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# Temperature at night affects the genetic control of acclimation to cold in maize seedlings

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## Abstract

Although suboptimal temperatures during maize (*Zea mays* L) seedling growth are known to result in decreased photosynthetic efficiency due to a combination of temperature and light stress, details remain scant on the impact of low night temperatures on photosynthetic activity. To better understand the role of night temperature on the acclimation of the photosynthetic apparatus to suboptimal temperature, a QTL experiment was conducted with the IBM302 population. Seedlings were grown under optimal temperature (24/22°C, day/night) or under suboptimal temperatures (17°C day and 6 or 13°C night). The two parental lines, B73 and Mo17, responded somewhat differently to suboptimal temperatures, as revealed by measurements of the operating quantum efficiency of PS II ($F_q'/F_m'$), the maximum quantum efficiency of PS II primary photochemistry ($F_v/F_m$) and leaf greenness (SPAD). While Mo17 showed very little change in response to the temperature at night, B73 exhibited a lower photosynthetic performance at 13°C than at 6°C at night. At 17/6°C the photosynthetic efficiency of both genotypes was similar. These observations were supported by QTL analyses. A major QTL for photosynthesis-related traits was detected on chromosome 5 with the favorable allele contributed by Mo17. This QTL showed a lower additive effect at a temperature of 6°C than at 13°C during the night and appeared to be the major factor explaining the differential response of the parental lines to changes in the temperature at night. As potential candidate genes for this locus, *ivr2* (coding for an acid vacuolar invertase) and *a2* (coding for an anthocyanidin synthase) were identified. QTL analyses for invertase activity and anthocyanin content revealed a QTL for invertase activity near the *ivr1* gene and a QTL for anthocyanin content close to the *r1* locus, both, however, were not related to the major QTL for photosynthesis-related traits. Comparative QTL analyses of photosynthetic traits of this population and other pulished studies revealed conserved QTL regions on chromosomes 6 and 8.

**Keywords**: chlorophyll fluorescence, cold acclimation, growth, QTL, *Zea mays*

## Introduction

Studies of plant acclimation to extreme environments are of outstanding importance in agriculture, especially for important crops like maize (*Zea mays* L). Temperature is one of the most important factors determining maize cultivation in temperate regions. Here, maize is cultivated between spring and autumn and is, consequently, mostly affected by low temperature during germination, hetero-trophic growth, early autotrophic growth and later during the grain filling period (Lee et al, 2002a; Ying et al, 2002). In North America and Europe, the optimal temperature for growth, which is between 20 and 30°C, is rarely reached during seedling establishment. Sudden drops in temperature, which frequently occur during early seedling development, have a negative impact on the physiology of maize (Foyer et al, 2002). Furthermore, suboptimal temperatures retard germination and the growth of maize seedlings. Chlorotic leaves are the most visible indications of malfunction of the chloroplasts in seedlings that develop at suboptimal temperature (Nie and Baker, 1991; Nie et al, 1995; Haldimann et al, 1996). This is accompanied by inhibition of photosynthesis due to perturbations of photosynthetic electron transport (Haldimann et al, 1996), the carbon reduction cycle (Kingston-Smith et al, 1997) and the control of stomatal conductance (Allen and Ort, 2001). These types of chilling-induced damage to the photosynthetic apparatus have been described as being useful markers for estimating the chilling tolerance of maize seedlings (Fracheboud et al, 1999).

The central role of photosynthesis within the context of chilling stress is based on the fact that low temperatures induce a light intensity stress. Because of a cold-induced reduction in the activity of the carbon cycle, only a small part of the absorbed light energy can be used to drive carbon fixation. Indeed, many cold-regulated genes are (thought to be) induced by following this increase in excitation energy (Ndong et al, 2001). In order to safely dissipate the excess excitation, plants have developed mechanisms to down-regulate photosystem II, in which the xanthophyll cycle pigments play an important role (Adams III et al, 2006). In maize seedlings, growth at suboptimal temperature results in the accumulation of xanthophyll-cycle pigments in the leaves, especially when

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they develop at a higher light intensity (Haldimann et al, 1995). Such chilling-acclimated leaves are characterized by a lower photosynthetic efficiency; they are, however, better able to withstand photoinhibition and photo-oxidative stress than leaves that develop under more favorable conditions (Haldimann et al, 1996; Leipner et al, 1997). Other important factors that are involved in the acclimation of maize seedlings to chilling-induced light stress are the accumulation of antioxidants, scavenging enzymes (Kocsy et al, 1996; Leipner et al, 1997; Kingston-Smith et al, 1999) and anthocyanin (Pietrini et al, 2002). In addition, temperature *per se* seems to have an effect as well. In particular, changes in the lipid composition of membranes are modulated by temperature (Nishida and Murata, 1996) and seem to be associated with the chilling tolerance of maize seedlings (Kaniuga et al, 1999).

Although it is well known that most of these acclimation mechanisms are controlled by both temperature and light intensity, little is known about how night temperature affects the long-term acclimation of maize to chilling stress. There are indications that reduced temperature at night aggravates cold-induced damage (Ying et al, 2000; Saropulos and Drennan, 2002) and strongly affects leaf growth by increasing cell cycle time and decreasing cell production (Rymen et al, 2007). The extent of these alterations seems to be genotype-dependent, as demonstrated in grapevine (Bertamini et al, 2007). On the other hand, acclimation at low temperature during the night can increase the chilling-tolerance of photosynthesis and seems to be even beneficial for photosynthesis at high temperature (Pittermann and Sage, 2001).

