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**Sant'Anna**  
School of Advanced Studies – Pisa

# The Multi-parent Advanced Generation Inter-Cross (MAGIC) maize population



# Why MPPs are better than biparental RILs

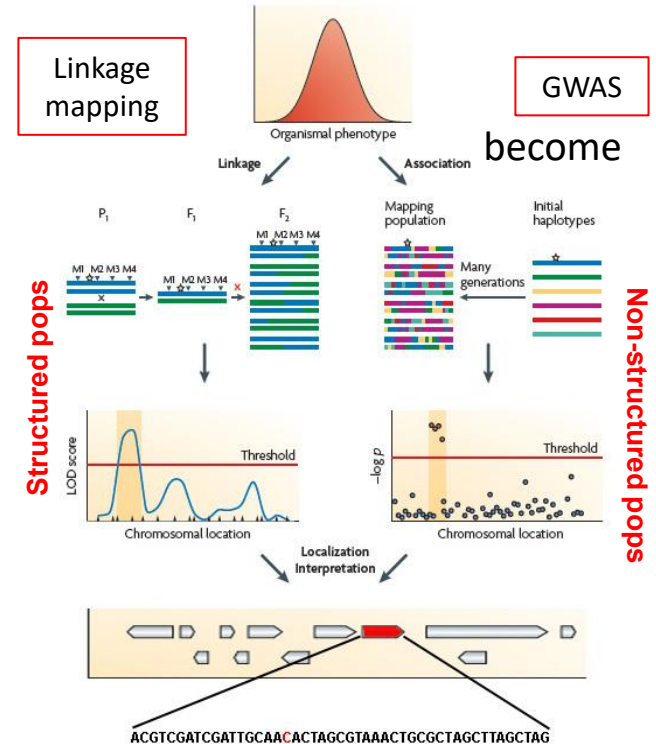
High-throughput DNA sequencing of individuals is becoming routine, but **linking complex phenotypes to their molecular basis remains a challenge**:

- **Marker density** ceases to be a limiting factor
- **Genetic diversity** and **recombination density** the limiting factor



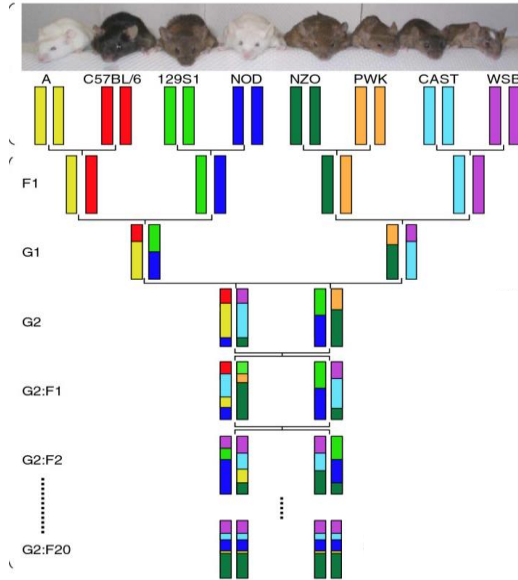
**Multi-parent cross designs** bridge GWAS and haplotype mapping, and increase QTL detection and characterization by:

- Incorporating **greater genetic diversity**
- Increasing the **number of crossing generations**

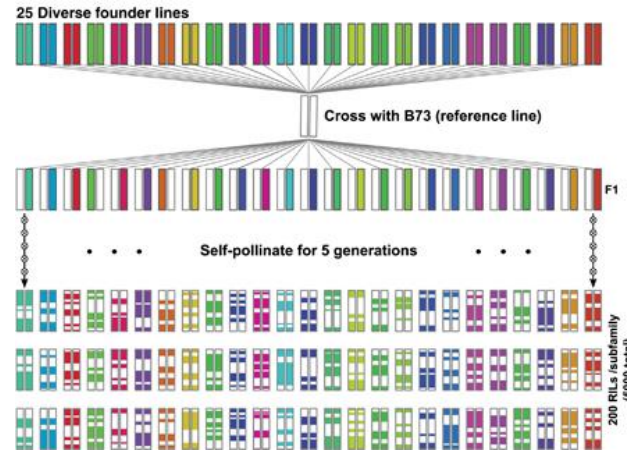


# Different types of MPPs

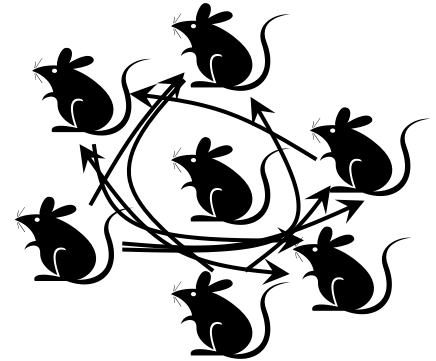
Multi-parental populations achieve **more diversity** and **more recombination events** than biparental populations with different designs



- Collaborative Cross (CC)
- Multiparent Advanced Generation InterCross (MAGIC)



Nested Association Mapping  
(NAM)



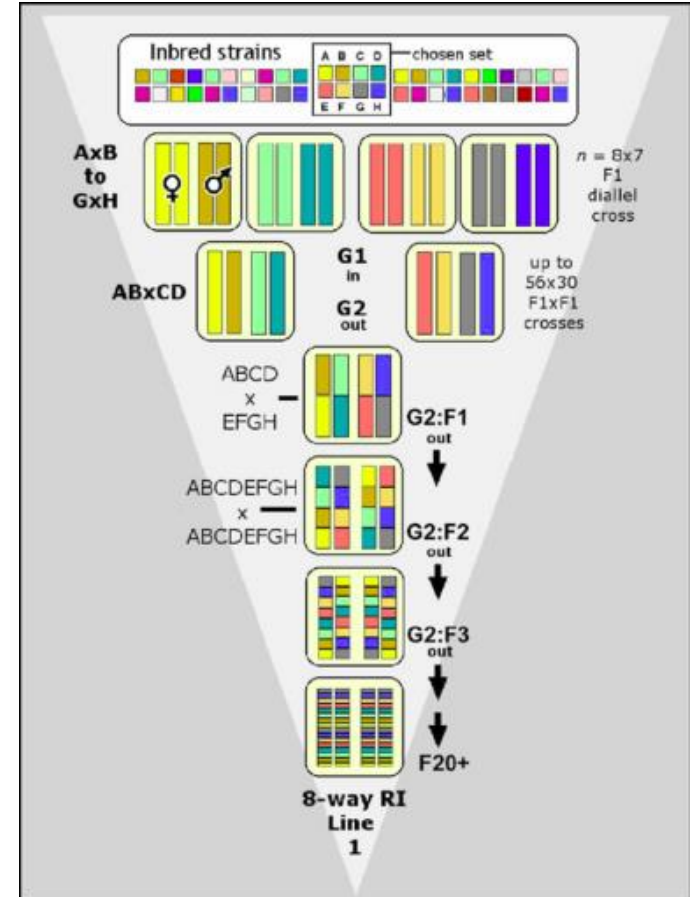
- Diversity Outbred (DO)
- Heterogenous Stock (HS)

# CC like, or MAGIC (Multiparental Advanced Generation InterCross)

- A set of diverse founders
- Multiple intercrossing generations
- Inbreeding to produce **Recombinant Inbred Lines (RILs)**

## Results:

- **Closed**, pooled population of RILs deriving from the founders
- **Small numbers** provide many recombination events and much diversity
- **Eternal**, immutable

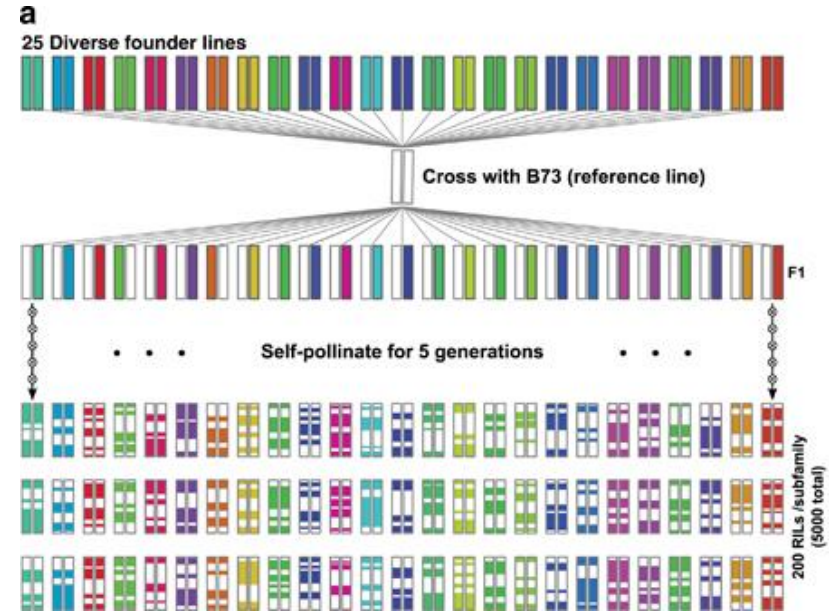


# NAM (Nested Association Mapping population)

- One recurrent founder, n diverse founders
- One intercross generation
- Inbreeding to produce **Recombinant Inbred Lines (RILs)**

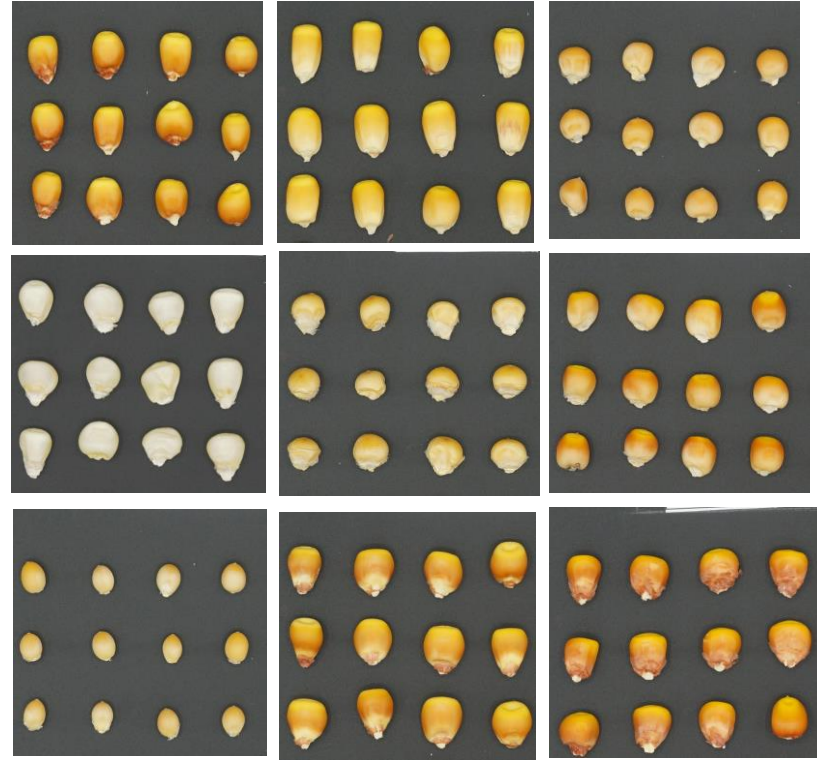
## Result:

- **Open** collection of biparental families
- **Higher numbers** provide many recombination events and much diversity
- **Eternal**, immutable

