

Generation of Plant Protein Microarrays and Investigation of Antigen–Antibody Interactions

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Summary

The application of proteomics methods, such as the protein microarray technology, in plant science has been strongly supported by the completion of genome sequencing projects of *Arabidopsis thaliana* and rice. In this chapter we describe a method to generate plant protein microarrays and to use them for characterizing monoclonal antibodies or polyclonal sera with regard to their specificity and cross-reactivity. The method starts with characterized *E. coli* cDNA expression clones encoding His-tagged plant proteins. After expression and purification of these recombinant proteins in high throughput, protein microarrays are generated utilizing a contact printer. For the detection of the recombinant proteins on the microarrays, an anti-RGS-His₆ antibody is used. To characterize specific antibodies, the microarrays are incubated with the respective antibody solutions followed by fluorescently labeled secondary antibodies. Signal detection is performed by means of an arrayscanner system. Protein microarrays containing the whole proteome of a plant will represent the ideal format to test antibody specificity and cross-reactivity in the future.

Key Words: Protein microarray; antibody and serum profiling; interaction studies; *Arabidopsis thaliana*; high throughput; proteomics.

1. Introduction

Protein microarrays consist of hundreds or even thousands of addressable protein samples, which are arranged in a systematic order in high density on coated glass slides (1–4). They allow parallel, fast, and easy analysis of proteins for expression (5–7) and modification (8–11) as well as for their molecular interactions with antibodies (12–15), other proteins (16,17), DNA (18,19), or small molecules (20,21). Using protein antigen microarrays, several studies have been performed to characterize specific antibodies (12–15) or to screen sera from patients suffering from diverse diseases (15,22,23). As an ideal for-

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mat to characterize a specific antibody, proteome arrays are envisaged (13). To analyze several antibodies on a single microarray, different multiplexing approaches may be applied (24,25). Compared with Western blotting, the application of protein microarrays for antibody characterization has several advantages (13) including a higher sensitivity of protein detection (13,23).

Proteomics methods, e.g., 2D electrophoresis/mass spectrometry (2-DE/MS) methods or protein microarray technology, are finding increasing applications in the plant field (26–32). Here we describe a method to generate plant protein microarrays and to utilize them for the investigation of antigen-antibody interactions. We successfully applied the method to generate the first plant protein microarrays containing 96 *Arabidopsis* proteins, which were detectable with a limit of approx 2 to 4 fmol per spot with an anti-RGS-His₆ antibody on FAST™-slides (14). Utilizing these arrays, we were able to show that a monoclonal anti-TCP1 antibody and polyclonal anti-MYB6 and anti-DOF11 sera recognized their respective antigens on the microarrays and did not cross-react with the other immobilized proteins including other DOF and MYB transcription factors.

For this method, RGS-His₆-tagged plant proteins are expressed and purified in high throughput using characterized cDNA expression clones. Purified proteins are robotically arrayed onto FAST-slides. The generated protein microarrays are then incubated with the respective antibodies, followed by fluorescently labeled secondary antibodies. Signal detection and evaluation allows one to identify proteins interacting with the tested antibody.

2. Materials

2.1. High-Throughput Protein Expression and Purification

1. Media for culturing cells: 2 YT or LB medium containing 100 µg/mL ampicillin, 15 µg/mL kanamycin, and 2% glucose.
2. 96-Well microtiter plates (Greiner bio-one, Frickenhausen, Germany).
3. 384-Well microtiter plates (Genetix, Christchurch, UK).
4. 96-Deep-well plates: 96-well microtiter plates with 2-mL cavities (Qiagen, Hilden, Germany).
5. Replicator with 96 pins (Nunc, Wiesbaden, Germany).
6. 4X SB medium: 48 g/L bacto-tryptone, 96 g/L yeast extract, 0.8% (v/v) glycerol, sterilized by autoclaving at 120°C for 20 min.
7. 20X PP buffer (potassium phosphate buffer): 17 mM KH₂PO₄, 72 mM K₂HPO₄, sterilized by filtration through a 0.2-µm pore size filter.
8. Thiamin.
9. Isopropyl-β-D-thiogalactopyranosid (IPTG; MBI Fermentas, St. Leon-Rot, Germany).
10. MultiScreen_{HTS} Vacuum Manifold (Millipore, Eschborn, Germany).
11. NiNTA-agarose (NTA: nickel-nitrilotriacetic acid; Qiagen).

12. Denaturing lysis buffer: 100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M GuHCl, pH 8.0.
13. Washing buffer: 100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 6.3.
14. Elution buffer: 100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 4.5.