The analysis of quantitative trait loci (QTL) is a useful tool for locating genomic regions responsible for the expression of traits of interest, like chilling tolerance. Furthermore, it enables us to unravel the interaction of complex traits. A number of QTL studies focused on the chilling tolerance of maize seedlings (Fracheboud et al, 2002; Fracheboud et al, 2004; Hund et al, 2005; Jompuk et al, 2005; Leipner and Mayer, 2008; Pimentel et al, 2005; Presterl et al, 2007; Rodríguez et al, 2008). However, the effects of temperature at night on the long-term acclimation of maize seedlings to chilling have not been subjected to a QTL analysis. Consequently, this study aimed to determine i) chromosomal regions that are important in the adaptation of maize to chilling stress and ii) the possible influence of night temperature on cold-acclimation. To achieve these goals, the IBM302 maize population (Lee et al, 2002b; Sharopova et al, 2002) was studied for the presence of QTLs in seedlings grown at optimal temperature and seedlings grown at suboptimal temperature with two different chilling treatments during the dark phases.

## Materials and Methods

### *Plant material and growth conditions*

The two parental lines, B73 and Mo17, and 295 of their 302 intermated recombinant inbred (RI) lines, known as the IBM302 population (Lee et al, 2002b; Sharopova et al, 2002), were kindly provided by the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). The seeds were first multiplied by selfing the RI lines in the field in Switzerland during summer 2003. The resulting seeds were used in the experiments described here.

The seedlings were grown in two growth chambers (PGW36, Conviron, Winnipeg, Canada) in pots (10 × 10 × 10 cm) containing a commercial mixture of soil, peat and compost (Topf und Pikiererde 140, Ricoter, Aarberg, Switzerland). Three seeds were sown in each pot and thinned to one plant per pot after eight days. The plants were grown until the third leaf was fully expanded. The control plants grew for 14 days at 24/22°C (day/night) at a photoperiod of 12 h at 400 µmol quanta $m^{-2}$ $s^{-1}$ and a relative humidity of 60/70% (day/night). Chilling-treated plants were first grown for eight days as control plants and then for 20 days at 17/13°C (day/night) or 24 days at 17/6°C (day/night); the other conditions were the same as for the control plants.

One data set consisted of one plant of each of the 295 RI lines and six to eight plants of each parental line, all grown in the same chamber. For each temperature regime, the experiment was performed twice (in different growth chambers) on the whole population.

For the verification of candidate genes, the 295 RI lines of the IBM302 population were grown and re-scored as described above, at 24/22°C and 17/13°C (day/night).

### *Measurements of traits*

The chlorophyll fluorescence parameters were measured on the third fully expanded leaf with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) equipped with a leaf-clip holder (2030 B). The PS II operating efficiency ($F_q'/F_m'$) was measured under growth conditions according to Genty et al (1989) with the frequency of the measuring beam set to 20 kHz. The $F_q'/F_m'$ was calculated as $(F_m'-F')/F_m'$. The minimal fluorescence of dark-adapted leaves ($F_o$) was determined after 30 to 60 min in the dark at a measuring light frequency of 600 Hz. The maximal fluorescence ($F_m$) was then recorded during a one-second saturation flash (> 8000 µmol quanta $m^{-2}$ $s^{-1}$) at a measuring light frequency of 20 KHz. The measurements were started 3 hours after onset of the photoperiod, and the $F_q'/F_m'$ values of the whole population were determined within less than 3 hours; a significant shift of $F_q'/F_m'$ did not occur over time of measurements. The maximum quantum efficiency of PS II photochemistry ($F_v/F_m$) was $(F_m-F_o)/F_m$ and was determined after the measurement of $F_q'/F_m'$. The signal of the fluorometer was adjusted prior to the measurements with a fluorescent card (LI-COR, Lincoln, NE, USA) for later comparisons of $F_o$ and $F_m$ of the different

temperature treatments.

The leaf greenness was measured with an SPAD-502 (Minolta) instrument. Four measurements were made at random locations of the middle part of the third leaf of each plant and the average was calculated.

The shoots were cut at the coleoptilar node and dried for three days at 65°C to estimate the shoot dry matter. The growth rate was determined by dividing the shoot dry weight by the number of days of growth.

To determine the activity of soluble acid invertase, leaf samples were taken from the middle part of the third leaf at about 2 to 4 hours after onset of the photoperiod, immediately frozen in liquid nitrogen and stored at -80°C until assay. Frozen leaf samples were homogenized to a fine powder and extracted in 50 mM HEPES NaOH (pH 7.0), 10 mM $MgCl_2$, 1 mM Na-EDTA, 2.6 mM DTT, 0.02% (w/v) Triton. The extract was centrifuged for 5 minutes at 10,000 x g (4°C) and the supernatant used for acid invertase assay according to Pelleschi et al (1997). For the assay, 50 µl extract, 50 µl 0.2 M Na-acetate buffer (pH 4.8) and 20 µl 0.6 M sucrose were incubated for 4 hours at 30 °C. The reaction was stopped by adding 100 µl 0.5 M Na-phosphate buffer (pH 7.0) and the sample was heated to 80°C for 10 minutes. The concentration of glucose and fructose was determined by HPLC (Jasco, Tokyo, Japan) using a Waters Sugar-Pak™ 1 column (6.5 x 300 mm) and 0.1 mM Ca-EDTA as solvent. The injection volume was 20 µl, the flow rate 0.6 ml $min^{-1}$ and the column temperature 60°C. Glucose and fructose were detected using a refraction index detector (RI-930, Jasco, Tokyo, Japan) and quantified by peak area integration.

The content of anthocyanin was determined based on the method of Neff and Chory (1998). Tissue from the same leaves used for the invertase assay was homogenized to a fine powder and extracted in 600 µl methanol acidified with 1% HCl. After 3 hours of extraction, 400 µl water and 1000 µl chloroform were added, the sample vigorously mixed and centrifuged for 5 minutes at 10,000 x g (4°C). Total anthocyanins were determined by measuring the absorption at 530 nm of the aqueous phase (U-2000, Hitachi, Tokyo, Japan). To account for interference with chlorophylls, the absorption at 657 nm was measured and subtracted from the value of A530. The content of anthocyanin was calculated using an extinction coefficient of 29,600 L $mol^{-1}$ $cm^{-1}$ (Pietrini et al, 2002).

