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Histone deacetylase inhibitors and cell proliferation in pea root meristems

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Abstract

The histone deacetylase (HDA) inhibitors, trichostatin A (TSA) and HC toxin halt mitosis in cultured root meristems of *Pisum sativum*, while the anti-protozoan HDA inhibitor apicidin is ineffective. Two-dimensional PAGE of proteins from root meristems exposed to TSA and HC toxin did not show significant differences compared to controls, although a previously tested HDA inhibitor, butyrate, exhibited dramatic variations in its protein profile. Northern analysis of butyrate- and TSA-treated root meristems indicated that non-proliferating cells are expressing significant amounts of transcripts of the known cell proliferation associated genes: histone H2A, MAP kinase, cycA2:1 and cdc2. Western analysis reveals the presence of hyperacetylated nuclear proteins in HDA-inhibitor treated cells. These results suggest that the HDA inhibitors, butyrate and TSA, halt mitosis without down-regulating genes that typically have low or nonexistent expression levels in non-dividing cells.

Keywords: *Pisum sativum*; Leguminosae; Root meristems; Histone deacetylase inhibitors; PAGE; Protein profiles; Northern blots; Western blots

1. Introduction

There has been a growing recognition of the importance of the variable level of acetylation of nuclear proteins in the regulation of transcription. One particular inhibitor of histone deacetylase (HDA), butyrate, also has the ability to inhibit cell proliferation in the root meristems of *Pisum sativum*. In this study we determine whether HDA inhibitors prevent cell division by altering the expression of cell proliferation-associated genes and subsequently the polypeptide profile of the meristem, by the hyperacetylation of nuclear proteins.

A long term objective of molecular biology has been an understanding of the role of chromatin structure in the regulation of transcription. The importance of core histone acetylation is suggested by the fact that heterochromatin in inactive X chromosomes contains histones that are underacetylated, while hyperacetylation is correlated with structural changes consistent with transcriptional activation (Grunstein, 1997).

In mammalian cells in culture a variety of cell cycle alterations occur after exposure to butyrate (Chabanas et al., 1985). After 16 h of butyrate treatment, erythroleukemic cells were only in G1 and G2. After 31 h exposure, almost all cells were in G1. Other experiments support the hypothesis that butyrate arrests cells in G1. Butyrate at 3 mM has been found to inhibit the entry of quiescent serum-stimulated 3T3 cells into the S phase. In these cells, butyrate was also shown to inhibit DNA, but not RNA, synthesis (Kawasaki et al., 1981).

Butyrate and its analogues had inhibitory effects on HDA in both mouse and human cultured cells (Lea

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and Tulsyan, 1995). HDA inhibition by butyrate and also trichostatin A (TSA), have been shown in murine lymphocytes (Yamamoto et al., 1996). A high level of transduced viral-promoter-driven lac Z expression has been shown in Hela cells treated with both butyrate and TSA (Chen et al., 1997). Another HDA inhibitor, apicidin, has been shown to have broad anti-parasite activities (Darkin-Rattary et al., 1996).

Studies on the action of butyrate have revealed that it acts similarly in plant and animal cells in culture. In the root meristems of *Allium cepa* bulbs, butyrate at concentrations of 4 mM and above arrested the meristem cells in mid-G1 or close to the S/G2 border (Lanazagorta et al., 1988). In *Lactuca sativa* meristems, butyrate caused a marked decrease in the incorporation of [^{3}H]-thymine deoxyribose (TdR). This decrease has been correlated with the ability of butyrate to induce cell arrest in G1 and G2. The effect is reversible, and cells return to normal when treatment with butyrate ceases (Chiatante et al., 1986).

Past experiments determined that butyrate had a similar effect on cells in the root meristem of *P. sativum*. Roots exposed to butyrate for 12 h and then transferred to fresh medium, provided evidence that pea cells recovered from the effects of butyrate and resumed cycling (Tramontano et al., 1990). In analogous experiments roots exposed to propionic or valeric acid resumed normal cycling, with a burst of mitosis 8 h after transfer, a result similar to that observed with butyrate (Tramontano et al., 1991). [^{14}C]-butyrate was also incorporated into the root meristem, and eventually moved to the nucleus (Tramontano et al., 1994).

