Dissecting the contribution of microtubule behaviour in adventitious root induction

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Abstract

Induction of adventitious roots (ARs) in recalcitrant plants often culminates in cell division and callus formation rather than root differentiation. Evidence is provided here to suggest that microtubules (MTs) play a role in the shift from cell division to cell differentiation during AR induction. First, it was found that fewer ARs form in the temperature-sensitive mutant mor1-1, in which the MT-associated protein MOR1 is mutated, and in bot1-1, in which the MT-severing protein katanin is mutated. In the two latter mutants, MT dynamics and form are perturbed. By contrast, the number of ARs increased in RIC1-OX3 plants, in which MT bundling is enhanced and katanin is activated. In addition, any1 plants in which cell walls are perturbed made more ARs than wild-type plants. MT perturbations during AR induction in mor1-1 or in wild-type hypocotyls treated with oryzalin led to the formation of amorphous clusters of cells reminiscent of callus. In these cells a specific pattern of polarized light retardation by the cell walls was lost. PIN1 polarization and auxin maxima were hampered and differentiation of the epidermis was inhibited. It is concluded that a fine-tuned crosstalk between MTs, cell walls, and auxin transport is required for proper AR induction.

Key words: Adventitious roots, Arabidopsis, auxin, cell wall, microtubule, oryzalin.

Introduction

Adventitious root (AR) formation helps plants respond to environmental stresses such as water logging. It is also exploited for the propagation of cuttings in agriculture and forestry, so there is intense interest in understanding the mechanisms that drive this complex process of root differentiation and regeneration from non-root tissues (Riov et al., 2013). AR formation in cuttings occurs in four steps, in each of which auxin plays a major role: (I) cell de-differentiation, (II) induction of cell division, (III) development of root primordia, and (IV) root emergence (De Klerk et al., 1999).

In many difficult-to-root plants the transition from step II to step III is inhibited and, instead of root primordia, clusters of cells or callus are formed (Ballester et al., 1999; Greenwood et al., 2001; Levy et al., 2014; Vidal et al., 2003). Whereas a functional root has 15 different types of cells that together coordinate root growth and development (Benfey and Scheres, 2000), the callus formed in recalcitrant species is an amorphous cluster of cells that fails to function as a root.

Microtubules (MTs) are assembled into the spindle apparatus and the phragmoplast to allow plant cells to execute mitosis, meiosis and cytokinesis (Rasmussen et al., 2011;
MTs also establish distinct cortical arrays in interphase cells, in which the parallel MT array usually matches the orientation of cellulose microfibril deposition (Paredez et al., 2006). This consequently dictates cell shapes (Wasteneys, 2004; Wasteneys and Collings, 2004; Wasteneys and Fujita, 2006). ARs are formed from cells in inner layers, such as the cambium (Blakesley et al., 1991; Fahn, 1990) or pericycle (Falasca and Altmura, 2003). In lateral roots that are also initiated from an inner cell layer of the root, the pericycle, primordial cells push against and influence the cell layers above them in different ways (Swarup et al., 2008; Vermeer et al., 2014). In turn, inhibition of cell layer separation above the lateral root primordia by specifically suppressing auxin signalling in these layers or by suppressing inflorescence deficient in abscission (IDA) abscission signalling, which together should increase pressure on the dividing cells, affects lateral root primordium morphogenesis and differentiation (Kumpf et al., 2013; Lucas et al., 2013; Vermeer et al., 2014). In rice it was shown that the force exerted by AR primordia together with ethylene signalling and reactive oxygen species leads to epidermal programmed cell death, which is necessary for AR emergence (Steffens et al., 2012).

It has long been known that MTs respond to mechanical signals (Hardham et al., 1980; Williamson, 1990; Wymer et al., 1996; Zandomeni and Schopfer, 1994). It is now understood that katanin-dependent MT severing is important for the ability of meristemetic cells (Uyttewaal et al., 2012) and pavement cells (Sampathkumar et al., 2014) to respond efficiently to mechanical signals. MTs can be affected by changes in tissue-level stress patterns (Hamant et al., 2008; Heisler et al., 2010; Uyttewaal et al., 2012; Wymer et al., 1996), and their directionality matches predicted maximal stress direction that is influenced both by local geometry and growth axis (Burian et al., 2013). MTs are also influenced by cellulose synthesis (Bringmann et al., 2012; Fisher and Cyr, 1998; Himmelbach et al., 2003; Mei et al., 2012; Paredez et al., 2008).

