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The *Arabidopsis* class I TCP transcription factor AtTCP11 is a developmental regulator with distinct DNA-binding properties due to the presence of a threonine residue at position 15 of the TCP domain

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The TCP domain is a DNA-binding domain present in plant transcription factors that modulate different processes. In the present study, we show that *Arabidopsis* class I TCP proteins are able to interact with a dyad-symmetric sequence composed of two GTGGG half-sites. TCP20 establishes symmetric interactions with the 5′ half of each strand, whereas TCP11 interacts mainly with the 3′ half. SELEX (systematic evolution of ligands by exponential enrichment) experiments with TCP15 and TCP20 indicated that these proteins have similar, although not identical, DNA-binding preferences and are able to interact with non-palindromic binding sites of the type GTGGGNCCNN. TCP11 shows a different DNA-binding specificity, with a preference for the sequence GTGGGCCNNN. The distinct DNA-binding properties of TCP11 are due to the presence of a threonine residue at position 15 of the TCP domain, a position that is occupied by an arginine residue in most TCP proteins. TCP11 also forms heterodimers with TCP15 that have increased DNA-binding efficiency. The expression in plants of a repressor form of TCP11 demonstrated that this protein is a developmental regulator that influences the growth of leaves, stems and petioles, and pollen development. The results suggest that changes in DNA-binding preferences may be one of the mechanisms through which class I TCP proteins achieve functional specificity.

Key words: *Arabidopsis thaliana*, DNA-binding specificity, EAR domain, plant development, systematic evolution of ligands by exponential enrichment (SELEX), TCP domain.

INTRODUCTION

The TCP domain is a DNA-binding domain present in a family of plant transcription factors. Its name comes from the first three characterized members of the family: TB1 (teosinte branched 1) from maize, CYC (cycloidea) from *Antirrhinum* and PCF from rice [1,2]. The TCP domain consists of a basic N-terminal region followed by two putative α-helices connected by a loop, which gives this domain a resemblance with the bHLH (basic helix–loop–helix) domain present in a variety of eukaryotic transcription factors. On the basis of sequence homology within the TCP domain, TCP transcription factors can be divided into two classes, named I and II [2]. Studies using mutants and overexpressing plants have shown that class II members regulate growth in different organs, mainly through the inhibition of cell proliferation or growth. CYC, for example, establishes the asymmetric development of *Antirrhinum* flowers through the inhibition of stamen and petal growth in the dorsal region of the flower [3]. TB1 and its *Arabidopsis* homologue BRANCHED1 inhibit axillary bud outgrowth, thus regulating branching [4,5]. CIN (cincinnata) from *Antirrhinum* and several class II members from *Arabidopsis* regulate leaf and petal development, also through the regulation of cell proliferation [6–9].

Comparatively much less is known about the function of class I TCP transcription factors. Thirteen out of 24 TCP family members from *Arabidopsis* belong to this class [2]. Among them, TCP16 is involved in pollen development [10], TCP14 participates in regulating embryonic growth potential [11], TCP20 regulates several aspects of plant development [12], and TCP21 (also called CHE) is a component of the circadian clock [13]. Class I members have also been implicated in the stimulation of cell proliferation, since two rice proteins from this class bind to elements required for expression of the *PCNA* (proliferating-cell nuclear antigen) gene [14]. From this, and the fact that *Arabidopsis* TCP20 binds to a cyclin B and ribosomal-protein gene promoters, Li et al. [15] proposed a model in which the antagonistic activity on cell growth and proliferation of class I and class II members regulate organ growth and shape.

In vitro selection experiments indicated that rice PCF2 (class I) prefers the binding sequence GGNCCCAC (GTGGGNCC in the complementary strand), whereas PCF5 (class II) prefers GGGNCCAC [16]. Thus proteins from different classes show distinct, although very similar, DNA-binding specificities. Whether these consensus sequences apply to all members of the respective classes or there are variations in DNA-binding specificities is not known. The class II protein AtTCP4 (*Arabidopsis thaliana* TCP4), for example, selects the sequence GGGACCAC, denoting a higher preference for A at the fourth position than PCF5 [17]. In addition, TCP20 binds *in vitro* and *in vivo* to promoter regions containing sequences defined as site II elements (TGGGCY) or related sequences present in ribosomal protein and respiratory chain component genes [15,18–20].

To gain insight into the DNA-binding properties and functions of class I TCP transcription factors from *Arabidopsis*, we have examined the interaction of four of these factors with DNA. Our results suggest that most class I TCP proteins are able to interact with palindromic and non-palindromic DNA sequences and possess similar, although not identical, DNA-binding preferences.

Abbreviations used: AtTCP, *Arabidopsis thaliana* TCP; bHLH, basic helix–loop–helix; CaMV, cauliflower mosaic virus; CYC, cycloidea; EMSA, electrophoretic mobility-shift assay; GUS, β-glucuronidase; MBP, maltose-binding protein; SELEX, systematic evolution of ligands by exponential enrichment; TB1, teosinte branched 1; UTR, untranslated region; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

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In particular, TCP11 binds to DNA with distinct specificity due to the presence of a threonine residue, instead of arginine, at position 15 of the TCP domain. In addition, heterodimer formation between TCP11 and TCP15 produces species with increased binding efficiency that are able to interact with DNA sequences bound only poorly by the respective homodimers. TCP11 functions as a developmental regulator, since plants expressing a dominant-negative form of this protein show characteristic abnormalities in organ growth and pollen production. The results suggest that class I TCP proteins may have acquired functional specificity through evolutionary changes that modified their DNA-binding properties.

EXPERIMENTAL

Cloning, expression and purification of recombinant proteins

The complete coding regions of TCP11 and TCP21 were cloned in-frame with MBP (maltose-binding protein) in the BamHI and SalI sites of plasmid pMAL-c2 (New England Biolabs). TCP15 was cloned in the EcoRI and SalI sites, and TCP20 was cloned in the BamHI and HindIII sites of the same plasmid. TCP11 and TCP20 mutants were constructed by overlap extension mutagenesis [21] using complementary oligonucleotides with the desired mutations (see Supplementary Table S1 at http://www.BiochemJ.org/bj/435/bj4350143add.htm). For the analysis of mutations in TCP20, a shorter form of the protein (amino acids 1–157) was used. All constructs were checked by DNA sequencing.

For expression, *Escherichia coli* cells bearing the corresponding plasmids were grown and induced as described previously [22]. Purification of recombinant proteins was performed as indicated by the manufacturers of the pMAL-c2 system.

DNA-binding assays

For EMSAs (electrophoretic mobility-shift assays), aliquots of purified proteins were incubated with labelled double-stranded DNA generated by hybridization of complementary synthetic oligonucleotides (Supplementary Table S1) as described previously [23]. DNA-binding assays were performed with the proteins fused to MBP. Controls made with proteins obtained after cleavage with Factor Xa indicated that the MBP moiety did not affect the behaviour of the recombinant proteins.

Binding site selection by SELEX (systematic evolution of ligands by exponential enrichment)

To select DNA molecules specifically bound by TCP11, TCP15 and TCP20, the random oligonucleotide selection technique [24] was applied, using procedures described by Blackwell and Weintraub [25]. A labelled 52-mer double-stranded oligonucleotide containing a 10 bp central core with random and fixed positions (5′-NNGGNNCCNN-3′) was incubated with purified proteins as described above. Bound DNA molecules were separated by electrophoresis and eluted from gel slices with 0.5 ml of 0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA and 0.1 % SDS. The selected DNA molecules were amplified using oligonucleotides R1 and R2 (Supplementary Table S1). Amplification reactions were performed using 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C. After purification through polyacrylamide gels, the amplified molecules were subjected to new cycles of binding, elution and amplification. Enrichment in sequences bound specifically by the different proteins was monitored by binding and competition analysis in EMSAs. After selection, the population of oligonucleotides was cloned into the pCR 2.1-TOPO vector (Invitrogen) and individual clones were sequenced. For TCP11, a selection experiment using an oligonucleotide mixture with 12 random positions was performed in a similar way as described above.

Footprinting analysis

For the analysis of hydroxy radical footprinting patterns [26], double-stranded oligonucleotides containing the respective binding sites with BamHI- and EcoRI-compatible cohesive ends were 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. The fragments were labelled at 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 PAGE. Binding of proteins to these oligonucleotides (200 000 c.p.m.) was performed as described for EMSAs in 15 μl of 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 22 ng/μl BSA, 10 ng/μl poly(dI-dC)·(dI-dC) and 2 μg of protein. After binding, bound and free oligonucleotides were subjected to hydroxy 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, and separated by EMSA. The corresponding fractions were excised from the gel, eluted and analysed on denaturing polyacrylamide gels.

One-hybrid analysis in yeast

To obtain a yeast strain carrying binding sequences inserted into the genome, tandem copies of the corresponding oligonucleotides were cloned in front of the *LacZ* reporter gene preceded by the *CYC1* minimal promoter in the pLacZi vector (Clontech). Plasmids linearized at their NcoI sites were introduced into the *URA3* locus of the yeast strain aW303 (*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 analysed by PCR with specific oligonucleotides. To express fusions of the TCP proteins with the GAL4 activation domain, fragments encoding full-length TCP15 and TCP21 were cloned in-frame in the EcoRI and XhoI sites of plasmid pGADT7 (Clontech). TCP20 was cloned in the BamHI and SalI sites of the same plasmid. Plasmids or DNA fragments were introduced into yeast using the standard lithium acetate transformation method [27]. The β-galactosidase activity assay was performed as described in [28] using *o*-nitrophenylgalactoside as the substrate.

Plant material and growth conditions

A. thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds. SALK insertional lines were obtained from the *Arabidopsis* Biological Resource Center. Plants were grown in soil in a growth chamber at 22–24 °C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approx. 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Alternatively, plants were grown in Petri dishes containing 0.5× Murashige and Skoog medium and 0.8 % agar.

