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Molecular characterization of profilin isoforms from tobacco (*Nicotiana tabacum*) pollen

23459

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Abstract Profilins are actin-binding proteins in eukaryotes which participate in the phosphoinositide pathway via binding to PIP₂. Using polyclonal rabbit sera raised against plant profilins, the occurrence of several profilin isoforms is demonstrated in two-dimensionally analyzed tobacco pollen extracts. The cDNAs coding for two novel tobacco profilin isoforms (ntPro2, ntPro3) were isolated from a pollen cDNA library by antibody screening. When the cDNA and deduced amino acid sequences of the two isoforms were compared with a previously isolated tobacco pollen profilin cDNA (ntPro1), significant differences were noted in the non-coding regions, whereas the coding sequences, in particular the functional domains, showed little variation. The cDNAs coding for the three tobacco profilin isoforms were expressed in *Escherichia coli* and shown to bind comparably to different anti-profilin antisera. The high degree of similarity among the different tobacco pollen profilin isoforms points to functional equivalence. Assuming that the presence of profilin is indispensable to the control of the large amounts of actin present in pollen, the occurrence of different profilin isoforms in pollen is interpreted to represent a protective mechanism against loss of profilin functions.

Key words Profilin · Tobacco pollen · *Nicotiana tabacum* · cDNA cloning · Isoforms

Introduction

Plant profilins were originally discovered as cross-reactive allergens (Valenta et al. 1991; Valenta et al. 1992; Vallier et al. 1992; van Ree et al. 1992) and were subsequently described as actin-binding proteins in dicots and monocots (Valenta et al. 1993; Staiger et al. 1993). The cDNAs coding for profilins from birch (Valenta et al. 1991), maize (Staiger et al. 1993), timothy grass (Valenta et al. 1994), tobacco (Mittermann et al. 1995) and wheat (Rihs et al. 1994) have been isolated and shown to be very similar. The cDNA and deduced amino acid sequences were highly conserved among the different plant profilins, whereas rather low sequence similarities were found to other eukaryotic profilins (Valenta et al. 1993). Plant profilins bound to plant as well as to mammalian actins, which could be demonstrated with *in vitro* (Valenta et al. 1993; Giehl et al. 1994; Ruhlandt et al. 1994) as well as *in vivo* experiments (Staiger et al. 1994; Rothkegel et al. 1996). The inhibition of phospholipase activity in isolated pea plasma membranes by recombinant birch profilin indicated that plant profilins, as well as other eukaryotic profilins, interact with polyphosphoinositides and hence may be involved in the signal transduction cascade in plants (Drobak et al. 1994).

Evidence that profilins have a prominent role during pollen maturation, pollen tube growth and sexual plant reproduction came from the finding that profilin expression is high in mature and germinated pollen (Mittermann et al. 1995). It is hence likely that profilin controls the actin assembly during pollen maturation and germination. Correct formation of actin bundles is required for pollen tube growth and it is tempting to speculate that profilin may control this process by capping and/or releasing actin filaments. The description of profilin isoforms in the monocot *Zea mays* (Staiger et al. 1993) prompted us to investigate whether or not functional profilin isoforms can also be found in tobacco pollen. For this purpose, tobacco pollen protein extracts were analyzed by two-dimensional electrophoresis/immunoblotting, and a tobacco pollen cDNA library was

The nucleotide sequences of ntPro2 and ntPro3 have been submitted to GenBank under the accession numbers X93465 and X93466, respectively

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screened with a serum pool from profilin-allergic individuals which contained polyclonal IgE antibodies with broad cross-reactivity to profilins. The cDNAs coding for three tobacco profilin isoforms showed high sequence conservation, and there was no difference in the recognition of the recombinant proteins by different profilin-specific antisera. Because of the observed similarities, it is suggested that different profilin isoforms may be functionally equivalent and that the occurrence of isoforms may protect pollen against a loss of profilin activity.

