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The Potato Transcriptional Co-activator StMBF1 Is Up-regulated in Response to Oxidative Stress and Interacts with the TATA-box Binding Protein

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To gain a better understanding on the function of the potato *Solanum tuberosum* Multiprotein Bridging Factor 1 protein (*St*MBF1) its interaction with the TATA box binding protein (TBP) was demonstrated. In addition we reported that *St*MBF1 rescues the yeast *mbf1* mutant phenotype, indicating its role as a plant co-activator. These data reinforce the hypothesis that MBF1 function is also conserved among non closely related plant species. In addition, measurement of *St*MBF1 protein level by Western blot using anti-*St*MBF1 antibodies indicated that the protein level increased upon H_2O_2 and heat shock treatments. However, the potato β-1,3-glucanase protein level was not changed under the same experimental conditions. These data indicate that *St*MBF1 participates in the cell stress response against oxidative stress allowing us to suggest that MBF1 genes from different plant groups may share similar functions.

Keywords: MBF1, *Solanum tuberosum*, Stress, Transcriptional co-activator

Introduction

The expression of a gene is dictated, in part, by the integration of cellular and environmental signals that control the activity of transcription regulatory proteins. Transcriptional regulation plays a major role in the expression of the genomic information during complex biological processes. The presence of the TATA-box binding protein (TBP) at the TATA box is a pivotal intermediary step in transcriptional activation of protein coding genes. When this step occurs, a number of other transcription factors form a complex that recruits the RNA polymerase II, allowing transcription to proceed. The accessibility and binding of TBP to the promoter can be regulated by different regulatory proteins (Pugh, 2000).

A category of eukaryotic proteins named co-activators, which enhance transcription by interacting with both general and gene-specific transcription factors, has been identified and isolated from fungi, metazoan and plants (Takemaru *et al.*, 1997; Zhu *et al.*, 2000; Tsuda *et al.*, 2004). Multiprotein Bridging Factor 1 (MBF1) was originally purified from extracts of the silkworm *Bombyx mori* and identified as a transcriptional co-activator that interacts with the TBP and the *Drosophila melanogaster* nuclear receptor FTZ-F1 (Li *et al.*, 1994). Yeast and mammal MBF1 proteins bind to different transcription factors of the basic leucine zipper (bZIP) family, including yeast GCN4 and human AP-1 (Takemaru *et al.*, 1998; Kabe *et al.*, 1999; Brendel *et al.*, 2002; Busk *et al.*, 2003). Jindra *et al.* (2004) reported that MBF1 is a positive modulator of AP-1. MBF1 prevents an oxidative modification (S-cystenyl cystenylation) of a critical cysteine and stimulates AP-1 binding to DNA, preserving the redox-sensitive AP-1 activity via a direct interaction with the basic region of *Drosophila* D-Jun.

Godoy *et al.* (2001) isolated and characterized a potato cDNA clone, *St*MBF1, which encodes a protein with high

Abbreviations: 3-AT, 3-aminotriazole; GST, glutathione S-transferase; MBF1, Multiprotein Bridging Factor 1; *St*MBF1, *Solanum tuberosum* MBF1; TBP, TATA-box binding protein

