REPORT of the SHORT TERM SCIENTIFIC MISSION
COST STSM Reference Number: COST-STSM-FA1208-27582

Title: "Gene expression of candidate genes involved in *Ustilago maydis* infection in contrasting maize inbred lines"

Start date: 01-10-2015 End date: 30-10-2015

Applicant: Mara Lisa Vieira Alves

Host: Prof Dr Gunther Döhlemann, University of Cologne CEPLAS / Institute of Botany, Cologne, Germany

• Background:

Nowadays local maize landraces can still be found in cultivation across Portugal. One of the reasons pointed by farmers to maintain this material is related to the quality of the bread produced using these landraces. Nevertheless, fungal diseases such as *Ustilago maydis* infection can affect both production and kernel quality. Preliminary field characterization has detected variability in the degree of susceptibility to *U. maydis* infection among Portuguese maize landraces. To address this problem we propose to search for sources of resistance to *U. maydis* in maize Portuguese germplasm and identify maize genetic polymorphisms associated with this resistance useful to support future precision breeding. For that a collection of inbred lines, including material completely derived from Portuguese maize landraces and previously genotyped with the Illumina 50K SNP array, will be phenotype for their resistance level during the Spring/Summer maize multiplication field trial. These data will then be used for association genetic studies. What is proposed in this STSM application will allow complementing this work by preforming a detailed expression analysis of potential candidate genes for *U. maydis* infection resistance.

• Objectives/ Purpose of the visit:

1. Characterization of responses to *U. maydis* infection in a selected set of maize inbred lines under controlled conditions. The number of accessions evaluated will depend on the seed availability from the Spring/Summer field multiplication trial (Portugal). Growth chamber conditions will be provided at the University of Cologne (UoC) to guarantee controlled experimental conditions. Fungal isolates, equipment and expertise for disease screenings are also available at the UoC.
2. Collection of inoculated plant tissue samples from contrasting accessions for resistance response and expression analysis of plant candidate resistance genes. Information on candidate genes sequences, equipment and expertise for gene expression are available at the UoC.

• Description of the work

A bottleneck to achieve the first objective was encountered because the establishment of the Spring/Summer maize inbred lines multiplication field trial in Portugal was unsuccessful. This trial was going to allow for the selection of a set of inbreds varying for the resistance/susceptibility response to *U. maydis* infection, which would serve as basis for this STSM. To overcome this problem another approach was implemented,

based on the previously obtained genotypic data from the Illumina 50K SNP array by the Portuguese team, and on a list of candidate genes made available by the host lab (Table 1, Doehlemann *et al.* (2008), Doehlemann *et al.* (2009), Hemetsberger *et al.* (2012)). The genes in this list where selected based on their responsiveness to *U. maydis* either in a compatible interaction (in this case they are also jasmonic acid marker genes) or in an incompatible situations (in this case the genes actually also serve as salicylic acid marker genes).

Table 1. Selected maize candidate genes involved in the response to *Ustilago maydis* infection.

Gene	Gene model B73 RefGen v.3	Signalling pathway marker genes
PR1	GRMZM2G465226	Salicylic acid
PR3	GRMZM2G453805	
PR5	GRMZM2G402631	
PRm6b	GRMZM2G065585	
PR10	GRMZM2G112488	
OPR2	GRMZM2G000236	
Atfp4	GRMZM2G160710	
POX12	GRMZM2G103342	
ZmGST2	GRMZM2G132093	
ZmOXR	GRMZM2G020631	
P450	GRMZM2G075461	
ZmPR4b	GRMZM2G117971	Jasmonic acid
ZmCCD1	AC225718.2_FG006	
ZmWIP1	GRMZM2G156632	
ZmSerPIN	GRMZM5G815098	
CC9	GRMZM2G312061	
BBi	GRMZM2G338693	
TPS6/11	GRMZM2G127087	

Starting from the 50K SNP array results, the SNPs that were localized within or near the genes from Table 1 were used to construct the unrooted Neighbour Joining tree of the genetic distance matrix of the *–log[proportion of shared alleles]* to look for genetic variability on those genes. The stability of the tree branches was tested by bootstrap (1000 replicates). Using the phenotypic information available from the literature, inbred line standards for the response to *U. maydis* infection were defined: Line W64A was taken as susceptible; B73 was taken as partial susceptible/resistant; and Mo17 was taken as resistant to *U. maydis* infection. Those lines were then localized in the unrooted tree and the inbred lines that clustered together with them were assumed to have a higher probability to behave in a similar manner regarding the phenotypic response to *U. maydis* infection. Portuguese inbred lines covering the major tree branches were selected in a total number of ten different inbreds.

