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Identification of barley CK2α targets by using the protein microarray technology

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Abstract

We have successfully established a novel protein microarray-based kinase assay, which we applied to identify target proteins of the barley protein kinase CK2α. As a source of recombinant barley proteins we cloned cDNAs specific for filial tissues of developing barley seeds into an *E. coli* expression vector. By using robot technology, 21,500 library clones were arrayed in microtiter plates and gridded onto high-density filters. Protein expressing clones were detected using an anti-RGS-His_6 antibody and rearrayed into a sublibrary of 4100 clones. All of these clones were sequenced from the 5′-end and the sequences were analysed by homology searches against protein databases. Based on these results we selected 768 clones expressing different barley proteins for protein purification. The purified proteins were robotically arrayed onto FAST™ slides. The generated protein microarrays were incubated with an expression library-derived barley CK2α in the presence of [γ-^{33}P]ATP, and signals were detected by X-ray film or phosphor imager. We were able to demonstrate the power of the protein microarray technology by identification of 21 potential targets out of 768 proteins including such well-known substrates of CK2α as high mobility group proteins and calreticulin.

Keywords: *Hordeum vulgare*; Protein microarray technology; Kinase assay; Casein kinase; CK2α; Phosphorylation of target proteins; Substrates

1. Introduction

Protein functionality is often dependent on post-translational protein modifications. For the understanding of complex biological systems it is therefore crucial to have information about the state of as many proteins as possible (Templin et al., 2002).

Post-translational modifications of proteins include cleavage (e.g., elimination of signal sequences), or addition of many simple chemical groups such as acetyl-, methyl-, and phosphoryl-groups and more complex molecules, such as sugars and lipids. More than a hundred different modifications are now known (Cahill, 2001). One of the most important modifications is the reversible phosphorylation of proteins. Phosphorylation is mediated by protein kinases, which transfer the γ-phosphate of ATP on amino acid residues of proteins. The phosphorylation can be reversed by protein phosphatases. This process is very important for the temporary regulation of the protein activity.

The phosphorylation state of a protein can have profound effects on its activity and interaction with other proteins (Stone and Walker, 1995). The phosphorylation of proteins in plants has been found to be connected with the reaction of the organism towards different internal and external factors, such as light, invasion of pathogens, hormones, temperature stress and shortage of nutrition (Hardie, 1999).

It is estimated that about one third of all eukaryotic proteins are phosphorylated during their existence (Dombradi et al., 2002; Zolnierowicz and Bollen, 2000). Furthermore, it is presumed that 1–3% of all

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eukaryotic genes encode protein kinases (Stone and Walker, 1995).

Protein kinases are typically classified by their ability to phosphorylate either serine- and/or threonine-, or tyrosine-residues. These kinases share a great sequence homology and are also called the eukaryotic super family of protein kinases. Members of this family have a catalytic domain of 250–300 amino acids, which can be subdivided in up to 12 subdomains. In plants, an additional group of protein kinases has been found, which phosphorylate histidine residues and seem to be closely related to the histidine kinases of prokaryotic two component systems (Chang et al., 1993; Kakimoto, 2003).

The task of identifying targets for different protein kinases can be facilitated and accelerated by the application of high-throughput methods (Kersten et al., 2002, 2004) and especially of the protein microarray technology. Hundreds or even thousands of proteins can be printed in high density on coated glass slides, and afterwards analysed in parallel (for recent review see Glökler and Angenendt, 2003).

In this study, we applied the protein microarray technology to identify targets for the barley casein kinase IIα (CK2α). As a source for the kinase as well as for the potential target proteins we used a barley cDNA expression library specific for the filial part of the developing grain. The library was generated in this study.

In plants, casein kinase II is found in two different forms, the heterotetrameric CK2, composed of two catalytic α subunits and two regulatory β subunits, and the monomeric CK2α, consisting of the catalytic α subunit (Stemmer et al., 2003). CK2 has been classified as a messenger-independent protein serine/threonine kinase with a broad specificity. Over 100 potential physiological targets of CK2 have been identified to date (Litchfield, 2003). The maize CK2α was the first plant protein kinase for which a crystal structure was available (Niefind et al., 1998). A special feature of CK2 is the ability to utilise GTP as well as ATP as a phosphate donor. We chose this well-investigated kinase to establish and validate our system.