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sequence homology to the human, yeast and *B. mori* MBF1 genes. *St*MBF1 also shows a high degree of conservation with other MBF1 sequences of plant species. However, most of them have not been yet functionally characterized (Tsuda and Yamazaki, 2004). Tsuda *et al.* (2004) demonstrated that all three *Arabidopsis* MBF1 genes can complement MBF1 deficiency in yeast. Tsuda and Yamazaki (2004) identified two plant groups (I and II) of MBF1 genes by comparison of deduced amino acid sequences of three AtMBF1 subtypes with those of other MBF1s. The extent of amino acid sequence identity indicated that AtMBF1 a (At2g42680) and AtMBF1 b (At3g58680) belong to the same group I, as *St*MBF1. However, AtMBF1 c (At3g24500) resides in the group II. The steady-state level of transcripts encoding AtMBF1 c is elevated in *Arabidopsis* in response to different stress conditions (Tsuda and Yamazaki, 2004). Contrary, AtMBF1 a and AtMBF1 b are developmentally regulated (Tsuda *et al.,* 2004). In the case of *St*MBF1, its expression is induced by wounding and *Fusarium eumartii* attack in a non photosynthetic organ, such as potato tubers. In addition, Zanetti *et al.* (2003) reported that *St*MBF1 is phosphorylated and the phosphorylation status is positively regulated after treatment of potato cells with *Phytophthora infestans* cell wall elicitors. The study of potato defense mechanisms against different environmental stresses is an important subject in the agronomical field; however, the role of *St*MBF1 as a potato transcriptional co-activator and its relative contribution to biotic and abiotic stress tolerance remains unknown.

Here, we report that *St*MBF1 protein is induced in potato cell suspensions growing under oxidative stress conditions. In addition, *St*MBF1 interacts with the potato TBP protein and it is able to complement mutant yeast lacking the *y*MBF1 gene. In plants, the functional conservation of MBF1 as transcriptional co-activators can be deduced from these observations. Finally, MBF1 proteins could, therefore, be good candidates to perform a protective role during oxidative stress.

Materials and Methods

Cell suspension cultures and stress treatments. Cell suspension cultures of *Solanum tuberosum* L cv Spunta, established from friable calli, were maintained in liquid MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and phytohormones: 2, 4-D (2 mg ml^{-1}) and NAA (2 mg ml^{-1}). Cell suspension cultures were grown in 50 ml of MS medium at 25°C in the dark, with shaking at 125 rpm. These cultures were transferred into fresh medium at 7-d intervals. For experiments, aliquots of 10 ml of cell suspensions in the log growth phase were treated with 20 mM H_2O_2; controls were treated with sterile distilled water. At different time points, cells were collected by vacuum filtration, washed with distilled water and stored at −80°C. The cell viability was determined by Evan's blue (Merck) staining under light microscope. Five hundred µl of 1% Evan's blue solution were added to 1 ml of cell suspension. After 20 min, the mixture was centrifuged at 750 × *g* for 2 min and washed with MS medium. Approximately 400 cells were counted per microscopy field.

Preparation of protein extracts. Potato cells were homogenized in a mortar with liquid nitrogen and suspended in one volume of cold extraction buffer: 60 mM Tris-HCl (pH 6.8), 0.1% bromophenol blue, 25% glycerol, 2% SDS and 5% mercaptoethanol. The cell lysates were incubated for 30 min at 4°C and centrifuged at 12,070 × *g* for 30 min. The supernatants were transferred into clean tubes and stored at −20°C.

Western blot assays. For Western blot assays, total proteins from potato cells (50 mg fresh weight) were extracted in sample buffer. Protein samples were boiled for 5 min and running on 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (BioRad). Bound *St*MBF1 and potato β-1,3-glucanase were detected by a Western blot analysis using the anti-*St*MBF1 antibody at a dilution of 1 : 3,000 (Zanetti *et al*., 2003) and the anti-β-1,3-glucanase at a dilution of 1 : 7,000 (Tonón *et al.,* 2002), respectively. The blots were allowed to react with goat antirabbit antibody (1 : 10,000) labeled with alkaline phosphatase (Sigma) and revealed with BCIP/NBT according to procedures recommended by the manufacturer.

