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Homeodomain-leucine Zipper Proteins Interact with a Plant Homologue of the Transcriptional Co-activator Multiprotein Bridging Factor 1

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***St*MBF1 (*Solanum tuberosum* multiprotein bridging factor 1) is a plant member of the MBF1 family of transcriptional co-activators. In an attempt to understand the role of *St*MBF1, we analyzed its interaction with plant transcription factors of the homeodomain-leucine zipper (Hd-Zip) family, a group of proteins with a typical leucine zipper motif adjacent to a homeodomain. *St*MBF1 is able to interact *in vitro* with the Hd-Zip protein Hahb-4 both in the presence and absence of DNA. Upon binding, *St*MBF1 increases the DNA binding affinity of Hahb-4, and of another plant homeodomain containing protein from the GL2/Hd-Zip IV family, HAHR-1. The biological role of interactions is discussed in this paper.**

Keywords: DNA-binding, Hd-Zip protein, Leucine zipper, Multiprotein bridging factor 1 (MBF1), Plant homeodomain

Introduction

A category of eukaryotic proteins termed co-activators, which enhance transcription by interacting with both general and gene-specific transcription factors, has been identified and isolated from several organisms, including yeast, parasites and animals (Lewin, 1990; Zhu *et al*., 2000). The 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 general transcription factor TBP (TATA box binding protein) and the *Drosophila melanogaster* nuclear receptor FTZ-F1 (Li *et al*., 1994). Later, the MBF1 homologues factors were identified in yeast, parasites, and humans (Takemaru *et al*., 1997; Kabe *et al*., 1999; Zhu *et al*., 2000). Takemaru *et al*. (1997) and Kabe *et al*. (1999) found that yeast and human MBF1 bind to different transcription factors of the basic leucine zipper (b-Zip) family, including yeast GCN4, bovine Ad4BP/SF1, and human ATF1, c-Jun, and c-Fos.

Godoy *et al*., (2001) isolated and characterized a potato (*Solanum tuberosum*) cDNA clone that encodes a protein (*St*MBF1) with high sequence homology with human, yeast, and *Bombix mori* MBF1. The expression analysis revealed that *St*MBF1 mRNAs accumulates in potato tubers upon fungal infection. More recently, Zanetti *et al.*, (2003) reported that *St*MBF1 is phosphorylated, and this phosphorylation status is positively regulated after treatment of potato cells with fungal elicitors.

Plants challenged with fungal pathogens respond by up-regulating the expression of various genes, including genes that contribute to the transcriptional activation (Somssich, 1994). A homeodomain-leucine zipper protein (Hd-Zip), PRHA, has been implicated in the regulation of developmental and pathogenesis-related genes (Korfhage *et al*., 1994; Plesch *et al*., 1997). In addition, PRHA-interacting proteins, KELP and KIWI, have been described as putative transcriptional co-activators that are probably involved in gene activation during pathogen defence and plant development (Cormack *et al*., 1998).

In an attempt to understand the role of *St*MBF1, in the present work, we analyzed its interaction with Hahb-4, a plant transcription factor of the homeodomain leucine zipper (Hd-Zip) family, which belongs to the group of proteins with the typical leucine zipper motif that is adjacent to the C-terminal end of the homeodomain (Ruberti *et al*., 1991; Chan *et al*., 1998). Since animals and yeast MBF1 interact with leucine-

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zipper transcription factors, members of this family would be good candidate-interacting partners for plant MBF1. Hahb-4 was selected because it is a representative member of the HD-Zip family, whose DNA binding activity was well characterized (Palena *et al*., 1999; Palena *et al*. 2001). In addition, Hahb-4 and *St*MBF1, are both involved in the regulation of plant responses against environmental stresses (Godoy *et al*., 2001; Gago *et al*., 2002; Zanetti *et al*., 2003). We report that *St*MBF1 interacts *in vitro* with the HD-Zip protein Hahb-4, enhancing its affinity for its DNA binding site. The conservation of MBF1 functions can be deduced from these observations.

