research articles Journal of proteome research

Development of a System for the Study of Protein−Protein Interactions *in Planta*: Characterization of a TATA-Box Binding Protein Complex in *Oryza sativa*

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Received March 14, 2003

We describe a simple, rapid method for protein complex purification *in planta*. Using a biotin peptide as an affinity tag with TATA-box binding protein (TBP), 86 unique proteins present in the purified complex were identified by tandem mass spectrometry. We identified proteins known to be associated with TBP, and many other proteins involved in pre-mRNA processing and chromatin remodeling. The identification of these novel protein−protein associations will upon further investigations provide new insights into the mechanisms of mRNA transcription and pre-mRNA processing.

Keywords: protein−protein interactions • tandem mass spectrometry • HPLC−MS/MS • plant • protein complex • TATA-box binding protein.

Introduction

With the recent advances in sequencing of entire genomes, the next major task will be to uncover the functions, modifications, and regulations of the encoded proteins. There have been reports devoted to ascertaining the biological function of proteins and their networks at a genome-wide or proteome-wide level.[1−4] Much progress has been made on genome-wide protein−protein interaction studies in *Saccharomyces cerevisiae*, largely due to the availability of *S. cerevisiae* genome sequence information, and the ease of use of this organism.[5,6] The yeast two-hybrid system is used to detect pairwise protein−protein interactions via transcriptional activation of one or several reporter genes.[1,7] The two-hybrid approach has also been applied to the systematic analysis of protein−protein interactions in *C. elegans*[8], mouse,[9] and rice.[10] Because it is a technique based on one-to-one protein interactions, the two-hybrid approach has several drawbacks when applied to large-scale high-throughput screening. First, if the protein tested is a transcriptional activator, it may activate the reporter gene(s) without any additional interacting proteins. Second, only two proteins are tested at a time, which means that this method cannot identify components of a complex involved in a pathway that do not directly interact with the target protein. Third, it only predicts possible interactions, which may not represent what is happening under physiological conditions.

One alternative strategy is to use a tandem affinity purification (TAP) tag to pull down associated proteins,[11] which are then analyzed by mass spectrometry. The TAP tag is a fusion cassette encoding calmodulin-binding protein, a protease cleavage site, protein A, and the fusion protein. Rather than relying on a one-to-one protein interaction approach, this strategy allows one to identify whole protein complexes interacting with a given target protein without any *a priori* knowledge of the complex composition, activity or function. The TAP strategy has since been applied to a large-scale analysis of protein−protein interactions in yeast[12] and adapted successfully for transient and stable transfected mammalian tissue culture systems.[13] Other strategies combining immunoaffinity tag purification and mass spectrometry have also been developed and used to systematically characterize protein complexes in yeast,[14,15] including analysis of regulatory protein modifications.[16] Such methods have the drawback of involving multiple steps that are time-consuming and may cause losses of some of the components of the complex during the process, but these are necessary for high purity purification. We are interested in mapping protein−protein interactions *in planta* on a genome-wide scale. The recent release of a draft sequence of the rice genome[17,18] highlights the increasingly urgent need to develop a system capable of determining protein−protein interactions *in vivo* on a multicellular level in order to better understand protein functions.

Protein affinity tags are widely used for the purification and detection of recombinant proteins. Commonly used tags include fusion protein tags such as maltose-binding protein,

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10.1021/pr034023z CCC: $25.00
Published on Web 07/01/2003

glutathione S-transferase, and antibody epitope-tags such as myc, HA, and FLAG.[19,20] For a specific protein of interest, it may be difficult to decide which fusion system is the best because there are drawbacks associated with the use of any tag. The large peptide or protein tags are suitable for large-scale protein purification but have a greater chance of interfering with the structural characterization of the resultant fusion protein. The small peptide tags have a lesser chance of interfering with the fusion protein and are capable of a higher degree of protein purification, but the yield of purified protein can be low and thus may not be suitable for some special purposes such as protein complex purification and identification by mass spectrometry. One recently developed epitope tag is the streptavidin-binding peptide (SBP) that is 38 amino acids in length.[19] This has been used to purify proteins under mild conditions. Removal of epitope tags can often be accomplished with a site-specific protease contained within the tag or by introducing a self-splicing intein.[21] Inteins are internal protein elements involved in posttranslational processing that removes an internal protein fragment from a precursor and ligates the external fragments to form a mature protein.[22] The most commonly used proteases of choice are enterokinase, tobacco etch virus protease (TEV), and thrombin.[20] For a recent review of fusion protein tags and epitope tags, see Terpe.[20]

In this report, we describe a system using a tomato Biotin peptide and a TEV protease cleavage site as an affinity tag to purify protein complexes from whole protein extracts of rice suspension cell culture and demonstrate the successful application of this system in the identification of TATA-box binding protein (TBP) complexes in rice by tandem mass spectrometry. This Biotin fusion tagging system provides a highly useful alternative in the fusion protein complexes purification and investigation of protein−protein interaction mapping underlying cellular function.

Biotin is an essential cofactor for a set of enzymes involved in diverse metabolic processes, such as lipid, amino acid, and carbohydrate metabolism. In this system, a conserved biotinylation site of the biotin-containing enzyme methylcrotonyl-CoA carboxylase (MCCase) gene was identified.[23] A fusion protein containing a 70 aa peptide from the MCCCase was successfully *in vivo* biotinylated in *E. coli* and purified through affinity chromatography with immobilized avidin.[23] The *in vivo* biotinylation of recombinant proteins in mammalian cells using a transcarboxylase domain from *P. shermanii* was also reported.[24] The Biotin peptide from tomato was thus chosen as a viable affinity tag to use in our system to purify protein complexes from plant cells.

The TATA-box binding protein (TBP) is well characterized in Drosophila,[25] yeast,[26] and mammalian cells.[27] TBP is known to be involved in the establishment of the transcription initiation complex.[28,29] Therefore, we chose to use this as the test case target gene for development and optimization of our interacting protein complex isolation method. Although we are aware that there are numerous epitope-tagging strategies available for studying a complex of interest, this report is focused on our investigation of the protein−protein interactions of the TBP complex in rice using the Biotin fusion tagging system we have developed.