Preparation of recombinant proteins. To produce *Schistosoma japonicum* GST-TBP fusion protein in *Escherichia coli*, the ORF of *Solanum tuberosum* TBP was amplified by RT-PCR using potato RNA as template. The following primer combinations were used: *St*TBPup (5'-CTCTGGATCCATGGCAGATCAGGGATTAGAG-3') and *St*TBPlw (5'-CTCTCTCGAGTGCTCCACAGTCCATCAAAT C-3'). 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 fragment was cloned into the *Bam*HI and *Eco*RI sites of pGEX-4T-3 (Amersham Pharmacia Biotech). The resulting construct was verified by sequencing. *E. coli* BL21 (DE3) cells bearing the pGEX-TBP construct were grown in 2X YT medium (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, and 5 g l^{-1} NaCl) with 2% glucose and 0.1 mg ml^{-1} ampicillin overnight at 37°C. 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 the addition of 0.1 mM isopropyl-1-thio-β-D galactopyranoside (IPTG) for 4 h at 25°C. The cells were harvested by centrifugation at 3,000 × *g* for 10 min and suspended in 0.5 vol of PBS (25 mM sodium phosphate buffer pH 7.2, 150 mM NaCl) containing 5 mM EDTA, 1 mM PMSF, 5 mg ml^{-1} leupeptin, 20 mg ml^{-1} pepstatin and 1 mg ml^{-1} lysozyme. After one freeze/thaw cycle and sonication, the lysates were clarified by centrifugation at 10,000 × *g* for 20 min at 4°C. GST and GST-TBP proteins were purified by affinity chromatography according to the manufacturer's instructions (Amersham Biosciences).

*St*MBF1 was also expressed as GST fusion protein in *E. coli* (Zanetti *et al*., 2003). After affinity purification, *St*MBF1 was released from the GST moiety by thrombin treatment according to the manufacturer's instructions (Amersham Biosciences).

Antibody production. Polyclonal antibodies against GST-TBP were raised in rabbits according to Harlow and Lane (1988). The titer and specificity of the antiserum were tested against *E. coli*-purified GST-TBP by Western blot analysis.

GST pull-down assay. Bacterially expressed GST or GST-TBP were incubated with 5 µl (packed volume) of GSH-Agarose 4B (Molecular Probes, Oregon, USA) for 1 h at 4°C. After washing, 1 µg of purified *St*MBF1 was added in 500 µl of protein-binding buffer (PBB) (20 mM HEPES-KOH pH 7.5, 20% glycerol, 0.1% Nonidet P-40, 0.5 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF). The mixture was rolled for 2 h at 4°C. The beads were washed twice with 500 µl of PBB and suspended in 10 µl of SDS-PAGE sample buffer. The bound proteins were eluted by boiling, loaded onto a 15% SDS-polyacrylamide gel, and blotted to a nitrocellulose membrane (BioRad, Hercules, USA). Bound *St*MBF1 was detected by a Western blot using the anti-*St*MBF1 antibody (Zanetti *et al.,* 2003).

Co-immunoprecipitation assay. Approximately 40 µl of Protein A Sepharose CL-4B (Sigma) was incubated for 2 h at 4°C with the anti-*St*MBF1 antibody at a dilution of 1 : 25. Approximately equivalent molar amounts of purified *St*MBF1 and GST-TBP proteins were preincubated in PBS overnight at 4°C and added to the antibody-loaded beads. After 2 h of incubation at 4°C, the beads were washed with buffer B (10 mM TRIS-HCl pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycolate, 1 mM PMSF), and subsequently with 1× TBS buffer and a dilution of 1 : 10 of TBS buffer. Bound proteins were eluted with SDS sample buffer, boiled for 5 min, resolved on 15% SDS-PAGE and analyzed by Western blots.

Yeast complementation assay. Wild-type strain KT130 and mutant strain Δ*mbf1*, KT131, were obtained from Dr Hirose's laboratory (Takemaru *et al.,* 1998). The DNA fragment corresponding to *St*MBF1 open reading frame (ORF) was amplified by PCR using the following primers: 5'-CCAGGAATTCATGAGTGGAATATCG CAAGAC-3' and 5'-CCAGGGATCCTTTCTTTCCTCGAAGTTT CG-3'. Then, the DNA fragment was cloned into *Eco*RI and *Bam*HI sites of pGBT9 vector (Clontech). pGBT9-*St*MBF1 and pGBT9 were introduced into yeast Δ*mbf1* competent cells by lithium acetate procedure as described by the manufacturer (Clontech).