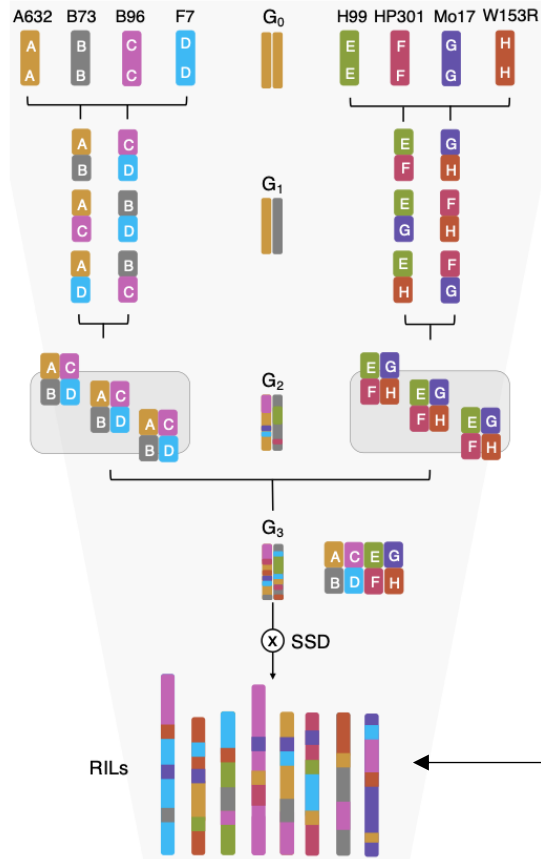


# Multi-parent populations in maize

A framework to combine **genomics**,  
**genetics**, and **diversity** in a multi-purpose  
tool for mapping and breeding



# The Multi-parent Advanced Generation Inter-Cross (MAGIC) maize population



Multi-parent population developed following a MAGIC crossing scheme inter-crossing **8 diverse lines** (founders)



Fig. Founders lines of MAGIC population

After 6 generations: 1,600 Recombinant Inbred Line (RIL)

Each RIL is a **mosaic of the founders' genomes**

➡ High genetic diversity, high recombination density

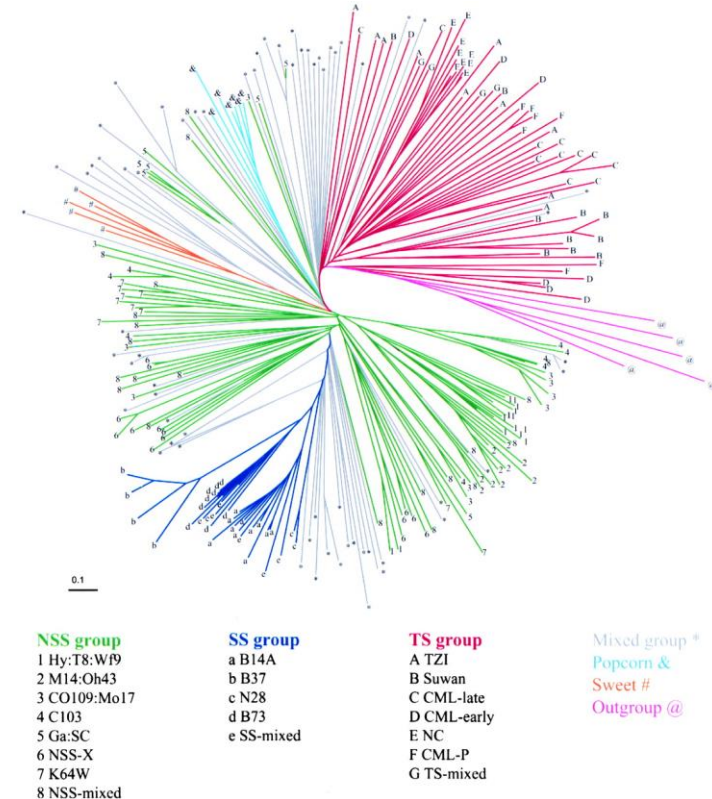


# Choice of parental lines

- Selection of **8 parental lines** maximizing variation (or incorporating useful traits)
- Effort to maximize **crossability** thanks to uniform phenology

Two main stages:

- 1) **MIXING STAGE:** progenitor lines are intercrossed to produce a foundation population
- 2) **INBREEDING STAGE:** randomly chosen individuals of the foundation population are inbred (by selfing or sibling)



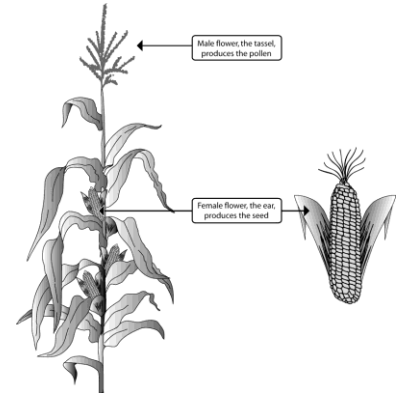


# 1) Mixing stage

- Production of **2-way hybrids** from every distinct pair of founders, generating a diallel cross
- Ideally, each cross could be the start of a bi-parental population

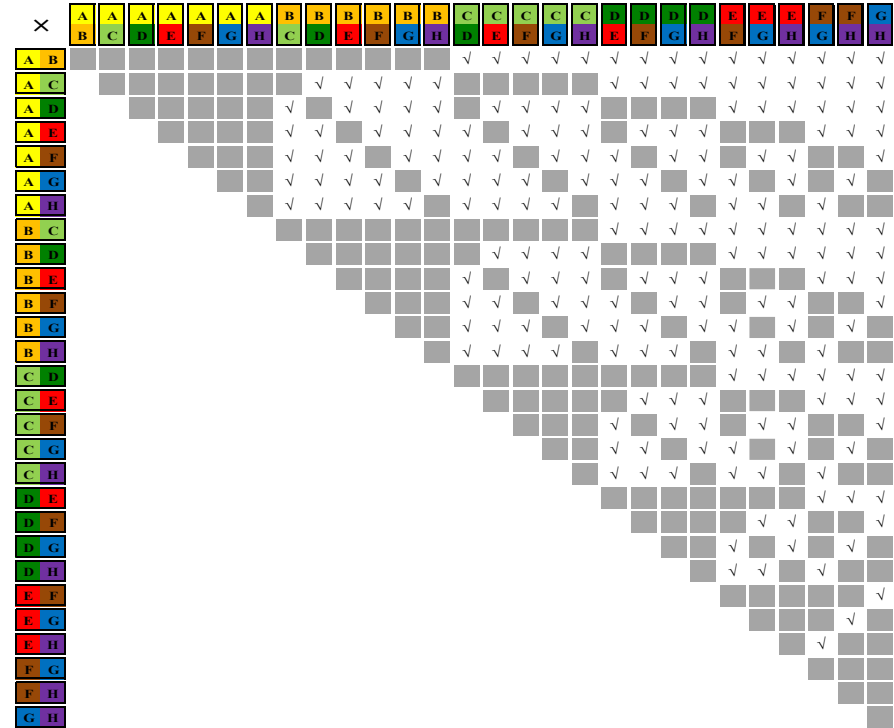
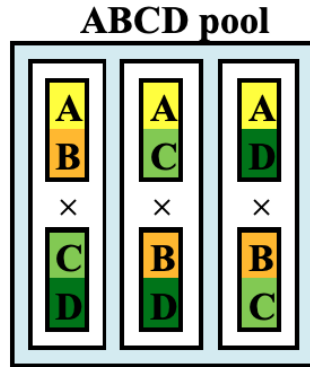
Inbred line	Group	Subgroup	×	A	B	C	D	E	F	G	H
A632	SS	B14A	▶	A	AB	AC	AD	AE	AF	AG	AH
B73	SS	B73	▶	B		BC	BD	BE	BF	BG	BH
B96	TS	Suwan	▶	C			CD	CE	CF	CG	CH
F7	Mixed	-	▶	D				DE	DF	DG	DH
H99	NSS	NSS-mixed	▶	E					EF	EG	EH
HP301	Popcorn	-	▶	F						FG	FH
Mo17	NSS	CO109:Mo17	▶	G							GH
W153R	NSS	NSS-mixed	▶	H							

Source: Liu et al. 2003



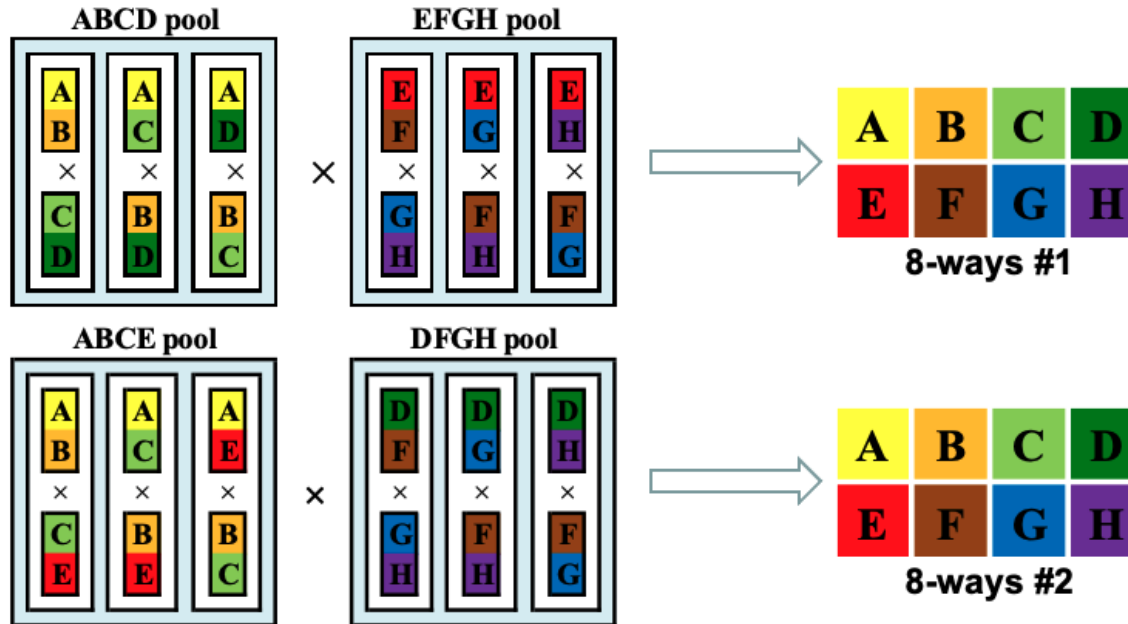
# 1) Mixing stage

- **4-way hybrids** are then produced
- To reduce complexity, only 2-way hybrids without founders in common were crossed
- **Triplets of 4-way crosses** containing the same parental alleles in different CIS combinations were pooled



# 1) Mixing stage

- Each **4-way pool** is **crossed** with its complementary pool to establish **8-way pools**



## 2) Inbreeding stage

		"genomic" He
Summer	2007 (IT)	F <sub>1</sub> (100%)
Summer	2008 (IT)	F <sub>2</sub> (50%)
Summer	2009 (IT)	F <sub>3</sub> (25%)
Winter	2010 (MM)	F <sub>4</sub> (12.5%)
Summer	2010 (IT)	F <sub>5</sub> (6.25%)
Winter	2011 (MM)	F <sub>6</sub> (3.125%)
Summer	2011 (IT)	F <sub>6</sub> Sib (3.125%)
Summer	2012 (IT)	PHENOTYPING & GENOTYPING

