

A Tandem Affinity Purification-based Technology Platform to Study the Cell Cycle Interactome in *Arabidopsis thaliana**[§]

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Defining protein complexes is critical to virtually all aspects of cell biology because many cellular processes are regulated by stable protein complexes, and their identification often provides insights into their function. We describe the development and application of a high throughput tandem affinity purification/mass spectrometry platform for cell suspension cultures to analyze cell cycle-related protein complexes in *Arabidopsis thaliana*. Elucidation of this protein-protein interaction network is essential to fully understand the functional differences between the highly redundant cyclin-dependent kinase/cyclin modules, which are generally accepted to play a central role in cell cycle control, in all eukaryotes. Cell suspension cultures were chosen because they provide an unlimited supply of protein extracts of actively dividing and undifferentiated cells, which is crucial for a systematic study of the cell cycle interactome in the absence of plant development. Here we report the mapping of a protein interaction network around six known core cell cycle proteins by an integrated approach comprising generic Gateway-based vectors with high cloning flexibility, the fast generation of transgenic suspension cultures, tandem affinity purification adapted for plant cells, matrix-assisted laser desorption ionization tandem mass spectrometry, data analysis, and functional assays. We identified 28 new molecular associations and confirmed 14 previously described interactions. This systemic approach provides new insights into the basic cell cycle control mechanisms and is generally applicable to other pathways in plants. *Molecular & Cellular Proteomics* 6:1226–1238, 2007.

Cell division is a fundamental biological process that shares conserved features and controls in all eukaryotes (1). Never-

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theless, plants have some special features that give control of cell cycle progression a particular importance and that might be the reason why plants evolved novel molecules orchestrating cell division (2). In *Arabidopsis thaliana*, more than 100 core cell cycle genes have been described (3, 4). As in other eukaryotes, CDKs¹ and cyclins govern the plant cell cycle. No less than 29 different CDKs and 52 cyclin-related genes have been identified so far (3). However, the molecular interface between the CDK-cyclin complexes and their substrates and interactions with other proteins are, to a large extent, unexplored in plants. Now that the *Arabidopsis* ORFeome is partially being cloned (5), a next step is the systemic proteomics study to decipher the cell cycle interactome that controls cell division in plants.

In recent years, new technologies have been developed to study protein-protein interactions under near physiological conditions. Especially tandem affinity purification (TAP) (6) combined with MS-based protein identification is a powerful approach that has led to the first genome-wide screens for protein complexes in yeast (7–10). TAP strategies have also been developed for transgenic plants (11, 12). However, plants contain only a minor fraction of dividing cells mostly concentrated in proliferating tissues of the meristems. Moreover cell cycle proteins are low abundance, and some of them have a cell cycle-dependent expression profile. Therefore, cell suspension cultures, rather than complete plants, offer an unlimited supply of protein extracts derived from dividing cells, expressing more than 85% of the core cell cycle regu-

¹ The abbreviations used are: CDK, cyclin-dependent kinase; Bis-Tris, bis(2-hydroxyethyl)amino-tris-(hydroxymethyl)methane; BN, blue native; CBP, calmodulin-binding peptide; CAK, CDK-activating kinase; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; GFP, green fluorescent protein; GO, gene ontology; MSMO, Murashige and Skoog basal salts with minimal organics; PMF, peptide mass fingerprint; TAP, tandem affinity purification; TEV, tobacco (*N. tabacum* L.) etch virus; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SNAPS, Simple Non-redundant Assembly of Protein Sequences; BLAST, Basic Local Alignment and Search Tool; BiNGO, Biological Networks Gene Ontology; GTA, galactosyltransferase-associated; TAIR, The Arabidopsis Information Resource; KRP, Kip-related protein.

lators (3). Furthermore cell suspensions can be synchronized, by for instance growth factor starvation (13), making it possible to focus on a specific cell cycle transition (e.g. G₁/S or G₂/M) to better understand the functional relationship between the different protein complexes during cell cycle progression. Therefore, the *Arabidopsis* cell suspensions are most suited to study the core cell cycle interactome.

Here we report on the implementation of a high throughput TAP platform for *Arabidopsis* cell suspension cultures combining different technologies to characterize protein-protein interactions in plants. New transformation vectors with high cloning flexibility were designed, allowing cloning of any promoter, ORF, and tag in one step. A streamlined transformation procedure for the fast generation of multiple series of transgenic cell suspension cultures was set up. The original yeast TAP protocol (6) was adapted for plant suspension cells. Tools were implemented for the high throughput identification of *Arabidopsis* proteins using MALDI-tandem MS and data analysis. Proof of concept for the methodology is shown by protein complex identification for six tagged core cell cycle proteins that were randomly chosen: four different CDKs (CDKA;1, CDKB1;1, CDKD;2, and CDKF;1) and two regulatory subunits (CKS1 and CYCD3;1). Finally data are provided on the activity of the isolated complexes via a functional assay, and incorporation of the expressed bait protein into physiological complexes is illustrated via blue native (BN)/SDS-PAGE.

EXPERIMENTAL PROCEDURES

Vector Construction for C-terminal Tagging—pKCTAP was constructed by cloning the 35S transcription termination signal of the cauliflower mosaic virus and a green fluorescent protein (GFP) expression cassette, recovered from pK7WG2D, into pKm43GW (14).

Vector Construction for N-terminal Tagging—pKNTAP was made by cloning three fragments separately in pUC19SX (15) between the appropriate restriction sites: a fragment containing the attR4/attR3 Gateway (Invitrogen) cassette amplified from pKCTAP, a fragment containing the NTAPi sequence preceded by the Ω leader and Kozak sequence amplified from the vector ntapi.289.gw.gck (11), and a fragment containing the attR1/attR2 Gateway cassette amplified from pKGW (14). The cloned fragments were checked by DNA sequence analysis and aligned in pK⁻² together with a fragment containing the 35S transcription termination signal and the GFP expression cassette cut from pKCTAP.

All entry and destination vectors were checked by sequence analysis. Expression vectors were obtained by MultiSite LR reaction (Gateway) and transformed to *Agrobacterium tumefaciens* strain C58C1Rif^R (pMP90) by electroporation. Transformed bacteria were selected on yeast extract broth plates containing 100 μ g/ml rifampicin, 40 μ g/ml gentamicin, and 100 μ g/ml spectinomycin.

Cell Suspension Cultivation—Wild-type and transgenic *A. thaliana* cell suspension cultures ecotype Landsberg *erecta* (PSB-D) were maintained in 50 ml of MSMO medium (4.43 g/liter MSMO (Sigma-Aldrich), 30 g/liter sucrose, 0.5 mg/liter α -naphthaleneacetic acid, 0.05 mg/liter kinetin, pH 5.7, adjusted with 1 M KOH) at 25 °C in the dark by gentle agitation (130 rpm). Every 7 days, the cells were subcultured in fresh medium at a 1:10 dilution.

Cell Culture Transformation with Callus Selection—The *Arabidopsis* culture was transformed by *Agrobacterium* cocultivation as described previously (16) with minor modifications. The *Agrobacterium* culture exponentially growing in yeast extract broth medium (A_{600} between 1.0 and 1.5) was washed three times by centrifugation (10 min at 3050 \times g) with an equal volume of MSMO medium and resuspended in cell suspension-growing medium until an A_{600} of 1.0. Two days after subcultivation, 3 ml of suspension culture was incubated with 200 μ l of washed *agrobacteria* and 200 μ M acetosyringone for 48 h in the dark at 25 °C with gentle agitation (130 rpm). Plant cells were washed three times with 50 ml of MSMO medium containing a mixture of three antibiotics (50 μ g/ml kanamycin, 500 μ g/ml carbenicillin, and 500 μ g/ml vancomycin). Cells were plated on MSMO agar (0.8%) containing the mixture of antibiotics and stored at 25 °C in the dark until callus formation was observed. After 2 weeks, stably transformed calli strongly expressing the fluorescent marker GFP were transferred to fresh MSMO agar plates containing 50 μ g/ml kanamycin and further grown for 4–6 weeks. Sixty milligrams of callus tissue was suspended in 10 ml of MSMO medium containing 50 μ g/ml kanamycin. After 1 week, 40 ml of fresh MSMO medium was added, and cultures were maintained as described previously. Two weeks later, transgene expression was analyzed in a total protein extract derived from exponentially growing cells, harvested 2 days after subculturing, by immunoblotting using the anti-calmodulin-binding peptide (CBP) antiserum (1:1000; Upstate, Bedford, MA). Cultures with high transgene expression were gradually scaled up to 200 ml (dilution ratio, 1:5) and 2 \times 1 liter (dilution ratio, 1:10) in 500-ml and 2-liter Erlenmeyer flasks, respectively. Finally cell material from 10 \times 1-liter cultures (dilution ratio 1:5) grown for 2 days was pooled, harvested on a sintered glass filter, frozen in liquid nitrogen, stored at –80 °C, and processed later for protein extraction and TAP.

