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ANALYSIS OF THE BARLEY (*Hordeum vulgare*) TIGHTLY BOUND DNA-PROTEIN COMPLEXES

Summary of doctoral dissertation

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MIEŽIŲ (*Hordeum vulgare*) TVIRTŲ DNR-BALTYMŲ KOMPLEKSŲ TYRIMAS

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INTRODUCTION

Eukaryotic chromosomes are thought to be organized into a higher-order structure consisting of discrete and topologically independent loop domains that are attached to the nuclear matrix. The loop organization of chromosomes may be important not only for compaction of chromatin fiber, but also for regulation of gene expression and replication. Polypeptides involved in specific structural organization and modeling of the chromatin fibres and chromosome territories belong, as expected, to the nuclear matrix proteins (for the most part insoluble) with a strong DNA affinity (Berezney, 2002; Cockell and Gasser, 1999; Foster and Bridger, 2005; Trinkle-Mulchany and Lamond, 2008). Functions and existence of the nuclear matrix is still debated object, but it is clear that in chromatin packaging and reorganization during cell cycle are involved proteins which tightly bound to DNA.

The polypeptides that are able to form permanent or transient tight complexes with DNA, including covalent ones, are of special interest. These proteins cannot be detached from DNA by standard deproteinization procedures or by treatment with strong dissociating agents such as sarkosyl, urea, guanidine chloride, etc. (Neuer-Nitsche and Werner, 1983). It has been demonstrated that serpins Spi-1, Spi-2, Spi-3 (Rothbarth et al., 2001b), 16 kDa protein C1D (Rothbarth et al., 1999), some polypeptides with phosphatase and kinase activities and other still not examined polypeptides (Avramova et al., 1994; Juodka et al., 1995) belong to TBP proteins of tightly bound DNA–protein complexes. Very similar group of TBPs were found in yeast and MEL TBP-DNA complexes (Borutinskaite et al., 2004; Labeikyte et al., 1999). These polypeptides also exhibited phosphatase and kinase activity.

At 1957-1959 was hypothesized that interaction between DNA and proteins can be covalent. It was shown recently that 0.1-0.3% of TBPs are bound to DNA with phosphotriester bond (Juodka et al., 1991; Labeikyte et al., 2002; Tsanev and Avramova, 1994). It is supposed that such polypeptides are important for differentiation and regulation of gene activity.

It has been shown earlier that the DNA presented in tightly bound DNA–protein complexes in higher eukaryotic organism's exhibit some properties of S/MAR. These sequences have a high AT content and belong to the Alu family of sequences (Neuer-Nitsche et al., 1988; Sjakste, 1997), however some heterogeneous sequences and

sequences, which correspond to the medium reiteration frequency MER sequences were also found (Avramova et al., 1994). It is known that DNA sequences harboring tightly bound proteins is actively transcribed. TBP distribution along the DNA depends on cell type (Razin et al., 1988). It is supposed that TBP-DNA complexes can bind temporalily to nuclear matrix which has some acceptor structures required for maintenance of this interaction (Labeikyte et al., 1995; Pfutz et al., 1992; Razin et al., 1995).

Despite a great deal of research, the functional significance of tightly bound DNA-protein complexes is not yet clear, therefore these complexes are perfect object for pioneering research. It is little known also about vegetal cell nucleus structure and functions. Plant cell TBP-DNA complexes were chosen as object for this investigation. Plant genomes possesses some peculiarities compared to animal genomes. The barley (*Hordeum vulgare*) is a useful model for plant development studies. Etiolated barley seedlings contain cell populations with different proliferation, differentiation and senescence status (Kirnos et al., 1983).

The goals of the present study was to isolate and characterize TBP proteins from barley (*Hordeum vulgare*) different developmental stages and different organs (first leaves, coleoptile and roots) and to characterize DNA fragments from barley first leaves tightly bound DNA-protein complexes.

THE MAIN OBJECTIVE

To investigate tightly bound DNA-protein complexes from barley (*Hordeum vulgare*) and to establish their role in chromatin function.

THE OBJECTIVES OF THE STUDY WERE:

1. To isolate and characterize tightly bound DNA-protein complexes from barley different developmental stages and different shoot organs: first leaves, coleoptiles and roots.

2. To characterize protein moiety of TBP-DNA complexes from different organs and at developmental stages.

3. To characterize specificity of interactions between TBP and DNA sequences in TBP-DNA complexes of different organs and at different development stages.

4. To isolate and characterize DNA sequences from barley first leave and grain TBP-DNA complexes.

5. To establish the redistribution of DNA sequences from grain TBP-DNA complexes along alpha amylase and beta amylase genes. To examine the role of these DNA sequences in expression of alpha amylase and beta amylase genes during grain development.

TOPICALITY OF THE RESEARCH

Despite a great deal of research, the functional significance of tightly bound DNA-protein complexes is not yet clear, therefore these complexes are perfect object for pioneering research. Very little is known about plant TBP-DNA complexes.

In this work we investigated barley TBP-DNA complexes from different organs (first leaves, roots and coleoptiles) at different developmental stages. We characterized individual components of tightly bound DNA-proteins complexes: polypeptides (TBP) and DNA. We isolated and characterized TBP proteins from barley first leaves, roots and coleoptiles of different age and differentiation stage. Also we isolated and characterized the DNA fragments from barley first leaves and water ripe and milky ripe grain TBP-DNA complexes.

We demonstrated that in different developmental stages of coleoptiles, first leaves and roots TBP-DNA complexes were identified as a group of 15-160 kDa proteins, most of TBPs are acidic. Some of barley TBPs (10, 25, 38, 40 and 55 kDa) exhibit phosphatase, maybe Ser/Thr activity. We have identified also that some of TBPs tyrosines were phosphorylated, this modification depends on organ and developmental stage. Identified barley TBPs were involved in fundamental genetic processes, as well as in chromatin rearrangement and regulation processes. Nuclear matrix proteins, enzymes, transcription factors, serpins, immunophilins, and transposon polypeptides were identified among TBPs. We demonstrated that expression of TBPs depends on organ and developmental stage. TBPs manifested high affinity to DNA fragments from the same organ TBP-DNA complexes.

We established that the DNA sequences from TBP-DNA complexes are similar to S/MAR, but have peculiar traits. Most of sequences are localized in subtelomeres, telomeres, long arm of chromosomes (which chromosomes?). DNA sequences forming

TBP-DNA complexes are homologous to various transposons, retrotransposons and repeat sequences. These sequences are heterogeneous, but have some motifs, that are common for all of them: ori motifs, topoisomerase II binding sites, short A+T rich stretches, curved DNA stretches. These sequences are rich in transcription factors binding sites. Our results show that examined sequences are specific. They have some characteristics of S/MAR sequences and they may be important for maintenance of DNA high-order structure and for regulation of gene expression.

By means of hybridization between the DNA fragments of TBP-DNA complexes and microarray of barley *Amy32b* and *Bmy1* genes we demonstrated that barley TBPs interact with *Amy32b* gene promotor and with *Bmy1* gene Exon III, Intron III 1.5 kb region. In case of both genes, interaction with TBP proteins decreases in the course of grain development (during transition from water ripe to milky ripe stage). The changes in TBPs distribution in the genes were coupled to changes in their expression during water ripe and milky ripe stages. Effect of TBPs binding on gene expression are different – *Amy32b* expression increases, but *Bmy1* expression decreases.

We suppose that obtained results about tightly bound DNA-protein complexes can help to answer some important questions, for example, TBPs and nuclear matrix interaction, TBPs localization in nucleus, TBPs composition and functions, tightly bound to TBP DNA sequences composition and functions in genome. Performed research allows us to better understand plant cell nucleus and interactions between nuclear proteins and DNA.

MATERIALS AND METHODS

Bacterial strains and plasmids. To prepare competent cells was used *E. coli* strain JM 107 (F'tra D 36 pro AB[+] lacY lacZ δM15/δlac–pro end A1 gyr A 96 thi-1 hsd R17 sup E44 re/A1). As cloned DNA vector was used *E. coli* plasmid pUC 19 (GenBank/EMBL accession L09137).

Genes. GenBank accession X05166 sequence information was used to develop the microarray and to analyze data on the barley *Amy32b* gene (Whittier ir kt., 1987). GenBank accession AF061203 sequence information deposited for *Adorra* cultivar was used in this study for *Bmy1* gene. The previously reported data (Sjakste ir Zhuk, 2006)

indicate the identity of the selected gene sequences in the *Adorra* and *Balga* cultivars used in this study.

Table 1. *Amy32b* array oligonucleotide sequences.

Nr.	Sequences
1	5'-CCTCCCACGTTTATCTTCAATTTGTCAAAAAAATCATGTTCGGACCGTT-3'
2	5'-CAAAAGGTATATCCTGCGTAATATTTCTGTTACTGCACCACATTAAGAACAGTTTATATG-3'
3	5'-CAACGCTGGGTGATCCCAGCTTGGATAGTGCTATCTTTTCCCATGGAATTTGTGCCGGCC-3'
4	5'-TATCCATGCAGTGCCTCCAAGCAACACTCCACGGGGACGTAGCTCGTGTT-3'
5	5'-CAGTCTTGTGAATCATTCATCCACAGAACAAGAGTGCAGCGAACAGTGTAGATC G-3'
6	5'-CAACGAAGGTCCCTCTTCACACTAAAATCATTCGTGTCTCAACTGAACATC-3'
7	5'-AACGTGCAAATACGATCAAACAAGTATACAGTATACTGTACAAACTAAAAC-3'
8	5'-GACCACCTCAACGACCGCGTCCAGCGCGAGCTCAAGGAGTGGCTCCTCTGGCTCAAGAGC-3'
9	5'-CATGTGGCCATTCCCCTCCGACAAGGTCATGCAAGGCTACGCATACATCC-3'
10	5'-AATCATTCAGGAAACTAAAAATCTCTTGTCTTGTCGGTTTGCAGTTCTACGACCATTTCTTT-3'
11	5'-AAGCCTCCACTCATCCACCATTCAATCGAGCATGCATGAATTTTCCAAAATAATG-3'

Table 2. *Bmy1* array oligonucleotide sequences.

Nr.	Sequences
1	5'-ACGTTTGAACATTAACGTGTGTTTTTGGTGAAAGTGAAAAAATAGTTGAC-3'
2	5'-ATAATTGGTGAGGCACATTCTCATTTGATTGGTTAGTTTAACTTCCTTGTCACATTATTT-3'
3	5'-AAAAAAAAACAAATTTAGGATGATATTTTGGGGTAACTTTTGGTGTTCAATTTGTTTTTTT-3'
4	5'-ATGTGTGCGTCTTCACTTCGTATAGGGTGCCGTTTGGTTGAGAGTTGAGA-3'
5	5'-ACTATTTCAAGGATCTAGTGCACACATATACATTATTGTTGTACATATAACATTGATACT-3'
6	5'-CACCACTCTAGTTCTCTGATGCATATTTATATAGAAGTTCAAGATGACACCAAATACAAGC-3'
7	5'-TGGTGTTATCGTCGACATTGAAGTGGGACTTGGCCCAGCTGGAGAGATGAGGTACCCATC-3'
8	5'-AGAAATATATAGGATTCATCTGTGCAACTTAAATACTTAAAATGATTTTT-3'
9	5'-TTAAATTTTTAAATTGAGTGTCTTGGGTCTTGAATTTAAGACCTTTTGACTCGGATACCA-3'
10	5'-CTATGCATTTATACTTCAACAATAAGAATAGTGAGGTAGCAC-3'
11	5'-AACAACAAAAATACACAAAAC TATCCAGGCTAAGGGAACTCGCATTGCTTA-3'
12	5'-ATTTGTTGATTTGCAGGTGCCTATTATATACTAATAATTTAATTTTATTGTTTTCAGCCT-3'
13	5'-AGGCTGAAGGCCCCACCTGTGGCATGGGTGGGCAAGTTAAAGGCCCTACT-3'

Plant material. Seeds of the barley cultivar "Auksiniai 3"were obtained from the Botanical Garden of Vilnius University (Kairėnai, Lithuania). Etiolated shoots were grown for 3–5 days at a constant temperature (24°C) in the dark. Coleoptiles, first leaves and roots were dissected from shoots of Zadoks stage 07 and 10 developmental stages. Dissected coleoptiles, roots and first leaf tissue was combined into one sample for each tissue at both developmental stages and used for bulk DNA extraction. Seeds (barley cultivar *Balga*) of watery ripe (Zadoks 71) and medium milk development (Zadoks 75) stages were collected in the field of the Latvian State Priekuli Crop Breeding Institute. 70 – 100 mg of seed tissue was used for bulk DNA isolation (DNA isolation of Zadoks stages 71 and 75 was performed by Olga Sugoka according to chlorophorm-isoamylic alcohol extraction (Plaschke et al., 1995)). The classification here and further has been done according to Anderson (Anderson et al., 1995).

DNA isolation from barley Zadoks 07 and Zadoks 10 development stages. Plant tissues were frozen in liquid nitrogen and ground in a mortar up to a fine powder. DNA from plant material was extracted according to the previously described protocol of a chlorophorm-isoamylic alcohol extraction (Plaschke et al., 1995) with some modifications. Cells were suspended with (1: 1.6 V/V) extraction buffer (100 mM Tris/HCl, pH 8.0; 500 mM NaCl; 50 mM EDTA; 1.25% SDS) and incubated at 65 °C for 30 min. Then extraction with chlorophorm-isoamylic alcohol (24:1) (1:1 V/V) was performed, the mixture was centrifuged at 4 °C for 15 min at 2800 g. DNA was precipitated with cold ethanol (1:2 V/V), centrifuged for 30 min at 2800 g, rinsed with 70% ethanol and air-dried. Dry DNA was dissolved in 3 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Then 10μg/1ml of Rnase A (Serva) solution was added, and digestion was performed for 3 h at room temperature. In the following step, DNA solution was extracted with 3 ml of chlorophorm-isoamylic alcohol mixture (24:1) (1:1 V/V) and centrifuged at 2800 g in a cooled rotor (4 °C). DNA was precipitated with ethanol (1:2 V/V) and 3 M sodium acetate (1:1/10 V/V) and collected by centrifugation at 4 °C, 30 min/9000 g, then rinsed with 70% ethanol and air-dried. Dried DNA was dissolved in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). DNA solution was stored at 4 °C.

Purification of TBP-DNA complexes in dialysis condition. Isolated DNA (5 mg/ml) was diluted with benzonase buffer and then was added benzonase (Merck) (0.1 U/1 μg DNA). Reaction was continued in dialysis conditions 16 hours at room temperature against 1-1.5 l benzonase buffer (50 mM Tris-HCl, pH 8.0; 1 mM MgCl$_2$). Subsequently dialysis was continued 16-18 hours at 4^{0}C against 1-1.5 l TE buffer.