**35** 8-ways hybrids pools  
(5,000 individuals)

SSD

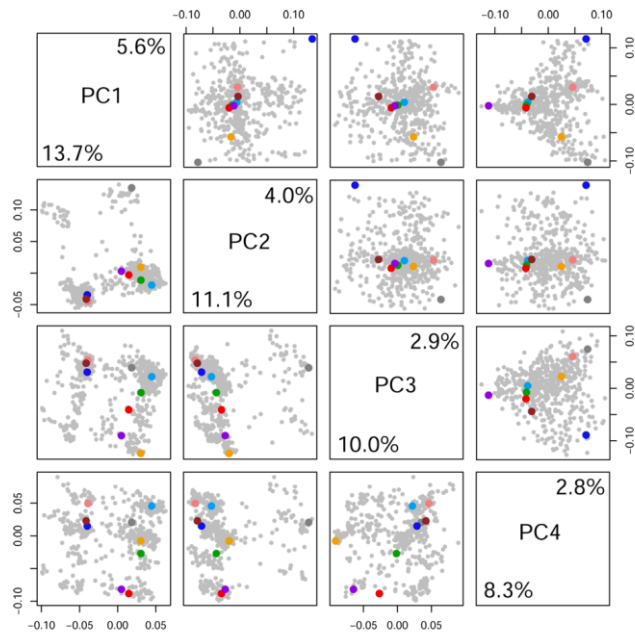
male sterility  
female sterility  
barreness  
human error

1,636 RILs-8W F<sub>6</sub>  
balanced in 35 families

529 RILs-8W F<sub>6</sub>

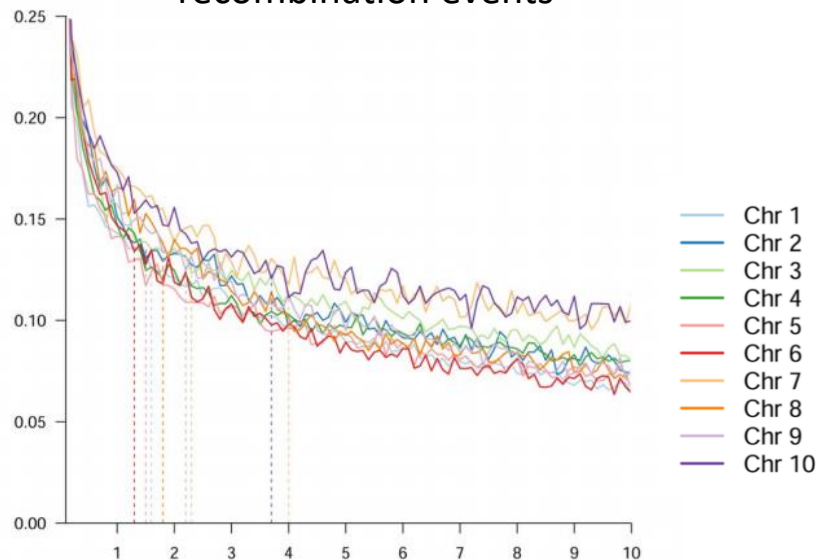
# Characteristics of the MAGIC population

- The MAGIC population is **highly diverse**
- Very **low genomic structure**, higher in centromeric regions



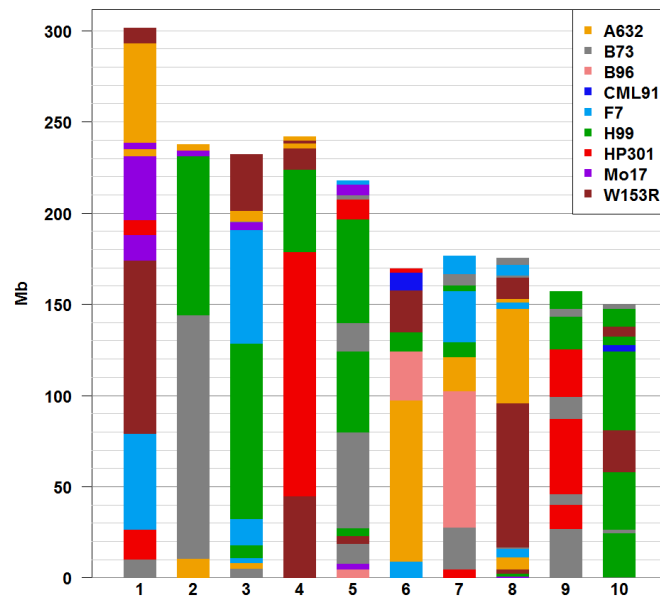
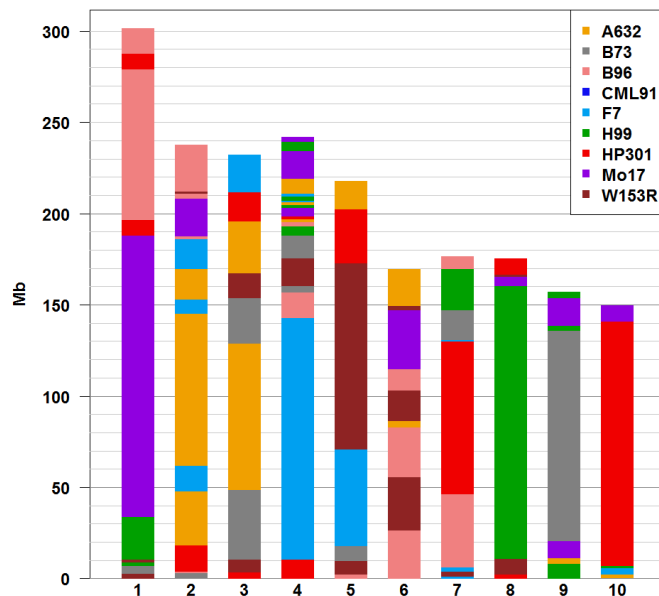
Telomeric regions don't show structure in relation to founders lines

- **LD decays fast**, indicating high density recombination events



# RIL genomes

RIL genomes are reconstructed via **hidden Markov model (HMM)** and founder haplotypes are assigned to each line accordingly



# Genomic data available

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DNA



**Whole genome sequencing** produced for:

A632, F7, H99, HP301, and W153R (Mo17 and B73 were already available)



RNA

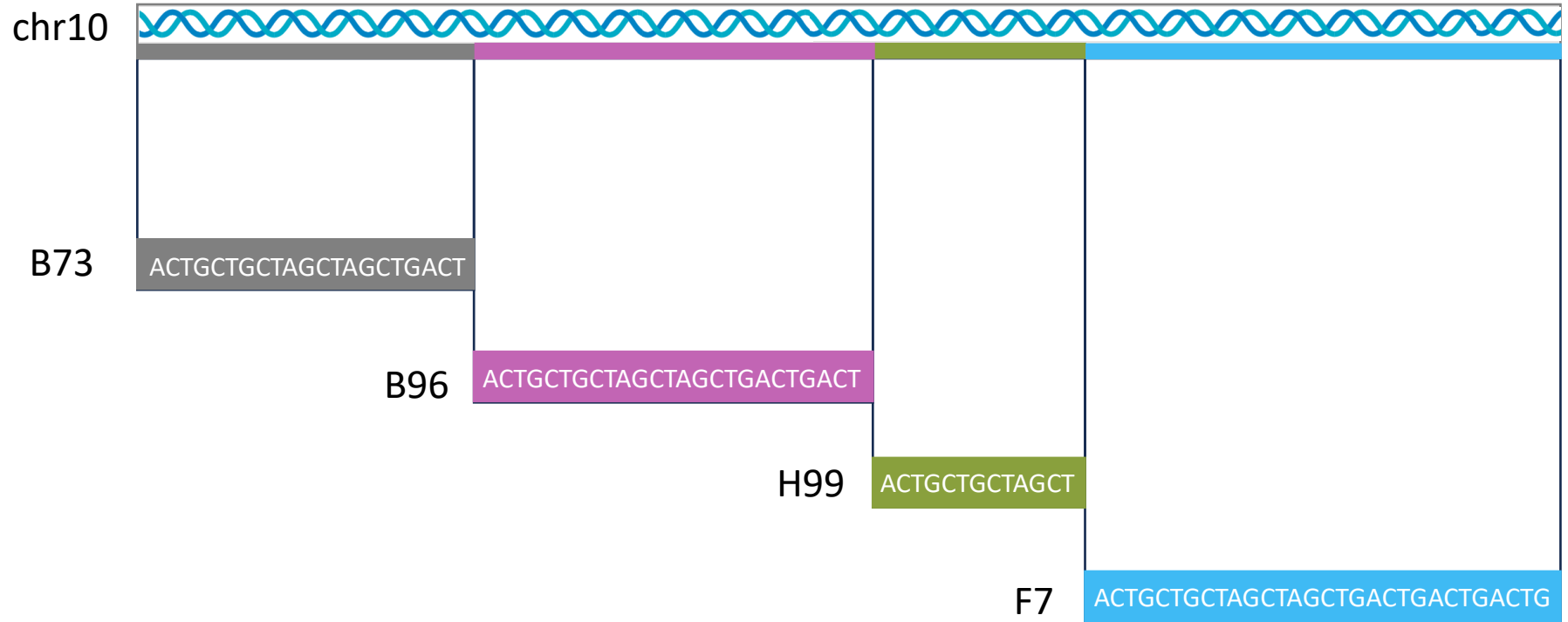


**Transcriptomics** data at the fourth leaf stage were produced for:

A632, B73, F7, H99, HP301, Mo17, W153R

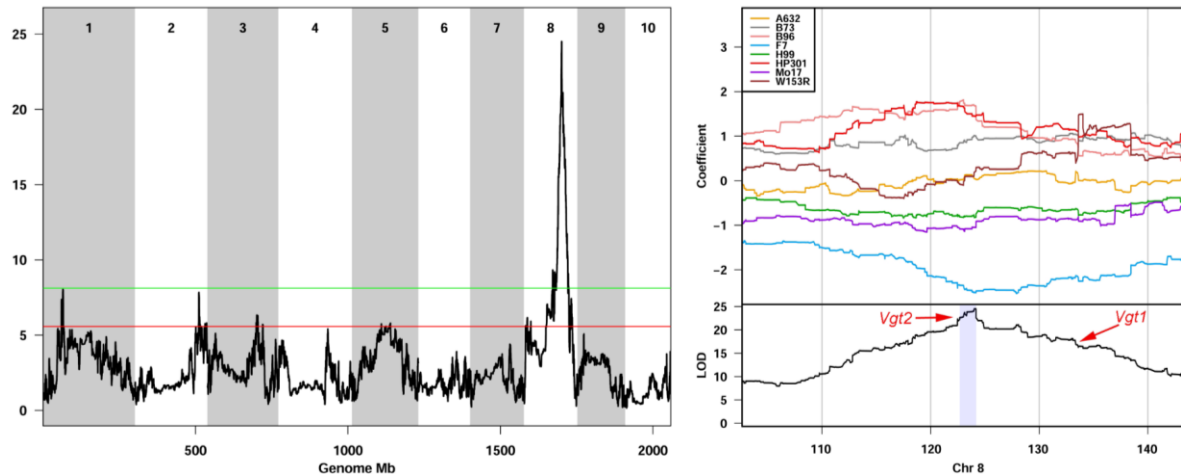


# We can project this information on RIL reconstructed genomes



# MAGIC maize as platform for QTL mapping

- QTL mapping can then be performed testing co-inheritance of **parental haplotypes** and **measured traits**
- Once a QTL is identified, the local effect of founder haplotypes can be estimated accordingly
- Data generated on parental lines (DNA seq, RNA seq, metabolomics, proteomics, ...) can be **projected on parental haplotypes**



# Current work on the MAGIC maize

- Broad, untargeted phenotypic diversity
- Oxford Nanopore Technology pangenome of parental lines
- Reconstruction of pan-transcriptome
- Integration of structural variation in forward genetics



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# Application of the MAGIC Maize Population to Uncover the Genetic Determinants of Drought Tolerance