When roots were treated with butyrate, two-dimensional gel electrophoresis revealed that the expression of a series of proteins in the *Mr* 27 and 33 kD range was enhanced with respect to controls. The pI of these proteins was slightly basic, between 7.0 and 7.5. Further experiments with propionic acid-treated roots showed identical results, with an increased expression of the *Mr* 27 and 33 kD proteins. Therefore, the appearance of these polypeptides correlates with the inhibition of mitosis and the repression of DNA synthesis (Tramontano and Barreiro, 1997).

Growth signal transduction pathways ultimately affect the activity of the cell cycle machinery. The core of this machinery consists of the cyclin dependent kinases (Cdks) and the cyclins. The Cdks phosphorylate enzymes and structural proteins enabling passage to the next stage of the cell cycle. The activity of the Cdks is dependent on their expression, the availability of the cyclins, activating phosphorylation, the lack of inhibitory phosphorylation, and the absence of Cdk inhibitors (Hirt, 1996). Cdk homologues have been identified in a variety of higher plants, while all known plant cyclins have been classified as either A-, B-, or D-types. In cells with multiple Cdks and cyclins, it is thought that specific complexes of Cdks and cyclins are active during only certain parts of the cycle (Renaudin et al., 1996). Cdc2 is a Cdk originally identified in *S. pombe*. Cdc2 and MAP kinase mRNAs are detected in some non-dividing plant cells, but the accumulation of high levels is correlated with proliferation, especially in pea bud meristems (Devitt and Stafstrom, 1995; Stafstrom, 1997). Histone H2A mRNA accumulation is a hallmark of the S phase in all eukaryotic cells (Rost and Bryant, 1996). Cyclin A2 mRNA production is tightly linked to cell proliferation in *Arabidopsis thaliana* (Doerner et al., 1996).

Experiments were conducted herein to first test if highly specific HDA inhibitors such as TSA, HC toxin and apicidin halt cellular proliferation and alter protein expression in a manner similar to butyrate. Next, via Northern blotting of specific cell proliferation genes, the question of whether butyrate-treated, butyrate-recovered and TSA-treated root meristems have significantly different gene expression patterns from control, non-treated roots was addressed. Lastly, via Western analysis we attempted to determine if HDA-inhibitor treated cells possess hyperacetylated nuclear proteins. This data will provide insight into the possible mechanism behind the ability of HDA inhibitors to alter cell proliferation capacity.

2. Results and discussion

Table 1 shows the effects of various HDA inhibitors on the mitotic index of isolated root meristems of *P. sativum*. Twelve h exposure to 10^{-5} M TSA resulted in a mitotic index of 0.2 ± 0.1. After 72 h the mitotic index was 1.4 ± 0.2, well below the normal value of 4.5. In a similar fashion, exposure to 10^{-5} M HC toxin resulted in a mitotic index of 0.9 ± 0.2 after 24 h and 0.1 ± 0.1 after 72 h. Of the three compounds tested, only apicidin, a protozoan HDA inhibitor, failed to reduce the mitotic index below control levels.

Both TSA and HC toxin were effective at concentrations 100-fold below butyrate, which significantly inhibited mitosis at 1 mM after 12 h (Tramontano et al., 1990). Effective concentrations of HDA inhibitors have been established to be in the μM range for TSA and HC toxin, and the mM range for butyrate using a variety of test systems (Darkin-Rattary et al., 1996)

Table 2 shows the mitotic index of root meristem cells taken at various time intervals after transfer of the cultured roots to fresh medium, following either 24 h exposure to TSA, or 48 h exposure to HC toxin. Both sets of meristems initially had low mitotic indices, but in the case of TSA, after 12 h the mitotic index returned to normal, and in the case of HC toxin, after 24 h normal mitotic indices reappeared. This data is again similar to that observed with butyrate, which

