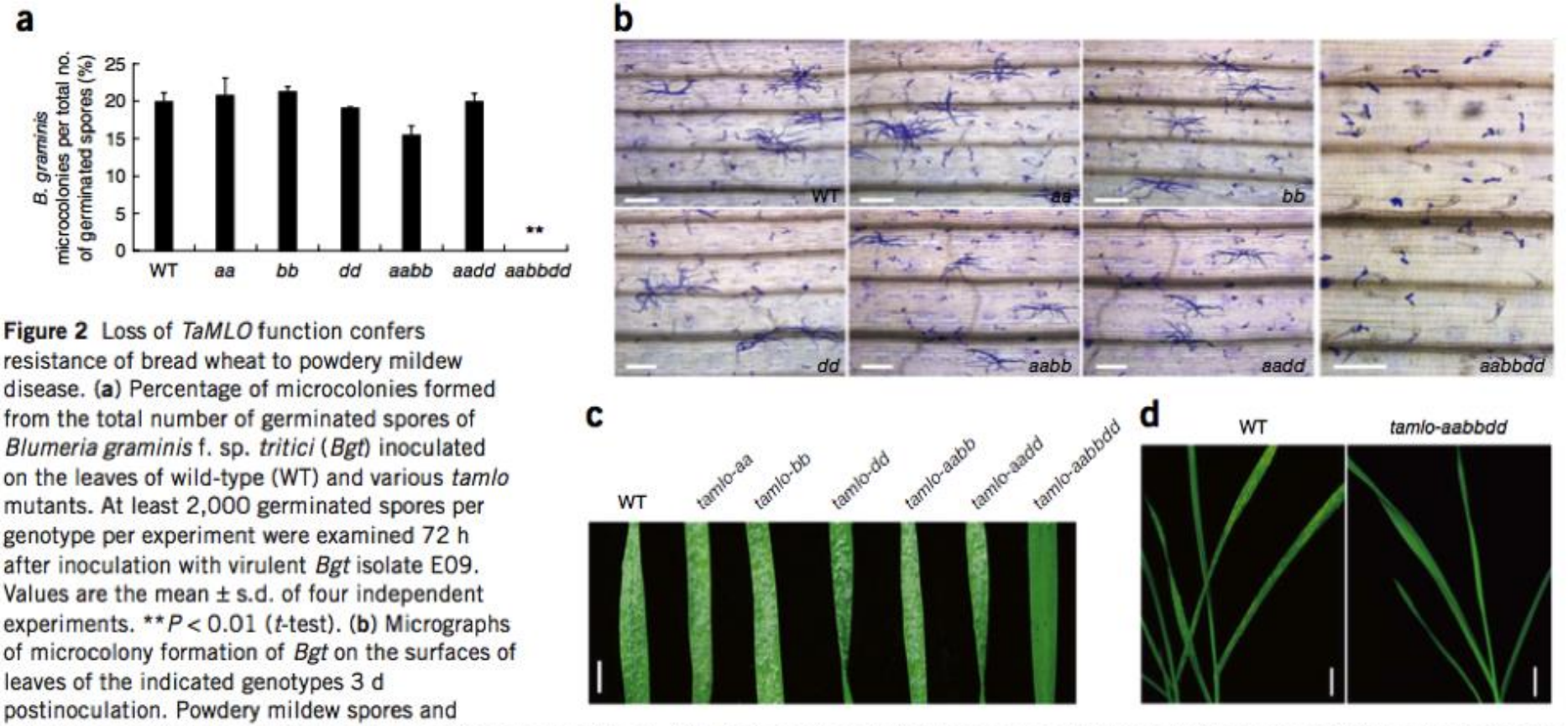


Figure 2 Loss of *TaMLO* function confers resistance of bread wheat to powdery mildew disease. (**a**) Percentage of microcolonies formed from the total number of germinated spores of *Blumeria graminis* f. sp. *tritici* (*Bgt*) inoculated on the leaves of wild-type (WT) and various *tamlo* mutants. 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.

