Analysis of proteins in tightly bound DNA–protein complexes from barley primary leaves

K. Bielskiene¹,
L. Bagdoniene¹*,
D. Labeikyte¹,
B. Juodka¹,
N. Sjakste²

¹Department of Biochemistry and Biophysics, Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius, Lithuania
²Faculty of Medicine, University of Latvia, Sharlotes iela 1a, LV-1001 Riga, Latvia

Tightly bound to DNA proteins (TBP) are a protein group that remains attached to DNA with covalent or non-covalent bonds after its deproteinisation [1, 2]. TBP have been found in DNA of numerous evolutionary distant species [3–5]. Some of these TBP proteins have been characterized in Ehrlich ascites [6–8] and yeast (Saccharomyces cerevisiae) [9] cells. The aim of this work was to characterize TBP proteins in the first leaves of barley (Hordeum vulgare) shoots by the MALDI TOF-TOF mass-spectrometry (MS) analysis. We have identified that most of these proteins (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) are transcription factors playing important roles in the development and coping with either biotic or abiotic stress in plants. Various barley TBP (above transcription factors, DEMETER-like protein 2, HAC12, RAD51, Ty3-gypsy retrotransposon, protein kinases, serpins) participate in chromatin rearrangement and the regulation of gene expression.

Key words: stable DNA–protein complexes, nuclear matrix

INTRODUCTION

Despite great research efforts, the basic questions regarding chromosome structure and gene expression mechanisms remain to be answered. Relationship between the spatial organization of the genome and the transcription machinery is one of the most important problems. Protein–chromatin interactions are transient, enabling fast scanning of the genome by transcription factors and leaving binding sites constantly available for scanning by other transcription factors [10]. Polypeptides involved in the cell type-specific structural organization and modeling of the chromatin fiber belong, as can be predicted, to the fraction of nuclear matrix proteins with a strong affinity to DNA [8, 11]. Individual proteins forming scaffolding structures for spatial genome organization remain to be characterized. The polypeptides that are able to form permanent or transient tight complexes with DNA, including covalent ones, are of special interest [1, 2, 6, 12, 13]. 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. TBP can be isolated only after DNA digestion with DNase I or benzonase [3, 5, 9, 12]. TBP distribution in the genome is site-specific. They are enriched in several reiterated sequences, but also in sequences of structural genes [13–16]. These sequences are similar to nuclear matrix attachment sequences (MAR) and are rich in transcription factor binding sites [17]. It has been demonstrated that serpins Spi-1, Spi-2, Spi-3 [7] and 16 kDa protein C1D [8] belong to TBP proteins of tightly bound DNA–protein complexes. C1D was also found to be associated with the transcriptional repressor RevErb and the nuclear corepressors N-cor and SMRT, which led to the conclusion that it could function as a component of the complex involved in transcriptional repression [8]. It has been demonstrated that some of TBP from Ehrlich ascites and yeast cells manifest phosphatase and kinase activity [5, 18]. 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 TBP participate in chromatin rearrangement and regulation processes [9]. Despite a great deal of research, the functional significance of TBP is not yet clear. It has been proposed that TBP proteins may participate in differentiation
and development processes [4]. 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.

MATERIALS AND METHODS

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 (26 °C) in the dark. First leaves were dissected from shoots of Zadoks 07 (coleoptile emerged stage) and Zadoks 10 (first leaf through coleoptile) development stages. The classification here and further has been done according to Anderson [19]. Dissected first leaf tissue from 50–100 shoots was united into one sample for each developmental stage. First leaf samples were subsequently used for bulk DNA extraction.

DNA isolation. 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 [20] 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. Then 10 μl of Rnase A solution (10 mg/ml) 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 cold ethanol (1 : 2 V / V) and 3 M sodium acetate (1 : 10 V / V) and collected by centrifugation at +4 °C, 30 min / 9000 g, then rinsed with 70% ethanol and air-dried. Dry DNA was dissolved in 1 ml of TE buffer. DNA solution was stored at +4 °C.