Table 1
Effects of various concentrations of the HDA inhibitors trichostatin, HC toxin and apicidin on the mitotic index of isolated root meristems of *P. sativum* grown in aerated White's medium. Three-day-old seedlings were employed. Mean and SE of at least three slides, 1000 nuclei were scored per slide

Exposure time (h)	Cells in mitosis (%)								
	No additives	Concentration of trichostatin			Concentration of HC toxin			Concentration of apicidin	
		10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-5} M	10^{-6} M
2	5.0 ± 0.1	2.7 ± 0.7	–	–	–	–	–	–	–
4	2.1 ± 0.4	1.0 ± 02	–	–	–	–	–	6.3 ± 0.7	4.1 ± 0.1
8	1.2 ± 0.5	0.3 ± 0.1	–	–	–	–	–	2.2 ± 0.2	4.0 ± 0.5
12	5.2 ± 0.5	0.2 ± 0.1	–	–	–	–	–	5.8 ± 0.9	5.0 ± 0.2
24	4.5 ± 0.1	0.6 ± 0.2	4.7 ± 1.1	5.0 ± 0.6	0.9 ± 0.2	5.6 ± 1.0	4.3 ± 0.8	7.0 ± 0.4	5.2 ± 0.2
48	5.0 ± 0.2	1.6 ± 0.3	5.1 ± 0.1	5.0 ± 0.2	0.7 ± 0.1	3.2 ± 0.4	3.9 ± 0.5	–	–
72	4.5 ± 0.3	1.4 ± 0.2	–	5.1 ± 0.2	0.1 ± 0.1	3.6 ± 0.1	2.9 ± 0.3	–	–

had recovered mitotic indices by 10 h (Tramontano et al., 1990).

In order to determine whether or not the presence of either TSA or HC toxin influenced the polypeptide profiles of treated meristems, two-dimensional gel electrophoresis was employed. Figs. 1–3 show the protein profiles of control, TSA-, and HC toxin-treated roots, respectively. The polypeptide profiles are quite similar. However, two basic peptides, one at *Mr* 20 kD, the second at *Mr* 27 kD disappeared when meristems were treated with TSA, as compared to controls. Previous experiments with butyrate showed the upregulation of two different groups of basic proteins, one at *Mr* 24–26 kD, the second at 35 kD (Tramontano and Barreiro, 1997).

Although exposure to butyrate, TSA and HC toxin produced a dramatic reduction in mitotic capability, the protein profile of butyrate-treated roots was markedly different from that observed following treatment with TSA or HC toxin. TSA and HC toxin are known to have a higher affinity for and greater specificity in inhibiting HDA (Darkin-Rattary et al., 1996), while butyrate requires higher concentrations to inhibit HDA, and has pleiotropic effects on cellular metabolism (Chen et al., 1997).

Table 2
Mitotic index of isolated root meristems of *P. sativum* which were initially grown in aerated White's medium with either 10^{-5} M trichostatin for 24 h or 10^{-5} M HC toxin for 48 h and then transferred to fresh medium without additives. Three-day-old seedlings were employed. Mean and SE of at least three slides per treatment, 1000 nuclei were scored on each slide

Recovery time after initial exposure (h)	Cells in mitosis (%)	
	Trichostatin	HC toxin
4	1.8 ± 0.2	1.3 ± 0.3
8	2.7 ± 0.5	0.1 ± 0.1
12	4.3 ± 0.4	0.3 ± 0.2
24	6.8 ± 0.4	4.1 ± 0.3
28	7.3 ± 0.5	3.6 ± 0.2
32	4.9 ± 0.2	4.4 ± 0.1

Four genes were tested for expression in control, butyrate-treated, butyrate-recovered and TSA-treated roots using Northern analysis. The genes tested were *P. sativum* homologues of histone H2A, MAP kinase, cycA2:1 and cdc2. Figs. 4–6 show that of the four genes tested, only histone H2A displayed a modest decrease in expression in butyrate-treated roots. The other three tested genes exhibited uniform expression across treatments. All roots recovering from butyrate exposure showed expression levels similar to control, non-exposed roots. Exposure to TSA concentrations that inhibit mitosis does not affect the expression of the four test genes. Thus, genes usually down-regulated in non-proliferating cells are still highly expressed. Other data with pea axillary bud meristems shows that non-proliferative cells usually down-regulate histone H2A, MAP kinase and cdc2. (Devitt and Stafstrom, 1995; Stafstrom, 1997). As a cyclin, cycA2:1 would also be expected to be down-regulated in non-proliferating cells. Our results indicate that butyrate and TSA halt proliferation without the down-regulation of several genes that participate in cell proliferation.