Characterization of responses to U. maydis infection in ten maize inbred lines under controlled conditions

Plant material and experimental design

A collection of ten Portuguese maize inbred lines was evaluated for *U. maydis* resistance under phytochamber conditions at UoC within the group of Gunther Döhlemann. The experiment consisted of three consecutive repetitions (biological replicates). In each repetition, twenty seeds from each of the ten selected maize inbred lines were sown in 1.5 L plastic pots (15 cm diameter) filled with potting soil, with four seeds per pot. Pots were placed in a phytochamber in a 12 hours/12 hours light/dark cycle; light period started/ended with 1 hour ramping of light intensity. Temperature was 28ºC and 20ºC, relative humidity 40% and 60% during light and dark periods, respectively, with 1 hour ramping for both values. Water was supplied accordingly to keep the pots/plants well-watered.

Ustilago maydis strain preparation

Liquid culture of *U. maydis* strain SG200 (Kämper *et al.*, 2006) was grown at 28ºC in YEPS light medium (1% yeast extract [w/v], 0.4% Bacto-pepton [w/v], and 0.4% sucrose [w/v]; Tsukuda *et al.*, 1988), shaking at 200 rounds per minute (rpm) at 28°C, to an OD_{600} of 0.6 to 0.8. Cells were centrifuged at 5000 rpm for 15 min and resuspended in water to an OD_{600} of 1.0 for infection of maize seedlings.

Plant infection procedure and symptoms scoring

For plant infections, 7 days old seedlings (at late V1 growth stage) of the individual inbred lines are syringe-inoculated with approximately 2×10^6 fungal cells per plant and symptoms are scored at day 12 after infection. Categories for disease rating are as follows: (0) no symptoms; (1) chlorosis; (2) ligular swellings; (3) small tumours, less than 1 mm in diameter (4) tumours larger than 1 mm in diameter, not associated with bending of stem; (5) large tumours associated with bending of infected stems.

Expression analysis of candidate plant resistance genes in response to Ustilago maydis infection under controlled conditions

Because of the insufficient number of seeds a choice to cover just one time point instead of the initial two times points initially proposed needed to be made. To select the time point where the majority of genes responded differentially to the fungal infection a pre-experiment was settled on the maize cultivar commonly used by the host laboratory, *cv.* Early Golden Batam.

Plant material and experimental design

The experiment consisted on sowing forty Early Golden Batam seeds in 1.5 L plastic pots (15 cm diameter) filled with potting soil, with a maximum of four seeds per pot. Pots were placed in a phytochamber in a 12 hours/12 hours light/dark cycle; light period started/ended with 1 hour ramping of light intensity. Temperature was 28ºC and 20ºC, relative humidity 40% and 60% during light and dark periods, respectively, with 1

hour ramping for both values. Water was supplied accordingly to keep the pots/plants well-watered.

The 40 plants were divided in two groups: 20 plants to evaluate gene expression at the time point 1 day after-infection; and 20 plants to evaluate gene expression at the time point 4 days after-infection. The 20 plants of each group were further divided into two other sub-groups of ten plants each: one for the *U. maydis* SG200 infection and the other for the mock infection done by syringe-inoculated the plants with sterile water (control).

Ustilago maydis strain preparation

Liquid culture of *U. maydis* strain SG200 (Kämper *et al.*, 2006) was grown at 28ºC in YEPS light medium (1% yeast extract [w/v], 0.4% Bacto-pepton [w/v], and 0.4% sucrose [w/v]; Tsukuda *et al.*, 1988), shaking at 200 rounds per minute (rpm) at 28°C, to an OD_{600} of 0.6 to 0.8. Cells were centrifuged at 5000 rpm for 15 min and resuspended in water to an OD_{600} of 1.0 for infection of maize seedlings.

