Gibberellic acid stabilises microtubules in maize suspension cells to cold and stimulates acetylation of α-tubulin

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Abstract Gibberellic acid is known to stabilise microtubules in plant organs against depolymerisation. We have now devised a simplified cell system for studying this. Pretreatment of a maize cell suspension with gibberellic acid for just 3 h stabilised protoplast microtubules against depolymerisation on ice. In other eukaryotes, acetylation of α-tubulin is known to correlate with microtubule stabilisation but this is not established in plants. By isolating the polymeric tubulin fraction from maize cytoskeletons and immunoblotting with the antibody 6-11B-1, we have demonstrated that gibberellic acid stimulates the acetylation of α-tubulin. This is the first demonstrated link between microtubule stabilisation and tubulin acetylation in higher plants.

Key words: Microtubule; Acetylated tubulin; Maize cell suspension (BMS); Cold; Plant cytoskeleton

1. Introduction

The cortical microtubules (MTs) of plants display dynamic properties. They can reorientate from a transverse (relative to the cell's long axis) configuration to a longitudinal one in as little as 18 min [1]. In addition, the plant hormone, gibberellic acid, induces MTs to reorientate from longitudinal to transverse in a process in which some MTs grow whilst others shrink [2]. It is important, therefore, to know how plant hormones affect the stability of MTs and whether the MTs are marked in the process.

A connection between gibberellic acid (GA_3) and MT stability was established by Mita and Shibaoka [3] who reported that GA_3 allowed the MTs in onion leaf sheath epidermal cells to resist the depolymerising effects of cold and herbicide. Later, Hamada et al. [4] demonstrated that a 3-day pretreatment of maize seedlings with GA_3 stabilised MTs against cold or herbicide treatment. It is not known, though, whether GA_3-treated MTs are different from controls.

There is a significant body of work in which specific anti-tubulin antibodies are used to show that stability can correlate with various post-translational modifications of tubulin. The terminal tyrosine of α-tubulin may be an indicator of dynamic MTs since this terminal residue is lost in stable MTs. Most of this work is in animal cells but, in pea epidermal cells, Duckett and Lloyd [5] showed that the GA_3-induced reorientation of MTs was accompanied by detyrosination of the α_1 isotype. Although other post-translational modifications have been detected in plants (e.g. [6]) it is not known whether these correlate with MT stability. Many studies suggest that acetylation of α-tubulin is a marker for stable MTs (e.g. [7]) but reports are rare in plants. Astrom [8] showed that plant α-tubulin could be acetylated but did not actually demonstrate that cold-stable plant MTs might have this modification.

In this study, our aim was to see if GA_3 could stabilise the MTs in a cell suspension which would be more amenable to biochemical analysis than heterogenous plant tissues and organs. Using a maize suspension, this study establishes that stabilisation of cytoplasmic MTs by GA_3 is accompanied by increased acetylation of α-tubulin.

2. Materials and methods

2.1. Cells and protoplasts

Black Mexican sweetcorn (BMS) suspension culture cells were maintained in Murashige and Skoog's medium (0.441%, w/v), 2% (w/v) sucrose and 2 μg/ml 2,4-D. Five or six days after subculturing they were shaken for 3 h with 100 μM gibberellic acid (Sigma) dissolved in DMSO (0.5% final concentration). Controls contained DMSO only. Cells were then collected by centrifugation and converted to protoplasts by treating for up to 4 h with 1.5% (w/v) Onozuka RS cellulase, 0.1% (w/v) pectolyase Y-23 and 0.7% (w/v) hemicellulase in 0.5% MES (w/v) buffer (pH 5.8) containing 80 mM $CaCl_2$ and 0.35 M sorbitol.