For items 12 to 14, see Note 1.

15. 96-Well filter plate with a non-protein-binding 0.65-µm pore size PVDF membrane (Millipore Multiscreen MADVN 6550).
16. Bradford reagent (Bio-Rad, Munich, Germany).

2.2. Generation of Plant Protein Microarrays

1. 384-Well microtiter plates (Genetix).
2. FAST-slides (Whatman Schleicher & Schuell, Dassel, Germany).
3. QArray system (Genetix, New Milton, UK).
4. Mouse, rat, and rabbit IgG antibodies (Santa Cruz, Biotechnology, Santa Cruz, CA).

2.3. Antibody Screening on Protein Microarrays

1. Arrayscanner: 428 Arrayscanner System (Affimetrix, Palo Alto, CA) or ScanArray 4000 (PerkinElmer Life Science, Cologne, Germany).
2. Cover slide (Carl Roth, Karlsruhe, Germany).
3. GenePixPro4.0 software.

2.3.1. Monoclonal Antibody Screening on FAST-Slides

1. TBS + Tween-20 (TBST): 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20.
2. Blocking solution: 2% bovine serum albumin (BSA; Sigma, St. Louis, MO) in TBST.
3. Mouse anti-RGS-His₆ antibody (Qiagen).
4. Specific monoclonal antibodies, e.g., rat anti-TCP1 antibody (Affinity Bioreagents, Cholden, USA).
5. Respective Cy3-labeled conjugates as secondary antibodies, e.g., rabbit antimouse IgG for anti-RGS-His₆ or rabbit antirat IgG for anti-TCP1 (Dianova, Hamburg, Germany).

2.3.2. Polyclonal Antibody Screening on FAST-Slides

1. TBS + Tween-20 (TBST): 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20.
2. Fish gelatine (gelatine from cold water fish skin; Sigma).
3. Polyclonal rabbit sera, e.g., anti-DOF11 or anti-MYB6 sera (Pineda, Berlin, Germany).
4. Goat antirabbit IgG-Cy3 conjugate (Dianova, Hamburg, Germany).

3. Methods

3.1. High-Throughput Protein Expression and Purification

For the production of recombinant plant proteins, we use characterized *E. coli* cDNA expression clones (in *E. coli* SCS1/pSE111; 35) constructed in pQE30-derived expression vectors, which allow for expression of recombinant

plant proteins with an N-terminal RGS-His₆-tag. A detailed description of the generation of such expression clones is beyond the scope of this chapter. Therefore the authors provide only a short summary. Characterized plant expression clones in the described format may be obtained from the following ordered cDNA expression libraries: barley expression library (8) constructed in the *E. coli* expression vector pQE30NST (accession number: AF074376) and *Arabidopsis* library (33) in vector pQE30NASTattB (accession number: AY386205). These libraries have been constructed according to the protocol of Konrad Buessow's group using deoxythymidine oligonucleotides for priming to produce recombinant proteins with their complete N-terminus (34–36). As another source of characterized plant protein expression clones, we used full-length *Arabidopsis* clones generated by directional cloning of open reading frames (ORFs) via restriction-dependent cloning using gene-specific primers (14) or via recombination dependent cloning (33) utilizing the GATEWAY™ cloning technology (37,38).

To express and purify plant proteins from these resources in high throughput (96-well format) and small scale we apply the following protocols:

For expression:

1. 96-Deep-well plates with 2-mL cavities were filled with 100 μ L 2YT medium supplemented with 2% glucose, 100 μ g/mL ampicillin, and 15 μ g/mL kanamycin.
2. Cultures of recombinant clones were inoculated from 384-well or from 96-well microtiter plates that had been stored at -80°C . For inoculation, replicators carrying 96 pins were used.
3. After 16 h of growth at 37°C with vigorous shaking (320 rpm), 900 μ L of pre-warmed medium (1X SB medium, 1X PP buffer supplemented with 100 μ g/mL ampicillin, 15 μ g/mL kanamycin, 20 μ g/mL thiamin) were added, and the incubation was continued for 2 h.
4. To induce protein expression, IPTG was added to a final concentration of 1 mM, and incubation was continued for another 4 to 5 h.
5. Cells were harvested by centrifugation at 1500g for 10 min at 4°C , and the pellets were stored at -80°C .
6. Aliquots of the lysates of harvested cells can be used to control the efficiency of the expression on a 15% polyacrylamide gel (Fig. 1).

