Ectopic expression of TATA box-binding protein induces shoot proliferation in *Arabidopsis*

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Abstract The TATA box-binding protein (TBP) is an essential component of transcription initiation complexes of all three eukaryotic RNA polymerases. Increasing evidence suggests that the TBP activity and availability may be regulated to precisely control gene transcription and play an important role in cell type-specific regulation. *Arabidopsis* TBP-2 is up-regulated in apical shoot tissues. Overexpression of *TBP-2* in transgenic *Arabidopsis* induces apical shoot proliferation. The expression of some shoot meristem regulatory genes is altered. These data suggest that the *TBP* gene dosage and/or expression level may play an important role in controlling shoot production and plant morphology.

Key words: TFIID; TATA box-binding protein; Shoot meristem; Regulation; Gene transcription; *Arabidopsis*

1. Introduction

The TATA box-binding protein (TBP) is assembled with unique TBP-associated factors (TAF) into functionally distinct complexes that act specifically with RNA polymerase I (SL1), RNA polymerase II (TFIID) or RNA polymerase III (TFIIIB) on cognate promoters. It has been reported that TBP also interacts directly with many other proteins including specific activators and repressors, co-activators and general transcription factors such as TFIIA and TFIIB [1,2].

TBP is a highly conserved protein [3]. Yeast and animals have one TBP gene, while plants may have at least two [1]. *TBP-1* and *TBP-2* genes from *Arabidopsis* have been shown to be equally active with promoters utilized by polymerases II and III [4,5]. It is suggested that the gene dosage and expression level of TBP should be precisely determined for appropriate gene expression in an organism [1]. In vivo, TBP exists in at least two transcriptionally active forms: one associated with multiple TAFs and one without TAFs [6]. Each form recognizes a distinct class of promoters in yeast [7,8]. TBP is recruited to bind to promoter DNA only when the promoter is physiologically induced [9,10], suggesting that binding of TBP to specific promoter DNA is highly regulated and depends on the presence of specific activators and/or on its availability. The expression of TBP shows cell type specificity in animals [11–13]. Increased levels of TBP can stimulate the transcription of subsets of genes in transfected animal cells [14,15]. In this paper, we report that *Arabidopsis TBP-2* expression is increased in apical shoots and that constitutively increased TBP levels in transgenic plants seriously affected aerial vegetative development.

2. Materials and methods

2.1. Plant growth conditions

Arabidopsis thaliana (Columbia ecotype) plants were used in this study. Both wild-type and transgenic plants were grown in soil in the greenhouse under 16 h/8 h light cycles at 22°C for seed production and for plant material harvest. For transgenic plant screening, infiltrated *Arabidopsis* seeds were germinated in vitro on MS salts supplemented with vitamins and sucrose (1%) in a growth chamber under continuous light at 21°C.

2.2. Transgene construction and plant transformation

Arabidopsis TBP-2 cDNA isolated by polymerase chain reaction (PCR) [16] was put under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S) in a transformation vector (pBI101, Clontech). The resulting plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 strain by electroporation. *Arabidopsis* plants were transformed using the in planta infiltration method [17].

2.3. Northern blot and reverse transcription (RT)-PCR

Arabidopsis tissues were collected from plants grown in the greenhouse. Total RNAs were extracted using Trizol (Life Technologies) by following the manufacturer's instructions.