### *Data analysis*

The statistical analysis was made with the R 2.00 software (R Development Core Team, 2007, http://

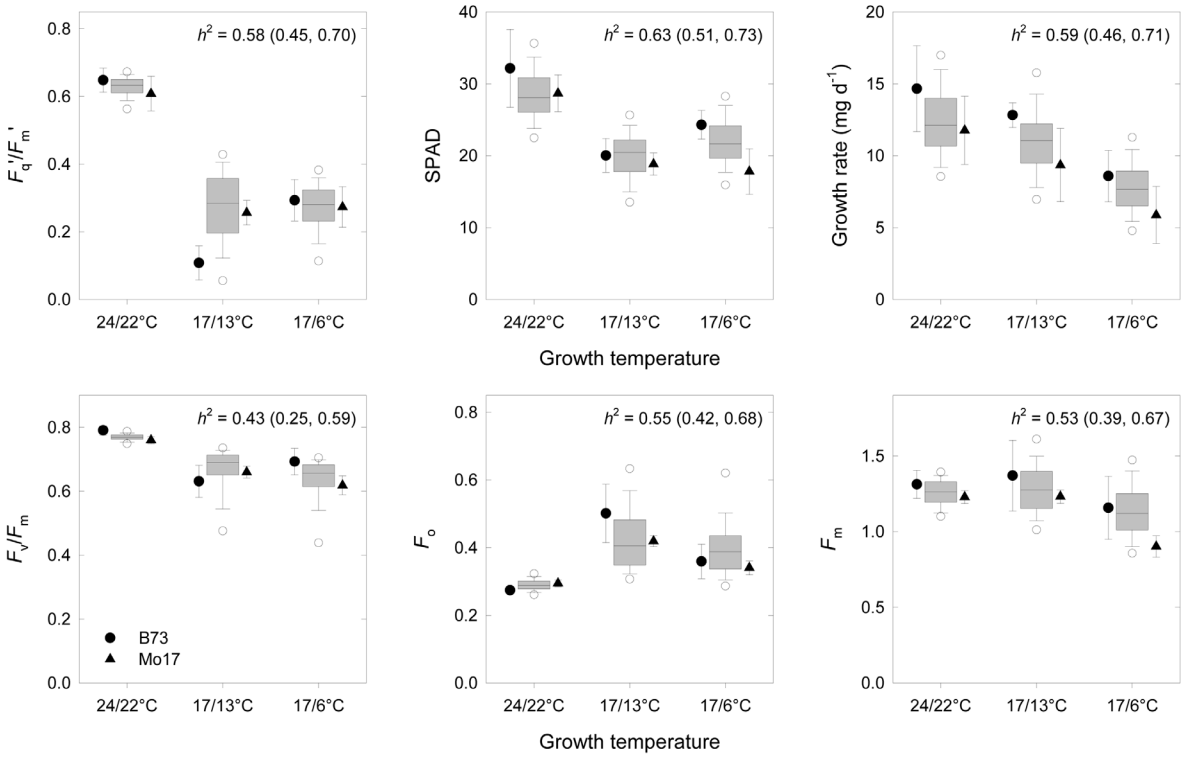


Figure 1 - Box-whisker plots for traits distribution. The horizontal lines near the centre of the boxes indicate the median values of the traits; the bottom and top sections of the boxes represent the values of the first and third quartiles. The whiskers at the top and bottom of the boxes indicate the 10th and 90th percentile. The 5th and 95th percentile is indicated by open circles. The heritability, $h^2$ (95% CI), based on the data of the RI lines is presented for each trait. The means (± SD) of the two parental lines B73 and Mo17 are indicated by closed circles and triangles, respectively.

Table 1 - ANOVA statistics of chlorophyll fluorescence parameters, leaf greenness (SPAD) and growth rate (GR) in the parental lines grown at 17/13 °C and 17/6 °C. *, **, *** indicate a significant effect at P < 0.05, 0.01 and 0.001, respectively; NS indicates that there is no significant effect.

| Factor | $F_q'/F_m'$ | $F_v/F_m$ | $F_o$ | $F_m$ | SPAD | GR |
|---|---|---|---|---|---|---|
| Genotype | ** | * | * | * | *** | *** |
| Temperature | *** | NS | *** | ** | NS | *** |
| Geno. × Temp. | *** | *** | NS | NS | * | NS |

www.r-project.org). The data for all the traits were corrected by adjusting the means of the two data sets.

The broad-sense heritability ($h^2$) was estimated over the two repetitions as described by Hallauer and Miranda (1981). Confidence intervals of heritability were calculated according to Knapp et al (1985).

The genetic map of the IBM302 population was based on the genotyping data of 1339 markers available from the Maize Mapping Project (http://www.maizemap.org). The distances between the markers were those in the frameworks that are available from the maize mapping project.

For the QTL analysis, outliers were removed from the dataset. The QTLs were identified by composite interval mapping using QTL Cartographer 1.17 (Basten et al, 1994), model 6 (Basten et al, 2005), with a blocking window size of 30 cM with the in and out thresholds set at a p-value of 0.01 using the QTL Cartographer source files for the IBM302 population deposited at the Maize Genetics and Genomics Database (http://www.maizegdb.org/qtl-data.php). The presence of a QTL was considered to be significant in single trait analysis when the likelihood of odds (LOD) value was larger than the critical value determined empirically by 1,000 permutations with a significant level of $\alpha = 0.05$ (Churchill and Doerge, 1994). The results of the composite interval mapping were verified by bootstrap analysis, conducting 200 bootstrap resamplings with the QTL Cartographer 1.17, as well as by joint analysis of both data sets using the *Jzmapqtl* procedure of QTL Cartographer 1.17.