Cell Culture Transformation without Callus Selection—*Agrobacteria* were cocultivated with PSB-D suspension cells under the same conditions as described above. Two days after cocultivation, 7 ml of MSMO medium containing a mixture of three antibiotics (25 μ g/ml kanamycin, 500 μ g/ml carbenicillin, and 500 μ g/ml vancomycin) was added to the cell cultures and grown further in suspension under standard conditions (25 °C, 130 rpm, and continuous darkness). The stable transgenic cultures were selected by sequential dilution in a 1:5 and 1:10 ratio in 50 ml of fresh antibiotics mixture-containing MSMO medium 11 and 18 days postcocultivation, respectively. After counterselecting the bacteria, the transgenic plant cells were subcultured weekly in a 1:5 ratio in 50 ml of MSMO medium containing 25 μ g/ml kanamycin for 2 more weeks. Thereafter the cultures were grown and scaled up as described above.

Protein Extract Preparation—Plant material (15 g) of exponentially growing cell cultures, harvested 2 days after subculturing, was ground to homogeneity in liquid nitrogen. Crude protein extracts were prepared in an equal volume (w/v) of extraction buffer (25 mM Tris-HCl, pH 7.6, 15 mM MgCl₂, 5 mM EGTA, 150 mM NaCl, 15 mM *p*-nitrophenyl phosphate, 60 mM β -glycerophosphate, 0.1% (v/v) Nonidet P-40, 0.1 mM sodium vanadate, 1 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml antipain, 5 μ g/ml chymostatin, 5 μ g/ml pepstatin, 10 μ g/ml soybean trypsin inhibitor, 0.1 mM benzamidine, 1 μ M *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane (E64), 5% (v/v) ethylene glycol) with an Ultra-Turrax T25 mixer (IKA Works, Wilmington, NC) at 4 °C. The soluble protein fraction was obtained by a two-step centrifugation at 36,900 \times g for 20 min and at 178,000 \times g for 45 min at 4 °C. The extract was passed through a 0.45- μ m filter (Alltech, Deerfield, IL).

Tandem Affinity Purification—Purifications were performed as described by Rigaut *et al.* (6) with some modifications. Briefly total protein extract was incubated for 1 h at 4 °C under gentle rotation with 500 μ l of IgG-Sepharose 6 Fast Flow beads (GE Healthcare) pre-equilibrated with 10 ml of extraction buffer. The IgG-Sepharose

² M. Karimi, unpublished data.

beads were transferred to a 1-ml Mobicol column (MoBiTec, Göttingen, Germany) and washed with 10 ml of IgG wash buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 5% ethylene glycol) and 10 ml of tobacco (*Nicotiana tabacum* L.) etch virus (TEV) buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 0.5 mM EDTA, 1 mM PMSF, 1 μ M E64, 5% (v/v) ethylene glycol). Bound complexes were eluted via AcTEV digest (2×100 units; Invitrogen) for 1 h at 16 °C followed by two wash steps with 750 μ l of calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 10 mM β -mercaptoethanol, 1 mM imidazole, 2 mM CaCl₂, 1 mM magnesium acetate, Complete EDTA-free protease inhibitor mixture (Roche Diagnostics), 5% (v/v) ethylene glycol). The CaCl₂ concentration of the IgG-eluted fraction was adjusted to 2 mM, and the fraction was incubated for 1 h at 4 °C under gentle rotation with 500 μ l of calmodulin-agarose beads (Stratagene, La Jolla, CA) pre-equilibrated with 10 ml of calmodulin binding buffer. The calmodulin-agarose beads were packed in a Mobicol column and washed with 10 ml of calmodulin binding buffer. Bound complexes were eluted with 2.5 ml of calmodulin elution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 10 mM β -mercaptoethanol, 1 mM imidazole, 25 mM EGTA, 5% (v/v) ethylene glycol) and precipitated with TCA (25%, v/v). The protein pellet was washed twice with ice-cold acetone containing 50 mM HCl, redissolved in sample buffer, and separated on 4–12% gradient NuPAGE gels (Invitrogen). Proteins were visualized with colloidal Coomassie Brilliant Blue staining.

Gel Filtration—Tandem affinity-purified protein complexes, eluted from calmodulin-agarose beads, were separated at 4 °C on a Superdex 200 HR (10/30; GE Healthcare) size exclusion column (at a flow rate of 75 μ l/min) pre-equilibrated in gel filtration buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 15 mM MgCl₂, 1 mM DTT, 5 mM EGTA, 5 mM β -glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, Complete EDTA-free protease inhibitor mixture) (Roche Diagnostics). Fractions of 500 μ l were collected. Histone H1 kinase assay was carried out by incubating 25 μ l of each fraction with 2 μ Ci of [γ -³²P]ATP in the presence of 1 mg/ml histone H1 (Sigma-Aldrich), cAMP-dependent kinase inhibitor, 50 mM Tris-HCl (pH 7.8), 15 mM MgCl₂, 5 mM EGTA, and 1 mM DTT. After 20 min of incubation at 30 °C, the reaction was stopped by heating the samples at 95 °C in the presence of SDS-PAGE sample buffer. Samples were analyzed by 12% SDS-PAGE, stained with Coomassie Brilliant Blue, and autoradiographed. Next 10 μ l of the sized fractions was analyzed by immunoblotting with anti-CDKA;1 antiserum.

BN/SDS-PAGE—Protein solutions were subjected to buffer exchange at 4 °C with the Ultra4 Amicon centrifugal device (Millipore, Bedford, MA) against BN sample buffer (30 mM HEPES, pH 7.0, 150 mM potassium acetate, 1% (v/v) protease inhibitor mixture, 10% (v/v) glycerol). Protein concentration was determined by Bradford assays (Bio-Rad). For BN separation, 400 μ g of protein was used from the total protein extract and the IgG-unbound fraction, and 55 μ g was used from the TAP eluate. Digitonin (Calbiochem) was added to a final concentration of 5 g/g of protein. Samples were incubated on ice for 30 min, and insoluble material was removed by centrifugation at $20,000 \times g$ for 45 min at 4 °C. Native 5–16% gradient gels with a 4% stacking gel were cast on the SE600 Ruby system (GE Healthcare). Running conditions were 45 min at 100 V, 7 mA and 10–15 h at 500 V (maximum), 15 mA in blue cathode buffer (50 mM Tricine, 15 mM BisTris, 0.2% Coomassie G-250, pH 7.0) and anode buffer (50 mM BisTris, pH 7.0) at 4 °C. For separation in the second dimension by 12% SDS-PAGE, lanes from the first dimension gel were cut out and incubated for at least 30 min in BN denaturing buffer (50 mM Tris, pH 6.8, 66 mM Na₂CO₃, 10% (w/v) glycerol, 2% (w/v) SDS, 2% β -mercaptoethanol). First dimension strips were placed onto the stacking gel (4%) and overlaid with 0.5% agarose. The second dimension separations were run on the Ettan Dalt Six system (GE

Healthcare) for 1 h at 600 V, 400 mA, 2.5 watts followed by 4.5 h at 16.6 watts/gel. Immunoblotting of the second dimension was performed according to a standard protocol.

Proteolysis and Peptide Isolation—Complete lanes from the protein gels were cut into slices, collected in microtiter plates, and further processed for MS analysis as described before with minor modifications (17). Dehydrated gel particles were rehydrated in 4 μ l of digest buffer containing 25 ng of trypsin (MS Gold; Promega, Madison, WI), 100 mM NH₄HCO₃, and 10% (v/v) CH₃CN for 30 min at 4 °C. After addition of 10 μ l of a buffer containing 25 mM NH₄HCO₃ and 10% (v/v) CH₃CN, proteins were digested at 37 °C for 3 h. The resulting peptides were concentrated and desalted with microcolumn solid phase tips (PerfectPure™ C₁₈ tip, 200-nl bed volume; Eppendorf, Hamburg, Germany) and eluted directly onto a MALDI target plate (OptiMALDI; Applied Biosystems, Foster City, CA) with 1.1 μ l of 50% CH₃CN, 0.1% CF₃COOH solution saturated with α -cyano-4-hydroxycinnamic acid and spiked with 20 fmol/ μ l Glu¹-fibrinopeptide B (Sigma-Aldrich).