The feedback loop between MTs and cell wall properties appears to be linked to auxin signalling (Landrein and Hamant, 2013). Auxin, by promoting proton pump activity, induces cell wall acidification (Rayle and Cleland, 1992). As a result, the cell wall protein expansin is activated so that cross links in the cell wall matrix are modified, leading to cell wall loosening (Cosgrove, 2005). Auxin has also been shown to trigger demethylterification of the pectin homogalacturonan and thereby lower cell wall rigidity (Braybrook and Peaucelle, 2013). Vice versa, cellulose synthesis (Feraru et al., 2011), cell wall properties (Braybrook and Peaucelle, 2013; Hamant et al., 2011; Heisler et al., 2010), and external forces (Nakayama et al., 2012) have been shown to affect the localization of the auxin efflux carrier PIN1 in the plasma membrane. In turn, auxin affects MT orientation (Blancaflor and Hasenstein, 1995; Chen et al., 2014; Dhonukshe et al., 2005; Fu et al., 2005; Lin et al., 2013).

Here it is shown that interference with MTs or the cell wall form can either increase auxin-induced AR induction, or lead to callus-like formation instead of ARs. The reciprocal relationships between auxin, MTs, and cell walls during AR formation are discussed.

Materials and methods

Materials

All materials were purchased from Sigma (Rehovot, Israel) unless otherwise mentioned. Alexa Fluor-conjugated antibodies were from Molecular Probes (http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html).

Arabidopsis plants, plasmids, and transformation

ARs were germinated and transformed as previously described (Clough and Bent, 1998). Plasmids containing DR5 Pro::VENUS, which was transfected into morl-1 plants, was kindly provided by the Meyerowitz laboratory (Heisler et al., 2005). Other plants used were wild-type Arabidopsis thaliana ecotype Columbia or A. Landsberg erecta. Mutants with MT-associated protein mutations were morl-1 (Whittington et al., 2001) and rid3 the latter provided by the Sugiyama laboratory (Konishi and Sugiyama, 2003). The DR5 Pro::VENUS seeds (Laskowski et al., 2008) were kindly provided by Prof. Ben Scheres. Seeds of pGL2::GFP, a root epidermis marker, were provided by Prof. John Schiefelbein from the University of Michigan (Lin and Schiefelbein, 2001).

Induction of AR formation in Arabidopsis plants

ARs were induced in intact plants as previously described (Abu-Abed et al., 2012; Gutierrez et al., 2009; Rasmussen et al., 2012). Briefly, seeds were germinated on Murashige and Skoog (MS) with 0.8% agar plates supplemented with 3% sucrose. The plates were kept in the dark for 2 d at 4°C, then in the dark for 4 d at 22°C, 3 d light, 2 d dark, and 5 d light. After 14 d, ARs (above the coleoptile) were counted. ARs were also induced in cut etiolated hypocotyls that were incubated in MS supplemented with 1% sucrose and 10 μM potassium-indole 3 butyric acid (K-IBA) in the dark. Oryzalin was dissolved in DMSO and applied at 25–300 nM. Isoxaben was dissolved in ethanol and applied at 0.1 nM, 1 nM, or 10 nM. Root induction was analysed during the first 3 d and after 6 d. Whole GL2:GFP seedlings (7 d old) were incubated in 10 μM K-IBA in the presence or absence of 10 nM isoxaben or 100 nM oryzalin for 3 d to determine the effect of the treatments on the GFP signal in primary root epidermis. From each treatment 50–70 primordia were scored for GFP in the epidermis.

