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Transcriptomic response of maize primary roots to low temperatures at seedling emergence

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Background. Maize (*Zea mays*) is C_4 tropical cereal and its adaptation to temperate climates can be problematic due to low soil temperatures at early stages of establishment. **Methods.** We report on a physiological and transcriptomic experiment on twelve maize varieties from a chilling condition adapted gene pool which identified four genotypes with significant contrasting chilling tolerance.These four varieties were subject to microarray analysis to identify up and down regulated genes under chilling conditions. **Results.** Stress induced by low temperature in the varieties Picker,PR39B29, Fergus and Codisco was reflected only in the expression profiles of the varieties Picker and PR39B29. No significant changes in expression were observed in Fergus and Codisco upon chilling stress. The overall number of genes up and down regulated in the two chilling tolerant varieties amounted to69. These two varieties exhibited two different trancriptomic patterns in which only four genes were shared, although not all with the same degree of regulation. Overall the expression pattern was similar between the two chilling tolerant varieties, indicating a common response to chilling stress. **Discussion.** Varieties with an enhanced root/shoot growth ratio under low temperature were more tolerant which could be an early and inexpensive measure for germ plasm screening. We have identified novel cold inducible genes in an already adapted maize breeding gene pool. This illustrates that further varietal selection for enhanced chilling tolerance is possible in an already preselected gene pool.

Transcriptomic response of maize primary roots to low temperatures at seedling emergence

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Abstract

Background. Maize (*Zea mays*) is C_4 tropical cereal and its adaptation to temperate climates can be problematic due to low soil temperatures at early stages of establishment. **Methods.** We report on a physiological and transcriptomic experiment on twelve maize varieties from a chilling condition adapted gene pool which identified four genotypes with significant contrasting chilling tolerance. These four varieties were subject to microarray analysis to identify up and down regulated genes under chilling conditions. **Results.** Stress induced by low temperature in the varieties Picker, PR39B29, Fergus and Codisco was reflected only in the expression profiles of the varieties Picker and PR39B29. No significant changes in expression were observed in Fergus and Codisco upon chilling stress. The overall number of genes up and down regulated in the two chilling tolerant varieties amounted to 69. These two varieties exhibited two different trancriptomic patterns in which only four genes were shared, although not all with the same degree of regulation. Overall the expression pattern was similar between the two chilling tolerant varieties, indicating a common response to chilling stress. **Discussion.** Varieties with an enhanced root/shoot growth ratio under low temperature were more tolerant which could be an early and inexpensive measure for germplasm screening. We have identified novel cold inducible genes in an already adapted maize breeding gene pool. This illustrates that further varietal selection for enhanced chilling tolerance is possible in an already preselected gene pool.

Introduction

Maize is the major cereal product in the world and Europe is third largest producer of maize grain after the United States and China (www.fao.org). However low temperatures along with water availability represent one of the major impediments for the plant productivity and geographical distribution in the world.

The most widespread germplasms of maize around the globe are Dent types, commonly grown in North America, and Flint types, widely grown in Asia, Europe and in Central and South America (Reimer, 2008). The difference between the two types lies in the type of kernel, which is harder in the outer layer of flint corn. Besides, flint germplasm has a very low water content, which makes it better suitable to chilling conditions (Revilla, 2016). Dent corn grows better in warmer climates and it has a high yield potential when grown near optimal temperature conditions.

Maize is a C_4 tropical plant whose growth range of temperature extends up to 30°C - 35°C (Presterl et al., 2007) and is significantly sensitive to low temperature, particularly in the early growth stages. Nevertheless, hybrids derived from highland maize can adapt to lower temperatures than the optimal range (Greaves, 1996). However, temperatures below the optimum cause a steady decline of growth of maize, which stops around 6-8°C. Prolonged exposure to low temperatures involves irreversible cellular and tissue injury (Greaves, 1996), and the effect is mainly marked in the early growth stages as it impairs several developmental and physiological processes (Marocco, Lorenzoni & Fracheboud 2005).

Chilling is responsible for yield losses and lower metabolisable energy content (starch, sugar) in maize (Frei, 2000); in particular, it affects photosynthesis due to an over excitation of the PSII reaction centres and a concomitant production of oxygen radicals (ROS), which are

63 demonstrated to produce injurious effects to the photosynthetic apparatus (Nie, Long & Barker,
64 1992).

65 In Northern and Central Europe maize is generally sown in the last week of April, when soil
66 temperature is warm enough for seeds to germinate, and harvested in autumn before the first air
67 frost occurs damaging the crop with temperatures below -2°C. The date of sowing and the date
68 of harvest determine the length of the growing season and therefore the level of maturity and
69 quality of the crop. Early maturing varieties reach maturity earlier; this means that the
70 development of the canopy occurs earlier and so does its closure reducing, this way, the risk of
71 yield losses that can be caused by the first autumn air frost at the end of the growing season.
72 However, despite the improvement in crop quality and yield, these cultivars are still dependent
73 on suitable soil temperatures for the initial establishment of the seedlings and they still benefit
74 from a longer growing season.

