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Short communication

Plant antigens cross-react with rat polyclonal antibodies against KLH-conjugated peptides

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Abstract

Keyhole limpet hemocyanin (KLH)-conjugated peptides are routinely used to raise polyclonal antibodies for biochemical or immunolocalization studies. Rats are suitable for producing antisera against plant antigens as they often lack non-specific response towards plant materials. We attempted to obtain rat antisera against peptides derived from several plant proteins. However, most antisera recognized the same background KLH-related plant antigen (KRAP) in *Arabidopsis* and tobacco. We characterized KRAP with respect to size and cellular localization and examined possible antigen-specific reasons for the failure of most immunizations. We also found no reports of successful use of rat anti-KLH-peptide antibodies in plant studies. We thus believe that the rat-KLH:peptide system is poorly suited for production of antibodies, especially against plant antigens, and should be used with caution, if at all.

Keywords: Keyhole limpet hemocyanin; Rat antisera; Synthetic peptides; Cross-reactivity; Plant antigens

1. Introduction

Polyclonal antibodies against synthetic oligopeptides are routinely used in biochemistry and molecular biology. Since molecules <12 kD do not efficiently elicit vertebrate immune responses, peptide antigens have to be conjugated prior to immunization to a suitable carrier, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). KLH is a large copper-containing glycoprotein from the marine mollusk, *Megatura crenulata*, that carries a number of lysine residues suitable for chemical coupling of peptides, and triggers a strong immune response producing antibodies with very low or no non-specific cross-reactivity (Dixon et al., 1966; reviewed in Harlow and Lane, 1988). Immunogenic peptide design is guided by several criteria, such as accessibility on the surface of the molecule (especially if the antisera are to be used for in situ immunolocalization), and good solubility, which often represents the limiting factor.

Polyclonal antisera from herbivorous animals, such as rabbits and chicken, often display non-specific background reaction towards plant antigens, while rat antisera usually exhibit minimal such cross-reactivity. This makes rats the animals of choice for production of antibodies in plant biology. Moreover, the amount of antigen needed for immunization is relatively small, the yield of sera is sufficient for most applications, and commercially available anti-rat secondary antibodies do not cross-react with anti-mouse primary antibodies, making double labeling experiments possible.

We have obtained rat antisera against a series of oligopeptides derived from the sequences of several plant proteins. Unexpectedly, we found that most of our antisera recognize the same conserved plant antigen unrelated to the peptides used for immunization, while no peptide-specific antibodies

Abbreviations: BSA, bovine serum albumin; FH2, formin homology 2; KLH, keyhole limpet hemocyanin; KRAP, KLH-related antigen of plants; PLD, phospholipase D; SEC, size exclusion chromatography.

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were detected. We present the results of the preliminary characterization of this background KLH-related antigen, and examine the possible peptide sequence-related causes of the systematic failure of specific antibody production.

2. Materials and methods

2.1. Peptide design and antibody production

Antigens and peptide sequences are summarized in Table 1.

For formin-derived antigens, sequence conservation and predicted 3D-structure were used as primary criteria. 3D models of the AtFH3 and AtFH16 FH2 domains were generated by threading the antigen sequence onto a known FH2 domain structure (PDB: 1UX5) as described elsewhere (Cvrčková et al., 2004; Grunt et al., 2008); for the PTEN domain, published models were used (Cvrčková et al., 2004). Hydrophilicity determined according to Hopp and Woods (1981) was considered as a secondary criterion. For the remaining antigens and for comparison purposes, predictions of hydropathy (Kyte and Doolittle, 1982) and antigenicity (Welling et al., 1985) were performed at http://www.expasy.org/tools/pscale, antigenicity predictions using Kolaskar and Tongaonkar's (1990) method at http://bio.dfci.harvard.edu/Tools/antigenic.pl. Secondary structure and exposed residues predictions, performed at the Predict Protein server (Rost et al., 2003) were taken into account as secondary criteria.