Gene cloning and plant transformation

For the expression of TCP11 fused to the EAR repressor domain [29], the full-length TCP11 coding sequence was amplified with primers T11EAR-F and T11EAR-R (Supplementary Table S1),

digested with SalI and ligated with a double-stranded synthetic oligonucleotide with a compatible end encoding the EAR domain. The fusion was amplified with primers T11EAR-F and EAR-XK and cloned in the binary vector pBI121 under the control of the 35S CaMV (cauliflower mosaic virus) promoter. For the fusion of the *AtTCP11* upstream sequence to *gus* (*β*-glucuronidase gene), a fragment spanning nucleotides −999 to −1 with respect to the ATG start codon was obtained by PCR amplification of *Arabidopsis* genomic DNA using primers pTCP11-F and pTCP11-R (Supplementary Table S1). The resulting fragments were cloned in vector pBI101.3 in front of the *GUS* coding region.

All constructs were checked by DNA sequencing and introduced into *Agrobacterium tumefaciens* strain LB4404. *Arabidopsis* plants were transformed by the floral dip procedure [30]. Transformed plants were selected on the basis of kanamycin resistance and genotyping. Twenty independent lines for each construct were further reproduced. Homozygous T3 and T4 plants were used to analyse *gus* expression. In the case of TCP11–EAR-expressing plants, transcript levels were determined by Northern blotting, and four independent lines with relatively high transcript levels were kept for further analysis.

GUS assays

GUS activity of transformed plants was analysed by histochemical staining using the chromogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid) as described by Hull and Devic [31]. Whole plants or separated organs were immersed in a 1 mM X-gluc solution in 100 mM sodium phosphate, pH 7.0, and 0.1 % Triton X-100 and, after applying a vacuum for 5 min, they were incubated at 37 °C for 12 h. Tissues were cleared by immersion in 70 % ethanol.

Miscellaneous methods

Total protein was measured as described by Sedmak and Grossberg [32]. RNA was prepared using the method of Carpenter and Simon [33]. For quantitative analysis, the amount of radioactivity in gels was measured using phosphor storage technology with a Typhoon scanner (GE Healthcare).

RESULTS

Class I TCP proteins establish symmetrical contacts with DNA

The resemblance of the TCP domain to the bHLH domain suggests that a TCP dimer is the binding unit that interacts with DNA. The formation of dimers has been documented for different class I and class II proteins from rice [16] and for the class II protein TCP4 from *Arabidopsis* [34]. Accordingly, it can be proposed that a homodimer of a TCP protein must be able to interact with a dyad-symmetric sequence conformed by two half-sites, each recognized by one of the monomers. To validate this proposition, we analysed the binding of four *A. thaliana* class I TCP proteins with an oligonucleotide containing a dyad-symmetric sequence formed by the consensus sequences preferred by PCF2 (GGNCCCAC) and PCF5 (GTGGNCCC) from rice [16]. The nucleotide positions of this sequence, hereafter named BS, were numbered from 1 to 10 (Figure 1A). TCP11, TCP15, TCP20 and TCP21 were able to interact with BS in a specific manner. Analysis of binding of these proteins to oligonucleotides containing variants of BS showed that, except for TCP11, single changes at positions 3, 4 or 5 produce a decrease in binding, whereas changes at positions 1 or 2 are almost ineffective (Figure 1A; it should be kept in mind that positions 1–5 are equivalent to positions 10–6 respectively, since this is a dyad-symmetric sequence). The relative importance of positions 3–5 was different, with the order 4>3>5. Double mutations in symmetrical positions (e.g. 5 and 6, and 3 and 8) almost completely abolished binding. In the case of double substitutions at positions 2 and 9, or 1 and 10, a decrease in binding was observed (Figure 1A), indicating that the proteins must recognize at least one side of the palindromic sequence for efficient binding. Of the proteins tested, TCP15 showed more relaxed specificity since it was less affected by single nucleotide changes than were the other two proteins. Binding of TCP15, TCP20 and TCP21 to BS was also observed *in vivo* in one-hybrid assays in yeast and, as observed *in vitro*, it was affected by a change at position 3 of the binding sequence (Figure 1B).

TCP11 binding to DNA was affected in a similar way with the exception of modifications at position 3 (BS3), which did not influence the interaction (Figure 1A). Analysis of binding of different protein amounts of TCP11 and TCP20 to BS and BS3 indicated that, in fact, TCP11 displays similar affinity for both sequences, whereas binding of TCP20 is severely affected by a change at position 3 (Figure 1C).

The interaction of TCP20 with BS was analysed further using hydroxy radical footprinting assays [26]. In these assays, the free and bound DNAs are subjected to attack by the hydroxy radical, which produces breaks in DNA at sites that are not in close contact with the protein. The footprinting patterns obtained indicate that TCP20 establishes symmetrical interactions with BS (Figure 2A). Contacts are established with the entire 10 bp sequence, but are stronger with the 5′ region of each strand containing the sequence GTGGG. In addition, a relatively stronger contact is established with the adenine nucleotide present in the 3′ side of each strand (Figure 2A). The results agree with a model in which each monomer of the dimer is able to interact in a similar way with one half-site of the palindromic binding site.

The hydroxy radical footprinting pattern generated by TCP11 upon interaction with BS was also symmetrical, but enhanced protection was observed in the 3′ side of each strand (Figure 2B). The footprinting pattern generated after binding to BS3 was essentially similar. A subtle diference was that the thymine nucleotide located at position 2 of the top strand was slightly more protected than its surrounding nucleotides (Figure 2B).

DNA-binding preferences of class I TCP proteins analysed by SELEX experiments

Single changes at positions 1, 2 or 5 of BS produce only mild effects on DNA binding by class I TCP proteins, indicating that nucleotide changes are allowed at these positions. We then decided to perform SELEX experiments to analyse the nucleotide preferences of TCP20, TCP15 and TCP11. For TCP20 and TCP15, a population of oligonucleotides containing the sequence NNGGNNCCNN (with fixed nucleotides at positions 3, 4, 7 and 8, which are important for binding) was used as the starting material. Both proteins were able to select specific sequences from this population, as indicated by the fact that binding efficiency increased upon progression of the experiment (Figure 3A). After three rounds of selection, when no further increase in binding efficiency was observed, the population of bound oligonucleotides was cloned and the sequences of 20 clones from each experiment were analysed. The results indicated that both proteins select a sequence of the type GTGGGNCCNN (NNGGNCCCAC if the complementary strand is considered; selected nucleotides underlined), with almost 100 % efficiency (Figure 3B). It is noteworthy that, for both proteins, efficient selection was observed in the three variable positions of the same half of the palindromic

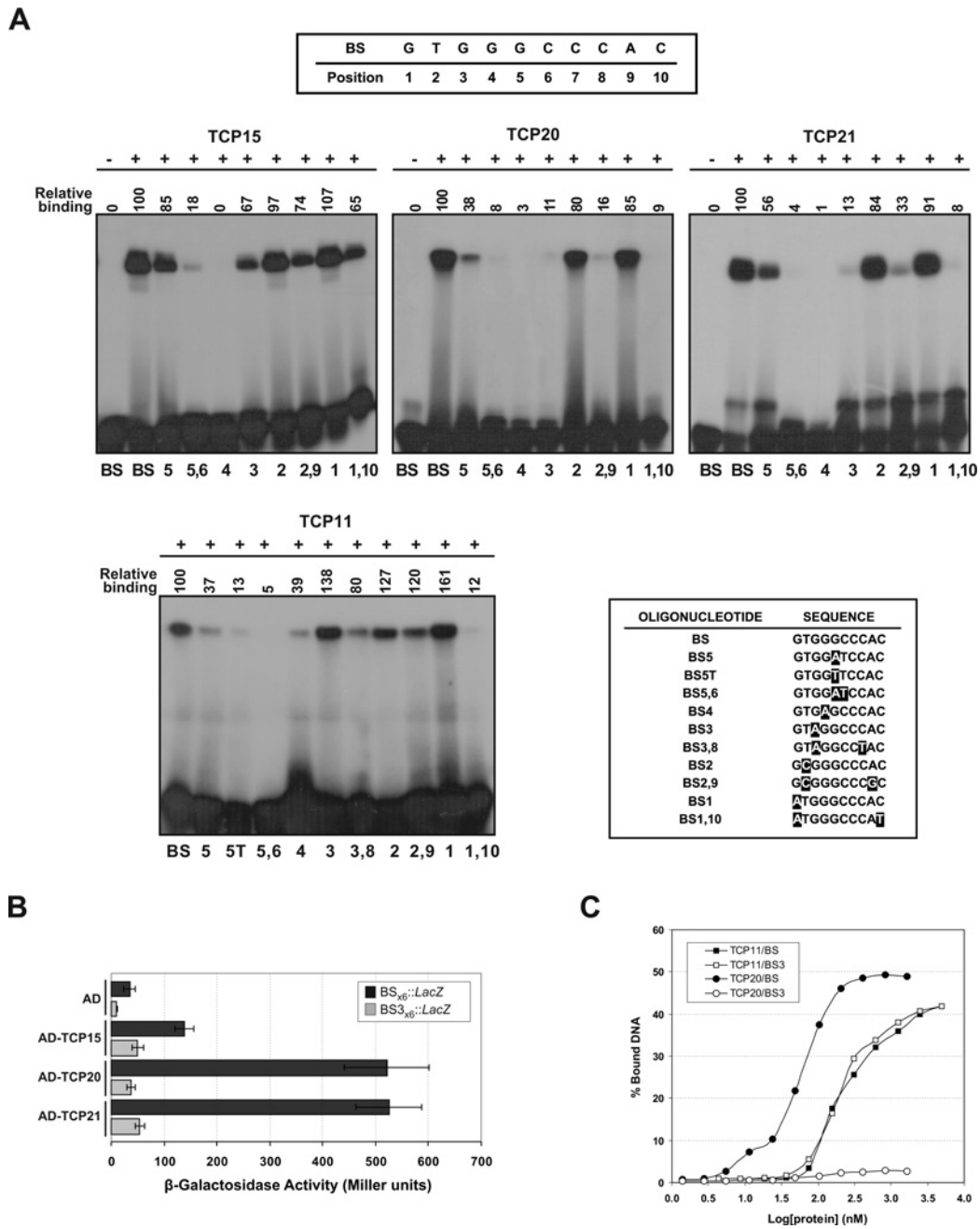

Figure 1 Class I TCP proteins interact with a dyad-symmetric sequence composed of two GTGGG sites