75 Maize root growth generally occurs between 9°C and 40°C, but it has been demonstrated that,
76 during the early phases of development, maize growth is dependent on soil temperature which
77 ranges from 10°C to 17°C according to the trait and variety (Blacklow, 1972). As the
78 temperature decreases, it is possible to observe morphological changes in the root system; roots
79 become swollen behind the tip and thicker, with a higher number of seminal roots (Farooq et al,
80 2009 – chilling tolerance in maize: agronomic and physiological approaches). The effect of low
81 temperature on roots may be indirectly reflected on shoot elongation and leaf formation.

82 The use of biodegradable polythene films distributed on the soil surface has helped solve the soil
83 temperature issue in practical crop husbandry giving maize growth a significant enhancement

(Keane, 2002), but further varietal improvements through breeding are required to make the maize crop more economical in Northern and maritime Europe.

A better understanding of the developmental stages that are particularly sensitive to low temperature will help improve maize adaptation to temperate climates. Each physiological and biological process can be more or less susceptible to suboptimal temperatures, depending on what is called thermal threshold, which is the sub optimal temperature at which the maize hybrid is able to maintain high rates of growth. The lower the thermal threshold the higher is the growth rate and the faster is emergence from soil under low temperatures (Greaves, 1996). The thermal threshold is controlled by specific genes which regulate specific processes at specific developmental stages. The combined processes with lower thermal thresholds will result, therefore, in an optimised growth under low temperature conditions.

Plant breeding is still dependant on phenotypic selection, where new hybrids are tested in yield trials and, therefore, selected on the harvestable yield rather than on their ability to cope with chilling temperatures. Chilling tolerance is controlled by genes that are not directly involved in yield, but they contribute to it by conferring tolerance and so aiding the plant to reach its full yield potential (Greaves, 1996).

The detection of the transcripts and the identification of the genes associated to them will lead, with an appropriate breeding programme, to the transfer of the traits of interest to new hybrids with an improved tolerance to low temperatures. Gene expression profiling can be viable with the employment of technologies like microarray and qRT-PCR capable to screen a large set of transcripts or even the entire transcriptome. Recently, a microarray analysis has been conducted to identify cold-regulated genes by chlorophyll fluorescence determination (Sobkowiak et al.,

2014), while in another work QTLs were linked to cold tolerance in temperate maize inbred lines (Revilla, 2016).

In this study, we aimed to investigate the physiological and transcriptomic response to low temperatures of primary roots at early seedling emergence in twelve commercially available chilling tolerant maize cultivars differing in kernel type and maturing time. As the genotypes were known to be cold tolerant and in order to identify specific genes that are regulated under lower thermal thresholds, both control and stress climate conditions were set to suboptimal temperatures. This was aimed at the identification of novel transcripts conferring enhanced chilling tolerance in an already adapted maize breeding gene pool.

Material and Methods

Plant material and growth conditions

Untreated maize seeds for the physiological experiment were provided by the seed companies Caussade (France), Pioneer (France) and Codisem (France), for a total of twelve varieties (Supplemental Materials Table 1). Varieties Algans, Justina and Picker were included in the Irish Recommended List 2008 of the cultivars that have shown a high yield performance under Irish climate conditions in trials. The experimental varieties also differed in the type of kernel (Flint, Dent, and Flint-Dent) and maturity type.

Two independent non seed coating treated 45-seed groups of each variety were germinated in growth chambers (Snijder Microclima 1750, The Netherlands) under control and low temperature conditions on a surface of capillary matting lying over two layers of blotting paper

soaked with 100ml of distilled water. The blotting paper and capillary matting were placed in 52cm x 42cm x 9cm seed trays, which were covered by another inverted seed tray to reduce water loss. The blotting paper was placed below the single layer of capillary matting. Each group of 45 seeds, were arranged as two sub-groups of 21 and 24 seeds in two separate seed trays. Seeds were used directly from the seed bag and placed under two controlled growth conditions. The control temperature regime was set at 18°C for 16 hours and 12°C for 8 hours; the low temperature regime was set at 12°C for 16 hours and 6°C for 8 hours. Control temperature conditions were chosen like at an ideal spring day when maize is being sown in temperate climates like in Ireland. This allowed us to target the identification of transcripts which are up- or down regulated in germplasm which has been bred to perform in the maritime climate of the northern hemisphere. The experiment was conducted in constant dark conditions with 40% of relative humidity.