Peptides were synthesized and polyclonal rat antibodies produced commercially by Moravian Biotechnology (Brno, Czech Republic). Peptides were prepared on a PE Biosystems Pioneer synthesiser using the Fmoc/tBu method (Chan and White, 2004). Acylation reactions were carried out using amino acids activated with HBTU in the presence of HOBt and DIPEA. Amino acids, resins and solvents were purchased from Novabiochem, Applied Biosystems and Biosolve, respectively. Cleavage of peptide from the resin and side chain deprotection were carried out by treatment of peptidyl resin with TFA/TIPS/water (95:2, 5:2,5). Synthetic peptides were coupled to KLH (for immunization) and BSA (for screening of animal response) using a single step coupling protocol (Harlow and Lane, 1988). The peptide CLN was conjugated to BSA via an added cysteine to improve solubility, as well as to KLH, for immunization. Peptide conjugation to KLH/BSA via cystein was carried out using MBS (*m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester) activated KLH/BSA (Pierce). For each peptide, 3 rats were immunized using standard procedures (Harlow and Lane, 1988). Animal response was screened using a dot blot assay as follows; peptides coupled to either BSA or KLH, as well as carrier-only controls (all at 20 μg/ml in PBS), were bound to nitrocellulose membrane strips for 2 h at 37 °C; the following steps were performed at room temperature. After blocking in 10% fetal calf serum (FCS) in D-MEM for 2 h, 2 μl aliquots of serially diluted sera were spotted on antigen-coated membranes, incubated for 2 h, washed 3× in PBS, incubated with horseradish peroxidase-conjugated goat anti-rat antibodies (Sigma A9037) diluted 1:100 in D-MEM plus 10% FCS, washed 3 × 5 min in PBS and developed using 1, 4-chloronaphtol.

To remove antibodies against the KLH carrier, selected antisera were commercially affinity-purified on a KLH-containing column (Hena s.r.o., Czech Republic).

2.2. Plant materials

Pollen of *Nicotiana tabacum* cv. Samsun was harvested, stored and germinated in a medium containing 10% sucrose and 0.1% H_3BO_3 for 1–2 h as described previously (Potocký et al., 2003). *Arabidopsis thaliana* suspension cultures, *N. tabacum* BY2 cells and *A. thaliana* Col-0 plants were maintained and harvested as described (Hála et al., 2005; Synek et al., 2006).

Table 1
Peptides used in this study, corresponding target antigens (with Uniprot or Genbank accessions in brackets), immunization outcomes (see Section 3.2) and relevant peptide parameters (see Section 3.4). N.A. – not available (tobacco has at least 2 isoforms; *Arabidopsis* homologue is 90 kDa); KT – method of Kolaskar and Tongaonkar (1990); * – possible specific reaction detected by dot blot, but only KRAP seen on Western blots (further characterization is in progress). For each peptide/recognized antigen combination, the number indicates the number of positive antisera (out of 3 rats). No major differences in probability of surface exposure or secondary structure were predicted

Peptide name	Peptide sequence	Target			Recognized antigens			Peptide parameters		
		Antigen	Predicted size (kDa)	Domain	KRAP75	KRAP90	Specific	Antigenicity		Hydropathy
								Welling	KT	
NVT	NVTTEEVVDAIKEGNELPVELL	AtFH3 (O23373)	65–70+	FH2 (specific)	3	1	1?*	−0.2	1.06	0.04
GRS	GRSSLTWPAERFLKIL				3	1	0	−0.18	1.07	0.04
DEL	DELQIQYGESQTAE	AtFH16 (Q9FF15)	79	FH2 (specific)	1	1	0	−0.49	1.01	−0.61
TED	TEDVFGGPDHNIDD				2	0	0	−0.22	0.97	−0.85
CLN	CLNRDEVDTLWHIKE	AtFH13 (Q9LVN1)	140	PTEN (conserved)	2	1	0	−0.13	1.02	−0.6
GEG	GEGGCRPIFRIYGQD	AtFH18 (Q9SK28)	123	PTEN (conserved)	1	0	0	−1	1.01	−0.42
LFL	LFLEFGNGDDSNSQLASVT	AtExo70A1 (NP_195974)	72	Internal	3	1	0	−0.38	1.00	−0.4
LER	LERLLGELFEGKSMNEPR	AtExo70A1 (NP_195974)		C-terminal	3	3	0	−0.36	0.98	−0.57
GDL	GDLELHIVHARHLPN	NtPLDδ	N.A.	N-terminal	1 (weak)	0	2	0.49	1.08	−0.06
QEL	QELKSSQLKDVHPSD	NtPLDδ	N.A.	Internal	0	0	1 (weak)	0.39	1.06	−0.83