Materials and Methods

Expression and purification of recombinant proteins in *E. coli* Different portions of Hahb-4, consisting of either the complete Hd-Zip domain, the homeodomain alone (ΔZip-Hahb-4), or the Hd-Zip domain without the N-terminal arm (ΔN-Hahb-4), as well as Hahr-1, were expressed in *E. coli* as fusion proteins with *Schistosoma japonicum* GST, and purified by affinity chromatography, as previously described (Palena *et al*., 1998; Palena *et al*., 1999; Palena *et al*., 2001). *St*MBF1 was also expressed as a GST fusion protein in *E. coli*, as described by Zanetti *et al.* (2003). After affinity purification, *St*MBF1 was released from the GST moiety by thrombin treatment according to the manufacture's instructions (Amersham Biosciences, Uppsala, Sweden).

GST fusion proteins pull-down assay Purified *St*MBF1 (500 ng) was incubated at 25ºC with 2 µg of either GST, or different GST-Hahb-4 fusion proteins that were immobilized on GSH-Sepharose 4B in 500 µl of a protein binding buffer (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). After 1 h of incubation, the beads were washed three times with 1 ml of a binding buffer and suspended in 10 µl of a 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 analysis using the anti-*St*MBF1 antibody at a dilution of 1 : 5000 (Zanetti *et al*., 2003) and an enhanced chemiluminiscence detection system, according to the manufacture's instructions (Amersham Biosciences).

Electrophoretic mobility shift assays Electrophoretic mobility shift assays were performed essentially as previously described (Palena *et al*., 1999) using the complementary oligonucleotides 5'-AATTCAGATCTCAATAATTGAGAG3' and 5'GATCCTCTCAATTATTGAGATCTG3'.

Miscellaneous methods The total protein was measured, as described by Sedmak and Grossberg (1977). SDS-PAGE was performed according to Laemmli (1970). For quantitative analyses, the radioactive bands were cut from the exposed gels and measured by scintillation counting. Data handling and curve fitting were performed using Graph Pad Incorporated Software, Version 2.0.

Results

In order to elucidate whether plant MBF1 has a similar function to those described for its homologues from animals and yeast, we examined its interaction with Hahb-4, a member of the Hd-Zip I subfamily of plant transcription factors (Ruberti *et al*., 1991; Chan *et al*., 1998). For this purpose, Hahb-4 and its derivatives (Fig. 1A), expressed as GST fusion proteins, and purified *St*MBF1 were used in combination in GST pull-down assays. Western blot analysis using anti-*St*MBF1 antibodies showed that a significant amount of *St*MBF1 bound to GSH-Sepharose when a fusion protein carrying the Hd-Zip domain of Hahb-4 was present (Fig. 1B). This probably reflects a physical interaction between Hahb-4 and *St*MBF1, since only faint bands were observed when either GST or buffer alone was used instead of the fusion protein. The results also indicated that the deletion of the N-terminal arm of the homeodomain, which affects DNA binding affinity (Palena *et al*., 2001), did not perturb the interaction (Fig. 1B). Interestingly, the deletion of the leucine zipper portion, which abolishes dimerization (Palena *et al*., 1999), caused a decrease in the interaction between Hahb-4 and *St*MBF1.

