Multiple isotypes of α- and β-tubulin in the plant *Phaseolus vulgaris*

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The tubulins of the plant *Phaseolus vulgaris* have been identified in cell extracts by a combination of western blotting of 1D and 2D gels and detection by monoclonal antibodies specific for α- and β-tubulin. Plant α-tubulin differs from mammalian tubulin in that it migrates ahead of the β-subunit on Laemmli SDS gels. The *Phaseolus* root tip extracts contain 4 α- and 4 β-tubulin isotypes which are electrophoretically separable on 2D gels under a number of preparative conditions. The results indicate a complex expression of multiple tubulin isotypes in plant tissues.

Tubulin Phaseolus *Microtubule* *Plant cell* *Monoclonal antibody* *Western blotting*

1. INTRODUCTION

Microtubules have become the focus of a large amount of biochemical investigation, however until recently much of this work has been restricted to the microtubules of animal cells. In this context early reports often emphasised the conservation of tubulins isolated from different sources. Recently, however, two aspects of tubulin diversity have become apparent. Firstly, although the tubulins are highly conserved polypeptides distinct differences are detectable, both at the level of the tubulin gene and polypeptide, between the tubulins of animals and those of eukaryotic microbes [1–6]. The use of limited proteolytic cleavage with *Staphylococcus aureus* protease and electrophoretic separation of the resulting polypeptides has allowed the identification and classification of cleavage patterns characteristic of α-tubulins from a variety of evolutionarily diverse sources [7,8].

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Abbreviations: CHES, 2-(*N*-cyclohexylamine)ethanesulfonic acid; DTT, dithiothreitol; IEF, isoelectric focussing

Tubulin diversity has also been observed as the phenomenon of multiple tubulins. Genetic and molecular evidence suggests that the α- and β-tubulin genes of many organisms are arranged as multigene families [9]. Other evidence suggests that multiple tubulins can be expressed between the different cell types found in the life cycle of some eukaryotic microbes [5,6] and within the different tissues of metazoan organisms [10]. There is also evidence that multiple tubulins can exist within an individual cell, raising the possibility that different microtubules or microtubule-based organelles might be composed of distinct tubulins. The best evidence for this latter phenomenon – specificity of tubulin location within one cell – comes from studies of *Chlamydomonas* [11,12] and the trypanosome, *Crithidia fasciculata* [13,14], where a specific α-tubulin is located in the flagellum. In both instances this flagellum-specific α-tubulin arises via a posttranslational modification.

Microtubules play obvious and important roles in plant cell morphogenesis and yet we have very little information about plant cell tubulin. Tubulin has been purified from plant cells and shown to be capable of assembly in vitro into microtubules [15]. Furthermore, there are indications that these purified plant tubulins appear to differ in certain

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respects from animal cell tubulins. In particular, they are similar to the previously reported tubulins of *Physarum polycephalum* [1,5,6] in that they exhibit a very low affinity for colchicine [1,16,17]. There is, however, no information on whether these purified tubulins are representative of the complete tubulin complement of plant cells nor on the organisation of the α- and β-tubulin genes in plants.

We show here that the tubulin polypeptides of plants can be identified using well characterised monoclonal antibodies to the α- and β-tubulin. The electrophoretic behaviour of plant tubulins on 1D and 2D SDS–PAGE reveals distinct differences from mammalian cell tubulin but certain similarities to the tubulin of eukaryotic microbes. We also present the first evidence for the existence of multiple α- and β-tubulins in plants.

2. MATERIALS AND METHODS

French bean seeds (*Phaseolus vulgaris* L.) were surface-sterilised in 1% sodium hypochlorite, washed in double distilled water and germinated in John Innes compost at 25°C. The apical meristems and root tips (0.5 cm) were collected from 20 germinated seeds and crushed in liquid nitrogen. The disrupted tissues were solubilised in 150 μl Laemmli sample buffer prior to separation on one-dimensional SDS-polyacrylamide gels [18]. Two-dimensional gel samples were prepared by a freeze-thaw method (Burland et al., 1983) and also via a protocol involving boiling in 5% SDS, 2% DTT and 0.1 M CHES buffer (pH 9.5). Two-dimensional gel electrophoresis was performed as in [5]. The slab gels were stained either with Coomassie brilliant blue R250 or silver using the protocol of Wray et al. [19].