For protein purification:

1. First, 150 μ L of denaturing lysis buffer were added to the thawed pellets. Pellets were resolved by extensive vortexing and afterward incubated for 30 min at room temperature on a shaker (approx 650 rpm) (see Note 2).
2. The lysated cells were centrifuged at 1900g. The supernatant was transferred to a 96-well filter plate (see Note 3) and immediately drawn into a fresh filter plate by using a vacuum manifold (see Notes 4 and 5).

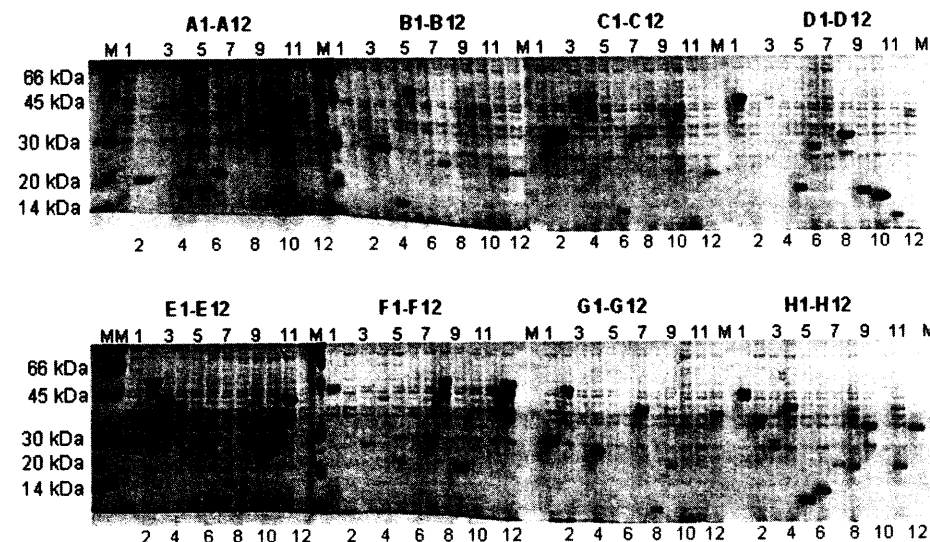


Fig. 1. SDS-Page (15%) of the lysates of 96 *Arabidopsis* expression clones after induction of expression. Expression clones were obtained from an *Arabidopsis* cDNA expression library (33). Lysates were separated using a 15% SDS-PAGE and then Coomassie stained. A1–H12, coordinates of the 96-well plate. M, marker. Approximate sizes of the protein markers are shown at the left in kDa.

3. Afterwards 30 μ L NiNTA-agarose (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 1 h at 300 rpm at room temperature.
4. The agarose beads were washed three times by resuspending in 100 μ L washing buffer, shaking for 5 min, and removing liquid on the vacuum manifold (see Note 5).
5. Finally, proteins were eluted from the agarose beads by incubation with 80 μ L elution buffer without shaking for 20 min and subsequent filtration into a fresh 96-well microtiter plate (see Note 6).
6. Proteins were stored at 4°C .
7. Purified proteins can be separated, e.g., on a 15% polyacrylamide gel (Fig. 2), and protein concentrations can be determined by the Bradford assay (39).

Using the methods described, we obtained purified proteins with an average concentration of approximately 100 μ g/mL (data not shown).

3.2. Generation of Plant Protein Microarrays

We used FAST-slides as the surface (see Note 7) to generate plant protein microarrays for antibody characterization (14) or for phosphorylation studies (8,33) (see Chapter 29 in this book).

