Running title: Low temperature affects cell cycle in maize leaves

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Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes\textsuperscript{[1]}

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ABSTRACT

Low temperature inhibits the growth of maize (*Zea mays*) seedlings and limits yield under field conditions. To study the mechanism of cold-induced growth retardation, we exposed maize B73 seedlings to low night temperature (25/4°C; d/n) from germination until the completion of leaf four expansion. This treatment resulted in a 20% reduction in final leaf size compared to control conditions (25/18°C; d/n). A kinematic analysis of leaf growth rates in control and cold-treated leaves during daytime showed that cold nights affected both cell cycle time (+65%) and cell production (-22%). In contrast, the size of mature epidermal cells was unaffected.

To analyze the effect on cell cycle progression at the molecular level, we identified through a bioinformatics approach a set of 43 cell cycle genes and analyzed their expression in proliferating, expanding and mature cells of leaves exposed to either control or cold nights. This analysis showed that: (i) the majority of cell cycle genes had a consistent proliferation-specific expression pattern; (ii) the increased cell cycle time in the basal meristem of leaves exposed to cold nights was associated with differential expression of cell cycle inhibitors and with the concomitant down-regulation of positive regulators of cell division.
INTRODUCTION

The growth of maize (*Zea mays*) seedlings is sensitive to low temperature, particularly during early spring in northern European and American environments. Delayed sowing to avoid this problem, reduces the length of the growth season and the potential yield (Lyons, 1973; Graham and Patterson, 1982). Improved performance at low temperature would result in greater light interception early in the growth season and increase productivity through a timely onset of grain filling (Dugan, 1944; Iba, 2002). Breeding programs for cold tolerant genotypes can benefit from improved knowledge of the plant’s growth response to low temperature at the cellular and molecular level (Wang et al., 2003).

Cold in early spring has several distinct effects on the establishment of maize seedlings in the field. Severe cold (chilling stress) impairs chloroplast function thereby reducing photosynthetic capacity (Allen and Ort, 2001), and may result in cell death (Gomez et al., 2004). Prolonged chilling stress eventually may arrest growth. Interestingly, even before visible chilling effects occur, suboptimal temperature inhibits leaf growth (Ben-Haj-Salah and Tardieu F, 1995). Monocotyledonous leaves have persistent developmental gradients along the leaf axis, with proliferating, expanding and mature cells located at increasing distance from the leaf base (Fiorani and Beemster, 2006). In different organs and species, the responses to unfavorable environmental conditions are a consequence of inhibition of both cell proliferation and cell expansion (Pahlavanian and Silk, 1988; Beemster et al., 1996; Schuppler et al., 1998; Tardieu et al., 2000; Sharp et al., 2004). Although we know the basic molecular control mechanisms of the cellular growth processes, cell proliferation and expansion, (for reviews see e.g. Fry, 2004; Inzé, 2005; Sampedro and Cosgrove, 2005), our understanding of the impact of abiotic stress on regulatory molecular interactions is limited.

There is evidence that cell cycle regulation plays an important role in growth responses under unfavorable conditions. The activity of A-type cyclin dependent kinases (CDKA), one of the central regulators of cell cycle progression, is associated with the decrease in leaf growth rate of some species under stress conditions. In maize grown at contrasting temperature and water supply there is a positive correlation between CDKA activity, cell division rates and leaf growth (Granier et al.,
Similarly, in wheat (*Triticum aestivum*) leaves grown under mild drought stress, a reduction in the size of the leaf basal meristem is associated with a reduced CDKA activity, possibly due to an inhibitory phosphorylation on Tyr 15 residue (Schuppler et al., 1998). In Arabidopsis roots, changes in the cell cycle machinery accompany a decreased cell production in response to salt stress. Severe salt stress transiently reduces the expression levels of the cyclins CYCA2;1 and CYCB1;1 (Burssens et al., 2000). Mild salt stress leads to loss of CDK activity and to reduced promoter activity of CYCB1;2 (West et al., 2004). Finally, the production of a CDK activity inhibitor, KIP related protein (KRP) is stimulated by abscisic acid (ABA) in Arabidopsis (KRP1) and alfalfa (*Medicago sativa*; KRPMt), suggesting a role for this gene in the ABA-mediated stress response pathway (Wang et al., 1998; Pettko-Szandtner et al., 2006).

In this paper, we studied a cold treatment that inhibits early leaf growth of maize, but does not cause significant chilling symptoms. We compared the daytime growth (at 25°C) of seedlings exposed to control (18°C) and cold (4°C) nights and studied the cellular growth mechanisms that inhibit growth. We found a specific inhibition of cell cycle activity in the leaf basal meristem (hereafter referred to as leaf meristem). Therefore, we identified 43 putative maize homologs of cell cycle regulatory genes based on sequence database information. Finally, we analyzed the transcription of these genes by constructing expression profiles along the leaf growth zone using real-time PCR. Our results reveal a link between the observed leaf growth inhibition and the expression of specific cell cycle genes.