2.3. Protein extracts and Western blotting

Fresh plant material was homogenized in ice-cold extraction buffer (70 mM Tris–Cl, pH 7.5, 250 mM sucrose, 3 mM EDTA, 5 mM DTT) + 1 × PIC (Sigma P9599) with sand in a mortar and centrifuged at 7500 × *g* for 10 min at 4 °C. Equal amounts of total proteins (25–30 μg per lane for the gels shown) were separated on SDS-PAGE (10–12.5% (w/v)) and transferred onto a nitrocellulose membrane (BioRad). Blot quality was verified by staining the membrane with Ponceau Red and by visual control of successful transfer of the pre-stained markers up to at least 200 kDa. For immunological detection, primary antibodies were diluted 1:1000 in TBS supplemented with 0.5% (v/v) Tween-20 and 5% (w/v) non-fat dry milk, secondary antibodies (Sigma A9037) were used at a dilution of 1:10 000. ECL detection was performed as recommended by manufacturer (Amersham).

2.4. Subcellular fractionation and size exclusion chromatography (SEC)

4.5 g of 7-day-old *Arabidopsis* suspension culture were ground in liquid nitrogen; all subsequent steps were performed at 4 °C. For fractionation, the homogenate was mixed with 2–3 ml of buffer TS (10 mM Tris–Cl, pH 7.4, 250 mM sucrose) and centrifuged subsequently at 800 × *g* for 5 min, 30,000 × *g* for 7.5 min, and 100,000 × *g* for 1 h yielding CW (cell walls and nuclei), M (plasma membrane), and P (microsomes) membrane fractions as well as the S (supernatant) fraction. For SEC, the homogenate was mixed with 5 ml of buffer (20 mM HEPES, pH 6.8; 150 mM NaCl; 1 mM EDTA; 1 mM DTT; 0.5% Tween-20) + 1 × PIC (Sigma P9599) and centrifuged at 30,000 g, 30 min. 80 mg of total protein were applied on a Superdex300 HiLoad 26/60 column (Pharmacia), flow rate 60 ml/min. 5 ml fractions were collected and subjected to Western analysis, starting from the fraction corresponding to the void volume of the column (120 ml); molecular weights of the eluted protein complexes were estimated based on spectroscopically documented elution of a set of standards (proteins and blue dextran) in the range between 67 and 2000 kDa.

2.5. In situ immunolabeling of pollen tubes and microscopy

Pollen tubes were fixed by mixing the culture with equal volume of 3.7% paraformaldehyde in 2× concentrated PEM buffer (100 mM PIPES, 10 mM EGTA, 10 mM $MgSO_4$, pH 6.9) followed by 5 min vacuum infiltration and 1 h of gentle shaking. Cells were washed 2× with PEM and 1× with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3), permeabilized by 0.3% cellulase, 0.3% pectinase and 0.05% driselase (Sigma) in PBS for 20 min on a rotator, followed by 3× washing in PEM and 1 wash in 0.1% Triton X-100 in PEM. After 3 washes in PBS, samples were incubated overnight at 4 °C with the primary antibody (1:200 in PBS with 1% BSA), washed 3× in PBS and incubated with the FITC-conjugated secondary antibody (Sigma F6258, 1:350 in PBS with 1% BSA) at room temperature for 1 h. Cells were mounted on slides in 50% glycerol with 0.1% para-phenylendiamine to avoid bleaching.