## Results

### *Effects of growth temperature*

To better understand how the temperature at night influences the photosynthetic activity of maize seedlings, seedlings were examined at two low-temperature regimes. Seedlings grown at 17/13°C or 17/6°C were characterized by a lower PS II operating efficiency ($F_q'/F_m'$) and a lower maximum quantum efficiency of PS II photochemistry ($F_v/F_m$), paler leaves (SPAD) and a slower growth rate than seedlings that developed at 24/22°C (Figure 1). The decrease in $F_v/F_m$ was due to an increase in the 'dark' level of the chlorophyll fluorescence ($F_o$) in seedlings grown at suboptimal temperatures (17/13°C or 17/6°C) and, additionally, due to a decrease in the maximal fluorescence ($F_m$) in seedlings exposed to 6°C at night (Figure 1).

In leaves of plants grown at 24/22°C, the trait values of both parental lines were similar. However, B73 was characterized by a slightly but significantly higher $F_v/F_m$, caused by a lower $F_o$, compared to Mo17. The traits differed markedly when seedlings were grown at suboptimal temperatures. The ANOVA of the effect of night temperature on the two parents grown at suboptimal temperatures indicated that the $F_q'/F_m'$, $F_v/F_m$ and SPAD of the two parental lines responded differently (Table 1). For $F_v/F_m$ and SPAD, B73 showed significantly higher values than Mo17 when the seedlings were grown at 17/6°C. The PS II operating efficiency ($F_q'/F_m'$) was also higher in B73 than in Mo17 at 17/6°C, but was significantly lower when seedlings were grown at 17/13°C. Similarly, $F_v/F_m$ was higher for Mo17 compared to B73 at 17/6°C. This suggests that the decrease in the photosynthetic performance, caused by the suboptimal growth temperature, was minimized in B73, but not in Mo17, due to the low temperature at night.

In the RI population, lowering the temperature at night from 13°C to 6°C significantly decreased the medians of $F_v/F_m$, $F_m$ as well as the growth rate of the plants; it increased leaf greenness but had no influence on $F_q'/F_m'$ and $F_o$ (Figure 1). The result was the same for the means of the traits, as revealed by t-tests (data not shown). All traits showed moderate values (around 0.5) for broad sense heritability ($h^2$), with values somewhat lower for $F_v/F_m$ ($h^2 = 0.43$) and higher for SPAD ($h^2 = 0.63$) (Figure 1). The correlation between replications was significant ($p < 0.1$) for all traits.

### *Quantitative trait loci analysis*

To investigate the genetic basis of the effect of temperature at night on cold acclimation, QTL mapping of the IBM302 RI population was carried out (Table 2). Figure 2 presents a map of the LOD scores. In total, 28 QTLs were detected above the LOD threshold of $\alpha < 0.05$, which was empirically determined by a permutation test for each trait at each environment and corresponded to a LOD score of around 6. Most of these QTLs could be confirmed by bootstrap analysis and joint analysis of both repetitions (Table 2). The number of QTLs was higher in seedlings grown at suboptimal temperatures, especially when they developed at 17/13°C, than in plants grown at 24/22°C. Whilst QTLs were found for all photosynthesis-related traits, the analysis did not reveal any significant QTL

Table 2 - Main characteristics of QTLs for PS II operating efficiency (Fq'/Fm'), maximum quantum efficiency of PS II photochemistry (Fv/Fm), minimal fluorescence (Fo), maximal fluorescence (Fm) and leaf greenness (SPAD) with a LOD score above a threshold of $\alpha < 0.05$ identified in the IBM302 population grown under different temperature regimes.

