# Deliverable 1.1 Report

A core part of the construction of the pancistrome is to analyze relative haplotype-specific variation in global transcription factor (TF) binding affinity in maize and teff F1 hybrids pangenome-wide. The goal is to detect a potential association between genetic/epi-genetic variation and TF binding affinity to generate a high-resolution map of functional variation in cis-element function and hence the location of cis-regulatory loci pangenome-wide (pancistrome.

The focus goal of deliverable D1.1 was to establish a protocol to analyze the cis-element occupancy in the maize and teff F1 hybrids and generate the pancistrome in later stages of the project

## Generation of maize germplasm

Maize F1 hybrids involving the European germplasm of the SeqOccIn population were produced during the 2023 Field season by both UDUS and CREA. Multiple crossing schemes were generated including a tester (nested omni-hybrid approach with a single shared mother genotype) and a variation of a double round-robin design (nested F1 hybrids with mixed crosses that share haplotypes across multiple crosses). The tester design allows direct relative interpretation of haplotype-specific TF footprints measured through MOA-seq to maximize the power to detect cis-acting genetic variants with a simplified population structure. On the other hand, the double round-robin design was chosen as it maximizes genetic diversity that can be captured with fewer crosses at the cost of more genetic and bioinformatic analysis complexity.

1st crossing scheme: Oh43 (drought tolerant tester) as a common mother (UDUS)

31 F1 hybrids with sufficient seeds for further analysis (>150 seeds) were generated:

Tester design crosses performed:

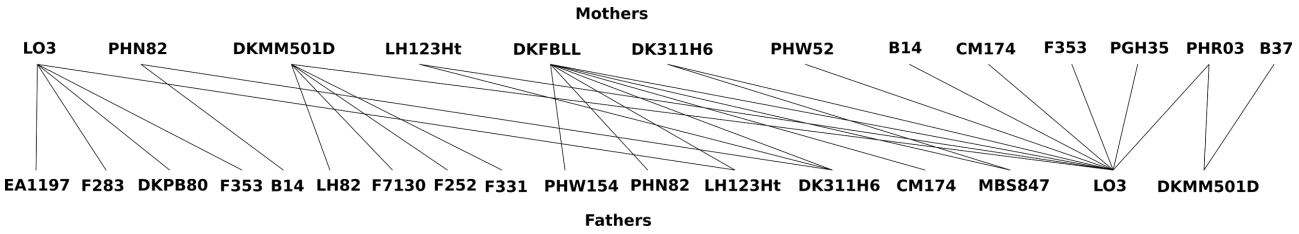
| **Mother** | | **Father** | **Source Population** |
|---|---|---|---|
| | | | |

| | | | |
|---|---|---|---|
| Oh43 | x | A632 | SeqOccIn |
| Oh43 | x | B14 | SeqOccIn |
| Oh43 | x | B37 | SeqOccIn |
| Oh43 | x | CM174 | SeqOccIn |
| Oh43 | x | CO255 | SeqOccIn |
| Oh43 | x | DK311H6 | SeqOccIn |
| Oh43 | x | DKFBLL | SeqOccIn |
| Oh43 | x | DKMM501D | SeqOccIn |
| Oh43 | x | DKPB80 | SeqOccIn |
| Oh43 | x | EM1197 | SeqOccIn |
| Oh43 | x | F120 | SeqOccIn |
| Oh43 | x | F7130 | SeqOccIn |
| Oh43 | x | FV252 | SeqOccIn |
| Oh43 | x | FV283 | SeqOccIn |
| Oh43 | x | FV331 | SeqOccIn |
| Oh43 | x | FV353 | SeqOccIn |
| Oh43 | x | FV4 | SeqOccIn |
| Oh43 | x | GR3 | SeqOccIn |
| Oh43 | x | LH123Ht | SeqOccIn |
| Oh43 | x | LH244 | SeqOccIn |
| Oh43 | x | LH82 | SeqOccIn |
| Oh43 | x | LO3 | SeqOccIn |

| | | | |
|---|---|---|---|
| Oh43 | x | MBS847 | SeqOccIn |
| Oh43 | x | PHG35 | SeqOccIn |
| Oh43 | x | PHN82 | SeqOccIn |
| Oh43 | x | PHPO2 | SeqOccIn |
| Oh43 | x | PHRO3 | SeqOccIn |
| Oh43 | x | PHW52 | SeqOccIn |
| Oh43 | x | CML228 | US NAM |
| Oh43 | x | CML52 | US NAM |

2nd crossing scheme: Double Round Robin (variant) crossing scheme combinations of SeqOccIN lines (CREA)

31 F1 hybrids with sufficient seeds for further analysis (>150 seeds) were generated:




## Optimized protocol for maize F1 hybrid population for haplotype-specific MOA/mRNA-seq

The next goal was to optimize growth conditions for the maize F1 hybrids to allow optimal conditions for a later harvest and analysis of haplotype-specific TF footprint across the population.