**RESULTS**

**Effects of low night temperature on maize leaf growth**

We investigated the effect of low night temperature on the growth of leaf four of maize seedlings by comparing plants grown under control conditions (25/18°C (d/n)) and plants exposed to cold nights (25/4°C (d/n)). This treatment did not cause leaf discoloring or premature senescence. We confirmed these visual observations with measurements of
leaf chlorophyll content, which was not significantly affected (Supplemental Fig. 1). Also, the transcript level of the large subunit of the ribulose bisphosphate carboxylase (RUBISCO) gene was similar in both control and treated leaves (data not shown). In contrast, severe cold stress, particularly occurring during the photoperiod, causes profound perturbation of chloroplast development and may lead to cell death (Allen and Ort, 2001, Gomez et al., 2004). Nevertheless, our treatment resulted in a 20% decrease of final length of leaf four (Table I; p<0.001, n=12).

To analyze the developmental basis of reduced leaf size, we measured leaf elongation rate (LER) of leaf four from emergence to maturity. The average LER over a 24 h period was approximately constant during the first 120 h after emergence in both treatments. However, growth rates were about 35% lower in leaves exposed to cold nights (P<0.001, n=12; Fig. 1A). In contrast, we observed minimal effects on the growth during the periods before and after steady-state growth: leaves emerged from the sheath of the previous leaf at a similar length and LER was negligible at 200h after emergence irrespective of the treatment. Our measurements allowed us to estimate the magnitude of the two components contributing to the observed temperature response in our experimental setup: a general temperature effect, presumably due to temperature effects on enzyme kinetics and general physiological processes (Granier and Tardieu, 1999), and a cold stress effect. At night, cold-treated plants showed a growth reduction (LER x duration of measurement interval) of 90% compared to the control caused by both effects (1.5mm vs. 14.9mm, p<0.001, n=12, Fig. 1C). During the photoperiod, this reduction was 20% due to the cold stress effect alone (45.9mm vs. 57.3mm, p<0.001, n=12, Fig. 1B). Assuming the cold stress effect also accounts for 20% of growth inhibition at night (i.e. 3.0 mm), the total contribution of the cold stress effect to the temperature response ((57.3-45.9)+3.0=14.4mm) was larger than that of the general temperature effect ((15-1.5)-3.0=10.5mm).

Kinematic analysis

Next, we set out to analyze the cold stress response at the cellular level by means of a kinematic analysis of leaf growth during daytime at steady-state growth (48h after leaf emergence). For a detailed spatial
and temporal characterization, we measured epidermal cell length as a function of position along the leaf growth zone. The cell length profiles of both control and cold-treated leaves were similar (Fig. 2A and 2B). For both treatments, the cell length profile had a typical shape characterized by distinctive domains. At the leaf base, there was a zone of approximately constant cell size, in which cell growth and division occur simultaneously (leaf meristem). At more distal positions, cells sharply increased their length by expansion in the absence of mitotic activity (elongation zone). Finally, cell size did not vary throughout the mature part of the leaf.

Epidermal cells expanded to the same mature cell length (124 ± 10 and 127 ± 5 in control and cold-treated leaves, respectively), implying that overall post-meristematic cell expansion was unaffected and that reduced cell production was responsible for the observed growth reduction. Consistently, cell production decreased significantly by 22% in cold-treated leaves (Table I).

Because total cell production depends on the number of meristematic cells and on their division rate, we measured the size of the leaf meristem, by staining nuclei and recording the position of the most distal mitotic figures (Fig 2C). We found a leaf meristem size of 16-17 mm, irrespective of treatment (Table I). In contrast, the size of the proliferating epidermal cells was strongly reduced by the stress, particularly in the basal 5 mm (24.0 ± 2.7 mm in control vs. 14.4 ± 0.8 mm in cold-treated leaves; Fig. 2A inset, p<0.05, n=3). Combining these observations, we calculate that cold nights increased the number of cells in the leaf meristem (Table I). We concluded that the reduction in cell production due to low night temperature was due to a slower cell cycle progression. Indeed, the calculated average cell cycle duration was 65% longer in these leaves (p<0.05).