Plant infection procedure and collection of plant material for RNA extraction

For plant infections, 7 days old seedlings (at late V1 growth stage) of the individual inbred lines were syringe-inoculated with approximately 2×10^6 fungal cells per plant and infection sites in third seedling leaves from 10 plants per inbred line were collected from water-injected (mock infection) and fungal-infected individuals after 1 and 4 dpi (days post-infection). From the third leaf, for the time point 4 dpi, the material was taken 2 cm after syringe wound and the 4 cm bellow leaf segment was collected and kept in liquid nitrogen; for the time point 1 dpi, the material was taken 1 cm after syringe wound and the 4 cm bellow leaf segment was collected and kept in liquid nitrogen. This material was used for RNA isolation and posterior expression of candidate plant resistance genes by RT-qPCR.

Samples:

1. Maize *cv.* Early Golden Bantam 1 dpi MOCK
2. Maize *cv.* Early Golden Bantam 1 dpi SG200
3. Maize *cv.* Early Golden Bantam 4 dpi MOCK
4. Maize *cv.* Early Golden Bantam 4 dpi SG200

RNA isolation from maize leaf tissue, cDNA synthesis, and RT-qPCR

For RNA isolation, material from the 10 plants from each time point and treatment was pooled, ground in liquid nitrogen and RNA was extracted with TRIzol® Reagent (Invitrogen, Karlsruhe, Germany) following manufacturer specifications and treated with Ambion® DNA-free™ DNA Removal Kit to remove contaminating DNA from RNA samples. RNA was quantified in a NanoDrop 2000c spectrophotometer and the samples were standardized to a concentration of 1000 ng/µL. RNA integrity was check

by running 1 µL of the RNA samples in a 0.8% agarose gel at 118 V for 30 min (Figure 1).

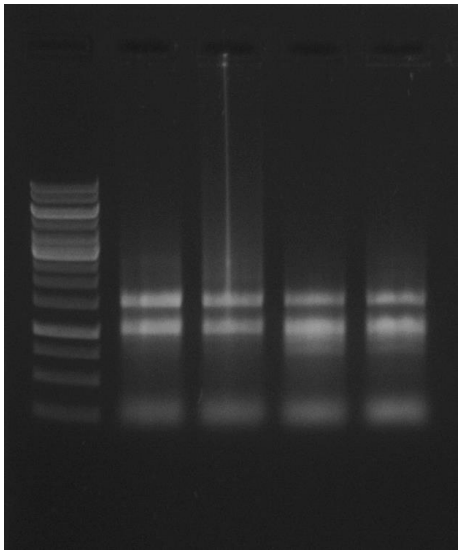

Figure 1. RNA integrity in a 0.8% agarose gel. Order of samples in the gel: DNA ladder 1Kb; Maize *cv.* Early Golden Bantam 1 dpi MOCK; Maize *cv.* Early Golden Bantam 1 dpi SG200; Maize *cv.* Early Golden Bantam 4 dpi MOCK; Maize *cv.* Early Golden Bantam 4 dpi SG200.

For cDNA synthesis, 1 µg of template RNA was used according to the cDNA synthesis protocol from iScript cDNA synthesis kit (Bio-Rad). The reaction program was adapted so that in step 2 instead of 30 min at 42°C, the samples were kept at 42ºC for 60 min.

Prior to the analysis by RT-qPCR, primers pairs were tested to check positive amplification with a simple PCR. Each PCR reaction consisted of 7.5 µL of GoTaq® Green Master Mix (Promega), 1 µL of the forward and reverse primers and 5.5 µL of cDNA (cDNA stock at 1 ng/µL). Cycling conditions were 2 min 94°C, followed by 35 cycles of 10 sec 94°C/10 sec 58°C/30 sec 72°C, and final extension at 72ºC for 2 min. Positive amplification was checked by running 15 µL of PCR products in a 2% agarose gel at 120 V for 30 min.