Isolation of grains TBP-DNA complexes by means of fractionation on nitrocellulose. For microarray experiments DNA was fragmented by sonication in a SONICS Vibra Cell^{TM} device (Sonics & Materials, 53 Newtown, USA) with 60% output for a period of time ranging from 30 seconds to 4 minutes. Completeness of DNA digestion or extent of fragmentation was tested by gel electrophoresis. DNA samples with average fragment sizes of about 500 bp were diluted in 3 ml of filtration buffer (0.5 M KCl; 5 mM EDTA; 10 mM Tris- HCl, pH 7.4) to a final concentration of 25-70 μg/ml. The solution was pressed through a nitrocellulose filter (HAWP 025 00, HA 0.45 μm, 25 mm, Millipore or NC 45, 0.45 μm, 25 mm, Whatman (Schleicher & Schuell)

presoaked with filtration buffer and supported in a Swinnex holder. The filter was washed up to five times with the same 3 ml volume of filtration buffer to avoid contamination between fractions. Filtration buffer wash off resulted in filtered DNA fraction (F). The filter retained a DNA fraction enriched in the tightly bound proteins; it was eluted with five washes of 3 ml each of 5 mM EDTA; 10 mM Tris HCl, pH 7.4 (low-ionic strength eluted fraction R1) and 3 ml of 50 mM NaOH (alkali-eluted fraction R2) in sequence (Werner and Neuer-Nitsche, 1989). The DNA content in the F, R1 and R2 fractions was measured spectrophotometrically. Then in the united R fraction was added proteinase K (Roth) to final concentration 0.5 mg/ml and 10% SDS to final concentration 0.5%. Solution was incubated at 37^0C 16 hours then extracted with phenol and chlorophorm. DNA was precipitated with addition of two volume ethanol (1:2 V/V). Obtained DNA fragments were analyzed with electrophoresis (in 1% agarose gels). Obtained R and F fragments were used for hybridization with oligonucleotide microarrays.

Isolation of DNA fragments from TBP-DNA complexes. Into solution of TBP-DNA complexes (2 μg/μl) (received after dialysis, sec. 2.5.) was added proteinase K (Roth) to final concentration 0.5 mg/ml and 10% SDS to final concentration 0.5%. Solution was incubated at 37^0C 16 hours then extracted with phenol and chlorophorm. DNA was precipitated with addition of two volume ethanol (1:2 V/V). Obtained DNA fragments were analyzed with electrophoresis (in 1% agarose gels).

Cloning of the DNA fragments from TBP-DNA complexes. The residual phenolyzed and ethanol-precipitated DNA were blunt-ended and ligated with the pUC19/*Sma*I vector. Transformation of *Escherichia coli* XLI-blue competent cells resulted in colonies containing recombinant plasmids with inserts which were analyzed by conventional techniques (Curr. Protocols in Mol. Biol., 1994; MBI "Fermentas", 2005). Eleven clones were picked at random and sequenced by the dideoxy chain-termination method.

Evaluation of DNA fragments. Cloned DNA fragments (1-8) were sequenced in UAB Fermentas. Remained DNA fragments (9-11) were obtained with the help of Dr. Tatjana Sjakste in Germany. GenBank accession numbers of these sequences are: GS504199, GS504200, GS504201, GS504202, GS504203, GS504204, GS504205, and GS504206.

Bioinformatics resources. Eleven DNA sequences were compared with known sequences from the GenBank by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/, with Advanced BLAST (Basic Local Alignment Search Tool 2.0 program), also these DNA sequences were analyzed with such databases like http://www.genomatix.de/ (GEMS Launcher 5.0), http://www.futuresoft.org/Mar-Wiz, http://www.tubic.tju.edu.cn/Ori-Finder/, http://zlab.bu.edu/repfind/. Transcription factor binding site localization in DNA sequences was analyzed with https://www.genomatix.de/cgi-bin/matinspector_prof/ (MatInspector program).

Analysis of DNA sequences from TBP-DNA complexes using DNA array method. After fractionation on nitrocellulose membrane, different DNA fractions (F, R1 and R2) were labeled with [α^{32}P]dATP (Hartmann Analytic) using a HexaLabel^{TM} DNA Labeling Kit (Fermentas). Unincorporated nucleotides were removed by selective precipitation of DNA with ethanol in the presence of ammonium acetate. *Amy32b* (GenBank accession X05166) and *Bmy1* (GenBank accession AF061203) genomic sequence information was used to design the arrays. The DNA array for each gene was developed according to the published approach (Bielskiene et al., 2008; Ioudinkova et al., 2005), and consisted of 50-60 bp oligonucleotides spaced along the whole gene. Sequences of the oligonucleotides (Metabion) are given in Table 1 and Table 2, their location in the structural genes is shown in Figures 9 and 10, for *Amy32b* and *Bmy1* genes, respectively. The oligonucleotides had a similar T_m. Prior to hybridization the oligonucleotides were analyzed *in silico* to avoid repetitive DNA sequences. The oligonucleotides were slot-blotted onto membrane Hybond Zeta Probe GT (BIO-RAD) with Minifold I Spot-Blot System (96-spots) (Whatman, Schleicher & Schuell) using 0.4 M NaOH and 2x SSC and fixed by baking at 80°C for 2h. The hybridization was carried out at 65°C in modified Church buffer (0.5 M phosphate buffer, pH 7.2; 7% SDS; 10 mM EDTA) overnight. The blot was washed subsequently in 2x SSC, 0.1% SDS twice for 30 min, then in 1x SSC, 0.1% SDS for 30 min. The blots were exposed to Phosphorimager Fuji FLA-5100 for 3-12 h. Data were analyzed with program Fuji Film Image Gauge 4.0. The average of three independent experiments (two hybridizations per experiment) is presented. The signal of the total DNA hybridization was subtracted from the F and R fraction hybridization signals. Data are presented as R/F ratio of hybridization signals.

SDS-PAGE gel electrophoresis. 12% SDS-PAGE gels were run at 120 V constant voltage for 4 h (Laemmli, 1970) and then stained with Coomassie brilliant dye.

Western blot. Samples of proteins were fractionated in denaturing polyacrylamide gels. After electrophoresis PAGE gel and 0.45 µm nitrocellulose filter (BIO-RAD) were incubated 15 min in transfer buffer (19 mM glycine; 25 mM Tris; 0.1% SDS, and 20% methanol). Then was prepared transfer "sandwich" – Whatman 3MM paper, nitrocellulose filter, gel, Whatman 3MM paper. Electroblott was processed 3 hours at room temperature by supporting constant 200 mA current.

Establishment of TBPs phosphatase activity using MUP. As substrate for phosphatases was used 2mM MUP (Boehringer Mannheim). Hydrolysis of MUP was observed fluorimetrically (stimulation and emission wave were 340 nm and 424 nm accordingly) in 300 µl (50mM Tris-HCl, pH 7.5) buffer 30 min.

Establishment of TBPs phosphatase activity *in situ*. TBP proteins were fractionated in denaturing polyacrylamide gels and electroblotted onto nitrocellulose filters (sec. 2.8.). Protein blots were incubated overnight at 4ºC in renaturation buffer (100 mM Hepes (Roth); 0.2% Chaps (Roth); 10 mM $MgCl_2$; 50 mM KCl; 1% BSA (Roth), pH 7.4). Phosphatase activity was established *in situ* by incubation of protein blots in solution containing 50 mM Tris-HCl, pH 7.0; 0.5 % agarose; 50 µg/ml BClP (Sigma) and 1 mg/ml NT (Sigma) at 2-3 hours. Proteins with phosphatase activity were colored in blue.

TBPs immunoblott with anti-phosphotyrosine (clon 4G10). After digestion with benzonase TBP proteins were electrophoretically separated in one dimensional 12% SDS-polyacrylamide gel. Proteins were electroblotted from the gel onto 0.45 µm nitrocellulose filter (BIO-RAD) in 19 mM glycine, 25 mM Tris, 0.1% SDS, and 20% methanol. Protein blotters were washed with water and blocked with fresh TBS-BSA solution (50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween; 5% BSA) 1 hour at room temperature. Subsequently blotters were incubated with 0.5-2 µg/ml primary anti-phosphotyrosine antibodies (Upstate Biotechnology/Millipore) diluted with fresh TBS-BSA solution (50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween; 5% BSA) overnight at 4ºC. Then blotters were washed with TBS without BSA one time 15 min, two times 5 min at room temperature and incubated with secondary antibodies – anti-

mouse, conjugated with horseradish peroxidase (Santa Cruz Biotechnology) (diluted 1:10000) in TBS-BSA solution (50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween; 5% BSA) 1.5 hours at room temperature. Next blotters were washed three times 15 min with TBS, one time 15 min with water. Detection was proceeded using ECL reagents (Amersham Biosciences) and autoradiographed.

TBP immunoblot with anti-topoisomerase II α. Immunoblot was proceeded as noted above with anti-phosphotyrosine. Were used primary anti-topoisomerase II α antibodies (Santa Cruz Biotechnology) (diluted 1:5000), secondary antibodies – anti-rabbit, conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Detection was proceeded using ECL reagents (Amersham Biosciences) and autoradiographed.

2DE gel electrophoresis (IEF/SDS-PAGE). First the samples (20-50 µg TBP proteins) were cleaned with 2-D Clean-Up Kit (Amersham) according to manufacturer's recommendation. The samples were dissolved in a sample buffer (12 g urea; 50 mg DTT; 0.13 ml pharmalit 3–10; 0.13 ml Triton X-100; water until 25 ml). For the first dimension, Immobiline Dry Strips 4–7 pH (13 cm) were used (Amersham Biosciences). Dry strips were rehydrated, reduced and alcylated according to manufacturers' recommendations. For the second dimension 8–18% Excell gels (Amersham Biosciences) were used. IEF / SDS-PAGE were performed with a Multiphor II device (Amersham Biosciences). SDS buffer strips: anodal (0.45 mol/l Tris-acetate, pH 6.6; 4 g/l SDS; 0.05 g/l Orange G), cathodal (0.08 mol/l Tris; 0.80 mol/l Tricine and 6 g/l SDS, pH 7.1). Gels were visualized with Coomassie brilliant dye and silver (Shevcenko ir kt., 1996).

Tryptic digestion in-gel. The areas of the gel that had been deemed to be of interest were cut out and subjected to in-gel tryptic digestion overnight (Shevcenko et al., 1996), the gel slides were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini, Epperdorf). The protein spots were rehydrated in 20 µl of 25 mM ammonium bicarbonate (pH 8.3) containing 20 µg/ml of modified trypsin (Promega). Once this solution had been fully absorbed by the gel, a trypsin-free buffer was added just enough to cover the slice, and the samples were incubated overnight at 37 °C. The tryptic peptides were subsequently extracted from the gel slides as follows. Any extraneous solution remaining after the digestion was removed and placed in a fresh tube. The gel slides were first subjected to an aqueous extraction

and then to organic extraction with 5% trifluoracetic acid in 50% acetonitrile, shaking occasionally. The digestion and extract solutions were then combined and evaporated to dryness.

Mass-spectrometry (MALDI TOF-TOF MS). For the MALDI TOF-TOF (Matrix-Assisted Laser Desorption/Ionization tandem Time-Of-Flight) analysis the peptides were redissolved in 3 μl of 50% acetonitrile and 0.1% trifluoracetic acid solution and then prepared with a matrix (α-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed on a 4800 Plus MALDI TOF-TOF™ analyzer (Applied Biosystems) (in Lithuanian Proteomic Centre) and externally calibrated using synthetic peptides with known masses (4700 Cal Mix 1, Applied Biosystems). The MS spectra were obtained in the positive ionization mode at 3.080 kV, and the MS / MS spectra were obtained in the positive ionization mode at 3.780 kV. The mass information generated from the composite spectrum was submitted to a search performed with the MSDB and UniProtKB-SwissProt databases, using the GPS Explorer™ software (Applied Biosystems, Canada) based on the Mascot search engine.

Mass-spectrometry (MALDI-TOF MS). For MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight) analysis, the peptides were redissolved in 3 μl of 30% acetonitrile and 0.01% trifluoracetic acid solution and were then prepared with a matrix (α-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed on a Voyager MALDI-TOF MS (Perspective Biosystems Inc., Town State) (in Sweden) and externally calibrated using synthetic peptides with known masses. The spectra were obtained in the positive ionization mode at 20 kV. The mass information generated from the composite spectrum was submitted to a search performed with the Protana or EXPASY database, using the MS-Fit algorithm or the PeptIdent search engine.

Comparison of TBPs expression. Expression of proteins in the same organ, but different development stages and in the same development stage, but different organs was analyzed from 2-DE gels with program Melanie 7.

Analysis of interaction between DNA fragments and TBP proteins The assay was performed according to Luderus (Luderus et al., 1992). TBP proteins were electrophoretically separated in one dimensional 12% SDS-polyacrylamide gel. Proteins were electroblotted from the gel onto 0.45 μm nitrocellulose filters (BIO-RAD) in 19

mM glycine, 25 mM Tris, 0.1% SDS, and 20% methanol. Protein blots were blocked overnight at room temperature in blocking buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 2 mM EDTA; 1 % blocking reagent (BSA)) and subsequently preincubated for 1 hour in binding buffer (10 mM Tris-HCl, pH 7.4; 50 mM NaCl; 2 mM EDTA; 0.05% BSA). Later, the membranes were transferred into a fresh portion of the binding buffer supplemented with 10 ng/ml of radioactively labeled DNA probe (10,000-70,000 cpm/ml) and 100 ng/ml of competitor DNA (*EcoR* I digested plasmid pUC19). TBP-bound DNA fragments obtained according to the proteinase K (Serva) digestion procedure (Sec. 2.6.1.) were used as probes. Binding was carried out overnight at 37°C in a hybridization oven under gentle agitation. The membranes were washed three times for 15 min in 100 ml of binding buffer and autoradiographed.

RESULTS AND DISCUSSION

1. Analysis of barley proteins from TBP-DNA complexes

Despite a great deal of research effort, basic questions about chromosome structure and gene expression mechanisms remain to be answered, including the relationship between the spatial organization of the genome and the transcription machinery (Trinkle-Mulcahy and Lamond 2008). Plant nuclear proteins functions, including the tightly bound to DNA proteins (TBPs), remain obscure. The main goal of this study was to evaluate tissue specific changes in the TBPs distribution in barley different phases of shoot development. We wanted to characterize the polypeptide spectrum of TBPs, investigate properties of TBPs, identify TBPs using mass spectrometry and characterize proteins affinity to TBPs-associated DNA.

The life cycle of the barley plant, especially shoot and seed provide an excellent model for plant development studies. Etiolated barley seedlings provide cell populations with different proliferation, differentiation and senescence status (Kirnos et al., 1983). In the present work we used coleoptile, first leaves and roots dissected from shoots of Zadoks 07 (coleoptiles emerged stage) and Zadoks 10 (first leaf through coleoptile) development stages (classification according to Anderson) (Anderson et al., 1995).

Isolation of TBP-DNA complexes. The TBP proteins fractionation by SDS PAGE. TBP-DNA complexes were isolated from coleoptile, first leaves and roots different developmental stages according to extraction with chlorophorm method

(Plaschke et al., 1995). After extraction with chlorophorm precipitated DNA still contains associated TBP proteins, which can be identified only after hydrolysis of DNA with Dnaze I or benzonase (Fig.1, A).

As shown in Fig. 1, B, a group of 15-160 kDa proteins were obtained in coleoptile, first leaves and roots TBP-DNA complexes of Zadoks 07 and 10 development stages. Different barley organs contain different amount and pattern of polypeptides. It is interesting to note, that polypeptides composition differ insignificant in different developmental stages of the same organ, differ just TBP expression level.