Finally, the reduced cell production in the leaf meristem of cold-treated leaves also affected the flux of cells passing through the expansion zone and resulted in altered cell expansion dynamics (Fig. 3). Except for the first 10 mm adjacent to the leaf meristem, cell elongation rates in cold-treated leaves were lower in most of the elongation zone. However, the longer residence time in the elongation zone in cold-treated leaves fully compensated for these lower expansion rates, resulting in identical mature epidermal cell size (Table I).
Flowcytometry

To investigate whether the cell cycle inhibition occurred during a specific cell cycle phase, we analyzed nuclear DNA content by flowcytometry. We sampled the growth zone in 10 mm segments and further subdivided the basal segment into two 5 mm segments. The distribution of nuclear DNA content was unaffected by the cold-treatment (Fig. 4). In the leaf meristem the 2C/4C ratio was similar (2.3 ± 0.2 vs. 2.5 ± 0.4, average ± SE, n=3). This result indicated that the increased duration of the cell cycle in cold-treated leaves is associated with a comparable increase in duration of both G1 and G2 phases.

Our data also indicate that, irrespective of treatment, endoreduplication occurred in the elongation zone and stopped when cells mature (at distances >70 mm from the base), consistent with similar observations for Arabidopsis leaves (Beemster et al., 2005). However, endoreduplication was limited (5% of the cells reached 8C nuclear DNA content) and unaffected by the temperature treatment.

Annotation of cell cycle genes

Because cold reduced growth through a prolonged cell cycle, we set out to investigate changes in the molecular cell cycle regulatory machinery (CDKs and cyclins, and their interacting proteins). In maize, there are only a few cell cycle genes described to date (Table II). To define a more comprehensive gene set, we performed a sequence homology search on available maize expressed sequence tags (EST)/cDNA sequences. We used the cell cycle genes identified in the fully sequenced species Arabidopsis (Vandepoele et al., 2002) and rice (Oryza sativa) (La et al., 2003; Barroco et al., unpublished data) expanded with previously described maize homologues. After defining open reading frames (ORFs) on all sequences available in the TIGR maize Gene Indices (release 16.0), we used remote homology detection to identify new cell cycle genes based on hidden Markov Model (HMM) profiles (Materials and Methods; Eddy, 2004; Vandepoele et al., 2002). Using this procedure, we identified 43 homologs of cell cycle genes representing all functional classes of cell cycle regulators (Table II). For all these classes we performed a phylogenetic analysis comparing previously characterized cell cycle genes of Arabidopsis and rice with
the newly identified maize ones (Supplemental Fig. 2). Finally, we used the phylogenetic trees to build a nomenclature based on the one adopted for Arabidopsis (Vandepoele et al., 2002).

**Effect of low temperature on cell cycle gene expression**

To identify the molecular changes underlying the slower cell cycle progression in cold-treated leaves, we determined the expression profiles of the 43 identified cell cycle genes along the leaf growth zone. We collected samples from the leaf meristem (segment 0 to 5 mm), the elongation zone (segment 40 to 50 mm) and the mature part (segment 90 to 100 mm; arrows Fig. 2A) during the steady-state growth of leaf four. For each zone, we determined the relative transcript abundance by real-time PCR using three independent biological replicates. The expression levels of most of the cell cycle genes varied between the three analyzed cell developmental stages: 70% of the transcripts showed a significant change (p<0.05, n=3), the remaining 30% had a more stable expression pattern (Table II, Class V). Hierarchical clustering of differentially expressed genes yielded four distinct classes and two outliers (Fig. 5, Table II). As expected, nearly all of these genes showed high expression in the leaf meristem and a pronounced decrease of transcript abundance towards the mature region. The inhibitor KRP2 represented an exception. The expression of KRP2 was highest in the elongation zone, suggesting a role in cell cycle exit. The four classes of genes with marked proliferation-specific expression displayed a different decline in transcript abundance in the elongation and mature zone. Cluster IV contained seven genes with the most abrupt decrease of transcript abundance in the elongation zone (Fig. 5, Table II). Although DPa displayed the same pattern, it was not included in this cluster, probably because of the slight increase in expression after cell elongation. Clusters I and III contained seven and eight genes, respectively, showing a more gradual decrease of their expression (Fig. 5, Table II). In contrast, cluster II contained six genes characterized by a relatively high expression both in the leaf meristem and elongation zone. These latter genes most likely play a role in both cell division and processes involved in cell elongation and differentiation (Fig. 4). The comparatively high expression in the leaf
meristem is in agreement with a regulatory role in cell proliferation for the identified cell cycle genes.