Three of four HDA inhibitors tested, namely butyrate, TSA and HC toxin, halted mitosis in the pea root meristem. We initially hypothesized that HDA inhibitors altered the expression of proteins that were part of the cell proliferation machinery or that guided the cell in and out of the proliferative state. Our results do not support this initial hypothesis, since the protein profiles of control, TSA and HC toxin were relatively similar. Only butyrate, noted for its widespread physiological effects, in addition to its HDA inhibitory activity, dramatically altered the expression of different peptides (Tramontano and Barreiro, 1997). More significantly, the non-proliferating butyrate- and TSA-treated cells did not down-regulate transcripts of four known cell proliferation-associated genes (histone H2A, cycA2:1, MAP kinase and cdc2). Alternative

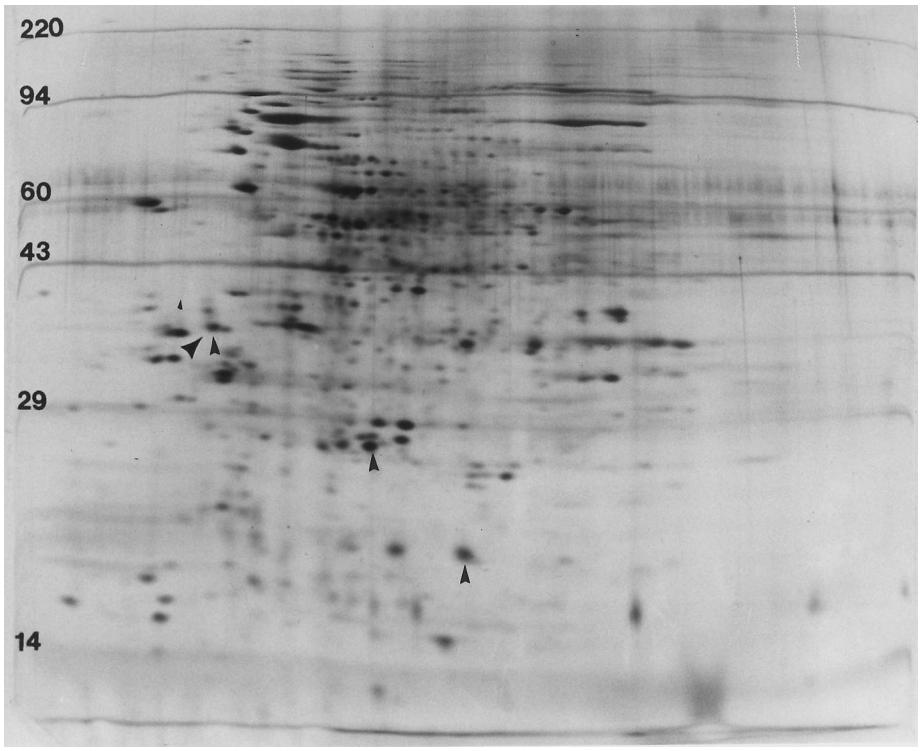

Fig. 1. Silver stained two-dimensional PAGE of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose for 24 h. *Mr* markers are listed on the left. Double arrows indicate 33 kD tropomysin internal standard. Arrows indicate where down-regulated trichostatin proteins are present.

