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Research paper

Binding properties of the complex formed by the *Arabidopsis* TALE homeodomain proteins STM and BLH3 to DNA containing single and double target sites

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ABSTRACT

We have analyzed the DNA-binding properties of the complex formed by the *Arabidopsis* TALE homeodomain (HD) proteins STM and BLH3 in comparison with those of the individual proteins. *In vitro* DNA-binding assays indicated that complex formation increases binding affinity for sequences carrying either a single target site or two such sites arranged in tandem. Complex formation is not correlated with the establishment of new detectable contacts as deduced from missing-nucleoside experiments. Increased binding was also observed when using BLH3 with a mutation that renders the HD unable to bind DNA, suggesting that only the STM functional HD is necessary for tight binding by the complex. Yeast one-hybrid assays using single or double target sites showed that the effect of complex formation is more dramatic for the double target site and that under these conditions competition for binding by the individual proteins is reduced. The results indicate that even if complex formation produces an increase in binding to DNA sequences containing either one or two target sites, the relative increase in binding produced after complex formation is dependent on the type of target sequence that is considered. This differential effect of complex formation on binding may have implications in the regulatory properties of these transcription factors within the cell.

1. Introduction

Homeodomain (HD) proteins constitute a vast family of transcription factors found in most eukaryotic organisms. The term HD defines a 60 amino acid conserved protein motif, composed of three alpha helices connected by a loop and a turn, which is involved in recognizing specific nucleotide sequences. Most HDs bind DNA sequences composed of a 4-nt core surrounded by nucleotides that are less important for binding [1–4]. Considering the reduced length of their target sequence and the fact that many HD proteins seem to have similar DNA-binding preferences, the question arises of what are the mechanisms these proteins use to achieve the regulation of specific sets of genes. Among these mechanisms, the interaction with other proteins, some of them HD proteins too, is frequently used to increase the specificity and/or the affinity of the interaction with DNA.

Plant HD proteins can be divided into different families according to sequence conservation within and outside the HD [5]. Proteins from two of these families, KNOX and BELL, belong to the TALE superclass, with members also in animals and fungi, since they contain three invariant extra amino acids in the loop that connects helices I and II [6]. KNOX and BELL proteins are developmental regulators participating in the establishment and maintenance of the meristematic identity and in the regulation of organ differentiation and architecture, among other processes [7–13], and recognize similar DNA sequences with a TGAC core [14–17]. Several reports have shown that KNOX and BELL proteins are able to interact in a DNA independent manner through conserved domains present in the N-terminal portion of the respective proteins [15,18–23]. The second half of the MEINOX domain of KNOX proteins (KNOX2) associates with the bipartite conserved MID domain of BELL proteins [19,20,24], composed of the BELL and SKY regions [18–20]. This led to the proposal that the complexes formed by KNOX and BELL proteins may be the actual entities participating in gene regulation. In addition, this interaction has been taken as an indication of the existence of ancient regulatory mechanisms since animal TALE proteins from the PBC and MEIS families are also known to interact through conserved domains present in similar locations [18].

Although many reports have shown that KNOX and BELL proteins are able to interact, the impact of this interaction on the DNA-binding properties of the complex has been addressed only in

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two systems involving proteins from maize and potato. *In vitro* studies have shown that the interaction of KN1 and KIP (KNOX and BELL proteins from maize, respectively) produces an increase in affinity for a DNA fragment containing a single TGAC binding site [15]. In the potato system, however, a DNA sequence with two tandem binding sites present in the *ga20ox1* promoter is required for the interaction with a complex formed by the KNOX and BELL proteins POTH1 and StBEL5 [25]. These results may indicate that complexes formed by different KNOX and BELL proteins have different properties.

In the present study, we have analyzed the interaction of the complex formed by the *Arabidopsis* proteins STM and BLH3 with DNA sequences containing one or two binding sites both *in vitro* and in one-hybrid assays in yeast. STM plays a central role in shoot meristem formation and maintenance during vegetative and reproductive development [7,13,26]. Lines with strong loss-of-function alleles of STM form embryonic structures but are defective in the establishment of a population of self-renewing stem cells [7,27]. BLH3 has been identified in a screen for interacting partners with STM and the combined overexpression of these proteins produces early flowering in plants, suggesting the existence of a functional interaction between them [28]. Our results indicate that complex formation between STM and BLH3 produces an increase in binding affinity for sequences containing either one or two binding sites but also that sequence-specific differences exist in the interaction of the complex and individual proteins with DNA.

2. Materials and methods

2.1. Cloning, expression, and purification of recombinant proteins

A 1236-bp BLH3 cDNA fragment encoding amino acids 169–524 was cloned in frame with the maltose binding protein (MBP) in the EcoRI and BamHI sites of plasmid pMAL-c2 (New England Biolabs). A BLH3-R55K mutant was constructed using complementary oligonucleotides (5′-GCGAGAGTTAAACTATGGAAAC-3′ and 5′-GTTT CCATAGTTTAACTCTCGC-3′; introduced mutations underlined) in combination with primers BLH31 (5′-AGCGGATAACAATTTCACA CAGGA-3′) and BLH32 (5′-GTTTTCCCAGTCACGAC-3′), respectively, to amplify partially overlapping N-terminal and C-terminal fragments. The resulting products were mixed in buffer containing 50 mM Tris–HCl (pH 7.2), 10 mM $MgSO_4$, and 0.1 mM DTT, incubated at 95 °C during 5 min, and annealed by allowing the solution to cool to 24 °C in approximately 1 h. After this, 0.5 mM of each dNTP and 5 U of the Klenow fragment of *Escherichia coli* DNA polymerase I were added, and incubation was followed for 1 h at 37 °C. A portion of this reaction was directly used to amplify the chimeric fragments with oligonucleotides BLH31 and BLH32.