2.2. Immunofluorescence microscopy

Protoplasts prepared from BMS cells (+/−GA_3) were washed with MES buffer twice, then PEM buffer (50 mM PIPES pH 6.9, 5 mM EGTA, 5 mM $MgSO_4$ containing 0.35 M sorbitol) and then divided into two parts. One part was placed on ice, the other at room temperature with gentle shaking. After 90 min, the protoplasts were attached to poly-L-lysine-coated slides for 15 min, fixed for 1 h in 3.7% (w/v) formaldehyde/PEM//0.25 M sorbitol containing DMSO (1.5%, v/v) and then extracted in 1% (v/v) Nonidet P40. After washing in PEM/sorbitol three times, protoplasts were incubated with antibody to β-tubulin (Sigma) then, after washing, with FITC-conjugated anti-mouse immunoglobulins (Dako, Denmark). Specimens were mounted in Citifluor anti-fade (City University Chemistry Department, University of London) and observed using a Bio-Rad MRC 600 confocal scanning microscope.

2.3. Cytoskeletal proteins and their analysis

Cytoskeletal fractions were isolated using the method of Chan et al. [9]. After washing, protoplasts were extracted in NP40 extraction buffer (10% (v/v) DMSO, 0.05% (v/v) Nonidet P40, 0.4 mM PMSF and 0.2 mM leupeptin in PEM). After incubation on a shaker for 20 min, the cytoskeletons were centrifuged for 5 min at $1200\times g$. The supernatant was discarded, the pellet resuspended in NP40 extraction buffer containing 2% (v/v) Triton X-100, and extraction carried out for a further 20 min with gentle agitation. After centrifugation at $1200\times g$ for 5 min, the uppermost layer of the pellet containing the cytoskeletons was resuspended in PEM containing 10% (v/v) DMSO and protease inhibitors, and the cytoskeletons collected by centrifugation for 5 min at $2000\times g$. Cytoskeletal pellets were taken up in tubulin isolation buffer (final concentrations, 5 mM imidazole-HCl, pH 7.5, 3 mM $CaCl_2$, 1 mM ATP, 1 mM dithiothreitol, 0.4 mM PMSF and 0.2 mM leupeptin) in order to extract tubulin and asso-

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This paper is dedicated to the memory of our friend Dr Richard Warn who was killed in a cycling accident in December 1998.

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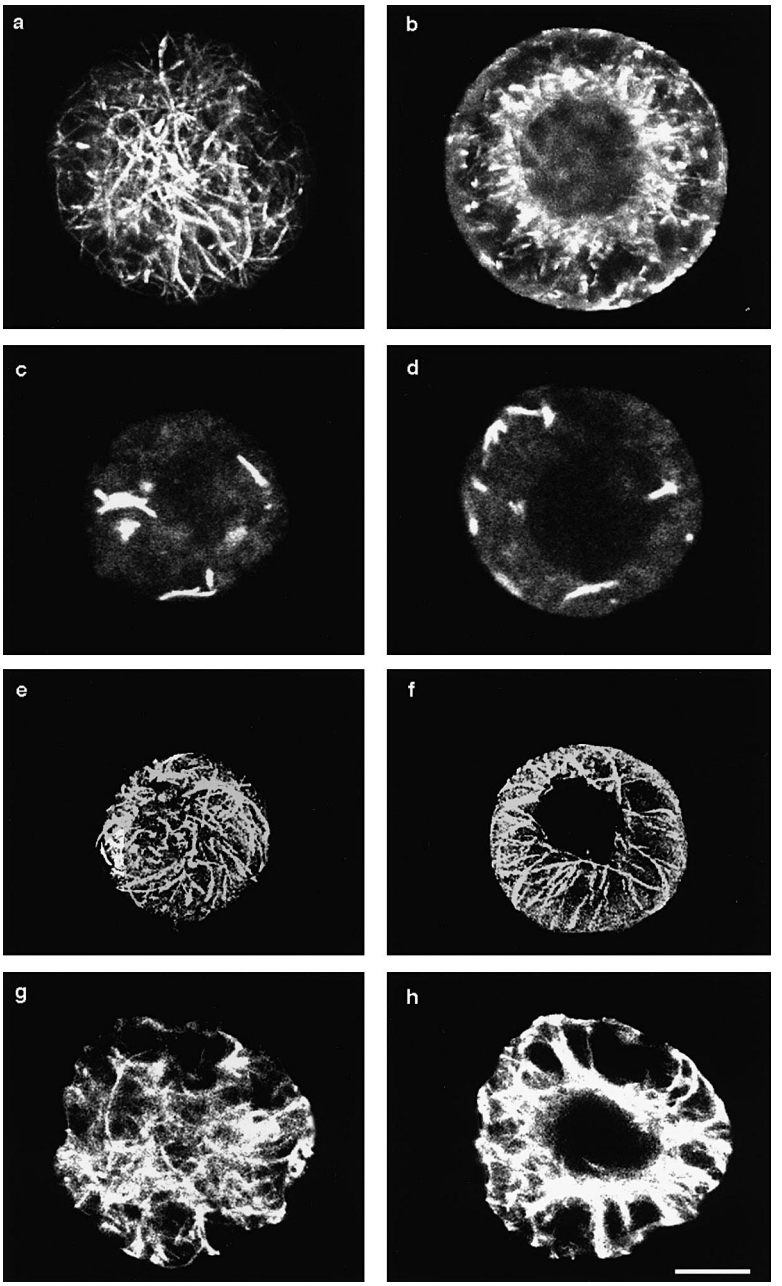