Materials and methods

Plant materials and protein extracts

Pollen was obtained from tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR 1) grown under controlled conditions. Freshly harvested pollen was ground under liquid nitrogen and extracted as described by Jarolim et al. (1989). Extracts were dialyzed, aliquoted and lyophilized. Protein concentration was determined according to Bradford (1976); and aliquots of the extracts were analyzed by SDS-PAGE (Fling and Gregerson 1986) after Coomassie blue staining.

Antisera

For immunoscreening of the tobacco pollen cDNA library, a serum pool was prepared by mixing equal volumes of sera from three profilin-allergic patients who had high levels of serum IgE against profilins from monocots and dicots (Valenta et al. 1992). The rabbit antiserum RP1, which was raised against purified celery root profilin, is described by Vallier et al. (1992) and Valenta et al. (1992, 1993). Rabbit antiserum RP5 was raised against purified recombinant tobacco pollen profilin ntPro1 (Mittermann et al. 1995).

SDS-PAGE, two-dimensional electrophoresis, and immunoblotting

Protein extracts were separated in a 12.5% SDS-PAGE (Fling and Gregerson 1986). For two-dimensional (2-D) separation of pollen proteins, extracts were separated in the first dimension according to pH by using Immobiline drystrips pH 3–10 and a Multiphor II apparatus (Pharmacia, Uppsala, Sweden), following the manufacturer's instructions. Strips containing the pH-separated proteins were then placed on a 12.5% SDS-PAGE for separation according to molecular weight (Fling and Gregerson 1986). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (Towbin et al. 1979). The immunodetection of proteins was performed as described by Mittermann et al. (1995).

Antibody screening of the tobacco pollen cDNA library; characterization of cDNAs coding for tobacco pollen profilin isoforms

Approximately 200 000 pfu of a cDNA library prepared from mature tobacco pollen were screened with serum IgE from profilin-allergic individuals (Mittermann et al. 1995). Six immunopositive λ ZAP phage clones were enriched and the corresponding plasmids pBSk+ were obtained by in vivo excision (Short et al. 1988). Plasmid DNA was prepared from the six clones using Qiagen tips (Qiagen, Hilden, Germany) to determine the DNA sequence of

their 5' and 3' ends according to Sanger et al. (1977) using ³⁵S dCTP (NEN, Stevenage, UK) and a T7 sequencing kit (Pharmacia). The 5' and 3' sequences were compared with a previously isolated tobacco profilin clone (ntPro1) (Mittermann et al. 1995) to select clones containing different isoforms (ntPro2, ntPro3) for detailed analysis.

Expression of profilin isoforms in *E. coli*

Escherichia coli XL-1 Blue, transformed with pBSk+ containing the cDNAs of ntPro1–ntPro3, was grown in liquid culture (LB containing 100 µg/ml ampicillin) to an OD 600 of 0.5 and induced by addition of IPTG to a final concentration of 3 mM for 4 h. *E. coli* cells were harvested by centrifugation and stored at –20°C until use.

Results

Different profilin isoforms are detected in 2-D analyzed tobacco pollen protein extracts

The occurrence of profilin isoforms has been described for *Acanthamoeba* (Pollard and Rimm 1991), *Dictyostelium* (Haugwitz et al. 1991) and humans (Honore et al. 1993). Those cDNAs coding for *Zea mays* profilin isoforms have been isolated (Staiger et al. 1993). To demonstrate the presence of tobacco pollen profilin isoforms at the protein level, pollen extracts were separated according to pH and molecular weight by 2-D electrophoresis. The 2-D gels were then blotted onto nitrocellulose, and profilin isoforms were detected with two different polyclonal rabbit antisera. The rabbit anti-celery root profilin antiserum RP1 (Fig. 1a) and the rabbit anti-recombinant tobacco profilin (ntPro1) antiserum RP5 (Fig. 1b) bound strongly to at least four distinct spots. Although the reactivity of RP5 was weaker, longer exposure revealed a binding pattern almost identical to that observed for RP1. It is noteworthy that although RP1 was raised against a somatic plant profilin (celery root profilin) and RP5 against one recombinant tobacco pollen profilin isoform (ntPro1), a similar reaction intensity to the individual spots was observed.