The full-length coding sequence of STM was amplified from clone RAFL09-36-A03 [29] and inserted in frame into the BamHI and EcoRI sites of the expression vector pGEX-3X [30]. Amplifications were performed using oligonucleotides STM1 (5′-CCGGGAT CCAGAGTGGTTCCAACAGCA-3′) and STM2 (5′-GGCGAATTCTCAAA GCATGGTGGAGGA-3′). The R55K mutation was introduced using oligonucleotides 5′-ACCAGAGAAAAAAGCACTGGAAG-3′ and 5′-CTT CCAGTGCTTTTTTCTCTGGT-3′ following a similar strategy as that described above. All constructions were checked by DNA sequence analysis.

For expression, *E. coli* cells bearing the corresponding plasmids were grown and induced as described previously [31]. Purifications of recombinant proteins were performed as indicated by the manufacturers of the pMAL-c2 system or as described by Smith and Johnson [30], with modifications described by Palena et al. [31].

2.2. DNA-binding assays

For electrophoretic mobility shift assays (EMSAs), the BS1 oligonucleotide (5′-CAGAATTCTACAGTGGCTGCTGACAGGTGATTGT CCAGGAAGCTTCATC-3′; binding sequence underlined) was amplified from clone E2-8 [17], purified on 10% polyacrylamide, digested with EcoRI and HindIII and labeled with [α-^{32}P]dATP by filling-in the 3′-ends using the Klenow fragment of DNA polymerase. In a similar way, the BS2 double-stranded oligonucleotide with BamHI and EcoRI compatible cohesive ends was cloned into similar sites of pBluescript SK$^-$. From this clone, DNA fragments were obtained by PCR using reverse and universal primers, followed by cleavage with HindIII and XbaI. Binding reactions (20 μl) containing purified proteins, the double-stranded DNA (30 000 c.p.m.), 20 mM HEPES (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM dithiothreitol, 0.5% Triton X-100, 22 ng/μl BSA, 50 ng/μl poly(dI–dC), and 10% glycerol, were incubated for 20 min on ice, supplemented with 2.5% Ficoll and immediately loaded onto a running gel (5% acrylamide, 0.08% bis-acrylamide in 0.5× TBE plus 2.5% glycerol; 1× TBE is 90 mM Tris–borate, pH 8.3, 2 mM EDTA). The gel was run in 0.5× TBE at 30 mA for 1.5 h and dried prior to autoradiography.

2.3. Missing-nucleoside experiments

For the analysis of the nucleosides required for STM and BLH3 binding, either alone or in combination, oligonucleotides BS1 and BS2 were obtained as described above. The fragments were labeled in one of their 3′ ends by incubation with the Klenow fragment of DNA polymerase and [α-^{32}P]dATP prior to cleavage with the second enzyme and were subsequently purified by non-denaturing polyacrylamide gel electrophoresis. The labeled oligonucleotide (15 μl) was then subjected to hydroxyl radical cleavage by the addition of 10.5 μl of 6.6 mM sodium ascorbate, 0.66 mM EDTA (pH 8.0), 0.33 mM $(NH_4)_2Fe(SO_4)_2$ and 0.2% H_2O_2 [32]. Binding of recombinant proteins to this oligonucleotide (200 000 c.p.m.) and separation of free and bound forms by EMSAs were performed as described above. The corresponding fractions were excised from the gel, eluted and analyzed on denaturing polyacrylamide gels.

2.4. One-hybrid analysis in yeast

For the *in vivo* experiments in yeast, a 1236-bp BLH3 cDNA fragment encoding amino acids 169–524 was cloned in frame in the EcoRI and BamHI sites of plasmids pGADT7, pGAD424, pGBKT7, and pGBT9 (CLONTECH). The full-length coding sequence of STM was cloned in the BamHI and XhoI sites of pGADT7 and pGAD424 and in the BamHI and SalI sites of vectors pGBKT7 and pGBT9. The cDNAs of ATH1 and BEL1 (from clones RAFL09-24-O21 and RAFL07-11-J17, respectively) were amplified with oligonucleotides 5′-GCCGAATTC GACAACAACAACAACAAC-3′ and 5′-GGCGAATTCCAAAGTTGGTAA ACACAAT-3′ (for ATH1) or 5′-GGCGAATTCGCAAGAGATCAGTTCT-3′ and 5′-GGCCTGCAG TTTAATCTCAAACAATATCAT-3′ (for BEL1) and cloned in the EcoRI and PstI sites of plasmids pGAD424, pGBT9 and pGBKT7.