2. Results and discussion

2.1. Construction and characterisation of a barley cDNA expression library

We constructed a cDNA library specific for the filial tissues of the developing barley grain (0–10 days after flowering, DAF) in the *E. coli* expression vector pQE30NST (GenBank accession no. AF074376). This vector allows the IPTG inducible expression of RGS-His_6-tagged proteins. After transformation of *E. coli* cells with the library, recombinant clones were arrayed into microtiter plates. The library consists of 21,500 clones with an average insert size of 1 kb. The arrayed library was gridded onto high-density filter membranes and screened with an anti-RGS-His_6 antibody for putative expression clones. Of 21,500 clones, 4100 (19.1%) gave a signal in the immunoassay (data not shown). This rate of yield corresponds with the 19.6%, which Büssow et al. (2000) obtained when they screened their human fetal brain library (hEx1), which was cloned into the same vector. The putative expression clones were rearrayed robotically into a sublibrary. All clones of this sublibrary were sequenced from the 5′-end. We removed 5′-and 3′-vector parts from the sequences (vector clipping). 5.4% bad sequences were removed from the set. All remaining sequences were submitted to GenBank and are accessible via http://gabi.rzpd.de.

A BLASTX search of the remaining 3878 sequences was performed against the TAIR plant protein database (http://www.arabidopsis.org/BLAST) (data not shown). Nearly 50% of the matches were found to be in the correct reading frame (the frame of the RGS-His_6-tag). This result is comparable to Lüking et al. (2003) who determined 55% of the rearrayed hEx1 library in the correct reading frame.

2.2. Generation of a frameset from the expression library

Seven hundred and sixty-eight clones of the expression library showing the correct reading frame were chosen and rearrayed robotically into another subset (frameset). Additional criteria for the selection of these clones were different match descriptions and an e-value equal or better than e^{-20}.

A table including the different clone IDs (e.g., the GenBank Accession) and the BLASTX results of all clones of the frameset is found under the following web address: http://www.molgen.mpg.de/~plant_protein_chips/barley_expression_frame_set.html. A motifscan of the sequences against the scansite database (http://scansite.mit.edu/motifscan_seq.phtml) resulted in a rate of 8.2% sequences with one or more CK2-target motifs. This relatively high rate corresponds to the broad specificity of the CK2 (for reviews see Meggio and Pinna, 2003; Litchfield, 2003).

2.3. Generation of protein chips and CK2α assay

All 768 clones were expressed in parallel in 96-well format. Proteins were purified by nickel chelate affinity chromatography under denaturing conditions. To control the quality of the purification 96 randomly chosen proteins out of the 768 proteins were separated using a 15% SDS–PAGE followed by Coomassie staining. 75% of the proteins were detected. The results for 24 proteins are shown in Fig. 1. After application of a similar purification technique, Kersten et al. (2003) detected 78%

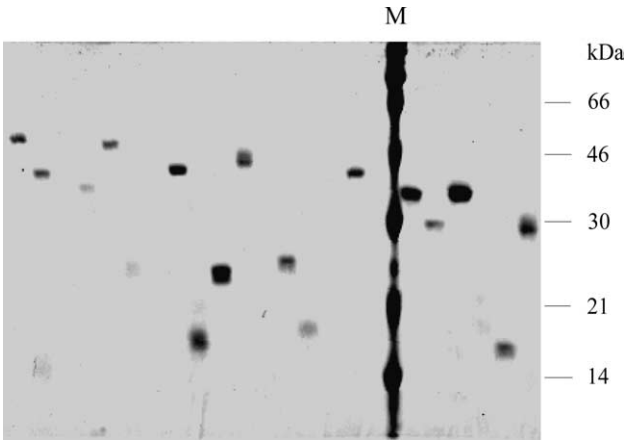

Fig. 1. SDS–PAGE of 24 proteins from barley (Coomassie-stained) after high-throughput expression and purification in a 96-well format. M, molecular weight marker; the approximate size of the proteins used as markers are shown on the right in kDa.

of 95 purified recombinant Arabidopsis proteins in a Coomassie stained SDS-gel.