The cDNA and deduced amino acid sequences of tobacco profilin isoforms are highly homologous

When the cDNA sequences of the three tobacco profilin isoforms (ntPro1–ntPro3) were compared (Fig. 2), significant differences were noted in the 5' and 3' non-coding regions. However, few differences were observed in the coding regions. Most of the differences were located in the third position of each codon and hence represented silent mutations. The coding regions of ntPro1 and ntPro2 were exactly the same length, whereas ntPro3 lacked one codon. An average molecular weight of 14.4 kDa was calculated for each of ntPro1, ntPro2 and ntPro3.

Comparison of the deduced amino acid sequences of the three tobacco profilin isoforms showed the carboxy-

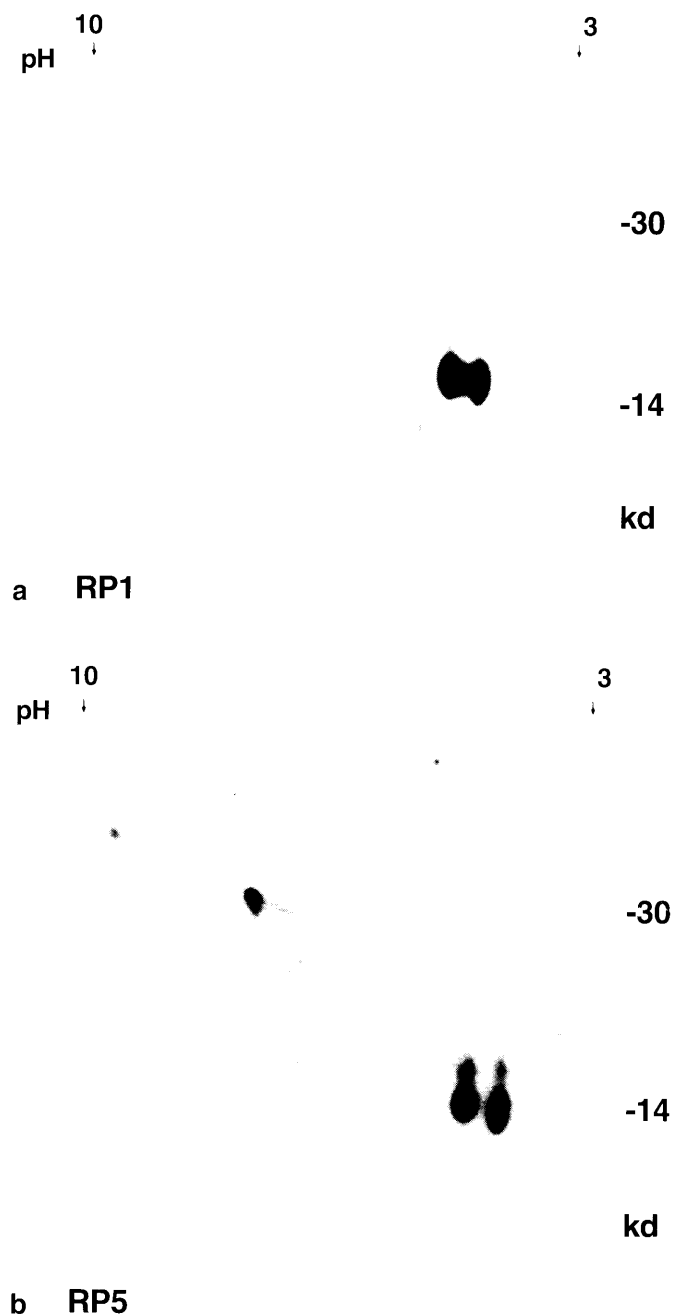


Fig. 1a, b Detection of profilin isoforms in 2-D separated tobacco pollen extracts. Tobacco pollen extracts were separated by 2-D gel electrophoresis and blotted onto nitrocellulose. Profilin isoforms were detected with **a** rabbit anti-celery root profilin antiserum (RP1) and **b** with rabbit anti-recombinant tobacco pollen profilin (ntPro1) anti-serum (RP5). Molecular weights are given on the right side of the figures and the pH range 3–10 is indicated on the top

