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Tyrosine phosphatase signalling in a lower plant: cell-cycle and oxidative stress-regulated expression of the *Chlamydomonas eugametos VH-PTP13* gene

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Summary

The first evidence for tyrosine phosphatase signalling pathways in plants is presented by characterizing a putative protein tyrosine phosphatase gene from the unicellular green alga *Chlamydomonas eugametos*. This cDNA, referred to as *VH-PTP13*, contains an open reading frame specifying a protein with a molecular weight of 30.3 kDa, that has significant homology with a distinct group of dual-specificity phosphatases. The highest homology is found with *CL-100*, a human stress-response gene that regulates MAPkinase activity. The purified *VH-PTP13* protein expressed in *E. coli* had phosphatase activity and inactivated MAPkinases from alfalfa and tobacco. Non-dividing *C. eugametos* gametes did not express the *VH-PTP13* gene whereas synchronously dividing vegetative cells only expressed *VH-PTP13* in the early G1-phase of the cycle, implying a function there. When vegetative cells were subjected to oxidative stress, expression of the *VH-PTP13* gene was strongly induced, analogous to the human *CL-100* gene. Its potential role in plant signalling pathways is discussed.

Introduction

In contrast to the vast body of knowledge on signal transduction pathways that regulate proliferation and differentiation in mammalian cells and yeasts, little is known about these cascades in plant cells. The key players in this game are enzymes that regulate the phosphorylation state of themselves and other proteins. In particular (de-)phosphorylation of tyrosine residues plays an important role in these signalling cascades (Egan and Weinberg, 1993; Walton and Dixon, 1993). While some plant kinases have been described that can phosphorylate tyrosine residues (Duerr *et al.*, 1993; Mizoguchi *et al.*, 1994; Mu *et al.*, 1994) nothing is known about the existence of the counteracting enzymes: protein tyrosine phophatases (PTPS). The family of PTPs is large, diverse and still growing (Charbonneau and Tonks, 1992; Walton and Dixon, 1993). There are transmembrane, receptor-like PTPs (CD45, Ralph *et al.*, 1987), cytosolic PTPs (PTP1B, Chernoff *et al.*, 1990) and nuclear-localized PTPs (PAC-1, Rohan *et al.*, 1993), which suggest that the localization of the PTP determines whether substrates can be modified *in vivo* (Mauro and Dixon, 1994). All PTPs share an active-site motif HCXAGXXR(S/T)G (where X is any amino acid), of which the cysteine appears to be essential for phosphatase activity (Walton and Dixon, 1993). Recently, the crystal structure of PTP1B has been elucidated (Barford *et al.*, 1994). It confirmed the previous biochemical data about the essential cysteine but also provided evidence for important, conserved amino acids outside the active site, which are thought to be necessary to present the substrate to the active site.

A special group of PTPs are the dual-specificity phosphatases, which can both dephosphorylate tyrosine and serine/threonine residues. The prototype of this family is the vaccinia virus H1(VH) gene, therefore these PTPs are often called VH-like PTPs (Guan *et al.*, 1991, 1992). VH-PTP genes appear to be regulated at the transcriptional level and are induced by a variety of factors; oxidative stress (Keyse and Emslie, 1992), proliferative stimuli (Rohan *et al.*, 1993) and even nitrogen starvation (Guan *et al.*, 1992). Independently, it has been shown that the human CL-100 (Alessi *et al.*, 1993), the human PAC1 (Ward *et al.*, 1994), the mouse MKP-1 (Sun *et al.*, 1993) and the yeast MSG5 (Doi *et al.*, 1994) proteins can dephosphorylate efficiently and thereby inactivate MAPkinase. It has been postulated that VH-PTPs can act as feedback inhibitors of the MAPK-pathway (Nebrada, 1994).

Using the plant cell with one of the best-studied signalling systems, *Chlamydomonas* (Musgrave, 1993), we screened for components involved in phosphotyrosine dephosphorylation. We report the cloning and characterization of a plant homologue of the dual-specificity VH-like phosphatases and describe the regulation of gene expression during the cell cycle and during oxidative stress.