Furthermore a CK2α clone (GenBank Accession of the 5′-EST: CK123377; Full-length sequence of the cDNA was submitted to GenBank) from the frameset was expressed and purified under native conditions.

The 768 purified barley proteins were arrayed on two FAST™ slides (384 different proteins on each slide) in quadruplicates. The chips were used for an antibody screening with an anti-RGS-His_6 antibody (see Fig. 2). Thus, for nearly all of the 768 spotted proteins of the frameset, a signal was detected in the immunoassay. This indicates that our selection of expression clones, due to filter and subsequent sequence analysis, was successful and that our purification technique is sufficient for this application. 16 controls were added to each chip. As positive controls library-derived barley proteins were used, which share a strong homology with different plant HMG (“high mobility group”) proteins. These proteins are well known targets of the CK2α (Grasser et al., 1989; Stemmer et al., 2002).

Two CK2α-assays with freshly spotted chips and two different preparations of the kinase were carried out independently. Fig. 2 shows an X-ray film with the results of the second experiment. The results of the two CK2α-assays were evaluated as follows: A protein was considered as positive in one experiment if all four spots of the corresponding quadruplicate gave a distinct signal on the X-ray film. A protein was regarded as potential target protein if it attained positive results in both experiments.

The intersection of positive-classified proteins from both experiments was generated and resulted in a list of potential target proteins, which were reassigned to their BLASTX matches (Table 1). Thus, 21 potential CK2α target proteins were identified. Some of these proteins