Images were obtained using the LSM 510 confocal laser microscope (Zeiss) with excitation at 477 nm using a band pass filter between 505 and 530 nm. Stacks of images were taken at an interval of 0.6 μm, with 4× line averaging, and maximum intensity projections were generated.

3. Results and discussion

3.1. Antigens and peptide design

We attempted to produce antibodies against 10 peptides derived from the sequences of 6 plant proteins. Our pilot antigen, the *Arabidopsis* class I formin AtFH3, is a putative actin nucleating protein bearing the conserved FH2 domain and a putative N-terminal transmembrane domain (Cvrčková et al., 2004), which is preferentially expressed in mature and germinating pollen according to microarray data in the Genevestigator database (Zimmermann et al., 2004). 2 of the remaining antigens also represent *Arabidopsis* formins, namely the cytoplasmic class II formin AtFH16 and a conserved portion of a PTEN-related domain shared by several class II formins (Cvrčková et al., 2004). The last 2 antigens were the *Arabidopsis* Exo70A1 subunit of the exocyst complex (Synek et al., 2006) and the *N. tabacum* phospholipase PLDδ, a newly identified member of the phospholipase D δ subfamily (MP, VŽ, and I Brabcová, unpublished).

In the case of formins, we used 3D models of the relevant domains in order to design peptides from accessible areas on the protein surface, as we intended to generate antibodies suitable for immunolocalization. To avoid cross-reactivity of the FH2 antibodies with other members of the large formin family, we chose peptides unique to each protein, even if their solubility and predicted antigenicity were suboptimal. Nevertheless, others successfully used a rabbit polyclonal antibody against a peptide of similarly low antigenicity for detection of a related protein, AtFH1 (Banno and Chua, 2000). On the contrary, conserved sequences were selected for PTEN peptide candidates with the aim of producing a wide-spectrum antibody against any PTEN-containing formin.

For Exo70A1 and PLDδ, peptides were selected from complete protein sequences, taking into account hydropathy, antigenicity, secondary structure and algorithmic prediction of surface localization. The sequences and other properties of peptides used for immunization are summarized in Table 1.

3.2. Antibodies against KLH-coupled antigens recognize a conserved protein in Arabidopsis and tobacco

Antibodies against 2 peptides derived from AtFH3 (GRS and NVT), but not the corresponding preimmune sera, detected a single band of approximately 75 kDa on Western blots of *Arabidopsis* and tobacco suspension cultures and tobacco pollen tubes (Fig. 1a,b). All 3 rats immunized by the GRS peptide exhibited a strong response to this protein, while sera against the