Experimental Section

Construction of Biotin Fusion Cassette. Total RNA was prepared from mature tomato leaves using an RNeasy Maxi kit (Qiagen, Chatsworth, CA) and was used to synthesize cDNA with a SuperScript Choice System kit (Invitrogen Life Technologies, Carlsbad, CA). The tomato Biotin gene was cloned by PCR from the tomato cDNA based on the sequences from nucleotide 1667−1880 of tomato methylcrotonyl-CoA carboxylase (TMC−B).[23] The oligonucleotide primer sequences were: 5′ primer CG*GGATCC*TTT*CCCGGG*GGTACTGTGATTGCACCCATGGC, 3′ primer CTATCC*GAGCTC*TCAGTCCTTGAGAGCAAAGAGTTTTATAC. Restriction enzyme sites BamHI and XmaI were added in the 5′ primer, SacI was added in the 3′ primer. The PCR product of the Biotin gene was cloned into vector pND0005 (Syngenta, contains the maize ubiquitin promoter and an NOS terminator). Two complementary oligonucleotides of the tobacco etch virus (TEV) protease cleavage site were synthesized by Sigma Genosys (5′-C*GGGATCC*AA*AGGCCTAC-CGGT*AAGATTCCAACTACTGCCAGCGAGAATTTGTATTTTCAGGGTGAGCTTAAAACCGCT*CCCGGG*GGTA-3′). After annealing the oligonucleotides, the TEV cleavage site was excised by digestion with BamHI/XmaI and inserted into the BamHI/XmaI sites of the tomato Biotin gene in pND0005, to give the construct pND05-Biotin. The multiple restriction sites BamHI, StuI and AgeI at the N-terminal of the TEV site were used for target gene cloning. Figure 1A shows the sequence of the TEV−Biotin cassette in the vector pND05-Biotin.

The maize TBP gene was cloned from a maize full-length cDNA library using PCR amplification based on the database sequence (Accession No. L13301, 5′ primer C*GGGATCC*ATGGCGGAGCCGGGGCTCGAGG, 3′ primer GCGC*ACCGGT*TTGCTGAACTTTTCGAAACTCTGCCAG), and inserted into the pND05-Biotin construct at the BamHI and AgeI sites to give the construct pND05-TBP-Biotin. The protein sequence of maize TBP is 94% homologous with the recently published rice OsTBP2.[30] As a result, the TBP gene is under the control of the maize ubiquitin promoter, and in-frame fused with the Biotin gene at the C-terminus, with the TEV cleavage site present as a linker region (Figure 1B). Using a similar strategy, a single translation starting codon ATG was added at the AgeI site of pND05-Biotin, in-frame with the TEV-Biotin peptide, to give an empty vector called pND05-ATG-Biotin used in control transformations to generate cell lines designated ATG-Biotin. All vector sequences were confirmed by sequencing. In addition, a Western blot was performed confirming the ATG-Biotin protein expression at the correct molecular weight (data not shown).

Rice Transformation and Transgenic Cell Maintenance. The constructs pND05-TBP-Biotin and pND05-ATG-Biotin were co-transformed with plasmid pCIB 7613 (Syngenta, ZmUbi-*hpt*-NOS), which contains the maize ubiquitin promoter driving the *hpt* gene for hygromycin B resistance, into rice suspension cells individually. The rice suspension cells were derived from mature seeds of *Oryza sativa L. japonica* cultivar Taipei 309 and transformed by particle bombardment.[31,32] Stable transformants were selected on hygromycin B-containing (50 mg/l) semisolid media and then resuspended in liquid medium for large-scale culturing as previous described.[31]

Protein Extract Preparation. Rice suspension cells were harvested in liquid nitrogen. Frozen tissues were homogenized to a fine powder with a mortar and pestle under evaporating liquid nitrogen. The resultant powder was resuspended in 2 volumes of chilled 10mM potassium phosphate (pH 7.4), 50mM NaCl, 5mM EDTA, 1mM PMSF, 0.5% protease inhibitor cocktail (Sigma). The mixture was filtered through 2 layers of Miracloth, centrifuged at 10 000 g for 20 min at 4 °C, and filtered through a 0.22 μm membrane (Millipore, Bedford, MA).

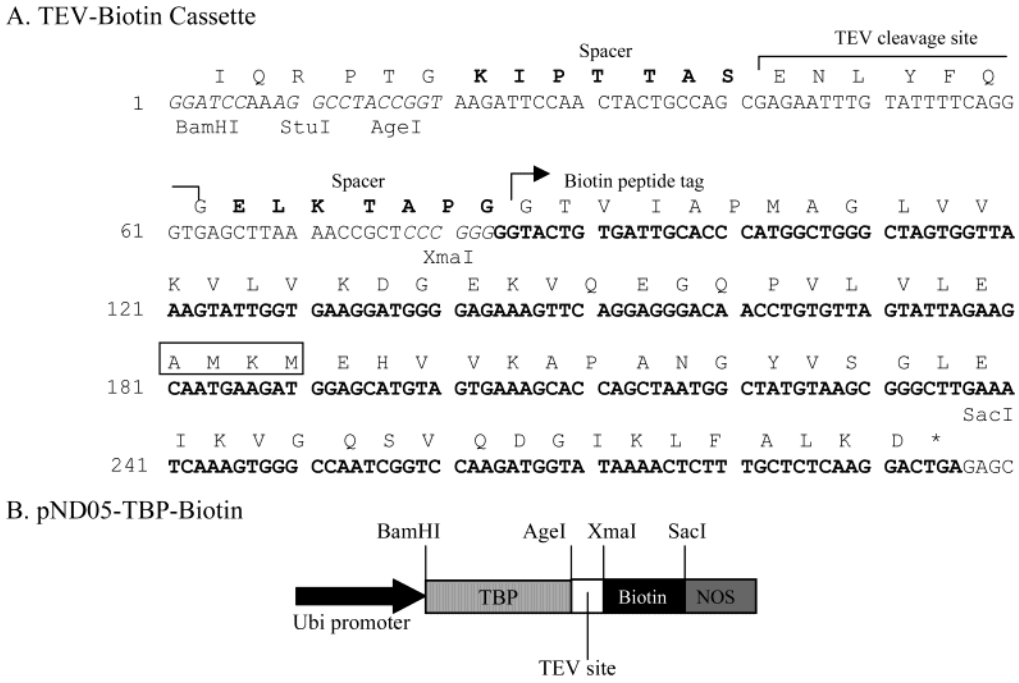

Figure 1. Construct pND05-TBP−Biotin. (A) Sequence and structure of TEV-Biotin cassette: DNA sequence and single-letter code for the translated amino acids of TEV-Biotin cassette are as shown, TEV protease cleavage site and Biotin peptide tag are indicated, spacer sequences for TEV protease cleavage are in bold. The start of Biotin peptide tag is marked with an arrow and the DNA sequence of the tag is bolded. The restriction enzyme sites used for construct cloning are indicated and the sequences are italicized. The conserved biotinylation site tetrapeptide A-M-K-M is boxed. (B) Diagram of construct pND05-TBP-Biotin: Maize TBP was cloned into BamHI/AgeI sites of TEV-Biotin cassette, in which the TBP gene is under the control of maize ubiquitin promoter and fused in-frame with TEV-Biotin cassette. NOS is the nopaline synthase terminator.