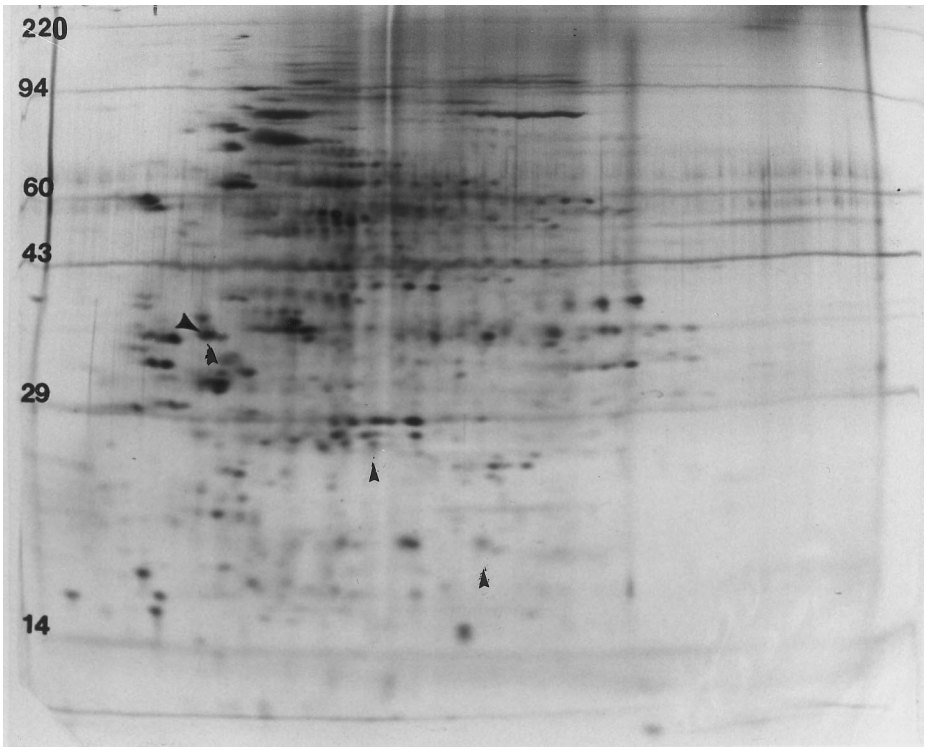

Fig. 2. Silver stained two-dimensional PAGE of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose and 10^{-5} M trichostatin for 24 h. *Mr* markers are listed on the left. Double arrows indicate 33 kD tropomysin internal standard. Arrows indicate down-regulated trichostatin proteins.