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Results

Cloning of a VH-PTP *cDNA from* Chlamydomonas eugametos

To investigate the presence of tyrosine phosphatases in the green alga *Chlamydomonas eugametos* we employed a PCR approach using primers matching the conserved active site motifs KCXXYWP and VHCSAGV/IG of yeast and mammalian PTPs (Freeman *et al.*, 1992). With cDNA template from different stages of the sexual and vegetative life cycle we were able to amplify approximately 300 bp fragments. The DNA and amino acid sequence of one of these PCR fragments, PTP13, showed high homology to the human PTP genes *CL-100* (Keyse and Emslie, 1992) and *PAC-1* (Rohan *et al.*, 1993) when compared with the EMBL/Genbank data base using the FASTA program (Pearson and Lippman, 1988). This PTP13 fragment hybridized to two transcripts of about 1.2 and 1.4 kb. Using the fragment as a probe, we isolated four recombinants from 1.10^5 p.f.u.s. of a cDNA library made from RNA of vegetative cells and sequenced two clones, 13.1 and 13.2 that are shown in Figure 1. They probably represent the two identified transcripts and differ only in the lengths of their 3′ untranslated regions. Accordingly, the 3′ *Ncol-Xhol* fragment from 13.2 (Figure 1) only hybridized with the 1.4 kb mRNA (data not shown). Restriction enzyme and sequence analysis of genomic clones isolated with the 13.2 cDNA probe indicated that the transcripts originate from a single gene (data not shown). In the largest cDNA (13.2), the *Chlamydomonas* polyA addition signal TGTAA is present 12 nt upstream from the polyA tail, while the shorter mRNA has a similar motif, TGTAG, 18 nt upstream from its polyA tail. The first 5′ ATG opens a reading frame of 276 amino acids specifying a polypeptide with a molecular mass of 30.3 kDa. It contains the conserved PTP active site sequence Ile/Val.His.Cys.X.Ala.Gly.X.X.Arg (Charbonneau and Tonks, 1992; Walton and Dixon, 1993) at position 170–178 (Figure 1). To be able to classify the *Chlamydomonas* gene in the large family of tyrosine phosphatases we constructed a phylogenetic tree using the PILEUP program. It is shown in Figure 2(a). For comparison we selected the prototype transmembrane PTP *CD45*, the cytosolic *PTP1B*, the mitotic inducer *cdc25* and the presently known members of the Vaccinia virus H1-related dual-specificty phosphatases. The *Chlamydomonas PTP13* gene clearly belongs to the latter group and only has partial homology with *CD45* and *PTP1B*. We therefore named it *VH-PTP13* to indicate its relation with this group of PTPs. Furthermore, it is unlikely that our gene is the plant homologue of *cdc25* because it has less than 15% identity with this gene. Figure 2(b) shows the alignment of the central part of the VH-PTP13 protein sequence with that of members from the group of VH-like PTPs. The VH-PTP13 amino acid

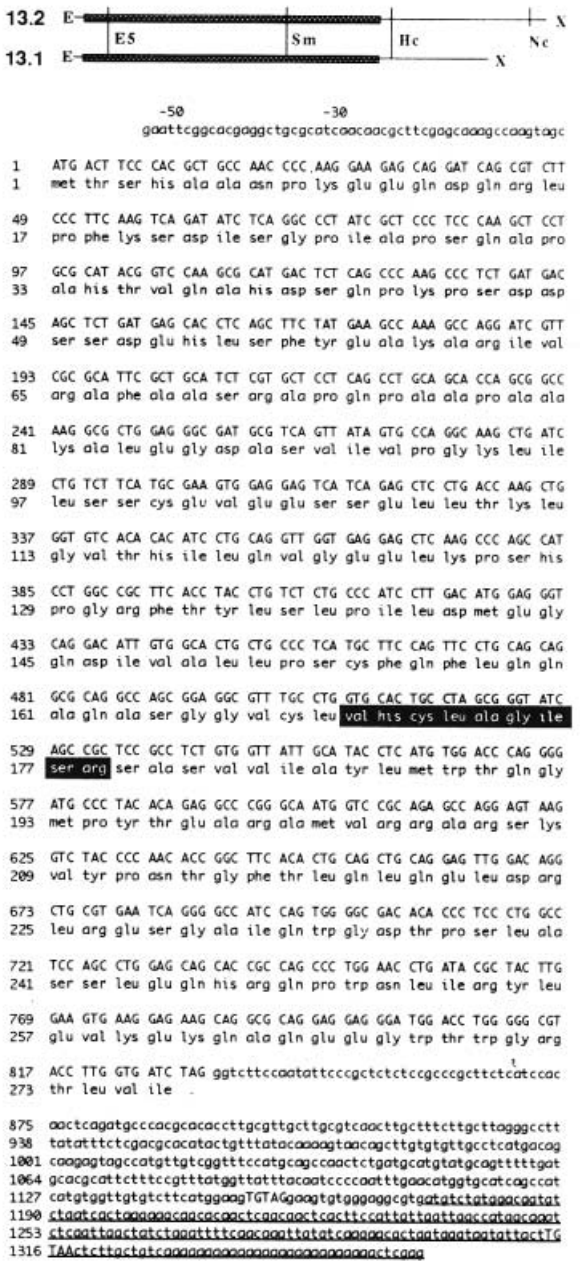

Figure 1. Physical maps, nucleotide and predicted amino-acid sequences corresponding to the *VH-PTP13* cDNAs 13.1 and 13.2.
Physical maps show the shared (E, *Eco*RI; E5, *Eco*RV; Sm, *Sma*I; Hc, *Hin*dII; X, *Xho*I) and unique (Nc, *Nco*I) restriction enzyme sites, while the predicted open reading frame is presented as a shaded box. Nucleotides are numbered on the left beginning at the first nucleotide of the deduced coding region. Amino acids are numbered on the left beginning at the first predicted Met. The termination codon is denoted by a full stop. The putative polyadenylation signals are in upper case, the 3′ UTR extension of the 13.2 cDNA is underlined. The position of the conserved active site at amino acids 170–178 is indicated by a black background.