Silver Staining and Western Blot Analysis. Protein extracts were separated on 10% NuPAGE gels (Invitrogen, Carlsbad, CA) and visualized with silver staining.[33] The biotin-containing peptides were detected using Pierce ImunoPure HRP−Streptavidin diluted at 1:50 000, and Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

Purification and Elution of Biotin-Tagged Protein. A 50-mg portion of protein extract was diluted to 45 mL in binding buffer (20mM sodium phosphate pH7.5, 100mM NaCl) and incubated with 300 μL (3 mg) of 1% BSA-saturated MPG streptavidin (CPG Inc., Lincoln Park, NJ) on a rotator at 4 °C overnight. The magnetic streptavidin coated beads and solution were then separated using a magnetic separator (Invitrogen, Carlsbad, CA). After washing the beads 3 times with 45 mL each of binding buffer, the beads and bound proteins were incubated with 1.5 μL (10 units/μL) of 6× His-tagged TEV protease (Invitrogen) in 300 μL of TEV cleavage buffer (50mM Tris, pH 8.0, 0.5mM EDTA, 1mM DTT) on a rotator at 4 °C overnight. The cleavage solution, separated from the magnetic beads by placing the tube on the magnetic separator, was collected and further incubated with 20 mM imidazole and 10 μL Ni-NTA magnetic agarose beads (Qiagen) on a rotator for 1 h at room temperature to remove excess TEV protease. The final purified protein supernatant was collected using the magnetic separator.

Sample Preparation for HPLC−MS/MS Analysis. The purified protein complexes were run on 1-D electrophoresis gels according to established methods using a BioRad mini-gel system and BioRad pre-cast gels. Protein bands from 1-D gels were visualized with silver staining,[33] excised manually and chopped into pieces of approximately 1 mm^2 with a clean scalpel, and transferred to 96-well plates (Thermo-Fast 0.2 mL skirted 96 well PCR plates, Abgene, Surrey, UK). The plates were transferred to a Massprep digestion robot (Micromass, Beverley, MA) for destaining[34] and in-gel digestion with trypsin.[35]

High Performance Liquid Chromatography−Tandem Mass Spectrometry. A microbore HPLC system (Surveyor, ThermoFinnigan, San Jose, CA) was modified to operate at capillary flow rates using a simple T-piece flow-splitter. Columns (10 cm × 75 μm I. D.) were prepared by packing 100 Å, 5 μm Zorbax C18 resin at 500 psi pressure into New Objectives Pico Frits (New Objectives, MA) columns with integral spray needles. Peptides were eluted in a gradient using buffer A (5% v/v acetonitrile, 0.1% formic acid) and buffer B (90% v/v acetonitrile, 0.1% formic acid), at a flow rate of 400 nl/min. Following an initial wash with buffer A for 10 min, peptides were eluted with a linear gradient from 0 to 100% buffer B over a 30 min interval. Samples were introduced onto the analytical column using a Surveyor autosampler (Surveyor, ThermoFinnigan, San Jose, CA) which first transferred the 100 μL peptide extract onto a C 18 (300 μm × 5 mm) cartridge (LC Packings, San Francisco, CA) and then used a switching valve to transfer the eluted peptides on to the analytical column. The HPLC column eluent was eluted directly into the electrospray ionization source of a ThermoFinnigan LCQ-Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Spectra were scanned over the range 400−1400 mass units. Automated peak recognition, dynamic exclusion, and daughter-ion scanning of the top two most intense ions were performed using the Xcalibur software, as described previously.[36]

Database Searching and Data Interpretation. MS/MS data were analyzed using SEQUEST.[37] In this study, the criteria for a preliminary positive peptide identification for a doubly charged peptide were a correlation factor (Xcorr) greater than

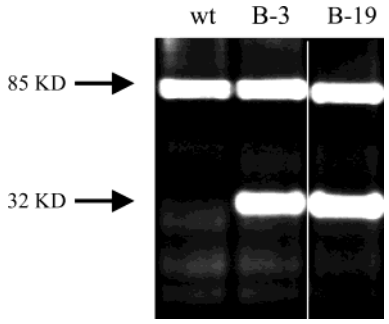

Figure 2. Detection of TBP-Biotin fusion protein expression in rice suspension cells by Western blot. 15 ug of crude protein extracts from different lines were separated on 10% NuPAGE gel. A single band of an endogenous biotin-containing protein was detected in both WT and the transgenic lines B-3 and B-19 at approximately 85 kDa. Transgenic lines B-3 and B-19 expressed the TBP−Biotin fusion protein at the expected size (32 kDa).

2.5, a delta cross-correlation factor (ΔXcorr) greater than 0.1, a minimum of one tryptic peptide terminus, and a high preliminary scoring.[38] For triply charged peptides, the correlation factor threshold was set at 3.5. All matched peptides were confirmed by visual examination of the spectra. All spectra were searched against a composite database containing a combination of the latest version of our proprietary rice database,[17] and the NRP nonredundant protein database from NCBI. In cases where peptides were identified from unannotated sequence data, protein function was assigned where possible by BLAST searching. The database entry that contained the identified peptide was searched using BLASTP against the latest release of the NCBI nonredundant protein database, and an expected value cutoff score of 10^{-7} was applied.[39]

Results

TBP-Biotin Fusion Protein Expression in Rice Callus. The construct pND05-TBP-Biotin, containing the maize TBP cDNA in frame with the coding region of the TEV protease cleavage site and the 70aa Biotin peptide of TMC-B (Figure 1), was transformed into rice suspension cells by particle bombardment and stable transformants were generated. Western blotting with HRP-conjugated streptavidin identified two stable transgenic cell lines (B-3 and B-19) expressing the 32 kDa TBP-Biotin fusion protein (Figure 2). The 85 kDa protein present in WT, and all of the transgenic lines, is an endogenous biotinylated protein, methylcrotonyl-CoA carboxylase subunit-A, the most prevalent of the biotin-containing enzymes in plants.[23] The detection of TBP-Biotin fusion protein in transgenic lines B-3 and B-19 with HRP-conjugated streptavidin demonstrated that the fusion protein was expressed and correctly biotinylated in rice suspension cells. In addition, the fact that the relative intensities of the two visible bands are approximately equal suggests that the expression level of the TBP-biotin fusion protein is comparable to that of an endogenously expressed protein.

Optimization of Conditions for Purification of the Biotin-Tagged Protein Complex. Once we had established that the TBP-Biotin fusion protein was properly biotinylated in rice suspension cells, the expressed fusion protein and its associated proteins were purified from rice suspension cell extracts by affinity chromatography with streptavidin coated magnetic beads. The optimum binding ratio of streptavidin beads to total protein extracts was determined using different ratios of protein extracts to beads and different incubation times. The complete

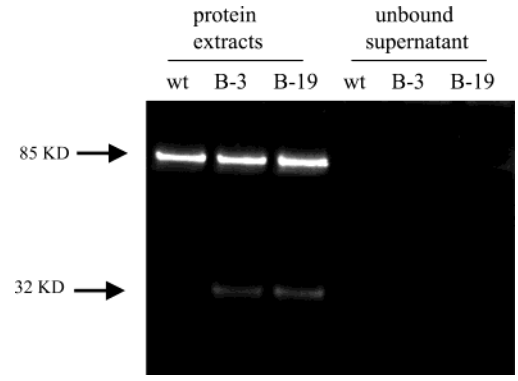

Figure 3. Binding capacity of MPG Streptavidin beads to protein extracts. 50 mg protein extracts from WT and transgenic lines B-3 and B-19 were incubated at 4 °C overnight with 3 mg MPG Streptavidin beads. The bound proteins and proteins remaining in the supernatant were detected by Western blot. Bands at 85 kDa represent the endogenous biotinylated protein. Bands at 32 kDa represent the expressed TBP-Biotin fusion protein.

