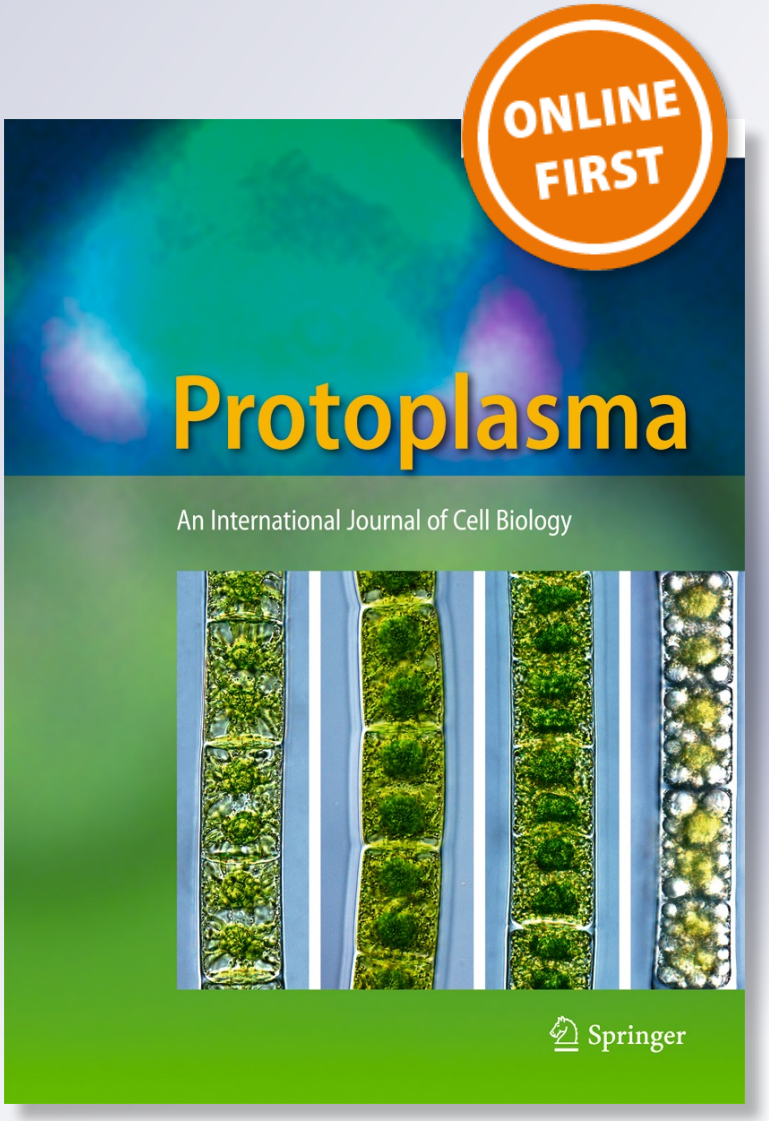
# *Involvement of DNA methylation in the control of cell growth during heat stress in tobacco BY-2 cells*

**Isabella Centomani, Alessandra Sgobba, Pietro D'Addabbo, Nunzio Dipierro, Annalisa Paradiso, Laura De Gara, Silvio Dipierro, et al.**

**Protoplasma**
An International Journal of Cell Biology

ISSN 0033-183X

Protoplasma
DOI 10.1007/s00709-015-0772-y




Springer

Protoplasma
DOI 10.1007/s00709-015-0772-y

ORIGINAL ARTICLE

# Involvement of DNA methylation in the control of cell growth during heat stress in tobacco BY-2 cells

**Isabella Centomani · Alessandra Sgobba · Pietro D'Addabbo · Nunzio Dipierro · Annalisa Paradiso · Laura De Gara · Silvio Dipierro · Luigi Viggiano · Maria Concetta de Pinto**

Received: 4 December 2014 / Accepted: 26 January 2015


**Abstract** The alteration of growth patterns, through the adjustment of cell division and expansion, is a characteristic response of plants to environmental stress. In order to study this response in more depth, the effect of heat stress on growth was investigated in tobacco BY-2 cells. The results indicate that heat stress inhibited cell division, by slowing cell cycle progression. Cells were stopped in the pre-mitotic phases, as shown by the increased expression of CycD3-1 and by the decrease in the NtCycA13, NtCyc29 and CDKB1-1 transcripts. The decrease in cell length and the reduced expression of Nt-EXPA5 indicated that cell expansion was also inhibited. Since DNA methylation plays a key role in controlling gene expression, the possibility that the altered expression of genes involved in the control of cell growth, observed during heat stress, could be due to changes in the methylation state of their promoters was investigated. The results show that the altered expression of CycD3-1 and Nt-EXPA5 was consistent with changes in the methylation state of the upstream region of these genes. These results suggest that DNA methylation, controlling the expression of genes involved in plant development, contributes to growth alteration occurring in response to environmental changes.

**Keywords** Cell cycle · Cell division · Cell expansion · DNA methylation · Heat stress · Tobacco BY-2 cells

Handling Editor: Peter Nick

**Electronic supplementary material** The online version of this article (doi:10.1007/s00709-015-0772-y) contains supplementary material, which is available to authorized users.

I. Centomani · A. Sgobba · P. D'Addabbo · N. Dipierro · A. Paradiso · S. Dipierro · L. Viggiano · M. C. de Pinto (✉)
Dipartimento di Biologia, Università degli Studi di Bari "Aldo Moro", via E. Orabona 4, 70125 Bari, Italy
e-mail: mariaconcetta.depinto@uniba.it

L. De Gara
Centro Integrato di Ricerca, Università Campus Bio-Medico di Roma, via A. del Portillo 21, 00128 Rome, Italy

M. C. de Pinto
Istituto di Bioscienze e Biorisorse, CNR, Via G. Amendola 165/A, 70126 Bari, Italy

## Introduction

Global climate changes, including very high temperatures, affect plant growth and productivity in many parts of the world, leading to alarming losses in crop productivity (Christensen and Christensen 2007). Heat stress can alter the normal intermolecular interactions required for correct growth, by impairing plant development. In order to maximize the chance of survival during adverse environmental conditions, since plants are sessile organisms, they need to activate signaling networks, which then results in strong physiological and molecular changes (Urano et al. 2010). Indeed, higher plants exposed to heat stress activate different cellular and metabolic responses, thus enabling them to withstand not optimal conditions (Saidi et al. 2011).