sequence from 130–219 is about 40% identical to the human *CL-100*, *PAC-1* and *VHR* genes, 31% identical to the yeast *YVH1* gene, 24% identical to the yeast *MSG5* gene and 19% identical to the vaccinia virus *H1* gene. Compared

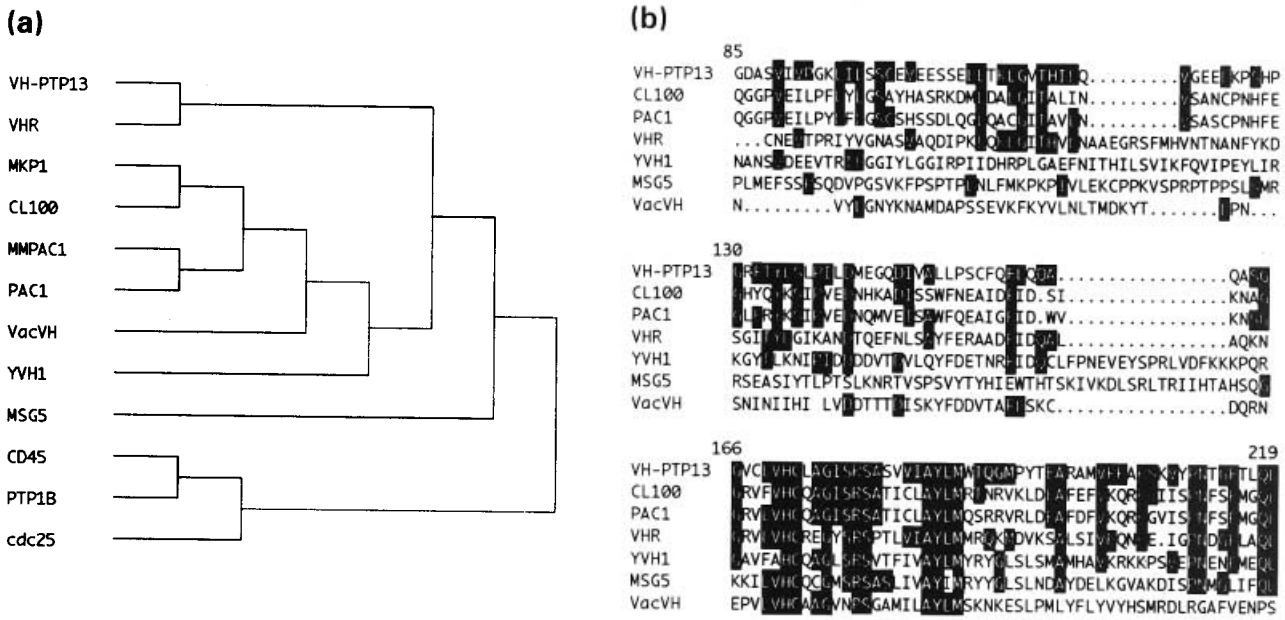

Figure 2. Phylogenetic tree and alignment of phosphatases related to *VH-PTP13*.
(a) Phylogenetic tree of several tyrosine phosphatases including *CD45* (Ralph *et al.*, 1987), *PTP1B* (Chernoff *et al.*, 1990), *cdc25* (Russel and Nurse, 1986) and members of the VH-like PTP family which includes *CL-100*, a human oxidative stress response gene (Keyse and Emslie, 1992), *MPK1*, its mouse homologue (Sun *et al.*, 1993); *PAC-1*, a mitogen-induced human PTP (Rohan *et al.*, 1993) *MMPAC1*, its mouse homologue (Rohan *et al.*, 1993); the human *VHR* gene (Ishibashi *et al.*, 1992); the nitrogen starvation-induced yeast *YVH1* gene (Guan *et al.*, 1992); the pheromone arrest associated yeast *MSG5* gene (Doi *et al.*, 1994) and the prototype vaccinia virus H1 sequence (Guan *et al.*, 1991).
(b) Alignment of the most conserved protein part, starting with amino acid 85 of the predicted *VH-PTP13* open reading frame, with those of human *CL-100*, human *PAC1*, human *VHR*, yeast *YVH1*, yeast *MSG5* and the vaccinia virus H1 gene (*VacVH*). Sequence identity between the *VH-PTP13* gene and the other VH-like tyrosine phosphatases is indicated by a black background. Full stops represent gaps introduced to maximize matches.

with the algal protein, the human VH-PTPs have an N-terminal extension of about 80 amino acids, while the yeast *YVH1* gene has a C-terminal extension of 150 amino acids. Although the VH-PTPs are thought to be localized in the nucleus (Rohan *et al.*, 1993), we were unable to find an obvious nuclear localization signal in the deduced amino acid sequence.