and amino-terminus to be identical (Fig. 3). As is known from X-ray analysis of *Acanthamoeba* (Schutt et al. 1993; Almo et al. 1994), human (Fedorov et al. 1994) and birch pollen profilin (Almo et al., unpublished data), both regions form alpha helices which are located close to each other and participate in the actin binding. Differing contents of histidines may be responsible for the pl

differences observed among the 2-D separated tobacco pollen profilins; pIs of 5.19, 4.74, and 4.93 were calculated for ntPro1, ntPro2 and ntPro3, respectively. The ntPro3 lacked one amino acid (glutamine) in position 18, which may explain the differences in molecular weight observed in the 2-D analysis.

Recombinant tobacco profilin isoforms are recognized by different profilin-specific antisera in a similar manner

Bluescript plasmids containing in-frame inserted cDNAs of tobacco profilin isoforms (ntPro1–ntPro3) were prepared by in vivo excision. *E. coli* extracts containing the profilin isoforms (Fig. 4a,b: lanes 1–3), as well as extracts from *E. coli* which had been transformed with an empty control plasmid (negative control) (Fig. 4a,b: lane 0), were separated by SDS-PAGE, blotted onto nitrocellulose and probed with two different anti-profilin antisera, RP1 (Fig. 4a) and RP5 (Fig. 4b). Both antisera reacted comparably with the recombinant tobacco profilin isoforms. Isoforms ntPro2 and ntPro3 bound strongly to RP1 and RP5 (Fig. 4a,b: lanes 1 and 2) whereas ntPro1 (lane 3) reacted much more weakly. No reactivity was observed at 14 kDa in the control *E. coli* extracts (lane 0).