For RT-PCR, total RNA were reverse transcribed into cDNA using Superscript (Life Technologies) with oligo dT as primer. The cDNAs were subsequently amplified by regular PCR using gene-specific primers. For Northern blots, equal amounts of total RNA from different samples were separated on a 1.5% denaturing agarose gel, blotted onto nylon membranes and hybridized with ^{32}P-labeled probes using the ULTRAhyb™ solutions (Amicon) and autoradiographed. DNA fragments corresponding to the coding region of *STM*, *KNAT1*, *CLV1*, *ZLL* and *CycD3* used as probes were obtained by PCR using gene-specific primer sets. The sequences of the primer sets used are for PCR: *TBP-2*: 5′-ATGACTGATCAAGGATTGGAAGG and 5′-ACCTATTGCTGTATCTTTCTGAATTC; *UBQ:* 5′-TAAAAACTTTCTCTCAATTCTCTCT and 5′-TTGTCGATGGTGTCGGAGCTT; *STM*: 5′-AAGATGGAGAGTGGTTCCAACAGC and 5′-CTTCTGACAATTGACATAAGCGGC; *KNAT1*: 5′-CCCGAGGGCCGAAGATCGGGAACT and 5′-TGGTGCGGGTGCTGCAGACCGAAC; *CLV1*: 5′-CGTGGAAGCTGCAAAGGGCTTGTG and 5′-CCTCGGGTCAACAATCGCAACAAC; *ZLL*: 5′-AACATGGCAAGATCCTGTTCGCGG and 5′-CGCATGGCTACAAAGGTAGAAGTC; *CycD3*: 5′-CAATGGCGATTCGGAAGGAGGAAG and 5′-AGAGACACGCGACAGAAACGAGCT.

2.4. Western blots

Leaf total proteins were extracted using Trizol (Life Technologies) and quantified by Bradford dosage. 20 μg of proteins per sample were separated by SDS–PAGE, blotted to PVDF membranes (Bio-Rad) and subsequently incubated with polyclonal antibodies prepared

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against the *Arabidopsis* TBP-2 [16]. After incubation with peroxidase-conjugated secondary antibodies, the reactions were visualized using Fast™ DAB tablets (Sigma).

2.5. Histochemical staining and light microscopy

2-week-old transgenic plants grown in soil in the greenhouse were fixed in 2% glutaraldehyde in 50 mM phosphate buffer at pH 7.4 for 4 h. After dehydration by a graded ethanol series, the samples were embedded in LR White (Polysciences, Warrington, PA, USA). The semi-thin sections (1 μm thick), cut with a Reichert–Jung microtome, were stained with periodic acid-Schiff's reaction for detection of insoluble polysaccharides (stained red) and naphthol blue for protein detection (stained blue).

2.6. Gel shift assays

DNA fragments corresponding to the KNAT1 promoter regions were obtained by PCR amplification using the following primer sets: 5′-CTTATCTCTTGTCCCTTCTC and 5′-AAGTGACATGGAAGAGAAG; 5′-CCTTGACGAATTCTATATAC and 5′-GGTCTCTGTAAGACTATAAC and ^{32}P-labeled by T4 polynucleotide kinase. Recombined TBP-2 production, binding reaction and gel electrophoresis were performed as previously described [18].

3. Results

3.1. Production of TBP-2-overexpressing transgenic plants

Semi-quantitative RT-PCR showed an induction of *TBP-2* expression in apical shoots of *Arabidopsis* seedlings (Fig. 1). In order to establish the relationship between TBP-2 level and plant gene expression and development, *Arabidopsis TBP-2* cDNA under the control of the CaMV 35S promoter was used to transform *Arabidopsis* plants by *in planta* infiltration. 28 kanamycin-resistant lines were analyzed for TBP expression and most showed much higher levels of *TBP-2* mRNA than wild-type plants. Some of them are shown in Fig. 2. Western blots detected also TBP-2 protein accumulation in those lines (Fig. 2).

3.2. Phenotype induced by TBP-2 overexpression

Fourteen independent *TBP-2*-overexpressing lines were maintained for phenotype examination at T1 and subsequent generations. During the growth of the cotyledons or the first pairs of leaves, no visible aberrant phenotype was observed in the transgenic plants. Subsequently, double or more pairs of leaves were produced simultaneously, forming two or more parallel shoots with an increase of total number of rosette leaves and, as a result, reduced or no apical dominance in nine of the 14 lines (Fig. 3). The abnormal plants with extensive branch ramification had decreased stature, smaller rosette leaves with margins curled toward the abaxial leaf surface (Fig. 3), delayed flowering time and senescence and reduced fertility (not shown). Transgenic lines with severer phenotypes