The composition of first leaves Zadoks 07 and 10 stages TBP proteins (Fig. 1, B, lanes 3, 4) differs significantly from the other organs TBPs, for example, 15 kDa prolypeptide was found in both developmental stages of first leaves TBP-DNA complexes, but this polypeptide was not identified in coleoptiles and roots complexes.

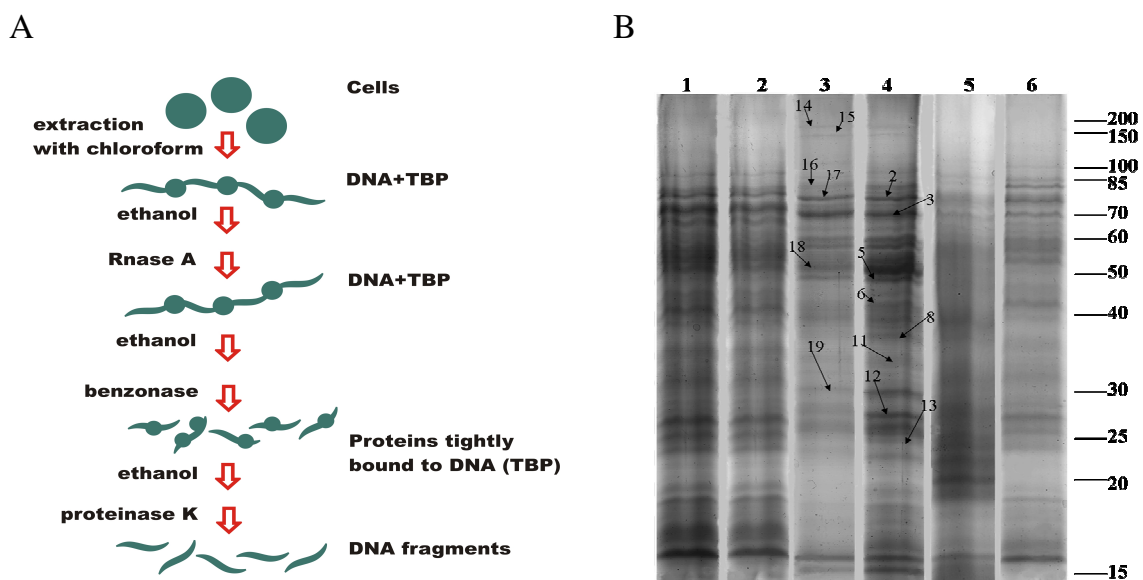A   B

Fig. 1. A – Isolation scheme of barley TBP-DNA complexes. B – Barley TBP proteins, obtained after 0.5 mg DNA digestion with benzonase, 12% SDS PAGE, stained with Coomassie blue G-colloidal dye. 1 – roots, Zadoks stage 10; 2 – roots, Zadoks stage 07; 3 – first leaves, Zadoks stage 10; 4 – first leaves, Zadoks stage 07; 5 – coleoptiles, Zadoks stage 10; coleoptiles, Zadoks stage 07. Positions of the molecular weight markers (kDa) are indicated on the right. Protein bands indicated with indexes and numbers were cut of and analyzed with mass spectrometry.

85 kDa protein band is faintly visible in first leaves TBP-DNA complexes (Fig.1, B, lanes 3, 4), but it is well visible in other organs. 50-55 kDa protein bands in first leaves TBP-DNA complexes are better visible in comparison with the same proteins in coleoptile and roots (Fig. 1, B, lanes 1, 2, 5, 6). Some high molecular weight proteins

(150-160 kDa) were identified in first leaves TBP-DNA complexes which are faintly visible in coleoptiles and roots complexes. 32-36 kDa proteins were identified in roots TBP-DNA complexes (Fig. 1, B, lanes 1, 2), but they are absent in first leaves complexes (Fig. 1, B, lanes 3, 4). 70 kDa polypeptide is identified in first leaves, but it is not so clear expressed in coleoptile and in roots it is absent.

Considerable differences between both developmental stages (Zadoks 07 and 10) TBP proteins were identified only in coleoptiles TBP-DNA complexes (Fig. 1, B, lanes 5, 6). During organ development or during change of organ function specificity (atrophy of coleoptiles is observed than first leaf through coleoptiles) novel polypeptides were synthesized, but other proteins were not expressed, for example, 15-24 kDa proteins is less expressed in Zadoks stage 07 coleoptile than in Zadoks stage 10 coleoptile. Expression of 20-23 kDa proteins is higher in coleoptile Zadoks stage 10, than in coleoptile Zadoks stage 07. Expression level of coleoptile 70-100 kDa TBPs is also different in both developmental stages (Zadoks 07 and 10) (Fig. 1, B, lanes 5, 6).

Thus, the composition of TBPs is specific for organ, but also was identified group of polypeptides with similar expression in different barley organs. TBPs composition of the same organ, but different developmental stages are similar, it is observed only different expression level of these proteins. Most significant differences were observed between TBP proteins in both developmental stages of coleoptile. It seems that it is associated with temporary function of coleoptile. Expression of Zadoks stages 07 and 10 first leaves TBPs is similar. Very similar is TBPs composition of different developmental stages of roots. This can be related with renovation and permanent function of root cells.

TBP 2-DE electrophoresis. We have identified that most of TBP proteins from various barley organs and different developmental stages TBP-DNA complexes are acid proteins, pI 4.0-6.8 (Fig. 2, A-F). We have also identified obvious inter-organ differences in TBPs composition in barley documented by 2D electrophoresis. These results will be discussed in pages 29-30.
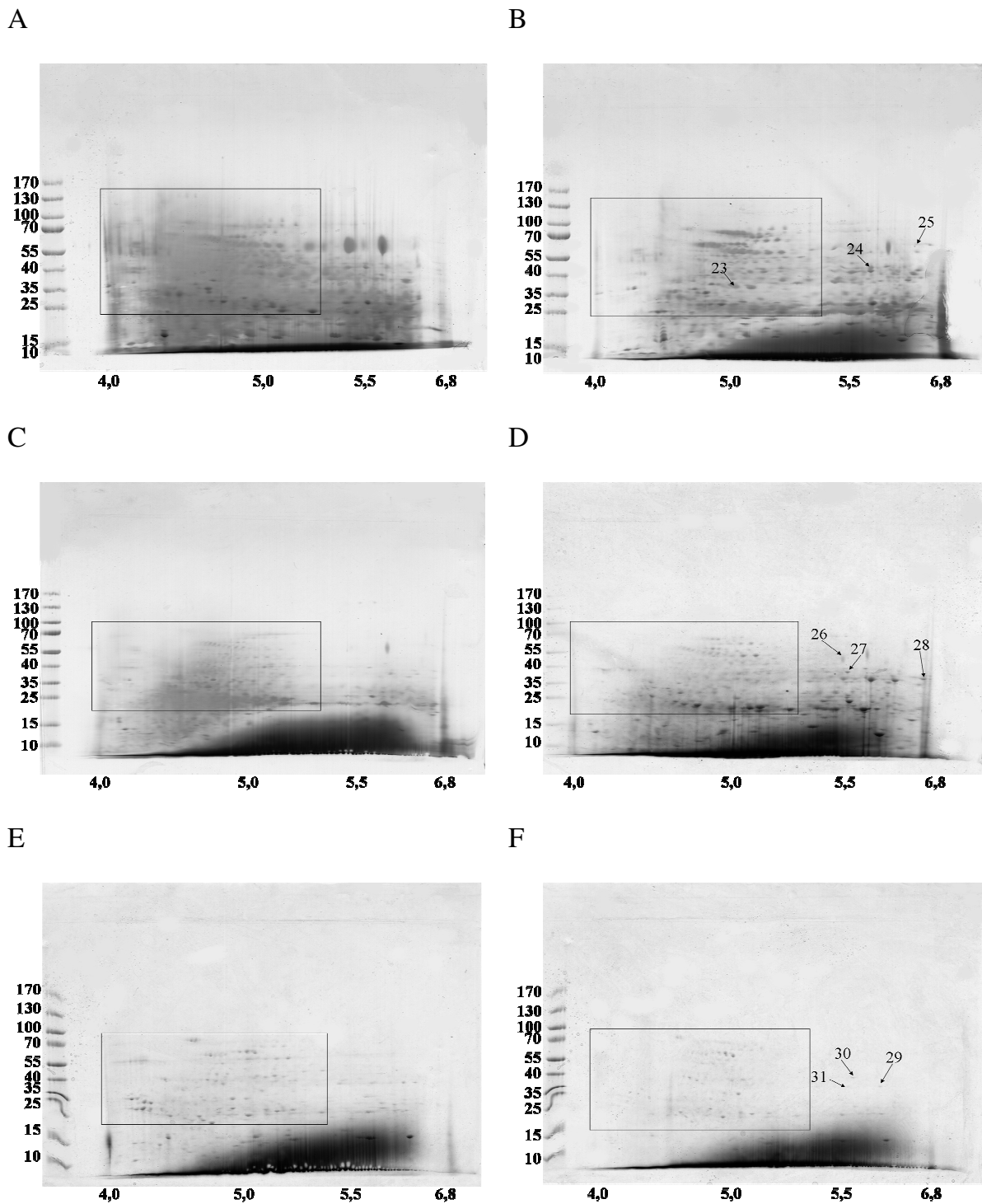
Fig. 2. Barley TBP proteins, obtained after 0.5 mg DNA digestion with benzonase, 2-DE, stained with Coomassie blue R-250 dye. A – coleoptile, Zadoks stage 07; B – coleoptile, Zadoks stage 10; C – roots, Zadoks stage 07; D – roots, Zadoks stage 10; E – first leaves, Zadoks stage 07; F – first leaves, Zadoks stage 10. Positions of the molecular weight markers (kDa) are indicated on the left, pI meaning are indicated below of the figures. Polypeptides indicated with indexes and numbers after digestion with trypsin were analyzed with mass spectrometry. Frame shows area of 2-DE gels, which were analyzed with Melanie 7 (TBPs comparison of the same organ, but different developmental stages).

Establishment of TBPs phosphatase activity *in situ*. 1/3 of total cell proteins are regulated during phosphorylation/dephosphorylation. These functions are performed by kinases and phosphatases. In cell nucleus kinases and phosphatases are involved in transcription and replication processes (Bolllen and Beullens, 2002). It was established that some of polypeptides from different organisms tightly bound DNA-protein complexes exhibit phosphatase activity (Borutinskaite et al., 2004; Labeikyte et al., 1999; Loeffler et al., 1996). We attempt to examine whether barley different developmental stages and organ TBP-DNA complexes TBP proteins exhibit phosphatase activity.

It should be pointed out that phosphatase activity of barley TBPs was detected only after hydrolysis of TBP-DNA complexes with benzonase (0.1 U/µg DNA) (control experiments show that benzonase hasn't impurity of phosphatase activity). Polypeptides with phosphatase activity were identified in all analyzed barley developmental stages (Zadoks 07 and 10) and organs: coleoptile, first leaves and roots (Fig. 3).

To establish the polypeptide responsible for phosphatase activity, we employed the SDS PAGE denaturation-renaturation scheme (Meikrantz et al., 1991). The SDS-PAGE-separated TBP proteins (Fig. 1, B) were blotted on a nitrocellulose membrane, renaturated overnight and exposed *in situ* to NT and BClP. As it is seen in Figure 3, different polypeptides show phosphatase activity in different organs, in case of coleoptiles and first leaves also in the same organ, but different developmental stages.
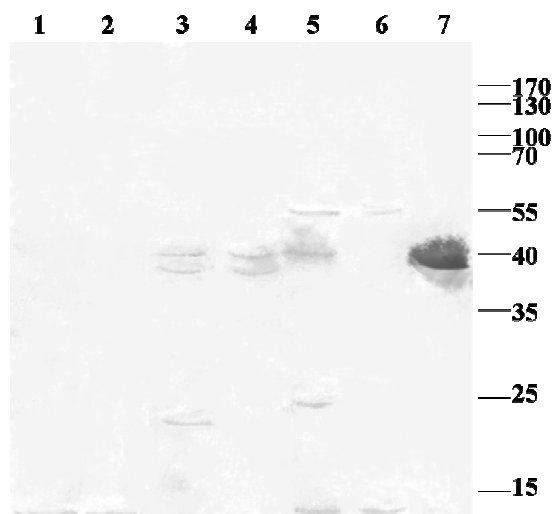

Fig. 3. Establishment of barley TBP phosphatase activity *in situ* with NT and BClP: 1 – roots phosphatase, Zadoks stage 10; 2 – roots phosphatase, Zadoks stage 07; 3 – first leaves phosphatases, Zadoks stage 10; 4 – first leaves phosphatases, Zadoks stage 07; 5 – coleoptile phosphatases, Zadoks stage 10; 6 – coleoptile phosphatases, Zadoks stage 07; 7 – alkaline phosphatase (CLAP), plus control. Positions of the molecular weight markers (kDa) are indicated on the right.

In barley roots of Zadoks stages 07 and 10 were identified ~10 kDa TBP (Fig. 3, lanes 1, 2), in first leaves - ~38 kDa and ~40 kDa TBPs (Fig. 3, lanes 3, 4), in coleoptile – ~70 kDa and 10 kDa polypeptides (Fig. 3, lanes 5, 6) possessing phosphatase activity. In coleoptile Zadoks stage 10 (Fig. 3., lane 5), like in first leaves TBP-DNA complexes 40 kDa and 25 kDa polypeptides indicated phosphatase activity. In first leaves of Zadoks stage 10 TBP-DNA complexes, differently than in other organs, 20 kDa polypeptide with phosphatase activity was found.

Thus, number of TBP proteins, which exhibit phoshatase activity in different barley organs, increase during development (transition from Zadoks stage 07 to Zadoks stage 10). This effect was not observed only in roots DNA-TBP complexes. We assume that it can be related with permanent function of roots during barley development.

TBPs tyrosine phosphorylation. Phosphorylation and dephosphorylation of cellular proteins are implicated in many biologically important processes such as cell growth, differentiation, and apoptosis. It has been shown that tyrosine phosphorylation is an early event in signal transduction through different cell surface receptors. Tyrosine phosphorylation of proteins is a process widely involved in the control of cellular processes (Hunter, 1995). However, tyrosine phosphorylation has not been systematically studied in higher plants (Barizza et al., 1999; Luan, 2002). It is supposed that proteins tyrosine phosphorylation is involved in plant responses to stress signals and in plant development (Barriza et al., 1999).

To establish tyrosine phosphorylation level of TBPs in different barley organs and developmental stages, we used SDS-PAGE separated TBP proteins (Fig. 1, B) blotted on a nitrocellulose membrane. Then immunoblot with anti-phosphotyrosine was proceeded, signal was detected with ECL reagent and autoradiographed (Fig. 4).