Electroblot transfer from the SDS gels was performed as described by Towbin et al. [20]. The nitrocellulose blots were stained with amido black for total protein patterns or probed with the anti-tubulin monoclonals. The antibodies used were YOL34, an anti-α-tubulin [21] and DM1B, an anti-β-tubulin [22]. Both antibodies show wide cross-reactivity with the tubulins of mammalian cells, plants and eukaryotic microbes. The second antibody used in most experiments was a goat anti-mouse IgG peroxidase conjugate. After protein transfer from the gels, the nitrocellulose blots were washed in 0.1% Tween 20 in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.4; 140 mM NaCl) for 30 min. Subsequent to incubation with the anti-tubulin monoclonal, the blots were again washed in this TBS–Tween 20 solution and then in a solution of 0.5% Tween 20 in HST (Tris/high salt – 10 mM Tris–HCl, pH 7.4, 1 M NaCl) to eliminate non-specific binding of the antibody. After incubation with the second antibody the blots were again washed for 45 min in 6 changes of 0.1% Tween 20–TBS, 2 changes of 0.5% Tween 20–HST and a final wash in TBS alone. Peroxidase-conjugate binding was developed by incubating with 0.3% of 4-chloro-1-naphthol (Sigma) in methanol diluted to 100 ml in TBS containing 20 μl of 30% hydrogen peroxide. The monoclonal antibodies were used at a dilution of 1:1000.

Mammalian brain tubulin was prepared from fresh sheep brain as in [7].

3. RESULTS

We have used two well characterised monoclonal antibodies to α- and β-tubulin to facilitate the detection of plant tubulins by Western blotting. One-dimensional SDS-polyacrylamide gels of tissue extracts from root tips and apical meristems of *Phaseolus vulgaris* were blotted onto nitrocellulose paper and probed with the monoclonal antibodies YOL34 (anti-α-tubulin) and DM1B (anti-β-tubulin) or a combination of both antibodies. Fig.1a,b show blots of the plant tissue proteins stained with amido black. Probing of such blots with both the anti-α-tubulin monoclonal (YOL34) and the anti-β-tubulin monoclonal (DM1B) revealed one band in each case (fig.1b–e). When blots were probed with a mixture of both anti-α- and anti-β-antibodies both bands were detected (fig.1g,h). Essentially similar results were obtained with the extracts of apical meristem and root tip. The α-tubulin band detected by YOL34 is the faster migrating band on these Laemmli gels. This is, of course, the reverse of the well characterised situation with mammalian cell tubulins where β-tubulin migrates faster than the α-tubulin. The peroxidase detection of α-tubulin with YOL34 gave a lighter staining reaction (fig.1). This was because YOL34 is a rat monoclonal and we used a peroxidase-linked anti-