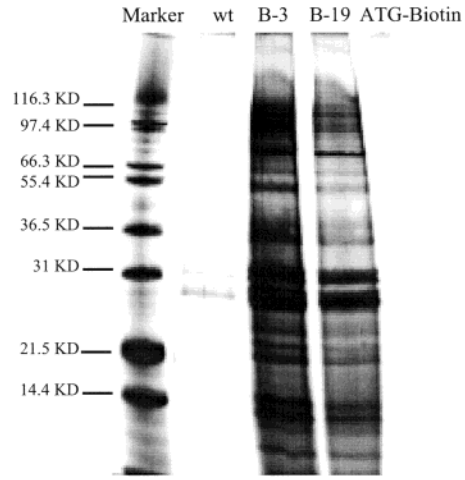

Figure 4. Detection of purified proteins by silver staining. The purified TBP complex from WT, ATG-Biotin, and transgenic lines B-3 and B-19 were separated on a 10% SDS-PAGE gel and visualized by silver staining. 25 protein bands were evenly excised from each of the transgenic lines B-3 and B-19, these were subjected to in-gel digestion with trypsin, and the resulting peptides were used for the identification of proteins by tandem mass spectrometry.

protein extracts, and unbound supernatants, were analyzed by Western blotting. The results showed that all of the detectable biotinylated proteins present in the protein extracts were bound to the beads when 50 mg of extract was incubated with 3 mg of streptavidin (Figure 3). These experiments enabled us to establish an optimized protein complex purification protocol consisting of five steps: protein extraction; streptavidin purification; TEV protease digestion to release purified proteins; Ni-agarose purification to remove excess TEV protease; and SDS-PAGE to visualize the purified proteins.

Purification and Visualization of TBP Complexes. To identify the proteins associated specifically with the TBP-Biotin expressing cell lines, the TBP-Biotin protein complexes were purified from 150 mg of protein extract from WT, ATG-Biotin, B-3, and B-19 lines. As shown in Figure 4, a large number of highly expressed protein bands were present in the TBP-Biotin transgenic lines B-3 and B-19, whereas few very faint bands

Table 1. Proteins Identified in the TBP Complex by Biotin-Tagging and Mass Spectrometry

	protein header/similarity[a]	UP[b]
	bait protein	
1	SW:TF21_MAIZE/transcription initiation factor TFIID	7
	known TBP associated proteins	
2	TBP associated factor (TAF-172)	5
3	putative TBP associated factor 24 kDa subunit	2
4	TBP-associated factor RNA polymerase 1-like	1
5	putative TATA binding protein-associated factor	1
	transcription/RNA processing	
6	plant transcriptional regulator Pbf-2	1
7	putative pur-alpha transcriptional activator	1
8	SW:GBLP_ORYSA/guanine nucleotide-binding protein beta subunit-like protein	1
9	single-stranded DNA-binding protein	1
10	putative single-stranded DNA-binding protein family	1
11	similar to myb family protein	2
12	similar to putative CHP rich Zinc-finger protein	1
13	GAR1 protein	2
14	putative RNA-binding protein	1
15	RNA-binding protein RZ-1	2
16	elongation factor ef-2	1
17	spliceosomal-like protein	23
18	putative spliceosome associated protein	1
19	U2 snRNP auxiliary factor, splicing factor-like protein	1
	chromatin remodeling associated proteins	
20	replication origin activator 2	1
21	replication factor c 40 kd	2
22	putative centromere/microtubule binding protein	2
23	putative histone deacetylase HD2-p39	1
24	DNA fragmentation factor	1
25	histone h2b	1
26	histone H1-like protein	1
27	putative Dnaj protein	1
28	putative mitotic checkpoint protein	1
	ribosomal protein	
29	60s ribosomal protein l18, putative	1
30	SW:RL9_ORYSA /60s ribosomal protein l9	1
31	SW:RL7A_ORYSA /60s ribosomal protein l7a	1
32	SW:RL6_MESCR /60s ribosomal protein l6	1
33	SW:RL5_ORYSA /60s ribosomal protein l5	3
34	60s ribosomal protein l26	2
35	60s ribosomal protein l24	1
36	60s ribosomal protein l2	2
37	60s ribosomal protein l19	1
38	60s ribosomal protein l18a	3
39	60s ribosomal protein l13	2
40	60s ribosomal protein l11a	1
41	SW:RL11_ORYSA/ 60s ribosomal protein l11	2
	ribosomal protein	
42	60s ribosomal protein l10a	3
43	SW:R101_ORYSA/60s ribosomal protein l10/	1
44	60s ribosomal protein l1	2
45	SW:RL7A_ORYSA/ 60s ribosomal protein 17a	1
46	60s ribosomal l18	2
47	60s ribosomal l6	1
48	SW:RLA0_ORYSA/60s acidic ribosomal protein p0	5
49	60s ribosomal protein l7	1
50	60S ribosomal protein l13A	4
51	60S acidic ribosomal protein P1	1
52	40s ribosomal protein-like	1
53	SW:RS8_ORYSA/ 40s ribosomal protein s8	3
54	40s ribosomal protein s7	8
55	40s ribosomal protein s6	2
56	40s ribosomal protein s5	2
57	SW:RS4_ORYSA/ 40s ribosomal protein s4	5
58	SW:RS3A_ORYSA/40s ribosomal protein s3a	1
59	40s ribosomal protein s3	3
60	40s ribosomal protein s2	1
	miscellaneous function and hypothetical proteins	
61	dihydrolipoamide s-acetyltransferase	3
62	dihydrolipoamide s-succinyltransferase homolog	2
63	2-oxoglutarate dehydrogenase E2	2
64	pyruvate dehydrogenase E1	4
65	dihydrolipoamide dehydrogenase	1
66	glyceraldehyde 3-phosphate dehydrogenase	2
67	branched chain alpha-keto acid dehydrogenase complex e2 subunit	8
68	OSA004965_1/cytosolic pyruvate orthophosphate dikinase	1
69	putative pyruvate kinase isozyme a	1
70	casein kinase ii, alpha chain	1
71	similar to pib, NBS-LRR type resistance gene	1
72	putative pol polyprotein	1
73	putative gag-pol protein	1
74	fibrillarin-like protein	1
75	TBB2_ORYSA/ Tubulin beta chain	1
76	70 kd heat shock-related protein	1
77	cold shock protein-like	1
78	33 kd secretory protein	6
79	SW:ENO_ORYSA/enolase/	2
80	polyubiquitin	1
81	Swp1 related gene	1
82	hypothetical protein	1
83	hypothetical protein	1
84	hypothetical protein	1
85	hypothetical protein	1
86	hypothetical protein	2

[a] Proteins identified from the Swiss-Prot database are indicated by the prefix SW: in the protein header. [b] Number of unique peptides identified.

remained in the WT and ATG-Biotin controls, indicating that nonspecific binding had been minimized. The pattern of bands seen in both transgenic cell lines was highly reproducible in duplicate experiments. These data indicate that all of the protein bands seen in the lanes for B-3 and B-19 in Figure 4 can be attributed directly to proteins associated with the TBP complex or components thereof.