Fig. 1. Confocal microscopy of immunostained microtubules in BMS protoplasts pretreated with GA_3 then challenged with cold. a, b: Controls showing the cortical (a) and nucleus-associated (b) microtubules of untreated protoplasts. c, d: after 90 min at 0°C only few microtubule bundles survive. e, f: Protoplast from cells pretreated for 3 h with GA_3. g, h: The extensive microtubule network at the cortex (g) and around the nucleus (h) of GA_3-pretreated cells, which survives 90 min at 0°C. Scale bar = 12 μm.

ciated proteins at 0°C for 1 h. The suspension was then centrifuged at $43\,000 \times g$ for 20 min at 4°C and the supernatant clarified by passing through a 0.2 μm pore size syringe filter before being stored in liquid N_2. Proteins were then prepared for 2-D gel electrophoresis [10], and run using Sigma p*I* standards. Western blotting was as described in [5]. We used anti-tubulin antibodies YOL1/34 (α-tubulin [11]), YL1/2 (tyrosinated α-tubulin [12]), or 6-11B-1 (acetylated α-tubulin [13]), and secondary anti-rat or mouse IgG antibodies conjugated to horseradish peroxidase (Dako, Denmark). The detection system was either chloronaphthol and hydrogen peroxide or ECL reagents.

3. Results and discussion

To study microtubule-based effects uncomplicated by possible influences from the cell wall, we converted BMS suspension cells to protoplasts. Pilot experiments established that protoplast microtubules were cold-sensitive. Based on Hamada et al. [4] the longest treatment with GA_3 was 36 h, but we also used 12 h and 3 h and found that 3 h was sufficient to stabilise protoplast MTs against cold. Like Hamada et al. [4] we found that exposure of protoplasts to 0°C for 60 min was insufficient to depolymerise fully the control MTs and increased the time to 90 min. Fig. 1a shows the MTs at the surface of un-pretreated protoplasts and Fig. 1b shows the nucleus-associated MTs which are a feature of this cell line. Fig. 1c,d illustrates that only a few, short, bundled MTs remain in these cells after 90 min at 0°C. The 3 h pretreatment

with GA_3 does not qualitatively affect the overall appearance of the MT array (compare Fig. 1e,f with Fig. 1a,b). MTs in GA_3 pretreated protoplasts survived the cold treatment, displaying significant numbers of MT bundles radiating from the nucleus (Fig. 1h) and at the cortex (Fig. 1g). Unlike control protoplasts (Fig. 1a), the GA_3/cold-treated protoplasts were not spherical (Fig. 1h). This was not due to the presence of residual cell wall as indicated by failure to stain these protoplasts with Calcofluor (not shown). The irregular shape of the GA_3/cold-treated protoplasts would appear to be due to the fact that the protuberances at the cell surface are on radii formed by the nucleus-associated MTs.