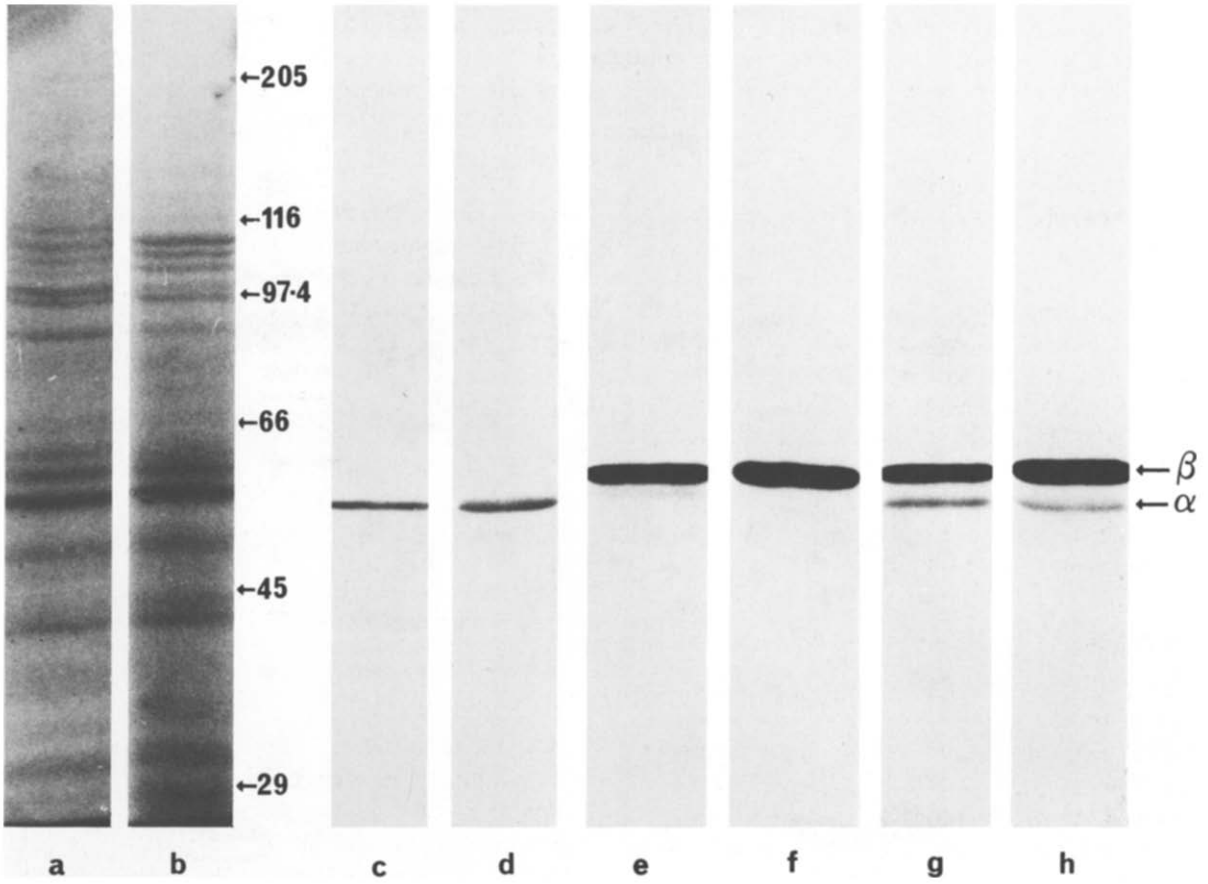

Fig.1. *Phaseolus* proteins from root tip (a,c,e,g) and apical meristem (b,d,f,h) were separated on 7.5% acrylamide gels, blotted onto nitrocellulose and stained with amido black to reveal total proteins (a,b); probed with the anti-α-tubulin monoclonal YOL34 (c,d); probed with the anti-β-tubulin monoclonal DM1B (e,f); probed with a mixture of YOL34 and DM1B (g,h). The α-tubulin bands detected by YOL34 are lighter than the β-tubulin bands detected by DM1B since YOL34 is a rat monoclonal detected, in this case, with an anti-mouse IgG peroxidase linked second antibody.

mouse antibody as the second antibody in the detection system. This lighter staining reaction was convenient in that it allowed us to differentiate between the YOL34 and DM1B reaction on the same blot (see fig.1g,h, figs 2–4). (Use of a peroxidase-linked anti-rat second antibody always gave the same staining pattern but with a stronger reaction).

Tubulin is easily detected as an abundant protein amongst the polypeptides of a mammalian cell separated by 2D gel electrophoresis. 2D gels of plant cell extracts showed no such abundant proteins at these gel coordinates. Consequently we applied the western blotting approach to determine which 2D gel spots represented the plant tubulins. Purified mammalian brain tubulin had characteristic coordinates on 2D gels, the α-tubulin has the slower migration in the SDS dimension and the more basic IEF position (fig.2a). To locate the likely region on our 2D gels occupied by the plant tubulins we mixed small amounts of the purified mammalian cell tubulin with the plant cell extracts and ran these mixtures on 2D gels, detecting the polypeptides by silver staining (fig.2b). We determined which of these plant cell polypeptides were tubulin by probing western blots of such mixed samples, separated by 2D gel electrophoresis, with the anti-tubulin monoclonals. The result of such an experiment is shown in fig.2c. The characteristic pattern of the mammalian α- and β-tubulin species was detected together with a series