Recombinant VH-PTP13 protein has in vitro *phosphatase activity*

To determine whether the VH-PTP13 protein is a phosphotyrosine phosphatase, the 13.1 cDNA was cloned into the pGEX-KG expression vector (Guan and Dixon, 1992) and after induction, the 61 kDa glutathione-S-transferase (GST)-fusion protein was purified by affinity chromatography. The result is illustrated in Figure 3(a). It hydrolysed *p*-nitrophenylphosphate (pNPP), a chromogenic substrate that is structurally related to phosphotyrosine (Figure 3b). The rate of hydrolysis was linear with time and under these conditions the specific activity was 20.4 μmol·min^{-1} mg^{-1} recombinant protein. In the presence of 200 μM sodium vanadate, an inhibitor of PTPs, the phosphatase activity was completely inhibited, confirming that VH-PTP13 must be regarded as a protein tyrosine phosphatase. It has relatively low activity compared with the CL-100 recombinant protein (Keyse and Emslie, 1992), but has approximately the same activity as the yeast MSG-5 recombinant protein (Doi *et al.*, 1994).

Expression of the VH-PTP13 gene is cell-cycle regulated

In searching for the function of this gene we profited from our knowledge of other VH-like phosphatases. Both *CL-100* and *PAC-1* are induced by mitogenic stimuli (Alessi *et al.*, 1993; Rohan *et al.*, 1993), implicating them in the release from growth arrest. A first indication that the algal VH-PTP gene plays a role in cell division came from comparing its expression in asynchronously dividing vegetative cells with that in non-dividing, nutrient-starved gametes, for as can be seen in Figure 4(a), the accumulation of *VH-PTP13* transcripts was repressed in gametes of both mating types. As a loading control, the blot was reprobed with a chlorophyll *a/b* binding protein probe, which is also illustrated. We have previously shown that synchronous growth of vegetative *Chlamydomonas* cells can be readily achieved by growing them in an alternating light/dark rhythm (Molendijk *et al.*, 1992). The phases of the cell division cycle are well characterized (John *et al.*, 1989; Molendijk *et al.*, 1992), making it a powerful tool for studying cell-cycle regulation. We synchronized a *Chlamydomonas* culture by growing the cells in a 16 h light/8 h dark cycle

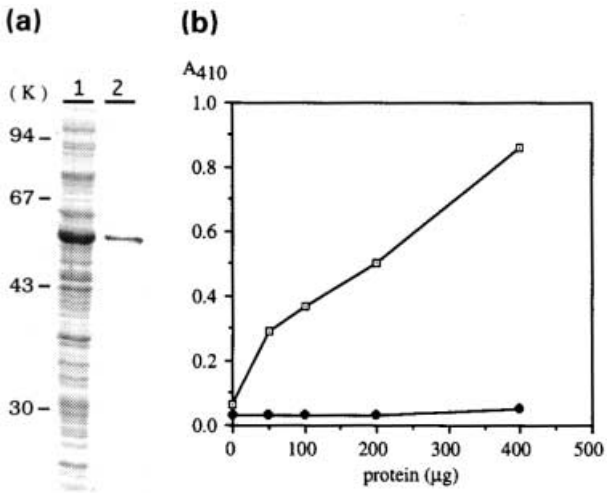

Figure 3. SDS–PAGE of the VH-PTP13 fusion protein and its phosphatase activity.
(a) SDS–PAGE of total *E.coli* lysate expressing the GST/VH-PTP13 fusion (lane 1) and purified protein (lane 2). Molecular weights (K=kDa) of standards are shown on the left side of the gel.
(b) The purified VH-PTP13 protein was assayed at the indicated protein concentrations for its ability to hydrolyse *p*-nitrophenolphosphate (pNPP) either in the absence (□) or in the presence (◆) of 200 µM sodium vanadate.