Purification of tight DNA–protein complexes. Isolated DNA (5 mg/ml) was diluted with benzonase buffer, and then benzonase was added (100 U/1 mg DNA). The reaction proceeded in dialysis conditions for 16 h at room temperature against 1–1.5 l benzonase buffer. Subsequently, dialysis was proceeded for 16–18 hours at +4 °C against 1–1.5 l TE buffer.

SDS-PAGE gel electrophoresis. 12% SDS-PAGE gels were run at 120 V constant voltage for 4 h [21] and then stained with Coomassie dye.

In-gel tryptic digestion and MALDI TOF-TOF MS. The areas of the gel that had been deemed to be of interest were cut out and subjected to in-gel tryptic digestion overnight [22], the gel slides were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini, Epperdorf). The protein spot was 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% trifluoroacetic acid in 50% acetonitrile, shaking occasionally. The digestion and extract solutions were then combined and evaporated to dryness. 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% trifluoroacetic acid 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, Canada) 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.

RESULTS AND DISCUSSION

Recently we have demonstrated that DNA isolated from different barley shoot organs (coleoptile, roots and leaves) by extraction with chlorophorm-isoamylic alcohol [20] still contains polypeptides which are resistant to SDS, urea, mercaptoethanol, phenol treatment and remain associated with DNA. It has been shown by SDS-PAGE that the composition of proteins differs in different shoot organs [4].

The goal of the present work was to characterize TBP proteins from first leaves of barley shoots (Zadoks stages 07 and 10) by the MALDI TOF-TOF MS analysis.

After digestion of DNA with benzonase, TBP proteins of the first leaves of barley shoots were fractionated in 12% SDS-PAGE (Figure). SDS-PAGE revealed a set of 15–100 kDa barley proteins. Some of these protein bands were cut from the gel, digested with trypsin and analysed by MALDI TOF-TOF MS.

The list of proteins identified employing the GPS Explorer™ software is presented in Table. The 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 found between them. The nuclear matrix is a three-dimensional

We have identified that protein in band 2 (75 kDa) is a putative nuclear matrix constituent protein NMCP1. It is found exclusively at the periphery of the nucleus during the interphase and is associated with the spindle during mitosis. NMCP1 proteins, like lamins, have a central coiled-coil domain flanked by a nonhelical short head and a larger tail domain; the pI of these proteins ranges from 5.6 to 5.8. Although they are roughly twice the size of lamins, they are currently the best candidates for lamin-like proteins in plants [23–25].

We have identified that eight barley TBP proteins manifest a homology with different transcription factors associated with development and either biotic or abiotic stress in plants. Proteins in bands 6 (42 kDa) and 16 (85 kDa) are homologous to WRKY transcription factors involved in many physiological processes including plant responses to biotic and abiotic stresses [26]. The WRKY family proteins contain one or two highly conserved WRKY domains characterized by the heptapeptide WRKYGQK and a zinc-finger structure distinct from the other known zinc-finger motifs. To regulate gene expression, the WRKY domain binds to the W box in the promoter of the target gene to modulate transcription [27, 28].