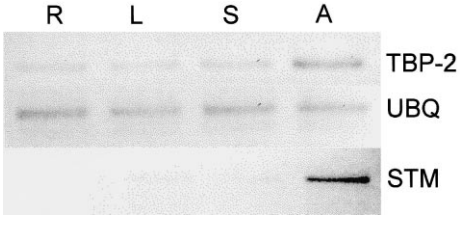

Fig. 1. Up-regulation of TBP-2 expression in apical shoots. Equal amounts (1 μg) of total RNA extracted from *Arabidopsis* young roots (R), adult leaves (L), siliques (S) and seedling apical shoots (A) were analyzed by RT-PCR (20 cycles) using primer sets corresponding to TBP-2, ubiquitin 10 (UBQ) and apical shoot-specific *STM* gene.

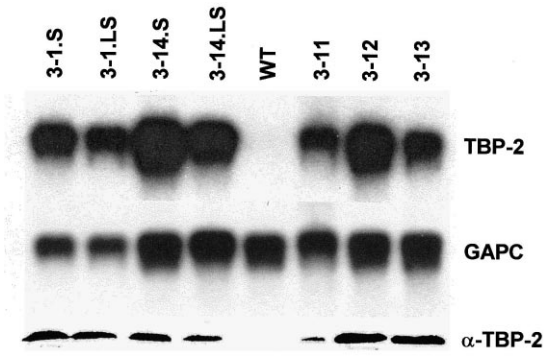

Fig. 2. TBP-2 overexpression in transgenic *Arabidopsis* plants. Upper panel: Northern blots of total RNA extracted from leaves probed first with *TBP-2* cDNA, then with the glyceraldehyde phosphate dehydrogenase sub-unit C (GAPC) cDNA as control. Plants from five independent transgenic lines (3-1, 3-11, 3-12, 13 and 13-14) at T2 generation were compared with wild-type plants. Two types of phenotypic plants (S: severe, as shown in Fig. 3D, SL: less severe, as shown in Fig. 3C) from lines 3-1 and 3-14 were used. Lower panel: Western blot analysis of protein extracts prepared from the same plants as in the upper panel.

(as judged by reduced leaf size and increased shoot number) showed higher expression levels of *TBP-2* (Figs. 2 and 3C,D). In addition, transgenic homozygous plants obtained from T2 and subsequent generations had severer phenotypes than heterozygous plants (not shown). No morphological abnormality could be observed in flowers and roots (not shown). These data indicate that elevated TBP-2 protein level affected mostly the aerial vegetative growth.

3.3. Shoot meristem proliferation in TBP-2-overexpressing plants

In order to know whether the shoot multiplication observed above was due to an abnormal shoot apical meristem (SAM) activity induced by *TBP-2* overexpression, 2-week-old plant shoot apices from three independent transgenic lines were sectioned and examined under light microscope. These plants had at least two vegetative shoots (Fig. 4A), indicating a loss of apical dominance. The secondary rosette was produced from precocious development of axillary meristems from the adaxial base of the subtending leaf (Fig. 4A). These precociously formed axillary meristems appeared to be normal with well defined cellular organization (i.e. L1 and L2 layers, corpus and leaf primordia). However, in some cases, groups of cells protruded out of the abaxial base of the youngest leaf primordium, forming meristem-like or growing structures (Fig. 4E). Cells in the leaf primordia had very dense cytoplasm without clear sign of differentiation (Fig. 4E). Fig. 4F,G shows more drastically disorganized leaf primordia with poorly differentiated or undifferentiated cells localized to the adaxial side. Secondary adventitious shoots also bulged out from growing ectopic shoots leading to drastic disorganization of the primary shoot apex (Fig. 4H) and plant morphology (Fig. 3).