# Maize and drought



**Global crop:** staple food, feed and bio-based products



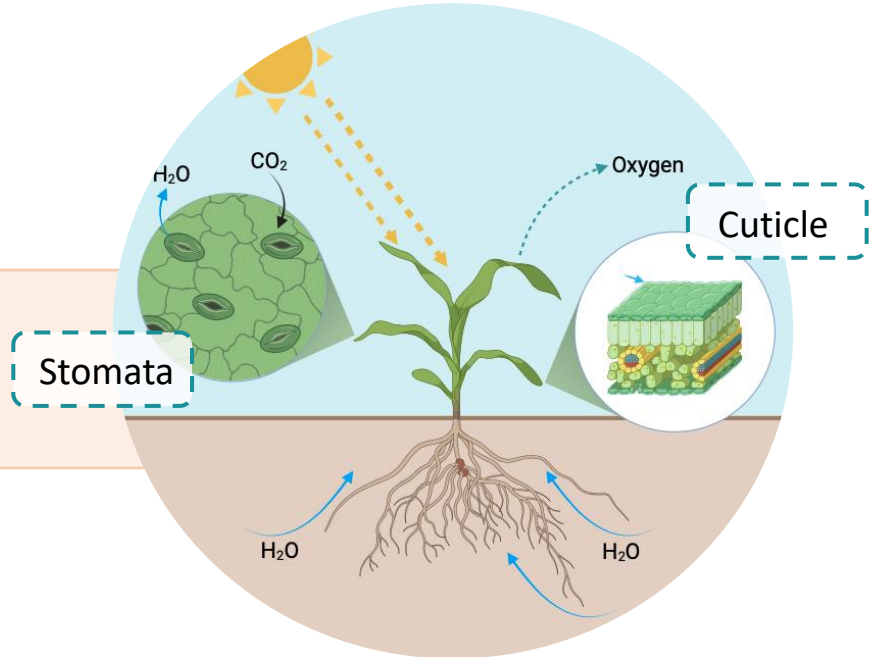
**Genetic model:** study of complex traits



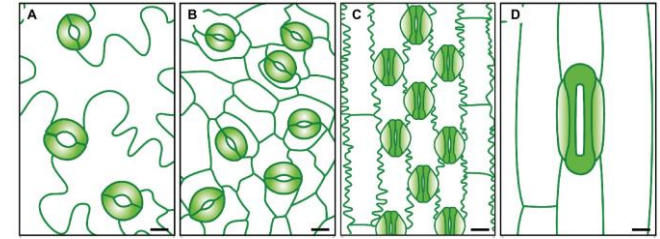
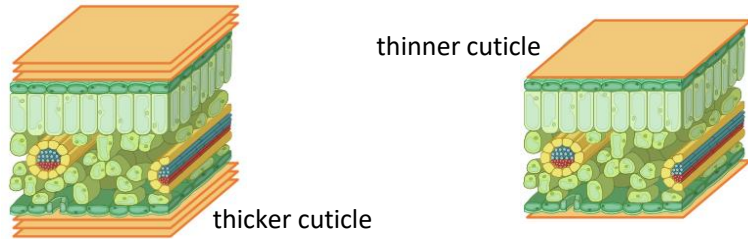
**Drought impact:** up to 15% global yield loss with effects on several agronomic traits

How can maize cope  
with this challenging  
environment?

Physiological and  
structural components  
traits of adaptation



# Cuticle and stomata



Bertolino LT et al. 2019

**Cuticle:** multi-layered hydrophobic structure **limiting** **water loss** and **protecting against dehydration**

**Cutin** and **cuticular waxes:** fatty acids

**Stomata:** tiny pores on leaf surface that **regulate gas exchange** and **minimise water loss**

**Stomatal Density:** n° of stomata / unit area (stomata/mm<sup>2</sup>)

**Affecting leaf permeability:** thicker cuticle and decreased stomatal density can **reduce the leaf permeability** and the water loss



Working hypothesis: **natural allelic variation** in maize for cuticular and stomatal traits can be used **to identify** **potential candidate genes** that are crucial for developing **maize varieties more adapted to drought**

# The Multi-parent Advanced Generation Inter-Cross (MAGIC) maize population

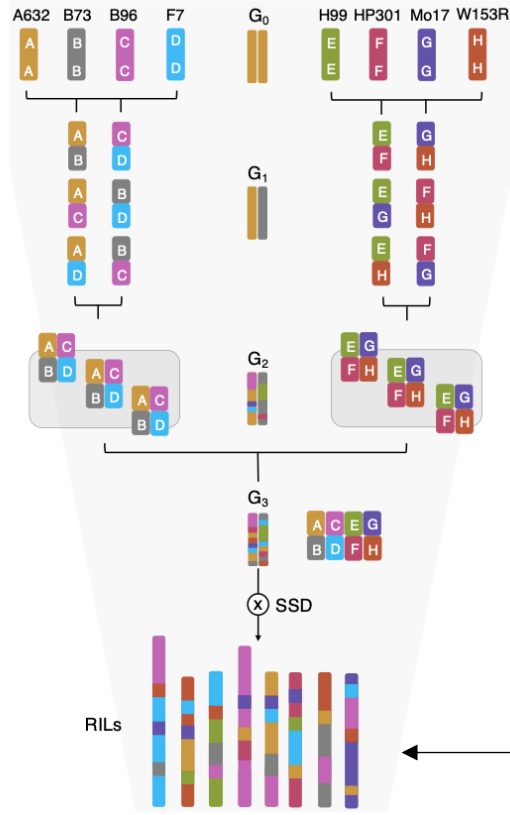


Fig. Breeding funnel of the MAGIC maize population (Dell'Acqua et al., 2015)

Multi-parent population developed following a MAGIC crossing scheme inter-crossing **8 diverse lines** (founders)



Fig. Founders lines of MAGIC population

After 6 generations: 1,600 Recombinant Inbred Lines (RILs)

Each RIL is a **mosaic of the founders' genomes**

➡ High genetic diversity, high recombination density



# The Multi-parent Advanced Generation Inter-Cross (MAGIC) maize population

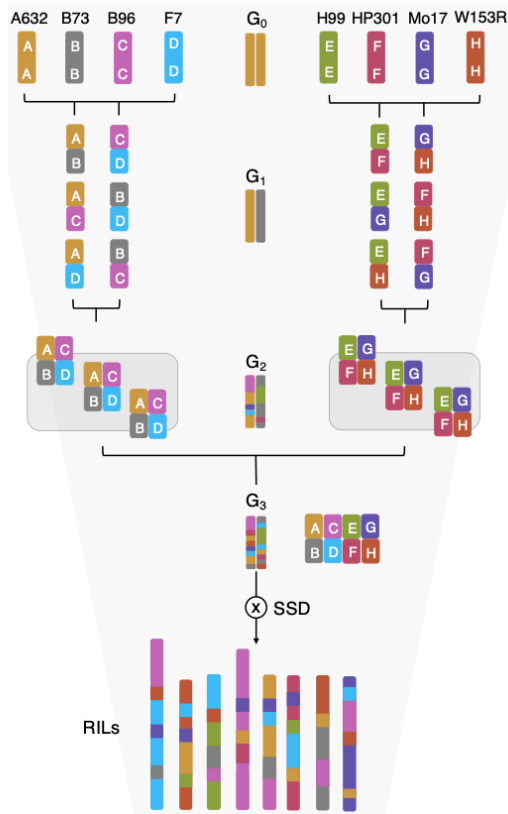


Fig. Breeding funnel of the MAGIC maize population (Dell'Acqua et al., 2015)

## Data used in the experiment:

- ✓ 285 RILs
- ✓ 75,000 SNPs markers from sequencing
- ✓ Oxford Nanopore Technology **pangenome** of founders
- ✓ **RNA-seq** in the growth chamber and in the field of founders and RILs

Data further available: 240 more RILs characterised



Fig. Founders lines of MAGIC population



Fig. Seed material of MAGIC population

# Experiment in greenhouse October-December 2024

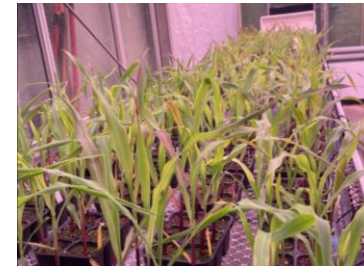
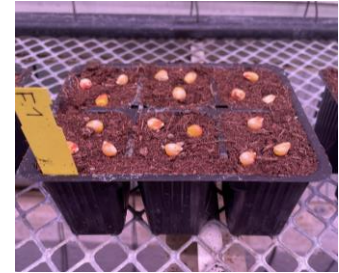
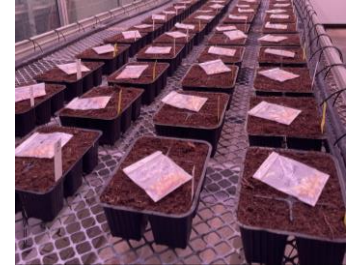
**285 RILs + 8 founders = 293 genotypes**

Up to 6 replicates for each genotype

**Augmented block design:** 4 batches with 4 blocks each one

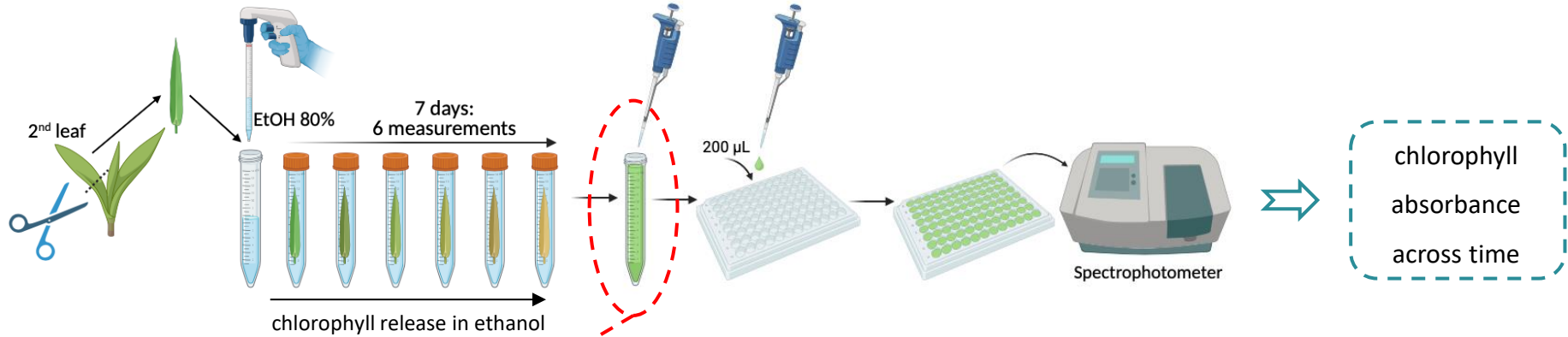
Environmental controlled conditions

BATCH 1	Block 4	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
	Block 3	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
	Block 2	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
	Block 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