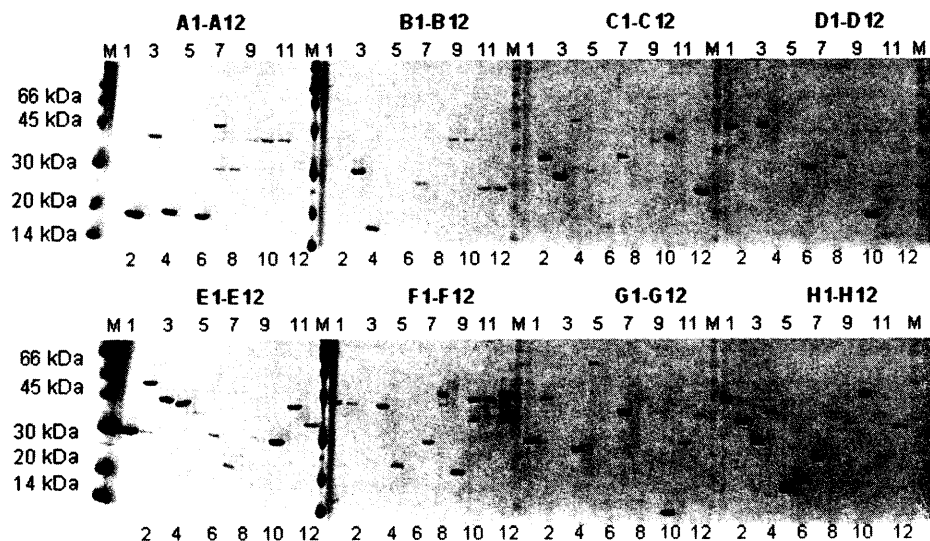


Fig. 2. SDS-Page (15%) of the 96 purified His-tagged *Arabidopsis* proteins. Proteins were expressed and purified from an *Arabidopsis* cDNA expression library (33). Proteins were separated using a 15% SDS-PAGE and then Coomassie stained. A1–H12, coordinates of the 96-well plate. M, Marker. Approximate sizes of the protein markers are shown at the left in kDa.

1. Prior to microarray production, 20 μ L of the purified plant proteins and of the controls (see Note 8) were transferred to 384-well microtiter plates.
2. FAST-slides were placed in a QArray system equipped with humidity control (65 to 70%) and 16-blunt end stainless steel print pins with a tip diameter of 150 μ m and a pin distance of 4.5 mm. We used a spotting depth of 120 μ m.
3. Each sample was loaded once, transferring a volume of 0.6 nL per spot (40).
4. After each transfer the spotting gadget was washed in ddH₂O (6 s), dried with a heat fan (2 s), washed in 80% ethanol (6 s), and dried again (3 s) to prevent cross-contamination.
5. Protein microarrays were stored at 4°C in a closed slide holder.

Proteins can be spotted in different spotting patterns, e.g., in 4 $\times$ 4, 8 $\times$ 8, 10 $\times$ 10 patterns, and so on (see Note 9). Also, the protein can be spotted in duplicates, quadruplicates, and so forth. For antibody-antigen interaction studies, we recommend patterns with horizontal duplicates spotted in two identical fields (see Note 10).

Plant protein microarrays produced by this protocol can be utilized for antibody characterization, giving reproducible results for at least 3 wk when stored at 4°C. Furthermore, such plant protein microarrays may be applied for phos-

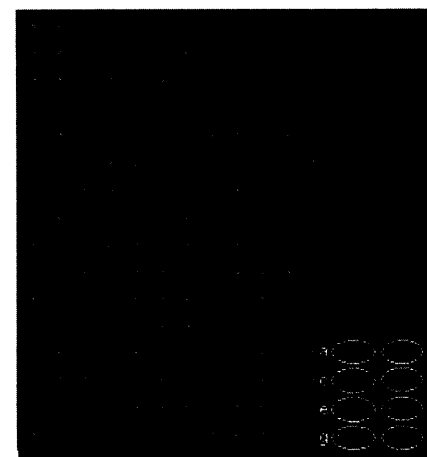


Fig. 3. Detection of 96 His-tagged *Arabidopsis* proteins on FAST-slides using an anti-RGS-His₆ antibody. Ninety six proteins were expressed and purified from an *Arabidopsis* cDNA expression library (33). The proteins and several controls (a–h) were spotted as horizontal duplicates in a 4 $\times$ 4 spotting pattern on FAST-slides. The microarrays were screened with anti-RGS-His₆ antibody. Controls: a, elution buffer; b, PBS; c, rabbit antimouse IgG-Cy3 conjugate, diluted 1:25 in PBS; d, BSA, 20 pmol/ μ L in PBS; e, normal rabbit IgG, diluted 1:10 in PBS; f, normal rat IgG, diluted 1:10 in PBS; g, mouse anti-RGS-His₆ antibody, diluted 1:10 in PBS; h, normal mouse IgG, diluted 1:10 in PBS.

phorylation studies with protein kinases (8,33), as also described in detail in this book (see Chapter 29).

For detection of the immobilized recombinant plant proteins, slides are incubated with an anti-RGS-His₆ antibody according to the following protocol (see Subheading 3.3.1.). As an example, Fig. 3 shows a microarray containing 96 *Arabidopsis* proteins, which we expressed and purified from a recently described ordered cDNA expression library (33). Proteins were spotted in a 4 $\times$ 4 spotting pattern with horizontally positioned duplicates in two identical fields. All the recombinant proteins gave a signal indicating that the efficiencies of protein expression, purification, and transfer were sufficient for detection of the tested recombinant proteins on the microarrays (Fig. 3).