To understand the differences in cell division rates identified by kinematic analysis, we measured transcript levels in the leaf meristem following the cold treatment. The expression patterns were consistent between the three biological replicates despite the relatively high variability for some of the transcripts (Supplemental table I). Based on this observation, we chose a three-fold cut-off threshold. By this criterion, 21% of the genes were differentially expressed (Fig. 6). Several members of the cyclin and CDK families, as well as members of the E2F/retinoblastoma-related (RBR) pathway were down-regulated (Fig. 6). Specifically, an A-type cyclin (CYCA3;1) showed the largest down-regulation upon treatment, more than twenty-fold on average, followed by CDKA1;1, CDKD1, DEL1, RBR2;2 and an E2F homolog. This group of transcripts includes members of the cell cycle activating CDK/CYC complexes and of E2f/RBR pathway, which controls cell cycle entry. Additionally, two KRPs show a more constitutive expression profile in our data set (KRP1 and KRP3). These were strongly up-regulated in the leaf meristem, on average eight and fourteen-fold, respectively. Combined with the observation that KRP2 was down-regulated by more than ten-fold in the leaf meristem, these results suggest functional divergence between the KRP genes. Taken together, these expression data indicate that a slower cell cycle progression in the leaf meristem of leaves grown at low night temperature was associated with concerted transcriptional changes of both positive and negative cell cycle regulators.

DISCUSSION

Low night temperature inhibits growth rates during the photoperiod.

Temperature is one of the most important environmental factors influencing leaf growth (Lyons, 1973). Temperature directly affects enzyme kinetics of many biochemical reactions and thereby the growth rate of plant organs. Experimental data show that temperature also determines the activity of the cell cycle regulatory kinase CDKA in maize (Granier et al., 2000).

Here we investigated whether cold has effects on growth and cell cycle
regulation, other than the general effects mediated by reaction kinetics. To interpret effects in a temperature-independent manner, growth rates are often modeled as a function of thermal time (Granier and Tardieu, 1998; and references therein). We used an alternative strategy to separate the general effects of temperature on enzyme kinetics from growth regulatory changes induced by cold stress. Only during nighttime, we lowered the temperature but maintained identical levels during daytime when we studied the growth effects. Using this method, we obtained results qualitatively similar to those obtained in previous studies in maize (Ben-Haj-Salah and Tardieu, 1995).

**Low night temperature inhibits cell cycle progression.**

Using a kinematic analysis based on cell length profiles (indirect kinematic analysis; Fiorani and Beemster, 2006), we show that the leaf growth inhibition by low night temperature is tightly linked to the reduction of cell production. In turn, this reduction is a consequence of prolonged cell cycle duration and not of a reduced cell number in the leaf meristem (Table I). This result is significant, because cell cycle duration is constant across a wide range of experimental conditions (Baskin, 2000). Relatively few studies reported prolonged cell cycle duration, either transiently in cell cultures (Reichheld et al., 1999) and roots (West et al., 2004) or more persistently in leaves (Schuppler et al., 1998; Granier and Tardieu, 1999) and roots (Sacks et al., 1997). More frequently, a reduction of cell production was associated with a reduced meristematic cell number, e.g. in response to soil compaction (Beemster et al., 1996) and to drought (Tardieu et al., 2000; Sharp et al., 2004). The flowcytometry data in our study indicate that low temperature apparently affects all cell cycle phases to a similar extent (Fig. 4). This finding is consistent with previous studies of temperature effects (Tardieu and Granier, 2000) and in contrast with other abiotic factors that act specifically on the G1-S transition (Sacks et al., 1997; Reichheld et al., 1999; West, et al., 2004). Moreover, growth reduction caused by abiotic factors is often due to a combination of both reduced cell production and mature cell size (Sacks et al., 1997; Granier et al., 2000; West et al., 2004), while our results show a specific effect on cell division only. A reduction of cell size at the base of the meristem accompanies this
effect on cell division (Fig. 2A, inset). These observations suggest that our treatment primarily affects the growth of meristematic cells and that those smaller cells require a longer time to progress through the cell cycle. However, the size of cells at the transition between the meristem and elongation zone is similar in both control and cold treated leaves, indicating that our treatment differentially affects cell division and expansion rates of actively dividing cells. To analyze the coordination between these two processes in more detail, local rates of cell division and expansion need to be measured throughout the meristem, which was not possible with the experimental approach used here.

In conclusion, reduced growth caused by low temperature treatment is associated with a prolonged cell cycle progression and not with a reduction of the meristematic cell number or a smaller mature cell size as reported for other abiotic stresses. Therefore, we postulate that specific cold-inducible mechanisms regulating cell proliferation exists, as hypothesized previously based on ecophysiological data (Grime and Mowforth, 1982).

Transcriptional analysis of cell cycle genes under environmental stress

In this work, we present a set of 43 putative maize cell cycle genes, of which only 13 previously annotated (Table II). This gene set shows overall the expected proliferation-specific transcription profile throughout leaf development, in agreement with a previous study in Arabidopsis (Beemster et al., 2005). Our choice of sampling by position, as opposed to the entire organ, is effective in capturing transcriptional gradients associated with cell developmental transitions encompassing proliferation, expansion and mature phases in monocot leaves.