The cold and GA_3 effects were analysed further using a semi-quantitative classification of immunostained MTs. Fig. 2a,b compares the MTs in protoplasts from untreated (a) and GA_3-treated (b) cells. This shows that, unchallenged by cold, GA_3 appears to exert no effect on the MT arrays. However, 90 min at 0°C (Fig. 2c) clearly has a negative effect: more than 50% of the protoplasts have no visible MTs, about 40% contain a few MTs, and only 5% contain a control-like array. This contrasts with the 50% of control-like arrays in Fig. 2d, showing that a 3 h pretreatment in GA_3 has a protective effect against the cold.

To investigate post-translational modifications, protein extracts were resolved by 2-D gel electrophoresis and immunoblotted with isotype-specific antibodies. Because whole cell homogenates contain tubulin isotypes found in the soluble pool (which may not necessarily be functional), in addition to those employed in functional MTs, we used protein samples prepared from detergent-extracted cytoskeletons from which the soluble fraction had been separated. Such cytoskeletons have been previously used by Hussey et al. [14] to study the tubulin isotypes in the polymeric fraction of carrot cytoskeletons except that, here, the cytoskeletal MT fraction was then depolymerised by the method of Chan et al. [9] to free it from nuclei. To establish the general pattern of tubulin isotypes, blots were probed with anti-β antibodies (not shown) and YOL1/34 for the α-isotypes (Fig. 3). No major differences in the overall pattern were seen to be induced by the GA_3 pretreatment. In both the untreated (Fig. 3a) and the GA_3-pretreated samples (Fig. 3b) the α-tubulin constellation consisted of a large complex spot (spot A, p*I* 5.1, which in various blots partially resolves into at least three isotypes), plus another well-resolved spot (spot B; p*I* 5.5). When these samples were probed with 6-11B-1 only a minor reaction was seen in spot A of the non-GA_3 control but no reaction could be found in spot B, even using the sensitive ECL detection method (Fig. 3c). However, following GA_3 pretreatment, spot B became reactive to 6-11B-1, and spot A had increased reactivity (Fig. 3d). The mAb 6-11B-1 is specific for acetylated α-tubulin (e.g. [13]) and so our data suggest first, that maize α-tubulin is acetylated but, perhaps more importantly, that GA_3 stimulates the post-translational acetylation of maize α-tubulin, particularly in the all-or-none reaction of spot B. Spot B is unlikely to have arisen by the post-translational modification and p*I* shift of a spot A isotype since α-tubulin already exists in that position as shown by the blotting with YOL1/34 (Fig. 3a,b) and YL1/2 (not illustrated).

Gibberellic acid has previously been shown to induce the reorientation of cortical MTs in epidermal cells of the dwarf pea [5]. This was accompanied by probable detyrosination of α-tubulin but we found no evidence in the present study for such a post-translational modification in BMS cells, using the YL1/2 antibody (not shown). In the present study, it is clear that GA_3 does induce the stabilisation of BMS MTs and allows them to resist 90 min at 0°C. The correlation with the increased acetylation of α-tubulin is interesting since this post-translational modification has been shown in diverse organisms to occur on stable MTs (e.g. [7,13,15]). In particular, Piperno and Fuller [13] showed that *Chlamydomonas* flagellar

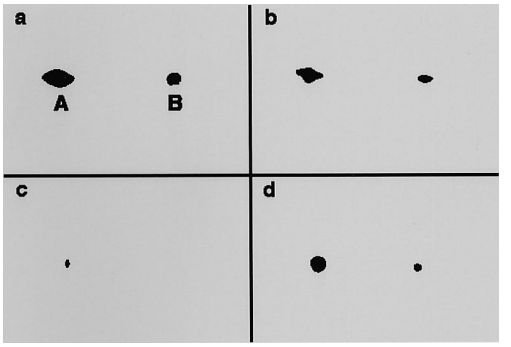

Fig. 3. GA_3 stimulates acetylation of α-tubulin. Cells were pretreated (or not) with GA_3, converted to protoplasts, then the microtubule-rich fraction of detergent-extracted cytoskeletons was isolated, separated by 2-D gel electrophoresis and immunoblotted. a: No GA_3 control, blotted with YOL1/34. b: GA_3-pretreated, blotted with YOL1/34. c: Same sample as in panel a but blotted with 6-11B-1. d: Same sample as in panel b but blotted with 6-11B-1. This shows that there is little acetylated α-tubulin in spot A (c) but that the microtubules in GA_3-pretreated protoplasts have increased levels of epitope, with spot B becoming acetylated in the process (d).

