*Journal of Experimental Botany, Vol. 54, No. 383, pp. 623–632, February 2003*
DOI: 10.1093/jexbot/erg061

Journal of Experimental Botany

RESEARCH PAPER

# Phosphorylation of a member of the MBF1 transcriptional co-activator family, *St*MBF1, is stimulated in potato cell suspensions upon fungal elicitor challenge

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Received 13 June 2002; Accepted 24 September 2002

## Abstract

***St*MBF1 (*Solanum tuberosum* multiprotein bridging factor 1) is a plant member of the MBF1 family of transcriptional co-activators. Previously, it has been described as being up-regulated at the transcriptional level by fungal and abiotic stress. To understand whether *St*MBF1 is also regulated at the post-translational level, *in vitro* as well as *in vivo* phosphorylation assays were performed. *St*MBF1 is phosphorylated under both experimental conditions and [$^{32}$P] incorporation into *St*MBF1 increases after treatment of potato cells with hyphal cell wall components (HWC) derived from *Phytophthora infestans*. The *St*MBF1-phosphorylating activity is strongly inhibited by the calcium-chelator EGTA and partially inhibited by calmodulin antagonists. Using bacterial purified *St*MBF1 as a substrate, a 57 kDa calcium-dependent protein kinase (p57) that is able to phosphorylate *St*MBF1 was detected. The *St*MBF1 kinase activity of p57 was higher in elicited than in non-treated cells. The role of the elicitor-dependent phosphorylation of *St*MBF1 is discussed.**

Key words: Calcium-dependent protein kinase, cell suspensions, elicitor, multiprotein bridging factor 1, protein phosphorylation, *Phytophthora infestans*, *Solanum tuberosum*, transcriptional co-activator.

## Introduction

Higher plants have developed several elaborate mechanisms to ward off pathogen attack. Whereas some of these defence mechanisms already exist and provide physical and chemical barriers to hinder pathogen infection, others are induced after recognition of specific signal molecules called elicitors (Kombrink and Somssich, 1995; Somssich and Hahlbrock, 1998; Blumwald *et al*., 1998). These last responses involve a network of signal transduction and the rapid activation of gene expression (Yang *et al*., 1997). Protein kinases and phosphatases have an important role in plant signalling mechanisms. Evidence supporting the involvement of protein phosphorylation in defence mechanisms was provided by an analysis of changes in the phosphoprotein profiles upon the application of elicitors to cell-suspension cultures (Dietrich *et al*., 1990; Felix *et al*., 1991; Viard *et al*., 1994). Later, several reports demonstrated the activation of protein kinases with characteristics of animal MAPK (mitogen-activated protein kinase) in plant signalling pathways that lead to the activation of the defence response (Hirt, 1997; Ligterink *et al*., 1997; Zhang *et al*., 1998). In addition to protein phosphorylation, an increase in cytosolic calcium concentration has been observed in plant cells immediately after elicitation, suggesting that calcium also plays a pivotal role in the regulation of inducible-defence responses (Dietrich *et al*., 1990; Xing *et al*., 1996). On the other hand, changes in calcium concentration can affect the activity of protein kinases directly or indirectly. In plants, the majority of calcium-stimulated protein kinase activity is performed by members of the calcium-dependent protein kinases (CDPKs) family (Roberts and Harmon, 1992).

In the cell nucleus, terminal biochemical signals lead to the transcriptional activation of a variety of pathogen-responsive genes (Rushton and Somssich, 1998). Activation and deactivation of transcription factors is an

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Abbreviations: aa, amino acid; CaMK, calmodulin-dependent protein kinase; CKII, casein kinase II; CDPK, calcium-dependent protein kinase; CPZ, chlorpromazine; DRB, 5,6-dichloro-1-(β-D-ribofuranosyl); GST, glutathione-*S*-transferase; HWC, hyphal cell wall components; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MBF1, multiprotein bridging factor 1; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; TFP, trifluoperazine.

additional level at which protein phosphorylation plays an important role (Sessa and Martin, 2000). Several reports have shown that the DNA-binding activities of various transcription factors involved in the activation of defence-related genes are regulated by phosphorylation events (Després *et al*., 1995; Sessa *et al*., 1995; Dröge-Laser *et al*., 1997).

Co-adaptors or co-activators are a new class of transcription factors, capable of connecting general and gene-specific transcription factors, allowing transcriptional activation to proceed (Roeder, 1991; Li *et al*., 1994; Guarente, 1995). MBF1 proteins have been identified as bridging molecules that fall under the category of transcriptional co-activators (Takemaru *et al*. 1997, 1998; Kabe *et al*., 1999). Godoy *et al*. (2001) reported the cloning and expression analysis of a plant member of the MBF1 transcriptional co-activator family, named *St*MBF1 (*Solanum tubersoum* multiprotein bridging factor 1). In mammalian and plant systems, the activity of some transcription machinery components is often regulated by phosphorylation/dephosphorylation events (Hill and Tresiman, 1995; Schwechheimer and Bevan, 1998). However, this type of post-translational modification has not been associated with any member of the MBF1 family. This report presents the first evidence of phosphorylation of an MBF1 protein. It also shows that the level of phosphorylation of *St*MBF1 is enhanced in potato cell suspensions after being challenged with *Phytophthora infestans* elicitors. Characterization of *St*MBF1-phosphorylating kinase activity indicates that a 57 kDa protein kinase acting in a calcium-dependent manner, participates in the phosphorylation of *St*MBF1 protein.

## Materials and methods

### *Biological material*

*Phytophthora infestans* race 0, obtained from INTA Balcarce, Argentina, was grown as described previously (Laxalt *et al*., 1996). The mycelia were harvested by filtration and washed with sterile distilled water. Hyphal-wall components (HWC) were prepared according to Ayers *et al*. (1976).

