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Jasmonic acid prevents the accumulation of cyclin B1;1 and CDK-B in synchronized tobacco BY-2 cells

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Abstract **Jasmonic acid (JA) plays a crucial role in plant fertility and defense responses. It exerts an inhibitory effect on plant growth when applied exogenously. This effect seems to be somehow related to a negative regulation of cell cycle progression in the meristematic tissues. In this report, we focus on the molecular events that occur during JA-induced G2 arrest. We demonstrate that JA prevents the accumulation of B-type cyclin-dependent kinases and the expression of cyclin B1;1, which are both essential for the initiation of mitosis. This feature suggests the existence of an early G2 checkpoint that is affected by JA.**
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Keywords: Jasmonic acid; Cell cycle; Cyclin B1;1; Roscovitine; G2/M transition

1. Introduction

Cyclin-dependent kinases (CDKs) play a central regulatory role during cell division in all eukaryotic cells [1]. Their activity is strictly controlled at the time of the G1/S and G2/M transitions of the cell cycle by means of phosphorylation and interactions with regulatory subunits [2]. One class of those subunits, called cyclins, determines the substrate specificity and subcellular localization of the kinase [3]. Cyclins are expressed at specific stages of the cell cycle and are rapidly degraded. There are three major types of cyclins in plants. D-type cyclins are expressed at the initial stage of the cell division, followed by A-type cyclins, which are thought to control DNA-replication and related events. Finally, the B-type cyclins accumulate prior to the onset of mitosis [4]. In a previous report [5], we demonstrated that jasmonic acid (JA) prevents the G1/S and G2/M transitions in synchronized tobacco BY-2 cells. We noted an interesting discrepancy: when JA was applied during S-phase, it prevented cells from entering mitosis without directly affecting their DNA synthesis. However, when applied during G2 phase, its effect on mitosis was negligible. There is approximately a 3-h interval between the S-phase and mitosis, during which exogenously added JA rapidly accumulates in the cells and is slowly converted to more hydrophilic derivatives. Yet, none of those compounds seem to have any effect on cell cycle progression between the S-phase and mitosis [6].

With the experiments described in this study, we examined the molecular mechanisms responsible for the anti-mitotic properties of JA. In order to pinpoint possible targets of JA action during G2, the expression of the mitotic markers cyclin B1;1 and B-type CDK upon JA treatment was studied. The resulting data provide evidence that JA induces G2 arrest in quite a unique manner. It decreases the histone H1 kinase activity of the CDK complexes and prevents the accumulation of cyclin B1;1 and CDK-B during G2. These features exhibit the characteristics of an early G2 checkpoint.

2. Materials and methods

2.1. Chemicals and equipment

All chemicals and basic materials were obtained from Sigma (Bornem, Belgium), unless mentioned otherwise.

2.2. Cell culture and synchronization

The BY-2 cells were maintained as described by Nagata et al. [7]. The culture was kept at 27 °C in constant darkness and rotating at 130 rpm. The synchronization protocol was based on the method of Nagata et al. [7]. The stationary culture was transferred in a proportion of 14:100 to the fresh medium and supplied with 5 mg L^{-1} aphidicolin (ICN Biomedicals, Asse, Belgium). After 24 h of incubation, the cells were released by extensive washing with fresh medium and were subjected to the treatments as indicated in the text. The pCycB1;1::GUS [pCDG] construct (a gift from P. Doerner) was introduced into disarmed *Agrobacterium tumefaciens* C58pMP90 by electroporation and the resulting *Agrobacterium* strain was used to transform the BY-2 cells according to [8].

2.3. Protein extraction and histone H1 kinase assays

Kinase activity was measured according to [9] with modifications as described [5]. Hundred μg of protein was incubated at 4 °C for 2 h with $p13^{Suc1}$ agarose beads (Oncogene, San Diego) or anti-CDK-B antibody [10]. The latter was afterwards precipitated using protein A or G agarose (Amersham, Freiburg, Germany). The samples were analyzed by SDS–PAGE in 17.5% (w/v) acrylamide gel. The incorporated radioactivity of histone H1 was visualized and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Western blots were performed according to [11].