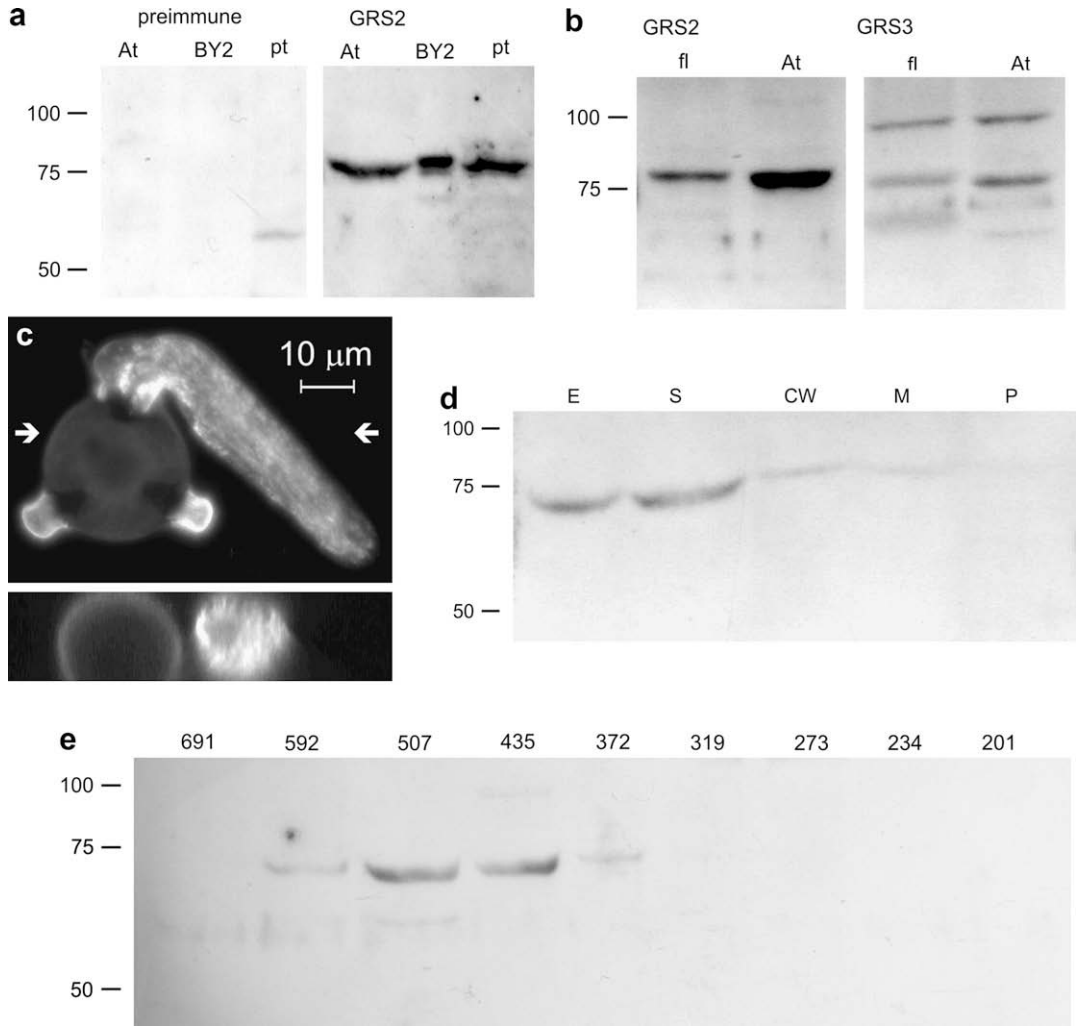

Fig. 1. (a) The GRS2 antibody but not its preimmune serum detects a 75 kDa signal (KRAP75) on Western blots of extracts from *Arabidopsis* suspension cells (At), tobacco BY2 cells (BY2) and tobacco pollen tubes (pt). (b) Western blots of extracts from *Arabidopsis* flowers (fl) and suspension (At) probed with GRS2 and GRS3 antibodies (showing KRAP90 signal in GRS3). (c) GRS2 decorates intracellular structures in tobacco pollen tubes; preimmune serum detected no signal (not shown). Top: maximum intensity projection of a stack of images; bottom – a reconstructed cross-section at the plane indicated by arrows. (d) Distribution of KRAP75 in subcellular fractions of *Arabidopsis* suspension cells: E – whole extract, S – cytosol, CW – cell walls, M – 30,000 g pellet, P – 100,000 g pellet. (e) KRAP75 participates in a single protein complex of approx. 450 kDa in SEC fractions from *Arabidopsis* suspension. Calculated mid-fraction molecular weights (in kDa) are shown above the lanes. Western blots (d) and (e) were probed by GRS2; positions and sizes (in kDa) of marker proteins (BioRad 161-0373) are indicated next to all blots (also in Fig. 2).