3.3. Antibody Screening on Protein Microarrays

3.3.1. Monoclonal Antibody Screening on FAST-Slides

1. Plant protein microarrays were blocked for 1 h at room temperature with 2% BSA/TBST.

2. Mouse anti-RGS-His₆ (1:2000 dilution) or plant-specific antibodies (e.g., rat anti-TCP1 antibody; 1:1000 dilution) were diluted in blocking solution (*see Note 11*) and then applied onto the arrays for 1 h at room temperature (*see Note 12*).
3. Two 10-min wash steps with TBST were performed.
4. The slides were further incubated for 1 h at room temperature with the respective Cy3-labeled secondary antibody (rabbit antimouse IgG for anti-RGS-His₆ primary antibody; rabbit antirat IgG conjugate for anti-TCP1 antibody), which were applied at a 1:800 dilution in blocking solution (*see Notes 13 and 14*).
5. Then three wash steps of 30 min each were performed in TBST (*Note 15*).
6. Prior to scanning, we dried the microarrays using a microtiter plate centrifuge (e.g., Eppendorf, Hamburg, Germany, cat. no. 5810R) or by manual fanning.
7. Signal detection was performed by means of a 428 Arrayscanner System or a ScanArray 4,000.

All antibody incubation steps were carried out in a 200- μ L volume underneath a cover slide.

To exclude nonspecific binding of the secondary antibody, we performed a parallel incubation of the protein microarrays with blocking solution without primary antibody followed by incubation with the respective secondary antibody, including all washing steps just described.

Figure 4 shows an example of the fluorescence image after incubation of an *Arabidopsis* protein microarray with a monoclonal anti-TCP1 antibody. Ninety six *Arabidopsis* proteins obtained from full-length cDNA expression clones (**14**) were immobilized on FAST-slides using a 4 $\times$ 4 spotting pattern with horizontally positioned duplicates in two identical fields. The image shows that the anti-TCP1 antibody specifically recognizes only the duplicate of TCP1 protein spots and does not cross-react with the other immobilized *Arabidopsis* proteins (**Fig. 4**).

3.3.2. Polyclonal Antibody Screening on FAST-Slides

1. The slides were blocked for 1 h at room temperature in fish gelatine (10% in TBST).
2. Rabbit sera were diluted in blocking solution (e.g., 1:1000 for anti-DOF11 or 1:500 for anti-MYB6 serum [**14**]) and then applied to the arrays for 1 h at room temperature.
3. Two 10-min wash steps with TBST were performed.
4. The slides were further incubated for 1 h at room temperature with goat antirabbit IgG-Cy3 conjugate, which was applied at a 1:800 dilution in blocking solution (*see Note 13*).
5. Then three wash steps of 30 min each were performed in TBST.
6. Prior to scanning, we dried the microarrays using a microtiter plate centrifuge (e.g., Eppendorf, cat. no. 5810R) or by manual fanning.
7. Signal detection was performed by means of a 428 Arrayscanner System or a ScanArray 4000.

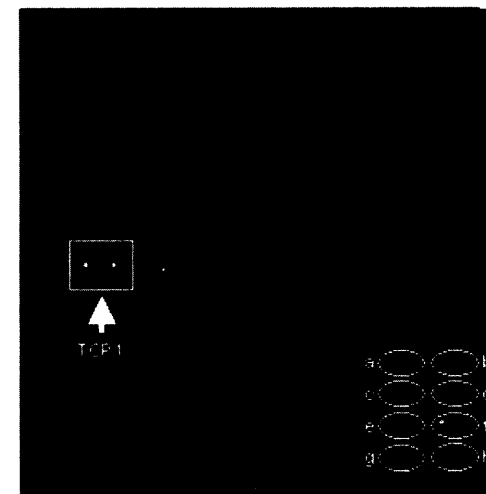


Fig. 4. Screening of an *Arabidopsis* protein microarray containing 96 proteins with an anti-TCP1 antibody. Proteins were expressed and purified from *Arabidopsis* full-length cDNA expression clones (**14**). The proteins and several controls (a–h) were spotted as horizontal duplicates in a 4 $\times$ 4 spotting pattern on FAST-slides. The microarrays were screened with anti-TCP1 antibody. For controls, see **Fig. 3** legend.

All antibody incubation steps were carried out in a 200- μ L volume underneath a cover slide.

3.4. Image Analysis

The median spot intensity data (local background subtracted) were obtained with GenePixPro4.0; for further comparison, the average values of the intensities obtained from both spots of the duplicates were calculated.