2.4. β-Glucuronidase assay and mitotic index determination

Protein extracts were prepared by grinding cells to a fine powder in liquid N_2 with mortar and pestle. Frozen powder was suspended in a 50 mM sodium phosphate buffer of pH 7.0, supplied with 10 mM β-mercaptoethanol, 10 mM EDTA and 0.1% Triton X-100. The samples were cleared by centrifugation at 4 °C and 13 000 rpm for 15 min. β-Glucuronidase (GUS) activity was measured according to [12]. Reaction progress was monitored with a SpectraMAX (Molecular

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Abbreviations: JA, jasmonic acid; CDK, cyclin-dependent kinase; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride; GUS, β-glucuronidase; MU, 4-methyl umbelliferone

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Devices, Sunnyvale, CA, USA) fluorescence plate reader (excitation 355 nm, emission 450 nm). Prior to the mitotic index measurements, the cells were fixed in ethanol:acetic acid 3:1 [v/v], stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) and examined under a fluorescence microscope (Nikon Instruments, Analis SA, Ghent, Belgium).

3. Results

3.1. Jasmonic acid targets S-phase

In order to determine which part of the cell cycle is specifically targeted by JA, we tested whether the removal of JA after 1 or 2 h of treatment during S-phase could restore mitotic activity (Fig. 1). A synchronization experiment was performed, during which the aphidicolin-released culture was divided into two pools. One was left untreated, while the other one received 100 μM JA immediately after aphidicolin release and was subsequently divided into three sub-cultures. The untreated control sub-culture reached a mitotic peak of 39% 5 h after aphidicolin release. The continuous presence of 100 μM JA caused a delay and reduction of the mitotic peak to 21% after 7 h. Removal of JA after 2 h or even after 1 h did not reverse the JA-induced decrease or delay of the mitotic peak. In a separate experiment, we demonstrated that washing in itself had no effect on the mitotic index of the synchronized culture, and that the washing procedure removed more than 90% of the JA taken up by the cells (data not shown). Those facts and our earlier observations [5] suggest that JA-induced arrest in G2 is imprinted during the first hour of the experiment, when cells are engaged in S-phase, which under our laboratory conditions, is completed within 2 h after aphidicolin release of tobacco BY-2 cells [5].

3.2. Jasmonic acid negatively regulates CDK activity

Taking this into consideration, we examined the effect of JA on the expression of the A- and B-type CDK complexes and their histone H1 kinase activity during S, G2 and mitosis in synchronized tobacco BY-2 cells (Fig. 2). At 200 μM concentration, JA completely abolished mitosis. The protein content of both types of CDK was examined by Western blot (Fig. 2A). The CDK-A level remained constant throughout the experiment and was unaffected by the JA-treatment. In accordance with previously reported data [10], the CDK-B level gradually increased, reaching its maximum level at 6 h after aphidicolin release (1 h before the peak of the mitotic index), and then slightly decreased. JA treatment strongly reduced the overall protein level of this kinase, although a similar accumulation profile with a peak at 6 h was still clearly visible. The total histone H1 kinase activity of both CDK complexes was measured upon affinity purification with $p13^{Suc1}$ beads (Fig. 2).

The kinase activity remained unaltered during the first two hours following JA treatment, after which we observed an increased activity in the untreated control and a reduced activity in the JA-treated cells. Upon immunoprecipitation, using a specific anti-CDK-B antibody, the kinase activity of the B-type CDKs was measured after 6, 7 and 8 h, corresponding to the highest accumulation of the CDK-B protein (Fig. 2). In the untreated control cells, the activity was the highest after 7 h, at which time the synchronized culture displayed the peak of mitotic activity. Concomitant with the decrease of the B-type kinase protein level, a treatment with 200 μM JA reduced the B-type CDK activity by approximately 60%.

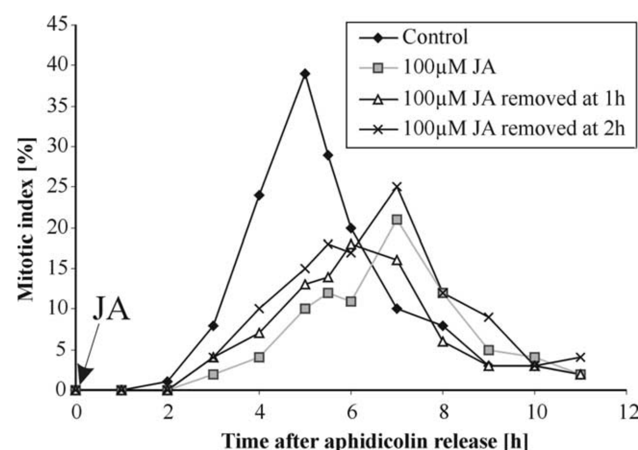

Fig. 1. The duration of JA treatment and the mitotic activity of BY-2 cells, synchronized using aphidicolin. Hundred μM JA was applied immediately after the release and subsequently removed by washing after 1 or 2 h of incubation. The mitotic index was compared to the untreated control and the continuously treated culture.