Microscopy

Immunostaining was performed as previously described (Chaimovitsh et al., 2011). Images of primordium MTs are projections of several optical sections, 0.5 μm apart, filtered by the rolling ball filter. For MT orientation quantification, the images were analysed by FiberScore (Lichtenstein et al., 2003) and coloured maps of MT orientation were prepared. Cells with parallel and oblique or random MTs were counted manually. From each treatment, 100–250 cells were counted from 7–20 primordia. A representative movie showing the sequential optical sections was also prepared from each treatment. Staining of PIN1 was performed using an antibody kindly provided by J. Friml. Primordia of plants expressing the DR5 Pro::VENUS seeds were fixed and stained. The Venus signal was preserved during fixation. The DR5 signal was measured using FV500 software (Olympus, Hamburg, Germany); the average fluorescence was measured from an equal circle placed on the nuclei, one by one. Each stage V primordium or a cluster of similar size was divided into two: the proximal half closer to the pericycle layer of the hypocotyl and the tip half that is the distal portion. DR5 fluorescence was expressed as the ratio between the average fluorescence of nuclei present in these two halves for each primordium. Measurements included approximately 120 nuclei from five primordia for each treatment. For PIN1 quantification, cells were divided into two groups; those that showed polarized localization of PIN1 at the cell face towards the tip and those that did not. Cells were counted manually and included approximately 200 cells from 7–10 primordia from each treatment.
An Olympus IX81/FV500 laser-scanning microscope was used to observe fluorescently labelled cells with the following filter sets: for enhanced GFP, 488-nm excitation and BA505–525; for Alexa Fluor 555, 543-nm excitation and BA610. The objectives used were PlanApo 60X1.00 WLSM /0.17. When enhanced GFP and Alexa Fluor 555 antibodies were detected in the same sample, dихroic mirror 488/543 was used. In all cases in which more than one colour was monitored, sequential acquisition was performed.

Light retardation was investigated using an LC-PolScope image processing system (CRI, Inc., Woburn, MA, USA) mounted on a microscope (Nikon Eclipse 80i, Tokyo, Japan) equipped with Plan Fluor 920/0.5 OFN25 DIC N2, Plan Fluor 940/0.75 OFN25 DIC M/N2, Fluor 960/100w DIC H/N2 ∞/0 WD 2.0 objectives. The system includes a computer-controlled universal compensator made of two liquid crystal variable retarders. Retardation images were taken by a cooled charge-coupled device camera at high optical resolution. Retardation values were extracted manually using Abrio software tools (CRI, Inc.) from 150 to 400 cells from each sample.

Histological preparations
For light retardation analysis, tissue was fixed in formalin/acetic acid/alcohol fixative (FAA; 50% ethanol, 5% acetic acid and 10% formaldehyde in H2O) overnight at room temperature. Tissues were gradually dehydrated with an ethanol series (50%, 70%, 95%, 100%, 100%; 1 h each), and then ethanol was gradually replaced by Histo-Clear in five 1-h steps in a ratio of Histo-Clear to ethanol of 1:3, 1:1, and 3:1, with two final steps of pure Histo-Clear. The Histo-Clear was then gradually replaced by paraaffin (Paraplast X-TRA). Sections (10 µm) were cut with a rotary microtome (Leica RM2255), deparaaffinized, and mounted in Eukitt mounting medium (Kaltek, Padova, Italy) under a coverslip.

Results
AR induction rate is affected by mutations in MT-associated proteins
In this study, AR formation was induced either in intact seedlings by two sequential transfers from dark to light as previously described (Gutierrez et al., 2009; Rasmussen et al., 2012) or by application of K-IBA to cut etiolated hypocotyls. To investigate the possibility that perturbation of MT dynamics affects ARs, AR formation was followed in various mutant plants. First tested were rid5 and mor1-1, mutant alleles of the MORI gene that encodes an orthologue of the XMAP215 class of MT-associated proteins (Whittington et al., 2001). The mutant rid5 was isolated in a screen for temperature-sensitive mutants with aberrations in AR formation (Konishi and Sugiyama, 2003), whereas mor1-1 was identified in a screen for temperature-dependent disruption of MT organization (Whittington et al., 2001). At the restrictive temperature, 29°C, mor1-1 MTs become short and lose parallel orientation (Whittington et al., 2001) as a result of reduced MT plus-end dynamics (Allard et al., 2010; Kawamura and Wasteneys, 2008). At permissive temperature (22°C), there are subtle but statistically significant reductions in MT plus-end dynamics (Kawamura and Wasteneys, 2008) but these changes have no obvious effect on array organization or plant form (Whittington et al., 2001). AR formation in these plants was induced by twice transferring intact seedlings from dark to light. Compared with wild-type seedlings, mor1-1 plants produced significantly fewer ARs at permissive temperature (22°C) and almost no ARs at the restrictive (29°C) temperature (Fig. 1A and Fig. S1). This significant reduction in AR formation at permissive temperature suggests that very subtle changes in MT dynamics can affect AR induction despite there being no changes in overall array organization.