Selected genes were analysed by quantitative RT-qPCR (Table 2). Maize glyceraldehyde dehydrogenase (*gapdh*; NM001111943) gene was used as reference gene for normalization. Quantitative RT-PCR was performed on a CFX96 Real-Time PCR Machine (Bio-Rad) using the iQ SYBR Green Supermix (Bio-Rad), following the reaction mixture stated on table 3. Cycling conditions were 1.5 min 95°C, followed by 45 cycles of 15 sec 95°C/15 sec 60°C/30 sec 72°C. After each PCR, the specificity of the amplified product was verified by analysis of the melting curve obtain by decreasing the temperature from 65ºC to 95ºC, with an increment of 0.5ºC per cycle, and the threshold cycle above background was calculated using Bio-Rad iCycler software.

Fold change on the gene expression levels were calculated relative to *gapdh* expression levels using the cycle threshold (Ct) $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

Table 2. Primer sequences of the genes analysed by simple PCR and further analysed by RT-qPCR (genes underlined).

Gene	Accession	Primer sequences (5'-3')
PR1	NM_001111929.1	Fw: ACTACGTGGACCCGCACAAC
		Rev: CGGAGTGGATCAGCTTGCAGTC
PR5	NM_001112232.2	Fw: TATCGGCCGGAATAGGCTCTG
		Rev: CGCGTACATACAAATGCGTGC
PR10	XM_008656464.1	Fw: CAAGCTCATCGCAGACCAC
		Rev: CGATCTCAACAGTCCAGCTGTT
OPR2	NM_001112435.2	Fw: GACCGACCGAGAGCAAATAG
		Rev: ATCTTGTAAGGCGTCAGCAG
Atfp4	NM_001158939.2	Fw: CAGCCTGTGGACATATGC
		Rev: GCACATGCCCTTAACCTC
POX12	NM_001137958.1	Fw: CTGAACAAGTTCTTCGCGG
		Rev:AGGTCCACGTAGTACTTGTTG
ZmGST2	NM_001111896.2	Fw: TGTGCTTGATTAGTTAATTGG
		Rev: CGTGGAGAAAGCAGCAAAAT
ZmOXR	NM_001147585.1	Fw: TGATGTTTGGTACAGAGTG
		Rev: TCATCCCGTCAGAGGTTTTA
ZmWIP1	NM_001154892.1	Fw: GTCCGAGACCATGAAGAGC
		Rev: AGGAGAAGTTGCAGTTGGTG
CC9	NC_024462.1	Fw: TATGGGTCCTTGACGTTCTC
		Rev: GGATCATCCGTAGCCATCTG
BBi	XM_008679647.1	Fw: CCGACATCCTCTTCAACTTCTG
		Rev: TTCTCTGAAGCGGCACAC
BAX1	NM_001147388.1	Fw: TCACCCTCTTCACAGACTTC
		Rev: TCTTCTTGTCCTCCGACTTG
TPS6	NM_001112204.1	Fw: CAATGGATGAAGCGTGTGAG
		Rev: CGTTCGTGGCACAATTATCG

Table 3. RT-qPCR reaction mixture for a final volume of 15 µL.

Reaction component	STOCK concentration	1x reaction (µL)
iQ SYBR Green Supermix	2X	7.5
Forward primer	10 µM	0.5
Reverse primer	10 µM	0.5
cDNA	1 ng/µL	6.5

• Main results:

Selection of maize inbred lines

Following the inbred lines selection approach, fifty percent (9 out of 18 genes) of the initial list of candidate genes had at least one polymorphic SNP localized near or within its sequence covered by the genotypic data from the 50K SNP array (Table 4). BBi gene had 3 SNPs scored and TPS6 was the gene with the higher coverage of

polymorphic SNPs, with 11 SNPs located within or nearby the gene. The remaining 7 genes were only scored with one SNP position.