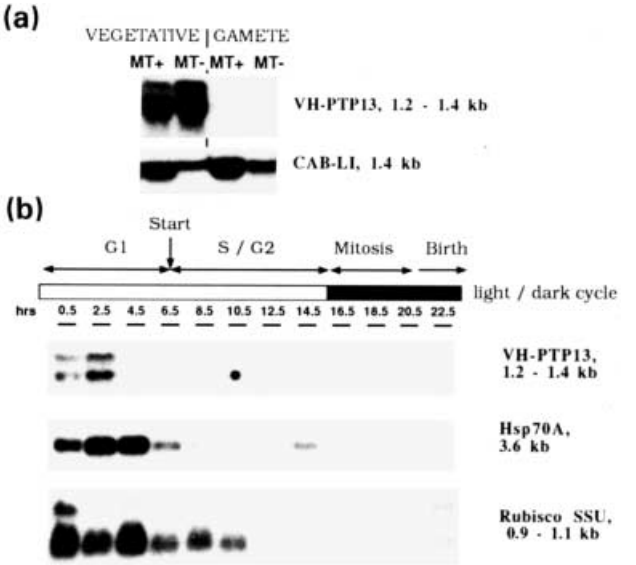

Figure 4. Cell-cycle regulated expression of *VH-PTP13* in *C. eugametos*.
(a) Nutrient starvation represses expression of *VH-PTP13*. The accumulation of *VH-PTP13* mRNAs in vegetative cells of both mating types (mt^+, mt^-) grown in continuous light was compared with that in sexually competent, growth-arrested cells induced by nutrient starvation. The blot was reprobed with a chlorophyll *a/b* binding protein probe (Gagné and Guertin, 1992) as a loading control.
(b) Accumulation of *VH-PTP13* mRNAs during the vegetative life cycle of *C. eugametos* (mt^-). Time points of sampling are indicated above the lanes. Approximate phases of the division cycle are depicted above the bar that illustrates the light/dark period of the synchronized culture. The blot was reprobed with the genomic *HSP70A* (Müller *et al.*, 1992) fragment and the *rbcS* cDNA (Goldschmidt-Clermont and Rahire, 1986) fragment to illustrate the diurnal rhythm of the culture.

at low density ($< 3\times10^5$ cells ml^{-1}). After two cycles we visually analysed the synchronicity of the culture and isolated RNA at 2 h intervals starting 30 min into the light period. Under these conditions all cells initiated cytokinesis between 17.00 and 17.45 h. The synchronicity of the culture was further confirmed by RNA gel blot analysis which indicated that maximum expression of histone H4 and β-tubulin mRNA coincided with the initiation of cytokinesis (see Molendijk *et al.*, 1992, for the samples were harvested from the same cultures). Daughter cells were released between 21.00 and 22.00 h, confirming the synchronous completion of the cell division cycle. To illustrate that we are studying cell-cycle regulated and not light-regulated gene expression, the cyclic behaviour of *rbcS* and *Hsp70A* gene expression is shown in the bottom panel of Figure 4(b). The light-responsive *rbcS* gene (Goldschmidt-Clermont and Rahire, 1986) is switched off long before the dark period and its mRNA reappears more than 2 h before the start of the light period. The expression of the *VH-PTP13* gene in this synchronized culture was limited to the beginning of the G1-phase. This is the first demonstration of when VH-like PTPases function in the cell cycle. Because the expression of our *VH-PTP13* gene is downregulated more than 2 h before the commitment point and the $p34^{cdc2}$ activation window (John *et al.* 1989), it should not be regarded as a *cdc25* homologue, which is a phosphatase that regulates $p34^{cdc2}$ activity (Millar and Russell, 1992). Although clearly cell-cycle regulated, the expression of the stress-response gene *HSP70A* (Müller *et al.* 1992), which will be used as a reference in the next section, followed a different pattern.

Oxidative stress results in long-term induction of VH-PTP13

Several VH-PTPs are induced by stress conditions: nitrogen starvation (*YVH1*), oxidative stress and heat-shock (*CL-100*). As already illustrated, cells deprived of nutrients such as nitrogen undergo gametogenesis and repress the expression of *VH-PTP13* (Figure 4a). We also observed that heat shock and oxidative stress both paralysed motile *Chlamydomonas* cells, but when the stress was removed, the cells quickly recovered and resumed swimming. We therefore treated vegetative *Chlamydomonas* cells with 1 mM hydrogen peroxide for 20 min, washed the cells and allowed them to recover in fresh medium with constant illumination. Cells started swimming again after 60 min in fresh medium. The accumulation of mRNAs for *VH-PTP13* and a known stress-response gene, *HSP70A* (Müller *et al.*, 1992), is shown in Figure 5. Already during treatment, both *VH-PTP13* and *HSP70A* transcripts accumulated rapidly, however, while *HSP70A* mRNA returned to a basal level after removing the hydrogen peroxide, the *VH-PTP13* mRNA continued to accumulate, reaching a maximum (50-fold increase) after 1–2 h. Even after 3 h there was still a 40-fold higher level of *VH-PTP13* transcript compared with

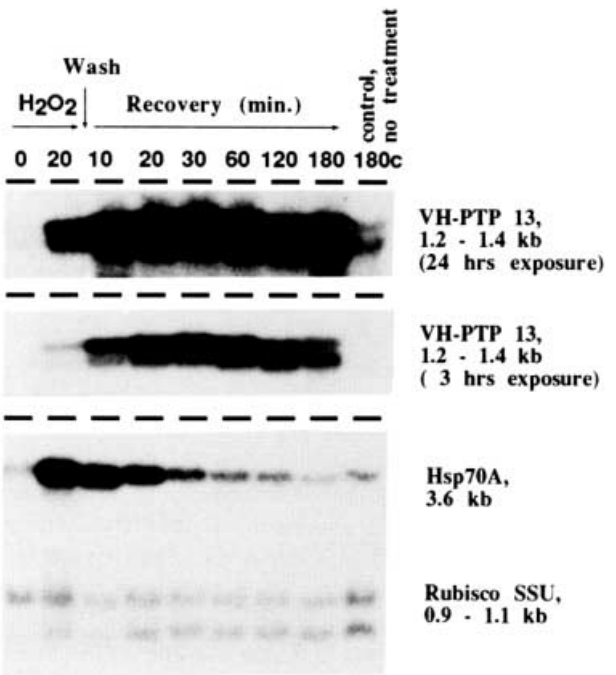

Figure 5. Induction of *VH-PTP13* mRNAs by treating vegetative *C. eugametos* mt⁻ cells with 1 mM hydrogen peroxide.
The shorter exposure of the blot illustrates the differential accumulation of the two *VH-PTP13* transcripts. For comparison the blot was reprobed with the heat-shock *HSP70A* gene while the *rbcS* gene functioned as loading control.