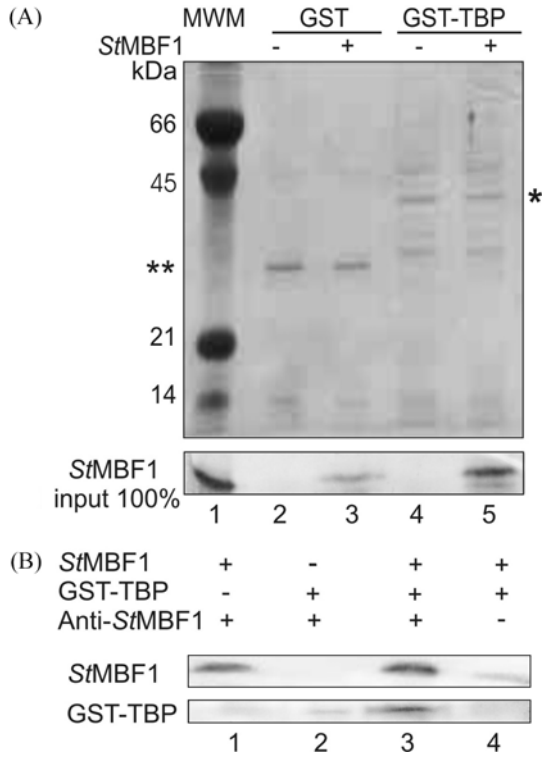

Fig. 1. In vitro protein-protein interaction. (A) GST pull-down assay for the interaction between *St*MBF1 and TBP. *St*MBF1 (1 µg) was incubated with total protein extracts containing GST (lanes 2 and 3) or GST-TBP (lanes 4 and 5). The bound proteins were analyzed by 15% SDS-PAGE and detected by Coomassie blue staining (upper panel) or Western blot (lower panel) using anti-*St*MBF1 antibody. Lane 1 (upper panel): molecular weight markers. Lane 1 (lower panel): input *St*MBF1. GST-TBP (*), GST (**). (B) Co-immunoprecipitation assay. *St*MBF1 was incubated with various combinations of proteins and subjected to immunoprecipitation with anti-*St*MBF1 antibodies immobilized to Protein-A Sepharose. Immunoprecipitates were analyzed by Western blot using anti-*St*MBF1 (upper panel) or anti-GST-TBP (lower panel) antibodies.

Results and Discussion

Previously, we have shown that *St*MBF1 interacts with Hd-Zip protein Hahb4 increasing the affinity of these proteins for their DNA target sites (Zanetti *et al*., 2004). This raises the possibility that *St*MBF1 can also interact with TBP in a conserved manner. In this work, we examined *St*MBF1 and potato TBP interaction. For this, both proteins were expressed in *E. coli* as translational fusions to GST. In order to reduce nonspecific binding, *E. coli* protein extracts containing the GST-TBP were used as described by Swaffield and Johnston (1996). Same amounts (900 µg) of total protein extracts containing GST or GST-TBP were used in combination with purified *St*MBF1 in GST pull-down assays. Thereafter, Fig. 1 (upper panel) shows that equivalent amounts of GST and GST-TBP proteins were eluted from the GSH- Agarose. Western blot analysis of the affinity-purified protein-complexes showed that a significant amount of *St*MBF1 protein was bound to GSH-Agarose when the GST-TBP protein was present (Fig. 1A, lower panel, lane 5). By contrast, only a faint band was observed when extracts containing GST were used (Fig.1A, lower panel, lane 3) and no band was detected when *St*MBF1 was omitted (Fig. 1A, lower panel, lanes 2 and 4). This result indicates that both proteins, *St*MBF1 and TBP, interact *in vitro*. Then, the *St*MBF1 and TBP interaction was confirmed by a co-immunoprecipitation assay. The protein complexes were analyzed by Western blots using both anti-*St*MBF1 and anti-GST-TBP antibodies. GST-TBP was co-immunoprecipitated with *St*MBF1, as evidenced by the detection of a prominent band of GST-TBP (Fig. 1B, lower panel, lane 3). A very faint band was detected in the absence of *St*MBF1, suggesting a weak cross-immunoreactivity between the TBP and the anti-*St*MBF1 antibody (Fig. 1B, lower panel, lane 2). As control, Fig. 1B (upper panel) shows the presence or absence of