NVT peptide showed somewhat weaker reaction (Table 1 and not shown). In addition, 2 antibodies (GRS3 and NVT9) recognized also a 90 kDa protein in *Arabidopsis* extracts (Fig. 1b). The apparent size of the shared antigen corresponds to that expected for AtFH3 (over 65–70 kDa depending on gene structure prediction, which may lack N-terminal exons). We therefore started a more thorough characterization of the 75 kDa antigen using the GRS2 antiserum, while waiting for the outcome of immunization with the remaining peptides.

As shown below, most antisera against the remaining antigens recognized the same two predominant bands on Western blots, regardless of different size of the targets (Table 1). In particular, the 75 kDa antigen always exhibited a strong signal, apparently resulting from cross-reactivity of antibodies against the carrier (KLH) with an unknown protein present in plant tissues and pollen. We will continue to refer to the antigens recognized by our antisera as KRAP (*K*LH-*R*elated *A*ntigen from *P*lants) 75 or 90, respectively (according to size).

Höglund et al. (2002) described a similar observation, as they found an identical pattern of immunocytochemical staining in *Zinnia* with several rabbit antisera against mutually unrelated peptides coupled to KLH. However, they provide neither sequences of the peptides, nor data on the number and size of cross-reacting antigens that would allow direct comparison with our KRAP75 or KRAP90. Based on limited sequence similarity of the tomato tyrosinase (Uniprot: Q08307) and related proteins with KLH, they suggest that the endogenous antigens may be members of plant tyrosinase or catechol oxidase families. However, *Arabidopsis* has no significant relatives of the proposed candidate proteins, whose size (66 kDa), moreover, does not fit our observations. Thus, other epitopes on the KLH molecule are apparently responsible for the cross-reactivity, including possibly

also carbohydrate determinants. Some of them are already known to cross-react with glycoproteins of *Schistosoma mansoni*, a feature utilized successfully in diagnostics (Grzych et al., 1987).

3.3. Intracellular localization of the KLH-related antigen KRAP75

The GRS2 antiserum decorated intracellular fibrous or membraneous structures in chemically fixed tobacco pollen tubes (Fig. 1c). Unexpectedly there was no signal at the plasmalemma, as is usual in a plant Class I formin and has already been observed for several members of the family (e.g. Banno and Chua, 2000; Cheung and Wu, 2004). Instead, our antibodies recognized discrete structures within the cytoplasm, morphologically resembling elements of the endomembrane system. This also alerted us to the possibility that the GRS2 antibody recognizes something other than AtFH3.

In cell fractionation experiments, the majority of KRAP75 was detected in the cytosolic fraction of *Arabidopsis* suspension cells, although we cannot exclude the presence of a minority membrane fraction (Fig. 1d). Fractionation of the soluble proteins by size exclusion chromatography, where native protein complexes are sorted by size, revealed only one peak detected by GRS2, corresponding to a 450 kDa protein complex in *Arabidopsis* suspension (Fig. 1e). This indicates that only antibodies against KRAP75, but not against AtFH3, are present in the immune sera, as the presence of both non-specific background and a specific signal in the same fraction is extremely unlikely.

3.4. Only highly antigenic peptides induce specific rat antibodies in the presence of KLH

Generally, we found a variety of possible immunization outcomes among the peptides and animals (Fig. 2a). For 2 peptides derived from tobacco PLDδ, and possibly also for one AtFH3-derived peptide (NVT9), we obtained specific antibodies. In particular, the GDL5 antiserum recognized, in addition to a very weak KRAP75 signal, 2 bands which could correspond to PLDδ variants since they persist after removal of KLH-interacting antibodies by purification over a KLH affinity column (Fig. 2b). This finding is evidence that KRAP75 does correspond to an epitope cross-reacting with KLH, as its signal disappears upon purification. Absence of the presumed PLDδ signal in *Arabidopsis* extracts (Fig. 2c) further supports its specificity, since no *Arabidopsis* protein contains a sequence corresponding to the GDL peptide. Further characterization of the potential anti-PLDδ antisera is in progress.