The modulation of morphogenesis is a key feature of the acclimation to environmental stress which consists in the alteration of growth patterns due to an adjustment of the processes in both cell division and expansion and cell differentiation (Potters et al. 2007). One of the most common and important constituents of this morphogenetic response, which is activated in the presence of environmental stress, is the impairment of the cell cycle (Potters et al. 2009). The inhibition of expansion, at both phenotypic and cellular levels, represents another key event in the modulation of morphogenesis

Published online: 25 February 2015

induced by abiotic stress (Ortega and Taleisnik 2003; Pasternak et al. 2005a, b; Verma and Mishra 2005). Heat stress, like other environmental stresses, is also able to alter cell growth, thus causing changes in cell division and expansion (Smertenko et al. 1997; Jang et al. 2005; Potters et al. 2009).

The morphogenetic response observed under stress conditions, including heat stress, is the result of the perception of environmental cues and the transmission by many signal transduction pathways. This ultimately leads to the activation of genes encoding effector proteins that enable adaptation to environmental challenges (Madlung and Comai 2004; Bita and Gerats 2013).

Epigenetic modifications, including DNA methylation, may play a critical role in the alteration of gene expression occurring during plant development and in response to environmental changes (Madlung and Comai 2004; Wada et al. 2004; Ahmad et al. 2010; Mirouze and Paszkowski 2011). It has been reported that mutations in two genes involved in DNA methylation, methyltransferase1 and chromomethylase3, lead to an abnormal development of embryos with altered planes and numbers of cell division in *Arabidopsis* (Xiao et al. 2006). The level of DNA methylation can also change in response to environmental stress and in correlation with the activation of stress-responsive genes (Wada et al. 2004). In the context of stress responses, methylation has been gaining increasing interest because it can regulate the expression of genes that play a key role in acclimation responses (Boyko et al. 2007; Choi and Sano 2007; Huang et al. 2010).

It has been recently reported that a moderate but prolonged heat stress likely inhibits the growth of tobacco BY-2 cells, a well-standardized model system for biochemical and molecular studies (Sgobba et al. 2015). In the present work, we studied the effect of heat stress more deeply on the same model system, focusing on cell division and cell expansion. In addition, the involvement of DNA methylation in the heat-dependent regulation of genes, coding for proteins that control cell cycle progression and cell expansion, was also analysed.

## Materials and methods

### Cell culture, heat treatment and cell growth parameters

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell suspensions were routinely propagated and cultured according to Nagata et al. (1992). For the experiments, 2 ml of stationary culture (7 days) were diluted in 100 ml of fresh culture medium in 250-ml flasks and grown at 27 or 35 °C for 7 days. At the indicated times, aliquots of cell suspensions were collected for the determination of cell growth parameters.

Packed cell volume (PCV) and cell viability were determined as described in Sgobba et al. (2015). For the analysis of mitotic index, cells were stained with DAPI as described in de Pinto et al. (2002) and visualized under a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 340 to 380 nm and a barrier filter of 400 nm. Mitotic index was determined as the percentage of the cells in mitosis on the total cells.

Cell size was calculated by optical microscopy images of BY-2 cells using UTHSCSA Image Tool software as described in de Pinto et al. (2000).

Protein content was determined according to Bradford (1976) using BSA as a standard.

### Semi-quantitative RT-PCR

Total RNA was isolated from tobacco BY-2 cells using the InviTrap Spin Plant RNA Mini kit (Stratec molecular) according to the supplier's recommendation. Residual DNA was removed from the RNA samples using a DNase I, RNase-free (Fermentas). Synthesis of complementary DNA was performed from 2 μg of total RNA with RevertAid M-MuLV RT- (Fermentas) and random primers. The primer sequences utilized for RT-PCR and amplicon sizes are reported in Supporting Information (Supplemental Table 1).

The 18S rRNA was used as an internal control in order to normalize each sample for variations in the amount of initial RNA. Amplification was conducted at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; the number of amplification cycles was optimized for each sequence. The RT-PCR products were analysed on 1.5 % (*w*/*v*) agarose gels containing 0.5 mg $ml^{-1}$ ethidium bromide and scanned with GelDoc System (Bio-Rad). Densitometric analyses of PCR products were carried out with the Quantity One software (Bio-Rad).

### Reconstruction of upstream sequences

In order to reconstruct the upstream regions, the coding sequences of the five genes (NtcycA13–X93467; Ntcyc29–D50737; CycD3-1–AJ011893; CdkB1-1–AF289465; Nt-EXPA5 AF049354) have been utilized as queries in blast analysis in two different databases (N. tabacum Methylation Filtered genome TGI:v.1 Contigs and N. tabacum Methylation Filtered genome TGI:v.1 Processed) derived by the massive sequencing of the *N. tabacum* genome (http://solgenomics.net/tools/blast/index.pl?db id=73). The BLAST searches were carried out recursively, i.e. the matching sequences identified in each search have been used as new queries in subsequent rounds of search. The identified sequences are reported in Supporting Information (Supplemental Figure 1).

To confirm the accuracy of the reconstruction of genomic upstream regions of the genes in the analysis, different primer pairs specific for coding sequences and the reconstructed upstream regions were designed utilizing the Primer3 software (http://primer3.ut.ee/). The primer sequences utilized for the

genomic amplification are reported in the Supplemental Figure 2A. The obtained genomic amplifications (Supplemental Figure 2b, 2c) were sequenced and compared with sequences previously obtained using Basic Local Alignment Search Tool (Blast2seq; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

### McrBC assay

The McrBC assay allowed to identify the methylation pattern of the upstream sequences previously identified (Vanhees et al. 2011). This assay consisted in the digestion of DNA with the McrBC enzyme and in the successive quantitative PCR (qPCR) conducted with primers designed on the CG-enriched regions of the 5′ sequences. McrBC is an endonuclease which cleaves in the presence of methylcytosine and leaves unmethylated cytosine intact. As a consequence, the level of methylation state can be measured by qPCR.

Firstly, primers in CG-enriched regions of the reconstructed 5′ sequences were designed using MethPrimer software (http://www.urogene.org/methprimer/; Li and Dahiya 2002). Results of primer selection were delivered through a web browser in text and graphic view (Supplemental Figure 3).

Since it is well known that the GC-enriched regions are difficult to amplify (Kumar and Kaur 2014), for each upstream sequence, several primer pairs were designed (Supplemental Figure 3). Successively, the Tm and the secondary structure of primers were analysed by Primer3 and, through appropriate base fluctuations, only the best primer pairs for each upstream sequence were employed in the qPCR assay. The forward and reverse primer sequences utilized for the analysis are reported in the Supporting Information (Supplemental Table 2).