Germination assessment and growth ratio (GR)

Seed germination was classified as such when the radicle emerged from the meristem and was at least 1mm long. Seed germination was recorded in the number of days from sowing to radicle emergence. The length of the primary roots and the shoots were measured on germinated seeds with similar root length under the same growth conditions at 24-hour intervals over a period of five days post-germination (time points). Trays were daily watered with 100ml of distilled water. The response to low temperature was calculated at every time point and was expressed as growth ratio (GR). The GR was calculated dividing the average length of roots and shoots of each

variety at low temperature by the average length of root and shoot of each variety at control temperature. GR was expressed in percentage.

Analysis of the physiological response

The experiment was a randomised block design and was conducted as three independent experiments. A three-way ANOVA was fitted including a blocking factor (experiment) and three effects (genotype, treatment and time point) with all the possible interactions of interest (genotype, treatment, time point, genotype *x* treatment, genotype *x* time point and genotype *x* treatment *x* time point). The measurements over time were made on the same experimental units and a repeated measures analysis incorporating a covariance structure was used to model this lack of independence. P-values <0.05 were taken as significant. Residual checks were made to ensure that the assumptions of the analysis were met and responses were log transformed to correct skew and/or non-constant variance as appropriate. The analysis was performed with the GenStat statistical software package (VSN International, UK) and the Statistical Analysis System (SAS) software (SAS Institute Inc., USA).

RNA extraction

Total RNA was isolated of three independent biological replicates from 3cm maize roots, on days 1, 2, 3, 4, 5 post-germination. Roots were excised and snap frozen in liquid nitrogen and stored at -80°C. Frozen root samples were homogenised in 1.5ml microcentrifuge tubes with the use of tube pestles (Sigma-Aldrich®, Germany) and used as a starting material for RNA

169 extraction. The isolation of total RNA was carried out with the RNeasy© Plant Mini Kit
170 (Qiagen, UK). Samples were treated with DNase I from the RNase-free DNase set (Qiagen, UK).
171 Quantity and quality of RNA were determined with an Agilent 2100 BioAnalyzer (Agilent
172 Technologies, USA). Only RNA samples with an RNA integrity number (RIN) ≥ 8 assigned by
173 the BioAnalyzer were used for microarray analysis.

174

175 *Microarray hybridisation and analysis*

176 Three independent root tissue RNA samples on day 1 post-germination (time point 1) were used
177 for the microarray experiment, for a total of 24 samples. One µg of each RNA sample was
178 hybridised on a 46K 70-mer oligo array developed in the 'maize oligonucleotide array project'
179 by the University of Arizona, The Institute for Genomic Research (TIGR) and the University of
180 Wisconsin (Seifert et al., 2012). The 46k array was configured as 4 rows and 12 columns. The
181 intersection of a row with a column represented a subarray. Each subarray consisted in 31
182 columns and 31 rows. A two-colour microarray was used to compare each variety from the
183 control and the same variety from low temperature according to an interwoven loop design. The
184 array hybridisation with Cy3 and Cy5 dyes was conducted by the Institute of Genomic Research
185 in Arizona. RNA samples were sent in RNAstable® microfuge tubes (Biometrica, USA)
186 according to manufacturer's instructions. Image acquisition was conducted with a GenePix®
187 scanner (Axon, USA) as a service in Arizona.

188 The analysis of microarray was performed with CARMAweb (Comprehensive R-based
189 Microarray Analysis web service, https://carmaweb.genome.tugraz.at/carma/) a web application
190 based on the R (http://www.r-project.org/) programming language and environment for statistical

computing. CARMAweb implements the Bioconductor limma (Linear Model for Microarray Data) package for R, specifically designed for microarray analysis. Data were quality checked, adjusted and normalised before analysis to remove the technical variance and systematic errors without altering the biological variance within the data.

The data were $\log_2$ transformed, background corrected and normalised. Background optical noise of the hybridisation was corrected with the *minimum* method, which consists in giving the half the minimum positive corrected intensities for the array to any intensity that is equal to zero or negative. *Within-array* normalisation was performed with the *print-tip loess* method and *between-array* normalisation was carried out by *scaling* the expression values.

The false discovery rate (FDR) was set at 40% of the genes with the biggest variance across the samples. Differentially expressed genes were determined by subtracting the average expression of the gene in the control from the average expression of the gene under treatment. Bad array spots were excluded from the analysis. Adjusted p-values were generated using the Benjamini and Hochberg method to correct for multiple testing in the experiment (Benjamini & Hochberg, 1995). The analysis was performed using the limma paired moderated t-test statistics, based on the empirical Bayesian approach.

Microarray data were deposited to GEO (Gene Expression Omnibus) under accession number GSE72508.

Real Time qPCR

Microarray data validation was performed with three independent replicates on day 1 post-germination. Reverse transcription was conducted with 500ng total RNA, 1µl 10mM dNTPs (Bioline, UK), 1µl oligo-dT(20) primers (Invitrogen, UK) and incubated at 65°C for 5min in a volume of 11µl. After incubation the solution was chilled on ice for 1min and 4µl of 5X FS reaction buffer, 1µl 0.1M DTT, 1µl RNase free H2O (Qiagen, UK) and 1µl SuperScript® III reverse transcriptase (Invitrogen, UK) were added bringing the volume to 20µl. The reaction was incubated at 50°C for 60min and inactivated at 70°C for 15min. The solution was brought up to a final volume of 50µl by adding RNase free H_2O.