3.4. Meristem regulatory gene expression

We chose to compare three transgenic lines (two with the phenotypes shown in Fig. 3B,C and one without) to the wild-type plant for expression of meristem regulatory genes. Three independent sets of Northern blots for each probe were performed. One set is presented in Fig. 5. The steady mRNA

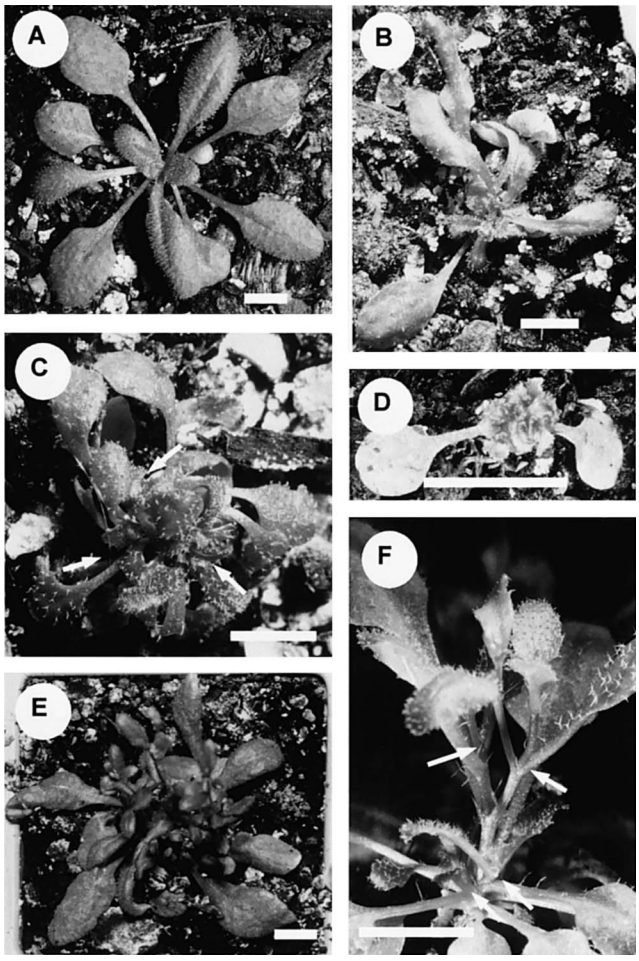

Fig. 3. Phenotypes of *Arabidopsis* plants overexpressing *TBP-2*. A: Wild-type plant control. B: A plant from line 3-16. Note the first pair of leaves appears normal. C: A plant from line 3-11. D: A plant from line 3-14, showing unaffected cotyledons and extremely small and deformed leaves. E: A plant from line 3-16 showing multiple branches. F: Lateral view of a plant from line 3-13. Arrows indicate axillary shoots. Bar = 0.5 cm.