| Trait | °T | Chr. | cM | Range | Nearest marker | LOD | R2 | Add. | QTL validation Bootstrap LOD (cM) | Joint LOD (cM) |
|---|---|---|---|---|---|---|---|---|---|---|
| $F_q'/F_m'$ | 24/22 °C | - | - | - | - | - | - | - | - | - |
| | 17/13 °C | 5 | 254 | 240-265 | php15024 | 25.86 | 23.0 | 0.051 | 21.3 (255) | 19.2 (255) |
| | | 7 | 161 | 147-193 | uaz187 | 8.75 | 6.3 | 0.028 | 7.2 (168) | 4.8 (161) |
| | 17/6 °C | 5 | 257 | 249-265 | ufg60 | 8.90 | 9.2 | -0.021 | 8.9 (255) | 11.9 (257) |
| $F_v/F_m$ | 24/22 °C | 1 | 696 | 677-739 | umc1446 | 7.16 | 7.2 | 0.003 | 10.4 (689) | 5.2 (714) |
| | 17/13 °C | 5 | 255 | 246-271 | php15024 | 18.87 | 18.3 | -0.025 | 20.0 (257) | 15.9 (257) |
| | | 9 | 411 | 399-432 | mmp132 | 6.19 | 6.4 | -0.014 | 6.3 (409) | 3.2 (420) |
| | 17/6 °C | - | - | - | - | - | - | - | - | - |
| $F_o$ | 24/22 °C | 6 | 395 | 393-408 | umc2170 | 9.35 | 9.7 | 0.005 | 5.3 (393) | 7.3 (397) |
| | | 6 | 424 | 416-430 | umc1490 | 11.97 | 9.3 | 0.006 | 5.7 (422) | 15.0 (424) |
| | | 8 | 250 | 242-259 | umc1457 | 23.55 | 19.6 | -0.008 | 13.3 (250) | 18.0 (249) |
| | 17/13 °C | 5 | 255 | 249-265 | php15024 | 20.80 | 18.9 | 0.039 | 10.6 (259) | 13.0 (254) |
| | | 5 | 360 | 347-374 | umc2026 | 7.15 | 6.3 | -0.022 | 3.3 (362) | 0.2 (351) |
| | | 6 | 483 | 472-487 | agp2 | 7.76 | 7.4 | 0.024 | 5.3 (483) | 4.2 (483) |
| | | 7 | 159 | 144-171 | bnlg1094 | 9.08 | 7.2 | -0.024 | 5.4 (165) | 2.9 (132) |
| | 17/6 °C | 5 | 254 | 246-261 | php15024 | 12.47 | 12.5 | 0.028 | 8.1 (255) | 1.9 (255) |
| | | 6 | 487 | 483-492 | umc2059 | 9.08 | 7.9 | 0.022 | 5.7 (487) | 1.4 (487) |
| $F_m$ | 24/22 °C | 6 | 422 | 414-436 | umc1490 | 10.19 | 13.7 | 0.033 | 4.2 (422) | 9.4 (426) |
| | | 8 | 250 | 242-259 | umc1457 | 18.63 | 20.0 | -0.042 | 10.3 (250) | 18.7 (250) |
| | 17/13 °C | 6 | 424 | 418-440 | umc1490 | 9.15 | 8.5 | 0.048 | 3.2 (426) | 6.6 (424) |
| | | 6 | 453 | 445-455 | umc1350 | 6.83 | 7.1 | 0.045 | 3.4 (449) | 3.2 (455) |
| | 17/6 °C | 4 | 180 | 165-188 | mmp111 | 6.17 | 7.1 | 0.046 | 10.1 (168) | 2.1 (168) |
| | | 7 | 242 | 218-250 | umc116a | 7.06 | 7.2 | 0.048 | 8.8 (224) | 1.8 (246) |
| | | 8 | 196 | 161-213 | mmp72 | 6.18 | 6.4 | -0.045 | 7.1 (180) | 3.1 (209) |
| SPAD | 24/22 °C | 2 | 306 | 286-323 | mmp119 | 6.37 | 6.6 | -0.9 | 8.2 (308) | 0.8 (305) |
| | 17/13 °C | 5 | 263 | 228-267 | bnlg1902 | 15.80 | 16.1 | -1.2 | 14.2 (263) | 9.5 (263) |
| | | 5 | 289 | 276-315 | bnlg1208 | 6.56 | 5.1 | -0.8 | 5.0 (287) | 10.3 (306) |
| | 17/6 °C | 4 | 373 | 357-389 | umc66 | 9.38 | 10.7 | 1.1 | 7.2 (368) | 5.4 (368) |
| | | 5 | 218 | 188-233 | umc1447 | 6.26 | 6.4 | -0.8 | 8.4 (220) | 2.5 (230) |
| | | 7 | 139 | 128-169 | gta101a | 6.23 | 5.6 | 0.8 | 7.4 (139) | 1.1 (161) |

°T = Growth regime; Chr. = Chromosome number; cM = Position of the peak of the QTL in centimorgan; Range = Range was defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half; $R^2$ = Percentage of the phenotypic variance explained by genotype class at LOD peak; Add. = Additivity (positive additivity = high values of the trait were inherited from B73; negative additivity = high values of the trait were inherited from Mo17); Bootstrap (cM) = LOD score in the bootstrap analysis including the position of the peak in centimorgan; Joint (cM) = LOD score in the joint analysis of both replications including the position of the peak in centimorgan

for the rate of shoot growth in the three temperature regimes. However, some peaks with a LOD around 4.0 to 4.5 were detected (Figure 2).

In plants grown at 17/13°C, PS II efficiencies $F_q'/F_m'$ and $F_v/F_m$ as well as the minimal fluorescence ($F_o$) were mainly controlled by one QTL, which was detected on chromosome 5 at ~255 cM. It explained about 20% of the phenotypic variance for these traits. The indication of additivity showed that Mo17 carried the favorable allele at this locus. This QTL was also present in seedlings that were grown at 17/6°C, but additive effects and phenotypic variance were considerably smaller compared to the situation in seedlings grown at 17/13°C. In plants grown at 24/22°C, this QTL was not significant.

Two independent QTLs were likely present on chromosome 6 between 420 and 490 cM, which could account for the genotypic variations in chlorophyll fluorescence. For both QTLs, the favorable allele was inherited from Mo17. The QTL at 424 cM was found in seedlings grown at optimal and suboptimal temperatures. In contrast, the QTL at about 480 cM was present only in plants that developed at suboptimal temperatures.

A strong QTL on chromosome 8 at 250 cM was detected for the fluorescence levels $F_m$ and $F_o$ in plants grown at 24/22°C, but not in plants grown at suboptimal temperatures. This QTL explained about 20% of the phenotypic variance. High trait values were inherited from Mo17.

### *Identification and testing of candidate genes*

Potential candidate genes were searched for the major QTL on chromosome 5. The positions of genes and markers associated with genes, which are located in the QTL region between mmp108a and mmp58, were obtained from the Maize Genetics and Genomics Database (http://www.maizegdb.org). Based on their position and their presumable function in the response to chilling stress, the invertase gene *ivr2* and the locus anthocyaninless2 (*a2*), coding for an anthocyanidin synthase, were identified as potential candidate genes. In order to examine whether the gene of one of these enzymes was responsible for the expression of the QTL on chromosome 5, the activity of soluble acid invertase and the amount of anthocyanin was analyzed in the RI lines of the IBM302 population