To gain a better understanding of *St*MBF1 and Hahb-4 interaction, the GAL4 yeast two-hybrid system was also used (Fields and Song, 1989). The full-length coding region of *St*MBF1 was fused to the DNA binding domain of GAL4 and Hahb-4 was fused to the transcriptional activation domain of GAL4, and vice versa. Even though the immunoblot analysis revealed that both proteins were expressed, still the yeast cells

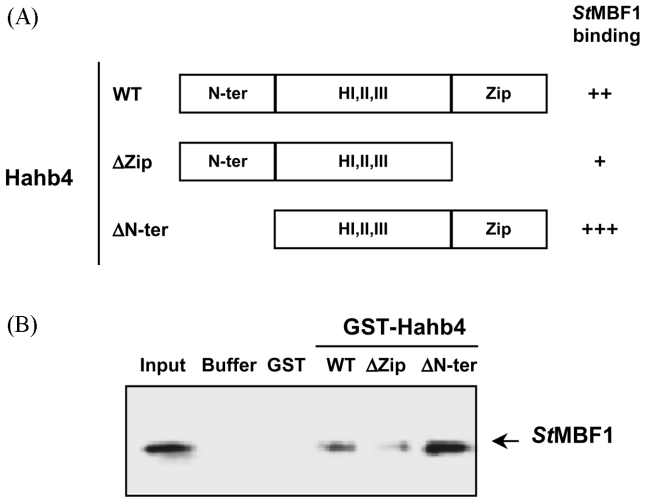

Fig. 1. Direct interaction between *St*MBF1 and Hahb-4. A: Schematic representation of Hahb-4 derivatives expressed by different constructs used in this study. B: GST pull-down assay for the interaction of *St*MBF1 with Hahb-4 derivatives. Different GST-Hahb-4 fusion proteins (2 µg) were incubated with 500 ng of purified *St*MBF1 and GSH-Sepharose. After several washes, the bound proteins were electrophoresed by SDS-PAGE and immunoblotted with anti-*St*MBF1 antibodies, as described in Materials and Methods. Input represents the *St*MBF1 mass included in the binding reactions.

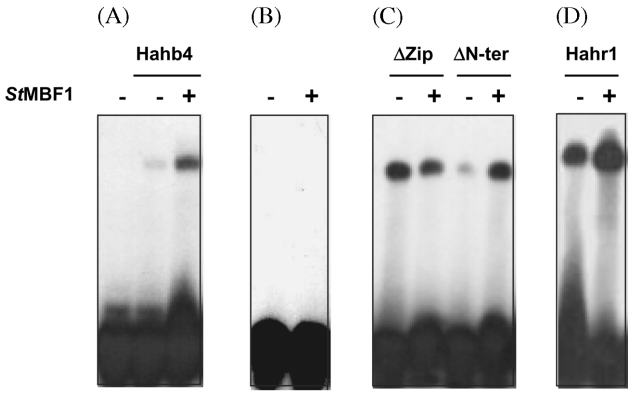

Fig. 2. *St*MBF1 increases Hahb-4 binding to DNA. A-C: Binding of Hahb-4 (15 ng) and its derivatives (100 ng) to a ^{32}P-labelled oligonucleotide carrying the Hahb-4 target sequence was analyzed by an electrophoretic mobility shift assay in the presence or absence of 150 ng of purified *St*MBF1. In A, first lane, the labeled oligonucleotide was incubated alone, in the absence of Hahb-4 or *St*MBF1. D, A similar assay with the GL2/Hd-Zip IV protein Hahr-1 (15 ng). "+ and –" signs indicate that the *St*MBF1 protein was added, or not, to the DNA binding assay, respectively.

containing both constructors neither activated the reporter gene nor grew under strong selective nutritional conditions (data not shown).

To measure the effect of *St*MBF1 on DNA binding by Hahb-4, electrophoretic mobility shift assays with oligonucleotides containing the target sequence 5'-CAAT(A/T)ATTG-3' bound by Hahb-4 were performed (Palena *et al.*, 1999). A significant increase in DNA binding by Hahb-4 upon addition of the purified *St*MBF1 to the binding assays was observed (Fig. 2A). This increase was not due to the binding of *St*MBF1 to DNA, since no binding was observed when Hahb-4 was omitted (Fig. 2B). This implies that *St*MBF1 must exert its effect through a direct interaction with Hahb-4. *St*MBF1 also increased the binding affinity of ΔN-Hahb-4, but had no effect on the binding of ΔZip-Hahb-4 (Fig. 2C). These results closely match those that were obtained in the direct interaction assay previously described (Fig. 1) and reinforce the view that, upon binding to Hahb-4, *St*MBF1 promotes an increase in the Hahb-4 DNA binding affinity. This effect does not seem to be unique for Hahb-4 since essentially the same results were obtained when Hahr-1, a homeodomain containing protein that belongs to the GL2/Hd-Zip IV family (Valle *et al*., 1997), was used (Fig. 2D).