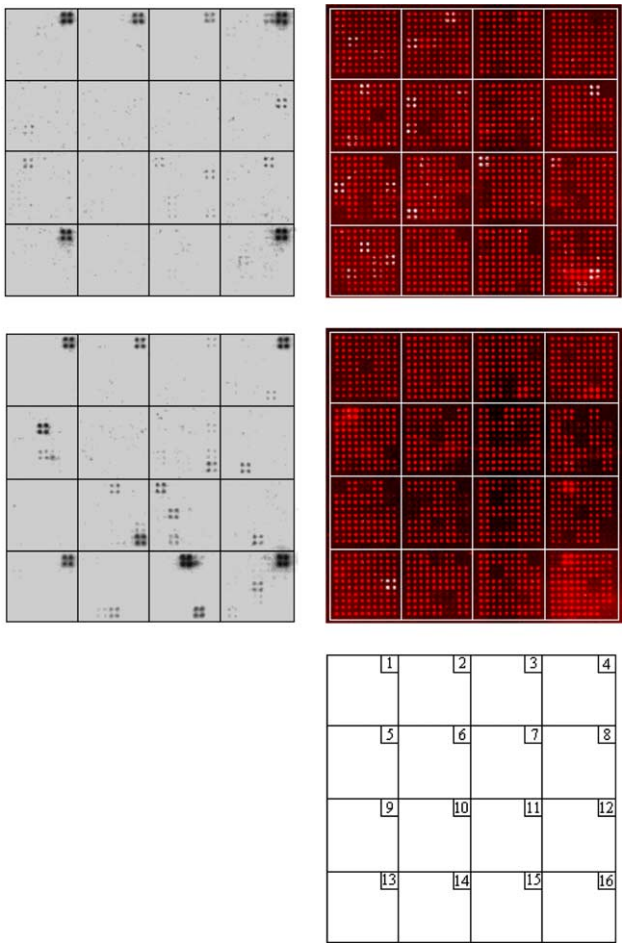

Fig. 2. Kinase- and immunoassay with 768 different barley proteins, immobilised on FAST™ slides (384 proteins on each slide). Left: X-ray film with the results of a representative CK2α-assay. Right: equal chips screened with an anti-RGS-His_6 antibody. The map below shows the positions of the 16 controls. The purified proteins and the controls were spotted in quadruplicates. Controls: 1, 4, 13, 16: HMG(1) (GenBank Acc.: CK124404), 0.29 mg/ml; 2: HMG(2) (GenBank Acc.: CK122788), 0.76 mg/ml; 3: HMG(3) (GenBank Acc.: CK124847), 0.28 mg/ml; 5: CK2α (GenBank Acc.: CK123377), 0.22 mg/ml; 6: other library kinase (GenBank Acc.: CK125548), 0.21 mg/ml; 7: human gapdh (His-tagged recombinant human glyceraldehyde-3-phosphate dehydrogenase), 0.36 mg/ml; 8: BSA, 20 pmol/μl in PBS; 9: native elution buffer; 10: denaturing elution buffer; 11: mouse anti-RGS-His_6 antibody, diluted 1:10 in PBS; 12: rabbit-anti-mouse IgG-Cy3 conjugate, diluted 1:25 in PBS; 14: H_2O; 15: PBS.

are well-known targets for CK2 and CK2α, respectively. HMG proteins are described as being phosphorylated by a maize CK2α (Stemmer et al., 2002). Evidence for phosphorylation by CK2 exists also for protein phosphatase 2C (PP2C) (Kobayashi et al., 1998), calreticulin (Baldan et al., 1996) and choline-phosphate cytidylyltransferase (Wieprecht et al., 1996). Meggio and Pinna (2003) have reviewed a list of 307 substrates for CK2 in eucaryotes. It contains the four known CK2 substrates, which we have found.

Table 1
Potential target proteins of barley CK2α identified in this study

Plate position of the clone in the frameset[a]	GenBank Accession of the library clone	GenBank Accession of the match	Match description
Plate 1 well B06	CK123797	AAO60033.1	Unknown protein [*Oryza sativa* (japonica cultivar-group)]
Plate 1 well F06	CK122753	BAC05614.1	P0458E05.14 [*Oryza sativa* (japonica cultivar-group)]
Plate 1 well F12	CK122788	BAB85204.1	High mobility group box protein 2 [*Oryza sativa* (japonica cultivar-group)]
Plate 1 well K08	CK122469	NP_172380.2	Expressed protein [*Arabidopsis thaliana*]
Plate 1 well M05	CK123128	AAP53374.1	putative protein phosphatase 2C [*Oryza sativa* (japonica cultivar-group)]
Plate 1 well O21	CK122269	NP_200931.1	Expressed protein [*Arabidopsis thaliana*]
Plate 1 well P17	CK123780	AAP44682.1	unknown protein [*Oryza sativa* (japonica cultivar-group)]
Plate 2 well A01	CK125075	NP_187977.1	Casein kinase – related [*Arabidopsis thaliana*]
Plate 2 well A13	CK124020	T07980	Probable choline-phosphate cytidylyltransferase (EC 2.7.7.15) (clone CCT2) – rape
Plate 2 well B06	CK125707	BAB89813.1	Putative protein kinase SPK-3 [*Oryza sativa* (japonica cultivar-group)]
Plate 2 well B23	CK125559	BAC01254.1	P0019E03.13 [*Oryza sativa* (japonica cultivar-group)]
Plate 2 well F06	CK124847	P40621	HMGL_WHEAT HMG1/2-like protein
Plate 2 well F23	CK124536	BAB89747.1	P0506B12.19 [*Oryza sativa* (japonica cultivar-group)]
Plate 2 well G05	CK124087	S48027	Ribosomal protein L34, cytosolic – common tobacco
Plate 2 well G16	CK125437	NP_174552.1	HAC13 protein [*Arabidopsis thaliana*]
Plate 2 well I11	CK124168	CAC24844.1	MCB1 protein [*Hordeum vulgare* subsp. vulgare]
Plate 2 well I14	CK124313	NP_173417.2	Expressed protein [*Arabidopsis thaliana*]
Plate 2 well J09	CK124581	AAP40415.1	Unknown protein [*Arabidopsis thaliana*]
Plate 2 well J15	CK124596	T05705	Calreticulin - barley (fragment)
Plate 2 well M14	CK124404	CAA90679.1	HMG1/2-like protein [*Hordeum vulgare* subsp. vulgare]
Plate 2 well P13	CK124732	BAC20622.1	Contains EST AU031225(E61165)-nhp2-like protein [*Oryza sativa* (japonica cultivar-group)]

[a] A table including the different clone IDs (e.g., the GenBank Accession) and the BLASTX results of all clones of the frameset is given under the following web-address: http://www.molgen.mpg.de/~plant_protein_chips/barley_expression_frame_set.html.