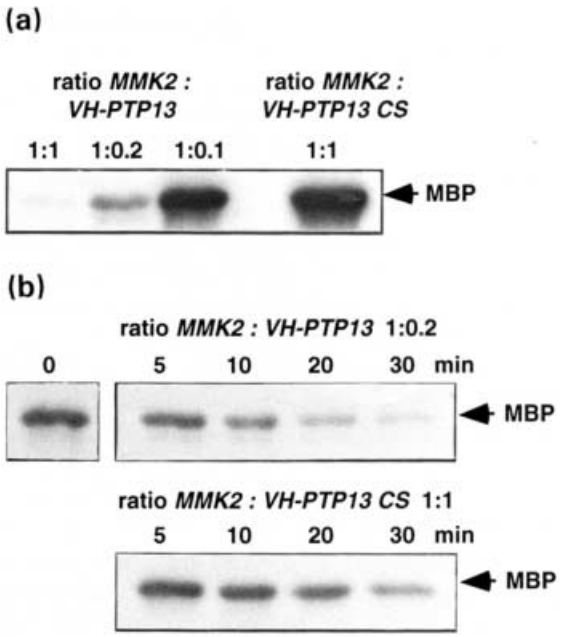

Figure 6. *In vitro* inactivation of alfalfa MAPkinase MMK2 by *Chlamydomonas* VH-PTP13 phosphatase.
(a) Concentration-dependent inhibition of MMK2 activity by VH-PTP13 or VH-PTP13 CS (purified as GST-fusion proteins). MMK2 activity was measured by its ability to phosphorylate myelin basic protein (MBP). The ratio MMK2:VH-PTP13 (μg) is indicated.
(b) Time course experiment in which 0.2 μg VH-PTP13 or 1 μg VH-PTP13 CS were incubated together with 1 μg MMK2 for the indicated periods of time.

the slightly elevated level of transcript at 3 h in the untreated control, implying that the inducing signal is maintained long after the removal of the causative agent. From the short-exposure autoradiograph, it is evident that the two *VH-PTP13* mRNAs accumulate with different kinetics. The blot was reprobed for the expression of *rbcS* to control the loading.

In vitro *inactivation of alfalfa MAPkinase MMK2 by the* Chlamydomonas *VH-PTP13 phosphatase*

Since the *Chlamydomonas* PTP gene has so much in common with the *CL-100* and *MPK-1* genes, we tested whether its phosphatase product could also use MAPkinase as substrate. MAPkinases have not yet been cloned from *Chlamydomonas* therefore a MAPkinase from a higher plant was used. GST fusions of the alfalfa MAPkinase MMK2 (Jonak *et al.*, unpublished data) and *Chlamydomonas* VH-PTP13 were expressed in *Escherichia coli* and affinity purified. Their integrity was checked by SDS–PAGE (data not shown). Activated MMK2 was incubated with different concentrations of purified VH-PTP13 protein and then assayed for its ability to phosphorylate myelin basic protein (MBP). The kinase activity of MMK2 was inhibited by treatment with VH-PTP13 in a concentration-dependent manner (Figure 6a). As a control we used a phosphatase negative mutant of VH-PTP13 in which the essential cysteine (residue 172) had been substituted by a serine. Incubation of MMK2 with equimolar amounts of VH-PTP13 CS did not alter the kinase activity, indicating that the inactivation of MMK2 by VH-PTP13 was dependent on phosphatase activity. Inactivation of MMK2 was linear with time, confirming the enzymatic nature of the inhibition by VH-PTP13 (Figure 6b). Treatment with a fivefold higher concentration of VH-PTP13 CS resulted in a slight decrease in MAPkinase activity. The rate of inactivation was three to fivefold slower than when using the lower amount of wild-type protein. Using the same strategy we have shown that VH-PTP13 can inactivate another alfalfa MAPkinase (MSK7) and two from tobacco (Jonak *et al.*, unpublished data; data not shown). These results demonstrate that the *Chlamydomonas* VH-PTP13 protein can inactivate higher plant MAPkinases *in vitro*.