Cell suspension cultures of *Solanum tuberosum* L. cv. Spunta, established from calli, were maintained in liquid Murashige and Skoog's medium containing 3 mg $l^{-1}$ NAA, 0.2 mg $l^{-1}$ kinetin and 30 g $l^{-1}$ sucrose (Murashige and Skoog, 1962). The cultures were grown in a rotary shaker (120 rpm) at 25 °C in the dark for 12 d. The viability of the cell suspensions was determined by Evans blue staining. For elicitation, the cells were treated with 1 mg $ml^{-1}$ of HWC. After the treatment, the cells were harvested by centrifugation, frozen in liquid nitrogen and stored at –80 °C until analysis.

### *Isolation of protein extract*

To prepare cytosolic extracts, cells were homogenized in a mortar with liquid nitrogen and suspended in 1 vol. of extraction buffer (50 mM TRIS-HCl pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 5 mM NaF, 1 mM $Na_3VO_4$, 20 mM β-glycerol phosphate, 0.5 μg $ml^{-1}$ pepstatin, and 0.5 μg $ml^{-1}$ aprotinin). After centrifugation at 16 000 *g*, supernatants were transferred into clean tubes and stored at –20 °C.

### *Plasmid construction and purification of StMBF1 recombinant proteins*

To produce *Schistosoma japonicum* gluthatione-*S*-transferase (GST)-*St*MBF1 fusion protein and its deletion derivatives in *Escherichia coli*, different regions of *St*MBF1 were amplified by PCR using *St*MBF1 cDNA cloned in pBC SK$^+$ (Stratagene) as a template. The following primer combinations were used: P1 (5′-CCAGGATCCATGAGTGGAATATCGCAAGAC-3′) and P2 (5′-CCAGGAATTCTTTCTTTCCTCGAAGTTTCG-3′) for the complete ORF of *St*MBF1, P3 (5′-CCAGGGATCCATCGAAA-CCGTTAAGAAGTC-3′) and P2 for *St*MBF1 Δ1–38, P1 and P4 (5′-CCAGGAATTCAATTGCCTTTCCCGACTCG-3′) for *St*MBF1 Δ118–139, or P4 and P5 (5′-CCAGGGATCCTTGTCTCATG-AAAAGGTACC-3′) for *St*MBF1 Δ1–70/Δ118–139. The PCR primers were designed to incorporate a *Bam*HI site in the 5′ end and an *Eco*RI site at the 3′ end of the PCR product. The amplified DNA fragments were cloned into the *Bam*HI and *Eco*RI sites of pGEX-4T-3 (Amersham Pharmacia Biotech). The resulting constructs were verified by sequencing.

*E. coli* BL21 (DE3) cells bearing the pGEX-*St*MBF1 constructs were grown at 37 °C overnight with shaking (170 rpm) in 2× YT medium (16 g $l^{-1}$ tryptone, 10 g $l^{-1}$ yeast extract, and 5 g $l^{-1}$ NaCl) with 0.1 mg $ml^{-1}$ ampicillin. The culture was diluted 100 times in fresh medium and the cells were grown under the same conditions until an $Abs_{600}$=0.8 was reached. Expression of the fusion protein was induced by addition of 0.4 mM isopropyl-1-thio-β-D galactopyranoside (IPTG) followed by additional incubation at 25 °C for 3 h. The cells were harvested by centrifugation at 5000 *g* for 10 min and suspended in 0.5 vols of PBS (25 mM sodium phosphate buffer pH 7.2, 150 mM NaCl) containing 1 mM EDTA and 1 mM PMSF. After treatment with one freeze/thaw cycle and sonication, the lysates were clarified by centrifugation at 15 000 *g* for 15 min at 4 °C. GST fusion protein purification and thrombin digestion were performed according to the manufacturer's instructions (Amersham Pharmacia Biotech).

### *Antibody production*

Polyclonal antibodies against *St*MBF1 were raised according to Harlow and Lane (1988). The preimmune serum was collected 1 week before the first inoculation. The titre and specificity of the antiserum were tested against *E. coli*-purified *St*MBF1 protein by Western blot analysis.

### *Western blot analysis*

Ten μg of whole extracts from potato cell suspensions were separated in 15% (w/v) SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated overnight at room temperature with anti-*St*MBF1 serum or the corresponding pre-immune serum at a dilution of 1:5000 and developed using enhanced chemiluminescence detection system (ECL) according to the manufacturer's procedure (Amersham Pharmacia Biotech).

### In vitro *phosphorylation of recombinant* St*MBF1*

Two μg of *St*MBF1or its truncated forms were incubated with 10 μg of potato cell extracts in 30 μl of kinase buffer (20 mM TRIS-HCl pH 8.0, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol) supplemented with 5 μM ATP and 5 μCi (6000 Ci $mmol^{-1}$, 150 mCi $ml^{-1}$) of [$\gamma$-$^{32}$P] ATP. Reactions were incubated at 30 °C for 5 min. To stop the reaction, sample buffer (Laemmli, 1970) was added and the samples were boiled for 5 min. The proteins were subjected to SDS-PAGE and stained with Coomassie Brillant Blue R 250 (Sigma). The gels were dried and analysed by autoradiography.

For experiments with inhibitors, potato cell extracts were preincubated for 15 min with the following compounds: 5 mM EGTA, 500 μM chlorpromazine (CPZ), 200 μM trifluoperazine (TFP), 200 nM staurosporine, 100 μM 5,6-dichloro-1-(β-D-ribofuranosyl) (DRB), 20 μg $\mu l^{-1}$ heparin, and 1% (v/v) DMSO. To start the reaction, recombinant *St*MBF1 and [γ-$^{32}$P] ATP were added. DRB was purchased from ICN and all other inhibitors from Sigma Chemical Co.