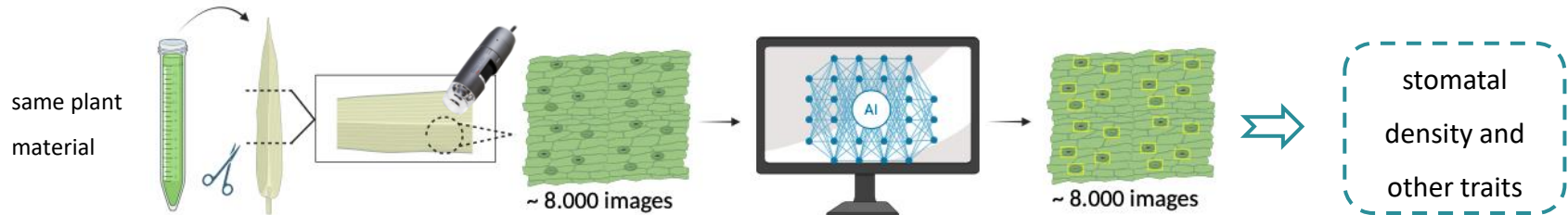


# Cuticle-stomata high-throughput phenotyping pipeline

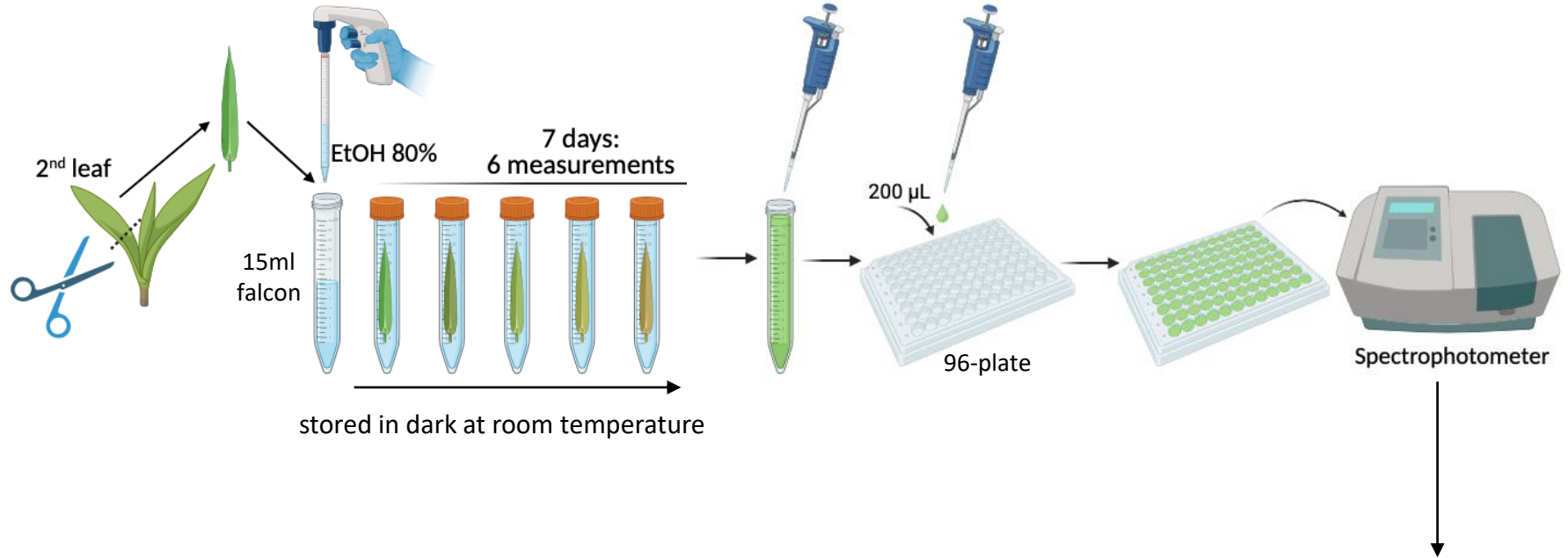
## ① Cuticle permeability by chlorophyll leaching assay:



## ② Imaging and stomata detection:



# Cuticle permeability by chlorophyll leaching assay:



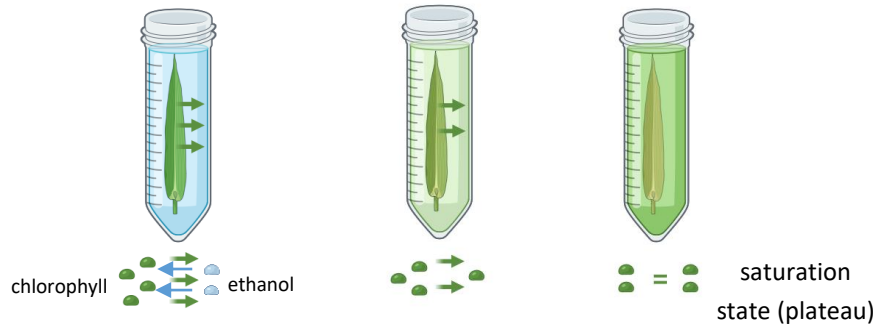
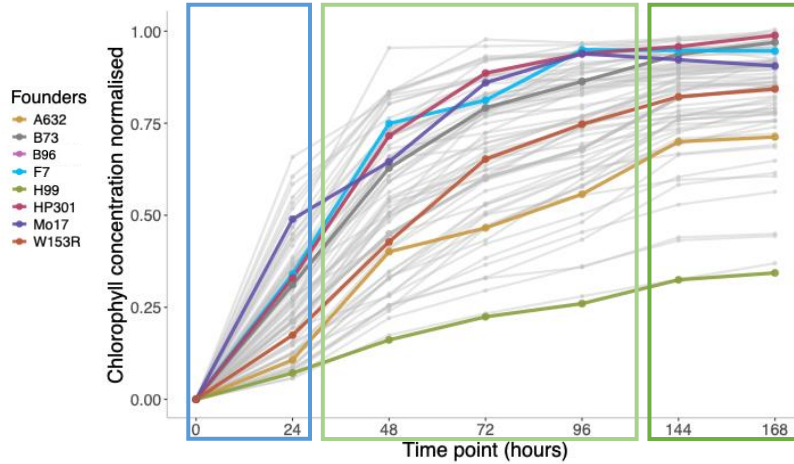
normalised per gram of  
fresh weight

$$\text{chlorophyll concentration } (\mu\text{M}) = (7.93 * A_{664}) + (19.53 * A_{647})$$

absorbance 664 nm  
absorbance 647 nm

# Cuticle permeability

Rate of the chlorophyll release:



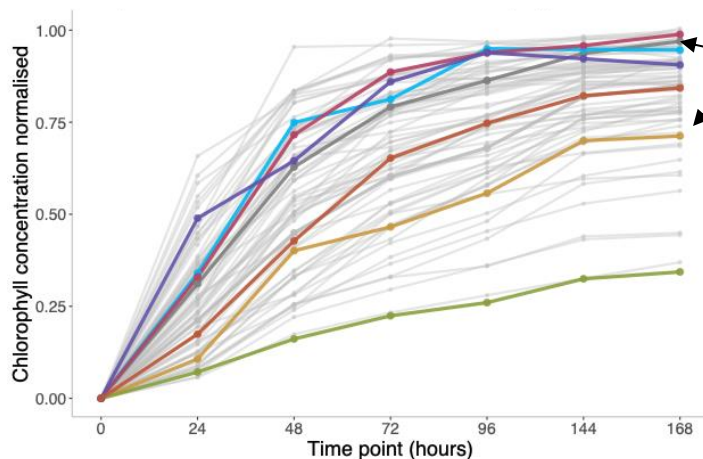
# Cuticle permeability

Rate of the chlorophyll release:

$$C(t) = C_{\max} \times (1 - e^{-k \times t})$$

**C<sub>max</sub>** = maximum chlorophyll concentration released  
How much chlorophyll a genotype can release

**k** = rate constant  
How fast a genotype releases chlorophyll



↓

$$\text{Cuticle leaf permeability} = \text{Speed}(t) = \frac{dC(t)}{dt} = C_{\max} \times k \times e^{-k \times t}$$

**Speed at different time points** → how fast different genotypes release chlorophyll over time

→ Traits for **QTL mapping**



# Deriving the trait(s) for mapping



Extraction of  $k$  and  $C_{max}$  for each genotype

Fitting a **non-linear mixed effects model**:

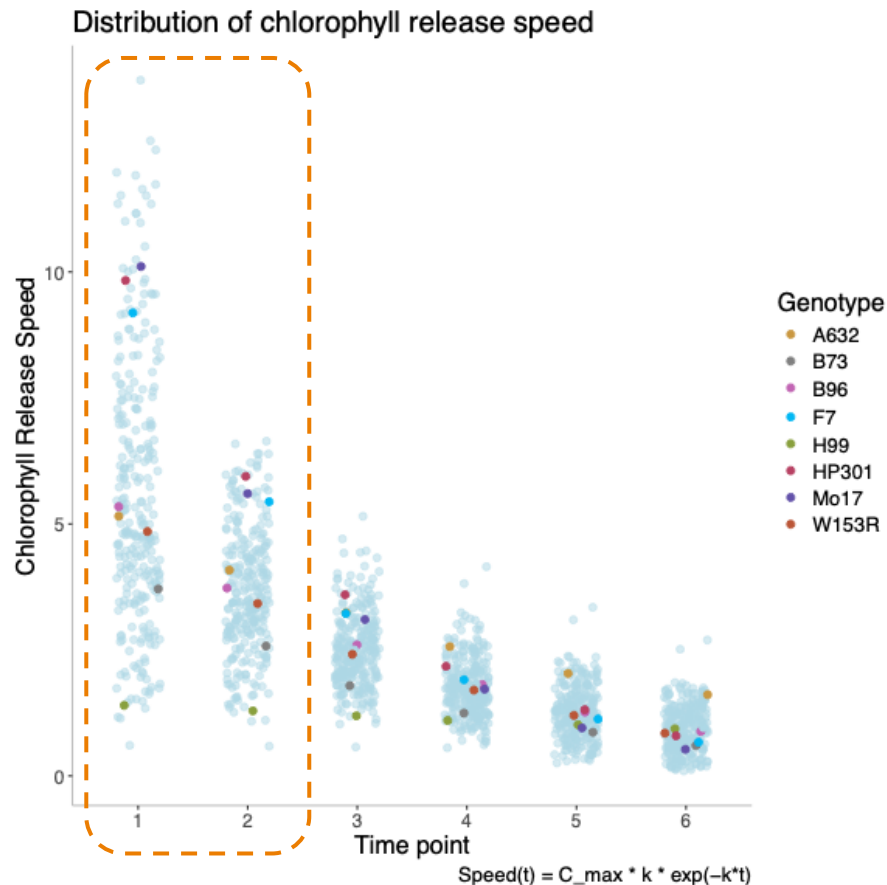
- fixed effects =  $C_{max}$  and  $k$
- random effect = genotype