To obtain a yeast strain carrying binding sequences inserted into the genome, tandem copies of the BS1 or BS2 oligonucleotides were cloned in front of the *LacZ* reporter gene preceded by the *CYC1* minimal promoter in the pLacZi vector (CLONTECH). Plasmids linearized in their NcoI sites were introduced into the *URA3* locus of the yeast aW303 strain (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*). The presence of the fragment of interest in the genome of transformants was analyzed by PCR with specific oligonucleotides. Plasmids or DNA fragments were introduced into yeast using the standard lithium acetate transformation method [33]. The beta-

galactosidase activity assay was performed as described in Ausubel et al. [34] using *o*-nitrophenylgalactoside as substrate.

2.5. Miscellaneous methods

Total protein was measured as described by Sedmak and Grossberg [35]. For quantitative analysis, radioactive bands were cut from exposed gels and measured by scintillation counting.

3. Results

3.1. The BLH3–STM complex binds DNA with higher affinity than the individual proteins

Overexpression studies in plants have suggested the existence of a functional interaction between the HD proteins STM and BLH3 [28]. Since these two proteins have also been shown to physically interact [23,28], it can be assumed that the phenotypes observed arise from the complex they form and its DNA-binding properties. Binding of these proteins to DNA has not been studied before. Only the STM HD has been analyzed and shown to recognize similar sequences as those bound by other KNOX and BELL HDs [17]. To confirm that the full-length STM protein has similar binding preferences as the isolated HD, we analyzed its binding to an oligonucleotide containing the STM binding site TGACAGGT described before [17] and variants thereof. In view of the conservation in putative DNA contacting amino acids, a similar approach has been undertaken to confirm that BLH3 also prefers sequences with a TGACAGG core, as has been described previously for other BELL proteins [15,17]. Single and double point mutations within the sequence TGACAGGT affect binding of STM and BLH3 proteins in a similar way as has been described for the HDs of STM and ATH1 (another BELL protein), respectively (Supplementary Fig. S1), suggesting that they have similar binding preferences. In the case of BLH3, a slight preference for TGACAGGA over TGACAGGT was evident, as is the case for ATH1 [17]. We used an oligonucleotide carrying the sequence TGACAGGT (here named BS1) to analyze binding when both proteins are present together. As shown in Fig. 1A, both proteins were able to bind this sequence, although higher protein amounts were required for STM to obtain a similar proportion of bound DNA. Binding intensity was nevertheless considerably lower than the one observed with an equimolar amount of the STM HD alone (not shown). When both proteins were included together in the binding assay, the amount of bound DNA increased considerably respective to the binding observed with the single proteins (Fig. 1A). This suggests the existence of a cooperative effect due to the formation of the complex between both proteins. This is supported by the presence of a shifted band with lower mobility than those observed with the individual proteins (Fig. 1A) and is in agreement with the fact that BLH3 and STM have been shown to interact in yeast two-hybrid assays and *in planta* [23,28]. Since the oligonucleotide used for binding contains a single target site, this implicates that specific binding by both proteins is not required for the increase in binding efficiency. A similar result was obtained when an oligonucleotide carrying the sequence TGACAGGA instead of TGACAGGT was used (Supplementary Fig. S2).

We also analyzed binding of BLH3 and STM to an oligonucleotide carrying the sequence TGACTTGACAGGT (BS2), similar to BS1 but with a preceding TGAC target site (Fig. 1B). The results observed were similar to those described for BS1 (i.e. a pronounced increase in binding efficiency was observed when both proteins were