4. Notes

1. pH values of the buffers have to be adjusted prior to every protein purification.
2. To yield extracts with higher content of specific recombinant proteins (e.g., for low-copy plasmids) for the subsequent purification, one can generate protein lysates from the pellets of two parallel 1-mL cultures using 100 μ L lysis buffer each, which are then combined to 200 μ L for the subsequent centrifugation (**15**).
3. Take off the supernatant very carefully. Only transfer clear supernatant to the filter plate to ensure that the plate will not occlude during the subsequent filtration.
4. Use a vacuum pump that is suitable for microtiter plates.
5. Ensure that no drops are retained underneath the filter plate after filtration; if so, they have to be transferred manually. To avoid this problem, the filtration should be carried out with enough pump power and as fast as possible. (The vacuum has

to build up quickly and should not be held up longer than necessary, because the filter plates become too leaky otherwise.)

6. Another possibility for performing high-throughput protein purification is to apply a Qiagen BioRobot 8,000 and the Ni-NTA Superflow 96 BioRobotKit (Qiagen) (14) using the same buffers for lysis, washing, and elution as in the case of manual purification (see **Subheading 2.1.**). Disadvantages of the robot method compared with the manual method are higher costs for consumables and the fact that the elution volume may not be adjusted to lower than 350 μ L. Using manual purification, elution volumes from 30 to 80 μ L may be used. Small elution volumes may be beneficial if rarely expressed proteins are purified.
7. FAST-slides are coated with a nitrocellulose-derived polymer. In terms of surface structure, they belong to the 3D microarrays, which have a higher immobilization capacity than 2D slides (2). For antigen-antibody interaction studies using monoclonal antibodies, we utilized self-made PAA slides in addition to FAST-slides (14). PAA slides have been prepared according to published protocols (15,41). Angenendt and co-workers compared different surfaces for antigen-antibody interaction studies (41,42). We reviewed different microarray surfaces for different microarray applications including antigen-antibody interaction studies (2).
8. Solutions without protein (elution buffer, PBS) as well as an unrelated protein without an RGS-His₆-tag (BSA) served as negative controls. As positive controls, we spotted the Cy3-labeled secondary antibodies (e.g., mouse IgG-Cy3 conjugate). To test the binding quality of the secondary antibodies we spotted respective monoclonal primary antibodies as well as IgG of the same species as the primary antibody (e.g., mouse IgG in the screening experiments with mouse anti-RGS-His₆ antibody or rabbit IgG in the screening experiments with polyclonal sera) as additional positive controls.
9. The spotting pattern used for antigen-antibody interaction studies on microarrays depends on the number of proteins and replicates to be analyzed. The maximum spot density at which individual spots can still be differentiated with fluorescence detection is highly dependent on the spotting method and the resolution of the microarray scanner. When we used our spotting protocol, a scanner with a resolution of 10 μ m, and spotting patterns of 14 $\times$ 14 (spot distance of 321 μ m) or 15 $\times$ 15 (spot distance of 300 μ m), we obtained sufficient resolution to evaluate the fluorescence signals (data not shown). We have not tested higher spotting patterns up to now. Lueking and co-workers (15) successfully applied a 13 $\times$ 13 spotting pattern for antibody-antigen interaction studies with fluorescence detection using a similar spotting protocol on PAA slides but loading each sample five times to create one spot. (We loaded each sample once.) Michaud and colleagues (13) used yeast proteome arrays (17) spotted at even higher densities (16 $\times$ 18) to characterize antibodies utilizing fluorescence detection. We reviewed different antigen-antibody interaction studies using protein microarrays besides other applications (2).

10. In our proof-of-principle study to characterize plant antibodies, we used a 4 $\times$ 4 horizontal duplicate spotting pattern with a spot spacing of 1050 μ m (14). Two identical fields were spotted on each slide.
11. The optimal antibody dilution, if not known from literature, may be determined prior to microarray studies by Western blotting experiments.
12. To prevent drying of the microarrays during antibody incubation, we put them on Whatman paper soaked with TBST.
13. Owing to fluorescence labeling of the secondary antibody, this incubation step and all subsequent steps have to be performed in the dark.
14. Fluorescently labeled conjugates are stored at 4°C or, after addition of glycerol (end concentration of 50%), at -20°C.
15. Alternatively to this protocol for screening of monoclonal antibodies on FAST-slides, we applied the following protocol (14): the FAST-slides were blocked with 1X PBS/0.5% (v/v) Tween-20 solution for 2 h at room temperature. A suitable dilution of monoclonal antibodies was then applied onto the microarrays for 2 h at room temperature, followed by two 10-min wash steps with blocking solution. The slides were further incubated with the respective secondary Cy3-labeled conjugate (species specific with regard to primary antibody) for 1 h at room temperature. The slides were washed two times with 1X PBS/0.5% (v/v) Tween-20 for 30 min and then two times with 1X PBS for 20 min at room temperature before the signal detection was performed.
In the case of the monoclonal anti-PhyB antibody Pea-25 (43), only the application of this protocol led to the detection of PhyB on FAST-slides (14). Using the protocol described in **Subheading 3.3.1.** we were not able to detect PhyB.