The survey of cell cycle gene expression in maize leaves provides a first insight into potential mechanisms that regulate the cell cycle machinery in response to low temperature. Although previous studies showed a link between meristem activity and decreased growth rate during stress treatments (e.g. Ben-Haj-Salah and Tardieu, 1995; Beemster et al., 1996; Tardieu et al., 2000), only few data are available on cell cycle gene regulation involved in tuning meristem activity to environmental signals (Burssens et al., 2000; Granier et...
al., 2000; West et al., 2004).

Our results show that transcriptional regulation of cell cycle gene expression plays an important role in the decreased cell production and growth during cold stress. The most striking effect was a decrease in the transcript level of an A-type cyclin (CYCA3;1) that might be an important regulator in response to stress. This observation is consistent with previous studies of A- and B-type cyclins (Burssens et al., 2000; West et al., 2004). Taken together, these observations are particularly interesting considering that the large number of cyclin genes in plants compared with other eukaryotes may reflect the higher plasticity of plant growth (Inzé, 2005).

In addition to altered expression of positive cell cycle regulators, some inhibitory proteins (KRP1, KRP2 and KRP3) are also differentially expressed. KRP1 and KRP3 have a strongly increased expression, in contrast to KRP2. Only two of these KRPs (KRP1 and KRP2) were previously described and characterized in maize endosperm. Interestingly, in vitro assays suggested that KRP1 had a stronger inhibitory effect on CDK activity than KRP2 (Coelho et al., 2005), leading to the possibility that cold treatment preferentially leads to activation of more effective KRPs to tune the cell cycle to the adverse conditions. Previous work indicated a possible role for KRPs in stress responses regulation. In Arabidopsis KRP1 is known to respond to ABA (Wang et al., 1998), and an alfalfa (Medicago sativa) KRP was induced both after mild salt treatment and ABA application (Pettko-Szandtner et al., 2006). Finally, we show a possible involvement of transcriptional regulation of the E2F/DP/RBR pathway in mediating responses to environmental conditions.

In conclusion, long-term exposure to low night temperature causes specific changes in the expression of cell cycle genes in maize leaves. Although the regulation of cell cycle progression involves several post-translational mechanisms (Inzé, 2005), these results highlight that also transcriptional changes play a role, at least in long-term responses. A combination of opposite effects on positive and negative cell cycle regulators lead to prolonged cell cycle duration involving all cell cycle phases and result in a decreased cell production. In turn, this decreased cell production leads to slower growth rates and shorter final leaf size. Future experiments will provide further insight into the role of cell cycle machinery in cold stress responses.
and clarify which molecular pathways are essential for the modulation of the observed transcriptional changes.

MATERIALS AND METHODS

Plant material and growth conditions

Maize (Zea mays cv B73) seeds (AgriObtention, INRA, St Martin-de-Hinx, France) were germinated in peat pellets (Jiffy International As, Norway) at 25°C and 70% humidity, a 16 hours photoperiod and a light intensity of 200 µmol m⁻² s⁻¹ PAR. Thereafter, the seedlings were transferred to 2L pots filled with soil (N°0, Structural, Kaprijke, Belgium) and placed in a growth chamber (type vb1014, Vötsch industrietechnik, Balingen, Germany) at 70% relative humidity, 400 µmol m⁻² s⁻¹ PAR at plant level provided by a combination of fluorescent tubes (Osram-77 and Osram-31-830, Osram, Munich, Germany) in a 16/8h (d/n) cycle with a gradual decrease and increase of radiation intensity over 0.5 h. Temperature was kept at 25°C during the photoperiod and decreased to 18°C (control) or to 4°C (treatment) during the last six hours of the night.

Growth analysis

To calculate LER during the photoperiod and the night, we measured the length of leaf four (12 plants per treatment) at the beginning and end of the photoperiod from leaf emergence to maturity, using the soil level as a reference point.