Relative Real Time PCR was conducted with an ABI 7500/7500 Fast Real Time PCR system (Applied Biosystems, USA) using the Fast Sybr® Green Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions, but in a reaction volume of 10µl.

All primers were designed using the Primer3Plus, an advanced Prime3 designer tool (Untergasser et al., 2007). Lyophilised primers were re-suspended in nuclease free water to a final concentration of 100pmol μl^{-1} (mM/L). Re-suspended primers were diluted to the working solution of 10pmol μl^{-1}. A five series dilution standard curve was used to test primer efficiency. As the efficiency of all the primers was ≥95%, the Livak's method (ΔΔCt method) was used to calculate the relative expression. Four genes (Supplemental Materials Table 2) were tested as housekeeping genes with the geNorm algorithm (Vandesompele et al., 2002). Adh and Ef1-α were finally used as housekeeping genes. The target genes used for Real Time qPCR were selected out of the top 100 up- or down regulated genes with the smallest p-values.

Three independent replicates on day 1, 2, 3, 4 and 5 post-germination were used to investigate the expression pattern of the target genes across five time points in a time series experiment.

Fold-change was calculated at every time point by subtracting the average expression of the gene in the control from the average expression of the gene under treatment. The relative expression across time points was calculated by subtracting the fold-change on day 1 post-germination from the fold-change of the other days post-germination.

Results

Physiology and genetics of maize roots grown at low temperatures

The physiological response of maize to low temperature was analysed in twelve maize varieties differing in kernel type and maturity group (Supplemental Materials Table 1). Overall all of these twelve varieties still had root and shoot growth under chilling treatment and thus all of them could be broadly considered as chilling tolerant (Figure 1). However for root and shoot growth, there was a significant *variety x treatment x time point* interaction (Table 1) resulting in an effect of variety on treatment and time point at which the measurements were taken. For this reason the physiological response was calculated as a chilling stress/control ratio (see Materials and Methods) and not simply the root and shoot growth under chilling stress. The twelve varieties exhibited a root response pattern different to the shoot pattern. In order to classify the varieties according to their combined root and shoot response, the Tukey's range test was performed as a multiple comparison method (Figure 1). The physiological response to low temperatures has led to the identification of two groups of genotypes with contrasting cold tolerance. The varieties Picker and PR39B29 showed the highest resistance to chilling stress in terms of both root and shoot growth, while Algans and Justina presented the lowest degree of tolerance. However, because of their poor ability to germinate under the stress conditions

applied, Algans and Justina were excluded for subsequent microarray analysis and substituted by the second most tolerant pair of varieties, Codisco and Fergus. Therefore, the four final genotypes used for the gene expression profiling were Picker, PR39B29, Codisco and Fergus.

Microarray analysis

The microarray analysis of root tissue showed that the most chilling tolerant varieties Picker and PR39B29 have, each, a set of differentially expressed genes (up and down regulated, p-value <0.05), while no genes were listed for the other two less chilling tolerant varieties Fergus and Codisco, indicating that no significant change in expression was found in any of the genes analysed (Table 2). The overall number of genes up and down regulated in the two more chilling tolerant varieties amounted to 69, which are, however, divided in a group of 39 genes in PR39B29 (Supplemental Materials Table 3) and 30 genes in Picker (Supplemental Materials Table 4), as the two varieties exhibited two different transcriptomic patterns in which only four genes were shared, although not all with the same degree of regulation (Supplemental Materials Tables 3 and 4). These four genes were a RNA binding protein (MZ00003507), a pathogenesis related protein-1 - maize (MZ00004486), a hypothetical protein (MZ00022876) and an unknown protein (MZ00041708). The Gene Ontology (GO) functions of the genes were available in the probe dataset. However, no information for MZ00003507 was available and the nearest match obtained for this sequence was an RNA binding protein in *Arabidopsis thaliana*. Microarray data of our hybridization experiments were deposited to GEO (Gene Expression Omnibus) under accession number GSE72508.

qRT-PCR for data validation

Nine differentially expressed genes, including the four in common, were selected to validate the microarray analysis with qRT-PCR. Real Time PCR was performed on three independent biological replicates. The $\log_2$ average expression values of the qRT-PCR at time point 1 were correlated to the $\log_2$ values (M-values) of the microarray analysis for validation. Spearman's correlation (rho) for non-parametric distribution was derived from the square root of R^2, which was 88%. This strong correlation between the two expression profiling techniques assessed that microarray data were successfully validated by qRT-PCR.

qRT-PCR was also performed to investigate the expression pattern of the genes of interest in Picker and PR39B29 across five time points corresponding to number of days post germination. Gene expression was not maintained over the time (Figure 2), but was subjected to fluctuation. Except for RBP, the gene expression pattern was similar between the two varieties, in particular for PRP-1.