Acquisition of Mass Spectra—A MALDI-tandem MS instrument (4700 Proteomics Analyzer; Applied Biosystems) was used to acquire peptide mass fingerprints (PMFs) and subsequent 1-kV CID fragmentation spectra of selected peptides. PMFs and peptide sequence spectra were obtained with the settings presented in Supplemental Tables 1 and 2. Each MALDI plate was calibrated according to the manufacturer's specifications. All PMF spectra were internally calibrated with the internal standard at *m/z* 1570.677 (fibrinopeptide B), resulting in an average mass accuracy of 5 ± 10 ppm for each analyzed peptide spot on the analyzed OptiMALDI targets. Using the individual PMF spectra, up to eight peptides exceeding a signal-to-noise ratio of 20 that passed through a mass exclusion filter (Supplemental Table 3) were submitted to fragmentation analysis. Fragmentation spectra were recorded according to the settings displayed in Supplemental Table 2 (no internal calibration was used).

MS-based Protein Homology Identification—PMF and peptide sequence spectra of each sample were processed with the accompanied software suite (GPS Explorer 3.5; Applied Biosystems) with parameter settings as summarized in Supplemental Tables 4 and 5. Data search files were generated according to the settings presented in Supplemental Table 6 and submitted for protein homology identification by using a local database search engine (Mascot 2.1; Matrix Science, London, UK). An in-house non-redundant database with acronym SNAPS (for Simple Non-redundant Assembly of Protein Sequences; version 0.2)³ for *Arabidopsis* (72,161 sequence entries, 28,697,815 residues) was compiled from eight public databases (Supplemental Table 7). Protein homology identifications of the top hit (first rank) with a relative score exceeding 95% probability were retained. Additional positive identifications (second rank and more) were retained when the score exceeded the 98% probability threshold. The probability-based MOWSE score (18), assuming that the observed match is significant (*p* value = 0.05), had to equal 61 when submitting PMF data to the database and 31 for individual peptide ions when submitting peptide sequence spectra. Peptide mass spectra of proteins that were solely identified on the basis of a PMF or a single peptide sequence were annotated in the supplemental data set. Proteins belonging to a multiprotein family were singled out based on the identification of unique and diagnostic peptides. To estimate the false positive rate of the protein homology data set, a decoy database from the *Arabidopsis* SNAPS (version 0.2) was generated. Each protein amino acid sequence was shuffled with the EMBOSS shuffle tool (19). The score distribution and false positive frequency of the spectra from the different TAP experiments with the decoy database was plotted (Supplemental Fig. 1).

Data Analysis—Homology-based searches of the identified pro-

³ K. Laukens, K. Henderickx, E. Bonnet, G. De Jaeger, and E. Witters, unpublished data.

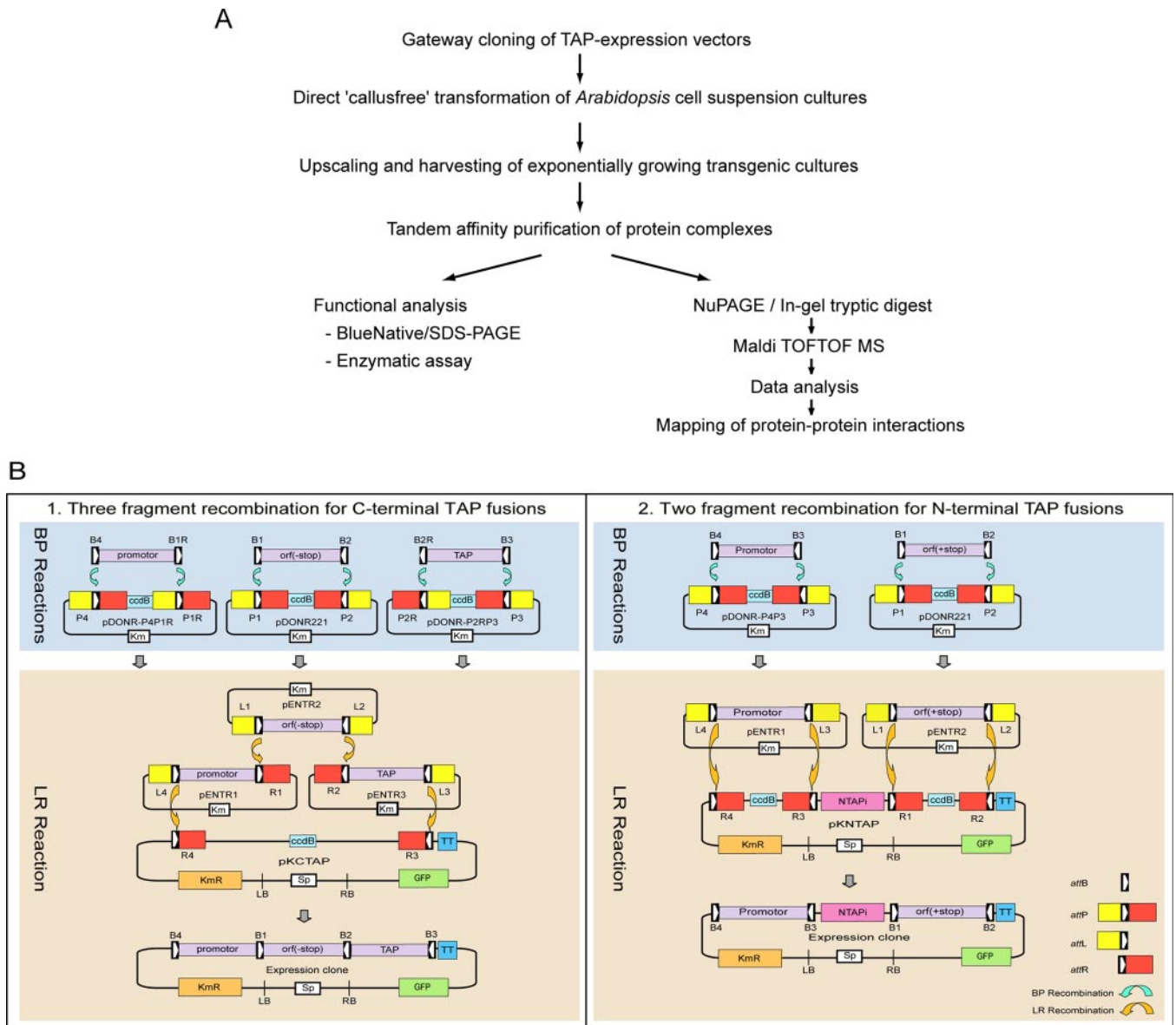


FIG. 1. Strategy followed to clone, express, purify, and identify TAP-tagged proteins and their interacting partners. *A*, summary of the technology platform implemented for screening of cell cycle-related protein-protein interactions in plant cells. *B*, overview of the TAP construct cloning strategy. For C-terminal TAP fusions, a three-fragment recombination strategy was used. Entry vectors pENTR1, pENTR2, and pENTR3 were produced in a BP Clonase reaction that transferred a PCR amplicon (promoter, ORF without stop codon, and TAP tag, respectively) flanked by the appropriate *att* sites (B4 and B1R, B1 and B2, or B2R and B3) in one of the three compatible donor vectors (pDonrP4P1R, pDonr221, or pDonrP2RP3; Invitrogen). Subsequently the three fragments were assembled into the pKCTAP destination vector in a single MultiSite LR Clonase reaction to produce an expression clone. Besides the Gateway gene cassette, pKCTAP contained between left (LB) and right (RB) T-DNA border sequences a kanamycin resistance gene for selection of transformed cells and a GFP expression cassette as a visible marker for transformation. For N-terminal TAP fusions, a two-fragment recombination strategy was used. The promoter was cloned by BP Clonase reaction in pDonrP4P3, and the ORF (plus stop codon) was cloned in pDonr221. Subsequently the two fragments were assembled into the pKNTAP destination vector, which contained the NTAPi tag (4), during a single MultiSite LR Clonase reaction to produce an expression clone. pKNTAP also comprised a kanamycin resistance gene for selection of transformed cells and a GFP expression cassette as a visible marker for transformation. TT, cauliflower mosaic virus 35S transcription termination sequence; CcdB, toxic killer gene for negative selection.

teins were performed with the BLAST and PSI-BLAST algorithms. Gene ontology (GO) annotation searches were done via the interface at The Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org). The Biological Networks Gene Ontology (BiNGO) (20) tool that is implemented as a plugin for Cytoscape (56) was used to

determine which GO biological process was statistically overrepresented in the set of identified interactors using the hypergeometric test combined with a Benjamini and Hochberg false discovery rate correction (p cutoff at 0.05) (20). A protein-protein interaction network was built with the Cytoscape software.