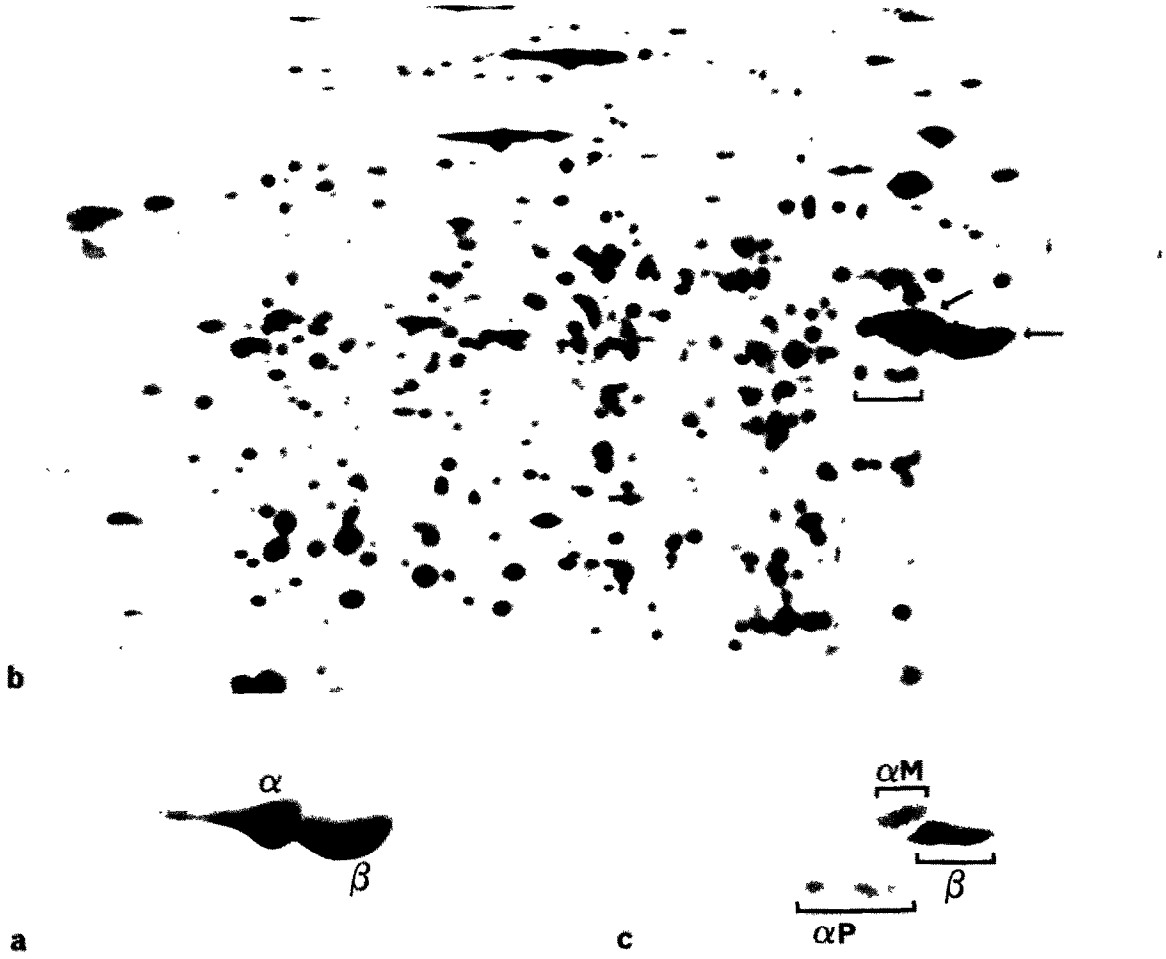

Fig.2. (a) Purified mammalian brain tubulin run on a 2D gel and stained with Coomassie blue. Loading was 10 μg tubulin, only the tubulin area of the 2D gel is shown. (b) Purified mammalian brain tubulin (1 μg) was mixed with *Phaseolus* root tip lysate and the mixture separated on a 2D gel and silver-stained. The two arrows denote the mammalian tubulin, cf. (a); the line denotes the plant α-tubulin species. (c) Western blot of the tubulin area of a gel similar to that in (b): a mix of 0.5 μg purified mammalian brain tubulin with *Phaseolus* root tip lysate. Probed with a mixture of YOL34 and DM1B. α_M denotes the mammalian α-tubulins; β denotes both the mammalian and plant tubulin area; αP denotes the 4 α-tubulin isotypes of *Phaseolus*. The mammalian and *Phaseolus* α-tubulins migrate to different coordinates on a 2D gel whilst mammalian and *Phaseolus* β-tubulins occupy the same coordinates.

of 4 discrete plant α-tubulin species. The 4 plant α-tubulins migrated faster than the β-tubulin species in the SDS direction and had a slightly more basic p*I* than that of mammalian α-tubulin. In contrast, on such gels the mammalian β-tubulins and those of *Phaseolus* migrated to essentially the same position. When 2D gels of plant extracts alone were run and probed with the monoclonals, a defined series of 4 α- and 4 β-plant tubulin species was detectable (fig.3). Comparison of these blots with the silver-stained 2D gels showed the plant tubulins to be minor components, yet each of the electrophoretically separable multiple tubulin species could be identified (e.g., cf. the α-tubulins of fig.2b,c).