Discussion

Investigation on the cold tolerance in maize has mainly focused on the early phases of growth, as it is known that plant establishment is fundamental for the crop to reach maturity and maximum development. The photosynthetic apparatus is susceptible to low temperatures (Stamp, 1984), therefore most of the attention has been focused on the effect of cold on leaf development and on photosynthesis. When maize is grown at sub-optimal temperatures, impaired chloroplast function

through photo inhibition as well as altered pigment composition and chlorophyll deficiency result in a damaged photosynthetic apparatus (Greaves, 1996; Marocco, Lorenzoni & Fracheboud, 2005). Besides the photosynthetic performance of seedlings is influenced by the different cold tolerance in root and shoot development (Stamp, 1984; Tollenaar & Lee, 2002). Germination and the heterotrophic phase of development are also considerably impaired when soil temperatures are too low for a functional root system to establish.

Studies on roots are usually performed on field grown maize, although this approach is not free from flaws, mostly due to the destruction of root material during sampling. The analysis is usually done by image acquisition, which can be biased because of the background noise due to soil (Dong et al., 2003). Over the years, hydroponic cultivation (Sanguineti et al., 1998) or the use of sand columns (Ruta et al., 2010) have been adopted to grow roots under controlled environments, but the sampling still presents limitations. The acquisition of data on root traits can be carried out in several ways, from recording roots using a simple camera or photocopier (Liedgens & Riechner, 2001; Collins et al., 1987), a scanner (Dong et al., 2003; Hund, Trachsel & Stamp, 2009) to X-ray imaging techniques (Gregory et al., 2003). Root image analysis has progressed to the development of advanced software for the quantitative analysis or root growth and the architecture of complex root systems (Lobet, Pages & Draye, 2011). However, these techniques can be invasive and damage the root samples as well as reduce the sample size under consideration. It is therefore necessary to use techniques that are not destructive and take repetitive measurements of the traits of interest, giving temporal information about root growth over a certain period of time. The growth of maize seedlings on blotting paper for root development analysis used in this work has been used previously, with the specific aim to

develop a phenotyping platform for non-destructive and repeated measurements of root growth for mapping studies of QTLs (Hund, Trachsel & Stamp, 2009).

In maize, quantitative trait loci (QTL) were associated to cold tolerance in photosynthesis (Fracheboud, 2002, Revilla 2016) and roots, where morphological traits of root development were associated to resistance to cold stress (Hund et al., 2004; Presterl et al., 2007). However, to date, no investigation at gene expression level in chilling stressed maize roots has been carried out. Maize genotypes can be distinguished in cold tolerant and cold sensitive varieties. Whereas low temperatures seriously injure the cold sensitive varieties , the cold tolerant genotypes adjust their metabolism to adapt to the environmental conditions in which they grow through the activation of metabolic mechanisms that increase the content of specific molecules, like cryo-protective compounds and antioxidants, but they also involve the down-regulation of some other gene products (Janska et al., 2010). In our study, such down regulated transcripts were also found, e.g. a glucose starvation-induced protein precursor (Supplemental Table 4).

Root and shoot growth was carried out in controlled environmental chambers in a range of temperature regimes, which are in accordance with previous studies (Blacklow, 1972; Farooq et al., 2009; Morocco, Lorenzoni & Fracheboud, 2005). Germination requires a minimum temperature of approximately 10°C (Levitt, 1980) and cellular and tissue damage occur when temperature is below 5°C (Greaves, 1996). Prolonged exposure below this temperature can seriously injure seedlings that are no longer able to recover (Theocharis, Clement & Barka, 2012). Both the temperature regimes used in the present work represent chilling conditions, as chilling defines a temperature range between 5-15°C (Nguyen et al, 2009). It is important to emphasise that the genotypes used in this investigation are cold tolerant because of their ability

342 to reach maturity when soil temperature is still warm enough to not impair plant tissues and
343 compromise the subsequent growth phases and final yield (Keane, 2002).

344 The stress induced by chilling temperature in growth performance was evidently reflected on the
345 transcriptome expression of the primary root of the four genotypes that were profiled with the
346 microarray. Genotypes with contrasting root and shoot growth performance under chilling stress
347 can help identify transcriptomic differences and, therefore, narrow down the number of genes
348 that could play a dominant role in response to chilling temperatures. The number of these
349 dominant genes can be furtherly reduced if we narrow our scope to those with lower thermal
350 threshold and whose expression is significantly regulated in more than one variety.