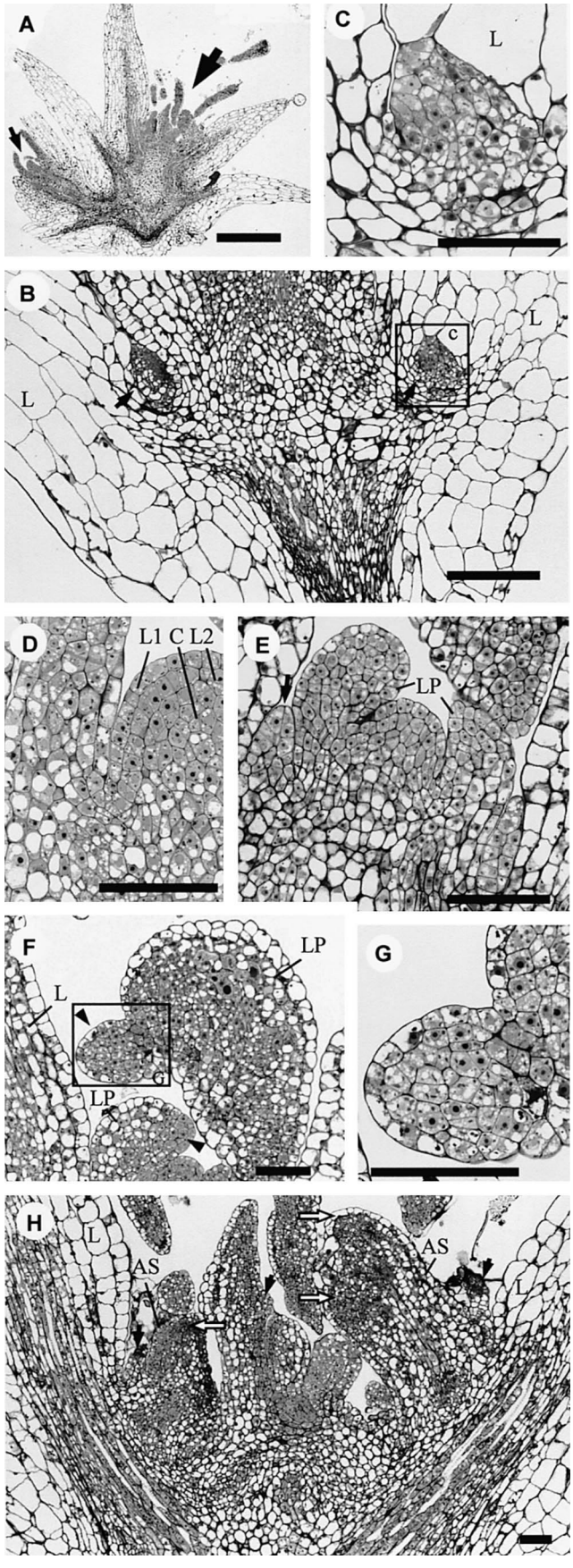

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Fig. 4. Shoot proliferation in *Arabidopsis* plants over-expressing *TBP-2* gene. A: Overall view of an apical section of a plant from line 3-14 showing the primary vegetative SAM (large arrow) and a precocious axillary shoot (small arrow) which was formed from the axial of one of the first leaves. Bar = 1 mm. B and C: Longitudinal section of a secondary rosette of the same plant as in (A) showing two developing tertiary axillary meristems (arrows). Bar = 0.5 mm (B) or 50 μm (C). D: Longitudinal section of a secondary shoot meristem of a plant from line 3-14 with normal cellular organization: layers 1 and 2 (L1, L2) and corpus (C). Bar = 50 μm. E: Longitudinal section of a secondary shoot meristem of a plant from line 3-17 with developing meristem-like structures (arrows) and poorly differentiated leaf primordia (LP). Bar = 50 μm. F and G: Longitudinal section of a secondary shoot meristem of a plant from line 3-11 with developing meristem-like structures (arrows) directly from disorganized leaf primordia (LP) with undifferentiated cells. Bars = 50 μm. H: Overview of the primary shoot apex of the same plant as in (A), showing two disorganized ectopic shoot (AS) on which secondary ectopic meristem-like structures form (open arrows). Arrows indicate supernumerary meristem-like activities. L: leaf. Bar = 50 μm.

level of *STM*, a homeobox gene required for meristem formation and maintenance [19], was actually reduced in highly *TBP-2*-overexpressing (or phenotypic) lines (Fig. 5). Similar results were obtained by RT-PCR (not shown). In the case of *KNAT1*, a *STM*-related homeotic gene, there are two species of mRNA produced from two transcription start sites [20]. Northern blot experiments revealed that the first start site was slightly inhibited, while the second one was strongly stimulated by *TBP-2* overexpression (Fig. 5).

The mRNA level of *CLV1* that negatively regulates the proliferation of meristem cells [21], was reduced in two of the transgenic lines. The reduction does not correlate with *TBP-2* expression levels and phenotype production. The mRNA level of *ZLL*, a gene required to maintain stem cells of the developing shoot meristem in an undifferentiated state [22], showed no clear difference between the samples (Fig. 5). In order to know whether *TBP-2* overexpression stimulated cell cycle-related genes, the expression of CycD3 [23] was examined. It was clearly activated in one of the examined transgenic plants (Fig. 5).

Taken together, *TBP-2* overexpression altered mostly the expression of *STM* and *KNAT1*, while had no major impact on the other tested genes.