The presence of multiple electrophoretically separable α- and β-tubulin species in *Phaseolus* does not appear to be an artifact of 2D gel electrophoresis. The pattern of multiple tubulins was very characteristic and reproducible. The pattern

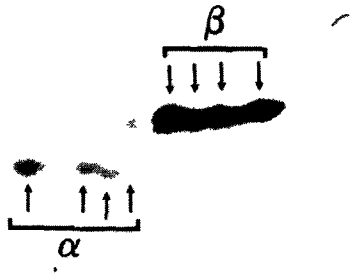

Fig.3. A 2D gel of a root tip extract of *Phaseolus* was prepared in the presence of a cocktail of protease inhibitors: pepstatin (5 μg/ml); benzamidine (5 μg/ml); chymostatin (5 μg/ml) and leupeptin (50 μg/ml). The gel was blotted onto nitrocellulose and probed with a mixture of YOL34 and DM1B. 4 α- and 4 β-tubulin isotypes were detected routinely. Only the tubulin area of the 2D gel is shown.

of 4 α- and 4 β-tubulins was found in samples prepared in either the absence of protease inhibitors or in the presence of a cocktail of such inhibitors including pepstatin, benzamidine, chymostatin and leupeptin (fig.3). We have also prepared 2D gels of *Phaseolus* tissues using a completely different gel sample preparation technique involving the rapid solubilisation of proteins in boiling SDS–CHES buffer. 2D gels of such samples again produced essentially the same pattern of 4α- and 4β-tubulin species (fig.4).

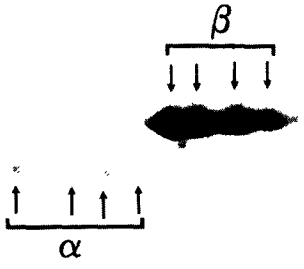

Fig.4. Tubulin area of an immunoblot of a 2D gel probed with a mixture of YOL34 and DM1B showing multiple tubulin isotypes. This sample of *Phaseolus* root tip proteins was produced by boiling the sample in CHES–SDS–DTT buffer.

4. DISCUSSION

The monoclonal antibodies used in this study are well characterised antibodies that recognise evolutionarily conserved epitopes on α- and β-tubulin. Use of these monoclonals with 2D gels has allowed us to define and characterise the tubulin polypeptides that are present in only small amounts in plant tissue extracts.

Electrophoretic separation of the plant proteins on standard one-dimensional Laemmli SDS gels showed that the plant α-tubulin migrated faster than the plant β-tubulin. This is the reverse migration pattern to that which is characteristic for animal cell tubulins where the β-tubulin is the faster migrating band. The α/β inversion of the tubulins was first described in *Physarum* [7] and has now been extended to *Dictyostelium* [23], *Paramecium* and *Tetrahymena* [24], and the trypanosome *Crithidia* [13,14]. Thus, in terms of its electrophoretic properties plant tubulin appears to resemble this group of lower eukaryotes more closely than it resembles mammalian tubulin. This finding is supported by results from a study of the peptide patterns generated by cleavage of α-tubulins with V8 protease [8,25]. Plant α-tubulin produced a similar peptide pattern to those produced from *Physarum, Tetrahymena* and *Chlamydomonas* α-tubulins, but this pattern was distinct from that generated from cleavage of animal α-tubulin [25]. In this content it is interesting that plant tubulin appears similar to *Physarum* tubulin [1,16] in that it has a reduced binding affinity for colchicine [17].

Our description of the α/β inversion of plant tubulin again emphasises the importance of assigning identity to the tubulin sub-types by criteria other than migrational position on one-dimensional SDS gels. In this context the use of the well characterised anti-tubulin monoclonals such as YOL34 and DM1B is extremely valuable.

The two-dimensional gel analysis of plant tubulins revealed that multiple isotypes of both α- and β-tubulin exist within root tip tissues of *Phaseolus*. This represents the first description of multiple tubulin isotypes in plants. We do not yet know whether all the 4 α- and 4 β-tubulins exist within every cell in the root tissue or whether there is a cell type dependent expression of selected isotypes. The origin of these multiple tubulin

isotypes remains to be investigated. However, the present study has revealed a complex pattern of tubulin isotype expression in higher plants.

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