Table 4. SNPs from the 50K SNP array localized within or near the candidate genes

Gene	Gene model	Chromosome	Gene position (base pairs)	Nearby SNP	SNP position (base pairs)	Excluded
PR1	GRMZM2G465226	7	3483732 - 3484794	none		
PR3	GRMZM2G453805	3	212009199 - 212010553	none		
PR5	GRMZM2G402631	1	178008019 - 178008996	rs131819131	178036171	
PRm6b	GRMZM2G065585	3	151971037 - 151972416	rs132040963	152010051	
PR10	GRMZM2G112488	1	47177906 - 47178782	none		
OPR2	GRMZM2G000236	9	7308153 - 7309906	rs131694665	7308829	
Atfp4	GRMZM2G160710	5	178737671 - 178738620	rs132250654	178784380	SNP 100% missing
POX12	GRMZM2G103342	3	146522696 - 146524904	rs132037735	146559140	
ZmGST2	GRMZM2G132093	10	90200516 - 90201775	none		
ZmOXR	GRMZM2G020631	1	195752951 - 195756226	rs131832282	195788746	
P450	GRMZM2G075461	2	213200367 - 213202390	none		
ZmPR4b	GRMZM2G117971	4	9680499 - 9681375	rs129633181	9686785	
ZmCCD1	AC225718.2_FG006	5	48531033 - 48531431	rs131517599	48531672	Monomorphic SNP
ZmWIP1	GRMZM2G156632	8	12273514 - 12274222	rs131648928	12276618	
ZmSerPIN	GRMZM5G815098	4	2296963 - 2327137	none		
CC9	GRMZM2G312061	4	15272109 - 15272574	none		
BBi	GRMZM2G338693	5	17193757 - 17195295	rs131498504	17249342	
				rs130001577	17249401	
				rs130001595	17251181	
TPS6/11	GRMZM2G127087	10	55603292 - 55606592	rs128526146	55579394	
				rs131764362	55599709	
				rs128526382	55603807	
				rs128526383	55603829	
				rs128526384	55603857	SNP close to fixation (MAF < 0.05)
				rs131764392	55684798	
				rs131764390	55685418	
				rs128526674	55690110	
				rs131764400	55766192	
				rs131764401	55775160	
				rs128527095	55804652	
				rs128527075	55807609	

A hierarchical clustering for the inbred lines from our association mapping panel was done using the 21 SNPs located near maize genes related to *U. maydis* infection response. Three main clusters could be visualized (Figure 2). The inbred line Mo17, considered as a resistant line, clustered away from the susceptible and partial susceptible/resistant lines. In order to maximise the hypothesis to have lines responding differently to the *U. maydis* infection lines from the different clusters were selected.