Mass Spectrometric Analysis of the TBP Complex. To identify the specific bands seen in the TBP-Biotin transgenic lines (Figure 4), and any proteins present in control preparations, the bands were excised from the gel, protease digested, extracted from the gel, and analyzed by HPLC−MS/MS. The peptides that produced the MS/MS spectra were identified by SEQUEST database searching. The only proteins positively identified in the control preparation lanes were the expected background contaminants of trypsin fragments, various keratins, and bovine serum albumin which is used in a pretreatment step of the beads. Table 1 shows the proteins identified with high confidence from the transgenic cell lines B-3 and B-19, along with the number of unique peptides determined for each. These data represent a composite of results obtained from duplicate experiments with the transgenic cell lines. A total of 86 distinct proteins were identified as being associated with TBP. The number of peptide matches per protein ranged from 23 for a spliceosomal-like protein to a number of proteins that were confidently identified on the basis of only one peptide. Figure 5 shows a representative example of MS/MS spectrum from a protein identified from one peptide. A table containing the 86 proteins as well as the unique peptides that were identified for each protein is found in the Supporting Information. The maize TBP bait protein was identified with seven unique peptides, and four known TBP associated factors

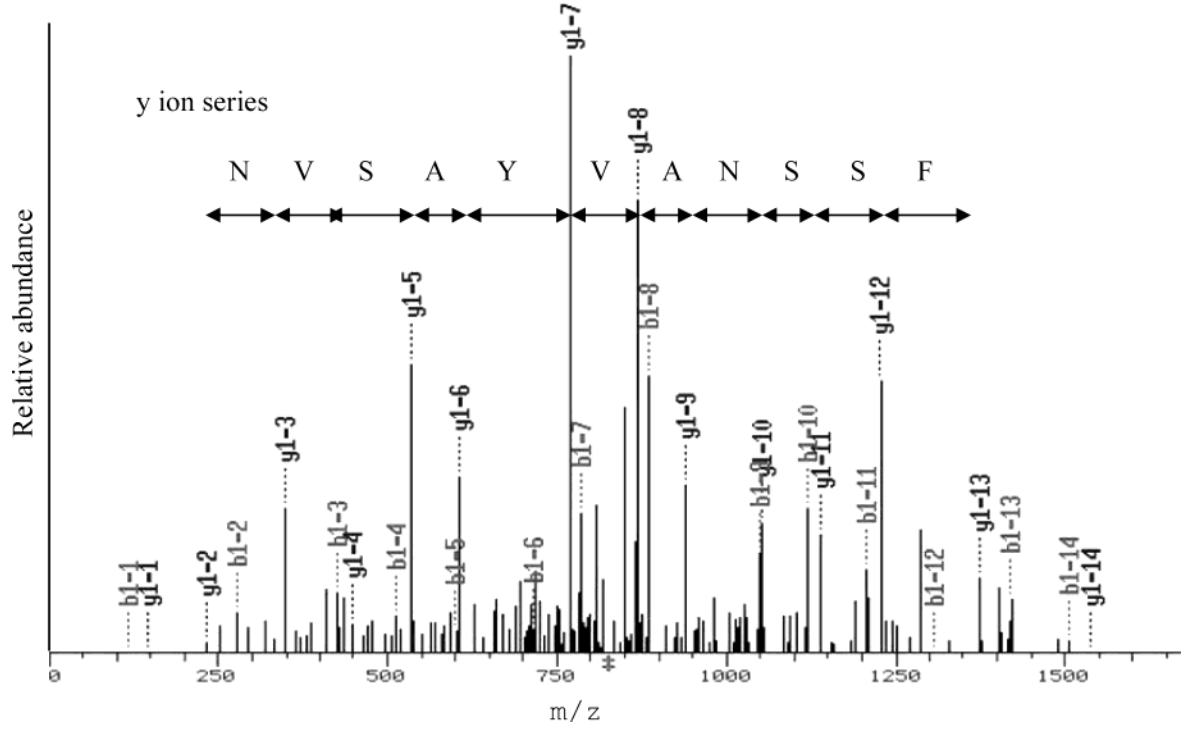

Figure 5. MS/MS spectrum of a peptide from TBP-associated factor, RNA polymerase 1-like. The SEQUEST output for the database search of this MS/MS spectrum identified the peptide as DYFSSNAVYASVNSK, with an Xcorr at 2.77 and delta Xcorr of 0.333. The y ions identified by Sequest are indicated and the corresponding amino acid difference between each ion is shown.

were also identified. Many transcription/RNA processing related proteins were co-purified with the TBP complex. These include several spliceosomal-like proteins, RNA binding protein, and elongation factor ef-2. Chromatin remodeling associated proteins identified included a putative centromere/microtubule binding protein and a putative histone deacetylase HD2. Translation related proteins were also identified including a number of ribosomal proteins. Other proteins with unclear or unknown function that were identified included the branched chain alpha keto acid dehydrogenase complex e2 subunit, and many hypothetical proteins.

To assess the reproducibility of the method, we compared the data generated from the B-3 and B-19 transformants in a single set of experiments. Considering all of the 86 proteins identified, 76% were found to be present in the data from both cell lines, while considering only those proteins identified from two or more peptides, 86% were found to be present in the data from both cell lines.

Discussion

We have described the identification of 86 different proteins that were purified as part of the complexes associated with TBP. Many of the proteins identified were represented by only one peptide and are, therefore, statistically more likely to be in error than those proteins identified from multiple peptides.[40] There are numerous reasons why proteins may be found only by single peptides, including low abundance levels in the gel, the presence of a large amount of another protein in the same gel region obscuring a less abundant protein, or as a consequence of incomplete gene assembly which is to be expected in a first draft of the rice genome.[17] In instances where the rice gene is incomplete, there may be only one peptide that hits the partial protein sequence. This will also make the annotation of each rice gene harder to predict because related family members may also contain a similar protein sequence. In addition, the possibility must also be considered that a protein may be present in our data set, and absent from our negative control data, but may represent a biological false positive as it is not part of the actual TBP complex *in vivo*. This may occur, for example, in the case of highly abundant cytoplasmic proteins that can interact with the nuclear-localized TBP complex members only when the cells are ruptured for purification purposes. This scenario would not occur in our negative control experiments as there are no TBP complex members with which the highly abundant proteins could form an association. In addition, a similar situation could also occur if the TBP bait protein was dramatically overexpressed by a strong promoter. Despite these caveats, we are confident that our rigorous data analysis, including manual inspection of all spectra assigned to peptides and manual inspection all BLAST search results, has ensured that our data set is correctly assigned.