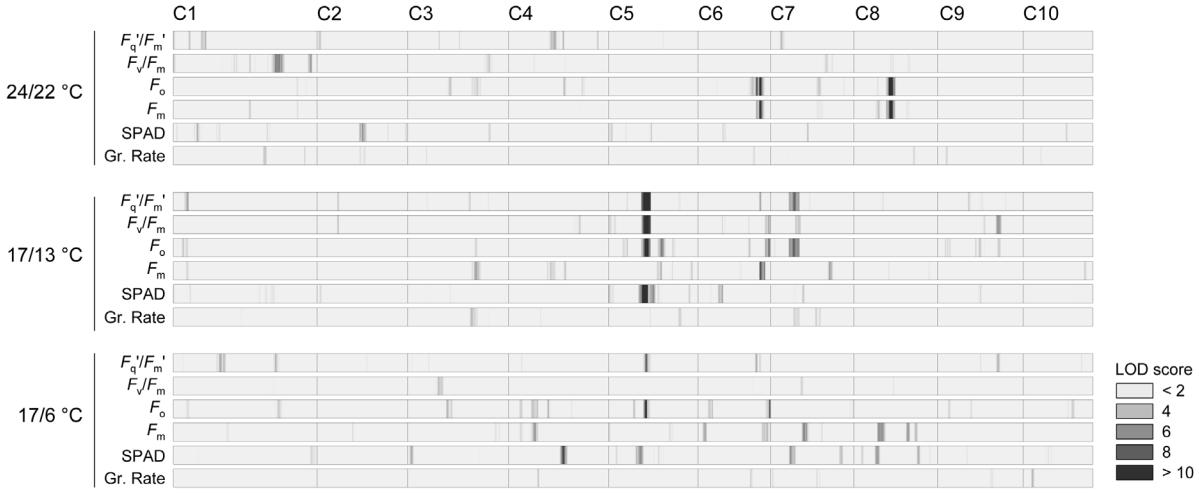


Figure 2 - Image of LOD scores for traits analyzed in the IBM302 population grown under different temperature regimes along the 10 maize chromosomes (C1-C10). The positions of the chromosomes are indicated along the X axes. The grey scale bar show the range LOD values from ≤ 2 (white) to ≥ 10 (black).

grown at suboptimal (17/13°C) and optimal temperature (24/22°C) and QTL analyses were conducted using a LOD threshold of $\alpha = 0.1$ determined by permutation test.

The activity of soluble acid invertase was lower in seedlings grown at 17/13 °C than in plants that developed at 24/22°C, on average 0.47 versus 0.58 µmol $m^{-2}$ $s^{-1}$ in the RI lines. The QTL analysis revealed that the QTL region on chromosome 5 was not involved in genotypic differences of invertase activity. As a control, the correlation between the chromosome 5 QTL and PS II efficiency was verified in this experiment (data not shown). Two major QTLs for soluble acid invertase activity were also detected on chromosomes 2 and 3 in seedlings grown at suboptimal temperature (Table 3). Although the QTL for invertase activity on chromosome 2 was more significant at 17/13°C than at 24/22°C, it did not co-localize with QTLs for chilling-tolerance of photosynthesis. These data show that the *ivr2* gene is not involved in protection from chilling stress under the experimental conditions.

The analysis of the anthocyanin content in the IBM302 population showed that suboptimal temperatures induced an accumulation of anthocyanin. In the RI lines, the anthocyanin content was in average 3.0 µmol $m^{-2}$ at 24/22°C and 8.0 µmol $m^{-2}$ at 17/13 °C. The QTL analysis revealed a major QTL for the content of anthocyanin in seedlings grown at suboptimal temperatures, which, however, was not localized on chromosome 5, but on chromosome 10 near marker bnl10.13a (Table 3). No significant QTL for the content of anthocyanin was detected in plants that developed at optimal temperatures.

## Discussion

### *Effects of night temperature on QTL expression*

Our study aimed to elucidate the effect of night temperature on the chilling acclimation of maize seedlings and to determine its genetic basis by QTL analyses. A decrease in temperature, from 24/22°C to 17/13°C, caused a reduction in photosynthetic performance, and in particular reduced the PS II operating efficiency ($F_q'/F_m'$) in genotype B73. The decrease of $F_q'/F_m'$ in B73 was less pronounced when the night temperature was 6°C. This genotype-dependent effect of night temperature on photosynthetic performance was also disclosed by comparing QTLs from both low-temperature stress conditions (17/13°C and 17/6°C). Even though there were no specific QTLs for $F_q'/F_m'$ and $F_v/F_m$ at 17/6°C, the additivity of the main QTL at ~255 cM on chromosome 5 of plants grown at 17/13°C was reduced by half at 17/6°C. Due to the lack of QTLs expressed solely at the lower night temperature (17/6°C), but not at 17/13°C, the QTL on chromosome 5 seems to be the major factor explaining the lower PS II efficiency of B73 compared to Mo17 at 17/13°C, whilst both genotypes exhibited a similar $F_q'/F_m'$ at 17/6 C. However, this does not explain why $F_q'/F_m'$ in B73 was lower at 17/13°C than at 17/6°C. Similar to the response of B73, a lower night temperature had a positive effect on photosynthesis in the $C_4$ grass *Muhlenbergia montana* (Pittermann and Sage, 2001); a lower $CO_2$ assimilation rate was found in plants grown at 24/16°C (day/night) than at 24/4°C, in particular when photosynthesis was measured at high temperature. The physiological cause of this behavior still remains to be elucidated. In maize, it appears that an unknown factor reduces the PS II operating efficiency in both genotypes at 17/13 °C compared to 17/6 °C, however, the Mo17 allele at the QTL on chromosome 5 seems to allow better com-

Table 3 - Main characteristics of QTLs for soluble acid invertase activity ($\mu$mol $m^{-2}$ $s^{-1}$) and anthocyanin ($\mu$mol $m^{-2}$) content with a LOD score above a threshold of $\alpha < 0.1$ identified in the IBM302 population grown under different temperature regimes.