351 Interestingly, the microarray analysis showed that the transcriptome expression was differently
352 regulated only in two (Picker and PR39B29) of the four varieties, precisely, the ones whose root
353 and shoot growth ratio was significantly higher, supporting the evidence that the regulation of
354 specific genes confers higher cold tolerance (Theocharis, Clement & Barka, 2012).

355 These results should not be unexpected as the growth temperatures used for this study were
356 suboptimal, meaning that common cold regulated genes like those controlling the
357 ICE_CBF/DREB1 regulatory pathways (Miura & Furumoto, 2013) could have been triggered in
358 both control and stress conditions and, therefore, no difference in expression could have been
359 detected by microarray for those genes. However, transcriptmic change was observed in two
360 varieties, meaning that these genes are probably regulated within a lower thermal threshold,
361 although none of the cold regulated genes that are frequently differentially expressed in response
362 to low temperature (Nguyen et al., 2009) were differentially expressed in the cold tolerant Picker
363 and PR39B29. Nevertheless, the analysis of these genes with Gene Ontology showed that the

majority of both the up and down regulated genes in two the varieties was involved in molecular functions involving proteins which have been shown to be induced by cold, like peroxidases and pathogenesis related proteins (ref, supplementary table with the list of all genes). Another significant aspect to consider is that the cold induced genes in the two varieties were not the same, with the exception of four genes (MZ00003507, MZ00004486, MZ00022876 and MZ00041708). The regulation of these four genes could represent a common response to cold stress and narrow down the gene pool responsibile for a greater cold tolerance.

The first of these four common genes encodes for the patogenesis related protein 1. PR-1 belongs to a group of PR-families of low-molecular proteins (6-43 kDa) that are present in all plant organs, like leaves, stems, flowers and roots (Edreva, 2005). PRs are constitutive expressed in plants and play a major role as preformed natural defence barrier (Edreva, 2005). However, PRs are also induced in response to several abiotic stressful environmental stimuli, including wounding and low temperatures (Van Loon, Rep & Pieterse, 2006). Nevertheless, specific gene expression patterns of these proteins in different tissues, cells, organs and developmental stages, suggest for them functions beyond defence, such as favouring seed germination in tobacco, embryogenesis in carrots and the transition to flowering and senescence (Edreva, 2005). Its role seems to be important for development and for coping with environmental stress.

The second shared gene encoded for a putative RNA binding protein. RBPs are known to be involved in the post-transcriptional regulation of RNAs, modulation of the expression pattern during development and in the adaption of plants in response to environmental stresses (Lorkovic, 2009). As per RP-1, the inability to regulate this gene at lower temperatures resulted

in poor growth. Nevertheless, the level of regulation of this gene is similar but opposite in the two cold tolerant genotypes, making its exact role in cold tolerance unclear.

The other two genes (MZ000022876 and MZ00041708) showed a similar response in the two varieties and at some extent also between them. These genes encode for hypotethical proteins whose function is unknown and PFAM analysis has failed to find a match for their protein sequences that could have helped understand their role in cold tolerance. If these are actually proteins, we might have identified novel cold induced transcripts in an already adapted maize breeding gene pool These genes could be the basis for more extensive research on transcripts involved in root tissue cold tolerance.

In order to understand how repression of RBP could induce cold tolerance, we have investigated the transcriptomic pattern of the four common genes over time by performing a qRT-PCR. The transcriptomic pattern of these genes showed that the expression was not maintained over time, but it fluctuated. Interestingly, the expression of RBP in PR39B29 went up, while for Picker went down. Even after this analysis the role of RBP remains to be established. As for the other genes, they showed a similar expression pattern between the varieties, suggesting a common way to respond to cold stress.

A practical outcome of this study, besides the in depth molecular insight into cold tolerance, is a practical metric to select germplams in cold tolerance screenings quickly and cheaply by using the growth ratio of root to shoots as demonstrated in this study. This metric differentiated more cold tolerant varieties very efficiently. This metric could inform breeding programmes where screening needs to efficiently happen at early stages of breeding generations in very large numbers.

407

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413 supervisor of MF.