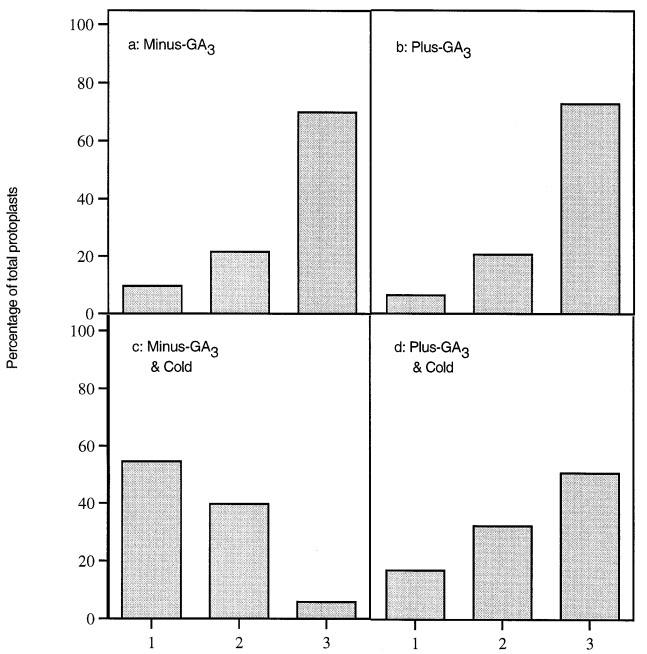

Fig. 2. Analysis of the effect of GA_3 and cold on protoplast microtubules. a: No GA_3 control. b: GA_3-pretreated. c: No GA_3 pretreatment, treated with cold. d: GA_3 pretreatment, treated with cold. Immunostained protoplasts were classified, 1 = no microtubules; 2 = few microtubules; 3 = control-level microtubules (see Fig. 1). This represents the average of four separate experiments, each counting at least 100 protoplasts for each treatment. BMS microtubules are cold-sensitive (c) but GA_3 pretreatment is able to stabilize microtubules against the cold (d).

MTs, which contained the acetylated epitope, were more stable to cold and colchicine than were the cytoplasmic MTs. Such studies show that differences in acetylation and stability exist between different microtubular structures but microinjection studies on human fibroblasts show that MTs can also differ within a single array. Cytoplasmic MTs containing acetylated domains incorporate tubulin much more slowly than do non-acetylated MTs within the same cell and the acetylated domains live longer [15]. According to these authors, tubulin acetyltransferase is likely to act upon tubulin polymer as a consequence, and not a cause, of stabilisation. They argue that the factors limiting acetyltransferase to specific domains could be MT-associated proteins.

Astrom's [8] appears to be the first full paper in which acetylated α-tubulin was detected with 6-11B-1 in higher plants. More recently, Smertenko et al. [6] showed that 6-11B-1 reacted with several, but not all, α-tubulin isotypes in tobacco cells. As for the functional implications of acetylation in plants, Astrom [8] suggested that it might correlate with stable arrays in tobacco pollen tubes and, by extrapolating from the staining patterns obtained previously with other non-acetylated antibodies, hypothesised that these *may* have been cold-stable. The present study now demonstrates this link between cold stabilisation and the level of α-tubulin acetylation. In onion leaf sheath cells [3], and in maize mesocotyl cells [4], gibberellins have been shown to stabilise MTs against the cold. The present study shows for the first time in a higher plant that this correlates with enhanced acetylation of α-tubulin.

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