Given the new population of F1 hybrids and our current gap in understanding the range of variation in growth and developmental timing the maize F1 hybrid populations may have,

we decided to focus on finding a protocol that would maximize suitability drought conditions for most F1 lines. After preliminary tests of pot sizes and the required growth stage to dry pots quickly, we therefore decided to test all F1 lines available in a large test trial, in favor of a limited number of F1 hybrids with more time points.

The following protocol was established after an initial test trial for the 31 hybrids of tester design crosses. Seeds of the double round robin cross design were not available in time for the condition trial experiment.

Maize drought

1. Pre-germination of seeds to ensure equal development and even numbers of plants in each pot:

- sterilize seeds in bleach (14% Hypochlorid) for 2.5 min, rinse with plenty of water
- place seeds on a wet paper towel in a closed container and germinate for 2.5 days at 28 degrees

2. Sowing of pre-germinated seeds

- use 2L round pots evenly filled with “Einheits Erde type ED 73” soil
- place 4 seeds in each pot, seven or 9 pots per hybrid (resulting in 3 (pilot) or 4 (final) pots per treatment)
- distribute pots in randomized block design, keep one spare pot for each hybrid on the side

3. Maintenance of plants

- plants are automatically watered for 5 minutes every day with fertilizer solution

4. Drought treatment

- start drought treatment the day after >75% of plants of a hybrid have reached the V4 stage

- pots are randomly assigned to well-watered (WW) or drought (DS) treatment and placed on separate benches
- well-watered plants are continuously watered each day with rain water (no fertiliser) while water is completely withheld for DS plants

5. Collection of material

- after 113h of water withholding, plants are cut above the roots, whole weight determined for all plants per pot, leaf blades of the oldest leaf without on auricle are harvested for molecular analysis and a 3 cm piece of rolled leaves from each plant is collected for relative water content measurements (Fig. 1).


**Figure 1: Illustration of harvesting procedure for WW and DS samples for the F1 maize hybrids.** All four plants in a pot are cut above the soil (red lines symbolize cuts), weighed for the whole plant weight, and then dissected further. One cut is placed at the last auricle and the oldest leaf that has not yet developed an auricle is harvested, the midvein is removed and the material is frozen for molecular analysis. For relative water content (RWC) measurements, a 3 cm piece of rolled leaves is cut from between leaf 3 and leaf 4.

**Results of the maize hybrid tester design trial experiment to identify optimal growth conditions:**

A pilot experiment was performed with all 31 Oh43 tester hybrids generated by UDUS. Water was withheld for 113hs, until visible drought responses appeared in the first hybrids. In terms of the relative water content (RWC), a broad range of drought responses is observed with the best-performing hybrids showing no difference in RWC and the most affected lines showing a reduction of about 35 percentage points (Fig. 2a). The soil water content (SWC) demonstrates that there was a strong reduction in soil water, but with slightly lower values for hybrids showing a stronger reduction in RWC (Fig. 2b). This suggests that at least for some hybrids the transpiration rate might contribute to the drought condition development. The fresh weight of four plants is reduced in most hybrids in drought conditions (Fig. 2c). The WW fresh weight is variable among hybrids but for the three most drought-susceptible lines a correlation between higher biomass and stronger drought symptoms (lower RWC) is indicated by the data. Both of these parameters (RWC and SWC) were in line with our requirements of an optimal window to analyze natural variation in the transcriptional drought response published previously, including natural variation with the DREB TF family in Liu et al., 2013 and Weng et al., 2020 for VPP1.

To test the drought response on a molecular level, we analyzed the mRNA levels of three genes shown to be transcriptionally regulated in response to drought conditions (Fig. 3) in five hybrids across the RWC spectrum. While the most drought-tolerant line according to RWC, DKMM501D, shows almost no upregulation of the analyzed genes under drought stress, already the second most tolerant line, LH82 shows some elevation of mRNA abundance under drought. This elevation is more pronounced the more differences in RWC are observed. Thus, the tested drought conditions are suitable to observe a broad drought response, both on the level of RWC and molecular level.