3.3. CyclinB1;1 accumulation is inhibited by JA

In order to examine the accumulation profile of cyclin B1;1, we used transgenic BY-2 cells harboring a *CYCB1;1* reporter line in which the *CYCB1;1* promoter and N-terminal destruction box are fused in-frame with *GUS* [13]. During the cell cycle progression of the synchronized BY-2 control culture, the GUS activity correlated well with the mitotic index (Fig. 3). It remained low until 5 h after aphidicolin release. Then, it increased approximately 20-fold at 7 h and rapidly decreased afterwards. In cultures treated with JA, the GUS activity was also in correlation with the mitotic index. Treatment with 150 μM JA completely abolished mitotic activity and the GUS activity remained low, not exceeding the background level. Compared to the control cells, in which we observed a mitotic peak of 32% after 7 h, the mitotic peak in the cultures treated with 75 μM JA was delayed with about 4 h, and decreased to 17%. The peak of GUS activity in the 75 μM JA-treated culture was also delayed for about 4 h, only to reach a maximum of merely 43% of the highest GUS activity detected in the control cells.

3.4. Comparison of the effects of roscovitine and JA on mitotic index and cyclinB1;1 accumulation

Because the decrease of CDK activity preceded the reduced accumulation of cyclin B1;1 upon JA treatment, we wanted to investigate whether these two phenomena are directly linked. Therefore, we applied roscovitine to mimic the inhibitory effect of JA on CDK activity. Analogous to the application of 100 μM JA during the S-phase, 50 μM of roscovitine caused a delay of about 4 h and a strong reduction of mitotic activity when applied 3 h after aphidicolin release (Fig. 1 and 3). The accumulation of CYCB1;1-GUS was delayed by the roscovitine treatment, suggesting that there is a certain relation between CDK activity and the induction of cyclin B1;1 expression. However, in contrast with the observed decrease in JA-treated cells, roscovitine caused CYCB1;1-GUS to accumulate, reaching values about two times higher than those observed in the control cells (Fig. 3). This further indicates that JA reduces the expression of cyclin B1;1 independently of the negative regulation of CDK activity.