**Speed** of release of chlorophyll across timepoints  $\rightarrow t = \text{from } 0 \text{ to } 6$

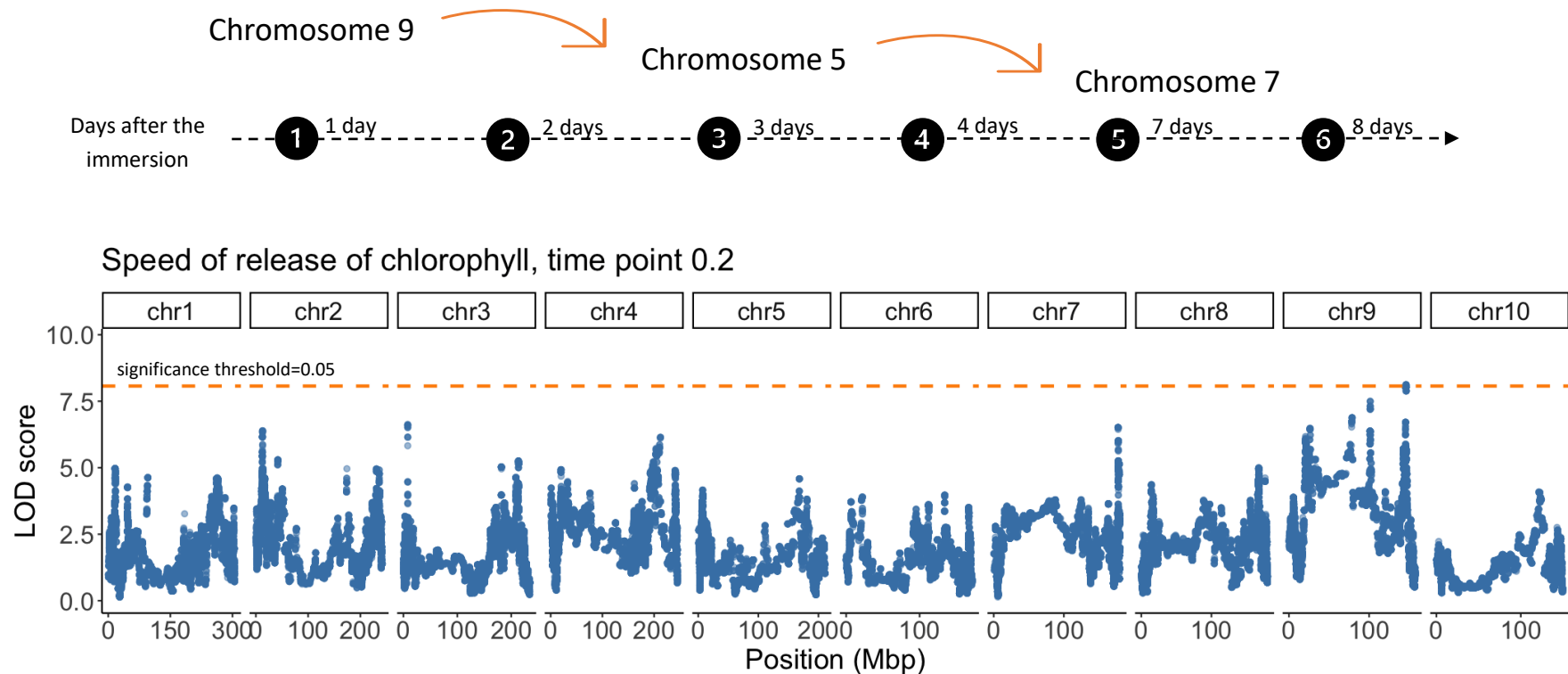
Traits for mapping:

- $k$
- $C_{max}$
- Speed of chlorophyll release for 6 timepoints





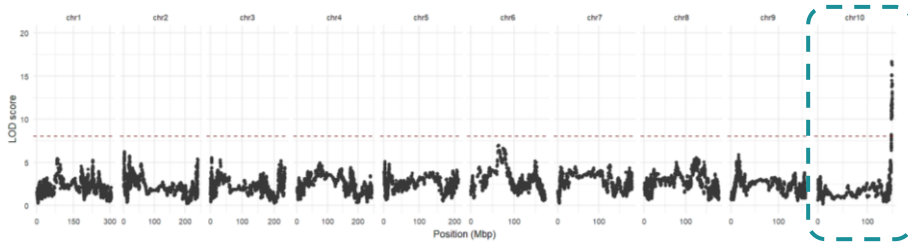
# QTL mapping



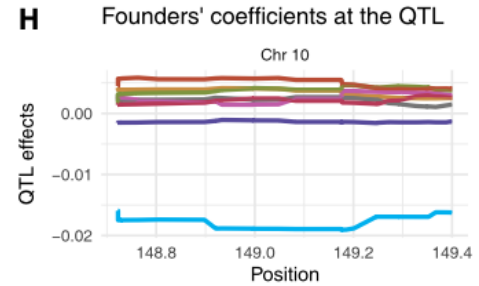
**Genotypic data:** 75,000 SNPs

**Covariate:** initial chlorophyll concentration inside the leaf

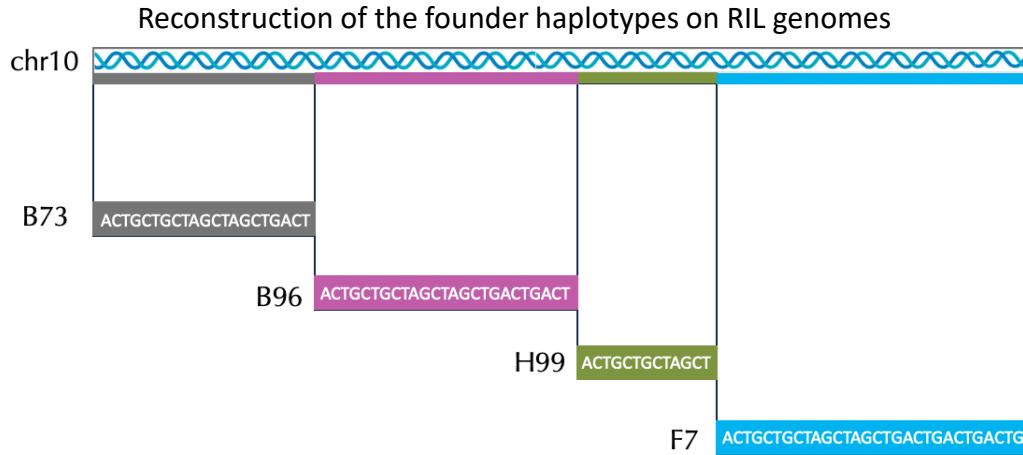
# Identification of candidate genes



(1) For each peak of each QTL detected

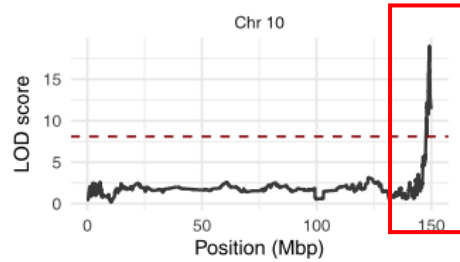


(2) Calculate founder allele effects on QTL

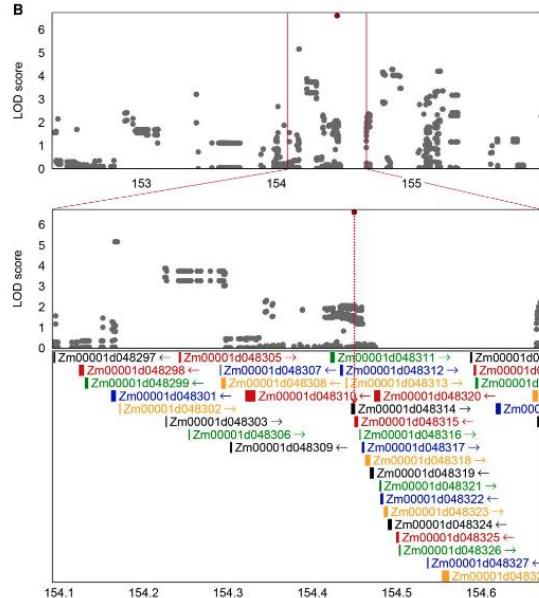


(3) Cluster founders by similarity of the effects (haplotype groups)

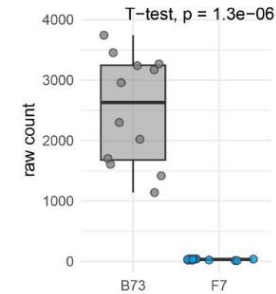
# Identification of candidate genes



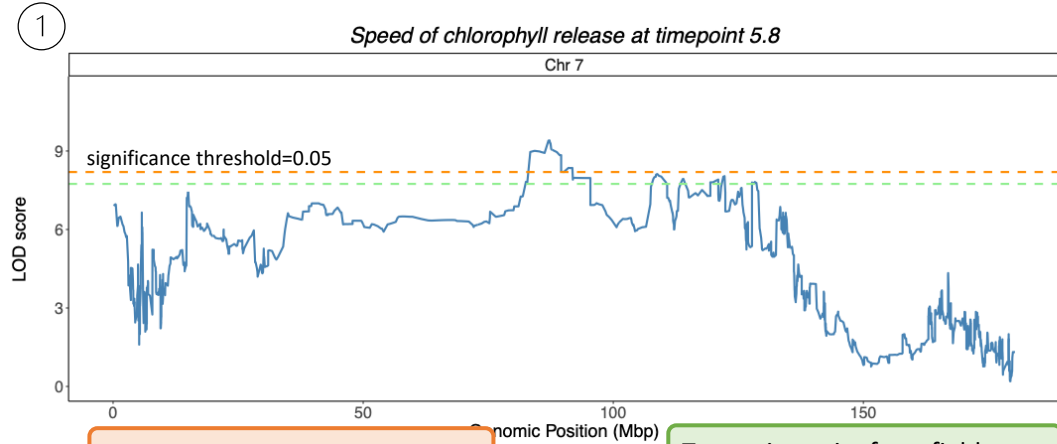
(4) Extract genes in the QTL interval



(5) Integration of RNA-seq data to test  
the genes for differential expression  
matching founder effects



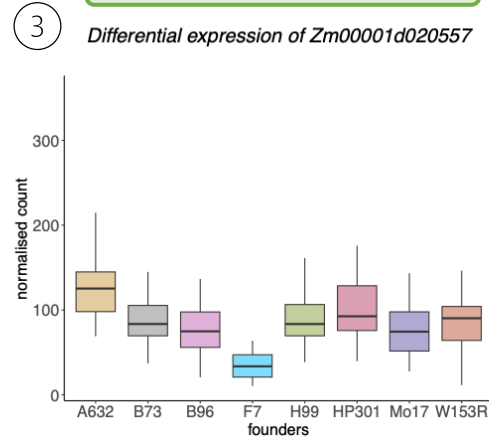
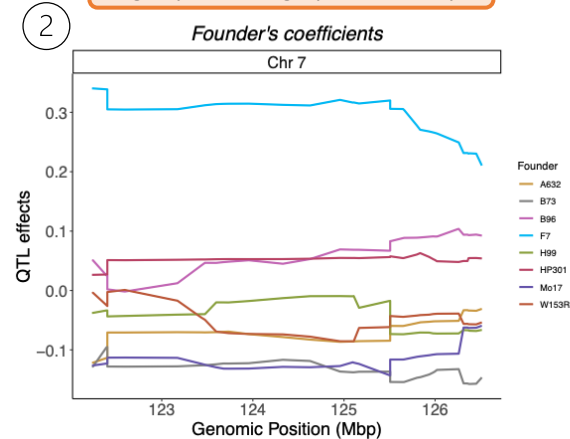
# Candidate gene on chr7



From 135 genes on this QTL to 2 candidates

*glossy1 - gl1*

Effects on the deposition  
of cuticular waxes on the  
surface of seedling leaves