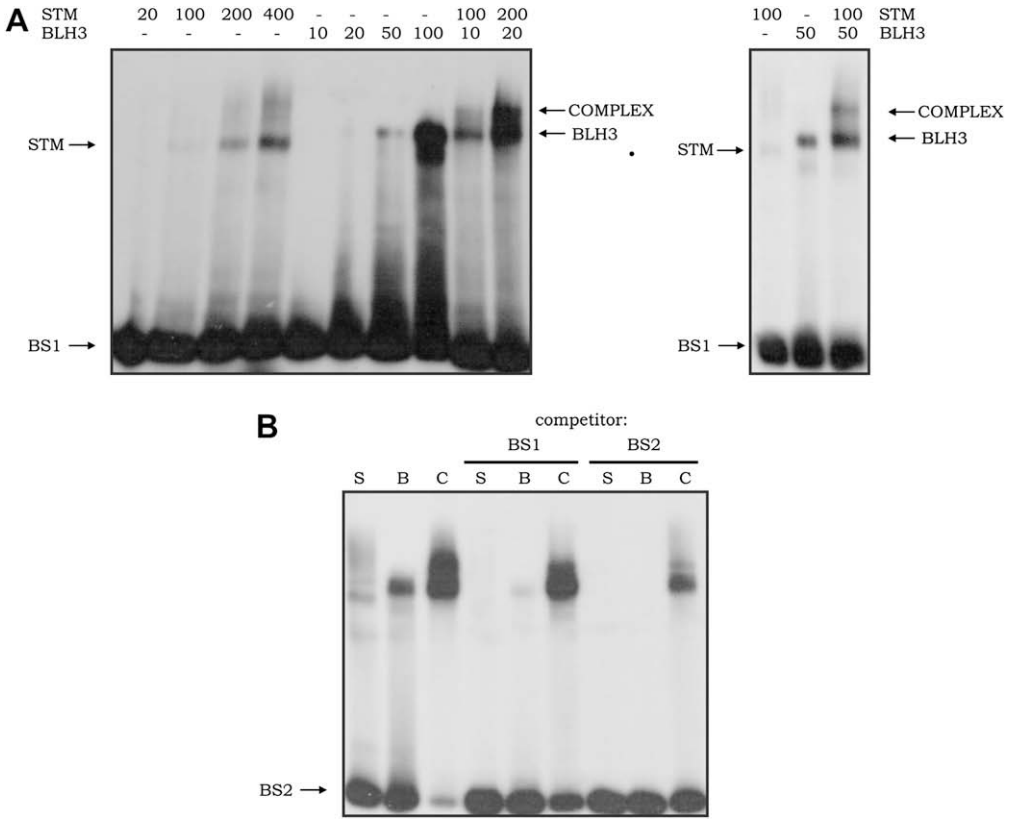

Fig. 1. Formation of the complex between BLH3 and STM increases binding efficiency to DNA molecules with one or two TGAC sites. (A) Binding of the indicated amounts of BLH3 and STM, either individually or in combination, to an oligonucleotide containing a single TGAC core (BS1) was analyzed by an EMSA. (B) Binding to BS2, containing two TGAC cores, was analyzed using 50 ng of BLH3 and/or 200 ng of STM, either in the absence or presence of a 25-fold molar excess of unlabelled BS1 or BS2, as indicated.

included together in the assay). However, BS2 was a more efficient competitor than BS1 (Fig. 1B and results not shown), suggesting that the complex binds with higher affinity to BS2.

To rule out any effect of the fusion proteins on the observed increase in binding efficiency, the effect of including a similar amount of MBP or GST was tested. As shown in Fig. 2, the inclusion of these proteins did not produce any effect on binding by either STM or BLH3. In addition, when only the STM HD, instead of the full-length protein, was used in combination with BLH3, binding by the individual proteins was not affected by the inclusion of the other (Fig. 2). This suggests that STM sequences located outside the HD, which are known to participate in complex formation, are required for increased binding.

3.2. Only one functional homeodomain is required for high affinity DNA binding

The fact that complex formation produces an increase in binding to DNA molecules with a single target site led us to investigate if two functional HDs are necessary for increased binding. For this purpose, we constructed the respective R55K mutants, with a lysine at position 55 of the HD replacing an arginine that has been shown to form an essential interaction with G of the TGAC core in a sunflower KNOX protein [16]. As expected, the mutant STM and BLH3 proteins were unable to bind DNA (Fig. 2). However, inclusion of mutant BLH3 produced a net increase in STM binding to oligonucleotides BS1 and BS2 (Fig. 2). We assume that in this complex the STM HD is the one that establishes specific contacts with DNA. Increased binding was not observed when using wild-type BLH3 and mutant STM. This indicates that, within the complex, specific binding of STM to its target site is necessary and, as judged by the results obtained with BS1, also sufficient for increased binding by the complex.

3.3. Analysis of DNA binding by missing-nucleoside assays

Information about the nucleotide positions that influence binding of STM, BLH3, and their complex to BS1 and BS2 was obtained in missing-nucleoside experiments. In these assays the DNA is treated with the hydroxyl radical to randomly remove nucleosides, and the population of modified molecules that have retained protein-binding capacity is resolved in an EMSA. After elution of the free and bound DNA species from the gel, the residues whose elimination interferes with protein binding can be identified as missing (or under-represented) bands in a sequencing gel electrophoresis. Fig. 3 shows that the pattern obtained with the complex is highly similar as the one obtained with BLH3 alone. For BS1, nucleotides required for binding include GACAGG in the top strand (the one containing the TGAC core) and TCACCTGTC, complementary to GACAGGTGA, in the bottom strand (Fig. 3). This interference pattern is similar to those reported for other KNOX and BELL proteins [16,17]. When BS2 was used, interference of nucleotides from both TGAC sequences was evident: GACTTGACAGG in the top strand and TaCCTgTCAAgTCAA in the bottom strand (lowercase nucleotides showing no or very weak protection). This would indicate that both TGAC sites are contacted by the protein(s) with similar efficiency and establishing similar contacts, considering the repetitive pattern obtained. With STM, slight differences were observed, mainly in the bottom strand of BS1, where a two-nucleotide shift towards the 3′ end was evident (Fig. 3). Nucleotides towards the 3′ end of the top strand also showed weak protection. These results indicate that the complex shows an interference pattern more similar to the one observed with BLH3 alone. If STM is responsible for specific binding within the complex, as suggested by the experiments with mutants described above, this would indicate that binding by STM undergoes a rearrangement within the complex. In any way, the interference patterns observed with BS1 and BS2 suggest that the stronger interaction of the complex with DNA is not related with the establishment of new detectable contacts respective to those observed with the individual proteins.