In roots during transition from Zadoks stage 07 to Zadoks stage 10 was observed increase of TBP proteins with phosphotyrosine (Fig. 4, lanes 1, 2), except one polypeptide, which was not found in Zadoks stage 10. Polypeptides with phosphotyrosine (27 kDa, 30 kDa and 55 kDa) were established in both developmental stages of roots TBP-DNA complexes, but 35 kDa, 40 kDa, 42 kDa, 68 kDa polypeptides with such modification were found only in Zadoks stage 10. 80 kDa polypeptide with phosphotyrosine was found in Zadoks stage 07 of roots TBP-DNA complexes, but this polypeptide was not found in complexes of the same organ Zadoks stage 10.
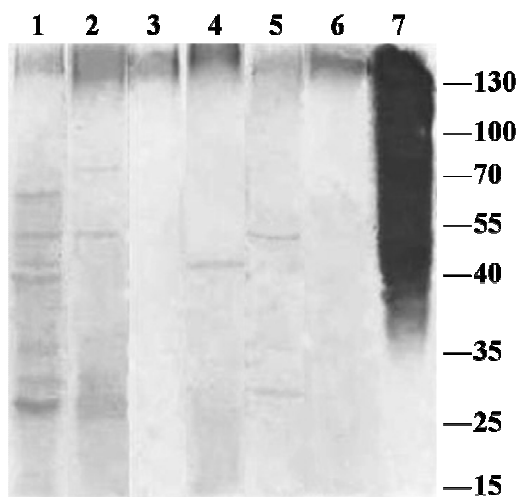
Fig. 4. Autoradiogram. TBPs immunoblot with anti-phosphotyrosine. 1 – roots, Zadoks stage 10; 2 – roots, Zadoks stage 07; 3 – first leaves, Zadoks stage 10; 4 – first leaves, Zadoks stage 07; 5 – coleoptile, Zadoks stage 10; 6 – coleoptile, Zadoks stage 07; 7 – cell lysate (plus control). Positions of the molecular weight markers (kDa) are indicated on the right.

In first leaves TBP-DNA complexes of Zadoks stage 07 only one protein (42 kDa) with phosphotyrosine was identified, which in later Zadoks stage 10 was not identified (Fig. 4, lanes 3, 4). Polypeptide (42 kDa) with such modification was also found in roots complexes, but in Zadoks stage 10.

In coleoptile TBP-DNA complexes, as in the case of roots, increase of tyrosinphosphorylated TBPs was observed during transition from Zadoks stage 07 to Zadoks stage 10 (Fig. 4, lanes 5, 6). Two tyrosinphosphorylated peptides (55 kDa and 29 kDa) were found in Zadoks stage 10 of coleoptile complexes, but in Zadoks stage 07 such polypeptides were not identified. 55 kDa tyrosinphosphorylated TBP is typical also for both developmental stages of roots TBP-DNA complexes (Fig. 4, lanes 1, 2). In roots of Zadoks stage 10 complexes ~30 kDa polypeptide with phosphorylated tyrosine also were identified.

Thus, TBPs tyrosine phosphorylation of barley roots and coleoptile differs from such TBP modification in first leaves. Increase of tyrosinphosphorylated TBP during organ development (during transition from Zadoks stage 07 to Zadoks stage 10) was observed in roots and coleoptile. Reverse process was observed in first leaves (in Zadoks stage 07 was identified only one polypeptide with such modification, but in Zadoks stage 10 this TBP is absent). It is evident, that barley TBPs tyrosine phosphorylation depends on differentiation and development.

Recently it was demonstrated that protein tyrosine phosphorylation patterns vary among different adult plant tissues or somatic embryo stages. Therefore it is supposed that protein tyrosine phosphorylation is involved in control of specific steps in plant

development (Barizza et al., 1999). Also, it was established that tyrosine phosphatase activity is involved in the regulation of stomata movement, a highly regulated process pivotal for plant survival (Luan, 2002). Based on the use of inhibitors of Tyr kinases and phosphatases generally used in animal systems, it was indicated that Tyr phosphorylation has an important role in plant signaling, particularly in disease-resistance signaling (van Bentem and Hirt, 2009). It is supposed that in plants like in animals exist complicated network of protein tyrosine phosphorylation (Barizza et al., 1996).

Thus, we establish that among the TBPs of different barley organ and developmental stages is found polypeptides with phosphorylated tyrosine. Tyrosine phosphorylation depends on tissue and developmental stages. We suppose that the TBPs with phosphorylated tyrosine can be involved in control of barley developmental stages.

Mass spectrometry analysis of TBPs. We have shown that barley first leaves, coleoptile and roots TBPs exhibit some interesting properties such as phosphatase activity and tyrosine phosphorylation. Therefore we wanted to identify TBP polypeptides and characterize their possible function in barley cells. In previous work it has been demonstrated that serpins Spi-1, Spi-2, Spi-3 (Rothbarth et al., 2001b) and 16 kDa protein C1D (Rothbarth et al., 1999) belong to the TBP proteins of tightly bound DNA–protein complexes. Recently, there have been identified some yeast TBP proteins, chromatin assembly factor 1, NNF1 protein, DNA repair protein RAD7, SOH1 protein among them. The identified yeast TBPs participate in chromatin rearrangement and regulation processes (Bagdoniene et al., 2008). It has been proposed that TBP proteins may participate in differentiation and development processes (Sjakste et al., 2005). Investigation of tightly bound DNA–protein complexes from different barley organs (coleoptile, leaf and roots) from Zadoks stages 07 and 10 can reveal some aspects of this assumption.

The goal of the further stage of work was to characterize the TBP proteins from first leaves, coleoptile and roots (Zadoks stages 07 and 10) by the MALDI TOF-TOF MS analysis.

We wanted to evaluate differences between the TBPs of the same organ, but different developmental stages. The TBP proteins from first leaves of barley shoots (Zadoks stages 07 and 10) were fractionated in 12% SDS-PAGE (Fig. 1, B, lanes 3, 4),

Some of these proteins bands were cut from the gels, digested with trypsin and analyzed by the MALDI TOF-TOF MS.

The list of the proteins identified employing the GPS Explorer™ software is presented in Table 3.

Table 3. MALDI TOF-TOF MS analysis of barley first leaves TBP proteins.

Band[1] No	Mw[2] Calculated	Mw[3] Experimental	Score[4]			The list of identified proteins	Organism	Accession No
			Expasy database	MSDB database	UniProtKB-SwissProt database			
2	121	75	9.93			Nuclear matrix protein NMCP1	*Arabidopsis thaliana*	Q9FLH0
3	21	72		31		AGAMOUS transcription factor homolog	*Hyacinthus oryentalis*	Q9ZPK9_9ASPA
5	40	50	0.65			Transcription factor TEOSINTE BRANCHED 1	*Zea mays*	Q93W12
6	44	45	1.00			Serpin-Z4	*Arabidopsis thaliana*	Q9M1T7
6	155	45			25	Probable WRKY transcription factor 16	*Arabidopsis thaliana*	Q9FL92
8	40	38	0.40			Putative F-box/Kelch-repeat protein At5g03000	*Arabidopsis thaliana*	Q9LYY5
8	36	38			14	DNA repair protein RAD51 B	*Zea mays*	Q9XED7
11	63	34	7.19			DEAD-box ATP-dependent RNA helicase 47B	*Oryza sativa*	Q10PV9
11	81	34	4.66			Scarecrow-like protein 9	*Arabidopsis thaliana*	O80933
12	>100	28		29		protein kinase F24O1.13	*Arabidopsis thaliana*	T01451
13	25	26	7.56			MADS-box transcription factor 26	*Oryza sativa*	A2YQK9
13	37	26			14	FKBP12-interacting protein of 37 kDa	*Arabidopsis thaliana*	Q9ZSZ8
14	194	160-170		20		Retrotransposon protein, putative, Ty3-gypsy subclass.	*Oryza sativa*	Q7XG10
14	190	160-170	6.08			Histone acetyltransferase HAC12	*Arabidopsis thaliana*	Q9FWQ5
15	148	150	1.63			DEMETER-like protein 2	*Arabidopsis thaliana*	Q9SR66
16	145	85			19	WRKY transcription factor 52	*Arabidopsis thaliana*	Q9FH83

1	2	3		4				
17	70	75		62		Heat shock protein 70	*Medicago sativa*	Q5MGA8
18	110	52	6.19			Squamosa promoter-binding protein 16	*Arabidopsis thaliana*	Q700C2
18	51	52	5.54			F-box/Kelch-repeat protein At5g15710	*Arabidopsis thaliana*	Q9LFV5
19	42	30	7.86			Transcription factor TGA4	*Arabidopsis thaliana*	Q39162

[1]The number of band in gels (Figure 1); [2]Mw calculated from the amino acid sequence in databases; [3]Mw calculated according to their migration in SDS PAGE gel; [4]MOWSE scores in ExPASy; MSDB and UniProtKB-SwissProt databases.

Most proteins identified by the MALDI TOF-TOF MS method are associated with DNA functions and participate in chromatin rearrangement and regulation processes. Several formerly identified nuclear matrix proteins were also found between them. The nuclear matrix is a three-dimensional network of insoluble proteins implicated in the spatial arrangement of pre RNAs and of transcription and splicing enzymes (Sjakste and Sjakste, 2001).

Mass spectrometry analysis enabled us to characterize TBP proteins of barley leaves. The barley genome is not completely sequenced, and MALDI TOF-TOF MS analysis data will be corrected in future. We can see today that most of the identified barley TBPs is various transcription factors (WRKY transcription factors 16 and 52, MADS-box transcription factor 26, Squamosa promoter-binding-like protein 16, Scarecrow-like protein 9, TGA4, TEOSINTE BRANCHED 1), histone acetyltransferase, RNA helicase, retrotransposon *Ty3/Gypsy* poliprotein, demethylase of plants, nuclear matrix protein NMCP1. All these proteins are known as nuclear matrix components that have certain functions and play an important role in transcription (Sjakste and Sjakste, 2001). Among TBP proteins we also identified DNA repair protein RAD51 homolog B, protein kinase, serpins and some other interesting proteins as immunophilins. It has been established previously that serpins were identified among the TBP proteins of Ehrlich ascites cells (Rothbarth et al., 2001b).

So, barley first leaves TBP proteins manifest homologies to functionally different enzymes and regulatory factors that participate in chromatin modification, reconstruction and repair. Several same polypeptides (WRKY transcription factor and F-box/Kelch-repeat protein) were identified among first leaves TBPs from different developmental

stages (Zadoks stage 07 and 10). Other characterized TBPs are different in both developmental stages. The TBPs specificity to developmental stage can be established only after detail analysis with mass spectrometry, when total barley TBPs proteome will be analyzed.

As mentioned above, we identified TBPs composition differences between barley organs using 2-DE. The previous performed comparison of TBPs isolated from different species and organs revealed that 62, 52 and 40 kDa proteins were common for all organisms, however TBP fractions isolated from two murine tumors (EAT and MEL cells), *Tetrahymena pyriformis*, and yeasts largely differed in polypeptide composition, moreover fish sperm and eggs contained very different TBPs spectrum (Labeikyte et al., 1999). In our study we have demonstrated a high diversity of the TBPs and significant differences between organs of barley shoots (Fig. 1, B; Fig. 2, A-F).

Then we wanted to evaluate differences between TBPs of the same developmental stages (Zadoks 10), but different organs. The goal was to characterize the TBP proteins from first leaves, coleoptiles and roots (Zadoks stage 10) by MALDI-TOF MS analysis. The TBP proteins of barley shoots were fractionated in 2-DE gels (Fig. 2, C, D, F)). Several predominant spots revealed by 2-DE were cut from the gels, digested with trypsin and analyzed by MALDI TOF-TOF MS. Barley TBPs manifest homologies to functionally different enzymes and regulatory factors (Table 4).

Finding of a protein homologous to serine/threonine-protein phosphatase in *A. thaliana* confirms that the phosphatase activity is one of the main features of TBPs (Borutinskaite et al., 2004; Juodka et al., 1991; Labeikyte et al., 1999; Loeffler et al., 1996). In this work we have also demonstrated that some of the TBPs from barley different organs and developmental stages manifest phosphatase activity.

Several identified polypeptides in first leaves and coleoptile TBP-DNA complexes are homologous to RNA helicases. It is known that these enzymes interact with double-stranded DNA and topoisomerase II (Fuller-Pace, 2006). Helicases were also identified by using MALDI TOF-TOF among first leaves TBPs (Table 3).

Table 4. MALDI-TOF MS analysis of barley first leaves, coleoptile and roots Zadoks stage 10 TBP proteins.

Band[1] No	Calculated		Experimental	Peptident		Protein name	Organism	Accession No
	pI	Mw[2]	Mw[3]	Score[4]	Sequence coverage (%)			
23	5.9	96	40	1.725	6	Serine/threonine-protein phosphatase BSL...	*Arabidopsis thaliana*	Q8L7U5
23	9.2	22	40	3.085	14	Metalloproteinase inhibitor 3	*Scyliorhinus torazame*	Q9W6B4
23	8.9	20	40	3.064	20	Cysteine and glycine-rich protein 1	*Coturnix japonica*	P67967
24	9.5	61	40	3.746	10	Maturase K	*Pseudotsuga menziesii*	Q9MV48
24	9.3	93	30	0.174	4	DEAD-box ATP-dependent RNA helicase	*Oryza sativa*	A2XVF7
26	6.9	168	50	0.084	2	DNA topoisomerase II	*Schizosaccharomyces pombe*	P08096
26	6	198	50	0.22	2	Transposon protein, putative, CACTA, En/Spm sub-class	*Oryza sativa*	Q109Z8
26	6.3	103	50	0.24	3	Putative chromosome associated protein	*Arabidopsis thaliana*	Q9SHT1
26	6.3	78	50	0.434	4	Replication origin activator MCM3	Ostreococcus lucimarinus	A4RT02
26	6.8	50	50	1.024	7	GAP-like zinc finger-containing protein	*Arabidopsis thaliana*	Q9FVH2
28	9.3	8	37	6.7	67	Uncharacterized protein SCRL20.	*Arabidopsis thaliana*	P82639
28	6.9	79	37	6.5	13	Cyclin-dependent kinase G-1.	*Oryza sativa*	A2X6X1
29	7.6	83	37	0.624	3	ATP-dependent RNA helicase MAK5	*Eremothecium gossypii*	Q757I6
30	6.9	168	50	0.24	2	DNA topoisomerase II	*Schizosaccharomyces pombe*	P08096
31	9.4	61	40	0.36		Maturase K (Intron maturase)	*Pinus pinaster*	Q8HQQ6

[1]The number of spot in gels (Fig. 2, B, D, F); [2]Mw calculated from the amino acid sequence in databases; [3]Mw calculated according to their migration in 2-DE; [4]MOWSE scores in UniProtKB-SwissProt database.

Finding of the enzyme DNA topoisomerase II among the TBPs is quite logical, as topoisomerases are considered to be a classical example of proteins covalently bound to DNA (Drygin, 1988). Also topo II motifs were found in DNA sequences tightly bound to TBP (Bagdoniene et al., 2005; Labeikyte et al., 2005).

However several authors failed to find topoisomerases among proteins remaining attached to DNA after deproteinization (Avramova, Tsanev, 1987).