(**A**) Binding of TCP15, TCP20, TCP21 and TCP11 to oligonucleotides containing the dyad-symmetric sequence (BS) and variants with single or double changes at defined positions was analysed using EMSAs. The sequence of BS is shown in the upper panel. Positions are numbered from 1 to 10. Note that positions located at a similar distance from the axis of symmetry (between positions 5 and 6) are equivalent. The table in the lower panel describes the sequences of the different oligonucleotides. Numbers refer to the positions of BS that were modified. In the upper part of each gel image, the amount of binding, relative to BS, is shown. (**B**) Binding of class I TCP proteins to BS (black) and BS3 (grey) *in vivo* in yeast. Specific β-galactosidase activity of yeast cells carrying a fusion of oligonucleotides BS or BS3 (six copies) to the *LacZ* gene containing a minimal promoter is shown. The strains were transformed with constructs expressing the corresponding TCP proteins fused to the GAL4 activation domain (AD). Cells transformed with a construct that expresses only the activation domain were used as controls. The mean activity value $\pm$ S.D. of three independent measurements is shown. (**C**) Binding curves of TCP11 (squares) and TCP20 (circles) with BS (black symbols) and BS3 (white symbols), as analysed by EMSAs. The amounts of free and bound DNA were calculated from signal intensities obtained with phosphor storage technology.

sequence, whereas poor selection was obtained in the other half. This is consistent with the consensus sequence obtained for rice PCF2 [16] and explains why double changes at positions 1 and 10, 2 and 9, or 5 and 6 markedly affect binding, whereas single changes are less effective. According to a model of DNA recognition by a TCP dimer, this would imply that one of the monomers establishes more specific contacts with DNA. The results also indicate that the interactions established with the different positions of the target site are not independent, since almost every time that a nucleotide is selected at position 5, selection is also observed at positions 1 and 2, but not at 9 and 10. The more logical explanation for this behaviour is that class I TCP

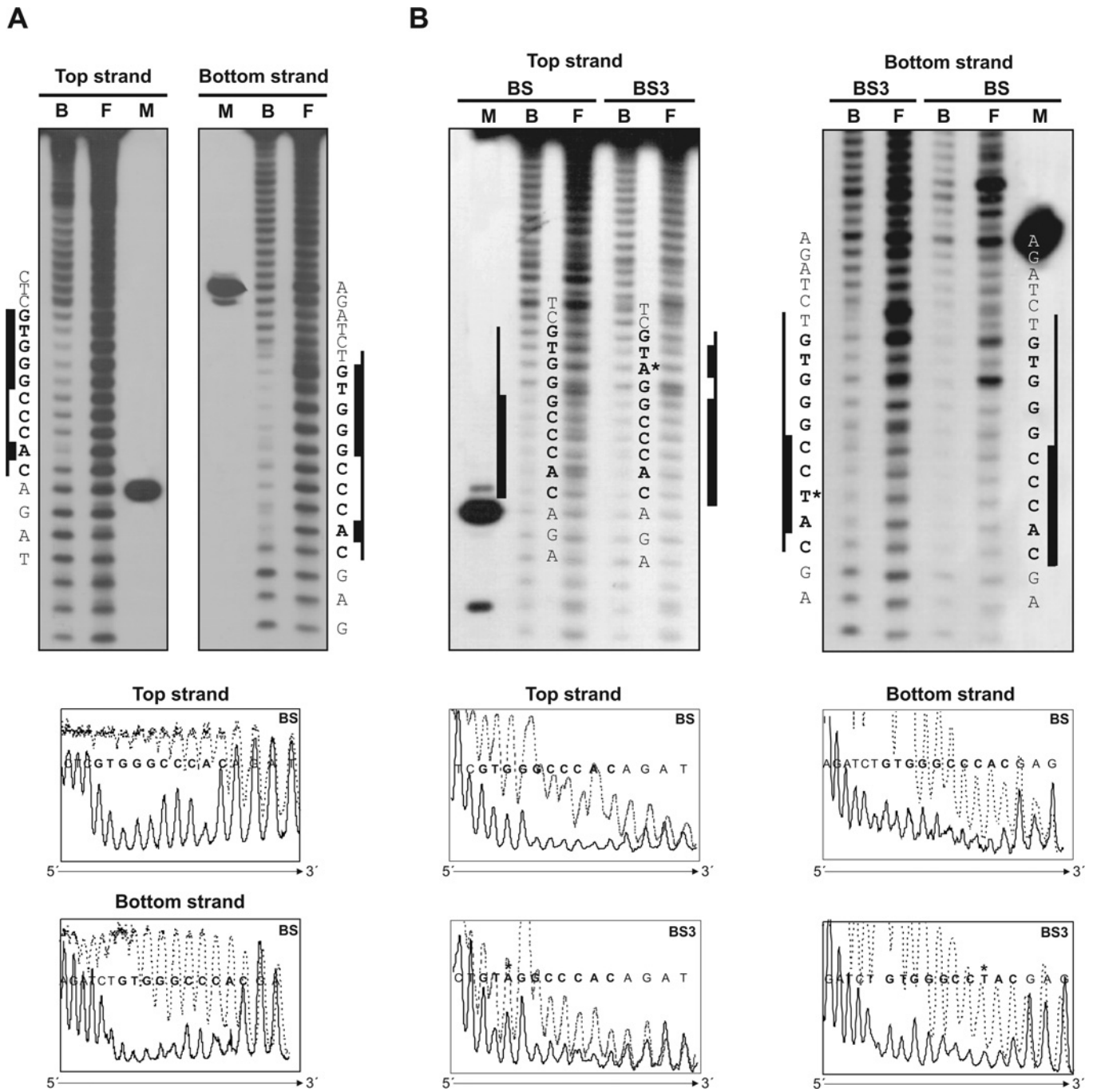

Figure 2 Hydroxy radical footprinting pattern of the complexes of TCP20 and TCP11 with BS

TCP20 (**A**) or TCP11 (**B**) were bound to BS labelled in the 3′ end of one strand (either the top or bottom strand, named arbitrarily). After binding, DNA was subjected to hydroxy radical attack. Free (F) and bound (B) DNA were separated and analysed. A portion of the same fragment digested with defined restriction enzymes was used as a standard to calculate the position of the footprint (M). Letters beside each panel indicate the DNA sequence (5′ end in the upper part) of the corresponding strand in this region. The vertical bars represent the relative intensity of the footprint in each region. Densitometric scans of the lanes corresponding to free (broken line) and bound (continuous line) DNA are shown. The footprint of TCP11 upon binding to BS3 is also shown in (**B**). The asterisks indicate the modified nucleotides at position 3 of the binding sequence.

proteins are able to establish asymmetric interactions with DNA and that, rather than the number of specific interactions established by the dimer, efficient binding by one of the monomers dictates binding behaviour. Binding to a palindromic DNA sequence would then reflect a special case among the different sequences that class I TCP proteins can recognize.

Another outcome of the SELEX results is that positions where a low level of selection was observed (6, 9 and 10) showed different nucleotide frequencies for TCP15 and TCP20 (Figure 3B), probably indicating the existence of subtle differences in binding preferences between these two proteins. These differences may originate a preferential interaction of certain class I proteins with specific target sites *in vivo*, thus providing a basis for functional specificity.

The results of the SELEX experiments were confirmed using synthetic oligonucleotides with the sequences selected by each

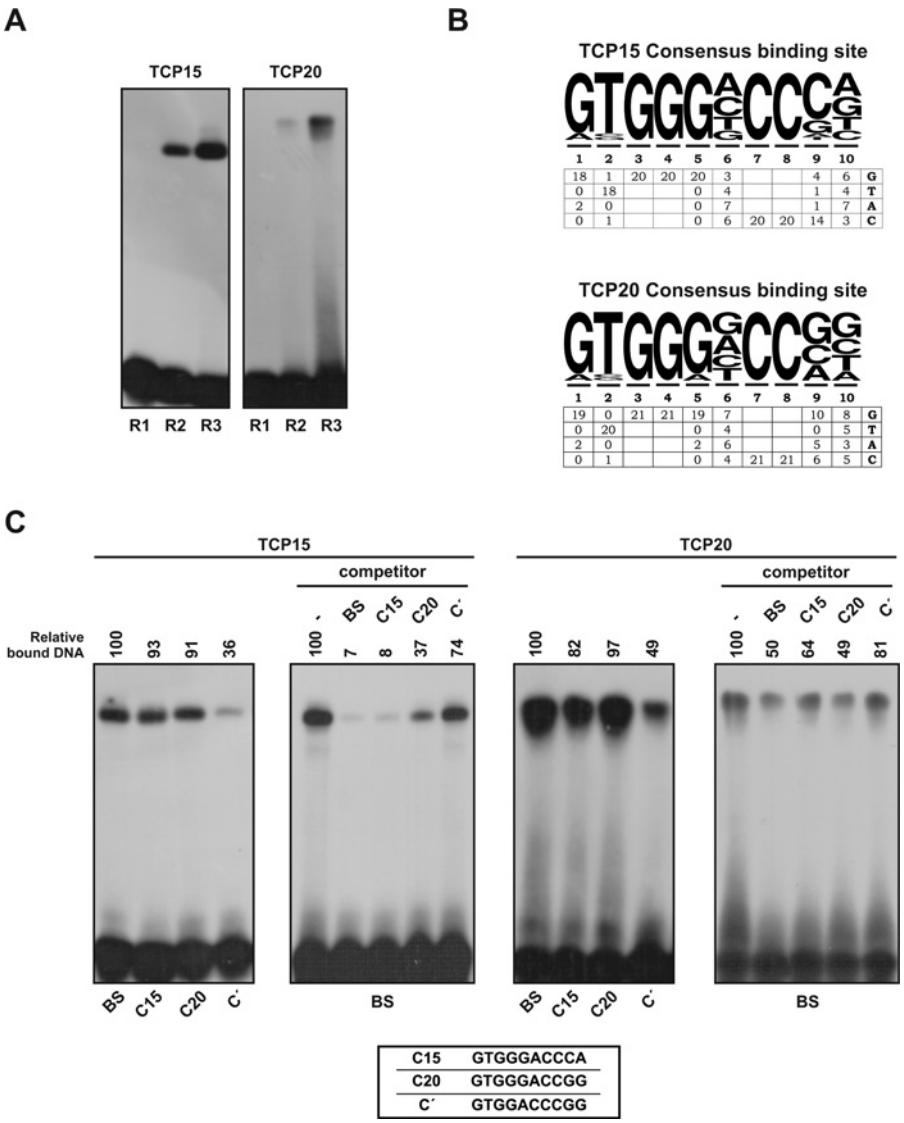

Figure 3 Binding-site preferences of TCP15 and TCP20

(**A**) Binding of TCP15 and TCP20 to oligonucleotide populations obtained after different rounds of selection (R1–R3) was analysed in an EMSA. (**B**) Nucleotide frequencies at different positions in the population of oligonucleotides selected by TCP15 and TCP20 are shown in sequence logos, below which the number of sequenced clones with each nucleotide is shown. (**C**) Binding of TCP15 and TCP20 to synthetic oligonucleotides containing the sequences selected by TCP15 (C15; GTGGGACCCA), TCP20 (C20; GTGGGACCGG) and a similar oligonucleotide with an exchange of G:C and A:T pairs at positions 5 and 6 (C′; GTGGACCCGG). Binding to BS is shown as a reference. In competition experiments, binding to BS was performed in the presence of a 30-fold molar excess of the indicated unlabelled oligonucleotides.