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References

1. LaBaer, J. and Ramachandran, N. (2005) Protein microarrays as tools for functional proteomics. *Curr. Opin. Chem. Biol.* **9**, 14–19.
2. Feilner, T., Kreutzberger, J., Niemann, B., et al. (2004) Proteomic studies using microarrays. *Curr. Proteomics* **1**, 283–295.
3. Templin, M. F., Stoll, D., Schwenk, J. M., Potz, O., Kramer, S., and Joos, T. O. (2003) Protein microarrays: promising tools for proteomic research. *Proteomics* **3**, 2155–2166.
4. Zhu, H. and Snyder, M. (2003) Protein chip technology. *Curr. Opin. Chem. Biol.* **7**, 55–63.
5. Miller, J. C., Zhou, H., Kwekel, J., et al. (2003) Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* **3**, 56–63.
6. Nielsen, U. B. and Geierstanger, B. H. (2004) Multiplexed sandwich assays in microarray format. *J. Immunol. Methods* **290**, 107–120.

7. Shao, W., Zhou, Z., Laroche, I., et al. (2003) Optimization of Rolling-Circle Amplified Protein Microarrays for Multiplexed Protein Profiling. *J. Biomed. Biotechnol.* **2003**, 299–307.
8. Kramer, A., Feilner, T., Possling, A., et al. (2004) Identification of barley CK2alpha targets by using the protein microarray technology. *Phytochem.* **65**, 1777–1784.
9. Zhu, H., Klemic, J. F., Chang, S., et al. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet.* **26**, 283–289.
10. Boutell, J. M., Hart, D. J., Godber, B. L., Kozlowski, R. Z., and Blackburn, J. M. (2004) Functional protein microarrays for parallel characterisation of p53 mutants. *Proteomics* **4**, 1950–1958.
11. Chan, S. M., Ermann, J., Su, L., Fathman, C. G., and Utz, P. J. (2004) Protein microarrays for multiplex analysis of signal transduction pathways. *Nat. Med.* **10**, 1390–1396.
12. Haab, B. B., Dunham, M. J., and Brown, P. O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* **2**, RESEARCH0004.
13. Michaud, G. A., Salcius, M., Zhou, F., et al. (2003) Analyzing antibody specificity with whole proteome microarrays. *Nat. Biotechnol.* **21**, 1509–1512.
14. Kersten, B., Feilner, T., Kramer, A., et al. (2003) Generation of *Arabidopsis* protein chips for antibody and serum screening. *Plant Mol. Biol.* **52**, 999–1010.
15. Lueking, A., Possling, A., Huber, O., et al. (2003) A nonredundant human protein chip for antibody screening and serum profiling. *Mol. Cell. Proteomics* **2**, 1342–1349.
16. Ramachandran, N., Hainsworth, E., Bhullar, B., et al. (2004) Self-assembling protein microarrays. *Science* **305**, 86–90.
17. Zhu, H., Bilgin, M., Bangham, R., et al. (2001) Global analysis of protein activities using proteome chips. *Science* **293**, 2101–2105.
18. Kersten, B., Possling, A., Blaesing, F., Mirgorodskaya, E., Gobom, J., and Seitz, H. (2004) Protein microarray technology and ultraviolet crosslinking combined with mass spectrometry for the analysis of protein-DNA interactions. *Anal. Biochem.* **331**, 303–313.
19. Snappyan, M., Lecocq, M., Guevel, L., Arnaud, M. C., Ghochikyan, A., and Sakanyan, V. (2003) Dissecting DNA-protein and protein-protein interactions involved in bacterial transcriptional regulation by a sensitive protein array method combining a near-infrared fluorescence detection. *Proteomics* **3**, 647–657.
20. MacBeath, G. and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science* **289**, 1760–1763.
21. Kim, S. H., Tamrazi, A., Carlson, K. E., and Katzenellenbogen, J. A. (2005) A proteomic microarray approach for exploring ligand-initiated nuclear hormone receptor pharmacology, receptor-selectivity, and heterodimer functionality. *Mol. Cell. Proteomics* **4**, 267–277.
22. Kim, T. E., Park, S. W., Cho, N. Y., et al. (2002) Quantitative measurement of serum allergen-specific IgE on protein chip. *Exp. Mol. Med.* **34**, 152–158.
23. Robinson, W. H., DiGennaro, C., Hueber, W., et al. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* **8**, 295–301.