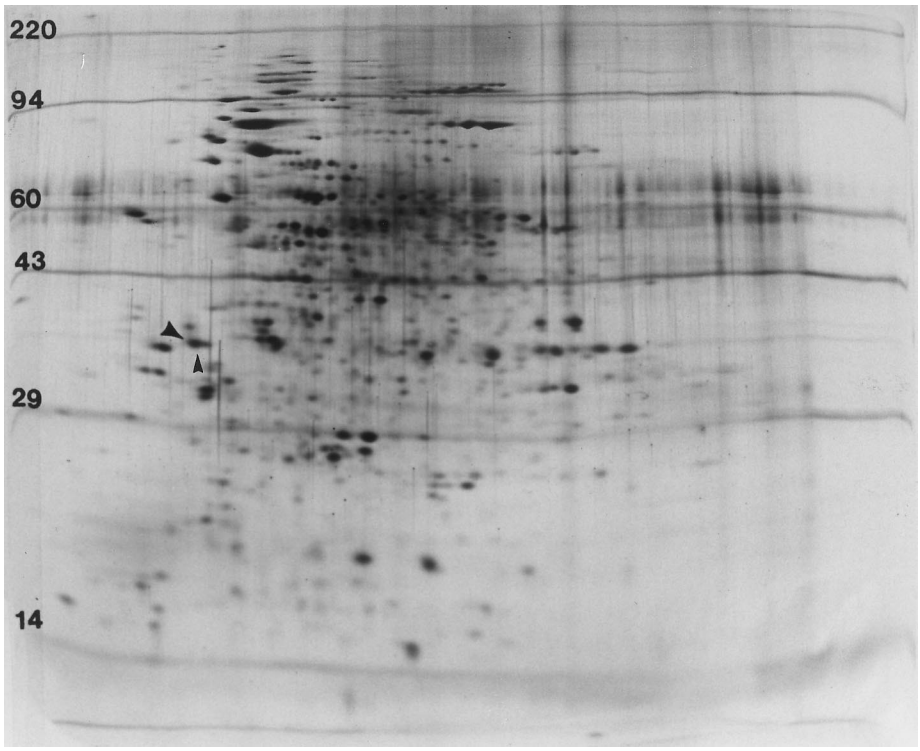

Fig. 3. Silver stained two-dimensional PAGE of proteins extracted from cultured pea root meristems (0–2 mm terminus) which have been grown in White's medium with sucrose and 10^{-5} M HC toxin for 48 h. *Mr* markers are listed on the left. Double arrows indicate 33 kD tropomysin internal standard.

mechanisms for the arrest of cell growth by these HDA inhibitors, including the prevention of chromosomal condensation that must occur prior to mitosis, should be considered.

With the recent availability of an acetylated lysine antibody, experiments were performed to determine whether nuclear proteins (histones) extracted from meristem cells were hyperacetylated after exposure to either butyrate or TSA. Fig. 7 shows an increase in the nuclear protein acetylation levels after treatment with butyrate and TSA, as detected via Western blotting. Acetylated nuclear proteins were found at approximately *Mr* 13, 45 and 60 kD. This supports the hypothesis that the test compounds do indeed alter protein acetylation levels, which may prevent the cells from dividing.

Future experiments will include subjecting RNA from roots treated with HC toxin and proprionic acid, another short-chain fatty acid capable of halting mitosis, to Northern analysis of known cell proliferation genes to determine their expression levels. Additional Western analysis will also be attempted to demonstrate acetylation levels in proprionic acid treated nuclear proteins. These experiments support the premise that the inhibition of cell division in pea root cells treated with HDA inhibitors is a consequence of alterations in the chromatin, not the down-regulation of certain genes.

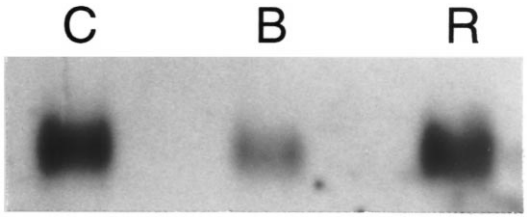

Fig. 4. Northern blot analysis of RNA extracted from control (C), butyrate-treated (B) and butyrate-recovered (R) *P. sativum* root meristems, probed with a biotinylated cDNA clone of *P. sativum* histone H2A. Each lane represents 10 μg of total RNA electrophoresed in 1.0% agarose/H_2CO. Equivalent amounts of RNA were loaded as determined by absorbance at 260 nm and confirmed by the intensity of the 18 S and 28 S ribosomal bands with ethidium bromide staining. Probe was a generous gift from Dr. Thomas Rost.

3. Experimental

3.1. General culture conditions

Seeds of *P. sativum* L. Cv. Alaska 2B (garden peas) were surface-sterilized in a sterile laminar flow hood with undiluted Clorox for 10 min, washed with sterile H_2O and germinated in the dark at 23° in sterile vermiculite.

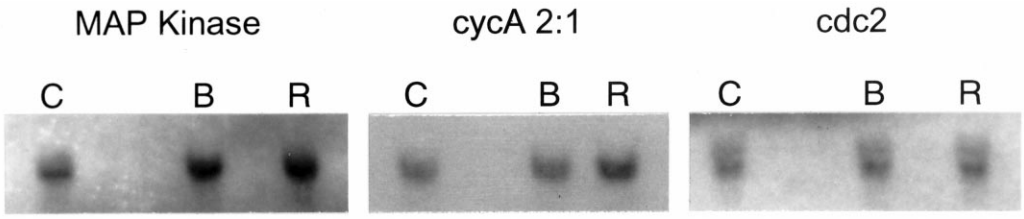

Fig. 5. Northern blot analysis of RNA extracted from control (C), butyrate-treated (B) and butyrate-recovered (R) *P. sativum* root meristems, respectively, probed with [^{32}P]-dCTP (Amersham) labeled cDNA clones generated by random priming for: MAP kinase, cycA2:1 and cdc2. All cDNAs were derived from *P. sativum*. Each lane represents 10 μg total RNA electrophoresed in a 1% agarose/H_2CO gel. Equal amounts of RNA were loaded as determined by absorbance at 260 nm and confirmed by the intensity of 18 S and 28 S ribosomal bands with methylene blue staining. MAP kinase cDNA was a generous gift of Dr. Joel Stafstrom; cdc2 and cycA2:1 cDNAs were generous gifts of Dr. Thomas Jacobs.