### In vivo *phosphorylation of* St*MBF1*

Potato cell suspensions (1 ml) were incubated with 0.3 mCi $ml^{-1}$ [$^{32}$P] sodium phosphate (285.5 Ci $mg^{-1}$, 5 mCi $ml^{-1}$) for 2 h at room temperature and then elicited with 1 mg $ml^{-1}$ HWC for 30 min. The cells were harvested by centrifugation at 1000 *g*, suspended in 500 μl of extraction buffer (50 mM TRIS-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 5 mM NaF, 1 mM $Na_3VO_4$, 20 mM β-glycerol phosphate, 0.5% (w/v) SDS, 0.5 μg $ml^{-1}$ pepstatin, and 0.5 μg $ml^{-1}$ aprotinin) and boiled for 5 min. Then, the cells were homogenized in a mortar and the extract clarified by centrifugation at 16 000 *g* for 30 min at 4 °C. One hundred μl of the supernatants (200 μg of protein) were diluted 5-fold with buffer A (12.5 mM sodium phosphate pH 7.2, 2 mM EDTA, 1.25% (v/v) Nonidet P-40, 1.5% (w/v) sodium deoxycholate, 0.2 mM $Na_3VO_4$, 50 mM NaF, and 0.5 μg $ml^{-1}$ aprotinin) and incubated overnight at 4 °C with anti-*St*MBF1 serum or the corresponding preimmune serum (diluted 1:50). Forty μl of protein A-sepharose (Sigma) were added and the mixture was further incubated for 2 h. The immunocomplexes were collected by centrifugation and washed sequentially with the following solutions: 1 ml of buffer B (10 mM TRIS-HCl pH 7.4, 0.1% (w/v) SDS, 1% (w/v) sodium dexycholate, and 1% (v/v) Triton X-100), 1 ml of buffer C (buffer B supplemented with 150 mM NaCl), again 1 ml of buffer B and finally 1 ml of TBS (10 mM TRIS-HCl pH 7.4 and 150 mM NaCl). The immunocomplexes were resuspended in 40 μl of sample buffer, boiled for 5 min and analysed by SDS-PAGE in 15% (w/v) polyacrylamide gel and autoradiography.

### *In-gel kinase assay*

Purified *St*MBF1 (0.1 mg $ml^{-1}$) was included into the polymerization mixture of resolving 12% (w/v) polyacrylamide gels. Thirty μg of potato cells proteins were loaded in each lane of the gel. After electrophoresis, the gel was washed twice with buffer A (50 mM TRIS-HCl pH 7.5, and 5 mM β-mercaptoethanol) containing 20% (v/v) isopropyl alcohol, and then re-equilibrated in buffer A at room temperature for 1 h. Proteins were denatured in 6 M guanidine hydrochloride in buffer A for 1 h. To renature proteins, the gel was incubated in buffer A containing 0.04% (v/v) Tween-20 at 4 °C overnight with several changes. Then, the gel was incubated in kinase assay buffer (40 mM HEPES pH 7.5, 2 mM DTT, 20 mM $MgCl_2$, 1 mM $CaCl_2$) for 30 min. The phosphorylation reaction was performed in 10 ml of the kinase assay buffer containing 50 μM ATP and 100 μCi (6000 Ci $mmol^{-1}$, 150 mCi $ml^{-1}$) [γ$^{32}$-P] ATP at room temperature for 2 h. The gel was extensively washed with 5% (w/v) TCA and 1% (w/v) sodium pyrophosphate until non-reacted radioactivity was removed. The gel was then dried and the signal detected by autoradiography.

### *Protein determination*

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

### *Statistic analysis*

Autoradiograms were scanned on a Genius colour–page HR5 scanner and densitometric analyses were performed with the TN-image Analysis Software 2.13 version. Data handling and statistic analysis were performed using Graph Pad Incorporated Software, version 2.0.

## Results

### In vitro *and* in vivo *phosphorylation of* St*MBF1 are stimulated in potato cell suspensions by* P. infestans *elicitors*

The complete *St*MBF1 cDNA sequence is available in the GenBank database under the accession No. AAF81108 (Godoy *et al*., 2001). To identify conserved motifs in the amino acid sequence of *St*MBF1, a standard search against the PROSITE databases was performed. Several motifs probably involved in signalling were detected in *St*MBF1: four protein kinase C (PKC) phosphorylation sites (**T**V**K**, aa 41–43; **S**N**R** aa 49–51; **T**R**K**, aa 61–63; **S**G**K**, aa 114–116), two casein kinase II (CKII) phosphorylation sites (**S**GA**E**, aa 35–38; **S**KL**E**, aa 125–128), a cAMP-and-cGMP-dependent protein kinase phosphorylation site (**K**KL**T**, aa 91–94), an N-glycosylation site (NLSH, aa 70–73), and an N-myristoylation site (GSNRAA, aa 48–53). Since some components of the transcriptional machinery are frequently modified by phosphorylation, an initial analysis to discover whether *St*MBF1 could be phosphorylated *in vitro* by potato protein extracts was performed. In order to test this, bacterial purified *St*MBF1 was used as an exogenous substrate in the presence of [γ-$^{32}$P] ATP and protein extracts from potato cell suspensions. As shown in Fig. 1A, lane 3, a band of 16 kDa, which corresponds to the appropriated electrophoretic mobility of *St*MBF1, was phosphorylated by potato cell extracts. By contrast, no signal was detected in that region when potato extracts were incubated without *St*MBF1 (Fig. 1A, lane 2). In addition, no $^{32}$P incorporation into *St*MBF1 was observed when the protein was incubated in the absence of potato extracts, indicating that *St*MBF1 is not autophosphorylated (Fig. 1A, lane 1). When potato extracts were incubated alone or with *St*MBF1 (Fig. 1A, lanes 2, 3), labelled proteins corresponding to approximately 35 and 45 kDa were observed. These bands might correspond to endogenous potato proteins that became phosphorylated under the experimental conditions.