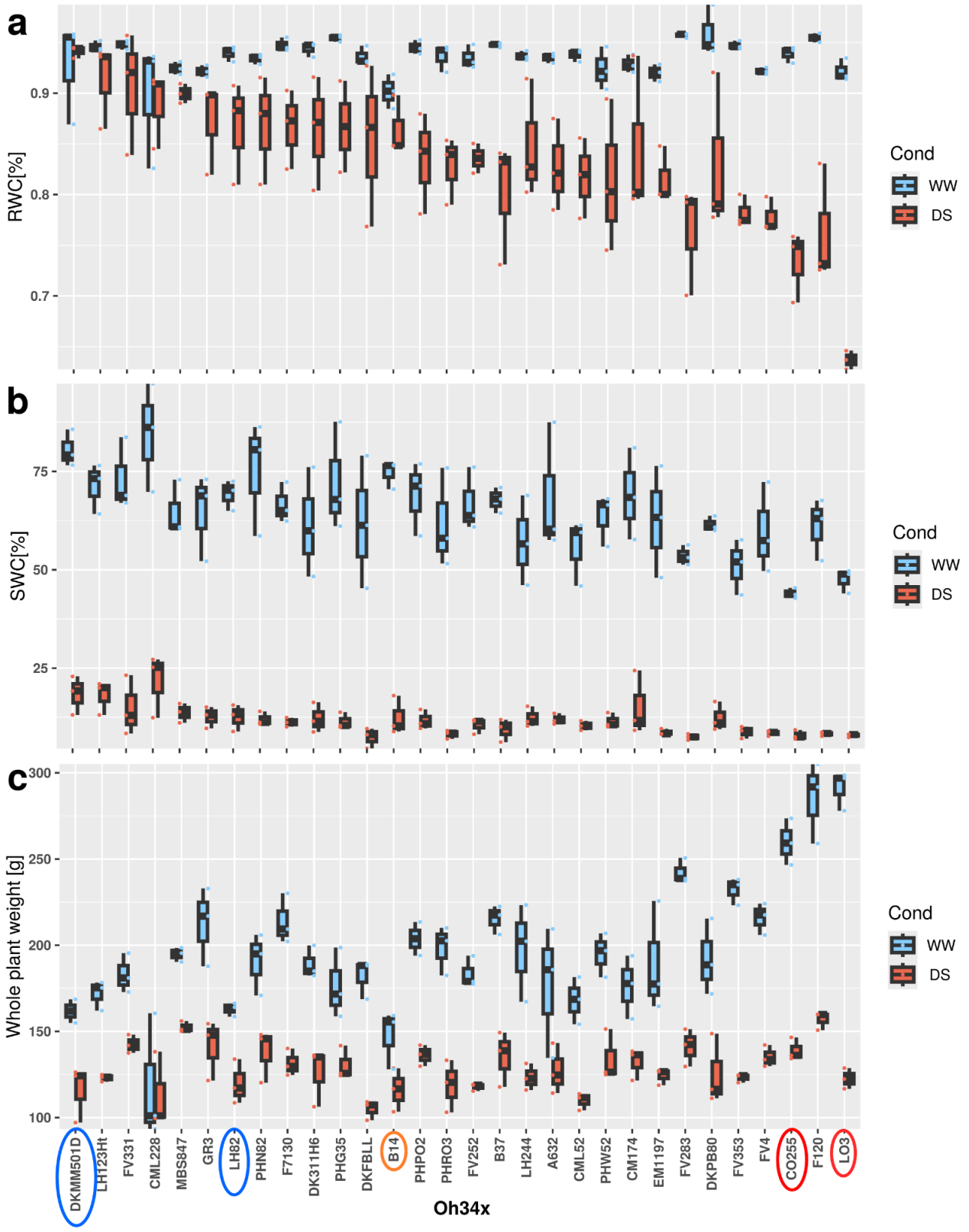



**Figure 2: Plant parameters under well-watered (WW) and drought (DS) conditions.** Relative water content (a), soil water content (b), and whole plant weight (c) for WW and DS in the pilot experiment. Three pots (each with four plants that were pooled) were analyzed here. DS corresponds to 113h of water withholding. Ellipses mark the five lines

that test drought-responsive gene mRNA levels (Fig. 3). F1 lines are sorted by their RWC in a for all graphs.