3.4. In vivo *binding of BLH3, STM and the BLH3–STM complex to single and double target sites*

The interaction of BLH3 and STM with DNA *in vivo* was studied using one-hybrid assays in yeast. For this purpose, two yeast strains were generated, containing tandem copies of oligonucleotides BS1 or BS2 inserted in its genome in front of the yeast *CYC1* minimal promoter fused to the *E. coli LacZ* gene. These strains were transformed with constructs that express BLH3 or STM fused to the GAL4 activation domain. Activation of transcription by either BLH3 or STM fused to the GAL4 activation domain was evident when using BS1, indicating that both proteins are able to interact with this sequence *in vivo* (Fig. 4A). If complex formation increases the

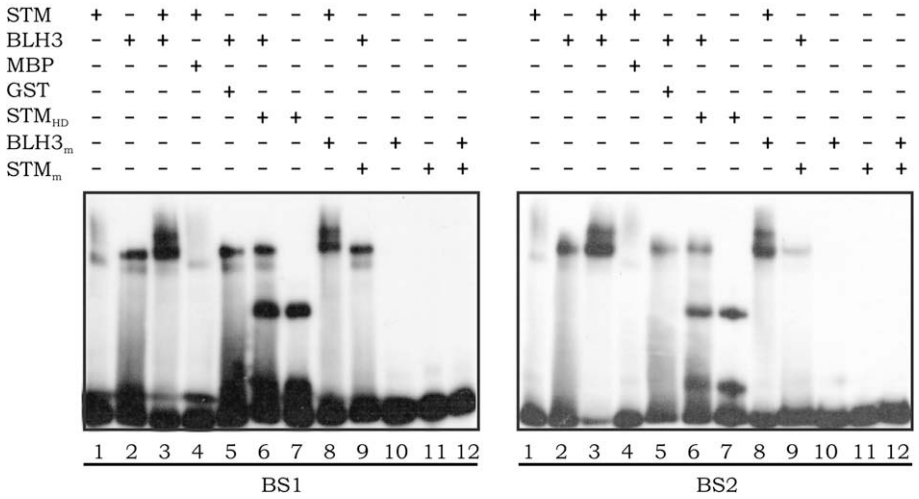

Fig. 2. A mutant form of BLH3 unable to bind DNA increases the DNA-binding efficiency of STM. Binding of wild-type and R55K mutants (BLH3$_m$, STM$_m$) of BLH3 (50 ng) and STM (200 ng), either alone or in different combinations, to oligonucleotides with a single or two TGAC binding sites (left and right panels, respectively) was analyzed by an electrophoretic mobility shift assay. The effect of the respective fusion partners (MBP and GST) and of the STM HD alone (STM$_{HD}$, 50 ng) was also analyzed.