Two recently published papers demonstrate the power of using quantitative proteomic approaches combining isotope tagging and mass spectrometry to distinguish specific from nonspecific protein−protein interactions.[41,42] In both cases, a protein−protein interaction study was performed along with an identical study using a nonligand containing control. The experimental and control sample were both labeled with a stable isotope tag of discernibly different mass, and samples were pooled and analyzed by mass spectrometry. The peptide pairs that were identified as highly abundant in the experimental sample compared to the control sample were considered specific. Combining affinity chromatography with isotope tagging and mass spectrometry in such an approach can therefore help eliminate nonspecific background interactions and further increase the experimental data confidence level.

TBP is a general transcription factor that is utilized for most preinitiation complexes. The basic mechanisms of transcription are highly conserved among different species in eukaryotic cells.[28,43,44] It is known that TBP is a universal transcription factor, required for initiation by all three eukaryotic RNA polymerases, and that TBP mediates promoter recognition through the sequence-specific binding of the TATA element found in many promoters.[45,46] Assembly of the two mega-complexes of general factor proteins, TFIID and RNA polymerase, on the core promoter results in formation of the preinitiation complex for transcription initiation. Although numerous data have been generated over the past three decades on the mechanism of the transcription complex, it is still not well understood how those transcription cofactors communicate or how many cofactors are involved in the mechanism, especially in plants. In addition, most reports are based on the knowledge of transcription complexes examined in animal and yeast cells, where the function or composition of protein components may differ from plants.[47] In this study, we have used rice callus as a model, because it represents a plant tissue that is relatively easy to manipulate, and because TBP is known to be expressed in callus, and therefore, interacting proteins would also be expected to be expressed. [44,30]

TATA-binding protein, which was the target protein, was observed with 7 unique peptides (Table 1). We also identified four TBP associated factors (TAFs), which are known components of the TBP complex in other species.[45] In contrast, 14 TAFs have been identified in yeast using immunopurification techniques with a polyclonal antibody directed against TBP.[26] It is possible that other known TBP associated factors that compose the TFIID complex were not detected in our study because of low abundance, weak interactions, or other technical limitations, and alternatively, it is also possible that some of the known yeast TAFs are not present in rice. In our initial draft sequence of the rice genome three genes were annotated as TAFs.[17] When *Nicotiana tabacum* TAF-2 (NCBI Z48602) was analyzed against the public nonredundant database using tblastx, five rice hits were found. In Arabidopsis, which is a model plant organism with a fully sequenced genome, 8 TAFs are annotated (www.ncbi.nlm.nih.gov). Taken together, these data suggest that there may be between five and eight TAFs in the rice genome, although that number may change as gene annotation continues. The four TAFs we have clearly identified in this report are thus likely to represent a significant fraction of the total number present, and this information will be helpful in the experimental annotation of the number and organization of TAFs in the draft version of the rice genome sequence.

However, the identification of these TAFs in our data indicates that the Biotin-tagging system is successful in determining known interacting proteins. In addition, this also suggests that some of the recombinant fusion protein was localized in the nucleus because we identified TAFs that are known to interact with TBP in the nucleus.

Interestingly, we identified a large number of proteins in the TBP complexes associated with RNA processing, chromatin remodeling, and translation. The co-purification of pre-RNA processing proteins GAR1, DNA binding protein, RNA binding protein, elongation factor ef-2, and spliceosomal-like proteins with TBP complexes suggests that pre-mRNA processing reactions may occur during, and not after, transcription. H1 and H2B histone-like proteins may be related to TAFs because several TAFs have sequence similarities with histones.[29] The identification of a putative centromere/microtubule binding protein, putative histone deacetylase HD2, and replication factor c in the complex may give some evidence that these proteins are involved in the chromatin decompaction process for facilitating the binding of activators and general transcriptional machinery to chromosomal DNA. In yeast, 9 known subunits of the chromatin remodeling complex were immunopurified with an anti-TBP IgG. [26] The machinery that transcribes protein-coding genes in eukaryotic cells must contend with repressive chromatin structures in order to find its target DNA sequences. The mechanism for the disruption of chromatin structure at a promoter has not yet been elucidated, but numerous data suggest several types of chromatin-directed activities play important roles in transcriptional activation. These include ATP-dependent nucleosome remodeling complexes and proteins that catalyze post-translational modification of histones.[45,48−50]

A number of ribosomal proteins were identified in the preparation, particularly for the 60S subunit. These proteins were not identified in the WT callus or ATG-Biotin transgenic lines. This suggests that these proteins may be associated with TBP complexes, but the significance of these data needs to be further evaluated.

Convincing data has been reported showing that the protein factors responsible for each individual step in the pathway from gene to protein are functionally, and sometimes physically, connected.[51,52] It is therefore not surprising that several proteins with different functions in the transcriptional machinery were co-purified in our streptavidin affinity purification. We found many proteins involved in chromatin remodeling, RNA processing, and translation. Many of these same proteins were also observed in recent reports where human pre-mRNA was used as bait to study spliceosomes.[51,53] The proteins reported were related to transcription, translation, and RNA processing, including TFIID 70 kDa subunit. The authors also concluded that the proteins involved in transcription and RNA processing may be coupled.[53] In yeast, evidence has also shown that a number of novel connections exist between TBP and the chromatin remodeling complex.[26] Our results, while preliminary, suggest that the chromatin structure remodeling, transcription and RNA processing may also be coupled in rice.

Five hypothetical proteins were also identified in our complex. These novel rice proteins were searched against the NCBI protein database using BLAST, and no proteins with a high similarity score were found. The fact that they were identified in a TBP complex, and not in the control lines, suggests that they may be functionally significant.

A number of different metabolic enzymes were identified in these complexes. Most of these proteins are in the glycolytic pathway and are subunits of a larger complex. For example, the mammalian alpha-ketoglutarate dehydrogenase complex is composed of the dihydrolipoamide dehydrogenase E1 and E2 components,[54] and dihydrolipoamide S-acetyltransferase is a subunit of pyruvate dehydrogenase complex.[54] Our data suggest the possibility that these proteins may have an alternative function in addition to carbohydrate metabolism. Enolase also has been shown to function as a heat shock protein and be able to bind to chromatin structures, indicating that it may play a role in transcription or chromatin remodeling.[55]

The technique used in this report identified many known and novel proteins in TBP complexes. Biotin is an essential cofactor for a set of enzymes involved in diverse metabolic processes and exists in many different organisms. The binding between biotin and streptavidin is specific and has a high

affinity.[23] The Biotin tag strategy can therefore potentially be applied to all kinds of organisms. This is the first report for an in vivo protein−protein interaction study successfully applied in the plant kingdom. In this system, where a single cell has the potential to contain more than 30 000 gene products,[17] only a few steps of affinity purification were required to isolate the proteins associated with the TBP-Biotin bait protein. The introduction of a TEV cleavage site into the Biotin construct greatly reduced the presence of contamination from endogenous biotin-containing proteins and, thus, contributed significantly to the specificity of the purification.