To determine cell length profiles, we collected the basal 100 mm of leaf four during steady-state growth from three plants per treatment and prepared 10 mm segments for microscopy as described previously (Piorani et al., 2000). In this segments we measured the cell length in two equivalent abaxial epidermal cell files adjacent to stomatal rows (Figure 2B) by imaging with differential interference contrast (DIC) microscopy (De Veylder et al., 2001). For samples of meristematic cells 40x (Achromplan, Zeiss, Jena, Germany, n.a. = 0.65) and for the elongating and mature cells 20x (Plan Apochromat, Jena, Zeiss, Germany, n.a. = 0.75) objectives were used, respectively.
The raw data obtained for individual leaves were smoothed and interpolated at an interval of 50 µm using the kernel smoothing function "locpoly" of the KernSmooth package (Wand and Jones, 1995) for the R statistical package (R Foundation for Statistical Computing), which allowed averaging between leaves and comparison between treatments. To estimate meristem size, we isolated the basal 30 mm of leaf four from three plants per treatment during steady-state growth. Samples were fixed as described previously (Fiorani et al., 2000), rinsed three times in a buffer containing 50mM NaCl, 5mM EDTA and 10mM TRIS-HCl (pH 7) and stained by incubating with buffer containing 1 µg/mL DAPI (4’, 6-diamidino-2-phenyindole) for five minutes. Fluorescent nuclei were observed with a microscope (Axioskop, Jena, Zeiss, Germany) equipped with an ebq 100 dc epifluorescent condenser. ScionImage software (Scion Corp., Frederick, MD) was used to measure the size of the leaf meristem defined as the distance between the base of the leaf and the most distal mitotic cell.

We calculated growth parameters through a kinematic analysis as described previously (Fiorani et al. 2000). Cell length profiles and LER were measured on separate batches of plants grown under identical conditions. To estimate variance for cell production, cell division and cell cycle duration, these parameters were calculated for each sample separately, based on their cell length profile (n=3) and the average of the LER data (n=12). Using the average LER rather than all possible combinations of the two sets of values avoids overestimation of the variance.

Chlorophyll content measurements

Four discs (113 mm²) were sampled from halfway along the blade of mature leaf four of different plants per treatment. They were placed in N,N-dimethylformamide (DMF) and stored at 4°C in darkness. After five days of incubation, we determined spectrophotometrically chlorophyll a and b content (wavelengths 646.8 nm, 663.8 nm using 750 nm as baseline; Porra, 2002).
Flowcytometry

The basal 100 mm of steady-state growing fourth leaves was cut in segments of 10 mm. The most basal 10 mm was subdivided further into two 5 mm segments. To release the nuclei, the segments were chopped with a razor blade in 2 mL ice cold buffer (200 mM Tris-HCl pH 7.5, 4 mM MgCl2 and 0.1% Triton X-100), filtered over a 30 µm mesh, stained with DAPI (De Veylder et al., 2001) and analyzed with a PARTEC Cyflow analyzer (PARTEC, Munster, Germany). For each sample, we counted at least 10000 nuclei and analyzed the relative abundance of nuclei with 2C and multiple DNA content using FloMax software (version 2.4d, PARTEC, Munster, Germany).

Annotation of putative cell cycle genes

For all sequences available in the TIGR maize Gene Indices (release 16.0), the coding ORF was determined with FrameD software (Oryza Interpolated Markov Model, parameters -E for eukaryotic EST analysis and -C for correcting frame shifts; Schiex et al., 2003). Subsequently, we constructed HMM profiles for all cell cycle gene families based on known dicotyledonous and monocotyledonous reference genes and used these profiles to screen the protein data set. Putative homologues were aligned to the reference sequences in the profile using CLUSTALW (Thompson et al., 1994) or T-coffee (Notredame et al., 2000). Family-specific signatures were analyzed to select valid maize homologs. We used the neighbour-joining algorithm for phylogenetic analysis with the software package TREECON (Van de Peer and De Wachter, 1994). Distance matrices were calculated using the Poisson correction and bootstrap analysis with 100 replicates was performed to estimate the significance of nodes. Based on the position in the tree, we classified genes according to the nomenclature rules for cell cycle genes (Renaudin et al., 1996; Joubès et al., 2000).
Transcriptional analysis of putative cell cycle genes

To analyze cell cycle gene expression, we extracted RNA from the fourth leaf of three representative plants at two days after emergence. Each of these leaves served as a biological replicate for subsequent analyses and was separated into the leaf meristem (0mm-5mm), elongation (30mm-40mm) and mature zone (90mm-100mm). Total RNA was isolated using TRI-reagent (Sigma-Aldrich, St. Louis, MO). First-strand cDNA synthesis was performed on 3 µg of total RNA with the Superscript RT II kit (Invitrogen, Carlsbad, CA) and oligo(dT)$_{18}$, according to the manufacturer's instructions. Based on the concentration of cDNA measured with a Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE), samples were normalized and 14 ng were used for each reaction.

We designed primers using the Beacon Designer 4.0 software (Premier Biosoft International, Palo Alto, CA; Tm = 59°C ± 1°C, amplicon length: 60bp-150 bp; Supplemental Table II). Primer specificity was assessed by performing a BLAST search against all known maize sequences available at GenBank and TIGR. For 15 selected primer combinations, sequencing of the PCR products yielded the target sequence, further confirming their specificity. The transcripts were quantified with an iCycler (Bio-Rad, Hercules, CA) with the qPCR core kit for SYBR green I (Eurogentec, Seraing, Belgium). PCR reactions were performed in triplicate technical replicates, following manufacturer guidelines. For each PCR reaction, we observed product melting curves by heating from 60°C to 95°C at 0.2°C/s. For all transcripts, this procedure allowed identification of a single product, which we confirmed by analysis on 2% agarose gels (data not shown).