Genomic DNA was extracted from 1 g of lyophilized plant cells using the DNeasy Plant Maxi kit (Qiagen), according to the supplier's recommendation. The concentration and quality of the DNA from the samples was determined in a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, USA). Successively, the McrBC digestion was performed in a 20 μl reaction mixture containing 1 μg of genomic DNA at 37 °C for 12 h and the reaction was stopped after incubation at 65 °C for 5 min. Digested DNAs were precipitated in 1/10 volume sodium acetate and two volumes 100 % ethanol at −70 °C for 30 min. Pellets were resuspended in 40 μl of distilled water. The concentration and quality of the DNA from the samples were determined in a NanoDrop spectrophotometer. qPCR was performed using ABI 7500 Real-Time PCR System (Applied Biosystems) with 20 ng of digested DNA for each sample.

The optimized qPCR was prepared in a volume of 15 μl, with 7.5 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.12 μM of each primer (Sigma Aldrich), 5.26 μl of ultrapure water (Invitrogen, USA) and 2.0 μl of DNA template, containing 10 ng of DNA. qPCRs were carried out in triplicate. The thermocycling conditions were set to 10 min at 95 °C, followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. To check contamination, a negative control was run by adding 2 μl of ultrapure water (Invitrogen, USA) to 13 μl of reaction buffer.

Ct values were normalized with the coding region of CDKB1-1 gene which does not contain 5-methylcytosine (ΔCt). The results were reported as relative methylation state ($2^{-\Delta\Delta Ct}$) obtained by the difference among ΔCt of samples at 35 °C and ΔCt of samples at 27 °C. An increase in PCR amplification products is indicative of hypomethylation, whereas a decrease in PCR amplification products indicates hypermethylation.

## Results

The exposure of tobacco BY-2 cells to moderate heat stress significantly inhibited growth, as highlighted by the huge decrease in the packed cell volume (PCV) observed in cells grown at 35 °C compared with cells grown at the optimal temperature (27 °C). The inhibition of growth, already evident after 3 days of heat stress, reached 70 % after 5 days of exposure to 35 °C (Fig. 1a). The heat stress also caused an increase in cell death which reached a maximum of 33 % after 7 days of exposure to 35 °C (Fig. 1b). However, the high decrease in PCV observed under heat stress cannot only be explained by the more moderate increase in cell death. Indeed, the treatment of tobacco BY-2 cells with 50 mM hydrogen peroxide, causing 50 % of cell death, was responsible for only 24 % of the PCV decrease (data not shown). Thus, two other parameters that influence PCV were determined: cell division and cell expansion.

As shown by the analyses of the mitotic index and cell number evolution, a marked inhibition in cell division occurred in heat-stressed cells (35 °C) compared with the control cells (Fig. 2). In cells exposed to 35 °C, a notable decrease in mitotic index was evident after 1 and 3 days of heat stress, that is when control cells showed the highest mitotic activity (Fig. 2a). Consistently, under heat stress, the number of cells in the suspension was significantly lower than observed in the control, from the third to the seventh day of culture (Fig. 2b).

To verify whether the block in cell division was due to an arrest in cell cycle progression, the expression of some genes encoding regulatory proteins of the cell cycle was studied. Although cell cycle is regulated by an extensive molecular-regulating mechanism, involving a great number of interacting genes (Komaki and Sugimoto 2012), only those genes where examined whose expression in response to abiotic stress during cell cycle progression had been previously demonstrated (Jang et al 2005). We analysed transcript levels of NtcycA13, a cyclin A that accumulates in the S phase; Ntcyc29, a cyclin B that accumulates in the late $G_2$ and during the M phase;

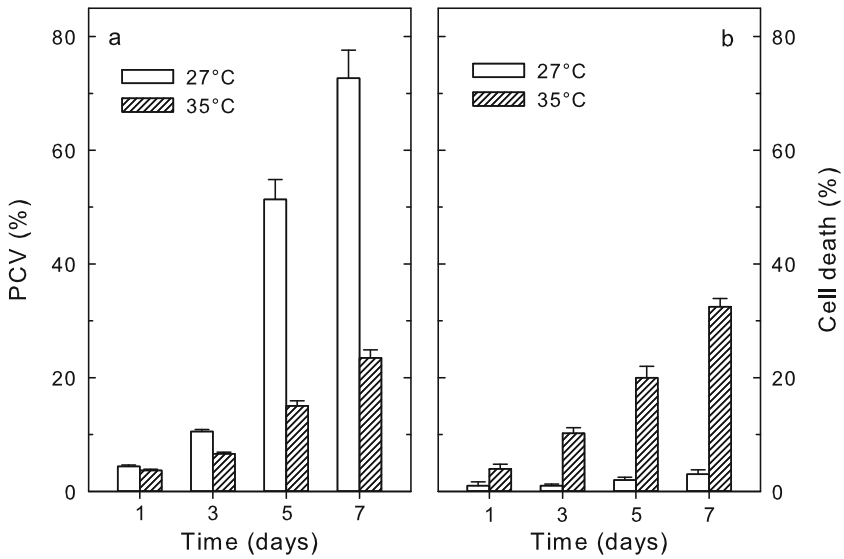



**Fig. 1** Time-dependent effect of heat stress on cell growth and viability. Packed cell volume (*PCV*) (**a**) and cell death (**b**) in tobacco BY-2 cells grown at 27 and 35 °C. Cell death was measured by trypan blue staining and phase contrast microscopy. The percentage of cell death was counted in a population of at least 1000 cells for each experiment. Values of PCV and cell death are the mean (± SE) of five independent replicates

CycD3-1 that accumulates in the late $G_2$ to M phase; and CDKB1-1, a cyclin-dependent serine/threonine kinase that accumulates in the M phase and promotes $G_2$-M transition (Sorrell et al. 1999, 2001; Jang et al. 2005).

Exposure to 35 °C determined a decrease in the transcripts of NtcycA13, Ntcyc29 and CDKB1-1 3 days after the start of the treatment, as shown by the representative images and densitometric analysis of the RT-PCR products. On the other hand, an increase in CycD3-1 transcript occurred 1 day after the beginning of the heat stress (Fig. 3).