| Trait | °T | Chr. | cM | Range | Nearest marker | LOD | $R^2$ | Add. |
|---|---|---|---|---|---|---|---|---|
| Invertase | 24/22°C | 2 | 205 | 191-209 | mmp42 | 5.96 | 8.6 | -0.079 |
| | 17/13°C | 2 | 205 | 198-211 | mmp42 | 15.18 | 14.2 | -0.091 |
| | | 3 | 553 | 539-563 | umc1273 | 10.90 | 12.3 | 0.082 |
| Anthocyanin | 24/22°C | - | - | - | - | - | - | - |
| | 17/13°C | 10 | 317 | 305-330 | bnl10.13a | 11.07 | 18.0 | -1.36 |

°T = Growth regime; Chr. = Chromosome number; cM = Position of the peak of the QTL in centimorgan; Range = Range was defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half; $R^2$ = Percentage of the phenotypic variance explained by genotype class at LOD peak; Add. = Additivity (positive additivity = high values of the trait were inherited from B73; negative additivity = high values of the trait were inherited from Mo17)

pensation for the loss of photosynthetic efficiency.

### *In-depth analysis of the major QTL for chilling tolerance of photosynthesis*

The major QTL on the short arm of chromosome 5 at about 255 cM was detected for most of the experimental traits ($F_q'/F_m'$, $F_v/F_m$, $F_o$ and SPAD) in plants grown at 17/13°C and showed a considerable phenotypic variance with regard to chlorophyll fluorescence parameters. Therefore, it is considered to be a major locus for chilling tolerance of photosynthesis in this population. Based on their position and their potential involvement in chilling acclimation, the invertase gene *ivr2* and the locus *anthocyaninless2* (*a2*), coding for an anthocyanidin synthase, were identified as candidate genes for this QTL. Besides these two genes, there was another interesting candidate gene within the QTL region. This gene was associated with the SNP marker pza01303 and codes for the *light-harvesting like 3* (*Lil3*) protein. The *Lil3* is supposed to be a temporary chlorophyll storage (Reisinger et al, 2008); its role seems to be in the prevention of the generation of potentially harmful oxygen species by free chlorophyll. Furthermore, the EST p-csu774, which shows high similarity with the maize chlorophyll a/b binding protein 6A gene, is present in this region. However, this EST is located more proximal from the QTL position for $F_q'/F_m'$ and $F_v/F_m$; it lies closer to the peak of the QTL for leaf greenness (SPAD) at 263 cM, which was identified in seedlings grown at 17/13°C (Table 2).

The rational for choosing the *ivr2* gene, which codes for the *vacuolar acid invertase 2*, as a potential candidate gene was that the vacuolar acid invertase is involved in soluble sugar metabolism and has been found to be induced in maize under drought stress conditions (Pelleschi et al, 1999). Furthermore, a cold-induced increase of the activity of acid invertase has been observed in wheat leaves (Vargas et al, 2007). On one side, the stress-responsiveness of invertase seems to be based on the fact that glucose, which is the product of sucrose cleavage by invertase, acts as a signaling molecule capable of inducing stress-responsive genes; on the other side, invertase plays a central role in the overall adjustment of carbohydrate metabolism, which is usually strongly affected when plants are exposed to stress conditions (Roitsch and González, 2004). Analysis of soluble acid invertase in the IBM302 population showed that its activity is lower in seedlings grown at suboptimal temperatures, as it was found also in cold-acclimated Arabidopsis (Klotke et al, 2004). The QTL analysis of the IBM302 population has shown that there is no QTL for soluble acid invertase activity on chromosome 5 and no co-localization of QTLs for chilling-tolerance of photosynthesis and invertase activity, indicating a minor role of soluble acid invertase for genotypic differences in chilling tolerance of maize seedlings. However, a major QTL for soluble acid invertase activity was found on chromosome 2 at marker mmp42. Aligning the genetic map of the IBM302 population with the IBM2 2008 Neighbors map showed that this marker is only 11 cM away from the *ivr1* gene. This makes it very likely that *ivr1* underlies the QTL for invertase at chromosome 2. Consequently, the IBM302 mapping population seems to be of high value for further analysis of the genetic control of the *ivr1* locus and the function of its gene product. This is underlined by the fact that this genomic region harbors QTLs affecting amylopectin and starch content in another maize mapping population (Séne et al, 2000).

Similar to *ivr2*, the locus *anthocyaninless2* (*a2*) is directly located at the position of the major QTL on chromosome 5. The *a2* gene, which codes for anthocyanidin synthase, a key enzyme of the anthocyanin synthesis, was found to be strongly induced in the cold (Christie et al, 1994). However, QTL analysis revealed no significant QTLs for anthocyanin content in the region of the major QTL for chilling tolerance of photosynthesis, ruling out that *a2* is the underlying gene of the QTL for photosynthesis-related traits on chromosome 5. As for the locus *a2*, the positions of several other genes involved in the accumulation of anthocyanin are known. Among these genes the locus *r1*, which shares sequence similarity with the MYC class of DNA binding proteins (Chandler et al, 1989) and which is known to be induced by cold (Christie et al, 1994), is located close to the QTL found for the content of anthocyanin on chromosome 10 and might