Discussion

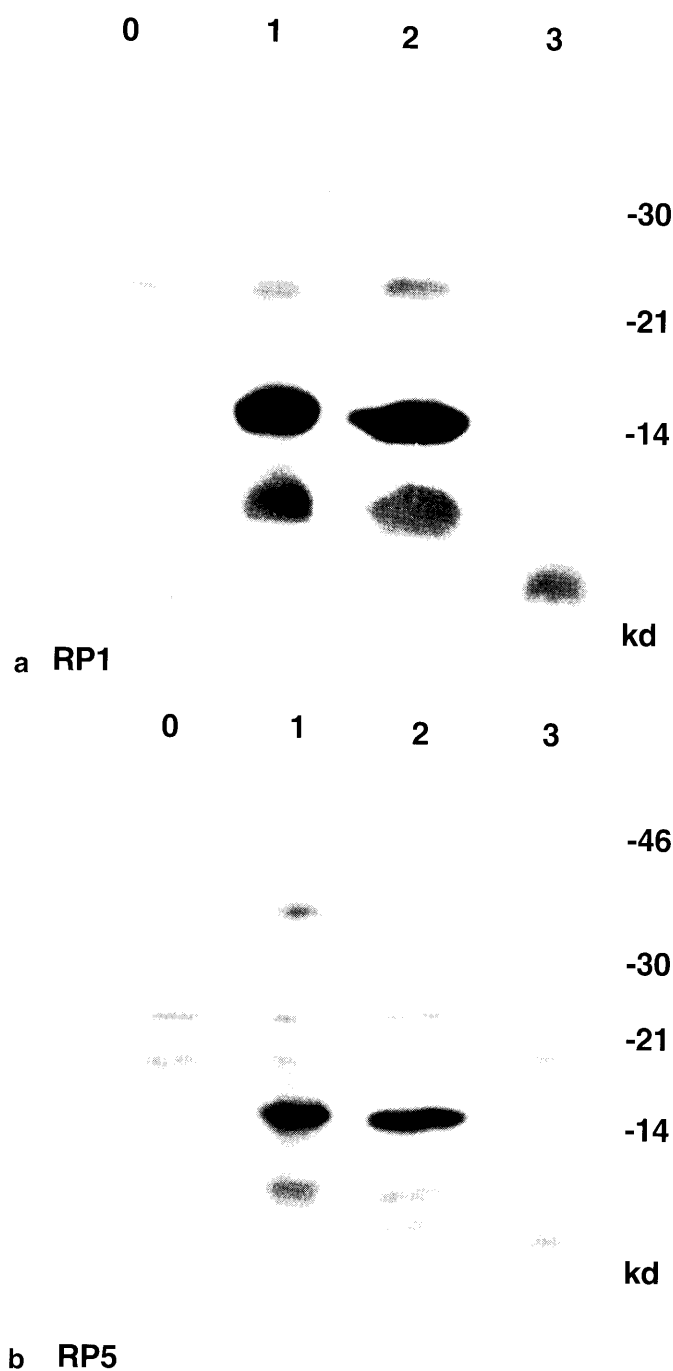
Plant profilins were first identified as cross-reactive allergens by screening a birch pollen cDNA library with serum IgE from a pollen allergic patient (Valenta et al. 1991). By using poly-L-(proline) affinity chromatography, profilins could be identified in many different plant species as cross-reactive allergens, which led to their designation as pan-allergens (Valenta et al. 1992). Actin- and PIP₂-binding properties of plant profilins were subsequently demonstrated (Valenta et al. 1993; Staiger et al. 1993; Drobak et al. 1994). The finding that large amounts of profilins are present in mature pollen compared to rather low levels in somatic plant tissues indicated that profilins play an important role in pollen biology (Mittermann et al. 1995). Consistent with these observations, profilins could be localized in resting and germinated pollen by immune electron microscopy (Grote et al. 1995). Plant profilins may function as actin-sequestering proteins which control actin polymerization in developing and resting pollen similar to what was described for the periacrosomal region of *Thyone briareus* sperm (Tilney and Inoue 1982). Perhaps profilins are even able to promote actin polymerization and stabilize dynamic actin structures during pollen germination, as was described for Chinese hamster ovary cells which were transfected to overexpress profilin (Finkel et al. 1994).

The importance of profilins in regulating the eukaryotic cytoskeleton requires that profilin functions be protected. One mechanism to protect cells from the loss of