In order to verify whether heat stress also influenced cell expansion, cell dimension was determined at 27 and 35 °C. Figure 4a shows that at 27 °C cell length decreased in the first 3 days of growth, when cells were actively dividing (Fig. 2). At the end of cell division, when the mitosis decreased and evolution of the cell number had stopped (Fig. 2), a gradual increase in cell size was observed (Fig. 4a), thus suggesting the occurrence of cell expansion. Continuous exposure to 35 °C blocked cell expansion, and the length of the cells remained lower than observed in control cells (27 °C) from the third to the seventh day of culture (Fig. 4a, b). Consistently, the protein content in cells grown at 27 °C progressively decreased starting from the third day of culture. This was as a consequence of the enlargement of the vacuole which, at the end of growth curve, occupied the majority of cell volume (Reisen et al. 2005). On the other hand, in the cells exposed to 35 °C, protein content remained high from the third to the seventh day of culture (Fig. 4c).

In order to gain more information on cell expansion during heat stress, we studied the expression of Nt-EXPA5, a tobacco expansin involved in cell growth (Link and Cosgrove 1998; Kuluev et al. 2013). The transcript level of Nt-EXPA5 was significantly reduced after 3 days in cells grown at 35 °C compared with control cells (Fig. 5), thus confirming that heat stress inhibited cell expansion.

In plants, the alteration of gene expression in response to environmental changes can be due to epigenetic modifications. We thus analysed whether the changes in the expression

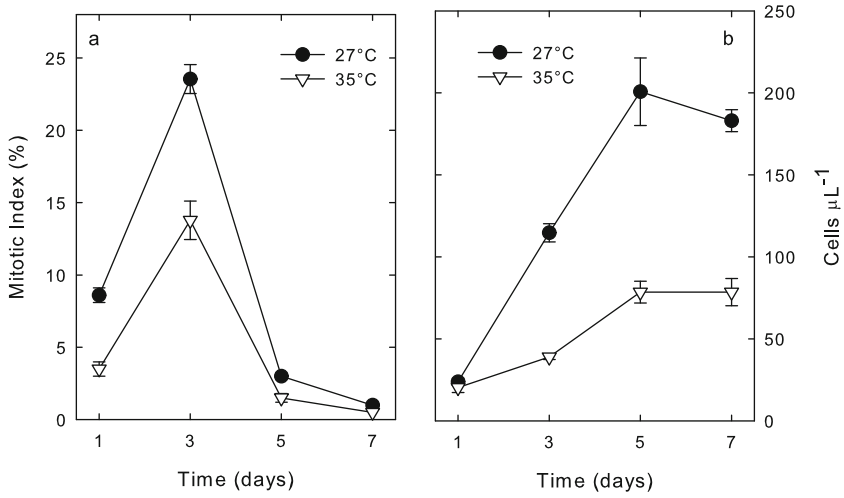



**Fig. 2** Time-dependent effect of heat stress on cell division. Mitotic index (**a**) and cell number (**b**) in tobacco BY-2 cell suspensions grown at 27 and 35 °C. The values are the mean (± SE) of five independent replicates in each of which at least 1000 cells were analysed

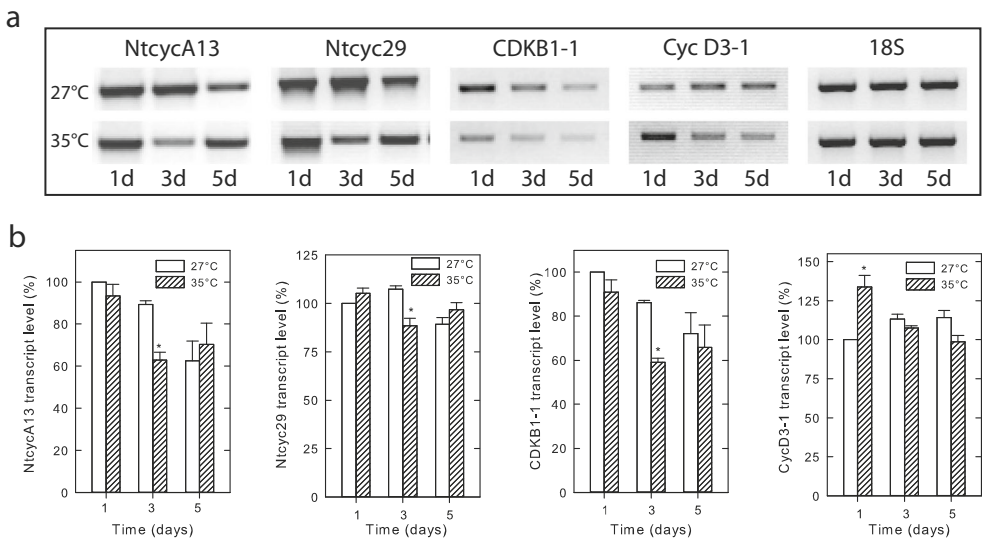



**Fig. 3** Gene expression analysis of genes involved in cell cycle progression in tobacco BY-2 cells subjected to heat stress. Representative semi-quantitative RT-PCR results for NtCycA13, NtCyc29, CDKB1-1 and CycD3-1 in control and heat-stressed cells after 1, 3 and 5 days of culture. Levels of 18S rRNAs were determined to normalize the results. **a** Percentage of transcript levels NtCycA13, NtCyc29, CDKB1-1 and CycD3-1 **b** obtained after densitometric analysis and normalization with 18S rRNA (see Mat and Meth). Values are the mean±SE of five replicates. *Asterisks* indicate values that are significantly different from the control (Student's *t* test with $P<0.05$)