3.5. *KNAT1* basal promoter analysis

Sequence analysis of the *KNAT1* promoter region found a TATA box 14 bp upstream of the start site of the smaller transcript (Fig. 6A). No obvious TATA box-containing sequence could be found upstream of the other start site, suggesting that a TATA-less promoter controls transcription from this site. In order to confirm this hypothesis, gel shift assays using recombined TBP-2 was performed. As shown in Fig. 6B, a 200-bp fragment containing the TATA box sequence was bound efficiently by TBP-2, while no binding signal could be detected with the region surrounding the upstream start site. This region is very rich in nucleotide T and C, which is characteristic for TATA-less promoters.

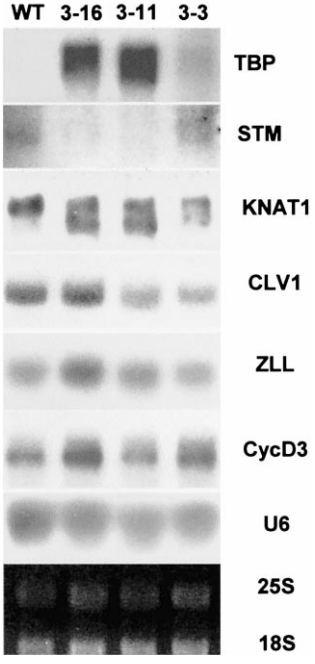

Fig. 5. Meristem regulatory gene expression in *Arabidopsis* plants overexpressing *TBP-2*. Equal amounts (20 μg) of total RNA extracted from aerial parts of wild-type plants and three transgenic lines: two (3-11 and 3-16) with severe phenotypes shown in Fig. 3C,D and one (3-3) without obvious phenotypes were hybridized with probes as indicated. U6 RNA (transcribed by RNA polymerase III) and rRNA (25S and 18S, transcribed by polymerase I) levels revealed either by Northern blot or by ethidium bromide staining were shown.