Moreover, the increase in Hahb-4 DNA binding affinity was dependent on the amount of *St*MBF1 that was added to the assay (Fig. 3). A 4-fold increase in binding was obtained with saturating levels (150 ng; 500 nM) of *St*MBF1 when 15 ng (20 nM) of Hahb-4 was used in the assay. Half-maximal affinity was reached with 250 nM of *St*MBF1. This value would represent an estimation of the dissociation constant of the Hahb-4/*St*MBF1 complex.

The observed *St*MBF1 effect could be due to either an

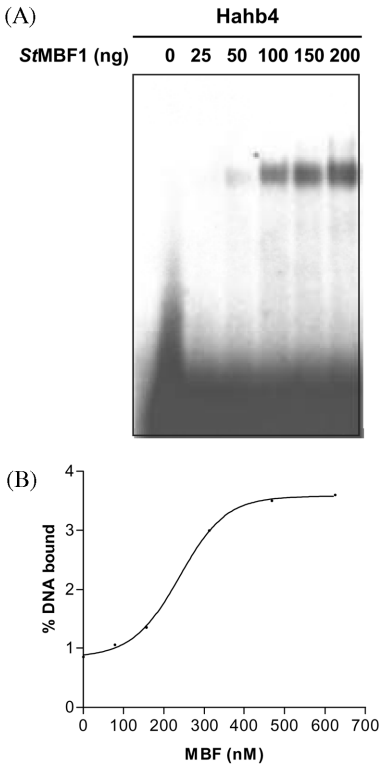

Fig. 3. The increase in Hahb-4 binding to its target DNA is dependent on the *St*MBF1 concentration**.** A: Binding of Hahb-4 (15 ng) to its target sequence was analyzed by an electrophoretic mobility shift assay in the presence of increasing amounts of *St*MBF1 (from 0 to 200 ng). In B: a graphical representation of the percentage of DNA bound as a function of the *St*MBF1 concentration is shown. The results are representative of at least three independent experiments.

increase in the rate of complex formation or stabilization of the complex once formed. To test these possibilities, we examined the time course of the complex formation in the presence or absence of *St*MBF1 (Fig. 4). More than half the final amount of complex was observed after 1 min of incubation in the presence of *St*MBF1, while several minutes were required to obtain a similar amount in the absence of the co-activator. Once the Hahb-4-DNA complex was formed, the presence of *St*MBF1 had no effect on its dissociation. This was concluded by the fact that the addition of cold DNA competed in a similar time-dependent manner either in the presence or absence of *St*MBF1 (data not shown).

Discussion

In the present study, we analyzed the interaction of the Hd-Zip protein Hahb-4 with *St*MBF1, a plant homologue of multiprotein bridging factor 1 (Godoy *et al*., 2001). Our studies indicated that *St*MBF1 interacts with Hd-Zip proteins increasing the affinity of these proteins for their DNA target sites. This effect is similar to that observed for *B. mori* MBF1

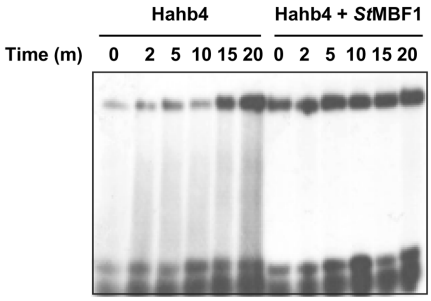

Fig. 4. Time course of Hahb-4 binding to its target sequence in the presence of *St*MBF1. The amount of DNA bound by Hahb-4 was analyzed by electrophoretic mobility shift assays at different times after the inclusion of Hahb-4 (100 ng), either in the presence or absence of *St*MBF1 (100 ng).