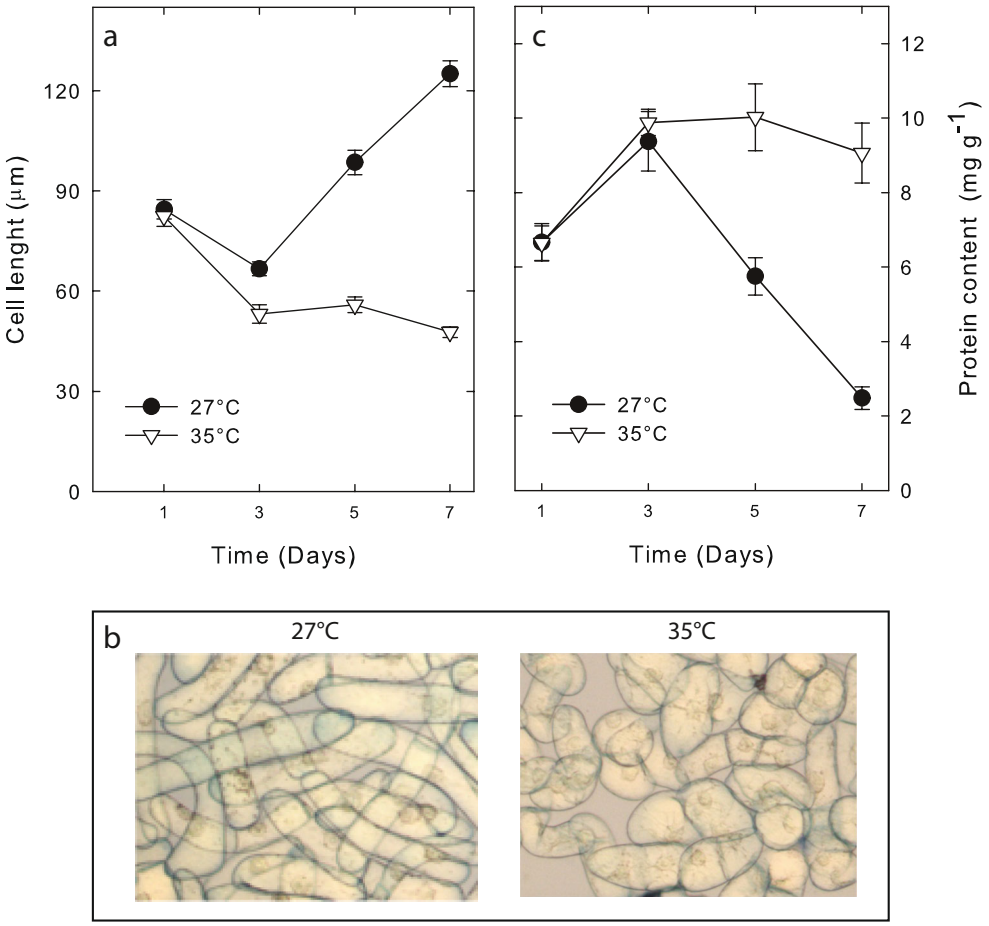



**Fig. 4** Time-dependent effect of heat stress on cell expansion. Cell length of tobacco BY-2 cells grown at 27 and 35 °C in different days of culture (**a**). Representative images of BY-2 cells grown at 27 and 35 °C after 5 days of culture (**b**). Protein content of tobacco BY-2 cells grown at 27 and 35 °C in different days of culture (**c**). Values of cell length and protein content are the mean (± SE) of five independent replicates. Cell length was measured in a population of at least 1000 cells in each separate experiment. *Bar*=20 µm

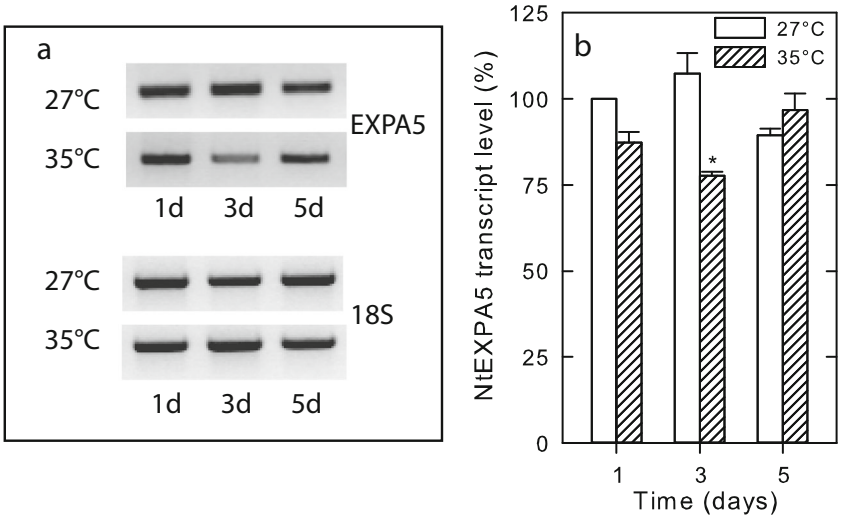



**Fig. 5** Gene expression analysis of Nt-EXPA5 in control and heat-stressed cells after 1, 3 and 5 days of culture. Representative semi-quantitative RT-PCR results for Nt-EXPA5 in control and heat-stressed cells after 1, 3 and 5 days of culture. Levels of 18S rRNAs were determined to normalize the results (**a**). Percentage of transcript levels of Nt-EXPA5 (**b**) obtained after densitometric analysis and normalization with 18S rRNA (see Mat and Meth). Values are the mean±SE of five replicates. *Asterisk* indicates values that are significantly different from the control (Student's *t* test with $P<0.05$)

of the investigated genes could be due to variations in the methylation state of the promoters of these genes.

To reconstruct the upstream regions of the five genes, the coding sequences were used as queries in blast search in two different databases (see Mat and Meth). The findings enabled us to elongate the 5′ regions of four genes (NtcycA13, CDKB1-1, CycD3-1, Nt-EXPA5; Supplemental Figure 1). In contrast, with this method, no elongation was possible for the 5′ region of Ntcyc29.

The methylation state of the four 5′ sequences identified was analysed by the McrBC assay. The primers were designed by the MethPrimer software (Li and Dahiya 2002) around the CpG-enriched regions identified in the reconstructed 5′ sequences of the genes. Total DNA, extracted from the cells grown at 27 and 35 °C after 1 and 3 days of culture, was digested with the McrBC, a restriction enzyme which only cuts the methylated cytosine, and then quantified by qPCR using the previously designed primers (Supplemental Table 2). The amount of amplified DNA was inversely proportional to the methylation level of the considered region. The results were reported as the relative methylation state (see Mat and Meth). In the cells exposed for 1 day at 35 °C, hypomethylation of the 5′ region of the CycD3-1 occurred. After 1 and 3 days of treatment at 35 °C, the 5′ sequence of Nt-EXPA5 was hypermethylated. Upstream sequences of NtcycA13 and CDKB1-1 did not show any significant differences in their methylation state due to heat stress (Fig. 6).

## Discussion

Under adverse environmental conditions, plants are able to significantly modify their growth pattern to trigger defence responses to overcome stress (Rymen and Sugimoto 2012). The morphogenetic response may be due to the inhibition of cell division or cell expansion; however, sometimes, both processes are affected (Lecoeur et al. 1995; Rymen et al. 2007; Skirycz and Inze 2010; Aquea et al. 2012). In line with the literature, our results show that the exposure of TBY-2 cells to a moderate but persistent heat stress caused a strong reduction in cell growth (Fig. 1), due to the inhibition of both cell division and cell expansion (Figs. 2 and 4).