By using anti-topoisomerase II we have identified topoisomerase II among the TBPs from different barley organs (Fig. 5)
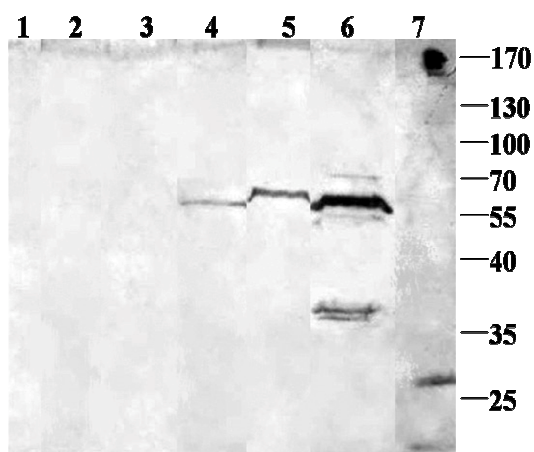
Fig. 5. Autoradiogram. TBPs immunoblot with anti-topoisomerase II. 1 – roots, Zadoks stage 10; 2 – roots, Zadoks stage 07; 3 – first leaves, Zadoks stage 10; 4 – first leaves, Zadoks stage 07; 5 – coleoptile, Zadoks stage 10; 6 – coleoptile, Zadoks stage 07; 7 – cell lysate (plus control). Positions of the molecular weight markers (kDa) are indicated on the right.

From our results we can see, that high molecular weight (~170 kDa) topoisomerase II is identified in both developmental stages (Zadoks 07 and 10) of different organs (Fig. 5, lanes 1-6). Polypeptides (37 kDa, 55 kDa, 70 kDa) which have epitopes of DNA topoisomerase II, are also detected in first leaves (Zadoks stage 07) and coleoptile (Zadoks stages 07 and 10). Thus, received results confirm MALDI-TOF data about DNA topoisomerase II presence in barley TBP-DNA complexes.

It is supposed that TBP proteins are a part of the replication complex. Presence of the replication origin activator MCM3 among TBP proteins confirms this suggestion. Ori motifs have been found also in mammalian and yeast DNA sequences of TBP-DNA complexes (Bagdoniene et al., 2005; Labeikyte et al., 2005) and in barley leaves TBP-DNA sequences (our results, page 31-37).

Finding among the TBPs polypeptides homologous to RNA maturases seems to be extremely interesting. These enzymes interact with both RNA and DNA. The DNA binding domains of the enzyme are protruded inside the DNA double helix, DNA forms a curvature in the site of interaction with the enzyme (Belfort, 2003). In previous work was observed enrichment of DNA fraction bound to TBPs with bent sequences and Z DNA (Bagdoniene et al., 2005; Labeikyte et al., 2005; Sjakste, 1997). In this work we also demonstrated that DNA sequences from first leaves TBP-DNA complexes contain curved DNA regions.

Among barley TBPs also was identified transposon CACTA protein. Terminal inverted repeats and conservative CACTA motifs are typical for transposon CACTA (Wicker et al., 2003). In this work we establish that DNA sequences from barley first

leaves TBP-DNA complexes show high homology with various transposons (and CACTA transposon among them), retrotransposons and repeat sequences.

Thus, identified TBP proteins from different organs appeared to be homologous to several replication proteins (DNA topoisomerase II, replication origin activator MCM3), proteins involved in regulation of transcription and RNA processing (cyclin-dependent kinase G-1, maturases, RNA-helicase), transposon binding proteins, phosphatase, metalloproteinase inhibitor 3, cysteine and glycine-rich protein 1, putative chromosome associated protein, GAP-like zinc finger-containing protein. Helicases, kinases, transposon proteins and zinc finger-containing proteins were identified in both cases: MALDI TOF-TOF MS (Table 3) and MALDI-TOF MS (Table 4). In different organs were found a group of the same TBPs, for example, helicase, maturase, DNA topoisomerase II, but other polypeptides differ between organs. We conclude that some TBPs are organs specific. It is necessary more detail analysis of barley TBP-DNA complexes proteins using mass spectrometry.

Identified proteins tightly interact with DNA and are important for the specific structural organization of DNA and proteins in the nucleus. Only part of these proteins has domains for interaction with DNA, and these domains are different: b (HLH) domain, various Zn finger-like structures. The question why these proteins are so tightly associated with DNA still remains open. Most of these proteins were identified in the nuclear matrix preparations. As DNA sequences of tight DNA–protein complexes are also similar to the nuclear matrix (MAR) sequences (Bagdoniene at al., 2005), we assent to the assumption (Razin et al., 1988; Sjakste et al., 2005) that tightly bound DNA–protein complexes are part of the inner network of the nuclear matrix.

Comparison of TBP proteins expression in different organs and development stages of barley. We have demonstrated significant differences of TBPs between same organ different developmental stages in 2-DE gels (Fig. 2, A-F). We perform detail comparison of proteins composition in different organs and development stages of barley with program Melanie 7. For analysis we choose particular region from 2-DE gels which are indicated with frames in Figure 2. Thirty six (36) TBP proteins from coleoptile Zadoks stages 07 and 10 coincided in this region. Thirty seven (37) same TBP polypeptides were found in roots of Zadoks stages 07 and 10, but in first leaves of both developmental stages were found only twenty nine (29) coincided proteins. We establish

that the TBPs expression differ in same organ different developmental stages. In coleoptile and roots expression of most TBPs is higher in Zadoks stage 07, in comparison with first leaves. In first leaves higher expression level of the TBPs was observed in Zadoks stage 10.

We also compared expression of TBPs in the same developmental stage, but in different organs (coleoptile, roots and first leaves). In analyzed particular region of coleoptile, first leaves and roots 2-DE gels were identified thirty nine (39) coincided TBPs. In different organs were found only part of similar TBPs. Some TBP polypeptides are typical only for two organs: coleoptile and first leaves or coleoptile and roots. The expression levels of coincided TBPs are different.

We also compared expression of TBPs identified by mass spectrometry. From our results we can see that 24th polypeptide (DEAD-box ATP-dependent RNA helicase) (Fig. 2, B) and 31th polypeptide (maturase K) were found in all analyzed organs. It is known that both enzymes interact with DNA. DNA forms a curvature in the site of interaction with these enzymes (Belfort, 2003). It is demonstrated that RNA helicases are involved in plant growth and development processes (Kim et al., 2008; Li et al., 2008; Linder et al., 2006).

25th polypeptide (Fig. 2, B) and 28th polypeptide (Fig. 2, D) were found only in coleoptile and roots. Mass spectrometry analysis didn't reveal homologous plant proteins to polypeptide 25th, but in 28th spot was identified kinase (Table 2).

Thus, in TBP-DNA complexes from different organs were found only part of the same proteins, but expression level of these proteins differs. We can conclude that expression of TBPs depends on the developmental stages.

Analysis of interaction between DNA fragments and proteins from TBP-DNA complexes. In further stage of work we analyzed specificity of interaction between DNA fragments and proteins from TBP-DNA complexes. The SDS PAGE separated TBPs from different barley organs and development stages were blotted on a nitrocellulose membrane and were hybridized with TBP-DNA complexes DNA fragments (obtained after digestion with proteinase K) labeled with [^{32}P]. Results are presented in Figure 6.

From obtained results we can see that highly strong interaction was observed between the TBPs and DNA fragments from the same organ TBP-DNA complexes. Different proteins are involved in interaction with DNA. In Zadoks stage 10 interactions

of some proteins with DNA become weaker, but other proteins of DNA-TBP complexes are emerged in interaction with the same DNA fragment.
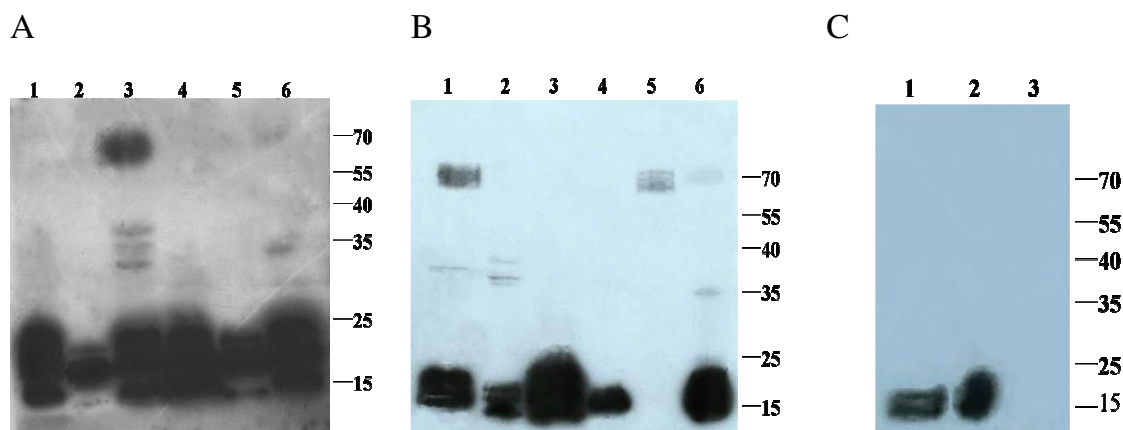

Fig. 6. Autoradiogram. Hybridization of DNA fragment from Zadoks stage 10 coleoptile TBP-DNA complexes and TBP proteins: 1 – roots, Zadoks stage 10; 2 – first leaves, Zadoks stage 10; 3 – coleoptile, Zadoks stage 10; 4 – roots, Zadoks stage 07; 5 – first leaves, Zadoks stage 07; 6 – coleoptile, Zadoks stage 07. B – Autoradiogram. Hybridization of DNA fragment from Zadoks stage 10 first leaves TBP-DNA complexes and TBP proteins: 1 – coleoptile, Zadoks stage 07; 2 – first leaves, Zadoks stage 07; 3 – roots, Zadoks stage 07; 4 – coleoptile, Zadoks stage 10; 5 – first leaves, Zadoks stage 10; 6 – roots, Zadoks stage 10. C – Autoradiogram. Hybridization of DNA fragment from Zadoks stage 10 roots TBP-DNA complexes and TBP proteins: 1 – coleoptile, Zadoks stage 10; 2 – roots, Zadoks stage 10; 3 – first leaves, Zadoks stage 10. Positions of the molecular weight markers (kDa) are indicated on the right.

Zadoks stage 10 first leaves TBP-DNA complexes TBPs are especially different from other proteins. In this case in interaction with DNA low molecular weight TBP proteins were replaced by 70 kDa polypeptide.

2. Analysis of DNA sequences from barley TBP-DNA complexes

Analysis of cloned DNA sequences from barley first leaves TBP-DNA complexes. The DNA-polypeptide complexes are not completely dissociated during nuclease digestion. After digestion with benzonase, 0.05–0.1% of undigested DNA was recovered, implying protection of DNA by the associated proteins. The samples were tested by using electrophoresis in 1.2% agarose gels. In 1.2% agarose gel start remain DNA fragments protected by TBP proteins. Following additional deproteinization with proteinase K (0.5 mg/ml, 37°C, 16 hours) 100–500 bp DNA fragments were obtained (Fig. 7).
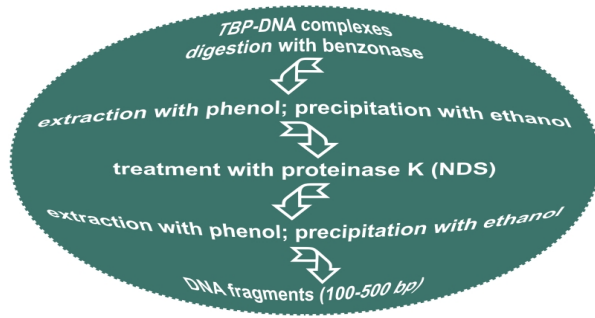
Fig. 7. Extraction scheme of the DNA fragments from barley first leaves tightly bound DNA-protein complexes.

Some obtained DNA fragments were cloned into *Sma* I site of pUC19 vector. Eleven clones of the transformants containing inserts were picked at random and sequenced. 1-8 cloned DNA fragments were sequenced in UAB Fermentas, and 9-11 fragments were obtained with the help of Dr. Tatjana Sjakste in Germany. Figure 8 represents the sequences of these DNA inserts.

Seq-1 (1-158)
TCNTTAANCANGAAGTGGCACTTTCNCCGAGTTCAAGACGAGGTTGGTATCTTCGCATCTCTNATAAGACTCGATCAAGGT
TGCTGAGGAAATCATCAAAGGAAGAACCGTAAACGGAGAAATCATCCATGAAAACCTCAACAATGTTTTCACAAAAG

Seq-2 (1-209)
GCTACGACAAAAACTGGATGCACTTCGTGTACAAAACGGACAATCTCTCTCGAAGTATCAGGGTTTCGAACGAGAACTCAT
CTCTTACAAAGGGATTTCATTTTTTTCGAACTTATTTGACCTCCATACTTTTTGTGTGTTCAAAATGCACCATTCAAAGGC
ACATCACAAAATTTCAACAATTTCTGACTTCATTTGGTATTCTTCGT

Seq-3 (1-243)
ccagcttCagGAGGAAAcCTGAAAcCACACGTGCCCCATCAAGTCCATAGGGTGCGACTAGGGGGTGGTCGCGCCCTGGTG
CGTTGGTGCTTCCCTGTGGCCCCTCTTTCCCTGTTCTGTGGCCTATAAATTCCCAAATATTCCAAAAATCCTAAATATCAT
ACGCAAACGCTTTTTACGCCGCTGCAAGTCTCTGTCCATAGCGATCCCATCTGGACCTCTGATGCGGCACCCTACCAAAGG

Seq-4 (1-156)
TGGGATGATCCTAACCTTTATAAAGAAGGAGTAGATGGTGTTATTAGACGTTGTGTACCTGAGCATGAACAGGGACAGATC
CTACAGAAGTGTCACTCCGAGGCTTACGGAGGACACCATGCGGGAGATAGAACTGCACACAAGGTATTGCAATCC

Seq-5 (1-165)
GCTTCATTGAANCAACACTTGATCCTTGAAATCCAGTCTGTTTTTACCGTGTCTCACTATGCCGGATTATTATTACTTTAT
ATTACCATCATAGAGTTTTTTCATGTACAACACTATTTTCCGGCTTATAAAAAAGTGCTCGGGTTATAAAGACATAATATT
GTC

Seq-6 (1-303)
NCTAGCTCTTGTATGACCTGTTTGGTGGGATTAAATTTTATTATGTCGCAGTTGCACTCTTCATAGCCCAACACCTCTCTC
CCTCTCTCTCACGCACATGCATACACACACTCCCTAGATAGTTTGGATCACTATGAGTATATCTCCCGCACACACACACAC
ACACACGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA
ATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT

Seq-7 (1-176)
NTCGGGGAGTTATAAAGATTCCCTAACAGATTATAATATCAAGTCGGGTACTTTTGGAATCATTGATGATCCCGATATATT
AAGAAAGGAGCTTTAACAAAATAAGTAAAGAAATAAAACAAAAGAACAAATANAACAAGAAAAAAACCANGGGTNGCCCCC
NCATTTTAACAAAA

Seq-8 (1-160)
CCTTTATTGAGGGAAAtCGGTGGANGTGGACAGCGAGCGCCAGCGTTCAAGATCGGCGCGAACCTGGTCCAGCTTGGCTTC
NACGACACTGAGCGCATCGCTGCCAGCGACGAAACGCAAGGGCGGTGCTGCCANANCCGCCAATTGCACAACCGCCAGG

Seq-9 (1-189)
CTGCTTGGGTGTGGGAAATCGGTGGGTTTGCATATGAAATCATATGCAAACCTCCCGTTTCTCCCGTAACCCTTGCTTTTC
CCAAACGTTGGCTCGGATGTCCCGTCGTTCTCCTGTCCCGTGTACGACTCATGCCAAATTCTGATCCGTCGGTCGAACGGC
TGTTCGGGTTGCGAGAAAAGTAC

Seq-10 (1-91)
ACAAACATGTGTGAAGATTATGAAAAGTTTCATGAAATTTCTCCCTTCAAAGTGAAGGCTAACCCAAGCTCCCAGGAGAGC
GATGAAGATT

Seq-11 (1-294)
CCTCCTCGTGTGATAGGGATGAGGGGCACGGCGGCGGCAGTGGTGCCCCTGGCAGGTCAGGATCCCCAGTGGTCAGGTCGG
AGTACGATTCCCCGGGTTCTGACTGACCATCGGCCCTCCCACCGAGTCCCGCTCACGCCTCTCCCCCGCCCGACGGGGGTA
TCTTTCGGAAAGTCGTGCCCGCGGTTATGTGGTAGACTTGGAAACTTTTTTTAACATACGGTTCTAGATAACTTGAAGTAC
ACTTAATTAAAGACTTGCCAACTGAGTGCGTAACCGTGATTGTCTCTTTCG

Fig. 8. Composition of barley leaves DNA fragments released from tightly bound DNA-protein complexes.