Discussion

A putative tyrosine phosphatase gene from the lower green plant *C. eugametos* has been cloned and partially characterized. Identity is based on the characteristic active site motif VHCXAGXGR in the amino acid sequence of VH-

PTP13, the ability of the recombinant protein to hydrolyse a phosphotyrosine analogue and inhibition of the phosphatase activity by vanadate. It is 40% identical to the human *CL-100* gene, a well-characterized VH-like PTP involved in mitogenic control. The fact that expression of the *C. eugametos* gene is strictly regulated in the cell cycle and, like *CL-100*, is strongly induced by oxidative stress, confirms their close relationship. Altogether, this report is a first step towards establishing tyrosine phosphatase signalling in plants and highlights the need to look for other such enzymes using a similar approach.

Apart from amino acids in the active site, several other residues are also conserved in all VH-PTPs, including the algal gene, which should be studied to elucidate structure/function relationships. In particular, conservation of the AYLM sequence at positions 185–188 is interesting because it is a potential tyrosine phosphorylation site that could regulate phosphatase activity (Guan *et al.*, 1992). Since the related phosphatases are known to be located in the nucleus, we looked for, but failed to find, the typical amino acid sequences that signal a nuclear destination. Consequently, we are presently generating antibodies against the *in vitro*-expressed VH-PTP13 protein to determine its subcellular location. The fact that two transcripts are produced from a single gene in both *C. eugametos* and *C. reinhardtii* (data not shown) together with the different kinetics of induction during oxidative stress, suggest a functional significance. However, with no clear difference in coding capacity or mRNA stability we can only state that alternative polyA addition signals can be used in these algae.

Our results illustrate that the expression of *VH-PTP13* is restricted to the early G1 phase of the cell cycle. However, because the cell cycle of *Chlamydomonas* is synchronized by light, the possibility exists that *VH-PTP13* expression in G1 is regulated by the shift from dark to light rather than by the cell cycle. Several lines of evidence suggest otherwise. First, we were unable to detect a difference in the expression of the *VH-PTP13* homologue in dark- or light-grown, asynchronous cultures of *C. reinhardtii*. Second, the level of *VH-PTP13* transcripts increased only twofold upon illumination of dark-grown cultures, while the fluctuation during the cell cycle was much higher. In contrast, the levels of light-induced *HSP70A* and *CAB* increased 20- to 50-fold within 1 h of illuminating dark-grown cultures (unpublished results).

We have shown that the algal *VH-PTP13* gene is strongly induced over a long period by a short treatment with hydrogen peroxide and therefore it could play a role in oxidative stress. Since this oxidant is implicated in wounding (Bradley *et al.*, 1992), pathogenic attack (Legendre *et al.*, 1993), salicylic acid induction (Chen *et al.*, 1993) and chilling tolerance (Prassad *et al.*, 1994) in higher plants, the importance of studying the involvement of *VH-PTP13* homologues in these processes is obvious. With the recent elucidation of the three-dimensional structure of PTPs and our knowledge of how they work (Barford *et al.*, 1994), the production of dominant negative mutations in these proteins can be readily achieved. This will allow the analysis of phenotypes resulting from the repression of their function.

It seems unlikely that the *VH-PTP13* gene is a functional homologue of the two cloned VH-PTP genes from yeast (Doi *et al.*, 1994; Guan *et al.*, 1992) because we were unable to complement the growth defect of a *YVH1*-knockout strain with the algal gene expressed from a GAL4 promoter, nor were we able to complement the MSG5 function in the mating pheromone signal transduction pathway (unpublished results).

While it remains to be determined what its function is, the strict cell-cycle expression of VH-PTP13 and its homology with CL-100, MPK-1 and MSG-5 implicate MAP-kinases as substrates. From our *in vitro* experiments we can conclude that this is a realistic assumption. The VH-PTP13 protein expressed in *E. coli* was able to inactivate MAPkinases from the higher plant alfalfa and tobacco. Inactivation of MMK2 by VH-PTP13 was dependent on the presence of a conserved cysteine in the active site of the phosphatase. When this cysteine was exchanged for a serine (VH-PTP13CS), the protein lost most of its inhibiting activity (Figure 6a). The residual inhibitory effect can be explained by the binding capacity that such PTPs retain even when the active site has been disturbed (Sun *et al.*, 1993). Similar results were obtained when the yeast FUS3-kinase was incubated with the yeast MSG5 CS phosphatase (Gartner, personal communication). Since four different plant MAPkinases (from alfalfa and tobacco) were shown to be inactivated by the VH-PTP13 protein, it seems probable that it recognizes a general conserved structural feature of MAPkinases. Because these VH-PTPs are signalling components that are transcriptionally regulated, they are excellent tools for studying regulation in transgenic plants using the established reporter genes and since plant MAP-kinases are known to be activated by auxin (Mizoguchi *et al.*, 1994), the possibility that they are involved in the transduction of phytohormone-stimulated cell proliferation deserves attention. Preliminary hybridization experiments indicate that higher plant species contain DNA fragments that cross-hybridize with the *Chlamydomonas VH-PTP13* probe, suggesting that it must be feasible to clone VH-PTPs from higher plants.