*Plant Physiology*®

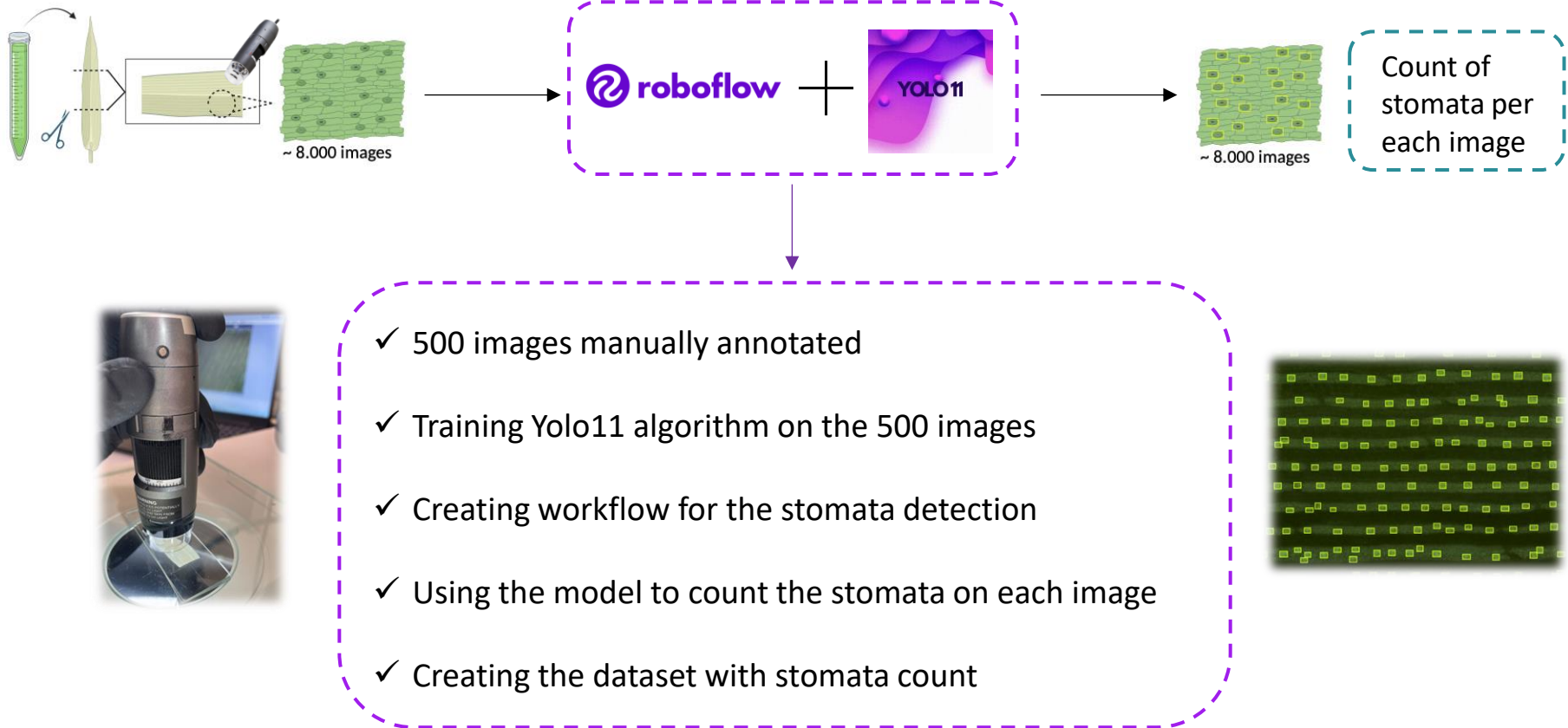
JOURNAL ARTICLE

**Cloning and Characterization of *GLOSSY1*, a Maize Gene Involved in Cuticle Membrane and Wax Production**

[Get access](#)

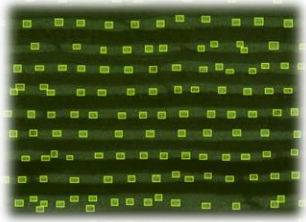
Monica Sturaro, Hans Hartings, Elmon Schmelzer, Riccardo Velasco, Francesco Salamini, Mario Motto

# Stomata imaging and detection with AI

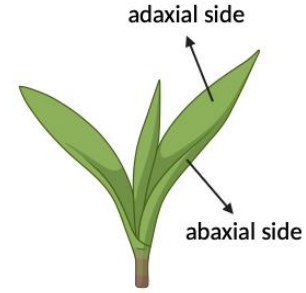




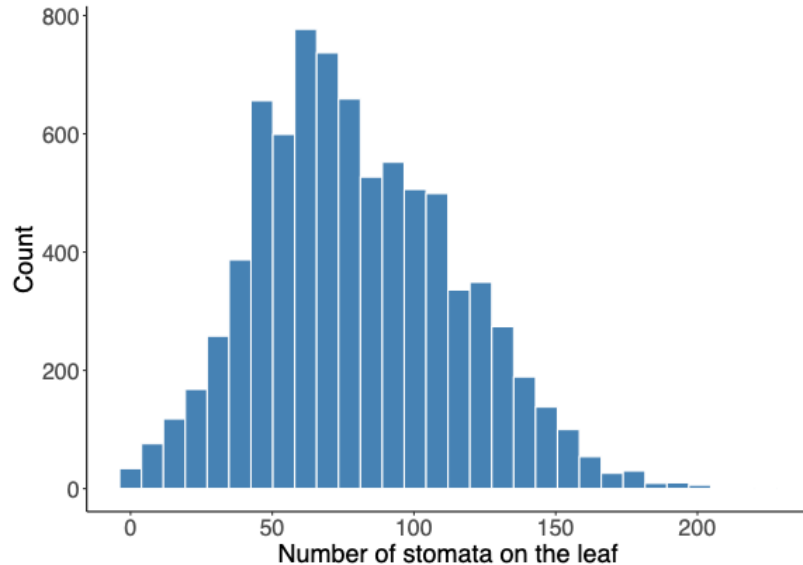
# Stomata imaging and detection with AI



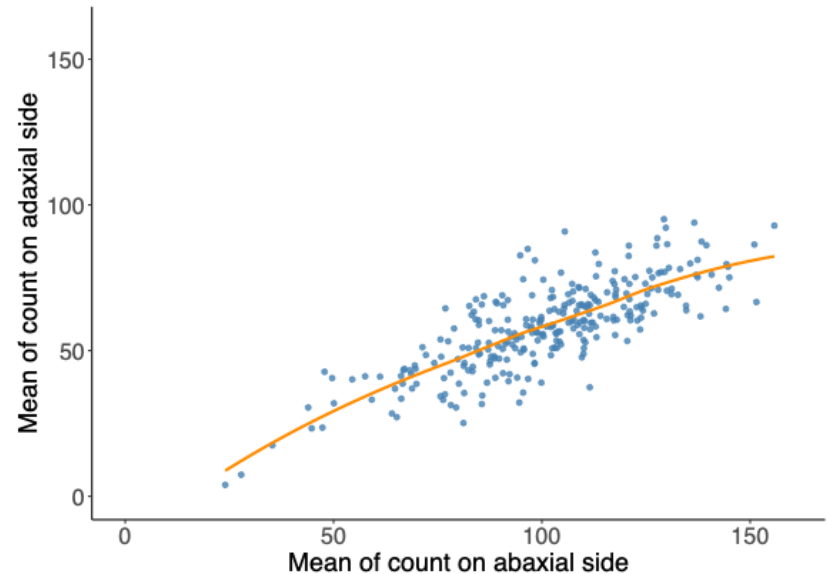
Count of  
stomata per  
each image



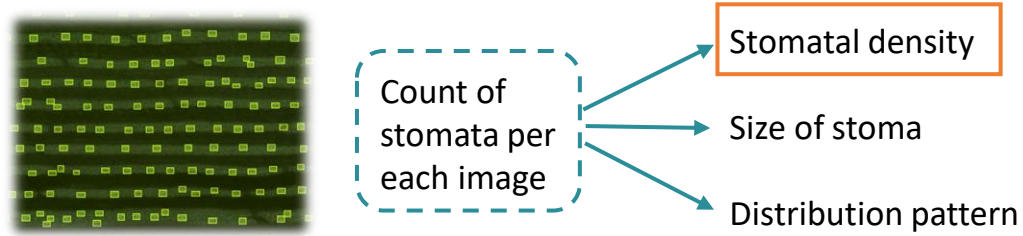
*Distribution of stomata count on the leaf*



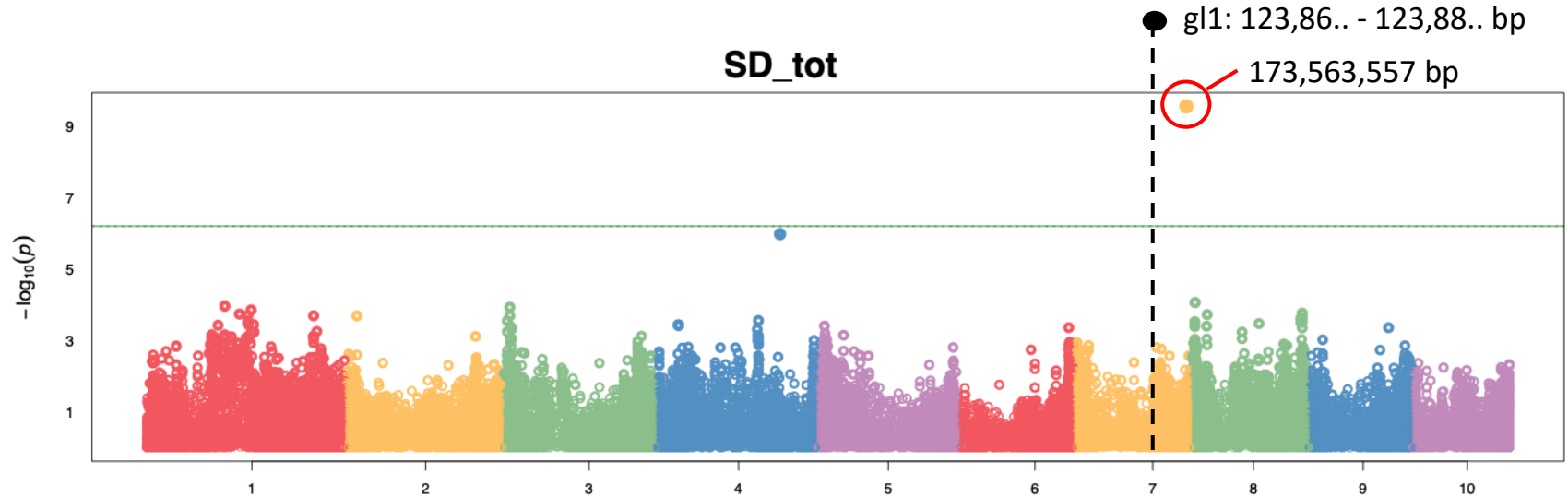
*Correlation between stomata count on abaxial and adaxial side*



# Preliminary results on stomatal density



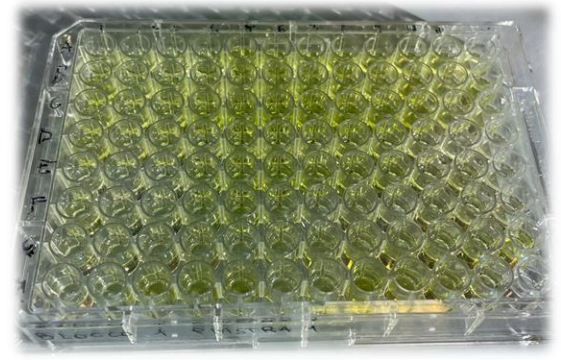
➡ **GWAS** on stomatal density (75,000 SNPs):





# Next steps

- ✓ Improvement of the model through **machine learning approaches** to derive other stomatal traits as size and distribution pattern
- ✓ **QTL mapping** of SD and other stomatal traits
- ✓ Integration of the **pangenome** data on other candidate genes
- ✓ Consolidation of the candidate genes through ***Arabidopsis thaliana* mutants**
- ✓ Test the effective **drought tolerance** on the MAGIC population



# Relevance for breeding

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Identifying the ***glossy1* gene** and other **candidate genes** could guide research on developing **new maize varieties** that have **lower permeability** and are **more drought-tolerant** through gene editing or molecular breeding techniques

## Take home messages:

- Modern breeding requires genomics + genetics + diversity
- Multi-parental populations are a powerful and enduring platform to combine agrobiodiversity and big data to support candidate gene mapping

# Acknowledgments



TRANSLATIONAL  
PLANT GENOMICS

Sant'Anna  
Scuola Universitaria Superiore Pisa

Martina Pallaoro  
...and the other colleagues!



Dr. Leonardo Caproni

Dr. Afewerki Yohannes Kiros

Prof. Matteo Dell'Acqua



Check our website!