protein. TCP20 showed a preference for its selected sequence (C20) over the one selected by TCP15 (C15; Figure 3C). Binding to C20 was otherwise similar to binding to BS. In the case of TCP15, only subtle differences in binding preferences were observed, in accordance with its more relaxed specificity. In competition experiments, however, C15 competed more efficiently for binding than C20 (Figure 3C). In addition, both proteins showed poor binding to a sequence in which the G:C and A:T pairs located at positions 5 and 6 were exchanged (C′; Figure 3C).

For TCP11, since it is not affected by a change at position 3 of BS (which is fixed in the oligonucleotide used for the SELEX experiments described above), we performed a SELEX experiment with an oligonucleotide containing 12 consecutive degenerate positions. After five rounds of selection, the consensus sequence GTGGGCC (GGCCCAC in the complementary strand) was obtained with almost 100 % efficiency (Figure 4A). This sequence is identical with positions 1–7 (or 4–10) of BS and is consistent with the fact that TCP11 is able to bind a sequence with a change at position 3 (or the equivalent position 8). This result also shows that TCP11 has relaxed specificity, and not a different preference, at this position. In addition, TCP11 displayed a clear preference for G:C pairs at both positions 5 and 6, unlike the other class I proteins tested. SELEX experiments conducted with the degenerate oligonucleotide containing fixed nucleotides at defined positions used for TCP15 and TCP20 produced a consensus binding sequence for TCP11 of the type GTGGGCCNN, in agreement with this observation (Figure 4B). This increased specificity at position 6 may originate from a compensatory change due to the loss of specificity at position 8. It is noteworthy that one of the central G:C pairs of BS (position 5 or 6) is protected

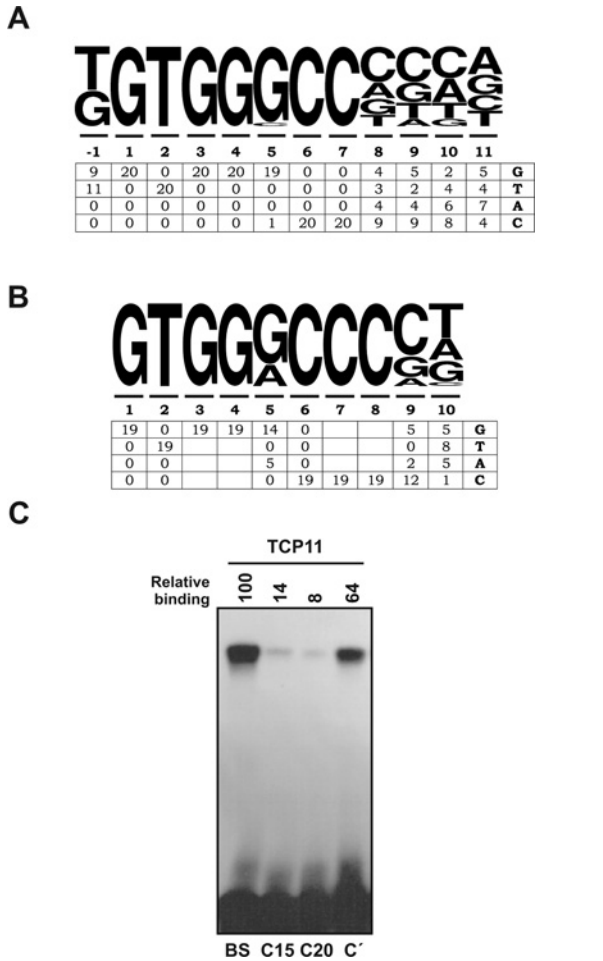

Figure 4 TCP11 has different DNA-binding characteristics compared with other class I proteins

(**A** and **B**) Binding-site preferences of TCP11 as deduced from SELEX experiments performed with oligonucleotides with 12 degenerate positions (**A**) or with fixed and degenerate positions (**B**). Nucleotide frequencies at different positions in the population of oligonucleotides selected by TCP11 are shown in sequence logos, below which the number of sequenced clones with each nucleotide is shown. (**C**) Binding of TCP11 to BS, C15, C20 and C′. In the upper part of the gel image, the amount of binding, relative to BS, is shown.

in both strands by TCP11 in footprinting experiments (Figure 2B). In addition, in both SELEX experiments, 100 % selection of a G:C pair was observed at position 6 and some changes were evident at position 5 (Figures 4A and 4B). This is also a difference found in other class I proteins, which select with high efficiency the G:C pair at position 5 (numbered in reference to the GT dinucleotide that is selected with 100 % efficiency by all the proteins) ([16] and the present work).

The SELEX results were confirmed by analysing binding to BS (with G:C pairs at positions 5 and 6), C15, C20 (with an A:T pair at position 6) and C′ (with an A:T pair at position 5). In agreement with the SELEX results, TCP11 showed a clear preference for BS, but preferred the presence of an A:T pair at position 5 rather than at position 6 (Figure 4C).

A threonine residue at position 15 of the TCP domain determines the DNA-binding properties of TCP11

Comparison of the basic regions of TCP11 and other TCP proteins revealed the existence of two non-conservative changes, namely Ser[11] instead of a glycine residue and Thr[15] instead of an arginine residue, in TCP11 (Figure 5A). Assuming that these residues may be responsible for the different DNA-binding properties of TCP11, we performed single mutations to convert positions 11 and 15 into glycine and arginine residues respectively. Introduction of glycine at position 11 did not produce noticeable changes in the DNA-binding behaviour of TCP11, other than a decrease in DNA-binding efficiency. The T15R mutant, in turn, displayed increased sensitivity to a change at position 3 of BS and a preference for C20 respective to a similar sequence with an A:T pair at position 5 instead of position 6 (Figures 5B and 5C). We also assayed the opposite changes in TCP20. Introduction of a threonine residue at position 15 of TCP20 produced a protein with reduced sensitivity to a change at position 3 of BS (Figure 5B). Thr[15]-TCP20 also showed a clear preference for BS and bound with greater efficiency the sequence with an A:T pair at position 5 in comparison with the one with an A:T pair at position 6 (Figure 5C). The results indicate that the presence of a threonine residue at position 15 of the TCP domain is responsible for the distinct DNA-binding properties shown by TCP11. Arg[15], present in other TCP proteins, may be a main determinant of the preference of a G:C pair at position 3, and probably also at the symmetrical position 8. Arg[15] also influences the nucleotide preferences at positions 5 and 6, the central positions of the binding sequence.

In addition to the changes described above, the nature of the residue present at position 15 also affects the binding efficiency of the protein. Mutation of Arg[15] to a threonine residue produced a significant decrease in binding of TCP20 to BS, whereas introduction of arginine at position 15 in TCP11 increased binding efficiency (Figure 5D). This suggests that an arginine residue at this position establishes more energetic contacts with DNA. Mutation of Thr[15] to an alanine residue in TCP11 completely abolished the interaction with DNA (Figure 5D), indicating that this threonine residue also participates in DNA binding.

The introduction of arginine at position 15 into TCP11 produced a relative increase in protection of the 5′ region of both strands of BS respective to the native protein (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350143add.htm), perhaps due to contacts established by Arg[15] in this region. For Thr[15]-TCP20, the footprint shifted towards the 3′ region (Supplementary Figure S1) in agreement with this observation.

Heterodimers formed by TCP11 and TCP15 show increased binding efficiency

The particular DNA-binding properties of TCP11 led us to analyse the formation of heterodimers between TCP11 and other class I proteins. TCP11 was able to interact with TCP15, TCP20 and TCP21 in yeast two-hybrid experiments (results not shown). Heterodimer formation was also indicated by the presence of a band of intermediate mobility of those of the individual proteins in EMSAs when TCP11 was included together with another class I protein. Similar results were obtained with Arg[15]-TCP11 (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/435/bj4350143add.htm), indicating that the presence of Thr[15] is not required for heterodimer formation. Figure 6(A) shows the binding of heterodimers between TCP11 and TCP15 to BS and BS3. Just as for TCP11, the heterodimer was not affected by a change at position 3 of BS. Assays with other oligonucleotides indicated that the binding specificity of the heterodimer is indeed similar to the one observed for TCP11.