Inhibition of cell division under adverse environmental conditions seems to depend on an arrest or a delay in cell cycle progression. It has been reported that stress conditions stop the cell cycle at $G_1$/S or $G_2$/M checkpoints or slow down the entire cell cycle (Granier and Tardieu 1999; West et al. 2004; Rymen

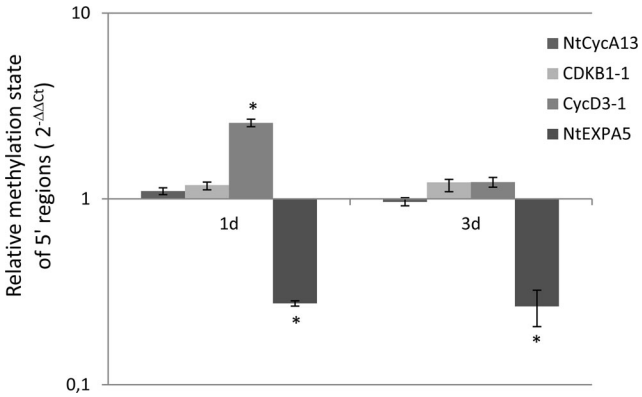



**Fig. 6** Effect of heat stress on the methylation state of upstream sequences of NtCycA13, CDKB1-1, CycD3-1 and Nt-EXPA5 after 1 and 3 days of culture. Reported values ($2^{-\Delta\Delta Ct}$) indicate methylation state of genes in cells grown at 35 °C compared to the same genes in cells grown at 27 °C. Values are the mean±SE of three biological replicates. *Asterisks* indicate values that are significantly different from the control (Student's *t* test with $P<0.01$)

et al. 2007). Cell cycle arrest under stress conditions can be due to the downregulation of cell cycle activators, such as specific cyclins and CDKBs (West et al. 2004; Rymen et al. 2007). The literature data show that the exposure of tobacco BY-2 cells at 30 °C cause a transient block of cell cycle progression (Jang et al. 2005). In line with the literature, tobacco BY-2 cells exposed at 35 °C showed an altered expression of genes encoding proteins of cell cycle regulation (Fig. 3) which could be responsible for a delay in cell cycle progression. After 1 day of exposure at 35 °C, there was an increase in CycD3-1 expression (Fig. 3). *Arabidopsis* CycD3-1 has been clearly indicated as the limiting factor for $G_1$-S transition (Menges et al. 2006). On the other hand, in tobacco BY-2 cells, CycD3-1 accumulates during the late $G_2$ to the M phase but is still present in the $G_1$ phase (Sorrell et al. 1999; Kawamura et al. 2006). The different behaviors of CycD3-1 in tobacco BY-2 cells could be a consequence of the indefinite proliferation potential and of the long-term culture of these cells or could be attributed to a different role of this cyclin in cell cycle progression (Sorrell et al. 1999). However, CycD3-1-associated kinases are able to phosphorylate the tobacco retinoblastoma-related protein with a peak at the $G_1$-S and a relatively constant level during the S to $G_2$/M phase (Kawamura et al. 2006). This data, together with studies conducted in mammalian cells showing that the phosphorylation of retinoblastoma proteins in $G_2$/M can cause an arrest of cells in $G_2$ (Taya 1997), enables us to speculate that the increase in CycD3-1 transcript observed in tobacco BY-2 cells after heat stress (Fig. 3) could accelerate $G_1$-S transition and simultaneously block the cells in $G_2$. It has also been shown that the over-expression of another tobacco CycD3, namely the CycD3-3, shortens the $G_1$ phase, lengthens the S phase and reduces the cell cycle in tobacco BY-2 cells (Nakagami et al. 2002). Also, synchronized *Arabidopsis* cells over-expressing CycD3-1 have shown an increase in $G_2$ length and a simultaneous delay in the activation of genes characterizing the M phase, such as those coding for B-type cyclins (Menges et al. 2006). Consistently, 3 days after the exposure at 35 °C, tobacco BY-2 cells show a reduction in the expression of NtCycA13, a cyclin A characteristic of S phase, NtCyc29 and CDKB1-1, a cyclin B and a cyclin-dependent kinase of B type, respectively, both required for the $G_2$-M transition (Sorrell et al. 2001; Jang et al. 2005). From these data, it is possible to hypothesize that the cell cycle block occurring in tobacco BY-2 cells exposed at 35 °C probably takes place at the $G_2$-M checkpoint (Fig. 7). Indeed, an increase in cycD3-1 should overcome the $G_1$-S checkpoint. On the other hand, a decrease in NtCycA13 and NtCyc29 and in CDKB1-1 could arrest the cell cycle in the pre-mitotic phases (Figs. 3 and 7). The delay in cell cycle progression that we found during the first 3 days of heat stress could be considered as a defence strategy, since it may increase the defence systems needed to protect the cells (Sgobba et al. 2015). The exposure of tobacco BY-2 cells to 35 °C also inhibited cell expansion, as indicated by the marked reduction in cell size

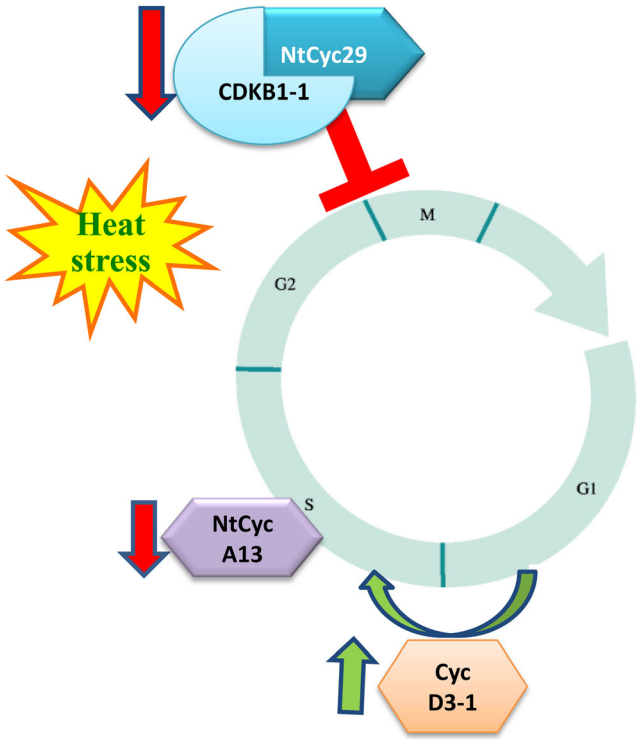



**Fig. 7** Schematic representation of the delay of cell cycle progression in tobacco BY-2 cells subjected to heat stress. The increase in CycD3-1 (indicated with *green arrow*) after 1 day from the heat stress permits to overcome the $G_1$-S checkpoint, whereas the decrease in NtCycA13, NtCyc29 and CDKB1-1 (indicated with *red arrows*) after 3 days of heat stress could stop the cells in the pre-mitotic phases