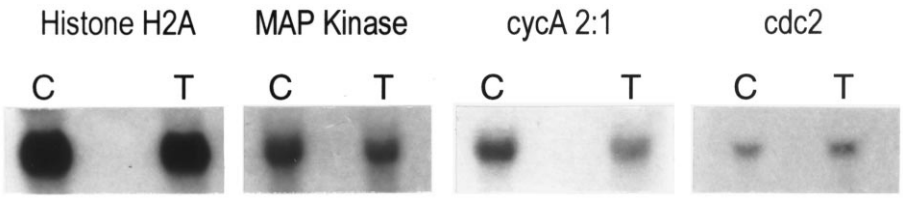

Fig. 6. Northern blot analysis of RNA extracted from control (C) and trichostatin-treated (T) *P. sativum* root meristems probed with [^{32}P]-d CTP (Amersham) labeled cDNA clones generated by random priming for: histone H2A, MAP kinase, cycA2:1 and cdc2. Each lane represents 10 μg total RNA electrophoresed in a 1% agarose/H_2CO gel. Equal amounts of RNA were loaded as determined by absorbance at 260 nm and confirmed by the intensity of 18 S and 28 S ribosomal bands with methylene blue staining.

3.2. Determination of mitotic index in pea root meristems exposed to various histone deacetylase inhibitors

Under aseptic conditions, roots were excised and cultured in 50 ml White's medium for 24 h. Roots were fixed in 95% EtOH–HOAc (3:1) for 30 min and transferred to 70% EtOH. The roots were then hydrolyzed with 5 N HCl for 20 min and stained with Feulgen. The roots were squashed onto slides, run through an EtOH series, and cover-slipped with Canada Balsam. At least three slides per treatment, 1000 cells per slide, were scored for mitotic index.

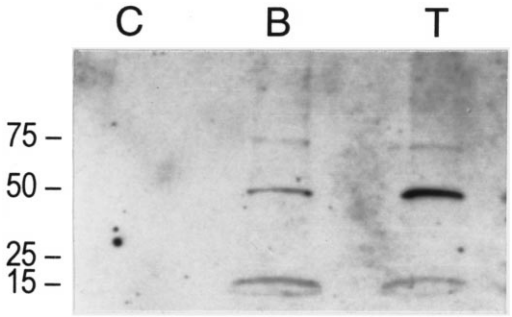

Fig. 7. Western blot analysis of nuclear proteins extracted from control (C), butyrate-(B) and TSA-treated (T) *P. sativum* root meristems probed with anti-acetylated lysine antibody. Each lane represents 0.50 mg nuclear protein electrophoresed on a 8–16% SDS-PAGE, transferred to nitrocellulose and probed. *Mr* markers are listed on the left.

3.3. Isolation of proteins

Excised 3-day-old roots were aseptically cultured in White's medium with various HDA inhibitors for the desired exposure period. 0–2 mm root tips were harvested, weighed and the total protein was extracted by grinding the samples in Webster buffer A (0.05 M Tris buffer (pH 8.0) containing 0.25 M sucrose, 1 mM EDTA, 0.7% β-ME, 0.005% PMSF, 1% Triton X-100) followed by centrifugation at 400 $\times g$ for 30 min. The supernatant was added to an equal volume of Webster buffer B (6 M urea, 1 M sucrose, 0.125 M Tris (pH 6.8), 2.5% SDS, 5% β-ME), and boiled for 3 min (Webster and Henry, 1987).

3.4. Protein assay

The Pierce BCA protein assay was employed (Pierce) to determine the amount of protein present. Samples were incubated at 37°C for 1 h, and protein content was determined spectrophotometrically.