### Table: MALDI TOF-TOF MS analysis of barley leaf TBP proteins

<table>
<thead>
<tr>
<th>Band No</th>
<th>Mw calculated</th>
<th>Mw experimental</th>
<th>Score</th>
<th>List of identified proteins</th>
<th>Organism</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>121</td>
<td>75</td>
<td>9.93</td>
<td>Nuclear matrix protein NMCP1</td>
<td>Arabidopsis thaliana</td>
<td>Q9FLH0</td>
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<tr>
<td>3</td>
<td>21</td>
<td>72</td>
<td>31</td>
<td>AGAMOUS transcription factor homolog</td>
<td>Hyacinthus oryentalis</td>
<td>Q9ZPK9_9ASPA</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>50</td>
<td>0.65</td>
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<td>Zea mays</td>
<td>Q93W12</td>
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<tr>
<td>6</td>
<td>44</td>
<td>45</td>
<td>1.00</td>
<td>Serpin-Z4</td>
<td>Arabidopsis thaliana</td>
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<tr>
<td>6</td>
<td>155</td>
<td>45</td>
<td>25</td>
<td>Probable WRKY transcription factor 16</td>
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<td>Q9FL92</td>
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<tr>
<td>8</td>
<td>40</td>
<td>38</td>
<td>0.40</td>
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<td>Q9LYS4</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>38</td>
<td>14</td>
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<tr>
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<td>63</td>
<td>34</td>
<td>7.19</td>
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<td>14</td>
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<tr>
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<td>20</td>
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<tr>
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<tr>
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<td>52</td>
<td>5.54</td>
<td>F-box / kelch-repeat protein At5g15710</td>
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<td>Q9LFV5</td>
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<tr>
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<td>30</td>
<td>7.86</td>
<td>Transcription factor TGA4</td>
<td>Arabidopsis thaliana</td>
<td>Q39162</td>
</tr>
</tbody>
</table>

1 The number of band in gels (Figure); 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.
In band 13 (28 kDa) and band 3 (72 kDa), we have identified the MADS-box transcription factor 26 and the AGAMOUS homolog transcription factor, respectively. These two proteins belong to the transcription factors that contain a conserved DNA-binding domain of 56 amino acids, namely a MADS box [29]. MADS-box transcription factors are major regulators of development in plants. The factors act in a combinatorial manner, either as homo- or heterodimers, and control floral organ formation and identity and many other developmental processes in leaves and roots through a complex network of protein–protein and protein–DNA interactions [30–32].

In band 18 (52 kDa), we have identified the Squamosa promoter-binding-like protein 16, a putative plant-specific transcription factor; these factors share a highly conserved DNA binding domain, the Squamosa promoter binding protein (SBP) domain of 76 amino acid residues [33]. The structural basis for this sequence-specific binding of DNA is two Zn-finger-like structures formed by the coordination of two zinc ions by conserved cysteine and histidine residues [34].

Protein band 18 (52 kDa) was identified as Scarecrow-like protein 9. Scarecrow (SCR) proteins are members of the plant-specific GRAS family of putative transcription factors involved in various aspects of plant development [35, 36]. In Arabidopsis, expression of the SCR gene was shown to be associated with the chromatin assembly factor-1 (CAF-1) [36].

In band 19 (30 kDa), we have identified the transcription factor TGA4. It belongs to a family of basic domain-leucine zipper (bZIP) transcription factors that are conserved in higher plants. The bZIP proteins are transcription factors which contain a basic region for specific DNA contact and a leucine zipper domain for dimerization. TGA transcription factors are implicated as regulators of pathogenesis-related (PR) genes because of their physical interaction with the known positive regulator of PR gene1 (NPR1) [37–39]. TGA factors not only interact among themselves, but also can cooperate with other DNA binding proteins [39].

In band 5 (50 kDa), we have identified the transcription factor TEOSINTE BRANCHED 1. The TCP domain protein TEOSINTE BRANCHED 1 (TB1) is a putative transcriptional regulator that represses bud outgrowth in grasses [40]. TB1 belongs to the plant-specific TCP domain group of proteins. The TCP domain (59–amino acid domain) has a noncanonical basic helix–loop–helix (bHLH) structure and is composed of 24 putative members [40]. Modeling suggests that the TCP domain allows nuclear targeting, DNA binding, and protein–protein interactions [41, 42].