Fruit Ripening in Tomato

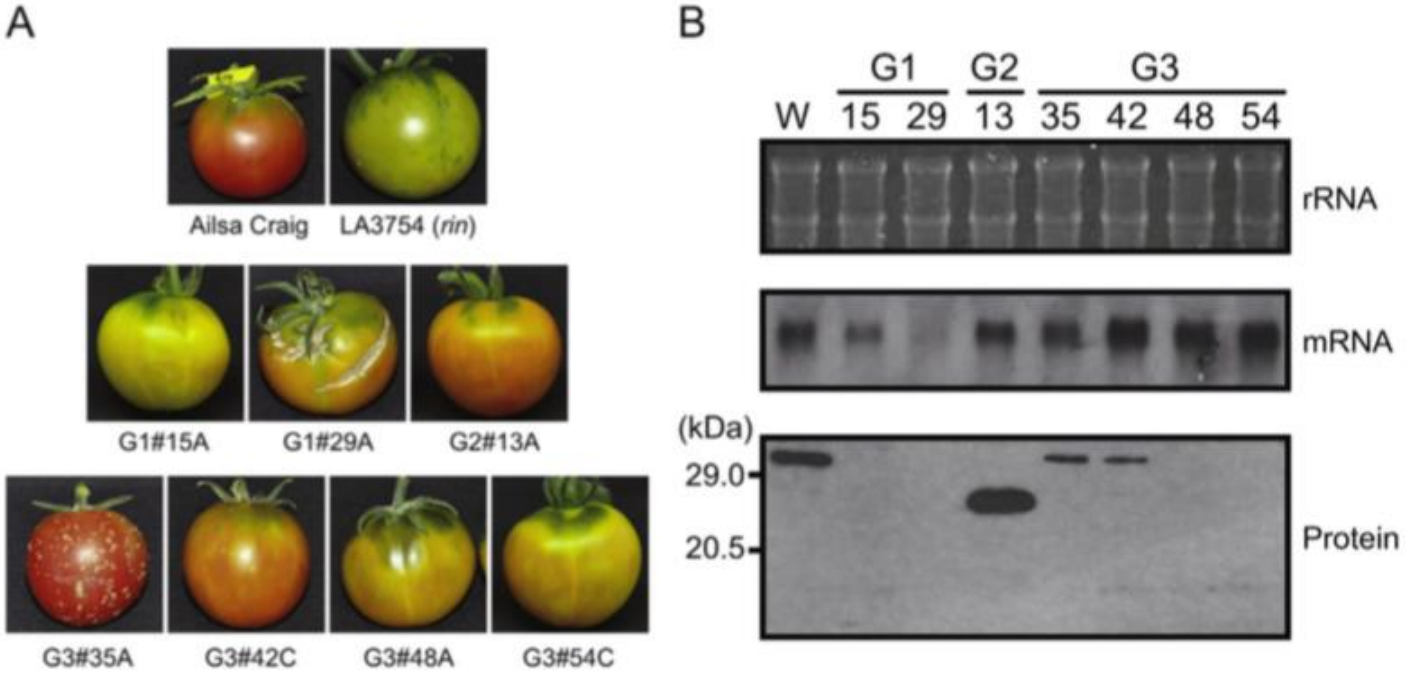


Fig. 3. Effects of the CRISPR/Cas9-induced mutations in ripening fruits. A) Appearance of mutant fruits harvested five days after the breaker stage. B) Expression analysis of *RIN* in mutant plants. Fruits of the mutants were harvested five days after the breaker stage and total RNAs and nuclear proteins 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