RESULTS

Expression of TAP-tagged Proteins in Cell Suspension Cultures—The strategy to purify and characterize protein complexes from *Arabidopsis* suspension cultures ectopically expressing TAP fusions is depicted in Fig. 1A. To maximize cloning throughput, Gateway-compatible vectors were designed for both N- (pKNTAP) and C-terminal tagging (pKCTAP) (Fig. 1B). The vectors give maximal cloning flexibility because the MultiSite Gateway cassette in pKCTAP enables simultaneous cloning of a promoter sequence, ORF, and a tag of choice. In pKNTAP, the promoter and the ORF can be cloned in one step via independent recombination. We used the TAP tag introduced by Rigaut *et al.* (6) consisting of two IgG-binding domains of the *Staphylococcus aureus* protein A (ZZ) and a CBP separated by a TEV protease cleavage site.

In yeast, the endogenous protein is replaced by the tagged version through homologous recombination in haploid cells (8). However, in higher eukaryotes, the tagged proteins are normally produced in the presence of the untagged endogenous version, which might compete for incorporation into multiprotein complexes. The accumulation level of the tagged protein might thus be an important parameter for complex isolation. Therefore, we first compared the expression levels for five different cell cycle proteins, all tagged C-terminally (CDKA;1, CDKB1;1, CDKD;2, CDKF;1, and CKS1) under control of either a strong constitutive 35S promoter of the cauliflower mosaic virus or their own promoter. The *Agrobacterium*-mediated transformation procedure combined with callus selection was used to generate transgenic *Arabidopsis* cell suspension cultures. Several cultures were made for each construct. Protein extracts of these cultures were screened by immunoblotting with anti-CBP, and for each construct the culture with the highest transgene expression was selected for further analysis. As expected, all recombinant proteins accumulated at higher levels when expressed under control of the constitutive promoter compared with the endogenous promoters (Fig. 2A). For CDKB1;1, CKS1, and CDKD;2, only very low levels or even no tagged proteins could be detected under control of their endogenous promoter (Fig. 2A). Next we compared the protein levels of the overexpressed fusion proteins, CDKA;1 and CKS1, to the corresponding endogenous proteins. Immunoblot analysis with polyclonal antisera against the two proteins revealed that the recombinant CKS1 concentration in the overexpressing culture was higher than that of the endogenous counterpart (Fig. 2B). However, for recombinant CDKA;1 under control of the 35S promoter, only cultures with accumulation levels lower than those of the endogenous CDKA;1 could be obtained (Fig. 2C) probably because of gene dosage effects, although a higher turnover of the TAP-tagged CDKA;1 proteins could not be excluded. Given the high ploidy level (8n) for the *Arabidopsis* cultures we used, the average transgene copy number per cell might be lower than that of the endogenous *Arath*;CDKA;1 gene, whereas for

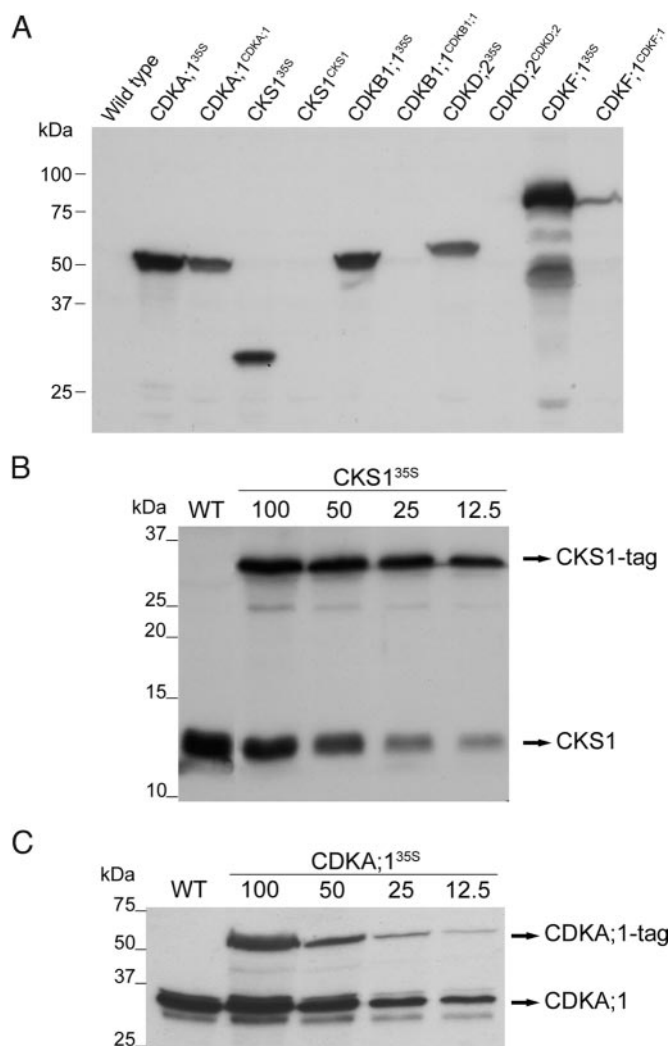


Fig. 2. Expression analysis of tagged proteins in transgenic cell suspension cultures. A, comparison of transgene expression under control of the constitutive 35S promoter versus the endogenous promoter. Expression was analyzed by immunoblotting with an anti-CBP antibody (1:1000). The total protein extract of 2-day-old cultures (60 µg) was separated by 12% SDS-PAGE and immunoblotted. Expected recombinant molecular masses are 54.6 kDa for CDKA;1, 31.1 kDa for CKS1, 55.9 kDa for CDKB1;1, 59.8 kDa for CDKD;2, and 74.4 kDa for CDKF;1. B and C, expression analysis of recombinant TAP-tagged CKS1 (B) or CDKA;1 (C) versus the corresponding endogenous protein by immunoblotting with an anti-CKS1 (1:2500) (B) or anti-PSTAIRES (1:2500) (C) antiserum. Of total protein from the untransformed cells, 50 µg was analyzed. For the transgenic cultures overexpressing the recombinant protein a series of 100, 50, 25, and 12.5 µg of total protein extract were separated. To prevent interaction between IgG present in the rabbit antisera used and ZZ domain of the TAP tag, thereby overestimating accumulation levels of the recombinant protein, human serum was added (1:250) to the blocking buffer and during incubation with primary and secondary antibodies. WT, wild type.

CKS1, there might be a compensation by the stronger 35S promoter. Because purifications of tagged proteins produced under control of a strong constitutive promoter followed by

MS provided the best coverage of protein complex detection (see below), we continued with the overexpression strategy.

Optimization of Cell Suspension Transformation—Protocols for *Arabidopsis* cell suspension transformations published so far are based on *Agrobacterium* cocultivation followed by transgenic callus selection on agar plates, growth of individual calli, and finally callus resuspension (16, 21, 22); the whole procedure takes ~3–4 months. Especially growing individual calli to sufficient biomass for resuspension is very time-consuming and laborious and hence less suited for a high throughput setup. Therefore, we changed the transformation procedure by fine tuning selection criteria of transformed cells and by eliminating callus selection (see “Experimental Procedures”). In this manner, transgenic cultures could be obtained in ~6 weeks. To assess large scale utility of the “direct callus-free” transformation procedure, 20 different randomly chosen cell cycle genes were overexpressed as TAP-tagged fusion proteins in *Arabidopsis* suspension cultures. Transgene expression was analyzed in total protein extracts of exponentially growing cultures by immunoblotting with an antibody against the CBP tag. In all transgenic cultures, the respectively tagged fusion proteins were detected and migrated on 12% SDS-PAGE at the correct molecular mass (Fig. 3A) except for *CycD2;1*, *CycD3;1*, *E2Fa*, *SMR2*, and *SMR3* whose migration was slower than expected. At least for *CycD3;1* and *E2Fa*, this aberrant migration had been reported previously (23, 24). For seven proteins, four of which were cyclins, additional protein bands with lower molecular masses were detected probably due to proteolytic degradation of these unstable proteins. Furthermore accumulation levels among the 20 different proteins varied largely despite these overexpression under control of the same 35S promoter. Independent transformations with the same transgene resulted in different cultures with similar expression levels; hence transformation variation could be excluded (Fig. 3B).