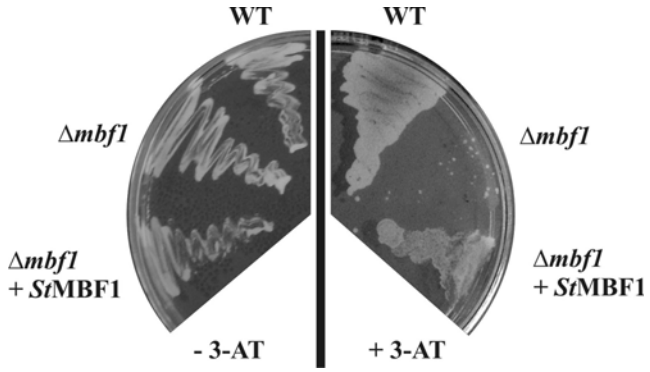

Fig. 2. Phenotype of the Δ*mbf1* strain. Cells of the yeast wild type strain (WT, KT130) and the Δ*mbf1* strain (KT131) were transformed with the empty vector (pGBT9); the Δ*mbf1* strain was transformed with pGBT9-*St*MBF1 (Δ*mbf1*+*St*MBF1). All yeast strains were grown on plates in the presence (+3-AT) or absence (-3-AT) of 50 mM AT for 3 days at 30°C.

*St*MBF1 in each reaction. All these results support the interaction between *St*MBF1 and TBP proteins indicating the presence of a common regulatory mechanism in two plant species, *Arabidopsis thaliana* and *Solanum tuberosum* which are not closely related to each other.

Takemaru *et al.* (1998) have described that *y*MBF1 mediates the GCN4-dependent transcriptional activation of the *His3* gene by bridging between GCN4 and TBP. The yeast mutant lacking either MBF1 or GCN4 are viable, but sensitive to 3-aminotriazole (3-AT), an inhibitor of the *His3* gene product. More recently, Tsuda *et al.* (2004) have demonstrated that *At*MBF1 proteins function as co-activators for GCN4-dependent transcriptional activation in yeast. Here we tested the function of *St*MBF1 as a co-activator by complementation of the yeast mutant Δ*mbf1* strain, KT131, with *St*MBF1. The Δ*mbf1* strain transformed with the empty vector, pGBT9, was sensitive to 3-AT (Fig. 3). Expression of *St*MBF1 cDNA (pGBT9-*St*MBF1) rescued the yeast Δ*mbf1* mutant phenotype upon histidine starvation in the presence of 3-AT. All yeast strains used in this experiment showed essentially the same normal growth rate on histidine-free medium in the absence of 3-AT (Fig. 3). These results indicate that *St*MBF1 also acts as a co-activator for GCN4-dependent transcriptional activation in yeast cells. Based on these data, we concluded that *St*MBF1 is an evolutionarily conserved transcriptional co-activator that connects the TATA element-binding protein and a regulatory transcriptional factor as it has been demonstrated for MBF1 of other species (Li *et al*., 1994; Takemaru *et al*., 1998; Kabe *et al*., 1999 and Tsuda *et al*., 2004). Taking together this new finding and our previous results we suggest that *St*MBF1 may serve as a bridging molecule between TBP and Hd-Zip transcriptional factors in the potato defense response (Zanetti *et al.,* 2004). Further, the characterization of other *St*MBF1 partners will allow us to understand its biological function.