Our results indicate that transcription factors of several families are found among TBP, thus it appears that bonds between the DNA and transcription factors are resistant to the deproteinization procedure. It seems that DNA–transcription factor bonds must withstand chloroform extraction. Interestingly, several “true” nuclear matrix proteins (other than transcription factors) possess DNA binding domains typical of transcription factors. Matrins F and G, classical nuclear matrix proteins, have zinc fingers [11 and references therein]. The well-known multifunctional protein NuMa binds with DNA through a leucine zipper, and lamins similarly form complexes with DNA [11 and references therein]. Thus, salt resistance is potentially inherent in transcription factor–DNA complexes; the same can be true also for resistance to deproteinization with organic solvents. A possible role of transcription factors in the spatial organization of chromatin domains has been analysed previously, and a hypothetical model has been proposed [11].

It has been previously demonstrated that DNA present in tightly bound DNA–protein complexes from yeast shares some properties with nuclear matrix (MAR) sequences; however, some features are specific of TBP-anchoring sequences [17]. These DNA sequences contain multiple motifs which are recognized by known transcription factors controlling the development processes and cycle of yeast cells [17].

We have identified also some other proteins known as components of the nuclear matrix among barley TBP proteins.

Protein in band 14 (170 kDa) was identified as histone acetyltransferase HAC12. Some transcription factors are known to interact with histone acetyltransferase which is
also fixed on the nuclear matrix; additional factors and RNA polymerase bind to the complex [11].

Histone acetyltransferases (HATs) play a critical role in the regulation of chromatin structure and gene expression. Arabidopsis HAC12 belongs to p300 / CRB HAT homologues. These proteins contain ZZ-type and TAZ-type zinc finger domains and a Cys-rich HAT domain at the C termini. Both ZZ-type and TAZ-type zinc finger domains have been implicated in protein–protein interactions with transcription factors, and the HAT domain confers HAT activity in vitro [43–45].

Protein in band 11 (34 kDa) was identified as DEAD-box ATP-dependent RNA helicase 47B. Most of the members in the DEAD box family possess a determined or putative ATP-dependent RNA helicase activity modulating the secondary and tertiary structure of RNA. An increasing evidence suggests that the DEAD-box RNA helicases play an important role in plant growth and development processes and participate in plant stress responses [46–50].

The 170 kDa protein in band 14 manifests a homology to the retrotransposon protein, the putative Ty3-gypsy subclass. In plants, transposable elements (TEs) are classified into two main classes. Class I TEs transpose via an RNA intermediate and include retrotransposons with long terminal repeats (LTRs) such as Ty1 / Copia-like and Ty3 / Gypsy-like, as well as non-LTR retrotransposons. Class II TEs transpose via a DNA intermediate [51–53].

The RTs (reverse transcriptase domains) of Ty3 / Gypsy elements and retroviruses were shown to be very similar. The expression of retrotransposons is controlled by hormonal and developmental factors [52, 54]. It has been observed for gypsy transposons that MARs (matrix attachment regions) sometimes act as insulators and prevent transcription factor binding and retrotransposon expression [11].

The protein in bands 15 (150 kDa) was identified as the DEMETER-like protein 2. In plants, DNA demethylation is carried out by bifunctional helix–hairpinhelix DNA glycosylases of the DEMETER (DME) family. The DME family consists of DME, DEMETER-LIKE 2 (DML2), DML3, and the repressor of silencing 1 (ROS1). DNA demethylation by DME occurs during reproductive development and is required for genomic imprinting and seed viability. DML enzymes demethylate approximately 179 loci, of which nearly 80% are genes [55–58].

The 26 kDa protein in band 13 was identified as the FKBP12-interacting protein of 37 kDa (AtFIP37). FKBP is a family of immunophilins with a peptidyl-prolyl cis-trans isomerase activity (PPiase), involved in the folding of target proteins. The FKBP12 immunophilin interacts with several protein partners in mammals and is a physiological regulator of the cell cycle [59, 60]. In plants, only one specific partner of AtFKBP12, namely AtFIP37 (FKBP12 interacting protein 37 kDa), has been identified [60]. Because AtFIP37 is expressed throughout plant development, the function of AtFIP37 is likely to be involved in fundamental aspects of plant cell life, such as cell growth or cell cycle [59, 60].