Inclusion of TCP11 together with TCP15 in DNA-binding assays produced a significant increase in binding respective to assays with the individual proteins (Figure 6B). This

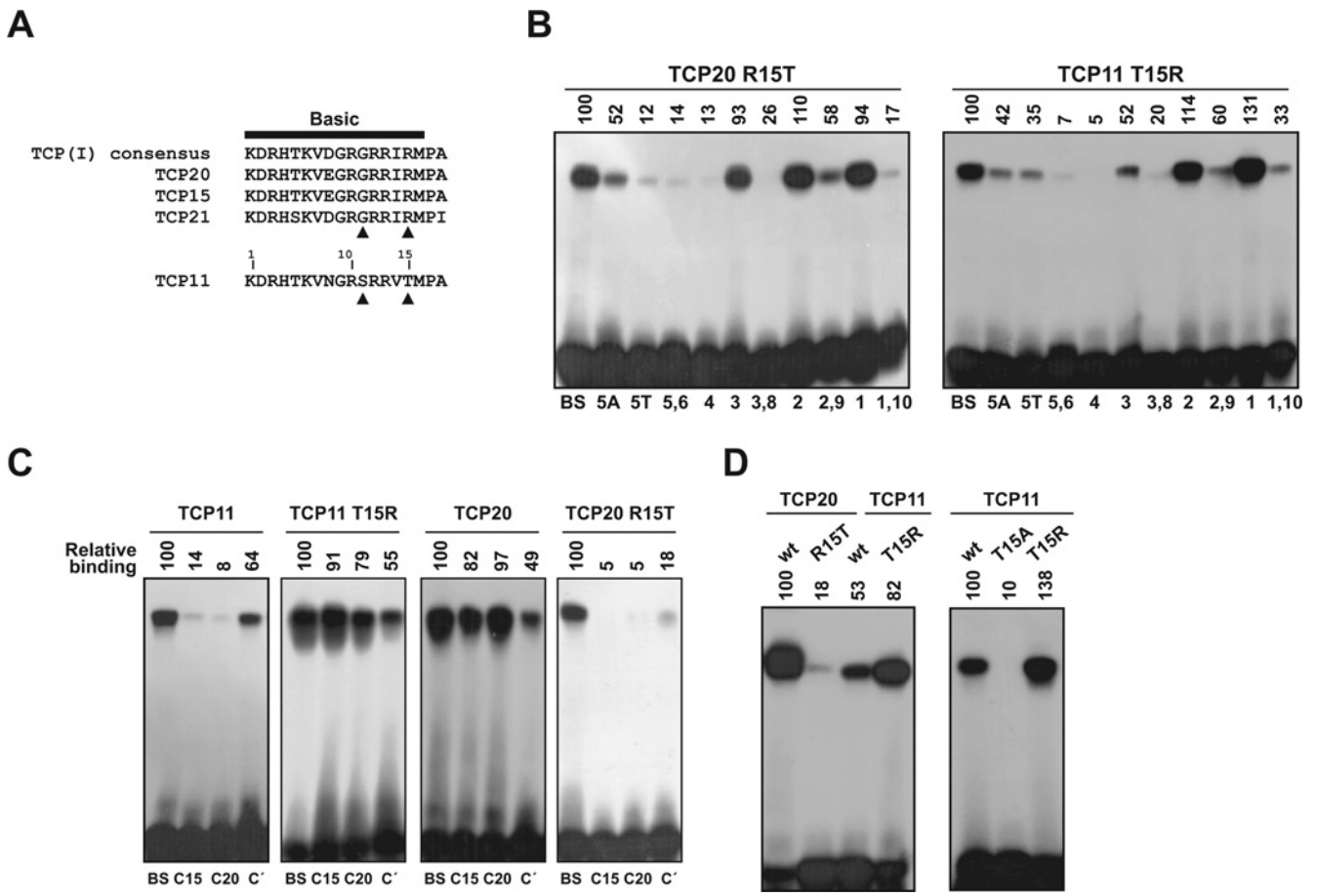

Figure 5 The residue at position 15 of the TCP domain determines the binding preferences of class I TCP proteins

(**A**) Consensus sequence of the basic region of class I TCP proteins [2], aligned with the corresponding sequences of TCP11, TCP15, TCP20 and TCP21. Numbering indicates residue position within the TCP domain. Arrowheads indicate positions with non-conservative substitutions in TCP11. (**B**) Binding of TCP11 and TCP20 with reciprocal mutations at position 15 of the TCP domain to oligonucleotides containing different binding sequences. The sequences of the different oligonucleotides are described in Figure 1. (**C**) Binding of TCP11 and TCP20 mutants at position 15 to BS, C15, C20 and C′. (**D**) Comparison of the binding efficiency of proteins with a threonine or arginine residue at position 15 of the TCP domain (left-hand panel) and effect of the mutation of Thr^{15} to an alanine residue in TCP11 (right-hand panel). Numbers in the upper part of each gel image show relative binding efficiency. wt, wild-type.

effect was particularly evident for BS3. The results suggest that heterodimer formation produces a species with distinct DNA-binding properties.

TCP11 is a developmental regulator

TCP11 activity was analysed in *Arabidopsis*. Analysis of four available SALK homozygous mutants with insertions in the 5′-UTR (untranslated region) or 3′-UTR (SALK_082324, SALK_003395, SALK_056047 and SALK_020124) indicated that they have normal or slightly reduced levels of *TCP11* mRNA and no obvious phenotypic differences compared with the wild-type. We then obtained plants that expressed a fusion of TCP11 to the EAR repressor domain, a strategy that has been previously used to study the function of other transcription factors, including TCP3 (class II) and TCP20 [8,12]. For this purpose, we transformed plants with a construct that expressed TCP11 fused to the repressor domain in its C-terminal part under the control of the 35S CaMV promoter. Several independent transformants were isolated and the expression levels were measured using Northern blotting experiments (see Supplementary Figure S3A at http://www.BiochemJ.org/bj/435/bj4350143add.htm). Four lines with different expression levels were further analysed.

Plants that express TCP11–EAR show curly rosette leaves whose margins tend to fold upwards (Figure 7E, arrowhead). Curling of rosette leaves was more pronounced in the distal part of the lamina and was stronger in plants with higher expression levels of TCP11–EAR. As opposed to leaves from wild-type plants, it was not possible to flatten leaves from over-expressing plants without making cuts in the borders. The observed phenotype is indicative of different growth rates in inner regions of the leaf lamina with respect to the margins, suggesting that TCP11 regulates cell growth or proliferation in leaves. In the most severe phenotypes, leaves of TCP11–EAR-expressing plants were smaller, suggesting that the fusion protein may act to repress growth preferentially at the margins of leaves. Cauline leaves were also affected. They were considerably smaller than those of wild-type plants and their lateral borders were curved upwards, forming rod-like structures with short petioles that surrounded the inflorescence stems at their base (Figure 7A, arrowheads). TCP11–EAR plants also showed reduced elongation of the primary inflorescence stem (Figures 7C and 7L). Unlike wild-type plants, elongation of the secondary stems in TCP11–EAR plants was comparable with that of the primary stem, thus giving the plants a bushy phenotype and reflecting a loss of apical dominance. Reduced stem elongation produced a more

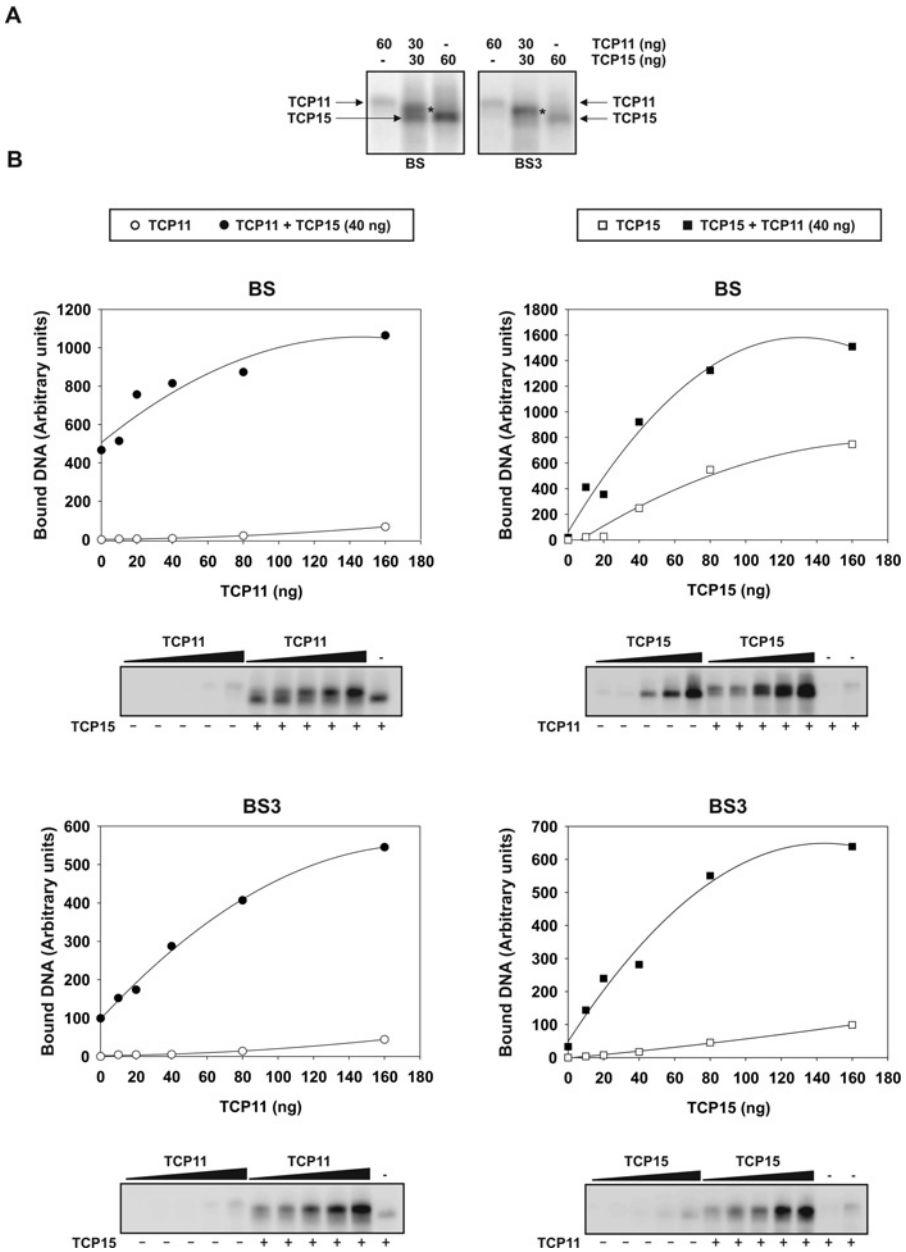

Figure 6 Heterodimers formed by TCP11 and TCP15 show increased binding efficiency

(**A**) Formation of heterodimers between TCP11 and TCP15 analysed in an EMSA. Only the fraction of bound DNA is shown. The presence of a band with intermediate mobility in the lane that contains the two proteins is shown by an asterisk. (**B**) Binding of different amounts of TCP11 (circles) and TCP15 (squares) to BS and BS3 in the absence (white symbols) or presence (black symbols) of the other protein was analysed in EMSAs. The amount of bound DNA was calculated from signal intensities obtained with phosphor storage technology. Below each graph, an image of the bound DNA fraction of the corresponding gel is shown.

compact inflorescence. Flowers appeared normal but had shorter pedicels (Figure 7C, arrowheads). Siliques were considerably shorter than those of wild-type plants (Figures 7C and 7N) and showed empty spaces with unfertilized ovules between the seeds. Accordingly, the number of seeds obtained from TCP11–EAR plants was significantly reduced and a higher proportion of seeds with abnormal morphology was observed (Figure 7M). Anthers of TCP11–EAR plants contained a reduced number of pollen grains and a high proportion of pollen of abnormal shape (Figure 7K, arrowhead). The reduced number of normal pollen grains may explain the presence of unfertilized ovules in the siliques of TCP11–EAR plants. In summary, expression of a repressor form of TCP11 affects the growth of leaves, stems and pedicels, suggesting that TCP11 participates in the regulation of organ growth. In addition, TCP11 seems to have a role in pollen development.