To investigate the possible post-translational regulation of *St*MBF1 by phosphorylation in response to fungal elicitors, an *in vitro* phosphorylation assay was performed using *St*MBF1 protein and cytosolic potato proteins. These extracts were obtained from potato cells after challenging them with HWC derived from *P. infestans* as the elicitor source. An increase in the incorporation of radioactivity from [γ-$^{32}$P] ATP into *St*MBF1 was detected when it was incubated with the extracts elicited during 30 min with HWC (Fig. 1B, upper panel and Fig. 1C). Such increase was steady, maintained up to 60 min and then, at 120 min, decreased to the initial values (data not shown). Since no differences in the mass of *St*MBF1 used in each reaction

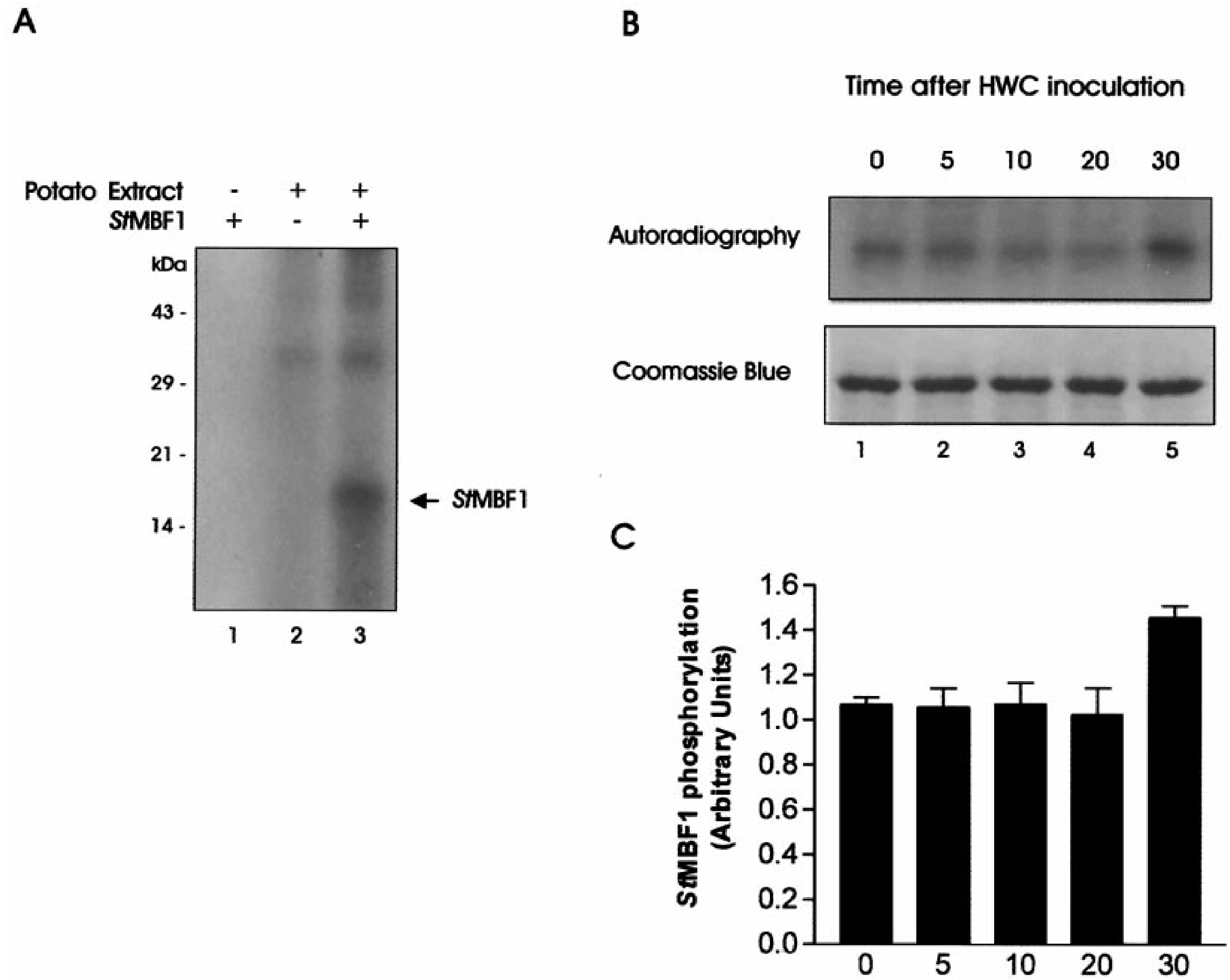


**Fig. 1.** *In vitro* phosphorylation of the *E. coli*-purified *St*MBF1. (A) Two μg of *E. coli*-purified *St*MBF1 were incubated alone (lane 1) or with 10 μg of potato cell extracts (lane 3) in the presence of [γ-$^{32}$P] ATP for 5 min at 30 °C. In lane 2, potato cell extracts were incubated alone. The arrow indicates the position of *St*MBF1. (B) Two μg of *E. coli*-purified *St*MBF1 were incubated with 10 μg of extract from potato cells non-treated and treated with HWC for 5, 10, 20, and 30 min as described in (A). After separation in 15% (w/v) SDS-PAGE, the proteins were analysed by Coomassie Blue staining and autoradiography. (C) The histogram represents the average of three independent densitometry measurements of different potato cells preparations. Error bars represent the SD.

were observed by Coomassie Blue staining (Fig. 1B, lower panel), the increase in the level of phosphorylation of *St*MBF1 after elicitor treatment is likely to reflect changes in some *St*MBF1-phosphorylating kinase activity/ies present in cell extracts.