TAP of Functionally Active Protein Complexes—Two-dimensional BN/SDS-PAGE was used to analyze the incorporation of tagged proteins into the corresponding physiological complexes. Protein extracts of the cell cultures overproducing C-terminally TAP-tagged *CDKA;1* under control of the 35S promoter were separated by two-dimensional BN/SDS-PAGE followed by immunoblot analysis of the bait and the endogenous protein with anti-*CDKA;1* polyclonal serum. Recombinant *CDKA;1* had a migration pattern similar to that of the endogenous *CDKA;1*, ranging from non-complexed monomeric *CDKA;1* to protein complexes with a molecular mass more than 669 kDa (Fig. 4A, panel 1), demonstrating that the tagged protein competes with the endogenous protein to be built into physiological protein complexes.

The original TAP procedure from yeast (6) was adapted to simultaneously purify TAP-tagged protein complexes from different plant cell cultures. Major changes were decreasing the incubation time with both affinity chromatography matrices; adding protease inhibitors to all buffers, thereby reducing

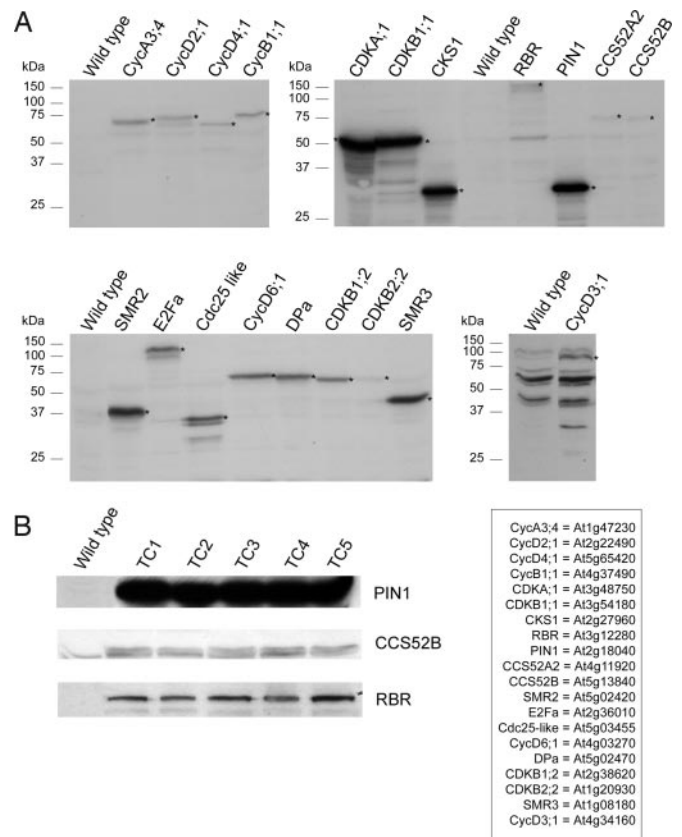


FIG. 3. Transgene expression analysis of cultures obtained by transformation without callus selection. A, detection of 20 different TAP-tagged proteins (with their corresponding accession numbers) in total protein extracts of exponentially growing cell suspension cultures. In each lane, 60 μ g of extract was loaded, and recombinant proteins were detected by immunoblotting with an anti-CBP antiserum (1:1000). Bands corresponding to full-length fusion proteins are indicated with an asterisk. B, evaluation of variation in expression levels due to different transformation events. For three different transgene constructs, five independent transgenic lines were made. Transgene expression was analyzed in 60 μ g of total protein extract through immunoblotting with an anti-CBP antiserum. TC, transformed culture; RBR, retinoblastoma related; PIN1, peptidylpropyl *cis/trans*-isomerase 1; DPa, dimerization partner a; SMR, SIAMESE related; CCS52, cell cycle switch 52. Accession numbers are from TAIR.

protein degradation of unstable cell cycle proteins; and increasing the EGTA concentration during the calmodulin-agarose elution. Typically 15 g of frozen cell suspension material was used to prepare a protein extract containing 200–300 mg of total protein. In a first affinity purification step, extracts were incubated with IgG resin, and bound complexes were eluted by tag cleavage with TEV protease. Co-eluting non-interacting proteins and the TEV protease were further separated from the tagged proteins and their associations in the flow-through of the second affinity step. The bait and interacting proteins were finally eluted from the calmodulin-agarose via EGTA-mediated removal of calcium. Two-dimensional BN/SDS-PAGE followed by immunoblot analysis was used to evaluate the purification procedure. Different steps of the purification

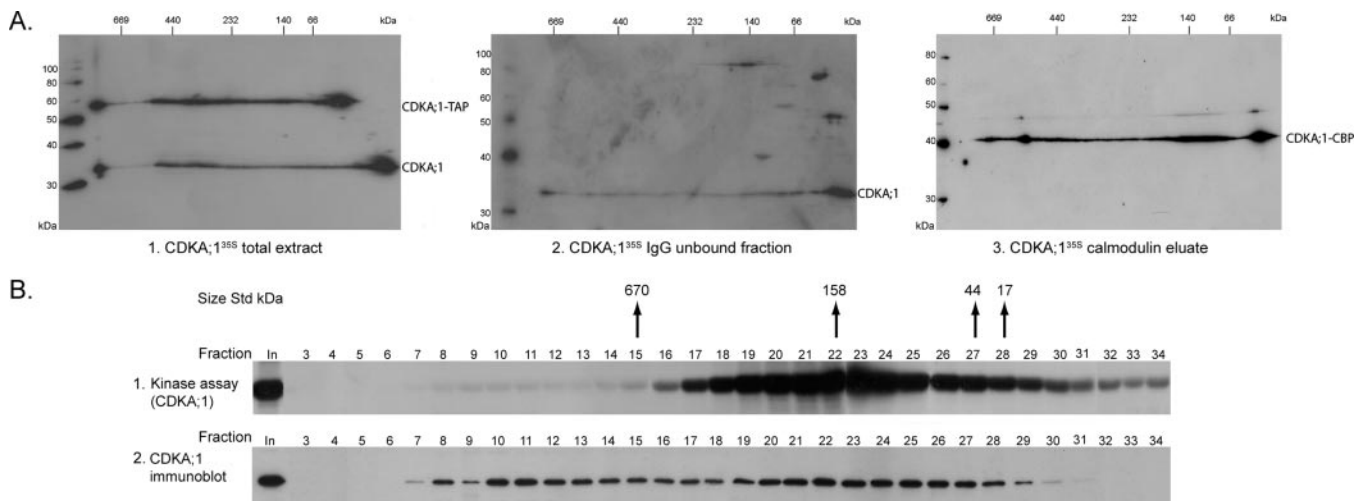


FIG. 4. **Protein complex analysis by BN/SDS-PAGE (A) and size exclusion chromatography (B).** A, protein complexes fractionated in the first dimension by BN-PAGE. In the second dimension, proteins were separated by SDS-PAGE, and gels were immunoblotted with the anti-CDKA;1 antiserum (1:5000). For CDKA;1^{35S}, 400 μ g of protein was analyzed from the total protein extract (*panel 1*) and the IgG-unbound fraction (*panel 2*), and 55 μ g was analyzed from the final calmodulin eluate (*panel 3*). Recombinant CDKA;1 and CDKA;1 fused to the complete TAP tag and the CBP tag are indicated as *CDKA;1-TAP* and *CDKA;1-CBP*, respectively. B, CDKA;1-TAP eluate (2 ml) fractionated on a Superdex 200 (300/10) size exclusion chromatography column. From each 500- μ l fraction, 25 μ l was assayed for kinase activity with histone H1 as a substrate (*panel 1*), and 10 μ l was analyzed for the presence of the bait protein by immunoblotting with an antiserum against CDKA;1 (1:5000) (*panel 2*). Arrowheads indicate the elution positions of marker proteins with their molecular masses (kDa). Std, standard.