We have also assayed the effect on plant development of expressing native TCP11 from the 35S CaMV promoter. Plants that contained similar *TCP11* transcript levels as those of TCP11–EAR lines described above (Supplementary Figure S3A) showed no obvious phenotype (results not shown), indicating that the effects observed in the repressor lines are not merely due to interference with the action of other TCP proteins, but rather result from the incorporation of TCP11–EAR into a transcriptional

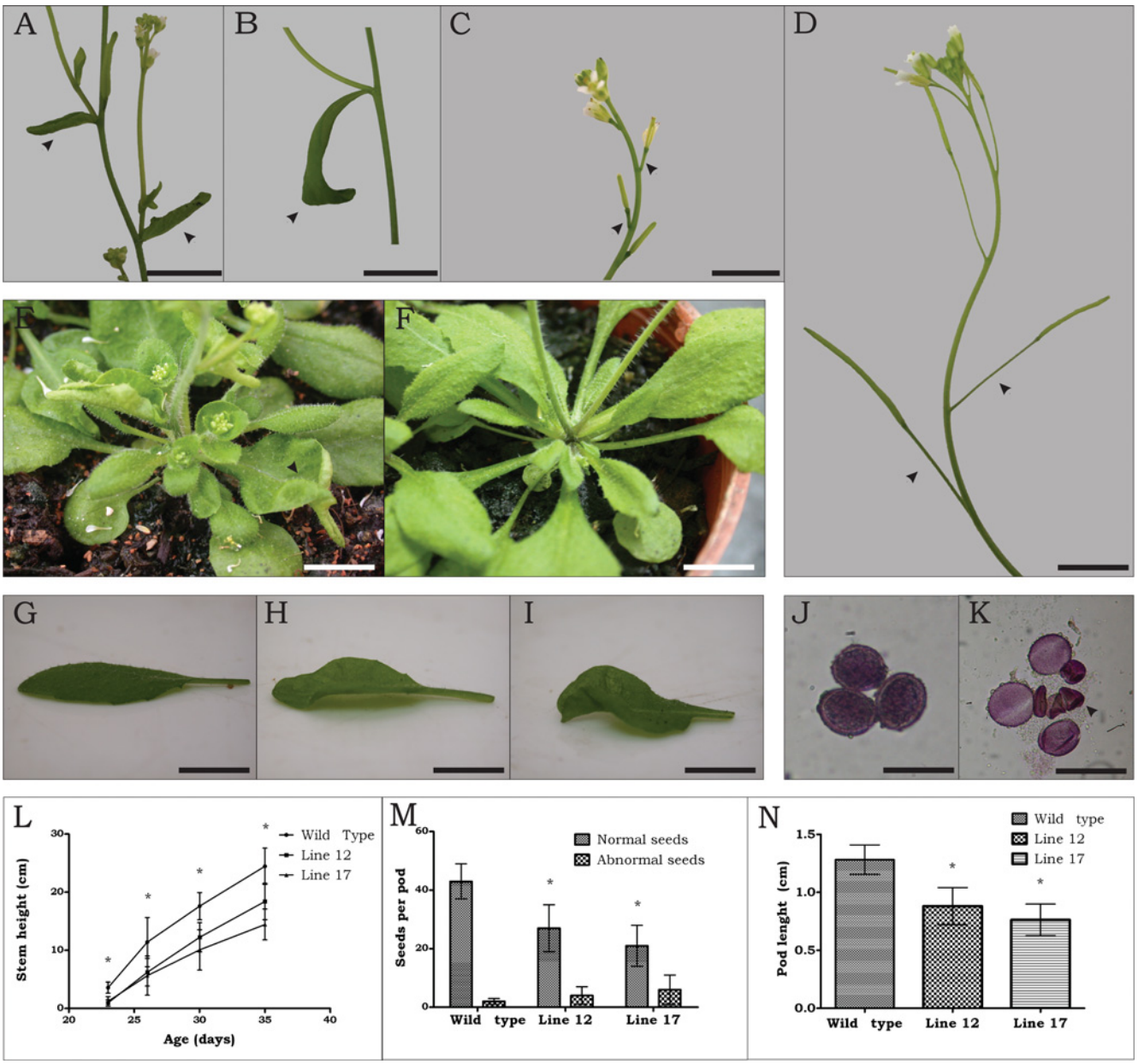

Figure 7 Expression of a repressor form of TCP11 alters several aspects of plant development

(**A**–**F**) Inflorescence stem and rosette of plants that express a fusion of TCP11 to the EAR domain (**A**, **C** and **E**) compared with wild-type plants of the same age (**B**, **D** and **F**) grown on soil. Arrows indicate differences in the phenotype of cauline leaves (**A** and **B**), pedicels (**C** and **D**) and rosette leaves (**E**). (**G**–**I**) Detached leaves from wild-type plants (**G**) and from lines with intermediate and high expression levels of TCP11–EAR (**H** and **I** respectively). (**J** and **K**) Pollen from wild-type plants (**J**) and plants that express TCP11–EAR (**K**) subjected to Alexander staining [38]. (**L**–**N**) Growth of the inflorescence stem (**L**), number of seeds of normal and abnormal appearance (**M**) and silique length (**N**) in wild-type plants and in two lines that express TCP11–EAR. Mean values $\pm$ S.D. ($n = 20$) are shown. Asterisks indicate significant differences with wild-type plants, according to Student's t tests ($P < 0.05$). Scale bars in (**A**–**I**) represent 1 cm; those in (**J** and **K**) represent 25 μm.

regulatory complex. It is also noteworthy that expression of Arg^{15}-TCP11 fused to the EAR domain produced plants with a normal phenotype. This is somewhat unexpected, since this protein binds DNA more efficiently than does TCP11, although with a different specificity. Expression analysis using antibodies raised against recombinant TCP11 indicated that these plants contain similar TCP11 levels to those of TCP11–EAR plants (Supplementary Figure S3B).

The expression pattern of the *AtTCP11* gene was analysed in plants that contain a fusion of the *AtTCP11* region located upstream of the translation start site to the *uidA* (*gus*) reporter gene. Analysis of independent transformants by histochemical staining revealed that in seedlings expression was present in the vascular system of cotyledons, leaves and hypocotyls (Figures 8A and 8B). Expression was also observed in root tips (Figure 8H) and in the shoot apical meristem, localized in spots in peripheral zones (Figure 8B, arrows), probably reflecting the initiation of leaf primordia. In adult plants, expression was observed in rosette and cauline leaves, preferentially in the vascular system (Figures 8E and 8F) and in the root vascular system (Figure 8G). Primordia present in the axils of cauline leaves with the inflorescence stem were also stained (Figure 8F, arrow). Expression was also evident in flowers, specifically in sepal veins, pollen and ovules (Figures 8C and

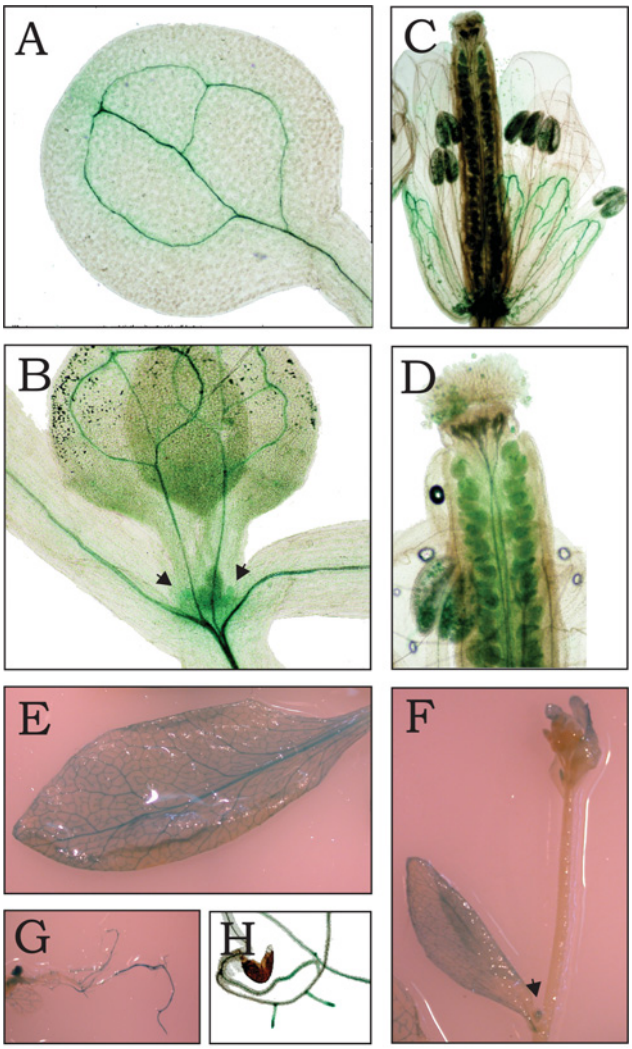

Figure 8 Expression pattern produced by the *AtTCP11* gene upstream region

Histochemical detection of GUS activity in plants transformed with the *AtTCP11* upstream region fused to *gus*. (**A** and **B**) Cotyledon, shoot meristem and primary leaves of a 12-day-old plant. (**C**–**G**) Flower (**C** and **D**), rosette leaf (**E**), inflorescence stem (**F**) and root (**G**) of a 5-week-old plant. (**H**) Root of a 12-day-old plant.