Six of the identified targets with a protein annotation in the match description (Table 1) are not described as CK2 target proteins so far (''casein kinase –related'', ''putative protein kinase SPK-3'', ''ribosomal protein L34'', ''HAC 13'', ''MCB1 protein'', ''contains EST AU031225(E61165)-nhp2-like protein''). Among them, we found two proteins, which seem to be related to known targets of CK2. In the case of the last protein in Table 1 the match description contains a reference to ''nhp2-like protein''. Nhp2 has been called ''HMG-like'' because of the physical/chemical properties it shares with the HMG proteins from higher eukaryotic cells (Kolodrubetz and Burgum, 1991), which are known substrates of CK2 as mentioned before. The MCB1 protein is a MYB-like transcription factor in barley (Churin et al., 2003) and contains a phosphorylation site for CK2. MYB factors have been identified previously as targets for CK2 (Meggio and Pinna, 2003). Therefore, it is probable that these two proteins are novel targets of barley CK2.

About half of the identified proteins (Table 1) lack a known functionality (''unknown proteins'', ''expressed proteins''). Therefore, no comparison with known targets is possible.

Our assay represents a screening for potential target proteins of kinases. In our test system, we used denatured proteins as targets. In the respective native folded protein potential phosphorylation sites are possibly not accessible for the kinase. Furthermore, it is possible that the target and the kinase cannot interact with each other in vivo, e.g., because they are localised in different cellular compartments. Nevertheless, the identification of well-known targets demonstrates the feasibility of our assay for the detection of possible new targets.

In order to assure that the identified potential target proteins are in vivo-targets of the chosen kinase, continuative experiments have to be performed; e.g., protein–protein interaction studies in yeast (yeast two hybrid studies, see Causier and Davies, 2002) or even in planta by using innovative microspectroscopic approaches such as fluorescence detected resonance energy transfer (FRET) or fluorescence lifetime imaging microscopy (FLIM) (Hink et al., 2002).

As expected, the rate of the sequences with a CK2 motif was significantly greater in the set of identified targets (38.1%) than in the frameset (8.2%). One explanation for the fact that not more than 38.1% of the sequences of our identified target proteins contain a CK2 target motif is that only the 5′-sequences were available for the motif scan. CK2 motifs, which are located in the 3′-region, may not be detected. Furthermore, it is not excluded that sequence motifs exist, which can be phosphorylated by the barley-specific CK2α but are

unknown up to now and, therefore, are not present in the scansite database.

In this study, we established a protein microarray-based kinase assay to screen for potential targets in a large set of recombinant proteins. We used FAST™ slides, which are coated with a nitrocellulose-derived polymer allowing for a non-covalent attachment of the proteins and have been used previously for other applications, e.g., the profiling of antibody and sera using the first plant protein microarrays (Kersten et al., 2003). In contrast to our study, Zhu et al. (2000) used PDMS coated microwells to carry out kinase assays with 119 yeast kinases and 17 different substrates. These microwells are useful if different components have to be added to the each sample. Such a feature is not necessarily required to screen for kinase targets as we showed in this study. Furthermore, specialised equipment is needed to load the microwells.

In this study, we used radioactively labelled ATP for the detection of the phosphorylation events. This is still the preferred and most sensitive method for the detection of phosphorylation of peptides or proteins. This detection method has been applied before in other protein-array based kinase assays (Zhu et al., 2000; MacBeath and Schreiber, 2000). We detected radioactive signals by phosphor imager as Zhu et al. (2000) or by X-ray film. MacBeath and Schreiber (2000) used a spot pattern with a similar density compared to our study. They detected the radioactive signals by incubating the chips with a photo emulsion followed by microscopic analysis of the slides. By this means they tried to improve the resolution to visualise the spots. In our study, we demonstrated that the resolution which can be obtained by using X-ray film or phosphor imager is sufficient for screening of up to 1600 spots in one field of a microarray.