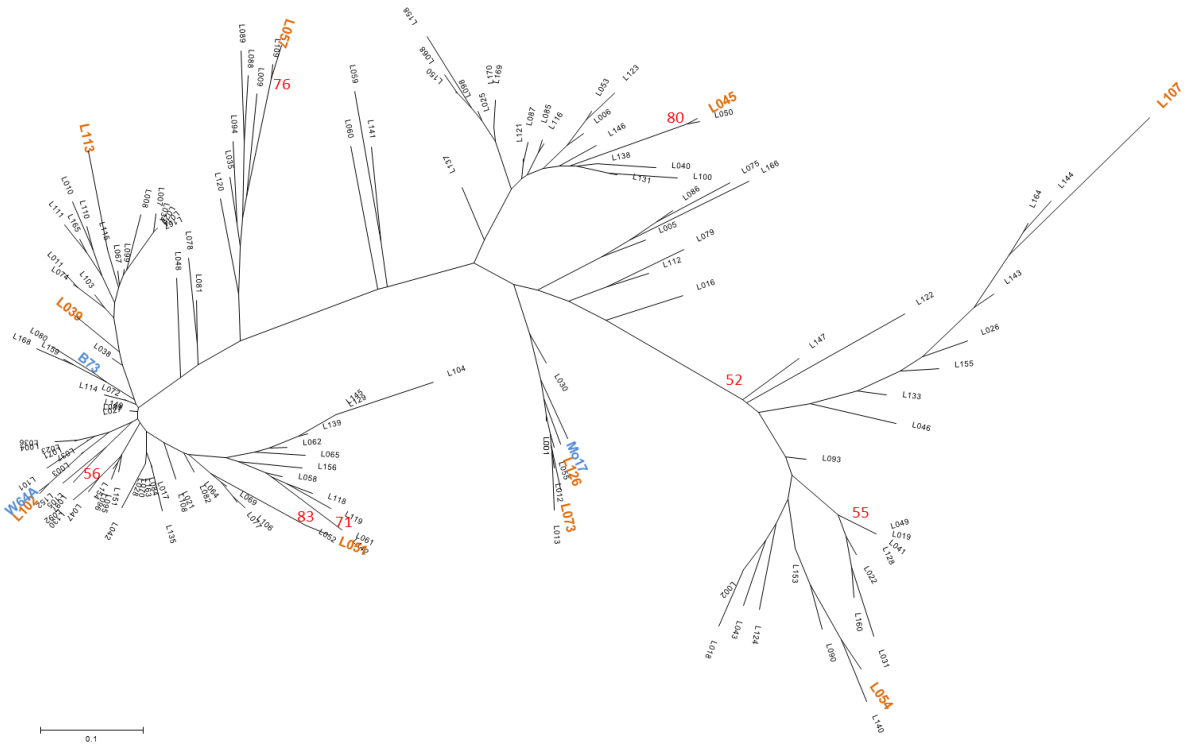

Figure 2. Unrooted Neighbour Joining tree of the genetic distance matrix of the –log [proportion of shared alleles] using 21 SNPs located near maize genes related to *Ustilago maydis* infection response (Table 4). Above the tree branches in red are the bootstrap values < 50% (1000 bootstrapped trees). In blue, inbred line standards from the literature for the response for *Ustilago maydis* infection: W64A, susceptible; B73, partial susceptible/resistant; and Mo17, resistant. In orange, selected Portuguese inbred lines.

1. Identification of quantitative resistant maize inbred lines to *Ustilago maydis* infection at seedling stage

Seeds from each of the inbred lines were sowed as scheduled however since the seeds from all 3 repetitions did not germinate the scoring of the symptoms upon infection was totally hampered.

2. Expression profile of candidate genes related to plant response to *Ustilago maydis* during infection

From the 13 genes tested one gene (PR1, NM001111929.1) fail to amplify even after new primer set synthesis was order. The remaining 12 genes were used for the RT-qPCR pre-experiment to select the time point to use on the Portuguese inbred lines. Six genes were early response genes, being upregulated at 1 dpi; and six genes were late response genes, being more upregulated at 4 dpi (Figure 3). In the category of early response genes, 3 genes were marker genes for salicylic acid signalling pathway (Atfp4, OPR2 and POX12) and 3 genes were marker genes for jasmonic acid signalling pathway (Bbi, ZmWIP1 and CC9). In the category of late response genes, the majority of them are considered marker genes from the salicylic acid signalling pathway (PR5, PR10, ZmGST2, ZmOXR). Taken these

results into consideration, the time point selected to further study the expression level of the candidate genes in Portuguese maize inbred was 1 dpi since at this time point marker genes from both jasmonic and salicylic acid signalling pathways are upregulated.

Taking into consideration the null germination rate of the tested Portuguese inbred lines, the seeds for the RT-qPCR experiment were surface-sterilized for 10 min in a 10% solution of sodium hypochlorite and then rinsed ten times with sterile water and put to pre-germinate in petri dishes at 22ºC in the dark. Unfortunately, even with this treatment no viable seedlings were obtained.