No significant similarities were found among the different DNA clones. Thus, it seems that the nucleotide sequence provides only limited information on the nature of DNA from TBP-DNA complexes.

It has been shown earlier that DNA presented in tightly bound DNA-protein complexes in higher eukaryotic organisms exhibits some properties of S/MAR. These sequences have a high AT content and belong to the Alu family of sequences (Neuer-Nitsche et al., 1988; Sjakste, 1997), however some heterogeneous sequences and sequences, which correspond to the medium reiteration frequency MER sequences were also found (Avramova et al., 1994).

Table 5. Nucleotide composition of barley leaves tightly bound DNA sequences (%). DNA composition was analyzed with GEMS Launcher 5.0 program (http://www.genomatix.de/).

Sequence	A (%)	C (%)	G (%)	T (%)	X (%)	A+T (%)	G+C (%)	TA+CA+TG (%)
1	34,18	20,25	19,62	22,78	3,16	56,96	39,87	18,31
2	29,67	22,01	15,31	33,01		62,68	37,32	17,68
3	22,22	31,28	22,22	24,28		46,50	53,50	18,5
4	30,77	19,23	27,56	22,44		53,21	46,79	21,79
5	28,48	19,39	13,94	37,58	0,61	66,06	33,33	22,41
6	26,07	28,38	18,15	27,06	0,33	53,14	46,53	24,42
7	44,32	14,77	15,91	22,16	2,84	66,48	30,68	16,38
8	22,50	29,37	30,62	15,00	2,50	37,50	60,00	15,0
9	18,38	27,03	25,41	29,19		47,57	52,43	15,12
10	36,26	18,68	20,88	24,18		60,44	39,56	16,38
11	18,71	28,23	28,23	24,83		43,54	56,46	15,0

The nucleotide ACGT composition of barley leaves tightly bound DNA has shown (Table 5) that most of the sequences (1, 2, 5, 6, 7, 10) are rich in AT, and sequences 3, 8, 9, 11 contain more GC than AT. Such composition is not typical to S/MAR sequences which are generally AT-rich, and this composition differs from mammalian (Neuer-Nitsche et al., 1988) and yeast (Bagdoniene et al., 2005) DNA fragments tightly bound to proteins. Maybe, heterogeneity of barley DNA sequences reflects its function. We demonstrated recently that barley TBP-DNA complexes DNA sequences are similar to many retrotransposons and repeated sequences.

It has been shown that CA, TA and TG dinucleotides are overrepresented in DNA sequences which are nuclear protein recognition sites (Trifonov, 1991). Some (not all) S/MARs display an unusual richeness in CA, TA and TG (Boulikas, 1993). As seen in Table 5, in most of analyzed barley TBP-DNA sequences, in contrast to murine

sequences (Labeikyte et al., 2005), were not found more CA, TA and TG as compared to randomly distributed sequences (in randomly distributed sequences CA, TA and TG content is 18.75%). Only sequences 4, 5, 6 contain more CA, TA and TG dinucleotides (Table 5).

Sequence	ori	Curved DNA	Topo II site	ATC rule
1	0	0	1	0
2	0	0	0	6
3	2	2	1	22
4	1	0	0	12
5	5	2	0	8
6	5	4	0	18
7	4	0	1	1
8	0	0	0	0
9	1	0	0	0
10	0	0	0	0
11	2	0	0	0

Table 6. Properties of barley leaves DNA fragments involved in tightly bound DNA protein complexes. Motifs specific to the S/MAR sequences were found with programs www.futuresoft.org/MAR-Wiz/ and www.tubic.tju.edu.cn/Ori-Finder/.

It is established, that curved DNA has generally been associated with the presence of copies of the dinucleotide CA, TA and TG that are separated by 2-4 or 9-12 nucleotides. Also intrinsically curved DNA has been identified at or near several matrix attachment sites (Yamamura and Nomura, 2001). Curved DNA is also considered to play an important role in nuclear processes that involve the interaction of DNA and proteins, such as site-specific recombination, replication, transcription and reparation (Devlin, 1997). Curved DNA was identified in the sequences 3, 5, 6 (Table 6). In sequences 5, 6 were also found more CA, TA and TG. Curved DNA and Z-DNA were found also in mammalian (Labeikyte et al., 2005) and yeast (Bagdoniene et al., 2005) DNA sequences involved in tightly bound DNA-protein complexes.

The origins of replication share the ATTA, ATTTA and ATTTTA motifs. These motifs are presented in most of sequenced (3, 4, 5, 6, 7, 9, 11) DNA clones (Table 6). This implies that TBP-DNA complexes can be involved in some development processes. In previous experiments ori sites were also identified in murine (Labeikyte et al., 2005) and yeast (Bagdoniene et al., 2005) cloned DNA sequences tightly bound to TBP.

Analyzed DNA sequences are not rich in TG nucleotides, but sequences 2, 3, 5, 6, 7 are rich in ATC rules (Table 6). Clustered A/T/C sequences found in MARs have a strong tendency to unwind by base unpairing and it is believed that this unwinding

property confers high affinity binding to the nuclear matrix. It is demonstrated that A/T/C sequences were found also in plant MARs (Avramova et al., 1998).

Some repeat sequences were found with REPFIND program (http://zlab.bu.edu/repfind/) in DNA sequences 6, 9, 11. In sequence 6 was identified CCTCTCTC repeat cluster at 74 bp to 89 bp. In sequence 9 is a specific repeat TCCCGT, located in region at 53 bp to 122 bp. In sequence 11 were found several specific repeats – ACTT at 198 bp to 259 bp and GGC - at 25 bp to 38 bp.

We have compared cloned DNA fragments with known sequences of plant genome by Blast program (www.nlm.nih.gov/blast/) (Altschul et al., 1997). Also we have performed search of transcription factor binding sites localization in analyzed DNA sequences by using MatInspector program (www.genomatix.de/cgi-bin/matinspector_prof/).

Computer analysis has revealed that all cloned DNA fragments are heterogeneous in their composition, but they have and some similarity (Table 7). Analyzed DNA sequences show identity with different sites of barley *Hordeum vulgare* chromosomes 1H, 2H, 3H, 6B, 7H. These sequences are localized in subtelomeres, telomeres, long arm of chromosomes, but all cloned sequences show no homology with centromeres. Eight DNA fragments (1, 2, 5, 6, 7, 9, 10, 11) were found in different repeated sequences between structural genes, remaining three DNA fragments (3, 4 and 8) show homology with both repeated sequences and structural genes. Most of these repeated sequences are general plant retrotransposones, transposones and tandem repeat sequences (retrotransposons: *BARE-2, BAGY-2, Vagabond, Lolaog, Sabrina, Sukkula, Wilma* and *Veju*, transposons: *Damocles, Hades, Tourist, Icarus* and *Caspar*, tandem repeat sequences: Afa family, direct and inverted repeat, intergenic spacers).

Structural genes that are homologous with cloned DNA fragments mostly belong to various plant disease resistant, stress response, developmental and environmental changes response genes (actin depolymerizing factor gene *Adf2,* stem rust resistance gene *Rpg5,* resistance protein gene *RGA1,* protein phosphatase 2C protein gene *PP2C,* peroxidase gene *P7X,* actin gene *ACT-1,* condensation factor gene *CCF,* nodulin-like protein gene *NLL*). These structural genes are localized in various identified contiguous loci of barley genome, composed of gene islands and a number of transposons and repeat regions. It is suggested that retrotransposons, transposons and various repetitive

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sequences are involved not only in plant genome "obesity", but also can influence the activity of genes. Because most of studied DNA sequences show identity with repeated sequences and retroelements, maybe, they can be involved in control of genes expression and chromatin rearrangement.

Table 7. Comparison of TBP-DNA complexes DNA sequences from barley leaves with known sequences of plant genomes, using Blast program (www.nlm.nih.gov/blast/).

Seq	Access number	Description	Score	Identity, %
1	AJ279072.1	*Hordeum vulgare BARE-2* and partially *BAGY-2* retrotransposons	219	91%
	AJ295124.1	*Hordeum vulgare BARGY-L3* retrotransposon	208	91%
	DQ445238.1	*Hordeum vulgare* retrotransposon *Vagabond_M19C-1*, retrotransposon *Lolaog_M19C-1*, transposons *Damocles_M19C-1* and *Hades_M19C-1*, transposon *Tourist_M19C-1*, retrotransposons *BAGY_M19C-1* and *Wham_M19C-1*	176	87%
2	AY853252.1	*Hordeum vulgare* chromosome 7H telomere region	2386	97%
	AF427791.1	*Hordeum vulgare* Mla locus	2863	98%
	Z54373.1	*H.vulgare* repeat DNA	297	96%
	AB003259.1	*Triticum urartu* tandem repeat DNA, Afa family sequence, clone pTuAfa1	268	89%
	X85384.1	*H. vulgare* direct and inverted repeat DNA sequences	305	93%
3	FM242578.1	*Triticum aestivum* storage protein activator (spa) locus region, D genome, clone BAC Ren2409K09	98.2	74%
	EU812563.1	*Hordeum vulgare Rpg4, RGA1 (RGA1), Rpg5, PP2C (PP2C)* ir *ADF3* genai	133	87%
	EU878778.1	*Hordeum vulgare Adf2 (Rpg4), RGA1*, NBS-LRR-S/TPK stem rust resistance (*Rpg5*) and *ADF3* genes	44.6	82%
	DQ445253.1	*Hordeum vulgare* retrotransposon *Sabrina_T8A-1*, retrotransposon *Sukkula_T8A-1* and retrotransposon *BARE_T8A-1_SoloLTR*	39.2	79%
4	AP009567.1	*Hordeum vulgare* genes for putative iron-deficiency specific 4 protein and putative ethylene-responsive transription factor	239	94%
	AF254799.1	*Hordeum vulgare* tonoplast intrinsic 1 (*TIP1*), tonoplast intrinsic 2 (*TIP2*) and *Rar1 (Rar1)* genes	221	91%
	DQ445252.1	*Hordeum vulgare* retrotransposon *BARE_T14B-1*, retrotransposon *Wilma_T14B-1*, transposon *Icarus_T14B-1*	181	87%
5	NC003076.7	*Arabidopsis thaliana* genominė DNR, 5 chromosoma	41.0	87%
6	NC003075.6	*Arabidopsis thaliana* chromosome 4, genomic DNA	46.4	100%
	NC008397.1	*Oryza sativa* chromosome 4 , genomic DNA	116	88%
7	EF067844.1	*Hordeum vulgare* vrs1 locus region and *Hox1* gene	211	78%
8		*Zea mays* P7X peroxidase gene		100%
9	X04075.1	Wheat 26S - 18S rDNA nontranscribed spacer region 2 (pTA 250), located between subrepeat region and 18S gene on chromosome 6B	50.0	77%
	AJ243071.1	*Triticum monococcum* intergenic spacer (IGS)	48.2	77%
10	EF560592.1	*Triticum turgidum* retrotransposon *Copia WIS-1*, transposon *Caspar-1*, transposon *Hades-1*, retrotransposon *Veju-1*, transposon *Caspar-2*, transposon *Hades-2*, transposon *Orpheus-1* and transposon *Isaac-3*	195	89%
	FJ477092.1	*Hordeum vulgare Rym4* and *MCT-1* genes	150	79%
11	DQ445253.1	*Hordeum vulgare* retrotransposon *Sabrina_T8A-1*, retrotransposon *Sukkula_T8A-1*, retrotranspoon *BARE_T8A-1_SoloLTR*	318	88%
	AF326781.1	*Triticum monococcum* actin (*ACT-1*)gene, putative chromosome condensation factor (*CCF*), putative resistance protein (*RGA-2*), putative resistance protein (*RGA2*) and putative nodulin-like-like protein (*NLL*) genes, retrotransposons *Josephine, Angela-2, Angela-4, Heidi, Greti, Angela-3, Fatima, Erika-1, Angela-6, Angela-5, Barbara, Isabelle, Erika-2* and *Claudia*	240	80%

Computer analysis using MatInspector program (Cartharius et al., 2005) has revealed that cloned DNA sequences contain multiple motifs which are recognized by known transcription factors (Table 8).

Table 8. Transcription factors binding sites in cloned DNA sequences of barley leaves TBP-DNA complexes (1-11), (+) - direct strand, (-) - reverse strand. For analysis were used program MatInspector (www.genomatix.de/cgi-bin/matinspector_prof/).