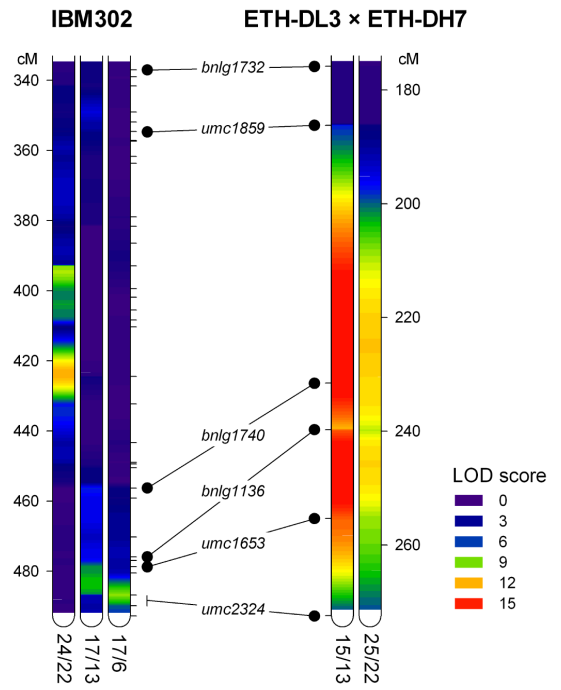


Figure 3 - Comparison of QTLs for the minimal fluorescence ($F_o$) in the telomeric region of the long arm of chromosome 6 in the IBM302 and ETH-DL3 × ETH-DH7 populations. The position (in cM) and the markers are given in the maps. Matching markers between populations are connected by lines. The colour scale bar show the range LOD values from 0 (blue) to ≥ 15 (red).

be, therefore, the underlying gene. Since this QTL for the content of anthocyanin was not associated with a QTL for chilling tolerance of photosynthesis, the role of anthocyanin in the acclimation to suboptimal temperatures seems to be insignificant, at least in the investigated maize material and under the experimental conditions.

*Comparative mapping*

The conservation of QTLs in different mapping populations exposed to similar stress conditions indicates the importance of specific genomic regions in the plant's response to stress. The QTLs found in IBM302 were compared with QTLs for chilling tolerance described in other studies and mostly in other mapping populations (Fracheboud et al, 2002; Fracheboud et al, 2004; Jompuk et al, 2005; Presterl et al, 2007; Rodríguez et al, 2008). The analysis revealed some co-localizations of QTLs between these populations. A QTL for leaf chlorosis and frost damage, which was identified in the SL × TH dent mapping population grown under field conditions in Germany and France (Presterl et al, 2007), overlapped with the major QTL on chromosome 5 found in the IBM302 population. No co-localizations were found between the QTLs detected in the present study and QTLs found under similar growth conditions in the Ac7643 × Ac7729 population (Fracheboud et al, 2002) and in the IBM302 population, which was, however, examined at an earlier growth stage (Rodríguez et al, 2008). By comparing the present results with the QTLs detected in the ETH-DL3 × ETH-DH7 mapping population (Fracheboud et al, 2004; Jompuk et al, 2005), two chromosomal regions were found, which showed an overlap of QTLs for similar traits. One of these chromosomal regions is located on chromosome 8 and was found to be significantly involved in the level of fluorescence ($F_o$ and $F_m$) at optimal temperature in the IBM302 population and, in the ETH-DL3 × ETH-DH7 population, in $F_o$ at 15°C (Fracheboud et al, 2004) and in $F_m$ under cool field conditions (Jompuk et al, 2005).

The major QTL for chilling tolerance of photosynthesis in the ETH-DL3 × ETH-DH7 population, which was detected in the telomeric region of the long arm of chromosome 6, seems to be located in the same region as the QTLs for $F_o$ and $F_m$ found in the IBM302 population. Re-analysis of the data from Fracheboud et al (2004) with an improved genetic map and alignment of the genetic maps of chromosome 6 from the ETH-DL3 × ETH-DH7 and the IBM302 population showed that the QTL for $F_o$, which was found in the IBM302 population grown at optimal temperature, corresponded to the QTL for $F_o$ (and other photosynthesis-related traits) found in the ETH-DL3 × ETH-DH7 population grown at suboptimal temperatures (Figure 3). The re-analysis of the ETH-DL3 × ETH-DH7 data revealed a second QTL close to the telomere that was in the same chromosomal region as the QTL for $F_o$ found in the IBM302 population grown at suboptimal temperatures (17/13°C and 17/6°C). The parameter $F_o$ is of great interest, since it is interpreted to be a good indicator of the integrity of the PS II reaction centre (Baker, 2008). Consequently, it demonstrates that this QTL region seems to be involved in the development of functional photosynthetic machinery under low temperatures. Due to the strong expression of this QTL for traits reflecting the integrity of the photosynthetic apparatus, genes, which seem to be directly involved in the photosynthetic light reaction, are likely to reflect the molecular basis of this QTL. Based on the previous and the present results, the search for candidate genes at the telomeric region of the long arm of chromosome 6, which could explain the observed phenotype, revealed *cab-m7* as a potential candidate gene. *Cab-m7* codes for the LHCII protein Lhcbm7 and is preferentially expressed in maize mesophyll cells and is strongly induced upon illumination (Becker et al, 1992). Co-localization of *Lhcb* genes with QTLs for cold-induced photoinhibition was also reported in another study of maize QTLs (Pimentel et al, 2005).

It is noteworthy that, although both B73 and Mo17 are not well adapted to chilling conditions, it was possible to find common QTLs with other mapping populations but at a much higher resolution, in contrast to the Swiss dent population (ETH-DL3 × ETH-DH7), in which one of the parents was characterized by a high

chilling tolerance. The comparison between cold-adapted and non-adapted genotypes may assist us in detecting genomic regions that have not yet been improved in the maize plant for adaptation to chilling stress. These regions could then be considered for a more exhaustive screening of different maize genotypes to obtain the best alleles in maize. Moreover, comparative mapping with more cold tolerant plant species, such as barley or wheat, would enable the identification of possible candidate genes for gene transfer to elite maize genotypes.

This study brings new insights into the complexity of studying the tolerance of maize to chilling stress. This is emphasized by the different genetic responses of photosynthetic traits when plants are exposed to different night temperatures. Therefore, low night temperatures must be considered when studying the acclimation of the photosynthetic apparatus of maize to chilling stress. The comparative QTL analysis of different mapping populations revealed chromosomal regions that are important for future research. Determining the gene(s) that explain the QTLs is relevant for a better understanding of the adaptation of maize to chilling stress and to the production of new genotypes with improved tolerance to chilling stress.

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