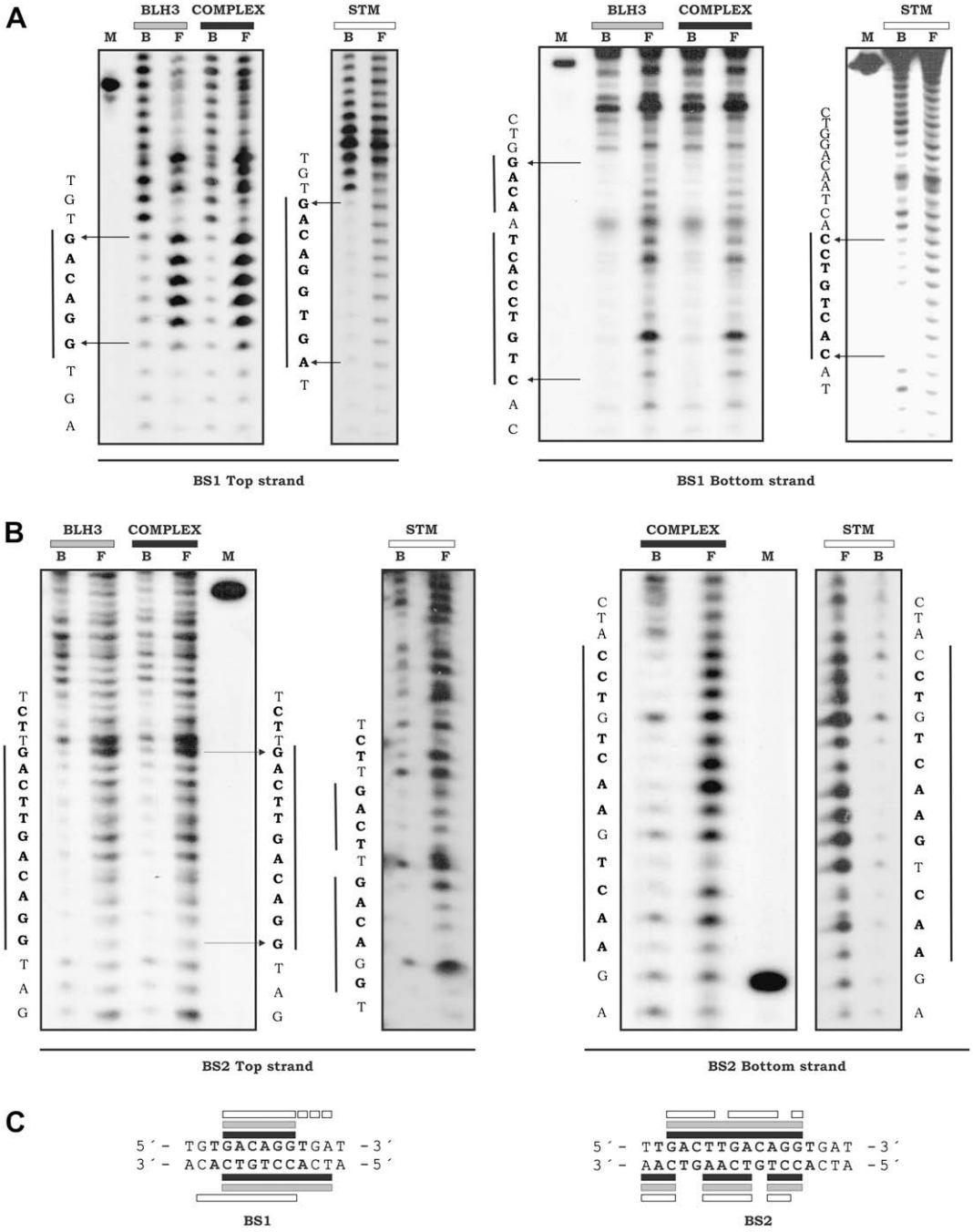

Fig. 3. Hydroxyl radical interference assays of the binding of STM, BLH3, and their complex with DNA. (A) An oligonucleotide containing a single consensus binding site (BS1) was labeled in the 3′-end of either strand (EcoRI or HindIII sites) and subjected to hydroxyl radical attack before binding to STM, BLH3, or a mixture of the two proteins, as indicated. Free (F) and bound (B) DNA were separated and analyzed. An aliquot of the labeled DNA fragment digested with the other restriction enzyme was used to locate the position of the footprint (lane M). Letters beside each panel indicate the DNA sequence (5′-end in the upper part) of the corresponding strand in this region. (B) An interference experiment was also performed with an oligonucleotide containing two TGAC binding sites (BS2). (C) The sequence of the binding sites is shown and the regions of interference in the top and bottom strands are indicated with white, gray, or black rectangles for STM, BLH3, and the complex, respectively.

affinity for DNA, we reasoned that co-expression of the two proteins may produce higher activation values. To analyze this, we co-transformed the yeast strains carrying the respective binding sites with constructs expressing the proteins fused to the GAL4 activation domain and others expressing fusions to the GAL4 DNA-binding domain (the only function of this domain would be to provide a nuclear localization sequence to the protein in yeast). Unexpectedly, co-expression of the two proteins produced beta-galactosidase activity values that were significantly lower than the ones observed with the activation domain fusion alone (Fig. 4A). We hypothesize that this is due to competition for binding by the non-complexed binding domain fusion which, upon binding to DNA, does not activate transcription. This is an indication that binding of individual proteins outweighs binding of the complex to BS1 under these conditions. Indeed, this effect was more evident with BLH3, in agreement with the higher affinity observed with this protein in EMSAs and the higher beta-galactosidase activity values obtained with the respective activation domain fusion in one-