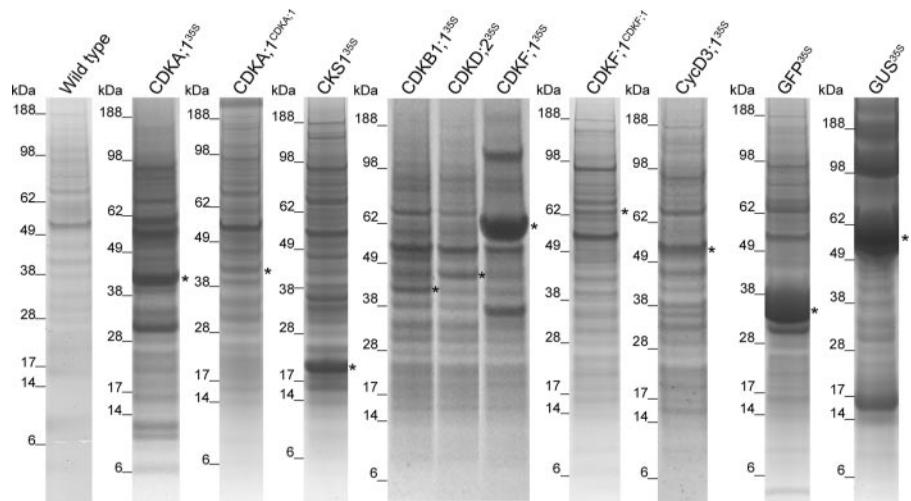
of the culture overproducing TAP-tagged CDKA;1 were analyzed in that manner. In the flow-through fraction of the first affinity chromatography step, only protein complexes with the endogenous CDKA;1 were detected (Fig. 4A, *panel 2*), demonstrating that the IgG resin bound all protein complexes containing the recombinant CDKA;1. In the final calmodulin eluate, the recombinant CDKA;1-containing protein complexes had a migration pattern similar to that of the starting material (Fig. 4A, *panels 1* and *3*), indicating that the TAP protocol allowed the isolation of intact CDK protein complexes.

To further analyze the functional activity of the purified complexes and thus their integrity, the CDKA;1 calmodulin eluate was fractionated by size exclusion chromatography. All fractions were analyzed for the presence of the bait protein by immunoblotting with an anti-CDKA;1 antibody and assayed for CDK activity with histone H1 as a substrate (Fig. 4B). The maximum of the histone H1 kinase activity migrated around 100 kDa (fraction number 23) and was associated with a maximum for CDKA;1 fusion protein. These observations are consistent with previously reported results in tobacco Bright Yellow-2 cells (25) and demonstrate that the overexpressed CDKA;1 fusion protein is incorporated into physiologically active complexes, which can be isolated through our TAP protocol. Immunoblot analysis of the sized fractions also showed that besides the active complex the recombinant CDKA;1 protein is part of inactive high molecular mass complexes (Fig. 4B). These data are consistent with the two-dimensional BN/SDS-PAGE results.

Identification of Copurifying Proteins by MS—To identify

protein interactions for the cell cycle baits CDKA;1, CDKB1;1, CKS1, CDKD;2, CDKF;1, and CYCD3;1, TAPs were performed on cultures of C-terminally tagged CDKA;1, CDKB1;1, CKS1, CDKD;2, and CDKF;1 and N-terminally tagged CYCD3;1 under control of the 35S promoter and cultures of C-terminally tagged CDKA;1 and CDKF;1 under control of the endogenous promoter. Each culture was at least purified twice independently. Eluted proteins were separated on 4–12% NuPAGE gels and visualized by colloidal Coomassie staining. In most cases, the tagged protein was detected as the most prominent band on the gel (Fig. 5). Protein bands were excised, subjected to tryptic digestion, and analyzed via MALDI-TOF-TOF-MS. The resulting peptides were assigned to specific proteins with the in-house SNAPS database.³ This non-redundant database combines all publicly available *Arabidopsis* protein sequences. By querying the complete set of available sequences at a time, the best possible hits were retrieved. Contaminating proteins due to experimental background were determined by purifications on wild-type and two transgenic cultures overexpressing TAP-tagged fusions of heterologous proteins, β -glucuronidase, and GFP (Fig. 5). Most contaminants are high abundant proteins such as chaperones, cytoskeleton proteins, ribosomal proteins, metabolic enzymes, or protein translation factors (Supplemental Table 8). Identical or similar proteins were found as common contaminants in other protein-protein interaction studies (7, 8, 26–29). Therefore, to increase the stringency of the data set, these proteins were systematically subtracted from the lists of purified proteins. The remaining identified proteins were divided into two groups: 43 proteins that could be confirmed

FIG. 5. Analysis of the TAP protein eluates. TAP-tagged protein complexes were purified from transgenic plant cell suspension cultures, precipitated with TCA (25%, v/v), separated on 4–12% NuPAGE gels, and visualized with colloidal Coomassie G-250 staining. Bait proteins are indicated with an asterisk. *GUS*, β -glucuronidase.



experimentally (Table I) and 186 proteins that were identified only once per bait (Supplemental Table 9).

The data set of confirmed interactors in Table I shows enrichment for proteins that are annotated as regulators of cell cycle progression (Table I, GO ID number). This enrichment was statistically confirmed with the BiNGO tool implemented as a plugin for Cytoscape (20). BiNGO analysis of the total list of interacting proteins (Table I and Supplemental Table 9) revealed a statistically significant enrichment for proteins involved in regulation of the cell cycle (corrected p value, $2.66E-11$; Supplemental Fig. 2a). When subtracting proteins that could not be identified in at least two experimental repeats, the p value decreased even more (corrected p value, $1.45E-17$; Supplemental Fig. 2b), suggesting that the non-confirmed data set contained proteins unrelated to cell cycle control and occurring occasionally and randomly due to experimental background. Nevertheless BiNGO analysis on the subset of nonconfirmed proteins clustered CKS1, CDKA;1, and two DNA mismatch repair proteins (MSH6-1 and MLH1) together as involved in DNA-dependent DNA replication (corrected p value, $2.14E-2$; Supplemental Fig. 2c). Moreover MSH6-1 and MLH1 copurified with CDKA;1 and CKS1, suggesting that they interacted albeit without confirmation. Therefore, the nonconfirmed identifications have to be evaluated with caution because they can represent weaker or more transitory associations between proteins or protein complexes. Especially those that are common between different baits are certainly interesting interactors to be followed up.

Depending on the expression level of the TAP-tagged fusion proteins, the number of identified protein interactions differs a lot. Under control of its endogenous promoter, we could only confirm TONNEAU 1b as an interacting protein; this in contrast to 19 proteins identified after purifications of the overexpressed CDKA;1 bait (Table I). TONNEAU 1b is a protein involved in cortical microtubule organization (30) and is similar to the human FGFR1 oncogene partner (FOP), a

protein implicated in centrosomal microtubule anchoring (31). Besides the fact that CDKA;1 is known to colocalize with microtubules of the preprophase band (32) and this plant-specific cytoskeleton structure is absent in the *ton1* mutant, there is no known correlation between the two proteins. Among the 19 interacting proteins identified after overproducing CDKA;1-TAP, six proteins had been previously reported as CDKA;1 interactors in plants: three D-type cyclins, CKS1, and two KRPs (33–38). Reciprocal purifications with CKS1 as bait validated this result and confirmed previously reported associations of CKS1 with A- and B-type CDKs (39). Besides CKS1, we also identified its close homolog CKS2 as an interacting protein of CDKA;1. CKS2 was also present in the purified fractions of overexpressed CDKB1;1 and CYCD3;1 and in cells expressing CDKA;1 under control of its endogenous promoter (Table I and Supplemental Table 9). In addition to KRP6 and CKS2, CDKA;1 was also identified as an interacting protein of the CYCD3;1-TAP fusion. Despite the isolation of KRP6 in the CDKA;1 data set, we could not identify CYCD3;1 in this complex (Table I) probably because of the highly unstable character of this specific D-type cyclin (23). We found also an expressed protein, similar to SIAMESE, as an interactor for CycD3;1 (Table I). SIAMESE is a plant-specific cell cycle regulator that controls endoreduplication onset in *A. thaliana* (40). It encodes a nuclear localized 14-kDa protein containing a cyclin-binding motif and a motif found in KRP cell cycle inhibitors. Furthermore it was found to associate with D-type cyclins and CDKA;1 (40).