24. Angenendt, P., Glokler, J., Konthur, Z., Lehrach, H., and Cahill, D. J. (2003) 3D protein microarrays: performing multiplex immunoassays on a single chip. *Anal. Chem.* **75**, 4368–4372.
25. Kersten, B., Wanker, E. E., Hoheisel, J. D., and Angenendt, P. (2005) Multiplexing approaches in protein microarray technology. *Expert Rev. Proteomics* **2**, 499–510.
26. Kersten, B., Bürkle, L., Kuhn, E. J., et al. (2002) Large-scale plant proteomics. *Plant Mol. Biol.* **48**, 133–141.
27. Kersten, B., Feilner, T., Angenendt, P., Giavalisco, P., Brenner, W., and Bürkle, L. (2004) Proteomic approaches in plant biology. *Curr. Proteomics* **1**, 131–144.
28. Agrawal, G. K., Yonekura, M., Iwahashi, Y., Iwahashi, H., and Rakwal, R. (2005) System, trends and perspectives of proteomics in dicot plants. Part III: Unraveling the proteomes influenced by the environment, and at the levels of function and genetic relationships. *J. Chromatogr. B Anal. Technol. Biomed. Life. Sci.* **815**, 137–145.
29. Agrawal, G. K., Yonekura, M., Iwahashi, Y., Iwahashi, H., and Rakwal, R. (2005) System, trends and perspectives of proteomics in dicot plants Part II: Proteomes of the complex developmental stages. *J. Chromatogr. B Anal. Technol. Biomed. Life. Sci.* **815**, 125–136.
30. Agrawal, G. K., Yonekura, M., Iwahashi, Y., Iwahashi, H., and Rakwal, R. (2005) System, trends and perspectives of proteomics in dicot plants Part I: Technologies in proteome establishment. *J. Chromatogr. B Anal. Technol. Biomed. Life. Sci.* **815**, 109–123.
31. Thiellement, H., Zivy, M., and Plomion, C. (2002) Combining proteomic and genetic studies in plants. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **782**, 137–149.
32. Canovas, F. M., Dumas-Gaudot, E., Recorbet, G., Jorin, J., Mock, H. P., and Rossignol, M. (2004) Plant proteome analysis. *Proteomics* **4**, 285–298.
33. Feilner, T., Hultschig, C., Lee, J., et al. (2005) High-throughput identification of potential *Arabidopsis* MAP kinases substrates. *Mol. Cell. Proteomics* **4**, 1558–1568.
34. Weiner, H., Faupel, T., and Bussow, K. (2004) Protein arrays from cDNA expression libraries, in *Methods in Molecular Biology* (Fung, E., ed.), Humana, Totowa, NJ, pp. 11–13.
35. Bussow, K., Cahill, D., Nietfeld, W., et al. (1998) A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. *Nucleic Acids Res.* **26**, 5007–5008.
36. Clark, M. D., Panopoulou, G. D., Cahill, D. J., Bussow, K., and Lehrach, H. (1999) Construction and analysis of arrayed cDNA libraries [Review]. *Methods Enzymol.* **303**, 205–233.
37. Heyman, J. A., Cornthwaite, J., Foncerrada, L., et al. (1999) Genome-scale cloning and expression of individual open reading frames using topoisomerase I-mediated ligation. *Genome Res.* **9**, 383–392.

38. Walhout, A. J., Temple, G. F., Brasch, M. A., et al. (2000) GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.* **328**, 575–592.
39. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
40. Soldatov, A. V., Nabirochkina, E. N., Georgieva, S. G., and Eickhoff, H. (2001) Adjustment of transfer tools for the production of micro- and macroarrays. *Biotechniques* **31**, 848–854.
41. Angenendt, P., Glokler, J., Murphy, D., Lehrach, H., and Cahill, D. J. (2002) Toward optimized antibody microarrays: a comparison of current microarray support materials. *Anal. Biochem.* **309**, 253–260.
42. Angenendt, P., Glokler, J., Sobek, J., Lehrach, H., and Cahill, D. J. (2003) Next generation of protein microarray support materials: evaluation for protein and antibody microarray applications. *J. Chromatogr. A* **1009**, 97–104.
43. Cordonnier, M. M., Greppin, H., and Pratt, L. H. (1986) Identification of a highly conserved domain on phytochrome from angiosperms to algae. *Plant Physiol.* **80**, 982–987.