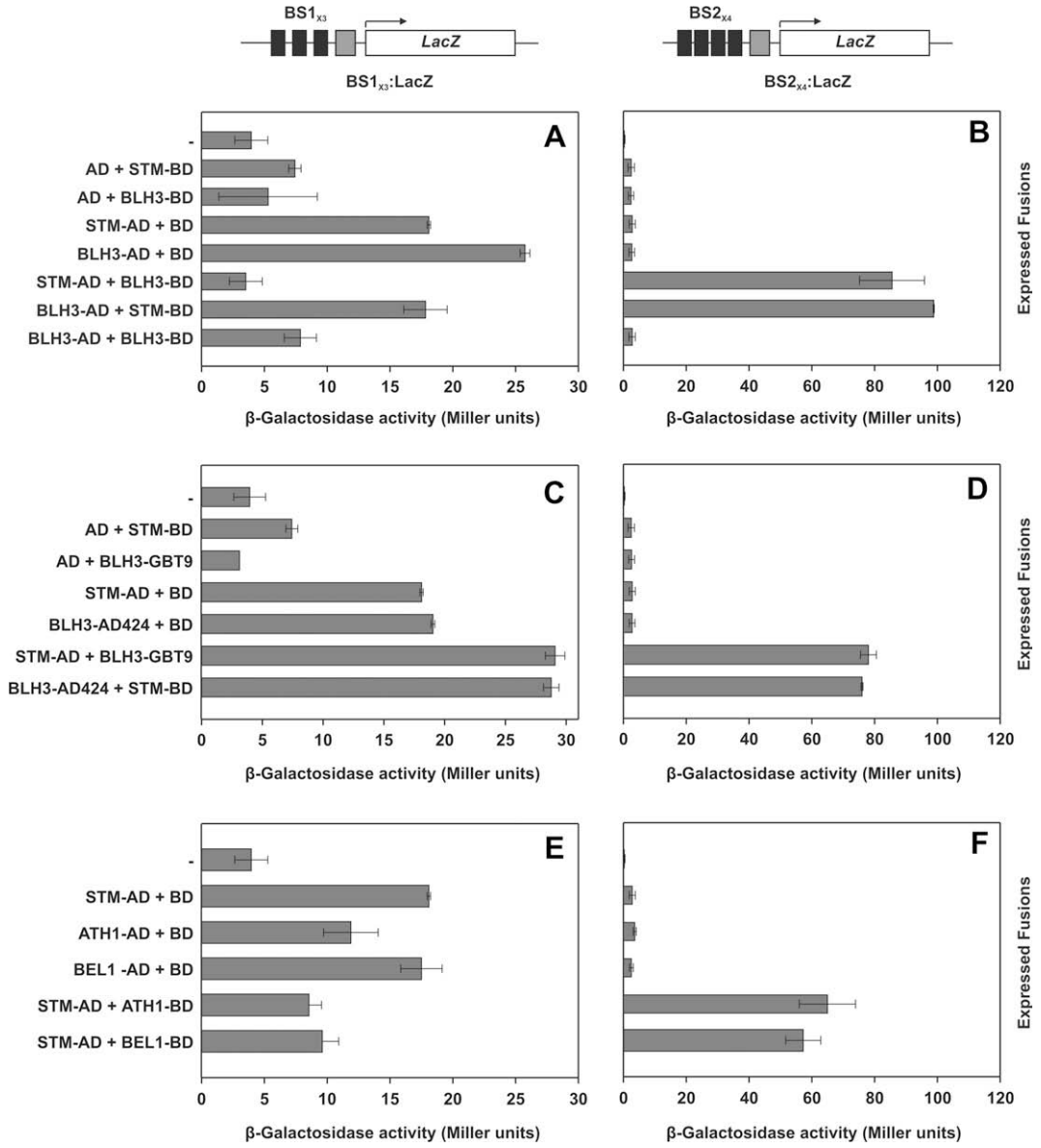

Fig. 4. Binding of BLH3 and STM to different target sequences *in vivo* in yeast. Specific beta-galactosidase activity of yeast cells carrying a fusion of oligonucleotides BS1 (A, C, E) or BS2 (B, D, F) to the *LacZ* gene containing a minimal promoter is shown. (A, B) The strains were previously transformed with different constructs expressing BLH3 or STM fused to the GAL4 activation domain (AD) or DNA-binding domain (BD) from plasmids pGADT7 or pGBKT7, respectively, as indicated. (C, D) Cells were transformed with similar constructs in plasmids pGAD424 or pGBT9. (E, F) Strains were transformed with different constructs expressing ATH1 or BEL1 fused to the GAL4 activation domain or DNA-binding domain from plasmids pGADT7 or pGBKT7. The mean activity value (±SD) of three independent measurements is shown.

hybrid assays. We then produced fusions of BLH3 from plasmids that originate lower expression levels, such as pGAD424 and pGBT9 (CLONTECH) to reduce the competition effect (Fig. 4C). The lower activation values observed with the BLH3 activation domain fusion when using this system indicate that, indeed, lower expression levels were obtained. In turn, co-expression of the STM-AD fusion with BLH3 in pGBT9 (DNA-binding domain fusion), originated higher beta-galactosidase activities than those observed with the STM activation domain fusion alone (Fig. 4C), most likely reflecting the formation of protein complexes with increased binding efficiency respective to the non-complexed forms. Similar expression levels were obtained when co-expressing BLH3 fused to the GAL4 activation domain and STM fused to the GAL4 DNA-binding domain (Fig. 4C), suggesting that, indeed, the observed activation is due to binding by the STM–BLH3 complex.

For BS2, rather low activation levels were observed with individual proteins (Fig. 4B and D). However, co-expression of both STM and BLH3 in different combinations produced considerably high beta-galactosidase activity values (Fig. 4B and D). Formation of the BLH3–STM complex originates then a significant increase in binding to BS2 *in vivo*.

We have also assayed the effect of the expression of two different BELL proteins, ATH1 and BEL1, on beta-galactosidase activities of yeast cells carrying the BS1 and BS2-LacZ reporters (Fig. 4E and F). The results were essentially the same as those observed with BLH3, suggesting that ATH1 and BEL1 are also able to interact with BS1 and to produce complexes with STM that have similar binding properties as those observed with BLH3.

The fact that competition for binding to DNA between BLH3 and STM was observed with BS1 but not with BS2 suggests that

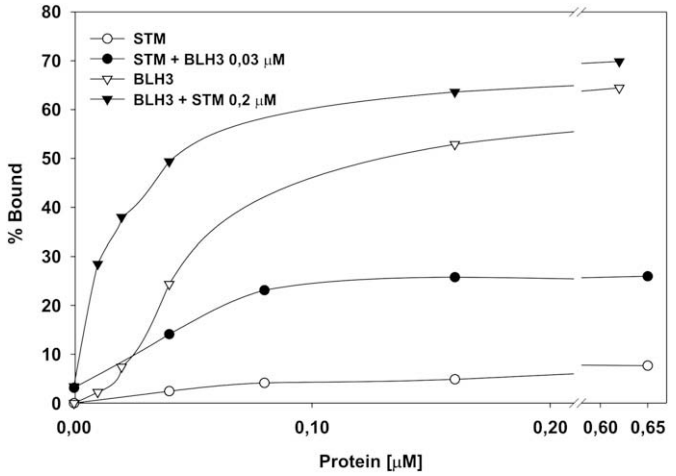

Fig. 5. Binding of different amounts of STM and BLH3, either alone or in the presence of a fixed amount of the other protein, to BS1. Binding of varying amounts (0.01–0.64 μM) of STM (circles) or BLH3 (triangles) to an oligonucleotide containing a single TGAC core (BS1) was analyzed by an EMSA either in the absence (white symbols) or presence (black symbols) of the indicated amount of the other protein. The amount of free and bound DNA was measured by scintillation counting after cutting the respective regions of the gel.