Other groups have tested the application of antibodies against phosphorylated peptide epitopes in combination with fluorescence detection (Lesaicherre et al., 2002). Not all phosphorylated epitopes are recognised by the antibodies. For this reason, this method is not suitable for our screening application in which every phosphorylation event should be detected.

Initial studies were performed in which fluorescent dyes were used for the detection of phosphorylated amino acids (Gast et al., 1999; Martin et al., 2003). However, the radioactive-based detection is still the most sensitive and robust detection method.

To identify target sequences for different protein kinases, peptide chips have been used successfully (Houseman et al., 2002). However, arrays containing recombinant proteins instead of peptides are less expensive. In contrast to peptide arrays which need some prior knowledge about the target sequence of the examined kinase, protein arrays allow to screen randomly for putative protein targets.

3. Conclusions

In this study, we established a novel protein microarray-based technique, which allows to screen for potential target proteins of kinases. We applied this technique to screen nearly 800 library-derived barley proteins for potential substrates of the barley kinase CK2α. We identified 21 targets including targets not yet described in literature.

For the further evaluation of the kinase assay, other kinases have to be tested to compare relative numbers and distribution of positives between CK2 and other kinases. The potential of this test system will be increased not only by testing different kinases, but also by upscaling the number of targets analysed.

Furthermore, the constructed barley protein expression library is a valuable source for potential substrates but also for the expression and purification of different kinases.

4. Experimental

4.1. Plant material and cDNA library preparation

Barley (*Hordeum vulgare*) cv. Barke plants were cultivated in growth chambers at 20 °C/18 °C under a 16 h light/8 h dark regime, respectively, until seed set. The developmental stage of the caryopses was determined from the mid-region of the ear as described in Weschke et al. (2000). Young caryopses were harvested from every day of seed development starting with anthesis till 10 days after flowering. Immediately after harvesting, the caryopses were hand-dissected in the maternal and the filial part of the grain (for tissue composition of the resulting seed fractions, see Sreenivasulu et al. (2002, 2004)) and the filial part of the grain (endosperm with embryo) was collected. Total RNA was isolated using the Purescript RNA Isolation kit (Gentra Systems, Minneapolis, USA). After photometric estimation of RNA concentrations, equal amounts of RNA from each sample were mixed together. 375 μg of mixed total RNA were taken to isolate polyA$^+$-RNA by using the Dynabeads mRNA Isolation kit (Dynal, Oslo, Norway). The pBluescript II XR cDNA Library Construction Kit (Stratagene GmbH, Heidelberg, Germany) was used for cDNA library construction following the manufacturer's protocol with the following modification: *Not*I linker primers and *Sal*I adaptors from Gibco (Gibco BRL, Karlsruhe, Germany) were applied instead of the *Eco*RI/*Xho*I system provided by Stratagene. The cDNA fragments were directionally ligated into the *Not*I/*Sal*I cloning sites of the vector pQE30NST (GenBank accession no. AF074376) and transformed into competent *E. coli* SCS1/pSE11 cells (Büssow et al., 1998).

Transformants were picked and kept in 384-well microtiter plates.

4.2. Generation of high-density protein filters and immunoassays

Protein filters were generated as described (Büssow et al., 1998). Filters were screened by incubation with an anti-RGS-His_6 antibody (Qiagen, Hilden, Germany)(dilution: 1:2000), followed by incubation with an anti-mouse-AP-conjugate-antibody (Sigma, Deisenhofen, Germany) (dilution: 1:5000) and Attophos detection (see Büssow et al., 1998). Images were taken with a high resolution CCD detection system and evaluated with the visual grid software (GPC Biotech, Martinsried, Germany).