Position	Strand	Sequence	Transcription factor family and characterisation
Seq-1 (1-158)			
67-83	(-)	caacc**TTGAtcg**agtct	W Box family
108-122	(+)	ccgta**AACGg**agaaa	M-phase-specific activator elements
122-138	(-)	aggt**ttTCATg**gat**g**at	Opaque-2 like transcriptional activators
Seq-2 (1-209)			
2-14	(-)	gttt**TTGTc**gtag	ID domain factors
18-40	(-)	tc**cgtt**ttgta**CACGa**a**gt**gcat	NAC-domain transcription factors
23-39	(+)	ctt**CGTGt**acaa**aacgg**	Calmodulin binding / CGCG box binding proteins
30-44	(+)	tacaa**AACGg**acaat	M-phase-specific activator elements
109-119	(+)	cttat**TTGAc**ctccata	W Box family
130-148	(-)	cct**TTGA**at	AS1/AS2 repressor complex
174-182	(-)	gaata**CCA**Aatgaa**g**tcagaa	MADS box proteins
Seq-3 (1-243)			
20-36	(-)	gggca**CGTG**tggtttca	Brassinosteroid (BR) response element
18-34	(-)	gca**CGTG**tggttt**cagg**	Calmodulin binding / CGCG box binding proteins
22-38	(+)	aaa**c**cac**ACGT**gcccca	ABA response elements
20-40	(-)	gatggggc**ACGT**gtggtttca	Plant G-box/C-box bZIP proteins
75-91	(+)	actaggg**GGTG**gtcg	Salt/drought responsive elements
128-141	(+)	ggcc**TATA**aattccc	Plant TATA binding protein factor
129-142	(+)	ggccta**TAAA**ttcccaa	L1 box, motif for L1 layer-specific expression
138-158	(+)	atatt**CCA**Aa**aa**tc**cta**aata	MADS box proteins
167-183	(-)	cggcgtaa**AAAG**cgttt	DNA binding with one finger (DOF)
Seq-4 (1-156)			
11-27	(-)	**tt**cttt**atAAAG**gtt**ag**	DNA binding with one finger (DOF)
15-29	(+)	cctt**TATA**aagaagg	Plant TATA binding protein factor
Seq-5 (1-165)			
4-12	(+)	**tca**TTGAan	AS1/AS2 repressor complex
47-59	(+)	ccg**TGTC**tcacta	Auxin response element
72-86	(-)	gtaa**TATA**aagtaat	Plant TATA binding protein factor
77-93	(-)	tatg**ATGG**taatataaa	GT-box elements
95-109	(-)	gtac**ATGA**aaaaact	GAP-Box (light response elements)
110-122	(+)	tatt**TTCCggc**ttat	E2F-homolog cell cycle regulators
124-140	(+)	cttataaa**AAAG**tgctc	DNA binding with one finger (DOF)
Seq-6 (1-303)			
10-26	(+)	tgta**tg**A**CCT**g**ttt**ggt	Opaque-2 like transcriptional activators
24-40	(+)	ggt**ggg**a**TTAA**attt**a**	GT-box elements
26-42	(+)	tggg**at**TAAA**t**tttatt	L1 box, motif for L1 layer-specific expression
68-92	(-)	tgag**agAGAGg**gagagagg**t**gttgg	GAGA elements
160-176	(-)	atccc**CGTG**tgtgtgtg	Brassinosteroid (BR) response element
Seq-7 (1-176)			
73-87	(+)	c**ga**tatat**ta**AGAAa	Heat shock factors
90-106	(-)	ctt**attTTGT**taaagct	MYB-like proteins
100-116	(+)	aaataagt**AAAG**aaata	DNA binding with one finger (DOF)
Seq-8 (1-160)			
29-49	(-)	cagg**TTCGcgc**cgat	E2F-homolog cell cycle regulators
52-66	(+)	ag**cttggc**ttc**NACGa**c**act**gag	NAC-domain transcription factors
117-131	(-)	cagc**ACCGccc**ttgc	Dehydration responsive element binding
120-132	(+)	caagg**g**c**GGTG**ctgc	Salt/drought responsive elements
Seq-9 (1-189)			
50-64	(-)	ggaga**AACGg**gaggt	M-phase-specific activator elements
71-87	(-)	gtttggga**AAAG**caagg	DNA binding with one finger (DOF)

121-137	(-)	tggc**aTGAGtcg**tacac	Opaque-2 like transcriptional activators
144-158	(-)	ttcga**CCGAc**ggatc	Dehydration responsive element binding factors
140-162	(-)	gc**cgtt**cgacc**GACGgatc**agaa	NAC-domain transcription factors
169-183	(-)	actt**TTCTcgc**aacc	E2F-homolog cell cycle regulators
Seq-10 (1-91)			
28-42	(-)	ag**a**aattt**caTGAA**a	Heat shock factors
Seq-11 (1-294)			
2-18	(+)	ctcct**CGTG**tgataggg	Brassinosteroid (BR) response element
22-38	(-)	gc**cg**ccg**cCGTG**ccct	ABA response elements
65-81	(-)	ccgac**cTGACc**actggg	W Box family
72-86	(-)	gtact**CCGA**cctgac	Dehydration responsive element binding
238-254	(-)	tttaat**TAAGtgt**actt	L1 box, motif for L1 layer-specific expression
244-260	(+)	acttaatt**AAAG**acttg	DNA binding with one finger (DOF)
267-283	(-)	atc**acgGTTAc**gcactc	GT-box elements

Most of these transcription factors are general regulator factors (GRFs) controlling the development processes, cycle of plant cells and stress response: AS1/AS2 repressor complex, GAGA-binding protein (GBP), DNA-binding with one finger (Dof) motive, 2F-homolog cell cycle regulator, mitosis-specific activator (MSA), abscisic acid (ABA), salt/drought, dehydration and brassinosteroid (BR) response elements, also MADs, W box family and NAC domain transcription factors etc.

It is important to note, that among TBP proteins from barley primary leaves were identified proteins homologous to some of transcription factors involved in development and biotic/abiotic stress response in plants: WRKY, MADs, Squamosa, Scarecrow, TGA4, TEOSINTE BRANCHED 1. We suppose that DNA sequences from barley primary leaves TBP-DNA complexes somehow participate in regulation of such processes as plant development, growth and stress response.

Computer nucleotide sequence analysis of eleven DNA fragments involved in tightly bound DNA protein complexes shows, that these sequences are heterogeneous, but have some motifs, that are common for all of them: topoizomerase II binding sites, short A+T rich stretches, curved DNA stretches. These sequences are rich in transcription factors binding sites. Our results show that examined DNA sequences may be important for rearrangement of DNA structure and for regulation of gene expression.

Analysis of DNA sequences from barley TBP-DNA complexes using oligonucleotide DNA arrays. In order to study transcription, tissue and development dependent changes in TBPs distribution in barley genes at different phases of seed development, we have used oligonucleotide DNA array technique. We have applied this technique in our recent work (Bielskiene et al., 2008) to study the distribution of TBPs along the chicken alpha-globin domain in cell lines that expressed the gene, did not

express it or conducted abortive expression. In this study we have shown profound transcription dependent changes in the TBPs-distribution pattern in the alpha-globin domain. Preliminary results of our team (Sjakste et al., 2005) also indicated the existence of tissue and development specificity in the patterns of TBPs distribution in barley (*Hordeum vulgare*) shoots.

To investigate DNA sequences from barley TBP-DNA complexes by apply to oligonucleotide DNA arrays, we used seeds of watery ripe (Zadoks 71) and medium milk development (Zadoks 75) (classification according to Anderson) (Anderson et al., 1995).

We need to choose also well characterized genes, that expression level is different in these stages of seeds. It is known that expression of several specific enzymes of carbohydrate metabolism in barley is development- or/and tissue specific and is restricted to well-defined stages of the plant development (Zou et al., 2008). It was reported that transcription of α-amylase genes is low and decreases during seed development, but β-amylase expression in endosperm is upregulated during its development (Green et al., 1997; Mundi et al., 1986; Zou et al., 2008).

We have formulated the goal of the present study - to investigate the distribution of TBPs along *Amy32b* and *Bmy1* genes in watery ripe and medium milk stages of seeds using oligonucleotide DNA arrays.

***Amy32b* and *Bmy1* genes expression at different phases of seed development.** It was found that *Amy32b* gene is highly expressed in watery ripe and silent in milky ripe seeds. On the contrary, expression of the *Bmy1* gene was detected exclusively in the milky ripe stage. Thus, the chosen barley genes at two stages of seed development represented a model system to be used in further analysis of TBPs distribution along the silent and expressed genes.

Microarray analysis. TBP-DNA complexes from seeds of watery ripe (Zadoks 71) and medium milk (Zadoks 75) stages of development were obtained as described previously (see Materials and Methods). DNA were disrupted by sonication and fractionated on nitrocellulose membrane. Leak out, TBP-free DNA (F) and retained on membrane, TBP-bound DNA (R) fractions were labeled with radioactive mark (see Materials and Methods) and used as probes for hybridization with DNA microarrays. *Amy32b* (GenBank accession X05166) and *Bmy1* (GenBank accession AF061203)

genomic sequence information was used to design the arrays. The DNA array for each gene was developed according to the published approach (Ioudinkova et al., 2005; Bielskiene et al., 2008), and consisted of 50-60 bp oligonucleotides spaced along the whole gene. Sequences of the oligonucleotides are given in Tables 1 and 2, their location in the structural genes is shown in Figure 9 and 10, for *Amy32b* and *Bmy1* genes, respectively.

The barley genome has not been sequenced completely yet, therefore we have checked whether the chosen oligonucleotides in the array contained repetitive sequences by hybridization with total barley DNA. It was shown that total barley DNA hybridizes almost equally to α-amylase and β-amylase genes DNA arrays, with exception of the oligonucleotides localized in positions 1 kb and 4.7 kb of β-amylase gene (positions 4, 12) and in positions 2 kb and 2.4 kb of α-amylase gene (positions 9, 11) (results is not show). These oligonucleotides may be represented by several copies in the genome or contain some short simple motifs. Received data of hybridization with F probe (probe derived from the TBP-free DNA) and R probe (probe derived from the DNA associated with TBP proteins) were quantified according to signal of total DNA hybridization. Data are presented as R /F ratio of hybridization signals.

Figure 9 and 10 present quantified results of hybridization between TBP-DNA complexes fractionated from seeds of watery ripe (Zadoks 71, panels A) and medium milk (Zadoks 75, panels B) stages and DNA microarray of *Amy32b* (Fig. 9.) and *Bmy 1* (Fig. 10.) genes correspondingly.

In samples from watery ripe seeds, ratios of hybridization intensities with R probe (probe derived from the DNA complexed with TBPs) and F probe (probe derived from the TBP-free DNA) and *Amy32b* microarray were approximately the same in oligonucleotide positions 1, 9, 10 and 11 reflecting an equal or similar amount of the corresponding DNA fragments in both TBP bound and TBP unbound fractions. In all other positions the ratio exceeded 1, indicating the enrichment in TBPs in the corresponding DNA fragments. Positions 3 (300 bp upstream the translation start codon), 7 (downstream part of Intron 2), and 8 (Exon III) were shown as the most TBP-enriched in samples from watery-ripe seeds. Transition to milky ripening was followed by the decrease of R vs F ratio in general along the whole *Amy32b* gene. Ratio much below 1 in positions 1, 5, 9 and 11 indicates predominant accumulation of corresponding DNA

fragments in the TBP- free F fraction. In positions 3 and 7 the ratio decreased from 6 and 4 to almost 1, and from 4 to 2.5 in position 8. The position of oligonuclotide 10, in which the ratio increased from 1 to 2, was an exception. Thus, during seed development, an overall decrease in TBP-DNA interactions (R → F transition) occurs along the *Amy32b* gene being most drastic in the promoter and Intron 2 gene regions.
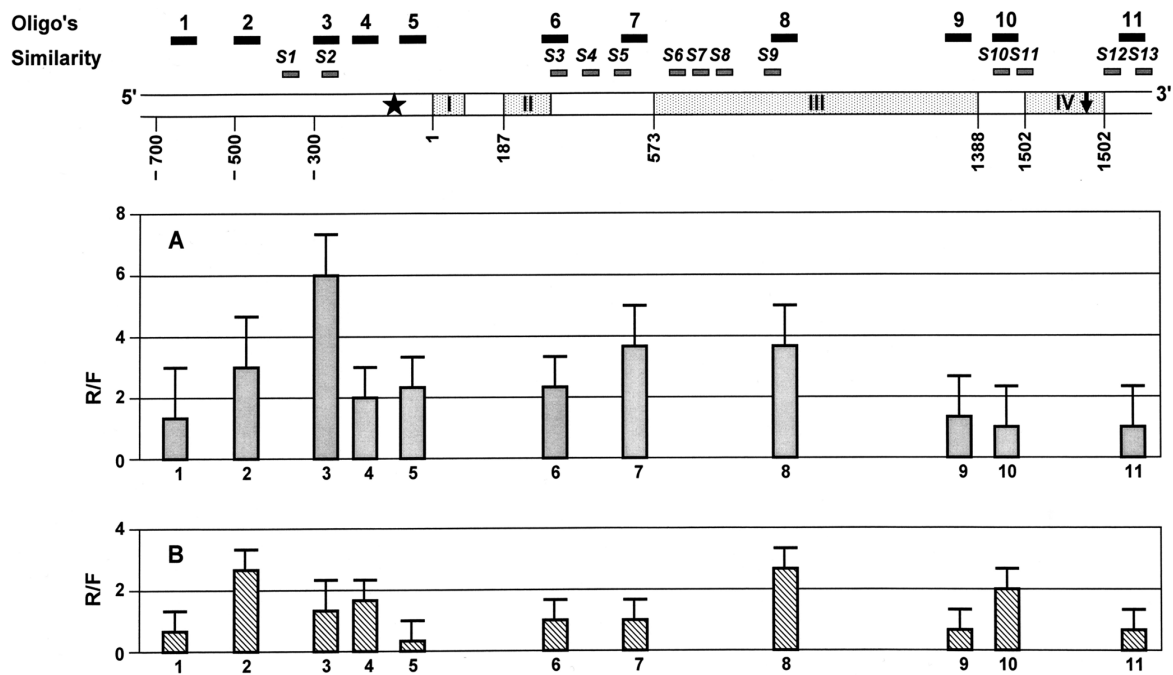

Fig. 9. DNA array based mapping of the TBPs distribution in *Amy32b* structural gene in watery ripe (A) and milky ripe (B) barley seeds. Upper panel presents the gene structure with positions of oligonucleotides of the array and regions of the similarity with *Bmy1* (S1 – S13). Star indicates position of the TATA box, black arrow in last exon indicates stop codon. Exon numbers are given as Roman numerals. The data in lower panels represent the ratio of hybridization of R vs F DNA fractions scored as an average of three independent experiments (two hybridizations per experiment). Error bars represent standard deviation.