For relative quantification, we set a threshold cycle at the same level for each reaction within the exponential amplification phase. For normalization, we used the amount of total RNA because all tested genes (including ‘housekeeping’ genes) showed significant differences in expression level both for developmental zones and/or treatment. We calculated efficiency and corrected the crossing point values as described previously (Ramakers et al., 2003).

We mean-centered, normalized and clustered average crossing points of significantly different genes along the leaf developmental gradient.
using hierarchical clustering algorithms (Eisen et al., 1998) implemented in TMeV software (Saeed et al., 2003).

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FIGURE LEGENDS

Figure 1: The effect of cold nights on leaf elongation.
LER of leaf four was determined under control (25°C/18°C (d/n); open circles) and low temperature (25°C/4°C; solid circles) conditions as a function of time from leaf appearance. (A) LER measured over 24 h, (B) LER during photoperiod and (C) during the night. Symbols are means ± SE (n=12).

Figure 2: Effect of cold nights on epidermal cell length.
(A) Cell length profiles of files adjacent to stomatal rows in the abaxial epidermis measured for control (open circles) and cold-treated (solid circles) plants. The data were obtained for leaf four from three plants for which the length of all cells in the basal 100 mm of two cell files adjacent to stomatal files were measured in function of position. To enable averaging across leaves, the data were smoothed and interpolated into 50 µm spaced data points as described in ‘Material and Methods’. For each condition the average and corresponding standard error is shown at each position (n=3). Inset: detail of the cell size in the meristem. The arrows indicate the sampling positions for the transcript profiling experiments. (B) Representative image of elongating epidermal cells on the leaf abaxial side. The arrow indicates the measured cell file. (C) Representative image of 4′,6-diamidino-2-phenylindole (DAPI)-stained epidermal cells in the meristem used to identify mitotic figures and estimate the size of the leaf meristem.

Figure 3: Effect of cold nights on cell expansion.
Profiles of cell expansion rates in leaf four of control (open circles) and cold-treated (solid circles) plants were calculated based on average LER during the photoperiod and the derivatives of the smoothed cell length profiles of cell files adjacent to stomatal rows in the abaxial epidermis. These calculations are only valid outside the meristem, the size of which was determined from observations of mitotic figures. Symbols are averages ± SE (n=3)
Figure 4. Effect of cold nights on nuclear DNA content.
Flowcytometry analysis of the ploidy level was performed at different positions of the growth zone. Fluorescence of nuclei was obtained from maize leaf segments (10 mm long, except the first 10 mm, which was divided in two 5 mm segments). (A) Fractions of the amount of nuclei at different ploidy levels are shown for both control and low temperature. Representative fluorescence intensity distributions of meristem (B), elongation zone (C) and mature (D) samples under control conditions.

Figure 5. Gene expression profiles of differentially expressed cell cycle genes in maize leaves.
Hierarchical clusters of the expression profiles of cell cycle genes with a significantly different expression along the leaf growth zone are shown (Table II, p<0.05, n=3). Transcript abundance in the meristem (me), elongation zone (el) and mature zone (ma) were determined using real-time PCR on leaf segments of 0.5 cm in the meristem and of 1 cm in the elongation and mature zone (see Fig. 2A).

Figure 6. Effect of cold nights on cell cycle gene expression in the meristem.
RNA samples were extracted from 0.5 cm leaf segments at the base of leaf four from control and cold-treated plants. Expression levels were determined by real-time PCR and fold-changes between averages of three biological replicates are shown. Vertical dotted lines indicate three-fold thresholds.
Table I. Effect of cold nights on cell division and cell expansion parameters during the photoperiod.

| Growth parameters          | Control            | Cold treated       | % change<sup>b</sup> |
|----------------------------|--------------------|--------------------|----------------------|
| Final leaf length (mm)     | 658±7              | 527±5              | -20                  |
| Leaf elongation rate (mm/h)| 3.7±0.2            | 2.9±0.2            | -21                  |
| Cell production (cells/h)  | 30±2               | 23±1               | -22                  |
| Division rate (cells/cell h)| 0.036±0.004     | 0.022±0.005        | -38                  |
| Cell cycle duration (h)    | 19±2               | 32±6               | +65                  |
| Residence time in the      |                    |                    |                      |
| meristem (h)               | 191±15             | 327±43             | +71                  |
| Number of dividing cells   | 821±94             | 1060±213           | +29                  |
| Meristem size (mm)         | 17±6               | 16±3               | NS                   |
| Mature cell size (µm)      | 124±10             | 127±5              | NS                   |
| Residence time in the      |                    |                    |                      |
| elongation zone (h)        | 28±1               | 37±1               | +30                  |
| Number of elongating cells | 705±59             | 737±22             | NS                   |
| Elongation zone size (mm)  | 50±2               | 45±2               | NS                   |

<sup>a</sup> Average ± SE; n=3, except for final leaf length and leaf elongation rate n=12. All parameters were determined at 48 h after leaf emergence.