4.3. Rearray of library clones

Clones, which should be rearrayed into a new sublibrary were summarised in a list. An in house-developed *mercury* -robot was used to rearray the clones according to this list. Fresh clones grown overnight were used for the rearray. These plates were put into the stacker of the robot and new plates with freezing media (2YT medium supplemented with 1x HMFM medium, 2% glucose, 100 μg/ml ampicillin, and 15 μg/ml kanamycin) were fixed in special plate holders on the working table of the robot. The robot was equipped with a gadget with 96 steel pins, which can be extended individually. According to the given list, the robot dips its 96 pins in succession into wells with clones, which should be copied into the new library, and inoculates 96 wells in the new plates simultaneously. After the run of the robot, the new cultures were incubated overnight at 37 °C.

4.4. Sequencing and sequence analysis

All sequencing was carried out by the AGOWA company (Berlin, Germany). Vector clipping of the sequences was carried out by removing 5′- and 3′-parts of the vector pQE30NST. By removing the 5′-parts of the vector, every sequence was cut at a defined position, which is important for the analysis of the reading frame. Bad sequences, which had too many undefined bases or were too short, were removed from the set.

BLASTX searches of the remaining sequences were performed against the TAIR plant protein database (http://www.arabidopsis.org/BLAST). The following parts of the BLASTX results were extracted from the HTML-files and were included into a table: (i) reading frame (important to determine the fitting of the insert, according to the reading frame of the RGS-His_6-tag); (ii) e-value (probability of error for a match); (iii) match description; (iv) match length; (v) start subject (start of the homology in the sequence of the match); (vi) start query (start of the homology in the sequence of the query).

To identify clones with potential target sequences of CK2α, a motif scan of the translated sequences was carried out against the scansite database (http://scansite.mit.edu/motifscan_seq.phtml). The settings were "Casein Kinase 2" for the motif and "medium stringency" for the stringency of the search.

Vector clipping, data submission to the Internet databases and data extraction from the result files were performed with in house-developed software.

4.5. Protein expression and purification in microtiter plates

Proteins were expressed in 1 ml cultures. 96-well microtiter plates were filled with 100 μl 2YT medium supplemented with 2% glucose, 100 μg/ml ampicillin, and 15 μg/ml kanamycin. Cultures of recombinant clones were inoculated from a 384-well plate (Genetix, Christchurch, UK) that had been stored at −80 °C. After 16 h growth at 37 °C with vigorous shaking (320 rpm), 900 μl of pre-warmed medium (2YT medium supplemented with 100 μg/ml ampicillin, 15 μg/ml kanamycin, 20 μg/ml thiamin) was added and the incubation was continued for 2 h. To induce protein expression, IPTG was added to a final concentration of 1 mM and incubation was continued for another 5 h. Cells were harvested by centrifugation at 1500 × g for 15 min at 4 °C and the pellets were stored at −80 °C.

For the purification of the proteins, 150 μl lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 6 M GuHCl, pH 8) were added to the thawed pellets. Pellets were resolved by vigorous vortexing and afterwards incubated for 30 min at room temperature. The resolved pellets were centrifugated at 1900 × *g*. The supernatant was transferred into a 96-well filter plate with a non-protein binding 0.65 μm pore size PVDF membrane (Millipore Multiscreen MADVN 6550, Eschborn, Germany) and immediately drawn into a fresh filter plate by using a vacuum manifold (Multiscreen, Millipore, Eschborn, Germany). Afterwards 25 μl NiNTA–agarose (NiNTA: nickel-nitrilotriacetic acid; Qiagen, Hilden, Germany), 1:2 diluted in lysis buffer, were added to each well, the plate was sealed with tape and His-tagged proteins were bound by shaking for 3 h at 300 rpm. The agarose beads were washed three times by resuspending in washing buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 6.3), shaking for 5 min, and removing of liquid on the vacuum filtration manifold. Finally, proteins were eluted from the agarose beads by shaking for 10 min in 80 μl denaturing elution buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 4.5) and by subsequent filtration into a fresh 96-well microtiter plate. Purified proteins were separated on a 15% polyacrylamide gel.