The R and F fractions obtained from watery ripe seeds hybridized also with different intensity with *Bmy1* gene microarray. The R vs F ratio was rather high in oligonucleotide positions 6, 9 (more than 2) and 13 (near to 3). Finally, in positions 7 and 10, the intensity of hybridization with R probe was 4 times stronger than with F probe. In the milky ripeness stage (Fig. 10, panel B) the R vs F ratio slighty exceeded 2 only in position 10 and 13. In all other oligonucleotide positions the R vs F ratio was around 1. Thus, similar to the results obtained with *Amy32b* gene, the overall decrease of

the R vs F ratio is revealed along the *Bmy1* gene during seed development. The process is more pronounced in Exon III and upstream the microsatellite locus in Intron III.
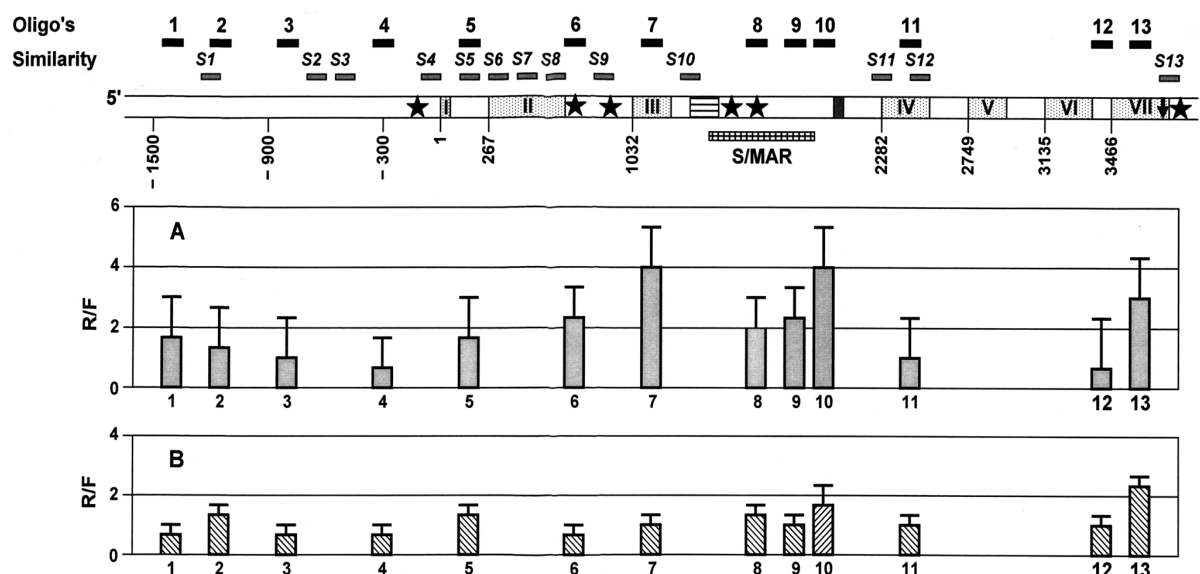

Fig. 10. DNA array based mapping of the TBPs distribution in *Bmy1* structural gene in watery ripe (A) and milky ripe (B) barley seeds. Upper panel presents the gene structure with positions of oligonucleotides of the array and regions of similarity with *Amy32b* gene (S1 – S13). Stars correspond to the positions of the TATA boxes; black arrow in last exon VII indicates the stop codon. Exon numbers are given in Roman numerals. Black and dashed squares in the Intron III indicate the positions of the microsatellite and MITE element, correspondingly. The predicted MAR position is indicated by a checked bar. The data on lower panels represent the ratio of hybridization of R vs F DNA fractions scored as an average of three independent experiments (two hybridizations per experiment). Error bars represent standard deviation.

Bioinformatic analysis of *Amy32b* and *Bmy1* gene sequences. Identification of specific areas of TBP binding in both genes raised the question of peculiarities in the gene sequences in these areas. With help of Latvian colleges (Sjakste T and Sjakste N) was performed bioinformatic analysis of *Amy32b* and *Bmy1* gene sequences. A search for nuclear matrix attachment regions (MARs) performed by the MatInspector program (Rel. 7.4), revealed a possible MAR in Intron III of the *Bmy1* gene (Fig. 9). No MARs were detected in the *Amy32b* gene. DiAlign TF, Release 3.1 revealed 13 homology regions (S1-S13) between the two genes (Figure 8 and 9), however no common features in the distribution of transcription factor binding sites along the genes and transcription factor binding modules were revealed. Comparison of the predicted MAR with the TBP binding sites in the *Bmy1* gene Intron III indicates that this site is enriched in TBPs.

Similar sequences of the two genes differed in their affinity to TBPs. The only exception was observed in the area of similarity S9; these sites were TBP-enriched in both genes.

Gene expression changes during seed development. In the present study we have revealed development dependent changes of the TBP distribution in *Amy32b* and *Bmy1* genes during transition of the barley seed from watery-ripe to middle milk ripe stage. Changes in TBP distribution in the genes were coupled to changes in their expression. β-amylase gene expression is linked to starchy endosperm development, but α-amylase gene is not expressed on late seed development stages (Mundy et al., 1986), moreover over-expression of α-amylase gene in developing seed leads to development defects (Green et al., 1997).

TBPs redistribution in the *Amy32b* gene. The barley *Amy32b* gene promoter contains at least five *cis*-acting elements that govern its GA-induced expression: O2S/W-box, pyrimidine box, GA response element (GARE), amylase box (Amy), and downstream amylase element (Lanahan et al., 1992). These motifs interact with various transcription factors controlling seed germination. Each of these elements may be bound by one or more transcription factors of R2R3 MYB, R1 MYB, DOF, and zinc finger protein families (Moreno-Risueno et al., 2007; Zou et al., 2008). However, it still remains unclear how these repressors and activators interact with each other in regulating gene expression. Mounting evidence suggests that each of the five *cis*-acting elements essential for GA induction of *Amy32b* can be bound by both transcriptional repressors and activators in barley aleurone cells. The pyrimidine box can be bound by the SAD activator and the BPBF and HvDOF19 repressors, which are DOF proteins (Moreno-Risueno et al., 2007). In addition to the transcriptional activator GAMYB, the repressor HRT, a zinc finger protein, can also bind to GARE. The Amy box can interact with the HvMCB1 repressor and the HvMYBS3 activator. Repressor HvWRKY38 interacts with the W-boxes. An activator for this element has not been reported, although RAMY, another zinc finger protein, also binds to this element (Zou et al., 2008).

It is amazing that we establish recently among barley TBPs WRKY transcription factors 16 and 52, W-box family, MYB-like proteins, and DOF proteins (see page 24-25). As mentioned above, MYB-like proteins are transcriptional activators of *Amy32b* gene, DOF proteins and WRKY38 are repressors. It is little known about function of other barley WRKY family members in GA signaling. It was established that the ratios

of repressors to activators, and more importantly, the cooperative binding of repressors or activators to the *Amy32b* promoter, determine the levels of *Amy32b* expression. The GA induction of *Amy32b* is modulated by two protein complexes, one for activation and the other for repression (Zou et al., 2008). Maybe some TBP are involved in *Amy32b* promoter inactivation/activation by formation of repressors/activators complexes, which bind to promoter.

Organization of the *Amy32b* gene 5' area appears to be favorable for TBP binding, as it contains several inverted sequences (Whittier et al., 1987). It was shown early that repeated sequences are often found in the TBPs anchoring sites (Avramova et al., 1994; Neuer-Nitsche et al., 1988; Pfutz et al., 1992; Werner and Neuer-Nitsche, 1989). Also we found that DNA sequences of barley TBP-DNA complexes show high homology with various transposons, repeat sequences, among them inverted sequences, which localized between structural genes (see page 31-37)

TBPs redistribution in the *Bmy1* genes. In *Bmy1* gene, besides the overall decrease of TBP binding coupled to up regulation of transcription we have also observed drastic changes of TBP binding in Exon III and Intron III, changes 1.5 Kb upstream the Exon I were also well-pronounced.

Taking into account that expression of the *Bmy1* gene is observed exclusively in the milky ripe stage, we can speculate that preferential TBPs binding to promoter and Intron III of dormant gene (watery ripe stage) somehow represses transcriptional activity. It was established recently that some sequences in Intron III and promoter region may serve as a binding sites for a negative transcription factor (Erkkila et al., 2001). Release of TBPs in the milky ripe stage can influence gene expression by binding transcriptional activators.

It was demonstrated that in *Bmy1* Intron III are sequences with various regulator element binding sites: G/C BZIP proteins, light specific elements, MYB and MYB-like proteins, ethylene responsive factor et al. (Sjakste and Zhuk, 2006). Light specific elements, MYB and MYB-like proteins binding sites were also found in barley DNA sequences of TBP-DNA complexes (see page 31-37).

Interestingly, Intron III of *Bmy1* gene harbors a microsatellite, MITE element, several other repeats and a predicted MAR (Fig. 10). Both microsatellites and mobile elements are often involved in tight DNA-protein complexes (Boulikas, 1995;

Nabirochkin et al., 1998) and hence these elements could also possess high affinity to TBPs. Indeed as position 10 of the microarray is very close to the microsatellite, sequences of the MITE element should hybridize with the oligonucleotide in the position 7 (Exon III at 3'side). Enrichment of the mobile genomic elements in complexes with TBP was revealed also in our study on the characterization of the TBP-associated DNA sequences (see page 31-37). On the other hand, the presence of a possible nuclear matrix attachment site (MAR) in Intron III of *Bmy1* gene and the absence of such sequences in other sites of the gene intensively binding TBPs indicate that TBP binding is not dependent on the presence of MAR.

TBPs binding and transcription. The overall intensity of the DNA association with TBP appears to be independent of transcription, as in medium milk seeds (Zadoks 75) we observed an overall decrease in the R vs F ratio along both genes, although one of them became expressed on this stage, but the other – remained dormant. Triggering or cessation of transcription is rather accompanied by redistribution of TBPs along the gene, but not overall accumulation or release of these proteins from the whole gene sequence. The same conclusion was made after a similar study on the chicken produced alpha globin gene domain (Bielskiene et al., 2008). Comparison of TBP distribution in the domain in erythroid cells differing in the degree of alpha-globin gene transcription revealed changes in distribution of the TBPs, however the TBPs were much more abundant along the domain in DNA from liver than in erythrocytes and erythroblasts. Thus, in animals overall enrichment in TBPs appears to be an organ-specific trait (Bielskiene et al., 2008). Our results indicate that in plants it could be characteristic of the development stage of an organ. Our data indicate the existence of development-dependent redistribution of the TBPs along *Amy32b* and *Bmy1*.

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CONCLUSIONS

1. Barley tightly bound DNA-protein complexes contain 15-160 kDa proteins whose composition varies in different organs (coleoptile, first leaves and roots) and different developmental stages. Most of TBPs are acidic.

2. Barley TBPs of different organs (coleoptile, first leaves and roots) and different developmental stages manifest phosphatase activity.

3. Barley TBPs tyrosine phosphorylation depends on organ and developmental stage.

4. Identified barley TBPs are involved in chromatin rearrangement and regulation processes. These are nuclear matrix proteins NMCP1 and topo II, some transcription factors, serpins, transposon polypeptide.

5. Interactions between TBP-DNA complex DNA sequences and TBPs are specific.

6. DNA sequences from barley first leaf TBP-DNA complexes show high homology to various transposons, retrotransposons and repeated sequences, localized between structural genes.

7. Examined sequences can be identified as specific S/MAR group with specific features.

8. Changes in TBP distribution in Amy32b and Bmy1 genes were coupled to changes in their expression during water ripe and milky ripe stages, but effects of TBPs binding to genes were different – *Amy32b* expression increased, but *Bmy1* expression decreased . Distribution of TBPs is specific for development stages.

9. Barley TBP-DNA complexes may be involved in chromatin structure rearrangement and regulation of gene activity.

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SANTRAUKA

Žinoma, kad pastovi nehistoninių polipetidų frakcija yra išgryninama kartu su eukariotine DNR ir sudaro labai tvirtus (galbūt kovalentinius) kompleksus tarp branduolio baltymų ir DNR. Nustatyta, kad Erlicho ascito tvirtuose DNR-baltymų kompleksuose yra baltymas C1D, baltymai, pasižymintys fosfataziniu ir kinaziniu aktyvumais, kai kurie proteazių slopikliai ir kiti, dar neištirti baltymai.

Nepaisant intensyvių tyrinėjimų, eukariotinių ląstelių tvirti DNR-baltymų kompleksai vis dar lieka menkai aprašyti ir yra objektas tolimesniems tyrimams. Augalų TBP-DNR kompleksai kol kas buvo tyrinėti labai mažai. Šiame darbe charakterizuojami miežių *Hordeum vulgare* tvirti DNR-baltymų kompleksai.

Mes tyrėme TBP-DNR kompleksus iš miežių skirtingų ūglių organų ir skirtingų vystymosi stadijų ląstelių: lapų, šaknų, koleoptilės. Norint ištirti tokių nukleoproteidų funkcijas, svarbu charakterizuoti individualius komplekso komponentus: polipeptidus ir DNR. Taigi, išskyrėme tvirtai su DNR sąveikaujančius baltymus iš miežių skirtingos diferenciacijos bei skirtingo amžiaus ląstelių: pirminių lapelių, šaknų, koleoptilės ir juos charakterizavome. Taip pat išskyrėme ir charakterizavome DNR fragmentus iš miežių pirminių lapelių bei vandeninės brandos ir pieninės brandos grūdų TBP-DNR kompleksų.

Parodėme, kad miežių TBP baltymai yra 15-160 kDa, dauguma baltymų yra rūgštiniai. Kai kurie iš miežių TBP baltymų (10, 25, 38, 40 ir 55 kDa) pasižymi fosfataziniu, galbūt, Ser/Thr aktyvumu. Nustatėme, kad tam tikrų TBP baltymų tirozinai yra fosforilinti, o ši modifikacija priklauso nuo organo ir jo vystymosi stadijos. Masių spektrometrijos metodu identifikavome tam tikrus miežių TBP baltymus. Dauguma jų yra susiję su DNR funkcijomis ir dalyvauja chromatino persitvarkymo bei reguliacijos procesuose. Tai branduolio matrikso baltymai, transkripcijos veiksniai, serpinai, imunofilinai, transpozonai. Parodėme, kad TBP baltymų raiškos lygis kinta priklausomai nuo organo ir vystymosi stadijos. Taip pat nustatėme, kad stipriausiai TBP baltymai sąveikauja su iš to paties organo išskirto TBP-DNR komplekso DNR fragmentais.

Nustatėme, kad tvirtai su TBP baltymais sąveikaujančios DNR sekos pagal savo savybes yra panašios į S/MAR sekas, tačiau turi ir savo ypatumų. Šios sekos nėra homologiškos tarpusavyje. Dauguma jų randama šalia telomerų arba telomerinėse chromosomų srityse. Šiose sekose randami bendri branduolio matrikso baltymų

atpažinimo motyvai: lenkta DNR, topo II, ori sritys, ATC seka. Randama daug transkripcijos veiksnių jungimosi vietų, o pačios TBP-DNR kompleksų DNR sekos yra homologiškos įvairiems transpozonams, retrotranspozonams bei kartotinėms sekoms. Gali būti, kad šios DNR sekos yra svarbios chromatino struktūros palaikymui ir genų veiklos reguliacijai vystymosi ir diferenciacijose procesų metu.

Tvirtų DNR-baltymų kompleksų DNR sekų hibridizacijos su miežių *Amy32b* ir *Bmy1* genų mikrogardelėmis metodu parodėme, kad miežių TBP baltymai sąveikauja su *Amy32b* geno promotoriaus sritimi, o *Bmy1* geno − egzonu III, intronu III bei 1,5 Kb sritimi virš egzono I. Abiejų genų atveju sąveika su TBP baltymais mažėja grūdų vystymosi metu (vykstant persijungimui iš vandeninės brandos į pieninę brandą). TBP baltymų persiskirstymas *Amy32b* ir *Bmy1* genuose yra susijęs su šių genų raiška vandeninės brandos ir pieninės brandos grūduose, tačiau TBP baltymų poveikis *Amy32b* ir *Bmy1* genų raiškai yra skirtingas − *Amy32b* geno raiška sustiprinama, o *Bmy1* geno raiška slopinama.

Manome, kad mūsų gauti rezultatai apie miežių tvirtus DNR-baltymų kompleksus padės atsakyti į tokius svarbius klausimus kaip TBP baltymų ir branduolio matrikso sąveika, TBP baltymų vieta branduolyje, TBP polipeptidų sudėtis ir funkcijos, tvirtai su TBP sąveikaujančių DNR sekų sudėtis ir funkcijos genome. Atlikti tyrimai leis geriau pažinti iki galo neištirtą augalų branduolį ir jame vykstančias sąveikas tarp branduolio baltymų ir DNR.

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