(Fig. 4). It is known that both the rate and the duration of cell expansion change according to turgor pressure and cell wall elasticity (Rymen and Sugimoto 2012). Expansins play a key role in determining the increase in cell wall flexibility, needed for cell expansion. They are proteins that cut hydrogen bonds between cellulose microfibrils and hemicellulosic polysaccharides (Kende et al. 2004; Sampedro and Cosgrove 2005). It has been reported that transcript level of some expansins has a positive correlation with cell expansion (Muller et al. 2007). Our data show that the Nt-EXPA5 transcript decreased in cells subjected to heat stress on the third day of growth (Fig. 5), which is when cell expansion started in the control cells as indicated by the increase in cell length over the subsequent days (Fig. 4). This result is also corroborated by the literature data showing that the reduction of hypocotyl growth observed in maize seedlings subjected to salt stress is closely related to the under-expression of three different expansins (Geilfus et al. 2010).

Changes in the expression of genes occurring during either normal plant development or in response to environmental changes may be due to epigenetic modifications (Wada et al. 2004; Causevic et al. 2006; Ahmad et al. 2010; Mirouze and Paszkowski 2011). DNA methylation, together with histone modifications, is involved in the determination of the

epigenetic state of the genome, affecting not only large chromosomal domains, but also individual genes (Suzuki and Bird 2008). Interestingly, only differential methylation in upstream genic regions seems to be strictly correlated with gene expression (Candaele et al. 2014).

A notable result emerging from our present work is that the alteration in the expression of two genes involved in the control of cell growth, cyclin D3-1 and Nt-EXPA5, subsequent to the exposure of cells to 35 °C, seems to be due to changes in the methylation state of the 5′ sequences of these genes.

The hypomethylation of the 5′ region of CycD3-1 1 day after heat stress exposure is consistent with the increase in its transcript level (Figs. 3 and 6). Literature data concerning both plant and animal systems suggest that changes in the methylation patterns of the upstream regions of different D-type cyclins can alter their expression. For instance, the silencing of the cyclin *D2* gene, which can be considered an early event in tumorigenesis, may be associated with the hypermethylation of the CpG island in the promoter of cyclin D2 (Evron et al. 2001). In addition, methylation-silenced cyclin D2 can be de-repressed by sulphoraphane, which decreases methylation in the cyclin D2 promoter (Hsu et al. 2011). In maize, the hypermethylation of the upstream sequence of CycD4, occurring in the transition from the dividing to the expanding zone of leaves, is correlated with the decrease in its expression (Candaele et al. 2014).

In our experimental conditions, the 5′ region of the CycD3-1 gene shows a rapid cyclical pattern of the methylation state, since hypomethylation was present only on the first day after heat stress, and the methylation state of this gene returned to the same state as that observed in control cells after 3 days of heat treatment. Although the epigenetic modifications of DNA methylation are usually associated with long-term memory genomics, new scenarios have recently been emerging. In human cells, the methylation pattern of the promoter regions of some genes may undergo rapid cyclical variations (Baccarelli et al. 2009). Although the mechanisms involved in the changes of methylation status of the CycD3-1 still need clarifying, we believe that our result is the first case of a rapid and dynamic role for DNA methylation in gene regulation in plant cells.

Our results also highlight that the hypermethylation of the promoter region of Nt-EXPA5 is in line with the decrease in its transcript level (Figs. 5 and 6). Candaele et al. (2014) also reported that changes in the methylation state of the upstream region of EXPB3 are inversely correlated with changes in the expression of this gene. This would thus seem to highlight that changes in DNA methylation could control cell division and cell expansion (Candaele et al. 2014).

In conclusion, our data demonstrate that a moderate but prolonged heat stress can affect cell growth by altering cell cycle progression and cell expansion. The results also highlight that heat stress induces changes in the methylation pattern of the upstream regions of CycD3-1 and Nt-EXPA5 and that these epigenetic changes could be responsible for the altered expression of these genes.

These results would seem to suggest that changes in the methylation state of genes involved in normal plant development contribute to the alteration of growth in response to environmental changes.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References


Ahmad A, Zhang Y, Cao XF (2010) Decoding the epigenetic language of plant development. Mol Plant 3:719–728

Aquea F, Federici F, Moscoso C, Vega A, Jullian P, Haseloff J, Arce-Johnson P (2012) A molecular framework for the inhibition of Arabidopsis root growth in response to boron toxicity. Plant Cell Environ 35:719–734

Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179:572–578

Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci 4:273

Boyko A, Kathiria P, Zemp FJ, Yao YL, Pogribny I, Kovalchuk I (2007) Transgenerational changes in the genome stability and methylation in pathogen-infected plants (virus-induced plant genome instability). Nucleic Acids Res 35:1714–1725

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Candaele J, Demuynck K, Mosoti D, Beemster GTS, Inze D, Nelissen H (2014) Differential methylation during maize leaf growth targets developmentally regulated genes. Plant Physiol 164:1350–1364

Causevic A, Gentil MV, Delaunay A, El-Soud WA, Garcia Z, Pannetier C, Brignolas F, Hagege D, Maury S (2006) Relationship between DNA methylation and histone acetylation levels, cell redox and cell differentiation states in sugarbeet lines. Planta 224:812–827

Choi CS, Sano H (2007) Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. Mol Genet Genomics 277:589–600

Christensen JH, Christensen OB (2007) A summary of the PRUDENCE model projections of changes in European climate by the end of this century. Clim Chang 81:7–30

de Pinto MC, Tommasi F, De Gara L (2000) Enzymes of the ascorbate biosynthesis and ascorbate-glutathione cycle in cultured cells of tobacco Bright Yellow 2. Plant Physiol Biochem 38:541–550

de Pinto MC, Tommasi F, De Gara L (2002) Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright-Yellow 2 cells. Plant Physiol 130:698–708

Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, Buluwela L, Weitzman SA, Marks J, Sukumar S (2001) Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. Cancer Res 61:2782–2787

Geilfus CM, Zorb C, Muhling KH (2010) Salt stress differentially affects growth-mediating beta-expansins in resistant and sensitive maize (Zea mays L.). Plant Physiol Biochem 48:993–998

Granier C, Tardieu F (1999) Leaf expansion and cell division are affected by reducing absorbed light before but not after the decline in cell division rate in the sunflower leaf. Plant Cell Environ 22:1365–1376

Hsu A, Wong CP, Yu Z, Williams DE, Dashwood RH, Ho E (2011) Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. Clin Epigenetics 3:3