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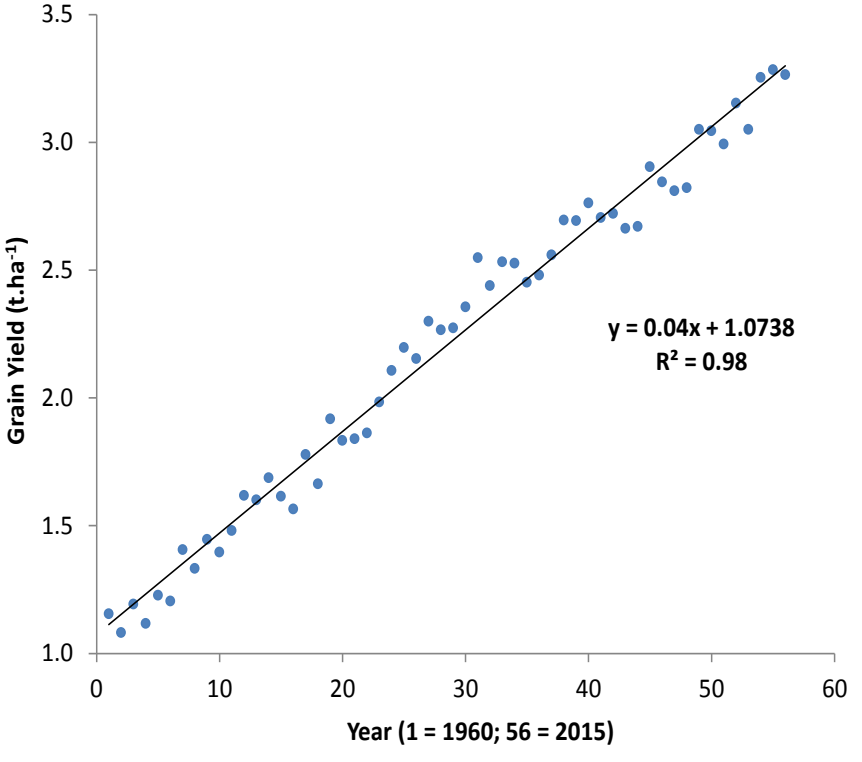
Table 2 Mutations in *CDKA* and *CDKB* genes in regenerated plants obtained from pZH_OsCas9, pZK_sgCDKB2-transformed calli #5, 11, 1 and 13

	#5										#11						#12	#13	
Regenerated plants	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**1**	**Mutated plant**
CDKB2	M	N	M	M	N	M	M	N	N	M	M	M	M	M	N	N	M	N	11/18
CDKA1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0/18
CDKA2	B	N	M	M	M	M	M	N	N	N	M	M	B	M	M	M	B	N	13/18
CDKB1	M	N	M	M	N	N	N	N	N	N	N	M	M	M	N	N	N	M	7/18

N, no mutation; M, monoallelic mutation; B, biallelic mutation.

Off-target editing can overcome the homeoallelic complexity of wheat

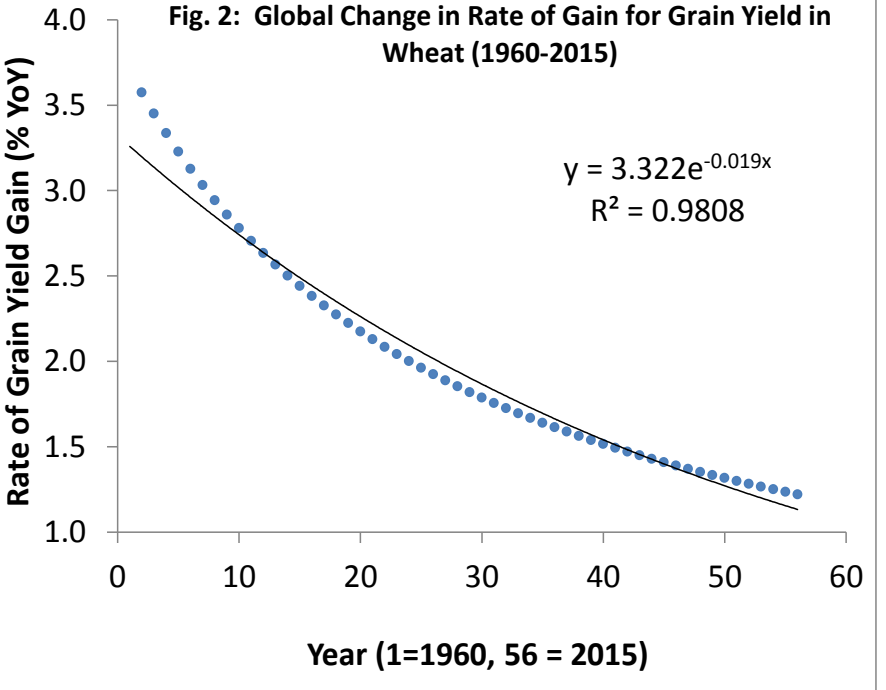
Fig. 1: Global Trend of Wheat Grain Yield Over 56 Years



Data Source: USDA, Foreign Agricultural Service, Production, Supply, and Distribution Database.

Average yield increase globally has been ~40 $kg.ha^{-1}.yr^{-1}$ over the last half century and about the same in four blocks each of of 14 year.

Fig. 2: Global Change in Rate of Gain for Grain Yield in Wheat (1960-2015)



Total rate of yield gain was calculated as $(Y_t-Y_{t-1})*100/Y_{t-1}$, 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, 3,000 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**