ntPro1	ggcacgagacaacttaatttagggcaacaaa															
ntPro2	cacgagacaaggagaaaaacaaa-acaa-----agac-aca-ctta-tt---															
ntPro3	gattaagaacaaagggactaggaa-a-ac--aac-acattcttg-a--t															
ntPro1	ATG	TCG	TGG	CAA	ACA	TAT	GTC	GAT	GAT	CAT	TTG	ATG	GCT	GAC	ATT	45
ntPro2	---	---	---	---	---	---	--G	---	---	---	---	---	---	---	---	
ntPro3	---	---	---	---	--C	--C	---	--C	---	--C	---	---	-TC	---	T--	
ntPro1	GAA	GGT	CAG	CAA	GGT	CAC	CAT	CTC	GCT	GCT	GCA	GCT	ATT	CTT	GGC	90
ntPro2	---	---	---	---	---	A--	---	---	---	---	---	---	--C	---	---	
ntPro3	---	--C	--A	...	--C	--G	---	--T	--C	--G	--G	---	---	--C	--A	
ntPro1	CAT	GAT	GGC	AGT	GTC	TGG	GCT	CAG	AGT	TCC	ACT	TTC	CCT	AAG	TTT	135
ntPro2	A--	---	---	---	---	---	---	---	---	A--	---	---	---	---	---	
ntPro3	---	---	---	--C	--T	---	---	--A	--C	C-G	CAC	---	---	---	---	
ntPro1	AAA	CCA	GAG	GAA	ATT	ACT	AAT	ATC	ATG	AAA	GAT	TTC	GAT	GAA	CCT	180
ntPro2	---	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	
ntPro3	---	---	--A	---	---	--G	---	---	---	---	---	--T	---	---	---	
ntPro1	GGA	CAT	CTA	GCT	CCA	ACT	GGT	TTA	TTC	CTT	GGC	GGG	GCA	AAA	TAC	225
ntPro2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
ntPro3	---	TT-	--T	---	--C	---	--G	C--	--T	---	-CT	--A	AT-	--G	---	
ntPro1	ATG	GTA	ATC	CAA	GGA	GAA	CCT	GGC	GCT	GTC	ATC	CGT	GGG	AAG	AAG	270
ntPro2	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	
ntPro3	---	---	--A	---	---	---	---	--T	---	---	---	---	---	--A	---	
ntPro1	GGA	TCT	GGT	GGA	ATA	ACC	ATC	AAG	AAA	ACT	AAC	CAG	GCT	CTG	ATT	315
ntPro2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
ntPro3	--T	--A	---	---	---	--A	---	---	---	---	---	---	---	---	---	
ntPro1	TTT	GGT	ATC	TAT	GAG	GAA	CCA	GTG	ACC	CCA	GGA	CAG	TGC	AAC	ATG	360
ntPro2	---	---	---	---	---	---	---	---	--T	---	---	---	---	--T	---	
ntPro3	---	---	T-A	---	--A	--G	---	---	---	---	---	---	--T	---	---	
ntPro1	GTG	GTT	GAG	AAA	ATT	GGT	GAC	TAT	CTT	GTT	GAC	CAG	GGC	TAT	TAA	405
ntPro2	---	---	---	---	---	---	---	--C	---	---	---	---	---	---	---	
ntPro3	--T	---	---	---	---	--A	---	--C	--C	---	---	---	--T	---	---	
ntPro1	attaattcccttgttttaaatTTgattgttcgcaatattgaattttgttgatttggTg															
ntPro2	-----ct--aa--t-gattA-tgcaaa-t-gaattt-g----at-tgg--a-t															
ntPro3	-a-ttga-atc-ca-aatt-aaatt-----aatttggtgattgca-ctcca-c--ata-a															
ntPro1	attgcatttgTatcttctagggggccaaaattggcactggaggTTTTtcttttcttt															
ntPro2	gcatttg-atctt--agggccaaattggcac---tgt-ttttcc---cct---ct---															
ntPro3	gccaaG-g-cac-gggt-ttttt-----a-aattaacttt-cc-aa-gcggcctac-															
ntPro1	tatggggaaaataacaagcaacatgagacattttctcttttaggattgctatttcccat															
ntPro2	-t-t-aa---ta-ca-gcagcatgagacat---ctctg--agaat-----															
ntPro3	a-c--ttgct--tt-ctctttgt-ata-at--a-gga-a-atTTtactgaaaaaaaa															
ntPro1	tttgctctaaaagttactttaactaaaaaaaaaaaaaaaa															
ntPro2	-----atattgtgtattcatcattttctagacatttctag															
ntPro2	atattttatttctagaaaaaaaa															