In the set of six tagged cell cycle proteins, we also analyzed the CDK-activating kinases (CAKs), CDKF;1 and CDKD;2, which have recently been proposed to represent two types of CAKs in *Arabidopsis*, playing a major role in phosphorylation of CDKs and the C-terminal domain of the largest subunit of RNA polymerase II (41), respectively. We showed that overexpressed CDKD;2 fusion protein forms a stable trimeric complex with the regulatory subunit CYCH;1 and the assembly factor MAT1 (Table I). Both proteins have been reported

TAP to Study the Cell Cycle Interactome in *A. thaliana*

TABLE I

Proteins identified by MALDI-TOF-TOF-MS after a TAP procedure from cultures producing CDKA;1 and CDKF;1 under control of their endogenous promoter and CDKA;1, CDKB1;1, CKS1, CDKD;2, CDKF;1, and CYCD3;1 under control of the 35S cauliflower mosaic virus promoter

Proteins copurifying with the bait proteins are shown only when they were confirmed in more than one experimental repeat. The GO annotations or references related to cell cycle control mechanisms are given as GO ID or reference number. Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

Accession number ^a	Protein name	GO ID ^b or Ref.
CDKA;1^{35S}		
At2g27960	CKS1	GO: 74, 278, 4693, 42023
At2g27970	CKS2	GO: 7049, 4693
At2g20580	26 S proteasome regulatory subunit S2 (RPN1)	GO: 74
At5g23540	26 S proteasome regulatory subunit, putative	54
At1g64520	26 S proteasome regulatory subunit, putative (RPN12)	55
At2g22490	CYCD2;1	GO: 74, 80, 16538
At5g40460	Hypothetical protein	
At3g19150	KRP6	GO: 4861, 45736, 30332
At1g23190	Phosphoglucomutase, cytoplasmic, putative	
At4g28470	26 S proteasome regulatory subunit, putative	
At5g63610	CDKE;1	3
At5g65420	CYCD4;1	GO: 80, 74, 16538
At5g10440	CYCD4;2	GO: 74, 4693
At1g10690	Expressed protein	
At2g32710	KRP4	GO: 45736, 4861, 30332, 45786
At3g49240	Pentatricopeptide repeat (PPR)-containing protein	GO: 9793
At2g28000	Rubisco subunit-binding protein α subunit, chloroplast	
At3g17020	Universal stress protein (USP) family protein	
At1g78900	Vacuolar ATP synthase catalytic subunit A	GO: 9555
CDKA;1^{PCDKA;1}		
At3g55000	TONNEAU 1b	
CDKB1;1^{35S}		
At2g27970	CKS2	GO: 7049, 4693
At2g28000	Rubisco subunit binding-protein α subunit, chloroplast	GO: 9790
At1g64520	26 S proteasome regulatory subunit, putative (RPN12)	55
CDKD;2^{35S}		
At5g27620	CYCH;1	GO: 74, 4693
At5g08690/At5g08670	ATP synthase β chain, mitochondrial	
At4g30820	CAK assembly factor-related protein (Mat1)	
At4g16143	Importin α -2, putative (IMPA-2)	
CDKF;1^{PCDKF;1}		
At3g16270	Expressed protein similar to cyclin G-associated kinase (O14976)	GO: 74
CDKF;1^{35S}		
At1g67580	CDKG;2	3
At1g66750	CDKD;2	GO: 79
CKS;1^{35S}		
At3g48750	CDKA;1	GO: 8284, 9574, 4693
At3g54180	CDKB1;1	GO: 4693
At1g47230	CYCA3;4	GO: 74, 16538
At4g14310	Peroxisomal membrane protein-related	
At1g20930	CDKB2;2	GO: 87, 4693, 7346
At2g22490	CYCD2;1	GO: 80, 74, 16538
Arath05g16630	Unknown	
At3g53880	Aldo/keto reductase family protein similar to chalcone reductase	
At1g76540	CDKB2;1	GO: 307, 86, 4693
CYCD3;1^{35S}		
At3g48750	CDKA;1	GO: 8284, 9574, 4693
At2g27970	CKS2	GO: 7049, 4693
At5g02220	Expressed protein similar to SIAMESE	40
At3g19150	KRP6	GO: 4861, 45736, 30332

^a Accession numbers are from TAIR, with the exception of Arath 05g16630, which is from the EuGène database (see Supplemental Table 7).

^b 74, regulation of progression through cell cycle; 79, regulation of cyclin-dependent protein kinase activity; 80, G₁ phase of mitotic cell cycle; 86, G₂/M transition of mitotic cell cycle; 87, M phase of mitotic cell cycle; 278, mitotic cell cycle; 307, cyclin-dependent protein kinase holoenzyme complex; 4693, cyclin-dependent protein kinase activity; 4861, cyclin-dependent protein kinase inhibitor activity; 7049, cell cycle; 7346, regulation of progression through the mitotic cell cycle; 8284, positive regulation of cell proliferation; 9555, male gametophyte development; 9574, preprophase band; 9790, embryonic development; 9793, embryonic development; 16538, cyclin-dependent protein kinase regulator activity; 30332, cyclin binding; 42023, DNA endoreduplication; 45736, negative regulation of cyclin-dependent protein kinase activity; 45786, negative regulation of progression through the cell cycle.

previously as interactors (29, 41). In mammals and rice (*Oryza sativa*), the trimeric CAK complex is part of a protein complex that forms the general transcription factor TFIIF and is responsible for the C-terminal domain phosphorylation (29, 42). This complex could not be identified; in contrast we did identify Xeroderma pigmentosum D (UVH6), a helicase that links the trimeric CAK to TFIIF complex, albeit without confirmation (Supplemental Table 9) and another interactor of CDKD;2, importin α -2 (Table I). The human homolog of importin α -2 (importin α/β) has been described recently in mammalian cells as a nuclear import receptor that binds one of the nuclear localization signals of cyclin H, thereby regulating the nuclear translocation of the CDK7-cyclin H complex (43). Our data show for the first time that a similar regulatory mechanism might be active in plant cells.

CDKF;1 is a known CAK-activating kinase that phosphorylates other CAKs in a cyclin H-independent manner (44). CDKD;2 was copurified from extracts overproducing the CDKF;1 tagged protein (Table I), demonstrating that the method allows us to purify the physiologically active CAK-activating kinase and its associated substrate. However, expression of CDKF;1 under control of its own promoter did not yield CDKD;2 (Table I); instead an expressed protein with similarity to the human cyclin G-associated kinase was identified. In the cell suspension cultures overexpressing CDKF;1, we also identified CDKG;2 as a new interacting protein. Together with its close homolog CDKG;1, CDKG;2 has been described recently as a new member of the CDK family of proteins in *Arabidopsis* (3) that are characterized by a PLTSLRE motif and have the highest sequence homology to the human galactosyltransferase-associated (GTA) protein kinase p58/GTA. p58/GTA is a member of a p34^{cdc2}-related kinase subfamily that interacts with cyclin D3 (45) and might act as a negative regulator of cell cycle progression in mammalian cells (46). The human p58/GTA isoform is specifically induced during G₂/M phase of the cell cycle and is posttranslationally regulated by phosphorylation (47). Sequence comparison of the plant CDKG;2 revealed that the activating Thr phosphorylation site in the T-loop, known to be regulated by CAKs, is conserved.

Finally we mapped the identified interactions around the six baits (Fig. 6) with the Cytoscape software. The interactions are depicted as *arrows* from the baits to the identified associations, both represented as *orange* and *blue nodes*, respectively. The mapping revealed an extensive network around CDKA;1, the primary CDK for cell division, including its known cyclin partners, CDK inhibitors (KRP4 and KRP6), and the small CDK-binding subunits CKS1 and CKS2 as well as new interactions. In various cases, the detected interactions were bridged by intermediary partners, implying that many interactions, such as CKS1-cyclin or KRP6-CYCD3;1, occur through the CDK subunit.

DISCUSSION

In eukaryotes, cell division is controlled by a large set of genes whose expression and function are both transcriptionally and posttranscriptionally regulated. Although DNA arrays continue to provide information regarding changes at the mRNA level, posttranslational modifications, changes in cellular localization, or interactions within complexes can only be assessed at the protein level. Here we report the successful setup of a TAP technology platform that allows us to unravel protein-protein interaction networks in plants. The methodology is based on an integrated approach comprising the fast generation of transgenic cultures overproducing tagged fusion proteins, TAP adapted for plant cells, high throughput protein identification by tandem MS, data analysis, and functional assays.