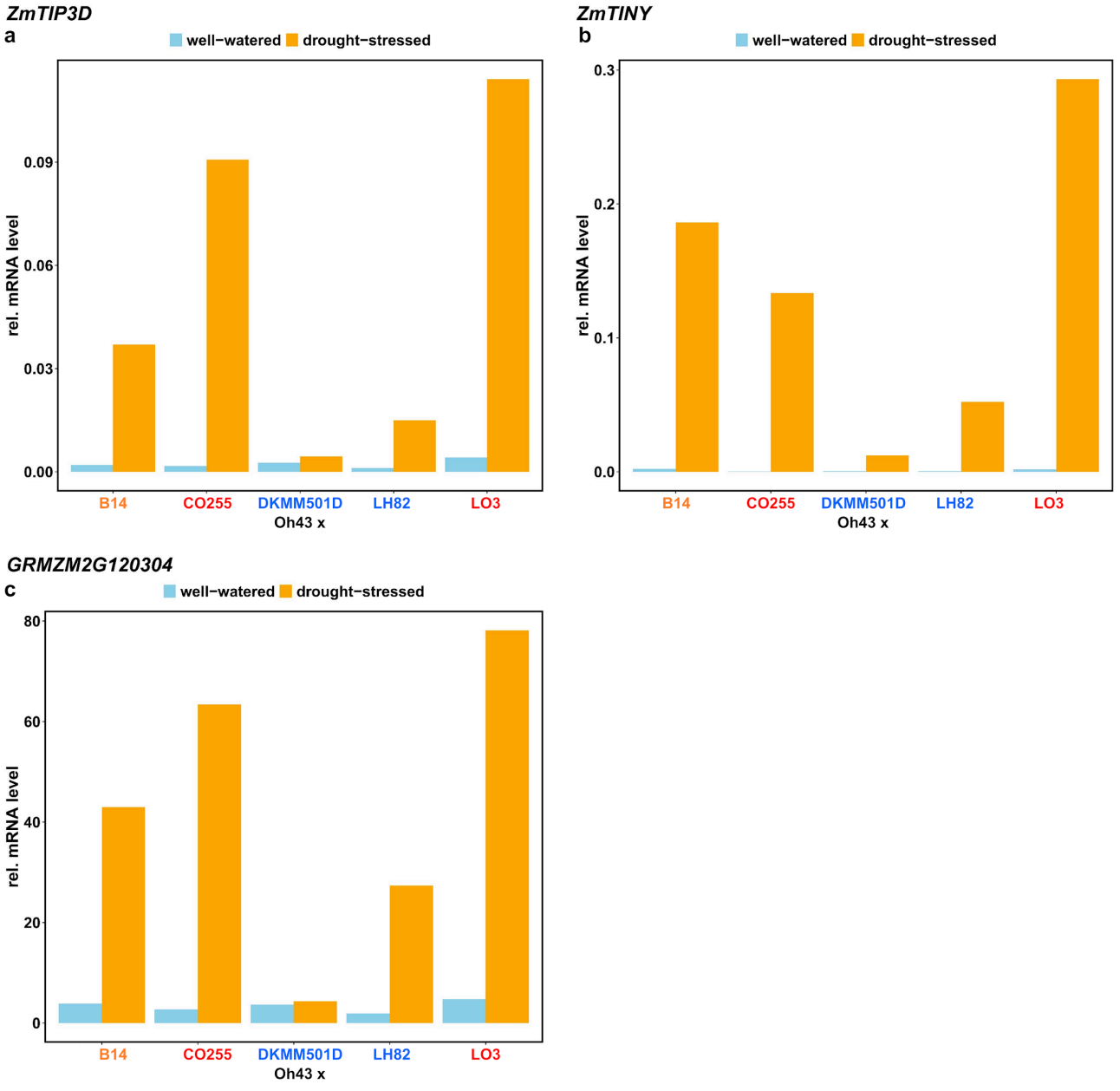



**Figure 3: qRT-PCR of drought-responsive genes in five selected hybrids.** According to their relative water content in DS conditions (Fig. 2) two rather drought-resistant hybrids (blue labeling), one medium (orange labeling), and two rather susceptible hybrids (red labeling) were tested for the mRNA abundance of genes with known drought response: (a) the homolog of aquaporin *BETA-TONOPLAST INTRINSIC PROTEIN 3* (*ZmTIP3d*, Johansson *et al.* 2000), (b) ZmTINY, a gene containing drought QTL region (Shikha *et al.* 2017,

Engelhorn/Hartwig data unpublished) and a hydroxyproline-rich glycoprotein family protein-encoding gene found to be strongly upregulated under drought by Zhang et al. (2018).

References

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**Shikha M, Kanika A, Rao AR, Mallikarjuna MG, Gupta HS, Nepolean T**. **2017**. Genomic Selection for Drought Tolerance Using Genome-Wide SNPs in Maize. *Front. Plant Sci.* **8**: 550.

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