Fig. 3 Comparison of the deduced amino acid sequences of three tobacco pollen profilin isoforms. Amino acids which are identical between ntPro1 and the other two profilin isoforms (ntPro2, ntPro3) are indicated as *dashes*. The *asterisk* in the ntPro2 sequence indicates the lack of one amino acid

ntPro1	MSWQTYUDDHLMADIEGQQGHHLAAAAILGHDGSUWAQSSTFPKFKPEEI	50
ntPro2	-----N-----N-----T-----	
ntPro3	-----U-----*--Q-----PH-----	
ntPro1	TNIMKDFDEPGHLAPTGLFLGGAKYMU IQGEPGAUIRGKKSGGGITIKKT	100
ntPro2	-----	
ntPro3	-----F-----A-I-----	
ntPro1	NQALIFGIYEEPUPGQCNMUVEKIGDYLUQGY	135
ntPro2	-----	
ntPro3	-----L-----	



profilin could be the occurrence of several isoforms of the same protein which may be able to replace each other. Indeed, in *Acanthamoeba*, *Dictyostelium* and humans, several profilin isoforms have been described which differ markedly in sequence, pI, function and expression pattern (Pollard and Rimm 1991; Haugwitz et al. 1991; Honore et al. 1993). In contrast to these isoforms, the only known profilin isoforms described for maize pollen (Staiger et al. 1993) were highly homologous; birch pollen and timothy grass pollen profilin (Giehl et al. 1994) had a rather acidic pI.

In this study, profilin isoforms from *Nicotiana tabacum* pollen were examined by 2-D electrophoresis and by analysis of their cDNA sequences. Two rabbit antisera, RP1 raised against a profilin from a somatic plant tissue (celery root profilin) (Vallier et al. 1992) and RP5 raised against a recombinant tobacco profilin isoform (ntPro1) (Mittermann et al. 1995) reacted comparably with 2-D separated tobacco profilin isoforms. The different isoforms were detected at comparable intensities by both antisera and were within an acidic pI range. Among six profilin cDNA clones isolated by immunoscreening, two novel cDNAs (ntPro2, ntPro3) were found which showed sequence differences when compared with a previously isolated cDNA (ntPro1). The sequence differences were, however, mostly confined to the non-coding regions, whereas the coding regions contained primarily silent nucleotide exchanges; the functional domains, in particular the carboxy- and amino-terminus were identical. It is possible that tobacco pollen contains more divergent profilin isoforms; however, no such variant could be isolated even though a serum pool of IgE antibodies from allergic individuals was used that displayed a broad cross-reactivity. In addition, it could be demonstrated that RP1 and RP5 reacted comparably with all three recombinant tobacco profilin isoforms.

Fig. 4a, b Binding of anti-plant profilin antisera to nitrocellulose-blotted recombinant tobacco profilin isoforms. Protein extracts from *E. coli* XL-1 Blue transformed with tobacco profilin isoforms ntPro1-ntPro3 (lanes 2, 3, 1) or with an empty Bluescript plasmid (negative control: lane 0) were separated by SDS-PAGE and blotted onto nitrocellulose. Membranes were probed with two different anti-profilin antisera: **a** RP1; **b** RP5

The high degree of sequence similarity of the three isoforms and their similar reactivity with different anti-profilin antisera indicates that they may be functionally equivalent. Based on the high degree of similarity of plant profilin isoforms (maize, Staiger et al. 1993; tobacco, this study) it is conceivable that different plant profilin isoforms might be able to replace each other in function. This hypothesis is supported by data showing that profilins from unrelated species (plants and mammals) which share much less sequence identity (20%) can fulfill similar functions (Valenta et al. 1993; Giehl et al. 1994; Ruhlandt et al. 1994) and even substitute for each other in living cells (Staiger et al. 1994; Rothkegel et al. 1996). These observations are also supported by the structural similarities observed between *Acanthamoeba* and mammalian profilins (Schutt et al. 1993; Fedorov et al. 1994; Almo et al. 1994).

The importance of having sufficient amounts of profilin available in cells such as pollen, which are rich in actin, is further supported by the finding that profilin can protect yeast cells which overexpress actin (Magdolen et al. 1993). In contrast, deletions of profilin created lethal or almost lethal phenotypes in yeast (Haarer et al. 1990), *Dictyostelium* (Haugwitz et al. 1994) and mouse (Witke et al. 1993). The presence of several profilin isoforms in pollen is, hence, interpreted as a mechanism that may protect profilin functions in pollen.

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