Our generic MultiSite Gateway-based transformation vectors enable high throughput cloning of large sets of TAP-tagged fusions with the cloning of the ORF of the genes of interest as the only time-limiting factor. In contrast to formerly published TAP vectors for plants (11, 12, 29), we are able to construct fusions of any ORF with any tag under control of any promoter in one cloning step as long as they are cloned in the proper Gateway donor vectors. Comparing accumulation levels of a set of randomly chosen tagged cell cycle proteins revealed a large variation despite overexpression under control of the same constitutive promoter. Possible explanations could be either counterselection of transgenic cells expressing aberrant levels of essential cell cycle genes or dependence on the nature of the proteins and on posttranslational regulation controlling the abundance of the corresponding endogenous protein. One could expect that relatively unstable proteins, such as cyclins, had lower accumulation levels than more stable and constitutive proteins, such as CDKA;1 and CKS1. The level at which the tagged bait accumulates has a major impact on the yield of purified complexes and hence the identified interactors. Data sets of interactions from ectopically expressed CDKA;1 and CDKF;1 under a strong constitutive or the endogenous promoter clearly showed this prominent difference. In our system, the tagged protein is produced in the presence of the endogenous protein that might contend for incorporation into multiprotein complexes. Given the high ploidy level of the *Arabidopsis* cell suspension culture used, only overexpression under control of the strong 35S promoter resulted in sufficient amounts of bait protein to compete with the endogenous protein. The use of an overexpression system probably also facilitates detection of weaker or more transitory associations between proteins. Indeed cyclins that have a cell cycle-dependent protein accumulation level controlled via ubiquitin/proteasome-dependent degradation could only be isolated when the bait was overproduced. Additional factors determining the number of possible identified protein interactions depends on the nature of the bait itself. For example, the number of interactions for

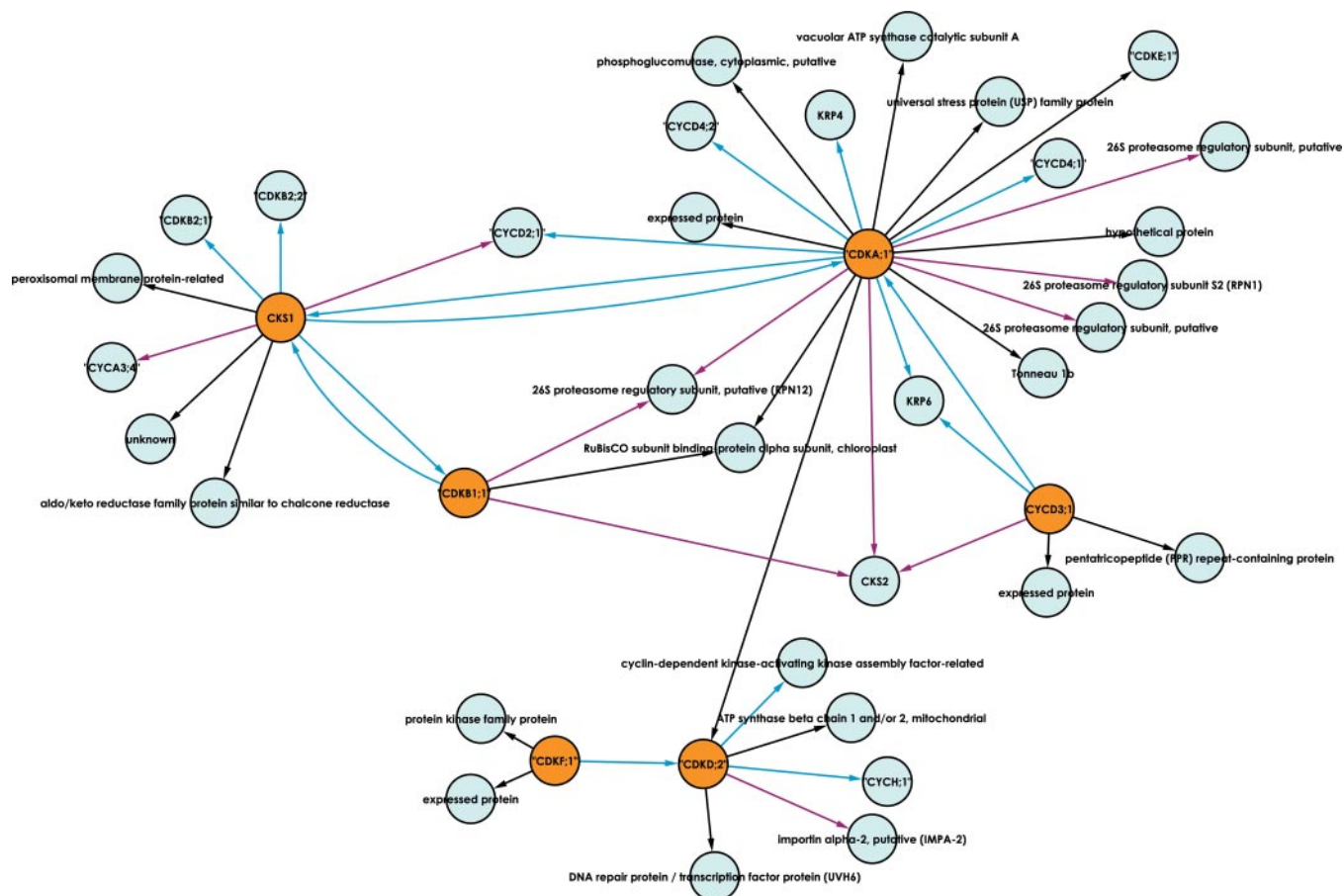


FIG. 6. Interactome map for the protein interactions (blue nodes) detected with six core cell cycle proteins (orange nodes). Interactions are represented as arrows pointing from the bait to the interactor. Known interactions, links described previously in the literature in orthologous systems, and unknown interactions are represented in blue, purple, and black, respectively.

CDKA;1, which is a kind of hub within the cell cycle interactome and is generally accepted to play a central role in cell cycle control, is much higher compared with proteins that have a more specific function. This observation has also been reported in other studies (7, 8). The likelihood of identifying protein associations with a cell cycle-dependent expression profile might also depend on the harvest time of the suspension culture. In this study, we worked with non-synchronized exponentially growing cells. Flow cytometric analysis of the harvested cell material showed an equal G₁/G₂ phase distribution (data not shown). Hence proteins with a cell cycle phase-specific expression pattern will yield fewer interactors as observed in our data set for CDKB1;1 that has a known G₂/M-specific kinase activity and expression profile (25). However, the amount of purified protein complexes does not solely depend on the expression profile of the transgene but also on the accessibility and integrity of the TAP tag and its possible steric hindrance during complex formation. Analysis by BN/SDS-PAGE of the different purification steps showed that the majority of the CDKB1;1-associated complexes bound only weakly to the first affinity resin (data not shown).

Gel filtration combined with kinase assays was integrated into the platform to analyze the physiological activity of the tagged proteins, thereby proving that our method allows the isolation of active CDK complexes. The activity peaks around 100 kDa, suggesting that these complexes probably consist of CDK and their regulatory subunit, cyclin. Besides the active complexes, the CDKA;1 fusion protein was incorporated into large, inactive complexes (>600 kDa). Identification of 26 S proteasome regulatory subunits in the TAP eluates suggests that part of these large complexes might be composed of CDKA;1 associating with the 19 S regulatory particle of the proteasome and possibly reflect the targeting of interacting proteins, such as CDK inhibitors or cyclins, to the degradation pathway. The ability of the ubiquitin/proteasome pathway to selectively degrade a single subunit of a multisubunit complex is a known regulatory switch, including those involving cyclins and CDK inhibitors. Mitotic CDK is inactivated at the end of mitosis by selective degradation of tightly bound mitotic cyclin (48, 49), and S phase CDK/cyclin is activated *in vivo* at the G₁/S transition upon degradation of its CDK inhibitor (50–53).

In conclusion, for the set of six different cell cycle proteins,

we identified and confirmed 42 protein-protein associations of which 28 were new interactions, demonstrating that the integrated TAP-MS platform that we developed for plant cells is a powerful tool to systematically identify cell cycle complexes and link proteins of unknown function to signaling pathways during cell division. Extension of the proteomics approach described in this study with the analysis of synchronized transgenic cultures should make it possible to investigate the variations in protein associations during cell cycle progression. Characterizing the global topology and dynamic features of the cell cycle interactome will be a major challenge for the future that certainly will provide further insights into developmental mechanisms.

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