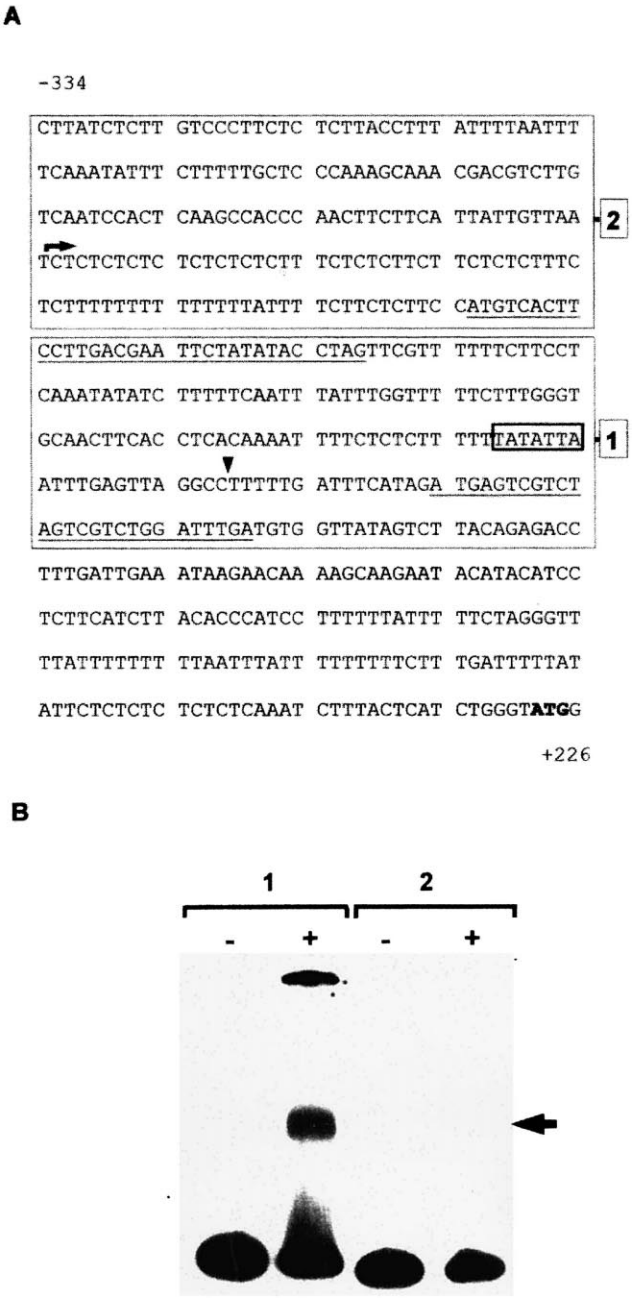

Fig. 6. *KNAT1* is controlled by a TATA-containing and a TATA-less promoter. A: DNA sequence upstream the coding region of KNAT1 (accession number: AJ131822). The initiation codon is indicated boldface type. The downstream transcriptional start site is indicated by a vertical arrow, the upstream site by a horizontal one. The small open reading frames are underlined. The TATA sequence is boxed. Sequences in zone 1 and 2 were used as probes in (B). B: Gel shift assays with sequences 1 and 2 described in (A) as probes in the presence (+) or absence (−) of recombined TBP-2 protein. Arrow indicates a TBP–DNA complex.

4. Discussion

Up-regulation of *TBP-2* in *Arabidopsis* apical shoots (Fig. 1) suggests that TBP-2 protein in a higher level might be needed for apical meristem function. In *TBP-2*-overexpressing plants, ectopic meristem-like structures arose essentially from highly undifferentiated leaf primordium (Fig. 4F,G) or later from young ectopic shoots during more advanced vegetative growth phases (Fig. 4H). These observations suggest that higher TBP-2 protein levels may prevent or delay cell differentiation in leaf primordium or young shoot cells and change their fate to develop into meristem-like structures, although their meristem cell identity is not confirmed. This effect is reminiscent of that induced by ectopic expression of *KNAT1* which induces shoot formation on the leaf surface in *Arabidopsis* [20,24]. This would correlate with the induction of *KNAT1* in TBP-overexpressing plants. However, *KNAT1* overexpression induces ectopic meristem formation in the sinus region close to veins. Furthermore, those plants produce lobed leaves [24] which were not observed in *TBP-2*-overexpressing plants. One possible explanation of the phenotypic differences is that the cell type-specific expression of additional factors needed for *KNAT1* function might be altered by *TBP-2* overexpression. Surprisingly, the *STM* mRNA levels were reduced in *TBP-2*-overexpressing plants (Fig. 5). The meaning of the reduction is not clear at this stage. It has been shown that weak alleles of *stm* also also the formation of extra branches due to meristem fragmentation [24]. The down-regulation of *STM* by *TBP-2* overexpression would be partially responsible for the phenotype of the transgenic plants.

Cytokinin is involved in the meristem activity. However, it is unlikely that *TBP-2* overexpression led to an increase of cytokinin level. First, *STM*, previously shown to be induced by increased levels of cytokinin [25], was not activated in *TBP-2*-overexpressing plants. Second, the expression of *CycD3*, a G1-specific cyclin gene that is involved in the activation of cell division in *Arabidopsis* and is stimulated by cytokinin [23], was not generally affected, though a clear induction was observed in one of the transgenic lines (Fig. 5).

TBP overexpression also altered the expression of many different genes as found by differential display (not shown). It is therefore highly possible that the phenotypes seen in the transgenic plants may be due to the altered overall gene expression profile induced by TBP overexpression.

4.1. TBP-2 protein and KNAT1 transcription

Differential activation of transcription from the two start sites of *KNAT1* by higher TBP levels indicates differences in the functioning of the corresponding promoters. For instance, the transcription from the downstream site seems to be more responsive to higher cytokinin levels [25]. This suggests that transcription from the downstream start site is more subject to a response to cellular and hormonal signals than the upstream one. Gel shift assays showed that TBP-2 binds to the sequences surrounding the downstream start site, but not the upstream one (Fig. 6), indicating that a TATA box-containing promoter directs transcription from the downstream site, while the upstream site is driven by a TATA-less one or an initiator. TATA-less promoters can be bound only by a TBP-containing TFIID complex but not by TBP alone [6]. It has been found that TBP can be rate-limiting in vivo for TATA-containing, but not TATA-lacking RNA polymerase II promoters [14,26]. Therefore, the TATA-containing promoter of *KNAT1* belongs to those whose activity depends on cellular TBP concentration. Higher levels of TBP protein would increase, presumably by interacting with a specific factor(s) which would respond to cell type signals or hormones, the recruitment of the RNA polymerase II to the downstream start site. This would lead to an increase in the transcription initiation rate in highly undifferentiated cells.