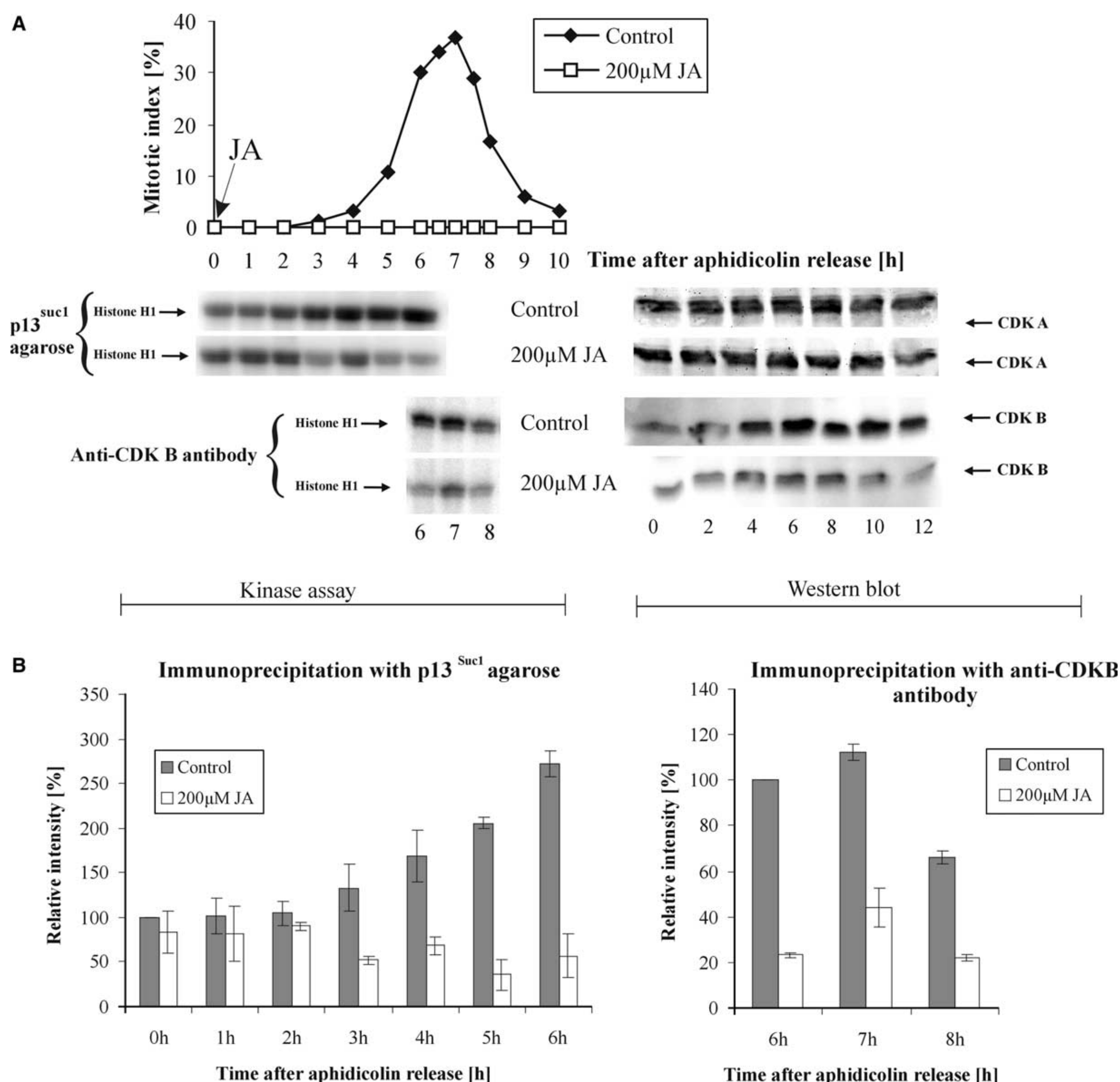

Fig. 2. Protein level and activity of CDKs after JA treatment. The cells were synchronized using aphidicolin and subjected to treatment with 200 μM JA, or kept untreated as a control. The cell cycle progression was analyzed by means of the mitotic index and both cultures were sampled at 1 h intervals. (A) Left: mitotic index aligned with histone H1 kinase activity in treated and untreated cultures; right: protein levels of CDK-A and CDK-B kinases, visualized by Western blot. (B) Quantified data from kinase assays shown in (A), data averaged from two independent measurements ± S.D.

4. Discussion

In synchronized tobacco BY-2 cells, the activity of CDKs gradually increases during cells cycle progression from S-phase to mitosis. However, in JA-treated cells CDK activity started to decrease significantly 2 h after aphidicolin release coinciding with the end of the S-phase. This fact was consistent with the observation that DNA synthesis, which requires CDK activity, progressed undisturbed in the JA-treated cells [5]. Since the bulk of JA was taken up very rapidly [6], we can conclude that the time lapse between the JA feeding, obligatory at the beginning of S, and the decrease of CDK activity were not related to the rate of JA uptake. Furthermore, the removal of JA in late S could not recover mitosis, suggesting that JA acts during early S and causes a subsequent decrease in CDK activity in G2. The short-term regulation of CDK-A activity is not dependent on protein turnover, as A-type CDK kinases have a long half-life [14] and are expressed in all plant cells that show competence for cell division, e.g. [15]. Their activities depend largely on several other factors: the phosphorylation of conserved residues, the presence or absence of inhibitor proteins from the KRP family, and the availability of their activating cyclin partners, which have a cell cycle-dependent expression pattern and are degraded in a strictly regulated fashion [2]. The CYCB1;1 protein is expressed starting from late G2 and the protein is rapidly degraded during the anaphase [16], making it a suitable marker for G2/M transition. JA treatment inhibited the accumulation of the chimeric CYCB1;1-GUS protein in a concentration-dependent manner. The inhibition was not due to increased cyclin degradation, because in an alternative system, where GUS expression was controlled by a *CYCB1;1* promoter and the protein stability