3.5. Two-dimensional protein separation

Isoelectric focusing was carried out on the protein samples in glass tubes of i.d. 2 mm using pH 4–8 ampholines for 9600 V h^{-1}. Fifty ng of an international standard, tropomyosin, was added to each sample. Tropomyosin migrates to a *Mr* of 33 kD with a pI of 5.2 (O'Farrell, 1975). After equilibration for 10

min in buffer, the tube gel was sealed to the top of a 10% acrylamide gel. SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA gel^{-1}. Gels were fixed in a solution of 10% HOAc and 50% MeOH, overnight. *Mr* standards were added to the tubes and the gel was silver-stained and dried (Oakley et al., 1980). Two-dimensional gel electrophoresis was carried out by Kendrick Labs in Madison, WI, who analyzed the gels and determined differences between the protein profiles in control versus treated root meristem extracts.

3.6. RNA extraction procedures

Total RNA was purified from cultured roots with the PLANT-RNeasy Kit from QIAGEN following manufacturer's instructions.

3.6.1. RNA gel blotting

Total RNA (10 μg/lane) was separated by denaturing formaldehyde gel electrophoresis and transferred to Biodyne A membranes (Gibco BRL) for the non-radioactive probe (butyrate histone H2A) and Nytran nylon membranes for all other radioactive probes (cdc2, MAP kinase, cycA2:1). Non-radioactive blots were hybridized, and detection of bound probes was carried out according to the protocol of the Photogene Nucleic Acid Detection System 2.0 (Gibco BRL), except solutions did not contain formaldehyde and reactions were carried out at 65°C. cDNA clones for histone H2A, MAP kinase, cdc2 and cycA2:1 were radiolabeled using Redivue [^{32}P]-CTP (Amersham, sp. act. 370 MBq/ml, 10 mCi/ml) using the random priming protocol (Feinberg and Vogelstein, 1983, 1984). Incubations in pre-hybridization and hybridization buffers (Sigma) were performed following manufacturer's protocols. After hybridization, filters were washed first at 23°C and then 60°C in 0.2X SSC, 5% SDS. Radioactive blots were exposed for 72 h to Kodak X-OMAT AR film at −70°C, and developed using Kodak D-19 Developer and Fixer.

3.6.2. Western analysis

Excised 3-day-old roots were aseptically cultured in White's medium with various HDA inhibitors for the desired exposure period. 0–2 mm root tips were harvested, weighed and the nuclear proteins were extracted by grinding the samples in 0.1 M triethanolamine (pH 8.2) for 30 s. The homogenate was filtered through two layers of cheesecloth, centrifuged for 2 min at 120 $\times g$, and the supernatant was saved and centrifuged at 27,000 $\times g$ for 15 min. The pellet was washed with a buffer containing 5% glycerol, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM MES and 5 mM DTT at pH 6. Proteins were removed via the addition of 100 μl 0.2 M H_2SO_4 and 100 μl $(CH_3)_2CO$.

For one-dimensional gel electrophoresis, samples were added to an equal volume of SDS gel electrophoresis loading buffer (3 ml glycerine 1.7 ml H_2O, 0.2 ml β-ME, 2 ml 10% SDS, 0.025% bromophenol blue) and loaded onto an 8–16% SDS gradient gel. Pre-cast gradient gels (Novex) were used to separate polypeptides in the *Mr* 10–150 kD range. Samples were electrophoresed using the Xcell II MiniCell gel apparatus (Novex), run with a Tris–glycine–SDS buffer, pH 8.3 at 125 V, until the tracking dye reached the bottom of the gel.

The gel was blotted overnight at 10 V onto nitrocellulose, washed with 1X TBS and incubated in blocking buffer for 1 h at 23°C. The membrane was incubated for 24 h at 4°C in the presence of anti-acetylated lysine antibody (New England Biolabs), washed 3X with TBST, and then incubated with HRP-conjugated secondary antibody (1:2000). Membranes were incubated with 10 ml LumiGLO for 1 min with gentle agitation, then exposed to Hyperfilm (Amersham) for 10 s and developed.

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