8D). The results indicate that TCP11 is expressed in structures that are altered in TCP11–EAR plants. The expression pattern generated by the *AtTCP11* promoter region is in agreement with RNA expression data available in the Genevestigator (https://www.genevestigator.com) and BAR (Bio-Array Resource) Expression Browser (http://bbc.botany.utoronto.ca/) web tools [35,36].

DISCUSSION

The fact that TCP proteins can form dimers and the resemblance of the TCP domain with the bHLH domain suggest a model in which each monomer of a TCP dimer interacts with a half-site of a dyad-symmetric sequence. Accordingly, we have observed that four *Arabidopsis* class I TCP proteins bind to a 10 bp dyad-symmetric sequence composed of two GTGGG half-sites. Footprinting experiments with TCP20 indicated that symmetric contacts are established with the entire 10 bp region in both strands, with enhanced protection in the 5′ half (GTGGG) of each strand. With the exception of TCP11, binding was affected by single changes at positions 3 or 4 and less affected by a change at position 5, suggesting that binding specificity relies mainly on the simultaneous interaction of both monomers with the G:C base pairs located at positions 3, 4, 7 and 8 (positions 3 and 4 of both half-sites). Modifications at positions 1 and 2 produce no effect on binding as long as only one half-site is modified, probably reflecting the fact that the interaction of only one of the monomers with this region is enough for efficient binding. This indicates that an asymmetric complex can be formed on DNA, depending on the sequence of the target site, and agrees with the results of SELEX experiments obtained by Kosugi and Ohashi [16] for PCF2 and by us for TCP15 and TCP20. The formation of an asymmetric complex on DNA has also been observed for homodimers of the bHLH protein E47 upon binding the sequence CACCTG [25,37]. In this case, one of the subunits establishes five specific contacts with one half-site, whereas the other subunit makes only two specific contacts with the other half-site of the target sequence [37].

Based on SELEX experiments and analysis of TCP domain sequences, we predict that most class I TCP proteins bind sequences of the type GTGGGNCCNN with subtle differences in binding preferences at variable positions that may be important for functional specificity. An exception to this rule is TCP11, which shows a preference for GTGGGCCNNN. According to mutagenesis studies, the different binding properties of TCP11 are attributable to the presence of a threonine residue at position 15 of the TCP domain, which is occupied by an arginine residue in all other *Arabidopsis* TCP proteins. Arg^{15} may be important for the interaction of the TCP domain with the G:C pair located at position 8 of the binding sequence (or the equivalent position 3 of BS), probably through the establishment of hydrogen bonds with the guanine nucleotide of this pair. This agrees with the fact that the preferential interaction with the 5′ side of both strands of BS, which contain this guanine, is lost in proteins with Thr^{15}.

Recently, Aggarwal et al. [34] presented a model of the interaction of the class II TCP4 domain with its target sequence based on a comparison with the bHLH domain. Arg^{64} (equivalent to Arg^{15} of the class I TCP domain) is not presented as a DNA-contacting residue in this model. However, mutation of Arg^{64} to a glycine residue abolishes DNA binding by TCP4 both *in vitro* and *in vivo* in yeast [34]. In the model, Arg^{62} or Leu^{65} is postulated to interact with a guanine nucleotide equivalent to G3 or G8 of BS, depending on the strand that is considered. One possibility is that class I and class II proteins have subtle structural differences that produce changes in the orientation of base-contacting amino acids. Another possibility is that Arg^{15}, rather than directly contacting DNA, influences the positioning of adjacent residues that directly interact with DNA. We note that Arg^{64} of TCP4 is predicted to be a base-contacting amino acid by two of the three methods used by Aggarwal et al. [34].

Heterodimers formed by TCP11 and TCP15 bind DNA more efficiently than the respective homodimers, especially when a target sequence with a change at position 3 is considered. According to their binding preferences, it can be envisaged that each of the monomers of the heterodimer would be able to interact productively with a specific half of the target sequence in this case. Increased binding to BS, on the other hand, may be related to structural changes that occur upon heterodimer formation. It can be speculated that *in vivo* the impact of heterodimer formation will be more important for target sequences that contain a change at position 3, since these sequences are less efficiently bound by class I TCP protein homodimers.

Arg[15] is almost absolutely conserved in TCP proteins from both classes. The only exceptions we found are TCP11 and its homologue from *Arabidopsis lyrata* (which contains Met[15]). Therefore this seems to be a recent evolutionary innovation. The participation of TCP11 as part of a transcriptional complex *in vivo* is supported by the fact that a fusion of this protein to the EAR repressor domain produces phenotypic changes when expressed in plants. Anthers from plants that express TCP11–EAR contain fewer pollen grains and grains with abnormal shape. This, and the observed increased activity of the TCP11 promoter in anthers, supports a role for TCP11 in pollen development, as has also been proposed for TCP16 [10]. Stems and pedicels of TCP11–EAR plants are shorter, indicating that TCP11 is a regulator of organ growth. In addition, leaves of plants that express TCP11–EAR show different growth rates in the margins and the distal part of the lamina, which produces a wavy phenotype, as observed in leaves with decreased levels of class II TCP proteins [6,7]. In the case of class II proteins, however, the margins grow more rapidly than the central part of the lamina. Li et al. [15] proposed a model in which class I TCP transcription factors promote cell growth and proliferation at early stages of organ development, and class II proteins repress these processes at more advanced stages. The expression pattern produced by the TCP11 promoter region (i.e. increased expression in meristem and leaf primordia) suggests that TCP11 may act to regulate cell proliferation or growth at early stages of organ differentiation and development. Detailed studies of these parameters in leaves of TCP11–EAR plants will be necessary to establish which processes are affected in these plants. Additionally, the extent of the generality of the proposed function for class I proteins is not clear. Fusion of TCP20 to the EAR domain, for example, produces much more severe alterations than those observed here for TCP11 [12]. In turn, loss of function of TCP14 and TCP16 lead to defects in germination and pollen development respectively [10,11], and TCP21 has been reported as a component of the circadian clock [13]. Some degree of specificity exists, then, in the action of class I TCP proteins. At least part of this specificity may rely on different DNA-binding preferences, as shown in the present study for three class I proteins.

AUTHOR CONTRIBUTION

Ivana Viola planned, performed and analysed DNA–protein-interaction experiments. Nora Uberti Manassero performed and analysed experiments involving *Arabidopsis* plants. Rodrigo Ripoll performed and analysed SELEX experiments with TCP11. Daniel Gonzalez planned and analysed experiments, and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

The *Arabidopsis* class I TCP transcription factor AtTCP11 is a developmental regulator with distinct DNA-binding properties due to the presence of a threonine residue at position 15 of the TCP domain

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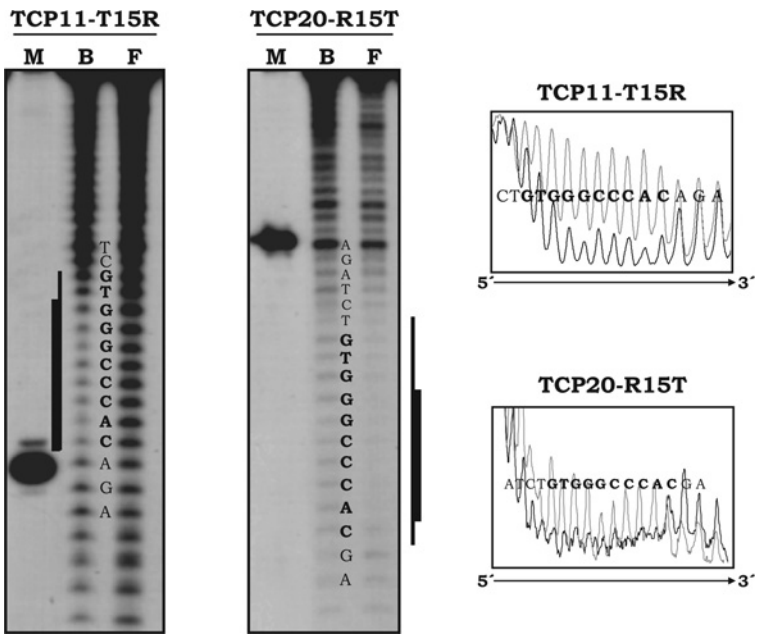

Figure S1 Hydroxy radical footprinting patterns of the complexes of Arg[15]-TCP11 and Thr[15]-TCP20 with BS

Footprinting analysis was performed essentially as described in Figure 2 of the main text (F, free DNA; B, bound DNA; M, size standard). Only the pattern on one strand is shown. Letters beside each panel indicate the DNA sequence (5′ end in the upper part) of the corresponding strand in this region. The vertical bars represent the relative intensity of the footprint in each region. Densitometric scans of the lanes corresponding to free (grey line) and bound (black line) DNA are shown in the right-hand panel.