Experimental procedures

cDNA library construction, screening and sequence analysis

Poly(A)$^+$ RNA was isolated from a synchronous culture of *Chlamydomonas eugametos* UTEX10 (mating type minus, mt$^-$) 0.5 h into

the light period and used to construct a cDNA library in λZAP-II (Stratagene, La Jolla, USA). Degenerate oligonucleotides to the catalytic domain sequences of known PTPs (Freeman *et al.*, 1992) were used to generate PCR products on 100 ng DNA from this library or 1% of a first-strand cDNA synthesis reaction (Gibco/BRL Life technolgies, The Netherlands). Products of 300 bp were cloned and sequenced. A PCR fragment with homology to the VH-like PTPs was used to isolate 4 cDNAs from 1×10^5 recombinants of the primary library. By restriction analysis three appeared identical, although one insert (13.1) was smaller. Inserts 13.1 and 13.2 were sequenced completely using the T_7 polymerase kit (Pharmacia) essentially as described by the manufacturer. Amino acid sequences were aligned using a combination of the PILEUP program of the CGC Wisconsin package and by visual inspection. The sequence will appear under accession number X77938 in the EMBL/Genbank data bases.

Expression and purification of GST-fusion proteins, construction of VH-PTP13 CS

The *VH-PTP13.1 Xba*I-*Xho*I fragment was gel-purified and inserted into the *Xba*I-*Xho*I digested pGEX-KG (Guan and Dixon, 1992) vector, and the fusion was verified by restriction enzyme analysis. This plasmid was used to express the GST/VH-PTP13 protein essentially as described before (Guan and Dixon, 1992). After cell lysis, proteins were solubilized in PBS/1% sarkosyl. Triton X-100 was added to the cleared supernatant (final concentration 2%) and the protein was purified by affinity chromatography (Pharmacia), dialysed against PBS, 1 mM PMSF and analysed by SDS–PAGE. The phosphatase assay was carried out as described by Keyse and Emslie (1992). A C172S mutation in *VH-PTP13* was introduced by PCR using the Bluescript forward primer and the primer 5'-TGCCTGGTGCACTCC-CTAGCGGG-3'. The fragment generated on a PTP13.1 template was sequenced, cut with *Apa*LI and *Xho*I and used to replace the same fragment in the *GST/VH-PTP13* expression plasmid. Expression was checked as described above and when used to hydrolyse pNPP, its activity did not register above the background (data not shown).

MAPkinase activity assays

The alfalfa MAP kinase MMK 2 (Jonak *et al.*, unpublished results) was expressed as a GST-fusion protein and affinity purified as described above. Aliquots of the fusion protein were stored at −80°C. Affinity-purified VH-PTP13 and VH-PTP13CS as GST-fusion proteins were immediately used for phosphatase assays. Protein concentration was determined using the Bio-Rad detection system (Bio-Rad, Richmond, USA). The quality of the purifications was confirmed by SDS-PAGE gelelectrophoresis. MKK2 was activated by autophosphorylation in kinase buffer (30 mM Hepes pH 7.5, 40 mM KCl, 4 mM $MgCl_2$, 0.06 mM ATP, 5.2% glycerol) at 30°C for 1 h. Decreasing amounts of VH-PTP13 (1, 0.2 and 0.1 μg) or 1 μg of VH-PTP13-CS were incubated together with 1 μg MMK2 in kinase buffer and 4 μCi [γ^{32}P]ATP at 30°C. After 10 min, 1 μg of myelin basic protein (MBP, Sigma) was added. Reactions were kept at 30°C for an additional 10 min, stopped with SDS-sample buffer and the proteins separated on 15% denaturing polyacrylamide gels. In time-course experiments, 1 μg MKK2 was activated by autophosphorylation in kinase buffer at 30°C for 1 h. At time-point zero, VH-PTP13 (0.2 μg) or VH-PTP13 CS (1 μg) and an appropriate volume of buffer were added. After 0, 5, 10, 20 and 30 min incubation at 30°C, 1 μg of MBP was added. After 5 min at 30°C, the reactions were stopped with SDS-sample buffer and the proteins separated on 15% denaturating polyacrylamide gels.

Growth conditions, RNA analyses

Vegetative cells and gametes were grown as described (Molendijk *et al.*, 1992). The UTEX 10 culture was synchronized by a 16 h light/8 h dark regime and synchronization was confirmed as described (Molendijk *et al.* 1992). RNA gel blot analysis was performed using standard techniques (Maniatis *et al.*, 1982). Vegetative *C. eugametos* mt$^-$ cells were harvested in mid-log phase and resuspended in medium at 10^7 cells ml^{-1}. Cells were mixed with an equal volume of 2 mM hydrogen peroxide, incubated in the light for 20 min, harvested, resuspended in fresh medium and allowed to recover in the light. They retained their flagella during the treatment, but became immotile, resuming swimming after 60 min. RNA was isolated at the time points indicated, followed by RNA gel blot analysis (Maniatis *et al.*, 1982). [^{32}P]dATP-labelled DNA probes were made by the random prime method (Feinman and Vogelstein, 1983) using gel-purified DNA fragments. Autoradiographs were exposed for 16–72 h.

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