To confirm whether *St*MBF1 was phosphorylated in potato cells, *in vivo* labelling was performed. Cell cultures were incubated with [$^{32}$P] phosphate, homogenized and immunoprecipitated using anti-*St*MBF1 or the corresponding preimmune serum. After the resolution of protein complexes by SDS-PAGE, a polypeptide of approximately 16 kDa, which corresponded to the electrophoretic mobility of *St*MBF1, was detected by autoradiography (Fig. 2A, lanes 3, 4). By contrast, this polypeptide was not observed when preimmune serum was used, confirming the specificity of the immunoprecipitation (Fig. 2A, lanes 1, 2). When elicited cells were used, an increase by approximately 1.5-fold in the incorporation of [$^{32}$P] into immunoprecipitated *St*MBF1 compared with non-treated cells was observed in two independent experiments. The autoradiographs were scanned and average densitometric values were 1.075±0.075 and 1.59±0.09 (in arbitrary units) for control and HCW-treated cells, respectively (Fig. 2A, lanes 3, 4). Since Western blot analysis revealed no differences in *St*MBF1 protein levels between control and elicited potato cells (Fig. 2B), the higher incorporation of [$^{32}$P] into *St*MBF1 detected in HWC-treated cells might derive from the activation of some kinase activity/ies.

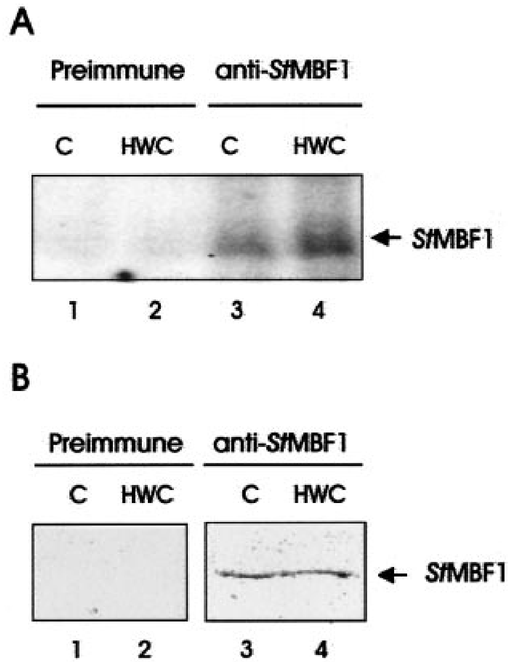


**Fig. 2.** (A) *St*MBF1 *in vivo* phosphorylation. Potato cell suspensions were incubated in the presence of [$^{32}$P] sodium phosphate for 2 h and then treated (lanes 2, 4) or not (lanes 1, 3) with HWC for 30 min. Cells were homogenized and immunoprecipitated with anti-*St*MBF1 serum or the corresponding preimmune serum. Immunoprecipitates were analysed by 15% (w/v) SDS-PAGE and autoradiography. (B) Western blot analysis of *St*MBF1 in potato cells. Ten μg of proteins from potato cell suspensions non-treated (lanes 1, 3) or treated with HWC for 30 min (lanes 2, 4) were separated in 15% (w/v) SDS-PAGE, transferred onto nitrocellulose and incubated with polyclonal antibodies against *St*MBF1 or the corresponding preimmune serum. The blots were developed as described in Materials and methods. Similar results were obtained in at least two independent experiments.

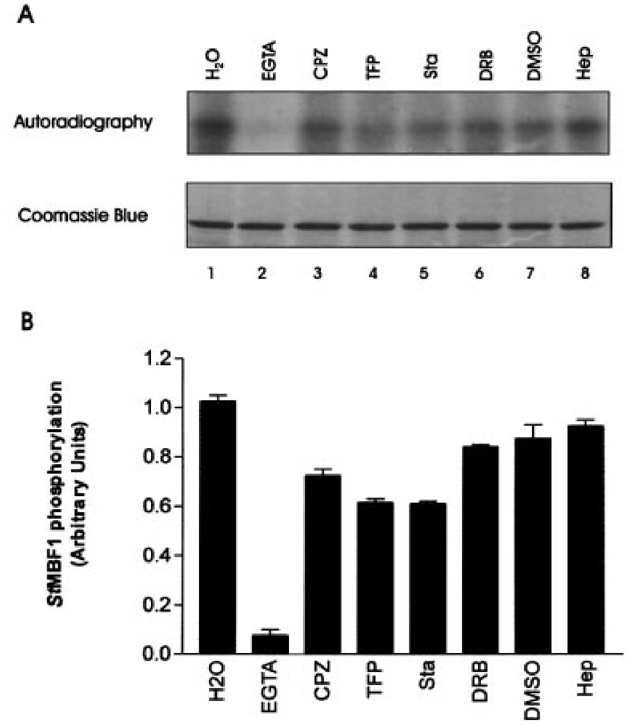


**Fig. 3.** Inhibition of *St*MBF1-phosphorylating kinase activity. (A) Ten μg of extract from cells treated for 30 min with HWC were preincubated for 15 min in the presence of inhibitors as indicated. After an additional incubation with 2 μg of *St*MBF1 in the presence of [γ-$^{32}$P] ATP for 5 min at 30 °C, the samples were subjected to 15% (w/v) SDS-PAGE and analysed by Coomassie Blue staining and autoradiography. (B) Densitometry quantification was performed. Error bars represent the SD. The effect of EGTA, staurosporine, DRB, and heparin was tested in six independent experiments and CPZ and TFP in two different experiments.