Several parameters that may affect the outcome of immunization have been determined either in the process of peptide design or subsequently (see Section 2.1 and Table 1). The successful peptides differed from the failed ones only in the antigenicity parameter computed according to Welling et al. (1985), while a newer approach of Kolaskar and Tongaonkar (1990) suggested comparably good antigenicity for both peptide groups. Remarkably, the 2 methods apparently differed by the initial selection of known antigen/antibody pairs used to derive characteristics of good epitopes. Although

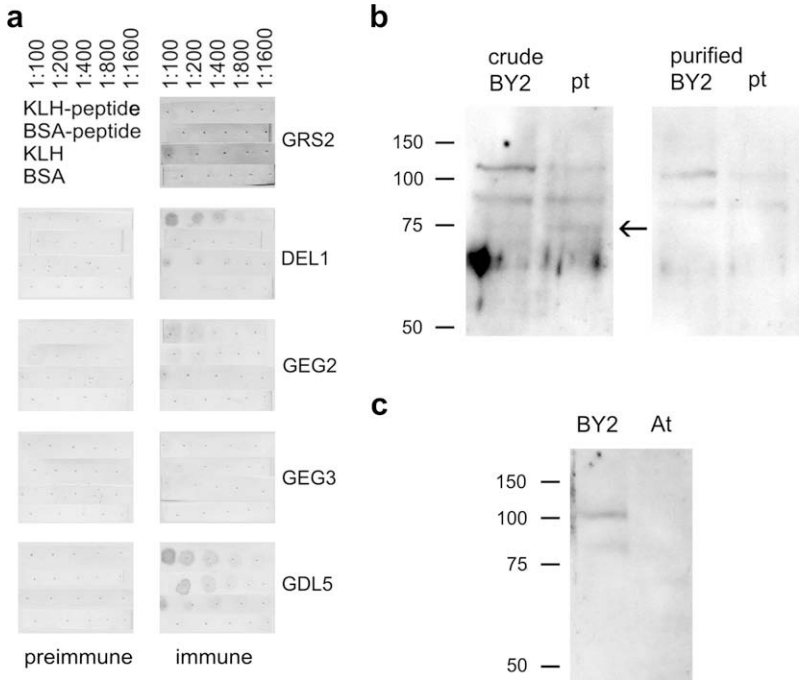

Fig. 2. (a) Representative immunization outcomes as detected by peptide dot blots: KLH cross-reactivity only (DEL1, and, to a weaker extent, GRS2), false positive reaction due to preimmune serum cross-reactivity to BSA-peptide (GEG2), no reaction (GEG3), and a putative specific reaction (GDL5). (b) Antibodies against the GDL peptide recognize putative tobacco PLDδ.Western blots of tobacco BY2 suspension (BY2) and tobacco pollen tubes (pt) probed with crude GDL5 antiserum (left) and GDL5 purified on a column with immobilized KLH (right). Arrow shows a weak KRAP75 signal that is removed by purification. (c) The antigen recognized by GDL5 is tobacco-specific: comparison of Western blots of tobacco (BY2) and *Arabidopsis* (At) suspension extracts.

we could not recover all the information on animals used for immunization in either case, a species bias cannot be excluded (human, goat, rabbit, mouse and chicken data were involved in the 1990 study). Although epitope antigenicity is long believed to be species-independent, no rat immunization studies have been included in the classical papers on this topic as far as we could establish (see Atassi, 1984 and references therein), and the results do indeed suggest that rats behave differently from more conventional immunization hosts.

A systematic failure to produce specific antibodies against a peptide antigen is costly both in terms of money and time. We thus believe that even negative results such as these should be reported if they could provide insight into possible causes of the failure, or hints for those considering similar immunizations in the future. Taken together, we do not recommend immunizing rats with KLH-coupled peptides derived from plant proteins; perhaps heterologously expressed proteins or fragments thereof may provide more reliable antigens. However, if a peptide-based strategy is to be used at all, care should be taken to select highly immunogenic peptides even at the expense of other factors, such as position on the molecule.

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