References

[1] Lee, T.I. and Young, R.A. (1998) Genes Dev. 12, 1398–1408.
[2] Orphanides, G., Lagrange, T. and Reinberg, D. (1996) Genes Dev. 10, 2657–2683.
[3] Patikoglou, G.A., Kim, J.L., Sun, L., Yang, S.H., Kodadek, T. and Burley, S.K. (1999) Genes Dev. 13, 3217–3230.
[4] Gasch, A., Hoffmann, A., Horikoshi, M., Roeder, R.G. and Chua, N.-H. (1990) Nature 346, 390–394.
[5] Heard, D.J., Kiss, T. and Filipowicz, W. (1993) EMBO J. 12, 3519–3528.
[6] Goodrich, J.A., Cutler, G. and Tjian, R. (1996) Cell 84, 825–830.
[7] Kuras, L., Kosa, P., Mencia, M. and Struhl, K. (2000) Science 288, 1244–1248.
[8] Li, X.Y., Bhaumik, S.R. and Green, M.R. (2000) Science 288, 1242–1244.
[9] Kuras, L. and Struhl, K. (1999) Nature 399, 609–613.
[10] Li, X.-Y., Virbasius, A., Zhu, X. and Green, M.R. (1999) Nature 399, 605–609.
[11] Bell, P. and Scheer, U. (1999) Exp. Cell Res. 248, 122–135.
[12] Persengiev, S.P., Robert, S. and Kilatrick, D.L. (1996) Mol. Endocrinol. 10, 742–747.
[13] Perletti, L., Dantanel, J.C. and Davidson, I. (1999) J. Biol. Chem. 274, 15301–15304.
[14] Colgan, J. and Manley, J.L. (1992) Genes Dev. 6, 304–315.
[15] Trivedi, A., Vilalta, A., Gopalan, S. and Johnson, D.L. (1996) Mol. Cell. Biol. 16, 6909–6916.
[16] Le Gourrierec, J., Li, Y.F. and Zhou, D.X. (1999) Plant J. 18, 663–668.
[17] Bechtold, N., Ellis, J. and Pelletier, G. (1993) C.R. Acad. Sci. 316, 1194–1199.
[18] Li, Y.-F., Le Gourrierec, J., Torki, M., Kim, Y.-J., Guerineau, F. and Zhou, D.-X. (1999) Plant Mol. Biol. 39, 515–525.
[19] Long, J.A., Moan, E.I., Medford, J.I. and Barton, M.K. (1996) Nature 379, 66–69.
[20] Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K. and Hake, S. (1994) Plant Cell 6, 1859–1876.
[21] Clark, S.E., Jacobsen, S.E., Levin, J.Z. and Meyerowitz, E.M. (1996) Development 122, 1565–1575.
[22] Moussian, B., Schoof, H., Haecker, A., Jügens, G. and Laux, T. (1998) EMBO J. 17, 1799–1809.
[23] Riou-Khamlichi, C., Huntley, R., Jacqmard, A. and Murray, J.A.H. (1999) Science 283, 1541–1544.
[24] Chuck, C., Lincoln, C. and Hake, S. (1996) Plant Cell 8, 1277–1289.
[25] Rupp, H.M., Frank, M., Werner, T., Strnad, M. and Schmulling, T. (1999) Plant J. 18, 557–563.
[26] Malello, B., Napolitano, G., De Luca, P. and Lania, L. (1998) J. Biol. Chem. 273, 16509–16516.