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Answer&Question

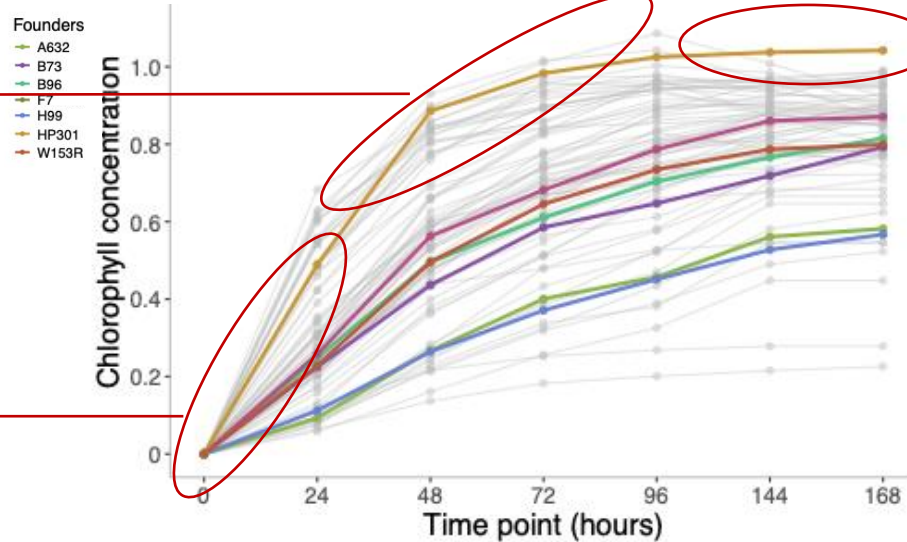
# Deriving the kinetics of the chlorophyll release:

The curve of the chlorophyll release is a **kinetics of a release** and follows a **non-linear model**

$$\text{Speed}(t) = \frac{dC(t)}{dt} = C_{\max} \times k \times e^{-k \times t}$$

As  $t$  increases, the speed decreases because of  $e^{-kt}$

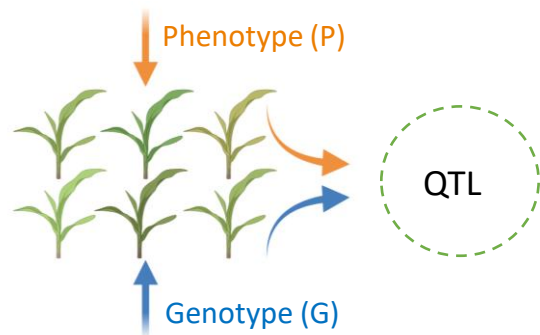
$0 < t < 1$ , speed is highest



At very large  $t$ , the speed approaches zero: no more chlorophyll is being released

Calculating the speed at different time points → **how fast different genotypes release chlorophyll over time**

# Quantitative Trait Loci (QTL) mapping



**Genotype:** 79,000 SNPs (founder and RILs)

**Phenotype:** k, Cmax, speed of release t(1-6)

**Environment:** controlled conditions

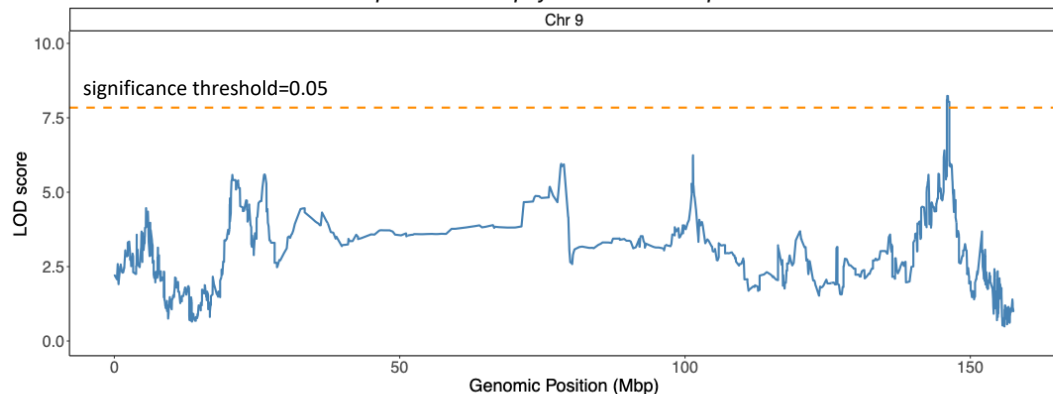
Peaks on different chromosomes

lodcolumn	chr	pos	lod	ci_lo	ci_hi
k	9	20.524041	7.83446998796123	20.173237	23.636085
k	9	26.23272	8.67548984873511	25.252052	26.661695
k	9	78.774982	9.87153723916841	32.620035	79.552722
k	9	101.377009	8.39956209475854	100.458234	146.070386
V_release_T0.4	9	146.066907	8.19872894141161	20.448291	146.766055
V_release_T0.6	9	146.066907	8.24842817826664	20.677708	146.759044
V_release_T0.8	9	146.066907	8.25744246004469	78.303794	146.432785
V_release_T1	9	146.066907	8.21238687538926	145.890735	146.325138
V_release_T1.2	9	146.066907	8.09810728512475	145.890735	146.325138
V_release_T1.4	9	146.066907	7.89884451773214	145.890735	146.325138
V_release_T3	5	50.23289	8.2478674937375	45.070079	60.080864
V_release_T3.2	5	50.23289	8.47364612208466	45.070079	59.686499
V_release_T3.4	5	50.23289	8.48430354022341	45.070079	55.894742
V_release_T3.6	5	50.451209	8.32292436168174	45.070079	55.894742
V_release_T5.4	7	87.03816	8.65052897370506	0.110398	128.990996
V_release_T5.6	7	87.03816	9.04252685501592	15.005919	128.990996
V_release_T5.8	7	87.249434	9.40556410694887	82.537038	128.614711
V_release_T6	7	87.249434	9.7347113666752	83.408208	93.518512
V_release_T6	7	108.770254	8.40832046175901	107.532652	110.984314
V_release_T6	7	122.234996	8.30564508528347	113.103042	128.990996



# Candidate gene on chr9

Speed of chlorophyll release at timepoint1

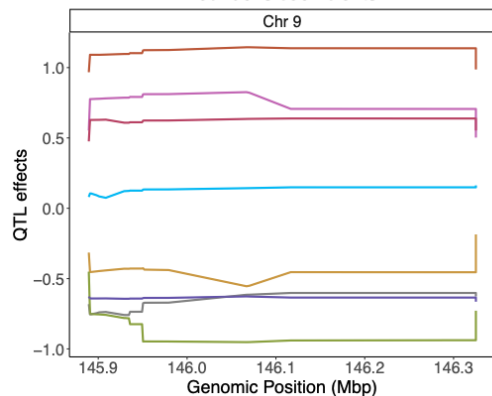


From 16 genes on this QTL to 1 candidate

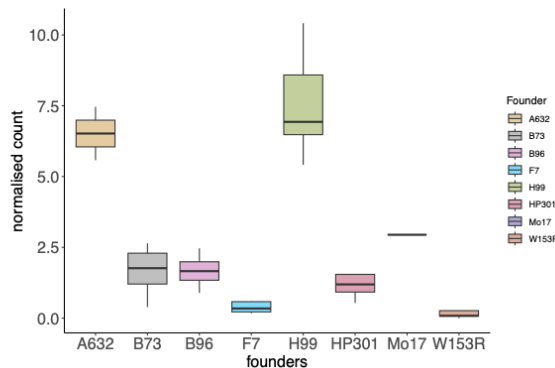
## Actin-depolymerizing factor 5

ADF5 involved stomatal closure and drought stress response (*A. thaliana*)

Founder's coefficients



Differential expression of Zm00001d047942



High speed=high permeability

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doi:10.1093/jxb/ery385 Advance Access publication 24 November 2018  
This paper is available online free of all access charges (see <https://academic.oup.com/jeb/pages/openaccess> for further details)

### RESEARCH PAPER

***Arabidopsis* ADF5 promotes stomatal closure by regulating actin cytoskeleton remodeling in response to ABA and drought stress**

Dong Qian<sup>1</sup>, Zhe Zhang<sup>1</sup>, Juanxia He<sup>1</sup>, Pan Zhang<sup>1</sup>, Xiaobin Ou<sup>2</sup>, Tian Li<sup>1</sup>, Lipan Niu<sup>1</sup>, Qiong Nan<sup>1</sup>, Yue Niu<sup>1</sup>, Wenliang He<sup>1</sup>, Lizhe An<sup>1</sup>, Kun Jiang<sup>2</sup>, and Yun Xiang<sup>1\*</sup>

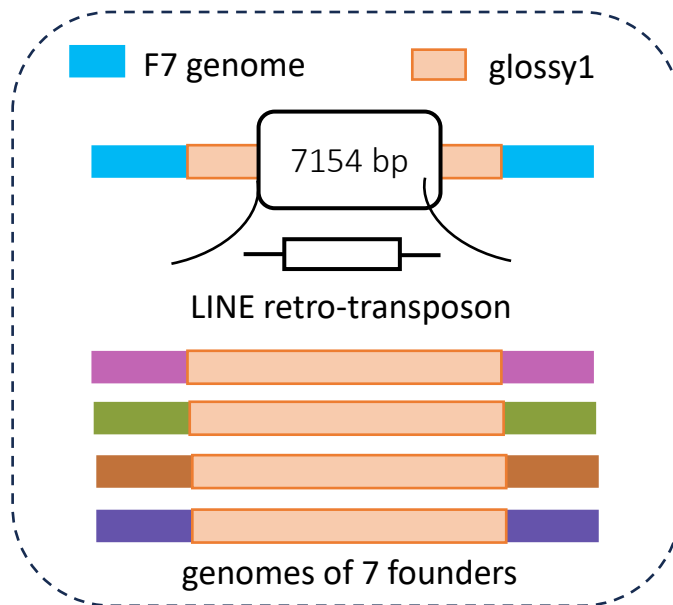
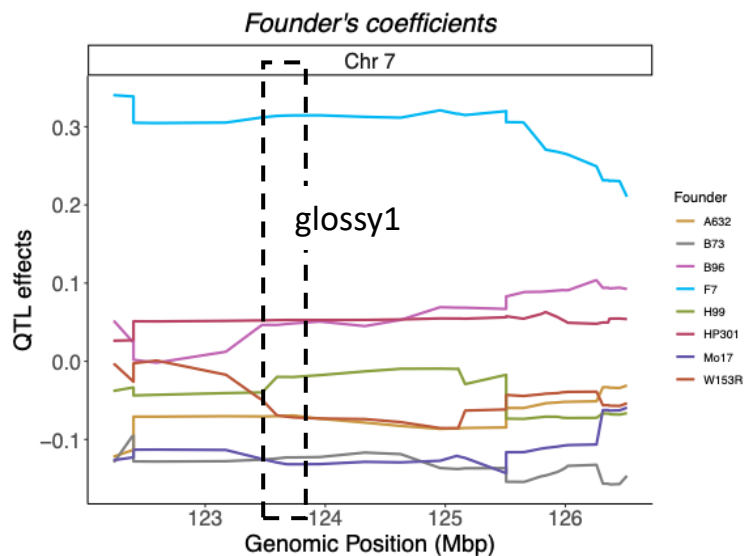


# Pangenome of MAGIC founders

**De novo genome assemblies:** ONT long reads (poster 1.25)

Pairwise alignment of each founder on F7: **unique F7 insertion** identified

TE annotation of the pangenome (PanETDA) : **LINE retro-transposon**





# Stomata detection



- ✓ 500 images manually annotated (+ oriented annotation)
- ✓ Training Yolo11 algorithm on the 500 images
- ✓ Creating workflow for the stomata detection
- ✓ Using the model to count the stomata on each image
- ✓ Creating the dataset with stomata count