The Biotin tagging strategy described in this report can theoretically be used in conjunction with mass spectrometry for system-wide analysis of protein−protein interactions on a genome-wide scale, by incorporating high-throughput technology into the system. The high specificity and affinity of biotin-streptavidin binding, the simple procedure for the protein complex purification, and the ease of rice suspension cell culture and maintenance make automation for the whole procedure feasible. We envisage that in the future the Biotin strategy we describe here will be a highly useful alternative tool in the mapping of protein−protein interaction networks underlying cellular function in plants.

Supporting Information Available: A table listing the proteins and peptides identified for each protein studied. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Legrain, P.; Selig, L. *FEBS Lett.* **2000**, *480*, 32−36.
(2) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101−2105.
(3) Kumar, A.; Snyder, M. *Nature* **2002**, *415*, 123−124.
(4) Tong, A. H.; Drees, B.; Nardelli, G.; Bader, G. D.; Brannetti, B.; Castagnoli, L.; Evangelista, M.; Ferracuti, S.; Nelson, B.; Paoluzi, S.; Quondam, M.; Zucconi, A.; Hogue, C. W.; Fields, S.; Boone, C.; Cesareni, G. *Science* **2002**, *295*, 321−324.
(5) Uetz, P.; Giot, L.; Cagney, G.; Mansfield, T. A.; Judson, R. S.; Knight, J. R.; Lockshon, D.; Narayan, V.; Srinivasan, M.; Pochart, P.; Qureshi-Emili, A.; Li, Y.; Godwin, B.; Conover, D.; Kalbfleisch, T.; Vijayadamodar, G.; Yang, M.; Johnston, M.; Fields, S.; Rothberg, J. M. *Nature* **2000**, *403*, 623−627.
(6) Ito, T.; Tashiro, K.; Muta, S.; Ozawa, R.; Chiba, T.; Nishizawa, M.; Yamamoto, K.; Kuhara, S.; Sakaki, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1143−1147.
(7) Fields, S.; Song, O. *Nature* **1989**, *340*, 245−246.
(8) Walhout, A. J.; Sordella, R.; Lu, X.; Hartley, J. L.; Temple, G. F.; Brasch, M. A.; Thierry-Mieg, N.; Vidal, M. *Science* **2000**, *287*, 116−122.
(9) Suzuki, H.; Fukunishi, Y.; Kagawa, I.; Saito, R.; Oda, H.; Endo, T.; Kondo, S.; Bono, H.; Okazaki, Y.; Hayashizaki, Y. *Genome Res.* **2001**, *11*, 1758−1765.
(10) Fang, Y.; Macool, D. J.; Xue, Z.; Heppard, E. P.; Hainey, C. F.; Tingey, S. V.; Miao, G. H. *Mol. Genet. Genomics* **2002**, *267*, 142−153.
(11) Rigaut, G.; Shevchenko, A.; Rutz, B.; Wilm, M.; Mann, M.; Seraphin, B. *Nat. Biotechnol.* **1999**, *17*, 1030−1032.
(12) Gavin, A. C.; Bosche, M.; Krause, R.; Grandi, P.; Marzioch, M.; Bauer, A.; Schultz, J.; Rick, J. M.; Michon, A. M.; Cruciat, C. M.; Remor, M.; Hofert, C.; Schelder, M.; Brajenovic, M.; Ruffner, H.; Merino, A.; Klein, K.; Hudak, M.; Dickson, D.; Rudi, T.; Gnau, V.; Bauch, A.; Bastuck, S.; Huhse, B.; Leutwein, C.; Heurtier, M. A.; Copley, R. R.; Edelmann, A.; Querfurth, E.; Rybin, V.; Drewes, G.; Raida, M.; Bouwmeester, T.; Bork, P.; Seraphin, B.; Kuster, B.; Neubauer, G.; Superti-Furga, G. *Nature* **2002**, *415*, 141−147.
(13) Westermarck, J.; Weiss, C.; Saffrich, R.; Kast, J.; Musti, A.; Wessely, M.; Ansorge, W.; Seraphin, B.; Wilm, M.; Valdez, B. C.; Bohmann, D. *The EMBO J.* **2002**, *21*, 451−460.
(14) Honey, S.; Schneider, B. L.; Schieltz, D. M.; Yates, J. R., III.; Futcher, B. *Nucleic Acids Res.* **2001**, *29*, E24.
(15) Ho, Y.; Gruhler, A.; Heilbut, A.; Bader, G. D.; Moore, L.; Adams, S. L.; Millar, A.; Taylor, P.; Bennett, K.; Boutilier, K.; Yang, L.; Wolting, C.; Donaldson, I.; Schandorff, S.; Shewnarane, J.; Vo, M.; Taggart, J.; Goudreault, M.; Muskat, B.; Alfarano, C.; Dewar, D.; Lin, Z.; Michalickova, K.; Willems, A. R.; Sassi, H.; Nielsen, P. A.; Rasmussen, K. J.; Andersen, J. R.; Johansen, L. E.; Hansen, L. H.; Jespersen, H.; Podtelejnikov, A.; Nielsen, E.; Crawford, J.; Poulsen, V.; Sorensen, B. D.; Matthiesen, J.; Hendrickson, R. C.; Gleeson, F.; Pawson, T.; Moran, M. F.; Durocher, D.; Mann, M.; Hogue, C. W.; Figeys, D.; Tyers, M. *Nature* **2002**, *415*, 180−183.
(16) Cheeseman, I. M.; Anderson, S.; Jwa, M.; Green, E. M.; Kang, J.-S.; Yates, J. R., Iii; Chan, C. S. M.; Drubin, D. G.; Barnes, G. *Cell* **2002**, *111*, 163−172.
(17) Goff, S. A.; Ricke, D.; Lan, T. H.; Presting, G.; Wang, R.; Dunn, M.; Glazebrook, J.; Sessions, A.; Oeller, P.; Varma, H.; Hadley, D.; Hutchison, D.; Martin, C.; Katagiri, F.; Lange, B. M.; Moughamer, T.; Xia, Y.; Budworth, P.; Zhong, J.; Miguel, T.; Paszkowski, U.; Zhang, S.; Colbert, M.; Sun, W. L.; Chen, L.; Cooper, B.; Park, S.; Wood, T. C.; Mao, L.; Quail, P.; Wing, R.; Dean, R.; Yu, Y.; Zharkikh, A.; Shen, R.; Sahasrabudhe, S.; Thomas, A.; Cannings, R.; Gutin, A.; Pruss, D.; Reid, J.; Tavtigian, S.; Mitchell, J.; Eldredge, G.; Scholl, T.; Miller, R. M.; Bhatnagar, S.; Adey, N.; Rubano, T.; Tusneem, N.; Robinson, R.; Feldhaus, J.; Macalma, T.; Oliphant, A.; Briggs, S. *Science* **2002**, *296*, 92−100.
(18) Yu, J.; Hu, S.; Wang, J.; Wong, G. K.; Li, S.; Liu, B.; Deng, Y.; Dai, L.; Zhou, Y.; Zhang, X.; Cao, M.; Liu, J.; Sun, J.; Tang, J.; Chen, Y.; Huang, X.; Lin, W.; Ye, C.; Tong, W.; Cong, L.; Geng, J.; Han, Y.; Li, L.; Li, W.; Hu, G.; Li, J.; Liu, Z.; Qi, Q.; Li, T.; Wang, X.; Lu, H.; Wu, T.; Zhu, M.; Ni, P.; Han, H.; Dong, W.; Ren, X.; Feng, X.; Cui, P.; Li, X.; Wang, H.; Xu, X.; Zhai, W.; Xu, Z.; Zhang, J.; He, S.; Xu, J.; Zhang, K.; Zheng, X.; Dong, J.; Zeng, W.; Tao, L.; Ye, J.; Tan, J.; Chen, X.; He, J.; Liu, D.; Tian, W.; Tian, C.; Xia, H.; Bao, Q.; Li, G.; Gao, H.; Cao, T.; Zhao, W.; Li, P.; Chen, W.; Zhang, Y.; Hu, J.; Liu, S.; Yang, J.; Zhang, G.; Xiong, Y.; Li, Z.; Mao, L.; Zhou, C.; Zhu, Z.; Chen, R.; Hao, B.; Zheng, W.; Chen, S.; Guo, W.; Tao, M.; Zhu, L.; Yuan, L.; Yang, H. *Science* **2002**, *296*, 79−92.
(19) Keefe, A.; Wilson, D.; Seelig, B.; Szostak, J. *Protein Expression Purif.* **2001**, *23*, 440−446.
(20) Terpe, K. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 523−533.
(21) Xu, M.; Paulus, H.; Chong, S. *Methods Enzymol.* **2000**, *236*, 376−418.
(22) Perler, F. B. *Cell* **1998**, *92*, 1−4.
(23) Wang, X.; Wurtele, E. S.; Keller, G.; Mckean, A. L.; Nikolau, B. J. *J. Biol. Chem*. **1994**, *269*, 11 760−11 768.
(24) Parrott, M. B.; Barry, M. A. *Mol. Therapy* **2000**, *1*, 96−104.
(25) Yokomori, K.; Admon, A.; Goodrich, J. A.; Chen, J. L.; Tjian, R. *Genes Dev*. **1993**, *7*, 2235−2245.
(26) Sanders, S. L.; Jennings, J.; Canutescu, A.; Link, A. J.; Weil, P. A. *Mol. Cell Biol.* **2002**, *22*, 4723−4738.
(27) Furukawa, T.; Tanese, N. *J. Biol. Chem*. **2000**, *22*, 29 847−29 856.
(28) Lemon, B.; Tjian, R. *Genes Dev*. **2000**, *14*, 2551−2569.
(29) Green, M. R. *Trends Biochem. Sci.* **2000**, *25*, 59−63.
(30) Zhu, Q.; Ordiz, M. I.; Dabi, T.; Beachy, R. N.; Lamb, C. *Plant Cell* **2002**, *14*, 795−803.
(31) Zhang, S. P.; Song, W.-Y.; Chen, L.; Ruan, D.; Taylor, N.; Ronald, P.; Beachy, R.; Fauquet, C. *Mol. Breeding* **1998**, *4*, 551−558.
(32) Chen, L.; Zhang, S.; Beachy, R. N.; Fauquet, C. M. *Plant Cell Rep.* **1998**, *18*, 25−31.
(33) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. *Nature* **1996**, *379*, 466−469.
(34) Gharahdaghi, F.; Weinberg, C. R.; Meagher, D. A.; Imai, B. S.; Mische, S. M. *Electrophoresis* **1999**, *20*, 601−605.
(35) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem*. **1996**, *68*, 850−858.
(36) Haynes, P. A.; Fripp, N.; Aebersold, R. *Electrophoresis* **1998**, *19*, 939−945.
(37) Yates, J. R., III.; Eng, J. K.; Mccormack, A. L.; Schieltz, D. *Anal. Chem*. **1995**, *67*, 1426−1436.
(38) Andon, N. L.; Hollingworth, S.; Koller, A.; Greenland, A. J.; Yates, J. R., III.; Haynes, P. A. *Proteomics* **2002**, *2*, 1156−1168.
(39) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J. Mol. Biol.* **1990**, *215*, 403−410.
(40) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. *Anal.Chem* **2002**, *74*, 5383−5392.
(41) Ranish, J.; Yi, E. C.; Leslie, D. M.; Purvine, S. O.; Goodlett, D. R.; Eng, J. K.; Aebersold, R. *Nat. Gen.* **2003**, *33*, 349−355.
(42) Blagoev, B.; Kratchmarova, I.; Ong, S.; Nielsen, M.; Foster, L. J.; Mann, M., *Nat. Biotechnol.* **2003**, *21*, 315−318.