### *Characterization of the* St*MBF1 phosphorylating activity*

In order to characterize the kinase activity/ies responsible for *St*MBF1 phosphorylation, *in vitro* phosphorylation assays were performed in the presence of different protein kinase inhibitors. Staurosporine is a general inhibitor of serine/threonine kinases. This compound was used at a concentration of 200 nM and partially inhibited (40% of inhibition) the incorporation of [$^{32}$P] into *St*MBF1 (Fig. 3A, lane 5, B). At higher concentrations of staurosporine (2 μM) a similar inhibition effect was detected (data not shown). The effect of 500 μM DRB and 20 μg μl$^{-1}$ heparin, both inhibitors of the casein kinase II (CKII) family, was also examined. Neither of them significantly modified the incorporation of [$^{32}$P] into *St*MBF1 (Fig. 3A, lanes 6, 8, B) indicating that this protein kinase family might not be involved in *St*MBF1 phosphorylation. When the reaction was performed in the presence of 5 mM EGTA, [$^{32}$P] incorporation into *St*MBF1 was inhibited by 90% (Fig. 3A, lane 2, B), showing that the kinase activity involved in *St*MBF1 phosphorylation required the presence of calcium in the medium. CDPK activity is strongly inhibited by the presence of the $Ca^{2+}$-chelator EGTA. Although calmodulin is not required for its activity, calmodulin antagonists inhibit CDPK family in a dose–response manner (Sopory and Munshi, 1998). Thus, the effect of two calmodulin antagonists, CPZ and TFP, on the *St*MBF1-phosphorylating kinase activity was analysed. TFP and CPZ reduced the incorporation of [$^{32}$P] into *St*MBF1 by approximately 40% and 30%, respectively (Fig. 3A, lanes 3, 4, B). These results revealed that *St*MBF1-phosphorylating kinase activity was partially inhibited by calmodulin antagonists at the concentrations used in this assay.

The molecular properties of the *St*MBF1-phosphorylating activities were further characterized by an in-gel kinase assay. For this purpose, purified *St*MBF1 was included in the matrix of an SDS-PAGE gel. Extracts from control and elicited cells were separated in this gel, and the proteins were then renatured. Finally, the gel was incubated in a [γ-$^{32}$P] ATP-containing buffer, washed and analysed by autoradiography. As shown in Fig. 4, a kinase activity band corresponding to an electrophoretic mobility of 57 kDa

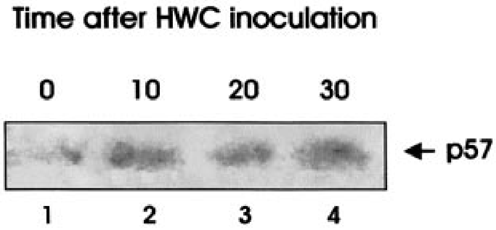


**Fig. 4.** Analysis of *St*MBF1-phosphorylating activity. Purified *St*MBF1 was included in the polymerization mixture of 12% (w/v) SDS-PAGE gels. Thirty µg of protein extract from non-treated potato cells (lane 1) or treated with HWC for 10, 20 and 30 min (lanes 2, 3 and 4, respectively) were separated in the gel. After performing a re-naturing procedure, the gel was incubated with a [$\gamma$-$^{32}$P] ATP-containing buffer, washed and dried. The signal was detected by autoradiography. The arrow indicates the band corresponding to the 57 kDa protein (p57).

was detected. This protein was named p57. At the times analysed, the activity was higher in elicited than in non-treated cells. By contrast, when *St*MBF1 was omitted in the SDS-PAGE matrix, no detectable signal was observed (data not shown). This indicated that the 57 kDa activity band was derived from *St*MBF1 phosphorylation and not from autophosphorylation activity. When the reaction was performed in the presence of 1 mM $CaCl_2$, p57 activity was detected (Fig. 4). However, when the assay was carried out in the absence of calcium, no *St*MBF1 kinase activity was observed (data not shown). This result agrees with that observed in Fig. 3 and supports the hypothesis that *St*MBF1 kinase activity is calcium-dependent.

### *StMBF1 is phosphorylated in the central region of the molecule*

To locate approximately which region of *St*MBF1 is phosphorylated by potato cell extracts, a small set of deleted proteins were tested. Various truncated polypeptides encompassing different regions of *St*MBF1 were expressed in *E. coli* and purified. *In vitro* phosphorylation assays were carried out in the presence of potato extracts, as described above. The deletion of 38 or 70 *N*-terminal amino acids had no effect on [$^{32}$P] incorporation into *St*MBF1 (Fig. 5, lanes 2, 4). The deletion of the 21 C-terminal amino acids (aa 118–139) did not affect the phosphorylation of *St*MBF1 either (Fig. 5, lanes 3, 4). These results indicated that *St*MBF1 phosphorylation occurs in the central 71–118 amino acid region of the protein. It is important to point out that, according to the colorimetric assay, equal amount of purified proteins were loaded in each lane of several independent gels. However, as it is shown in Fig. 5B, the Coomassie Blue pattern did not reveal an equal amount of protein. Since, Coomassie Blue dye recognizes amino groups of basic amino acids, the sensitivity of each protein could be different and, consequently, they were differentially stained. In addition, for the *N*-terminal deleted protein, *St*MBF1 $\Delta$1–38 a wide and clear band was detected in several experiments (Fig. 5B, lane 2, asterisk).

## Discussion

As far as is known, little has been reported about the role of plant coactivators during pathogen defence responses. Interestingly, two putative *Arabidopsis* transcriptional coactivators, KIWI and KELP, are believed to participate in gene activation during defence response and plant development (Cormack *et al*., 1998). Recently, Matsushita *et al*. (2002) reported that a member of the MBF1 transcriptional coactivator familiy, designed MIP24, interacts with the tomato mosaic virus movement protein (MP), raising the possibility that viral MPs could modulate host gene expression.