Huang NC, Li CH, Lee JY, Yen HE (2010) Cytosine methylation changes in the ice plant Ppc1 promoter during transition from C-3 to Crassulacean acid metabolism. Plant Sci 178:41–46

Jang SJ, Shin SH, Yee ST, Hwang B, Im KH, Park KY (2005) Effects of abiotic stresses on cell cycle progression in tobacco BY-2 cells. Mol Cells 20:136–141

Kawamura K, Murray JAH, Shinmyo A, Sekine M (2006) Cell cycle regulated D3-type cyclins form active complexes with plant-specific B-type cyclin-dependent kinase in vitro. Plant Mol Biol 61:311–327

Kende H, Bradford KJ, Brummell DA, Cho HT, Cosgrove DJ, Fleming AJ, Gehring C, Lee Y, McQueen-Mason S, Rose JKC, Voesenek LACJ (2004) Nomenclature for members of the expansin superfamily of genes and proteins. Plant Mol Biol 55:311–314

Komaki S, Sugimoto K (2012) Control of the plant cell cycle by developmental and environmental cues. Plant Cell Physiol 53:953–964

Kuluev BR, Safiullina MG, Knyazev AV, Chemeris AV (2013) Effect of ectopic expression of NtEXPA5 gene on cell size and growth of organs of transgenic tobacco plants. Russ J Dev Biol 44:28–34

Kumar A, Kaur J (2014) Primer based approach for PCR amplification of high GC content gene: mycobacterium gene as a model. Mol Biol Int 2014:937308

Lecoeur J, Wery J, Turc O, Tardieu F (1995) Expansion of pea leaves subjected to short water-deficit—cell number and cell-size are sensitive to stress at different periods of leaf development. J Exp Bot 46:1093–1101

Li LC, Dahiya R (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics 18:1427–1431

Link BM, Cosgrove DJ (1998) Acid-growth response and alpha-expansins in suspension cultures of Bright Yellow 2 tobacco. Plant Physiol 118:907–916

Madlung A, Comai L (2004) The effect of stress on genome regulation and structure. Ann Bot London 94:481–495

Menges M, Samland AK, Planchais S, Murray JAH (2006) The D-type cyclin CYCD3;1 is limiting for the G1-to-S-phase transition in Arabidopsis. Plant Cell 18:893–906

Mirouze M, Paszkowski J (2011) Epigenetic contribution to stress adaptation in plants. Curr Opin Plant Biol 14:267–274

Muller B, Bourdais G, Reidy B, Bencivenni C, Massonneau A, Condamine P, Rolland G, Conejero G, Rogowsky P, Tardieu F (2007) Association of specific expansins with growth in maize leaves is maintained under environmental, genetic, and developmental sources of variation. Plant Physiol 143:278–290

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell-line as the Hela-cell in the cell biology of higher-plants. Int Rev Cytol 132:1–30

Nakagami H, Kawamura K, Sugisaka K, Sekine M, Shinmyo A (2002) Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. Plant Cell 14:1847–1857

Ortega L, Taleisnik E (2003) Elongation growth in leaf blades of *Chloris gayana* under saline conditions. J Plant Physiol 160:517–522

Pasternak T, Potters G, Caubergs R, Jansen MAK (2005a) Complementary interactions between oxidative stress and auxins control plant growth responses at plant, organ, and cellular level. J Exp Bot 56:1991–2001

Pasternak T, Rudas V, Potters G, Jansen MAK (2005b) Morphogenic effects of abiotic stress: reorientation of growth in *Arabidopsis thaliana* seedlings. Environ Exp Bot 53:299–314

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12:98–105

Potters G, Pasternak TP, Guisez Y, Jansen MAK (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant Cell Environ 32:158–169

Reisen D, Marty F, Leborgne-Castel N (2005) New insights into the tonoplast architecture of plant vacuoles and vacuolar dynamics during osmotic stress. BMC Plant Biol 5:13

Rymen B, Sugimoto K (2012) Tuning growth to the environmental demands. Curr Opin Plant Biol 15:683–690

Rymen B, Fiorani F, Kartal F, Vandepoele K, Inze D, Beemster GTS (2007) Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiol 143:1429–1438

Saidi Y, Finka A, Goloubinoff P (2011) Heat perception and signalling in plants: a tortuous path to thermotolerance. New Phytol 190:556–565

Sampedro J, Cosgrove DJ (2005) The expansin superfamily. Genome Biol 6:242

Sgobba A, Paradiso A, Dipierro S, De Gara L, de Pinto MC (2015) Changes in antioxidants are critical in determining cell responses to short- and long-term heat stress. Physiol Plant 153:68–78

Skirycz A, Inze D (2010) More from less: plant growth under limited water. Curr Opin Biotechnol 21:197–203

Smertenko A, Draber P, Viklicky V, Opatrny Z (1997) Heat stress affects the organization of microtubules and cell division in *Nicotiana tabacum* cells. Plant Cell Environ 20:1534–1542

Sorrell DA, Combettes B, Chaubet-Gigot N, Gigot C, Murray JAH (1999) Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco bright yellow-2 cells. Plant Physiol 119:343–351

Sorrell DA, Menges M, Healy JMS, Deveaux Y, Amano C, Su Y, Nakagami H, Shinmyo A, Doonan JH, Sekine M, Murray JAH (2001) Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells. Plant Physiol 126:1214–1223

Suzuki MM, Bird A (2008) DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 9:465–476

Taya Y (1997) RB kinases and RB-binding proteins: new points of view. Trends Biochem Sci 22:14–17

Urano K, Kurihara Y, Seki M, Shinozaki K (2010) 'Omics' analyses of regulatory networks in plant abiotic stress responses. Curr Opin Plant Biol 13:132–138

Vanhees K, Coort S, Ruijters EJB, Godschalk RWL, van Schooten FJ, van Doorn-Khosrovani SBV (2011) Epigenetics: prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage. FASEB J 25:797–807

Verma S, Mishra SN (2005) Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. J Plant Physiol 162:669–677

Wada Y, Miyamoto K, Kusano T, Sano H (2004) Association between up-regulation of stress-responsive genes and hypomethylation of genomic DNA in tobacco plants. Mol Genet Genomics 271:658–666

West G, Inze D, Beemster GTS (2004) Cell cycle modulation in the response of the primary root of Arabidopsis to salt stress. Plant Physiol 135:1050–1058

Xiao WY, Custard KD, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL (2006) DNA methylation is critical for Arabidopsis embryogenesis and seed viability. Plant Cell 18:805–814