complex formation has a more dramatic effect on binding efficiency to BS2 under the conditions of the yeast one-hybrid assay. These data seem contradictory with those obtained in *in vitro* assays, where increased binding was observed upon complex formation with both oligonucleotides. To further investigate the DNA-binding characteristics of BLH3, STM, and the complex between them, binding of different amounts of BLH3 or STM to oligonucleotide BS1 was analyzed in the absence or presence of a fixed amount of the other protein (Fig. 5). For STM, relatively low amounts of binding were observed. This binding was considerably increased by the inclusion of a fixed amount of BLH3 at all concentrations tested. Binding by the complex reaches a plateau presumably when all BLH3 is complexed with STM, so that further increasing the amount of STM reflects binding of this protein alone (Fig. 5). For BLH3, binding was very inefficient at low protein concentrations (below 0.02 μM) and a pronounced increase was observed in the presence of STM. However, DNA binding by BLH3 increased pronouncedly at higher protein concentrations, reaching values similar to those observed in the presence of STM (Fig. 5). Considering that the interaction of BLH3 with itself has been previously documented [23], this increased affinity of BLH3 for DNA may be due to the formation of a complex between two BLH3 molecules. Under these conditions, the effect of complex formation on binding is negligible. The data obtained *in vivo* may reflect this behavior. Our data suggest that complex formation between the BELL protein BLH3 and the KNOX protein STM increases binding affinity for DNA in a manner that depends on the relative concentrations of the two proteins and on the sequence of the DNA-binding site.

4. Discussion

The establishment of interactions between different transcription factors is thought to be essential to increase the versatility of gene regulatory systems. Among HD proteins, known examples of protein–protein interactions include complexes formed by mammalian Pbx1 with either Hox or MEIS HD proteins [36–40]. In these cases, the two HDs present in the complex interact with adjacent binding sites located in tandem. The complex formed by Pbx1 and MEIS proteins has been presented as a hallmark of the interactions established by plant KNOX and BELL HD transcription factors, since they also belong to the TALE superfamily. In addition, in both cases protein–protein interactions were shown to require conserved domains present in the N-terminal portion of the respective proteins [15,18,37,38]. In the Pbx1-MEIS complex, the requirement of the sequence TGATTGAC, composed of the binding sites for Pbx1 and MEIS proteins, respectively, indicates that both HDs are necessary for the interaction with DNA and establishes the spatial arrangement of the HDs in the complex (i.e. Pbx1 binds to the left half of the sequence while MEIS proteins bind to the right half). In the case of plant TALE proteins, although many studies of protein–protein interactions exist, the effect of complex formation on DNA binding has been reported only for the maize proteins KN1 and KIP and the potato proteins POTH1 and StBEL5. With the maize proteins, increased affinity for a DNA molecule containing a single TGAC binding site was observed *in vitro* upon complex formation [15]. Similar studies with the potato proteins showed a requirement of a sequence containing two TGAC motifs (TGACTTGAC) since mutations in the central nucleotides of any of these motifs abolished binding [25].

In the present study, we have analyzed the DNA-binding properties of the complex formed by the KNOX protein STM and the BELL protein BLH3, that had been shown previously to functionally and physically interact *in planta* [28]. We have observed that the complex formed by these proteins does not require the presence of two TGAC motifs for efficient binding *in vitro*, suggesting that only one of the HDs makes specific contacts with DNA in the complex. This observation is reinforced by the fact that BLH3 with a mutation in the HD that renders this protein inactive for DNA binding also increases the affinity of wild-type STM, suggesting that STM has a more relevant role in the interaction of the complex with DNA. The mechanism by which an increase in affinity is produced may be related to the induction of conformational changes that relieve restrictions of the STM HD for binding to DNA. In this sense, we have observed that the isolated STM HD binds DNA with considerably higher efficiency than the full-length protein.

In vivo DNA-binding assays using the yeast one-hybrid system showed that the STM–BLH3 complex is able to interact with DNA containing a single TGAC site (BS1). An important difference, however, is that the increase in reporter gene expression originated by co-expression of both proteins was considerably higher with the construct containing two tandem sites (BS2). This may implicate that interaction of both HDs with DNA represents an advantage *in vivo*, perhaps due to the presence of internal factors that do not exist *in vitro*, and suggests that DNA sequence-specific differences exist in the interaction properties of individual proteins and the complex with DNA. Accordingly, it can be hypothesized that these species may differently interact with different sets of genes according to the sequences they contain in their promoters. Testing of this hypothesis will require the study of STM and BLH3 target genes in plants expressing different amounts of the respective transcription factors.

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Appendix. Supplementary data

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