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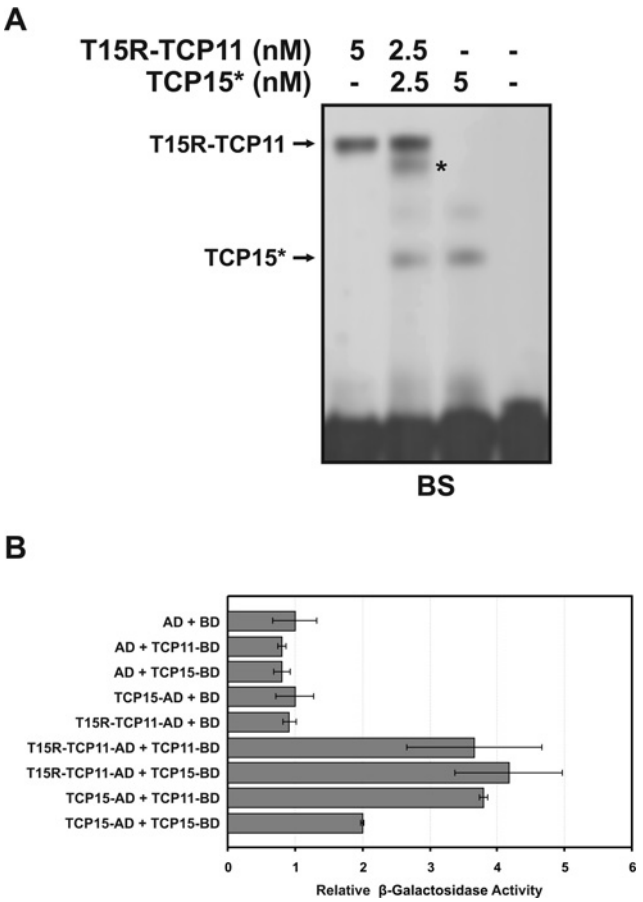

Figure S2 T15R-TCP11 forms heterodimers with TCP15

(**A**) Formation of heterodimers between T15R-TCP11 and TCP15 analysed in an EMSA. A shorter form of TCP15 (TCP15*), with the MBP moiety removed by Factor Xa cleavage, was used. The presence of a band with intermediate mobility in the lane that contains both proteins is shown by an asterisk. (**B**) Interaction of T15R-TCP11 and TCP15 analysed using two-hybrid assays in yeast. Specific β-galactosidase activity of yeast cells transformed with constructs expressing the corresponding TCP proteins fused to either the GAL4 activation domain (AD) or the DNA-binding domain (BD). Cells transformed with constructs that expresses only AD and/or BD were used as controls. The mean activity value $\pm$ S.D. of three independent measurements is shown.

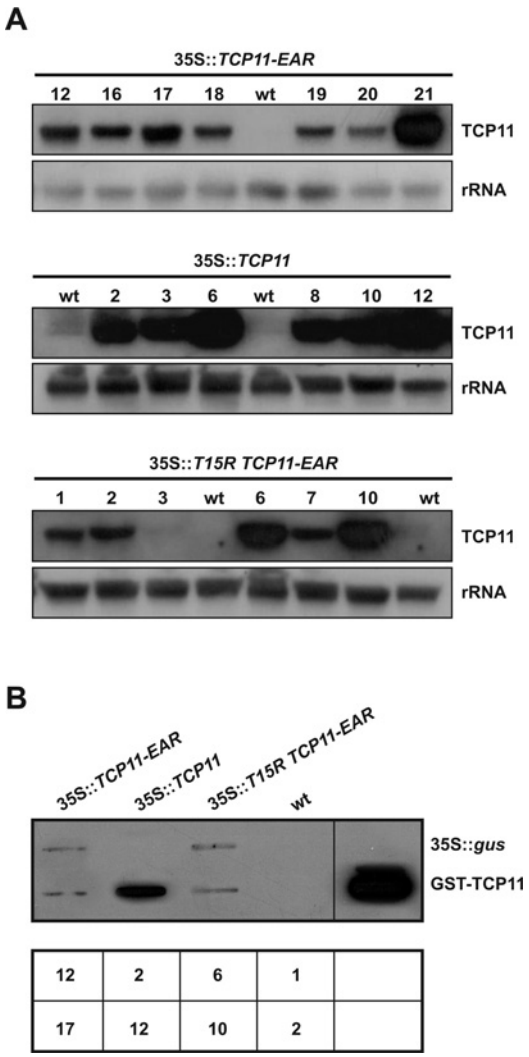

Figure S3 Analysis of transcript and protein levels in plants that express different forms of TCP11

(**A**) Northern blot analysis of plants that express different forms of TCP11, as indicated, using a probe specific for TCP11. Numbers indicate different independent lines. Total RNA extracted from non-transformed plants (wt) was used as a control. An rRNA probe was used as a control for RNA loading. (**B**) Slot blot analysis of expression levels of different forms of TCP11 using antibodies raised in rabbits against recombinant TCP11 (dilution 1:200). A total of 500 μg of protein extract was used. Extracts from non-transformed plants (wt) and from plants transformed with pBI121 (35S::*gus*) were used as controls. Recombinant TCP11 fused to GST (50 ng) was loaded as a positive control. In the lower panel, a scheme indicating the lines that were analysed is shown.

Table S1 List of oligonucleotides used

Underlining indicates restriction sites. Bold nucleotide sequences indicate the binding site of TCP proteins.

Name	Sequence (5′→ 3′)	Use
T15-F	GGGGAATTCCCGGATCATAACCATCGA	TCP15 pMALC2 and pGADT7 cloning
T15-R	GGCCTCGAGCTAGGAATGATGACTGGT	TCP15 pMALC2 and pGADT7 cloning
T20-F	CCCGGATCCCCCAAGAACCTAAATCGTC	TCP20-pGADT7 and ΔTCP20-pMAL C2 cloning
T20-R	CCCAAGCTTACGACCTGAGCCTTGAG	TCP20-pGADT7 cloning
T21-F	CCAGGATCCATGGCCGACAACGACGGA	TCP21 pMALC2 and pGADT7 cloning
T21-R	GGCGTCGACAACGTGGTTCGTGGTCGT	TCP21 pMALC2 and pGADT7 cloning
T11-F	GGCGGATCCTGATTTTTCAGAATGTGTG	TCP11 pMALC2 cloning
T11-R	CGGGTCGACTCGAGCTAATGGTGACGGCGTCT	TCP11 pMALC2 cloning
ΔT20-R	CGCAAGCTTGAGACCCACCTTGATGATGG	ΔTCP20 pMALC2 cloning
BS	AATTCAGATCT**GTGGGCCCAC**GAG	EMSAs
BSc	GATCCTC**GTGGGCCCAC**AGATCTG	EMSAs
R1	GATGAAGCTTCCTGGACAAT	SELEX cycle amplification
R2	CAGAATTCTACAGTGGCTGC	SELEX cycle amplification
P-40	GTT TTC CCA GTC ACG AC	Universal forward primer
PRLAR	AGC GGA TAA CAA TTT CAC ACA GGA	Universal reverse primer
K1A-F	AAGCTCTAACGCAGACAGACAC	K1A-ΔTCP20 cloning
K1A-R	GTGTCTGTCTGCGTTAGAGCTT	K1A-ΔTCP20 cloning
D2A-F	CTCTAACAAAGCCAGACACAC	D2A-ΔTCP20 cloning
D2A-R	GTGTGTCTGGCTTTGTTAGAG	D2A-ΔTCP20 cloning
R3A-F	TAACAAAGACGCACACACTAAA	R3A-ΔTCP20 cloning
R3A-R	TTTAGTGTGTGCGTCTTTGTTA	R3A-ΔTCP20 cloning
H4A-F	CAAAGACAGAGCCACTAAAGTC	H4A-ΔTCP20 cloning
H4A-R	GACTTTAGTGGCTCTGTCTTTG	H4A-ΔTCP20 cloning
E8A-F	CACTAAAGTCGCAGGTAGAGG	E8A-ΔTCP20 cloning
E8A-R	CCTCTACCTGCGACTTTAGTG	E8A-ΔTCP20 cloning
R12A-F	GGTAGAGGTGCACGAATTCG	R12A-ΔTCP20 cloning
R12A-R	CGAATTCGTGCACCTCTACC	R12A-ΔTCP20 cloning
R10A-F	GTCGAAGGTGCAGGTCGACG	R10A-ΔTCP20 cloning
R10A-R	CGTCGACCTGCACCTTCGAC	R10A-ΔTCP20 cloning
G11S-F	CGAAGGTAGAAGTCGACGAAT	G11S-ΔTCP20 cloning
G11S-R	ATTCGTCGACTTCTACCTTCG	G11S-ΔTCP20 cloning
R13A-F	TAGAGGTCGAGCAATTCGGATG	R13A-ΔTCP20 cloning
R13A-R	CATCCGAATTGCTCGACCTCTA	R13A-ΔTCP20 cloning
R15T-F	GTCGACGAATTACGATGCCTGCTC	R15T-ΔTCP20 cloning
R15T-R	GAGCAGGCATCGTAATTCGTCGAC	R15T-ΔTCP20 cloning
S11G-F	TAACGGACGAGGCCGTCGAGT	S11G-TCP11 cloning
S11G-R	ACTCGACGGCCTCGTCCGTTA	S11G-TCP11 cloning
T15R-F	CGTCGAGTTAGGATGCCGGCTC	T15R-TCP11 cloning
T15R-R	GAGCCGGCATCCTAACTCGACG	T15R-TCP11 cloning
C15	AATTCAGATCT**GTGGGACCCA**GAG	EMSAs
C15c	GATCCTC**TGGGTCCCAC**AGATCTG	EMSAs
C20	AATTCAGATCT**GTGGGACCGG**GAG	EMSAs
C20c	GATCCT**CCCGGTCCCAC**AGATCTG	EMSAs
C′	AATTCAGATCT**GTGGACCCGG**GAG	EMSAs
C′c	GATCCTC**CCGGGTCCAC**AGATCTG	EMSAs
pTCP11-F	GGCAAGCTTGCTACATCATTCAGAAAACTC	pTCP11 pBI101.3 cloning
pTCP11-R	GGCGGATCCCTCCGAATTCGTCGATTTTGT	pTCP11 pBI101.3 cloning
T11EAR-F	GGCTCTAGAATGATTTTTCAGAATGTGTG	35S–TCP11–EAR pBI121 cloning
T11EAR-R	CGGGTCGACAATGGTGACGGCGTCTAC	35S–TCP11–EAR pBI121 cloning
EAR-XK	GGCCTCGAGTACCTCAAGCGAATCCAAGTCTC	35S–TCP11–EAR pBI121 cloning
EAR-F	TCGACTTGATTTGGATCTTGAGTTGAGACTTGGATTCGCTTGAGCT	EAR motif
EAR-R	CAAGCGAATCCAAGTCTCAACTCAAGATCCAAATCAAG	EAR motif

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