MBF1 genes have been implicated in oxidative stress responses in different organisms (Jindra *et al.,* 2004; Tsuda and Yamazaki, 2004). Active oxygen species, especially H_2O_2 plays a critical role in the defense of plants. In this work, potato cell suspensions were subjected under oxidative stress conditions. First, the viability of potato cells exposed to H_2O_2 was monitored using Evan's blue staining. Evan's blue is excluded from viable cells but remains in dead cells (Yano *et al.,* 1998). Fig. 3A shows the presence of numerous dark cells consisting of dead cell clusters at 60 min after H_2O_2-treatment (right lower panel). Fig. 3B shows the time-dependent cell viability (Fig. 3B, black columns). At 180 min the cell viability dropped to 25% compared to non-treated cells (75%). In non-treated cultures the cell viability remained constant during the analyzed times (Fig. 3B, white columns). In addition, cells were treated with 20 mM H_2O_2 for 30, 60 and 180 min and *St*MBF1 levels were analyzed by Western blot. Fig. 4B shows that the *St*MBF1 level clearly increased at all analyzed times after initial treatment, whereas the level of acidic potato β-1,3-glucanase, *St*Gluc, did not change by the H_2O_2 treatments. Such β-1,3-glucanase has been described as a patatin-like protein associated with the resistance to *Phytophthora infestans*

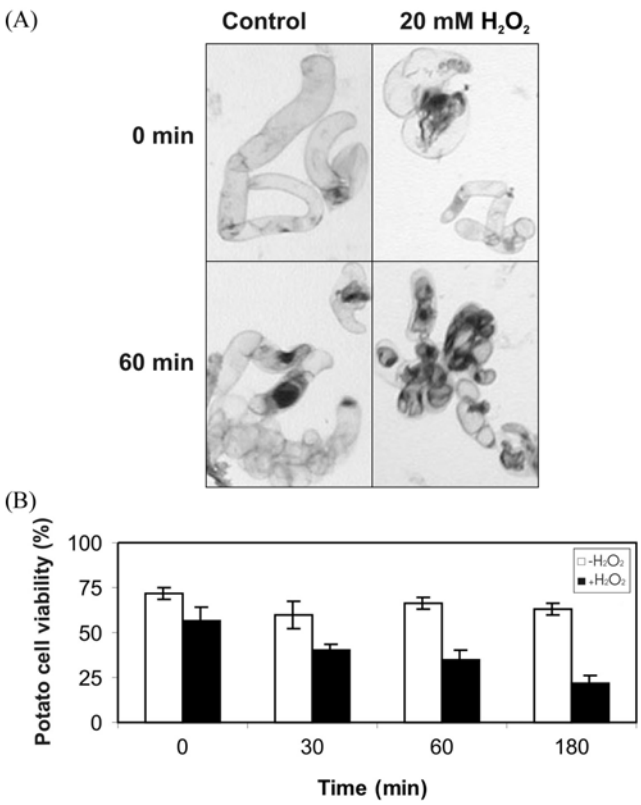

Fig. 3. Cell viability. (A) Light microscopy visualization of potato cells treated or not for 60 min with 20 mM H_2O_2 and evaluated by Evan's blue staining. (B) Time-dependent cell viability induced by 20 mM H_2O_2. Control cells (white columns), H_2O_2.treated cells (black columns). Values are the mean of at least three independent experiments.Vertical bars represent standard deviation. 100% of cell viability corresponds to the total number of cells visualized in each microscopy field.