Figures

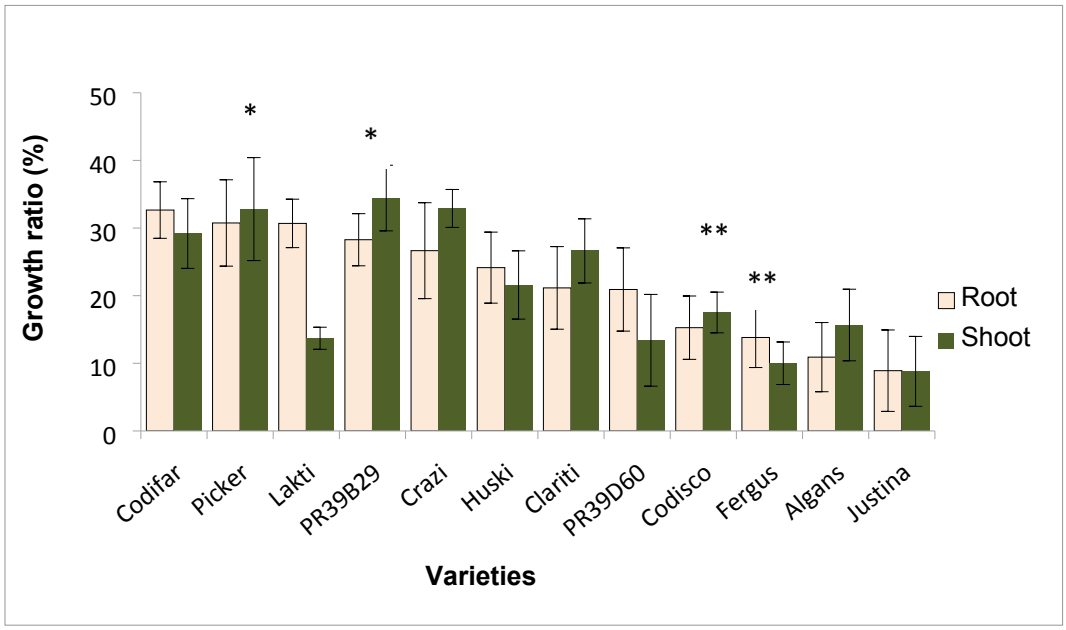
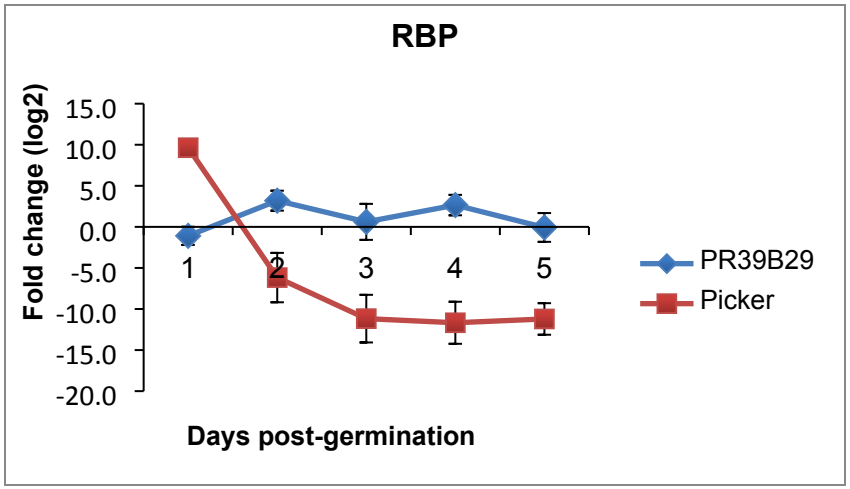
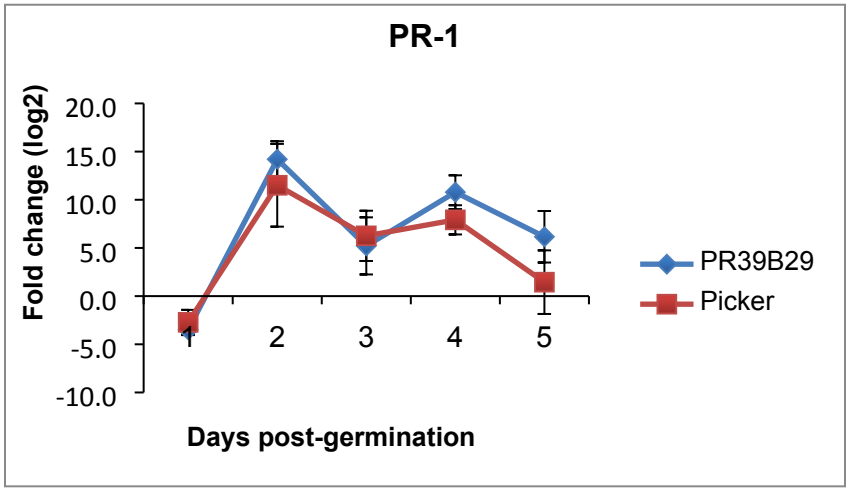


Figure 1. Growth ratio as response to cold stress. The growth ratio for roots and shoots was obtained by relating the average length of a root and shoot of cold treatment to the length of root and shoot of control, respectively. Tukey's range test was carried out to determine the two varieties with the highest combined root and shoot response to cold stress (indicated by *) and lowest combined root and shoot tolerance to cold stress (indicated by **). Note: varieties Algans and Justina were excluded from ranking list due to uneven germination rates. Vertical error bars represent mean ± SE (n=135). The difference between varieties was significant at $p<0.05$.