The expression of *St*MBF1 during wounding and infection of potato tubers with *F. eumartii* has previously been characterized (Godoy *et al*., 2001). The putative phosphorylation sites found in the amino acid sequence of *St*MBF1 led us to investigate whether the protein is actually phosphorylated in potato cells. *In vitro* phosphorylation assays revealed that *St*MBF1 is phosphorylated by potato cell extracts. In addition, immunoprecipitation of *St*MBF1 showed *in vivo* [$^{32}$P] incorporation, indicating that *St*MBF1 is a phosphoprotein. Treatment of potato cell suspensions with elicitors derived from *P. infestans* caused a reproducible increase of the radioactivity incorporated into *St*MBF1, as was revealed by both *in vitro* and *in vivo* phosphorylation assays (Figs 1B, 2B). A shift in the electrophoretic mobility of the induced phosphorylated *St*MBF1 protein was not observed, so it is suggested that the higher level of the phosphorylation signal is due to an increase in the amount of phosphorylated *St*MBF1 protein. However, taking into account that several putative phosphorylation sites are present in the *St*MBF1 sequence it is also possible that such increase was due to new phosphorylated sites. Dröge-Laser *et al*. (1997) reported changes in the phosphorylation of G/HBF-1in elicited cells by *in vivo* and *in vitro* assays, but there was no shift in the electrophoretic mobility. Unfortunately, the limited resolution of the gel was not sufficient to resolve phosphorylated proteins with very subtle changes in their molecular masses.

All this evidence indicates that *St*MBF1 phosphorylation is positively regulated in potato cells in response to fungal elicitors and it might reflect changes in the activities of protein kinases and phosphatases within potato cells. In-gel kinase assays using *St*MBF1 as the substrate allowed the identification of a 57 kDa polypeptide (p57), which was able to phosphorylate *St*MBF1 in the presence of calcium (Fig. 4). The activity of p57 was higher in elicited cells than in non-treated cells, supporting the idea that *St*MBF1-phosphorylating kinase activity is stimulated by elicitors. The *St*MBF1 phosphorylating activity detected at 0 time

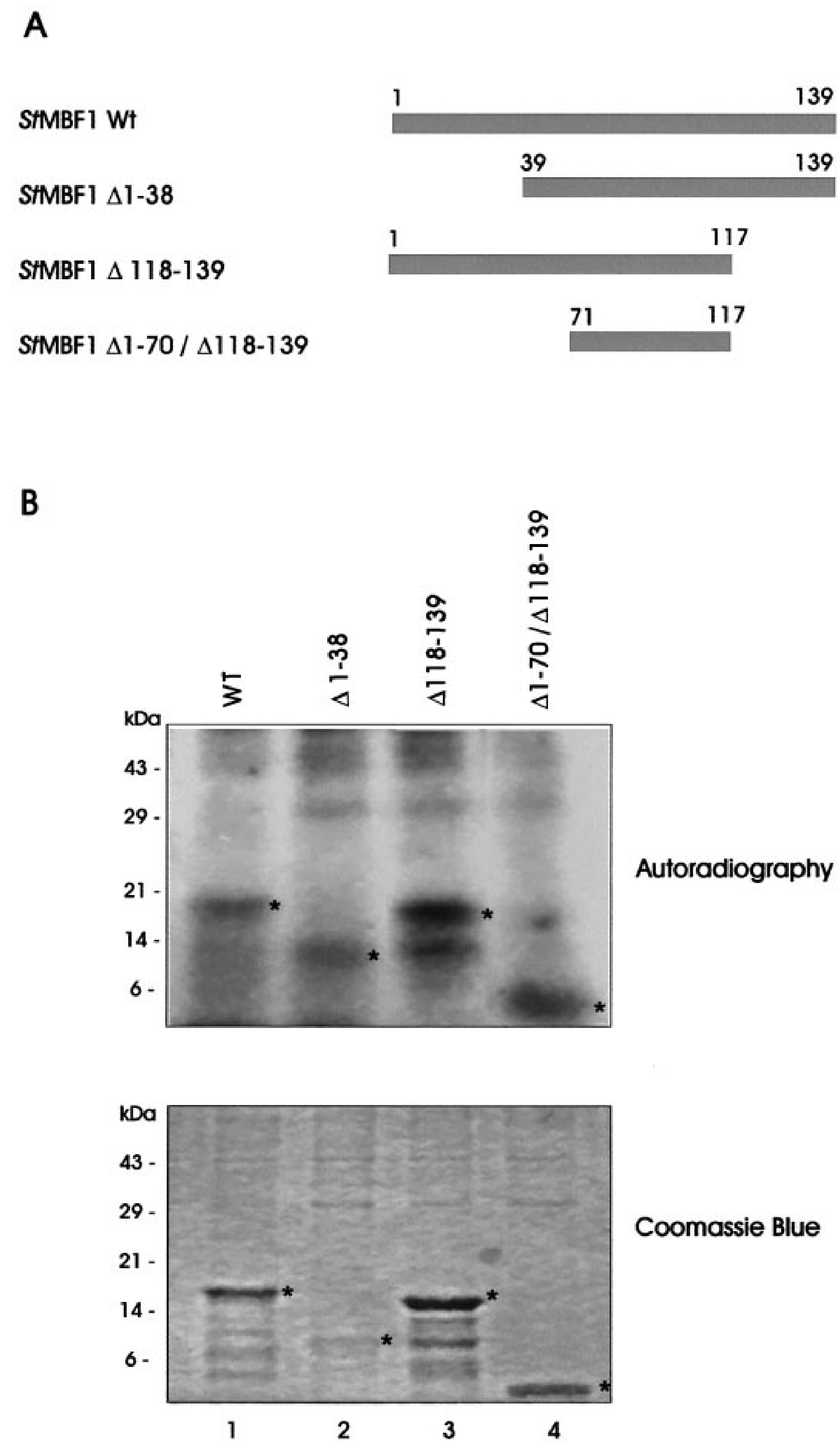


**Fig. 5.** Analysis of the region of *St*MBF1 phosphorylation. (A) Schematic representation of wild-type and truncated *St*MBF1. (B) *In vitro* phosphorylation assays were conducted using *E. coli*-purified wild type (lane 1) and truncated *St*MBF1: Δ1–38 (lane 2), *St*MBF1 Δ118–139 (lane 3) and *St*MBF1 Δ1–70/Δ118–139 (lane 3). Two μg of each protein were incubated with 10 μg of potato cell extracts in the presence of [$\gamma$-$^{32}$P] ATP for 5 min at 30 °C. After their separation in 18% (w/v) SDS-PAGE, the proteins were analysed by Coomassie Blue staining and autoradiography. Asterisks indicate the position of wild-type and truncated *St*MBF1 proteins. Similar results were obtained in four independent experiments.