We have also found that protein in band 8 (38 kDa) is a putative DNA repair protein RAD51 homolog B. RAD51 is a recombination protein that binds to single-stranded DNA, forming a nucleoprotein filament which then invades double-stranded DNA to form a heteroduplex joint. To accomplish heteroduplex formation, the RAD51 nucleoprotein filament has the ability to recognize DNA sequence homology. In vitro, the RAD51 protein can, even in the absence of other proteins, promote an extensive and efficient pairing of DNA molecules spanning several kilobases [61–63].

We have identified that protein in band 17 (75 kDa) is the plant heat shock 70 kDa protein. Heat shock proteins (Hsps) / chaperones are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes; they stabilize proteins, prevent aggregation and can assist in protein refolding under stress conditions. They can play a crucial role in protecting plants against stress by reestablishing a normal protein conformation and thus cellular homeostasis [64, 65]. Most of HSP70s are expressed under environmental stress. However, some HSP70s are also expressed under normal conditions; they are known as 70 kDa heat-shock cognates (HSC70s) and are often involved in assisting the folding of de novo synthesized polypeptides and the import / translocation proteins [66, 67]. Some members of Hsp70 are involved in controlling the biological activity of folded regulatory proteins and might act as negative repressors of heat-shock factor (HSF) mediated transcription [64].

Serpin-Z4 is a protein from band 6 (42 kDa). Serpins constitute a superfamily of serine proteinase inhibitors with regulatory properties. The majority of serpins inhibit serine proteinases, but serpins that inhibit caspases and papain-like cysteine proteases have also been identified. Rarely, serpins perform a noninhibitory function; for example, several human serpins function as hormone transporters, and certain serpins function as molecular chaperones or tumour suppressors [68–70]. Inhibitory serpins have been shown to function in the processes as diverse as DNA binding and chromatin condensation and apoptosis control [69, 70]. Most serpins may interact both as substrates and as suicide inhibitors forming inhibitor–proteinase complexes which are unusually stable toward SDS, urea, and other denaturants. It has been established that serpins Spi-1, Spi-2, Spi-3 belong to TBP of Erlich ascites cell tightly bound DNA–protein complexes [7].

A protein identified by the MALDI TOF-TOF analysis in band 12 (28 kDa) shows a homology to the protein kinase homolog F24. Despite the recent advances in research on higher plant protein kinases, the functions of protein kinases are much better understood in animals and in yeast. They are generally regulated by various mechanisms including protein phosphorylation by upstream kinases, controlled by regulatory subunits and autophosphorylation [71–73].

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 TBP are 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, demethylase of plants, nuclear matrix protein NMCP1. All these proteins are known also as nuclear matrix components that have certain functions and play an important role in transcription [11].

Among TBP, we also identified DNA repair protein RAD51 homolog B, protein kinase, serpins and some interesting proteins as immunophilins. It has been established previously that serpins are found among the TBP of Ehrlich ascites cells [7]. It has been demonstrated also that some of TBP from Ehrlich ascites and yeast cells exhibit protein kinase activity [5, 18].

Barley TBP proteins manifest homologies to functionally different enzymes and regulatory factors that participate in chromatin modification, reconstruction and repair. These 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 have 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 nuclear matrix preparations. As DNA sequences of tight DNA–protein complexes are also similar to nuclear matrix (MAR) sequences [17], we assent to the assumption [4, 12] that tightly bound DNA–protein complexes are part of the inner network of the nuclear matrix.

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References

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Hordeum vulgare miežių lapų stabilių DNR baltyminių kompleksų baltymų sudėties analizė

Santrauka


Be to, buvo nustatytas DNR reparacijos baltymas RAD51, baltymų kinazės, serpinas Z. Analogiški baltymai jau anksčiau buvo identifikuoti Erlicho ascito ir mielių ląstelių TBP–DNR kompleksuose.