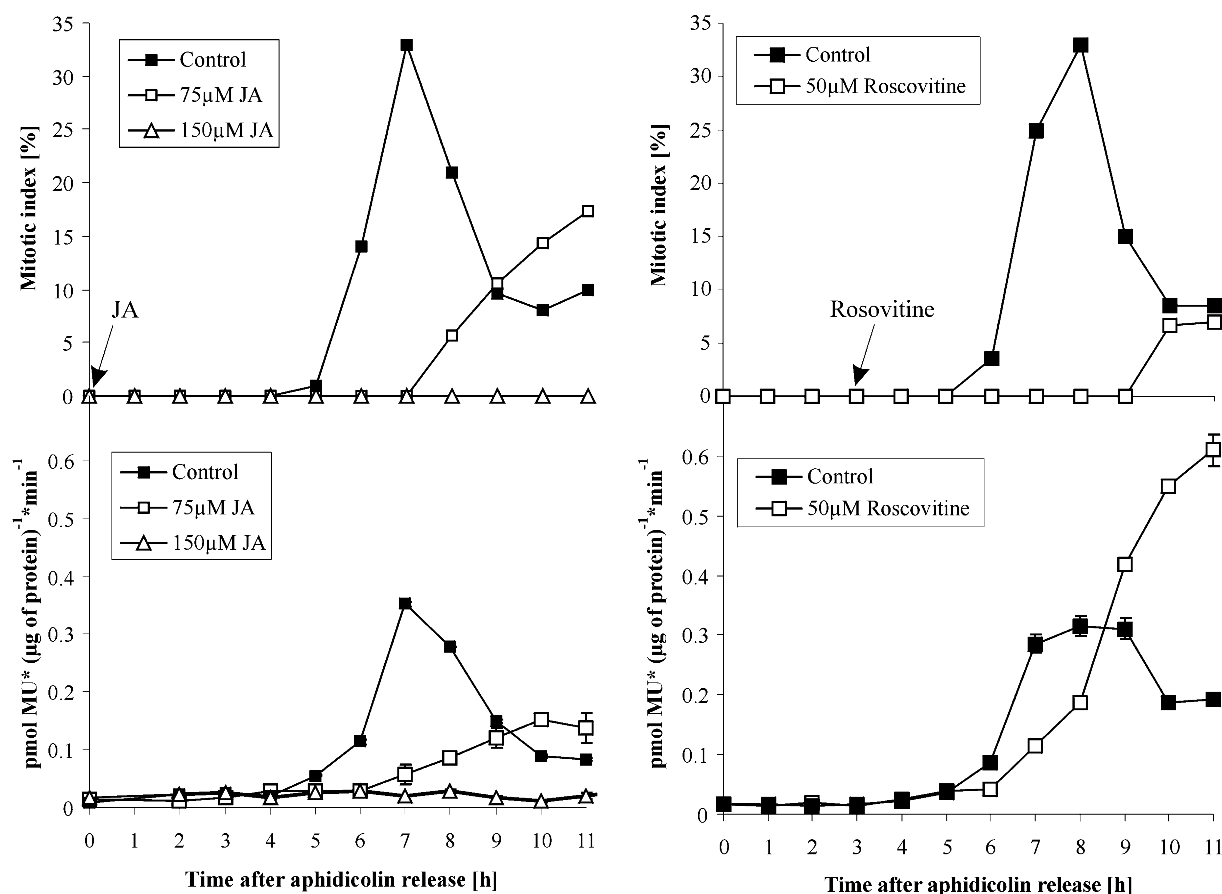

Fig. 3. The effect of JA and roscovitine on the expression of *CYCB1;1* in synchronized tobacco BY-2 cells, transformed with a *pCYCB1;1::GUS* construct. (Left) JA was applied immediately after aphidicolin release; (right) cells treated with 50 μM roscovitine, 3 h after aphidicolin release. The upper graphs represent mitotic index, the lower graphs represent GUS activity, averaged from three measurements ± S.D.

was not controlled by the destruction box, the pre-mitotic activation of expression was also prevented by JA (data not shown). The CDK-B kinase was another mitotic marker we studied. The observed reduction of the CDK-B protein level correlated with the decrease of its activity. Unlike the *CYCB1;1-GUS* expression, it did not decline to background levels, even after a treatment with 200 μM JA, which completely inhibited mitosis. The fact that the CDK-B activity was not completely abolished might seem somewhat surprising, because the accumulation of mitotic CYCB1;1 protein was completely blocked at this level of JA concentration. One would expect a complete loss of CDK-B activity as well, but it has been recently suggested that cyclin B2 [17], rather than cyclin B1, forms a complex with CDK-B, and its expression might be differentially regulated by JA. It seems significant, however, that during the JA-induced cell cycle arrest, both CYCB1;1 and CDK-B accumulation were prevented. Although both proteins accumulate at mitotic entry, their expression is probably regulated by different mechanisms; the MSA elements that mediate G2/M-specific transcription are only present in a *CYCB1;1* promoter and not in *CDK-B* [18,19]. Therefore, the levels of those two proteins are probably indirectly influenced by JA.