(Fig. 4) was not rigorously correlated with phosphorylation levels detected at this time by *in vitro* and *in vivo* assays (Figs 1B, 2A). However, it must be considered that only protein kinases that re-nature after SDS-PAGE remain active, and thus are detected by in-gel kinase assays. In order to characterize the kinase activity/ies responsible for *St*MBF1 phosphorylation, different specific inhibitors were used in *in vitro* assays (Fig. 3). *St*MBF1 phosphorylation was strongly inhibited by the calcium-chelator EGTA and partially inhibited by two calmodulin antagonists, CPZ and TFP. Since these calmodulin antagonist were used in the range of previously reported concentrations (Romeis *et al*., 2000; Chico *et al*., 2002) and considering that total protein extracts were used in the assays, it is possible that the partial inhibition reflects that more than one kinase acting in a calcium-dependent manner, participate in *St*MBF1 phosphorylation. In addition, it has been reported (Polya and Micucci, 1985) that TFP variously inhibit plant CDPKs and, moreover, some of them are partially inhibited, even at high concentrations (400 μM to 1 mM) of TFP. The general serine/threonine kinase inhibitor staurosporine also partially inhibited phosphorylation of *St*MBF1. Interestingly, protein kinases showing different sensitivity to staurosporine in response to elicitors in potato cells have been reported (Katou *et al*., 1999). In conclusion, the results presented in Fig. 3 suggest that *St*MBF1 might be phosphorylated by a serine/threonine kinase that requires calcium for its activity, but other kinase/s may also be involved in *St*MBF1 phosphorylation.

In mammals, PKC and calmodulin-dependent protein kinase (CaMK) have been extensively characterized as calcium-modulated (Sopory and Munshi, 1998). It has been postulated that the role of PKC in mammals might be fulfilled in plants by CDPKs (Roberts and Harmon, 1992). CDPKs have molecular masses that range between 52 kDa and 90 kDa, and a very conserved structure (Harmon *et al*., 2000). They are activated by micromolar concentrations of calcium and do not require calmodulin for their activation (Sopory and Munshi, 1998). CDPK isoforms were found in various subcellular locations including the cytosol, nucleus and plasma membrane (Roberts and Harmon, 1992; Schaller *et al*., 1992). In several species, it has been observed that CDPK activities are usually up-regulated under stress conditions such as cold and wounding (Li and Komatsu, 2000; Chico *et al*., 2002). In addition, the participation of CDPKs in plant defence responses has been suggested (Rudd and Franklin-Tong, 2001). Romeis *et al*. (2000) reported the activation of a CDPK in transgenic *Cf9* tobacco cell cultures elicited with *Avr9*. In addition, the transcriptional activation of a maize CDPK in response to fungal elicitors has also been observed (Murillo *et al*., 2001).

On the other hand, the specific substrates of this kinase family have been poorly characterized. Recently, it was reported that a two-component pseudo-response regulator, CPS1, is phosphorylated by a CDPK from *Mesembryanthemum crystallinum* (McCDPK1). Co-transformation experiments showed that CPS1 and McCDPK1 co-localized to the nuclei of NaCl-stressed plants (Patharkar and Cushman, 2000).

Several characteristics observed for *St*MBF1-phosphorylating kinase activity suggest that a member that belongs to the superfamily of serine/threonine CDPKs might be implicated in *St*MBF1 phosphorylation. First, the kinase activity showed a strong inhibition by EGTA and a partial inhibition by calmodulin antagonists (Fig. 3). Second, the polypeptide (p57) detected by the in-gel kinase assay, was able to phosphorylate *St*MBF1 only in the presence of calcium (Fig. 4). Third, the molecular mass (57 kDa) of *St*MBF1-phosphorylating activity matches the range of molecular mass described for the CDPK family. Using deleted proteins it was possible to identify that phosphorylation occurs in the central region (aa 71–117) of *St*MBF1 (Fig. 5). Within this region, there are several serine and threonine residues that might be phosphorylated. This evidence supports the hypothesis that *St*MBF1 might be a substrate for a serine/threonine protein kinase in potato cells. Further experiments have to be performed to identify the residues that are modified by phosphorylation.

In mammalian systems, as well as in plants, it is well documented that phosphorylation might control the activity of gene-specific transcription factors by modulation of their cellular distribution or changes in protein–protein or DNA–protein interactions in which they are involved (Hill and Tresiman, 1995; Schwechheimer and Bevan, 1998). By contrast, no evidence of such regulation has been reported for members of the MBF1 co-activator family. Interest is now focused on how phosphorylation affects the activity of *St*MBF1. It is possible to speculate that phosphorylation might induce changes in the subcellular distribution (between the cytosol and nucleus) of *St*MBF1 or affect its interaction with other components of the transcriptional machinery. Simultaneously, further investigations might also reveal the identity of the kinase responsible for *St*MBF1 phosphorylation.

## Acknowledgements

We thank Dr MT Tellez-Iñon for the help with the phosphorylation assays. The autohors thank Dr Liliana Busconi and Dr Verónica Beligni for their critical comments. This research was supported by grants from the IFS, Sweden (C2124), UNMDP, CONICET, ANPCyT, and Fundación Antorchas, Argentina. GRD is a member of CIC. CC is a member of CONICET, MEZ is a fellow of the same Institution.

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