(43) Pan, S.; Czarnecka-Verner, E.; Gurley, W. B. *Plant Cell* **2000**, *12*, 125−136.
(44) Zhu, Q.; Dabi, T.; Lamb, C. *Plant Cell* **1995**, *7*, 1681−1689.
(45) Naar, A. M.; Lemon, B. D.; Tjian, R. *Annu. Rev. Biochem.* **2001**, *70*, 475−501.
(46) Hampsey, M. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 465−503.
(47) Sugiura, M. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 383−398.
(48) Orphanides, G.; Reinberg, D. *Nature* **2000**, *407*, 471−475.
(49) Orphanides, G.; Reinberg, D. *Cell* **2002**, *108*, 439−451.
(50) Lomvardas, S.; Thanos, D. *Cell* **2001**, *106*, 685−696.
(51) Zhou, Z.; Licklider, L. J.; Gygi, S. P.; Reed, R. *Nature* **2002**, *419*, 182−185.
(52) Proudfoot, N. J.; Furger, A.; Dye, M. J. *Cell* **2002**, *108*, 501−512.
(53) Rappsilber, J.; Ryder, U.; Lamond, A. I.; Mann, M. *Genome Res.* **2002**, *12*, 1231−1245.
(54) Mccartney, R. G.; Rice, J. E.; Sanderson, S. J.; Bunik, V.; Lindsay, H.; Lindsay, J. G. *J Biol Chem* **1998**, *273*, 24 158−24 164.
(55) Pancholi, V. *Cell Mol. Life Sci.* **2001**, *58*, 902−920.

PR034023Z