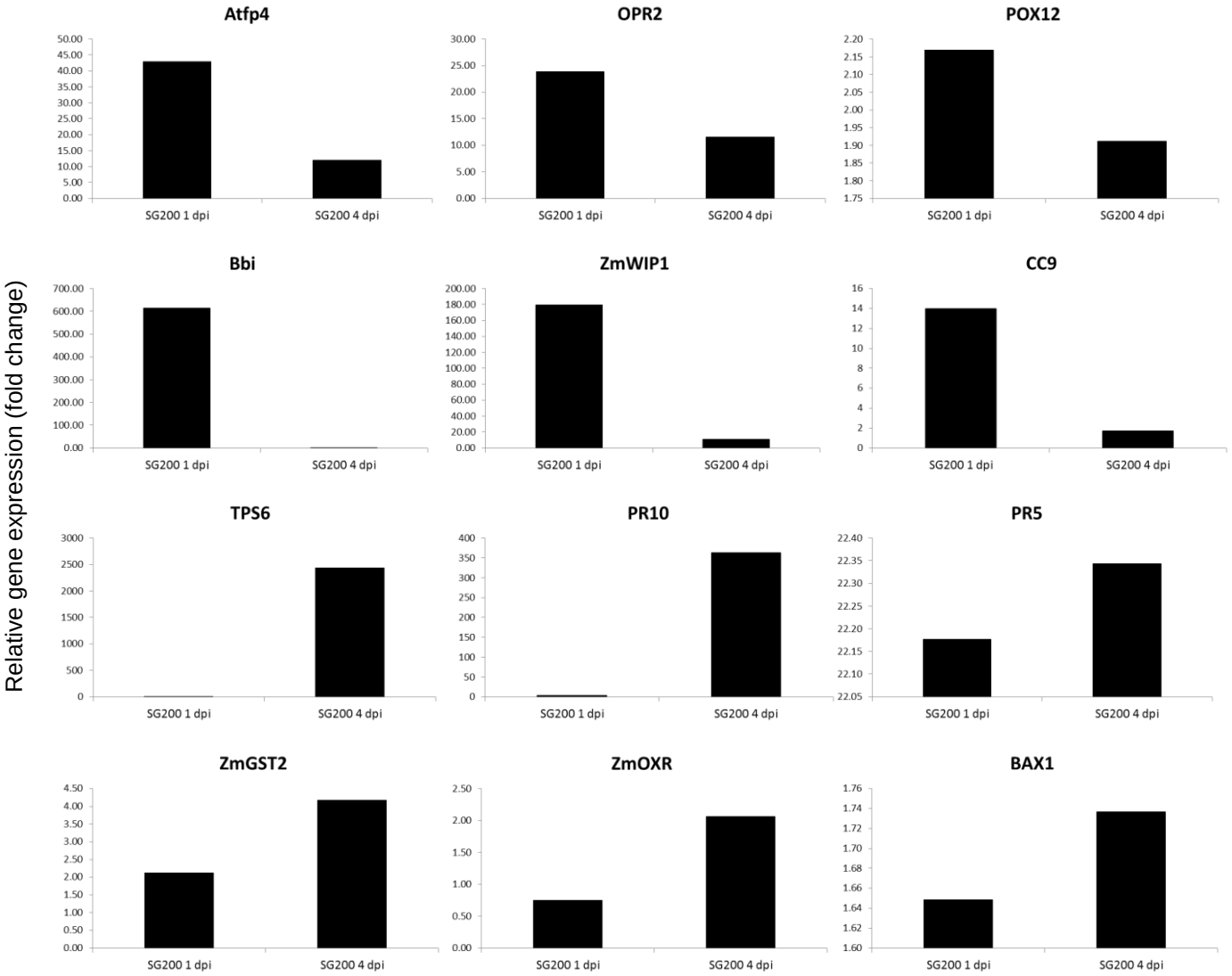

Figure 3. Gene expression level (fold change) for maize *cv.* Early Golden Batam seedlings after 1 dpi and 4 dpi with *Ustilago maydis* SG200. Values were normalized using *gapdh* expression levels.

• **Projected publications/articles related to or resulting from the STSM:**The techniques learned during this STSM will serve to support new lines of research on maize breeding for *U. maydis* resistance in my base Portuguese team.

• References

Doehlemann G., Wahl R., Horst R.J., Voll L.M., Usadel B., *et al.* (2008) Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. *Plant J* **56**:181–195

Doehlemann G., van der Linde K., Assmann D., Schwammbach D., Hof A, *et al.* (2009) Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. *PLoS Pathog* **5**:e1000290

Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G (2012) The *Ustilago maydis* Effector Pep1 Suppresses Plant Immunity by Inhibition of Host Peroxidase Activity. *PLoS Pathog* **8**(5):e1002684

Kämper J., Kahmann R., Bölker M., *et al.* (2006) Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **444**:97-101

Livak K.J. and Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* **25**:402–408

Tsukuda T., Carleton S., Fotheringham S. and Holloman W.K. (1988) Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. *Mol Cell Biol* **8**:3703–3709