<sup>b</sup> Statistical significance based on one-way ANOVA tests (p < 0.05); NS, not significant.
Table II: Overview of maize cell cycle genes.

| Gene name | Synonym(s) | Accession | Expr | Reference(s) |
|-----------|------------|-----------|------|--------------|
| CDC25     | TC300846   | V         |      |              |
| CDKA1;1   | Cdc22ma    | P23111*   | II   | (Colasanti et al., 1991) |
| CDKA2;1   | TC292977   | V         |      |              |
| CDKB1;1   | TC287283   | III       |      |              |
| CDKB2;1   | TC311057   | V         |      |              |
| CDK2      | TC304893   | V         |      |              |
| CDK1      | TC281529   | V         |      |              |
| CKS1      | TC292250   | III       |      |              |
| CKS2      | TC312708   | III       |      |              |
| CYCA1;1   | cycIIzm    | Q41732*   | IV   | (Renaudin et al., 1994) |
| CYCA1;2   | cycZm2w    | Q43693*   | IV   | (Hsieh and Wolniak, 1998) |
| CYCA3;1   | cycZme1    | P93646*   | IV   |              |
| CYCA3;2   | TC292456   | IV        |      |              |
| CYCA3;3   | TC298919   | IV        |      |              |
| CYCA3;4   | TC298903   | I         |      |              |
| CYCB1;1   | cycIazm    | Q41734*   | III  | (Renaudin et al., 1994) |
| CYCB1;2   | cycIbzm    | Q41733*   | III  | (Renaudin et al., 1994) |
| CYCB1;3   | cycZme1    | P93646*   | IV   |              |
| CYCB2;1   | cycIIIzm   | Q41731*   | III  | (Renaudin et al., 1994) |
| CYCB2;2   | TC300622   | III       |      |              |
| CYCD1;1   | CYCD2      | Q8S524*   | V    |              |
| CYCD4;1   | CYCD2;2, CYCD4 | Q8S522* | II   |              |
| CYCD4;2   | TC290220   | V         |      |              |
| CYCD5;1   | CYCD1      | Q8S523*   | III  |              |
| CYCD5;2   | TC289521   | I         |      |              |
| CYCH      | TC282470   | II        |      |              |
| DEL1      | TC295278   | IV        |      |              |
| DEL2      | TC306241   | V         |      |              |
| DPa       | BE511883   |           |      |              |
| DPb       | TC301910   | I         |      |              |
| DPC       | TC312155   | II        |      |              |
| DPd       | TC312156   | V         |      |              |
| E2F       | TC309263   | IV        |      |              |
| KRP1      | Q52PU9*    | V         |      | (Coelho et al., 2005) |
| Gene  | Accession Numbers | Class | References |
|-------|------------------|-------|------------|
| KRP2  | Q52PU8'          |       | Coelho et al., 2005 |
| KRP3  | TC306015'        | V     |            |
| KRP4  | TC301833'        | III   |            |
| RBR1;1| Q3LXA7'          | III   | Sabelli et al., 2005 |
| RBR2;1| O22344'          | V     | Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Rossi et al., 2003 |
|       | Q9LX9'           |       |            |
|       | Q7DLV4'          | V     |            |
| ZmRB  | Q41763'          |       |            |
| RBR2;2| Q8H0J6'          | II    | Ach et al., 1997 |
|       | O22346'          |       |            |
|       | Q41763'          |       |            |
|       | 022345'          |       |            |
| WEE1  | Q9SP28'          | I     | Sun et al., 1999 |

* Accession numbers for proteins were derived from UNIPROT.

* Accession numbers for assembled ESTs were derived from TIGR tentative consensus (release 16.0).

* Accession numbers for EST were derived from Genbank.

* Based on one-way ANOVA analysis, genes were constitutively (V, p>0.05, n=3) and differentially expressed (p<0.05, n=3) and divided in three different classes based on hierarchical clustering (I, II, and III; see Fig. 5).
Cluster I: 7 genes

Cluster II: 6 genes

Cluster III: 8 genes

Cluster IV: 7 genes

KRP2

DPa