425



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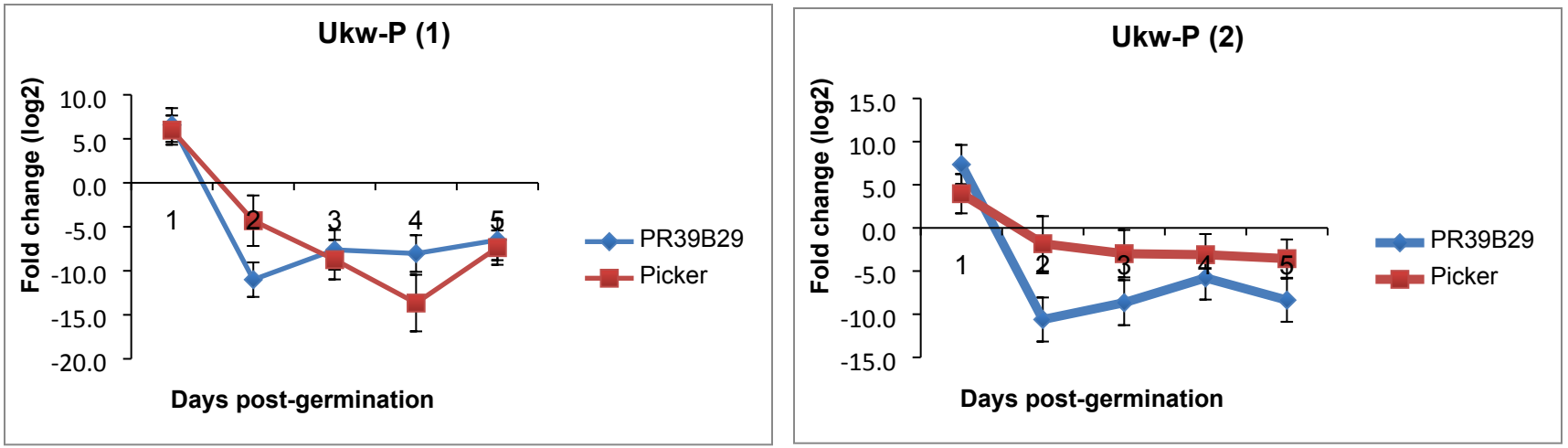

427

428 **Figure 2. Gene expression pattern of the significant four genes shared between the two most cold tolerant varieties Picker and**

429 **PR39B29.** Gene expression patterns were examined over five days post-germination, from day 1 to day 5. Vertical error bars represent

430 mean ± SE (n=3)

Tables

Table 1. Three-way Analysis of variance (ANOVA) tests of genotype, treatment and time point on growth in twelve maize varieties.

Effect	Root P value	Shoot P value
replicate	<.0001	<.0001
variety	<.0001	<.0001
treatment	<.0001	<.0001
variety *x* treatment	<.0001	<.0001
time point	<.0001	<.0001
variety *x* time point	<.0001	<.0001
treatment *x* time point	<.0001	<.0001
variety *x* treatment *x* time point	<.0001	<.0001

445 **Table 2. List of the nine most significant regulated genes in two cold tolerant maize varieties.** ID and Name are annotation of the
446 NSF Maize Oligonucleotide Array Project. MeanM and MeanA describe the average regulation and the average expression of each
447 gene, resulting from the mean of the values of the biological replicates. False discovery rate (FDR) with the Benjamini & Hochberg's
448 procedure accounted for the differential gene expression (FDR-adjusted p-value <0.05).

ID	Variety	Mean M	Mean A	Gene product	Abbreviation
MZ00003507	Picker	2.43	10.59	RNA binding protein *(Arabidopsis thaliana)*	RBP
	PR39B29	-2.78	10.81		
MZ00004486	Picker	-3.66	9.86	Pathogenesis related protein-1 – maize *(Zea mays)*	PR-1
	PR39B29	-2.42	9.35		
MZ00022876	Picker	2.61	8.66	Hypothetical protein (*Oryza sativa – japonica* cultivar-group)	Ukw-P (1)
	PR39B29	2.58	9.60		
MZ00041708	Picker	2.18	10.16	Unknown protein (*Oryza sativa – japonica* cultivar-group)	Ukw-P (2)
	PR39B29	2.46	13.30		
MZ00023411	PR39B29	2.46	11.87	22 kDa drought-inducible protein *(Saccharum hybrid* cultivar*)*	-
MZ00026737	PR39B29	2.55	11.62	Peroxidase *(Zea mays)*	-
MZ00029223	PR39B29	-2.47	11.38	Putative heat shock protein hsp22 precursor (*Oryza sativa – japonica* cultivar-group)	-
MZ00026029	Picker	-2.17	9.82	Probable lipid transfer protein – rice *(Oryza sativa)*	-
MZ00037140	Picker	-3.45	11.24	Glucose starvation-induced protein precursor (clone pZSS2) – maize *(Zea mays)*	-

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