We tried to find out whether a decrease in CDK activity, which was observed upon JA treatment, could negatively regulate the expression of *CYCB1;1*. However, in contrast to JA, a strong accumulation of GUS activity was observed after treatment with roscovitine, a potent inhibitor of the CDK activity. This is consistent with the fact that CDK-dependent phosphorylation targets CYCB1;1 for proteasome degradation [16]. Consequently, when the kinase is inhibited by roscovitine, the fusion protein that contains a cyclin destruction box accumulates. A very similar case has been observed in human fibroblast cells, where UV-C-induced DNA damage, which negatively regulates CDK activity, leads to cell cycle arrest and a decrease in *CYCB1* mRNA levels, yet also causes the accumulation of CYCB1 protein [20]. Another possibility is that roscovitine arrests cells in the phase where one of the yet unidentified factors required for the destruction of cyclin B1;1-GUS protein is not expressed. Taking all the data together, it seems that JA causes an arrest in early G2, before the induction of expression of *CYCB1;1* and *CDK-B* takes place. This was further corroborated by the fact that neither zeatin, which is thought to be a positive regulator of G2/M transition at the CDK level [21], nor caffeine, which overrides ATM-dependent G2 arrest [22], could reverse the effect of JA (Swiatek, unpublished data). It seems very likely that JA-treated cells arrest somewhere before the G2/M checkpoints that are targeted by these two agents.

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References

[1] Nurse, P. (2002) Science 289, 1711–1716.
[2] Mironov, V.V., De Veylder, L., Van Montagu, M. and Inze, D. (1999) Plant Cell 11, 505–522.
[3] John, P.L., Mews, M. and Moore, R. (2001) Protoplasma 216, 119–142.
[4] Dewitte, W. and Murray, J.A.H. (2003) Annu. Rev. Plant Biol. 54, 235–264.
[5] Swiatek, A., Lenjou, M., Van Bockstaele, D., Inze, D. and Van Onckelen, H. (2002) Plant Physiol. 128, 201–211.
[6] Swiatek, A., Van Dongen, W., Esmans, E.L. and Van Onckelen, H. (2004) Plant Physiol. 135, 161–172.
[7] Nagata, T., Nemoto, Y. and Hasezawa, S. (1992) Int. Rev. Cytol. 132, 1–30.
[8] Shaul, O., Mironov, V., Burssens, S., Van Montagu, M. and Inze, D. (1996) Proc. Natl. Acad. Sci. USA 93, 4868–4872.
[9] Reichheld, J-P., Vernoux, T., Lardon, F., VanMontagu, M. and Inzé, D. (1999) Plant J. 17, 647–656.
[10] Porceddu, A., Stals, H., Reichheld, J.P., Segers, G., De Veylder, L., Barroco, R.P., Casteels, P., Van Montagu, M., Inze, D. and Mironov, V. (2001) J. Biol. Chem. 276, 36354–36360.
[11] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[12] Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
[13] Colon-Carmona, A., You, R., Haimovitch-Gal, T. and Doerner, P. (1999) Plant J. 20, 503–508.
[14] Welch, P.J. and Wang, J.Y. (1992) Proc. Natl. Acad. Sci. USA 89, 3093–3097.
[15] Beeckman, T., Burssens, S. and Inze, D. (2001) J. Exp. Bot. 52, 403–411.
[16] Genschik, P., Criqui, M.C., Parmentier, Y., Derevier, A. and Fleck, J. (1998) Plant Cell. 10, 2063–2076.
[17] Lee, J., Das, A., Yamaguchi, M., Hashimoto, J., Tsutsumi, N., Uchimiya, H. and Umeda, M. (2003) Plant J. 34, 417–425.
[18] Ito, M., Araki, S., Matsunaga, S., Itoh, T., Nishihama, R., Machida, Y., Doonan, J.H. and Watanabe, A. (2001) Plant Cell. 13, 1891–1905.
[19] Menges, M., Hennig, L., Gruissem, W. and Murray, J.A. (2002) J. Biol. Chem. 277, 41987–42002.
[20] Vincent, F., Deplanque, G., Ceraline, J., Duclos, B. and Bergerat, J.P. (1999) Biol. Cell 91, 665–674.
[21] Zhang, K., Letham, D.S. and John, P.C. (1996) Planta 200, 2–12.
[22] Bartek, J. and Lukas, J. (2001) FEBS Lett. 490, 117–122.