on the *D. melanogaster* transcription factor FTZ-F1, yeast MBF1 on the b-Zip protein GCN4, and human MBF1 on several transcription factors of the b-Zip family (Li *et al*., 1994; Kabe *et al*., 1999). In all cases, the interaction was established in solution, and did not need the presence of DNA. The lack of a supershifted band in electrophoretic mobility shift assays suggested that the complex is disrupted upon loading the samples on the gel. A similar behavior was reported for MBF1 from other species, and it was explained by the dissociation of the complex during the electrophoresis since the gel and running buffer contained no Mg^{2+} (Takemaru *et al*., 1998; Zhu *et al*., 2000). All of these characteristics might suggest the existence of conservation in the mode of action of MBF1 across different kingdoms. Our results also indicated that the leucine zipper is a determinant of the *St*MBF1-Hahb-4 interaction. This was denoted because the Δzip protein barely interacted with *St*MBF1 in the GST-pull down assay, and the addition of *St*MBF1 did not increase the DNA-binding affinity of the ΔZip mutant protein. It is noteworthy that MBF1 co-activators interact with several leucine zipper proteins in yeast and humans (Takemuro *et al*., 1998; Kabe *et al*., 1999). The analysis of mutants, however, indicated that yeast MBF1 rather interacts with the basic region of GCN4 (Takemuro *et al*., 1998). These results may be concealed if it is assumed that the interaction takes place near the boundary between the DNA binding module and the dimerization motif. It can be predicted that the binding of *St*MBF1 in this region (near the recognition helix) can cause a conformational change in the homeodomain that increases its capacity for DNA binding (Gehring 1987; Gehring *et al*., 1994). This would also explain the effect of *St*MBF1 on DNA binding by Hahr-1, an unrelated member of the HD-Zip IV subfamily that contains a bipartite dimerization motif that resembles a leucine zipper interrupted by a loop (Palena *et al*., 1997). This primarily indicates that most or all members of the HD-Zip family may interact with this transcriptional co-activator. This promiscuous behavior of MBF1 seems to be conserved in eukaryotic since human and yeast MBF1 bind to different members of the bZip family of transcription factors (Kabe *et al*., 1999).

In vitro experiments strongly suggested that a direct physical interaction is established between Hahb-4 and *St*MBF1. However, no interaction between these partners was detected by two-hybrid assays. We previously demonstrated the phosphorylation of *St*MBF1 (Zanetti *et al*., 2003), but it is still unknown whether this regulation plays a role on the complex formation. In this regard, it has been described that interactions that are mediated by post-translational modification may not be detected by the two-hybrid system (Fields and Sternglanz, 1994).

Hd-Zip proteins participate at several stages of plant development, being able to induce changes in the already developed organs in response to environmental stresses and hormonal factors (Schena and Davis, 1992; Korfhage *et al*., 1994; Söderman *et al*., 1996; Gago *et al*., 2002). Other members of the MBF1 plant family are regulated under different physiological situations (Zegzouti *et al*., 1999; Matsushita *et al*., 2002). Since Hahb-4 and *St*MBF1 are both induced by stress and hormonal factors (Godoy *et al*., 2001; Gago *et al*., 2002), then we suggest that the *St*MBF1 and Hd-Zip proteins are part of the transcriptional machinery that is activated in plants in response to environmental and hormonal conditions or conversely; both proteins could participate in global transcriptional regulation. Clearly, further studies will be necessary to elucidate the role of the *St*MBF1 protein and the signal transduction pathways that are involved in its regulation.

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