4.6. Protein expression and purification under native conditions

Fifty milliliter falcon tubes were filled with 10 ml 2YT medium supplemented with 2% glucose, 100 μg/ml ampicillin, and 15 μg/ml kanamycin. Cultures were inoculated with bacteria from 384-well plates that had been stored at −80 °C. After overnight growth at 37 °C with vigorous shaking the cultures were transferred into 300 ml Erlenmeyer flasks and 90 ml prewarmed 2YT medium supplemented with 100 μg/ml ampicillin, and 15 μg/ml kanamycin were added. The incubation was continued until an OD of 0.7 was reached. To induce protein expression, IPTG was added to a final concentration of 1 mM, and incubation was continued for 4 h. Cells were transferred into 50 ml falcon tubes (two for each culture), harvested by centrifugation at $1900 \times g$ for 10 min and stored immediately at −80 °C.

The frozen pellets were thawed on ice. Cells were lysed in 0.5 ml native lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0) supplemented with 0.25 mg/ml lysozyme and 0.1 mM PMSF. Lysates were pooled and DNA was sheared with an ultrasonic homogeniser (Branson Ultrasonic, Danbury, USA) for 3×1 min at 50% power on ice. Lysates were transferred into 1.5 ml Eppendorf-tubes and centrifugated at $20,000 \times g$, 4 °C. Supernatants were transferred into fresh Eppendorf-tubes, 250 μl NiNTA–agarose were added and His-tagged proteins were bound by shaking for 1 h at 300 rpm. The mixtures were transferred to 1 ml polypropylen columns (Qiagen, Hilden, Germany). The columns were washed with 10 bed volumes native lysis buffer containing 20 mM imidazole. Proteins were eluted in 0.5 ml native lysis buffer containing 250 mM imidazole.

Protein concentration was determined by Bradford (1976) assay.

4.7. Generation of protein chips

FAST™ slides (Schleicher and Schuell, Dassel, Germany) were used for spotting experiments. The microscope slides were placed in a Q-array system (Genetix, New Milton, UK) equipped with humidity control (70%) and 16 blunt end stainless steel pins with a tip diameter of 150 μm. The samples were spotted using a 10×10 spotting pattern in quadruplicates and the spotting depth was 120 μm. Each spot was loaded once transferring a volume of 0.6 nl per spot. Mouse and rabbit IgG antibodies (Santa Cruz, USA) were used as controls in spotting experiments.

4.8. Immunoscreening of protein chips

The slides were blocked for 1 h at room temperature with 2% BSA (bovine serum albumin)/TBST (TBS/0.1% v/v Tween 20). Mouse anti-RGS-His_6 antibody (Qiagen, Hilden, Germany) was diluted 1:2000 in blocking solution and then applied onto the arrays for 1 h at room temperature, followed by two 10 min wash steps with TBST. The slides were further incubated for 1 h at room temperature with the respective Cy3-labelled secondary antibody (rabbit-anti-mouse-IgG conjugate, Dianova, Hamburg, Germany), which was applied with a 1:800 dilution in blocking solution. Then three wash steps of 30 min each were performed in TBST. The signal detection was performed by means of a ScanArray 4000 (Perkin–Elmer Life Science, Cologne, Germany).

All antibody incubation steps were carried out in a 200 μl volume underneath a coverslide and in the dark.

4.9. CK2α assay

Protein chips, which were spotted the day before, were washed in TBST for 1 h at room temperature at vigorous shaking to remove urea from the chips.

Chips were blocked for 1 h at room temperature with 2% BSA/TBST and were afterwards incubated with 13 μg/ml CK2α and 25 μCi/ml radioactive labelled [γ^{33}P]ATP in CK2α Buffer (25 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM DTT) for 1 h. Then six wash steps of 30 min each were performed in TBST. Chips were dried and transferred into a X-ray-cassette (Hypercassette, Amersham Pharmacia, Freiburg, Germany). Signal detection was performed by means of a X-ray-film (Kodak, Stuttgart, Germany). The film was laid on the chips and the cassette was stored for seven days at −80 °C. Afterwards the film was developed in a dark chamber. Furthermore, the chips were exposed to an imaging screen (BAS-SR 2025, Fujifilm, Japan) for 3 h and afterwards screened by a phosphor imager (BAS-Reader-5000, Fujifilm, Japan) for signal detection.

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