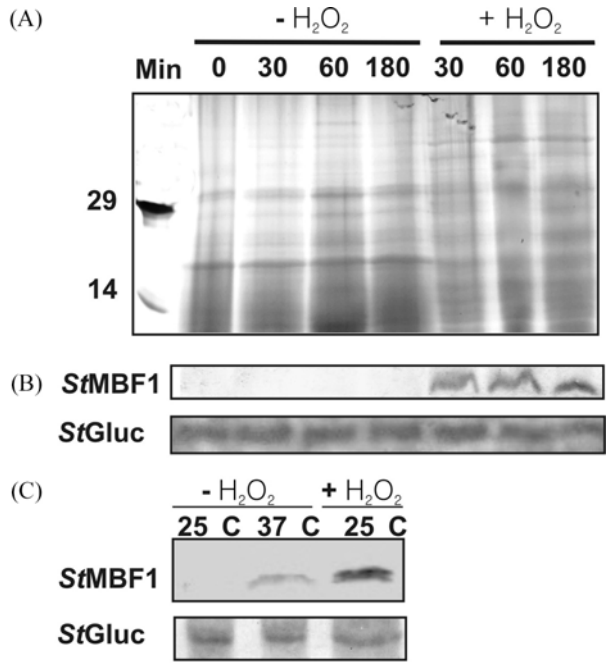

Fig. 4. *St*MBF1 level detected in potato cell suspensions after H_2O_2 or heat shock treatments. (A) 15% SDS-PAGE. Total protein extracts (50 mg fresh weight) from 4 day-old potato cell suspensions treated (+H_2O_2) or not (-H_2O_2) with 20 mM H_2O_2, were running in the gel and stained with Coomassie blue. (B) Western blot analysis using the anti-*St*MBF1 or the anti-β-1,3-glucanase antibodies (upper and lower panels, respectively). (C) Potato cell suspensions were incubated for 6 h at 25°C and 37°C or with 20 mM H_2O_2 for 3 h at 25°C. Western blotting was performed using the anti-*St*MBF1 or the anti-β-1,3-glucanase antibodies (upper and lower panels, respectively).

(Tonón *et al.,* 2001). β-1,3-Glucanases are considered to be one component of a broad generalized defense mechanism against pathogen attack and play different biological roles in a number of plant species (Tonón *et al.,* 2002). As control, Fig. 4A shows the loading for each sample by Coomassie blue staining. These results suggested that *St*MBF1 expression is strongly dependent on oxidative stress conditions. In general, organisms from prokaryotes to mammals have evolved a number of mechanisms to maintain redox balance and thus evade oxidative stress caused by naturally arising reactive oxygen species. Tsuda and Yamazaki (2004) have suggested that the AtMBF1 genes may be involved in the plant responses to multiple stresses, particularly to drought and heat shock stresses. Several reports have described that there is a link between the heat and oxidative stress responses (Banzet *et al*., 1998; Dat *et al*., 1998; Schett *et al*., 1999; Lee and Vierling, 2000). In our system, *St*MBF1 was almost undetectable in cell suspensions growing at 25°C (Fig. 4C, upper panel, lane 1). However, when cells were incubated for 6 h at 37°C, the *St*MBF1 level was higher than in control cells (Fig. 4C, upper panel, lanes 2 and 1, respectively). The β-1,3-glucanase level did not increase by heat shock and H_2O_2 treatments (Fig. 4C, lower panel). Fig. 4C, upper panel, also shows that the *St*MBF1 level is higher when cells are subjected to H_2O_2 than heat shock. Thus, it is reasonable to propose that H_2O_2 could be involved in the pathway of *St*MBF1 activation as a part of the orchestrated response of potato cells against severe oxidative stress conditions. Recently, Suzuki *et al.* (2005) have reported that the expression of *Arabidopsis* MBF1c in transgenic plants augmented the accumulation of a number of defense transcripts in response to heat stress. These results allowed us to suggest that the MBF1 genes from different plant groups (groups I and II, StMBF1 and AtMBF1 c, respectively) may share similar functions during the response against oxidative stress conditions. In addition, the data reinforce the hypothesis that MBF1 function is conserved between different plant species. Although still unclear is the role of *St*MBF1 in potato plants growing under environmental stresses, our findings provide fundamental bases to clarify regulatory genes involved in the potato defense mechanisms. It is an important subject in the agronomical field since potato represents the fourth horticole species in the world and its crop is strongly affected by biotic and abiotic stresses.

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