To test whether increased auxin concentrations lead to AR induction, cut etiolated morl-1 and rid5 hypocotyls were induced to form ARs in the presence of K-IBA. Fig. 1B shows that in the presence of ectopic auxin, morl-1 and rid5 mutants produced amorphous clusters of cells (hyperplasia) at the restrictive temperature, instead of the dome-like AR primordia seen in wild-type plants or in these mutants at the permissive temperature. To test AR formation in plants with distinct MT alterations, the katanin mutant bot1-1 (Bichet et al., 2001), was chosen. Fig. 1A and Fig. S1 show less AR formation in this mutant plant. By contrast, plants overexpressing the ROP GTPase effector protein RIC1 (RIC1-OX3), in which MT bundle formation (Fu et al., 2005; Fujita et al., 2011) and katanin-mediated MT severing activation (Lin et al., 2013) have been demonstrated, produced more ARs than control wild-type plants (Fig. 1 and Fig. S1). In addition, root induction in RIC1-OX3 etiolated hypocotyls was less sensitive to oryzalin, an MT-disrupting drug (Fig. S2), suggesting that the excess rooting is related to increased MT stability in these plants. Importantly, mild treatment of wild-type plants with oryzalin during AR induction led to increased formation of amorphous clusters of cells (hyperplasia) (Fig. S3), as in morl-1 and rid5 plants at 29°C.

For further analysis, six stages of AR development were determined (Fig. 2). Stage 0 is prior to root induction. In stage I, the four founder cells are formed after anticlinal cell division. Stage II comprises the first periclinal cell division to create two layers. During stages III, IV, and V, more periclinal and anticlinal cell divisions occur to create 3–4, 5–9, and 10–15 cell layers, respectively. In stage V the primordium acquires the classical dome shape and stage VI is when the root emerges. All further analyses were done on stage V primordia or cell clusters of similar size.

An immunolabelling method using GFP-tubulin expressing plants was used to document MT patterns because it is difficult to follow MTs in live root primordial. MTs were stained in stage V AR primordia and found to be organized in parallel arrays in wild-type and morl-1 plants at 22°C. In wild-type plants at 29°C there was a reduction in the number of cells with transverse or longitudinal parallel arrays. In morl-1 plants at 29°C and in wild-type plants after treatment with oryzalin or the cellulose synthesis inhibitor isoxaben, MTs lost parallel order and became randomly oriented or oblique in most cells (Fig. 3, Fig. S4, and Movies S1-6). Of note, previous studies have found hyper-alignment of MTs after short-term (a few hours) isoxaben treatment (Heisler et al., 2010; Paredez et al., 2008; Sampathkumar et al., 2014). By contrast in this study, long-term isoxaben treatment was applied and MTs were followed in cell clusters that formed in its presence over 3 d.

Accurate cell wall properties are important for AR induction
To determine if the properties of cell walls are involved in proper AR formation, AR induction was performed in any1
plants, which have a missense mutation in the CESA1 cellulose synthase. These plants have reduced anisotropic growth and reduced cell wall crystallinity (Fujita et al., 2013). More ARs were formed in this mutant compared to wild-type plants (Fig. S1). In contrast, when wild-type hypocotyls were treated with a constant amount of K-IBA and increasing concentrations of the cellulose synthesis inhibitor isoxaben, AR formation was inhibited in a dose-dependent manner (Fig. S5). Interestingly, after 3 d of treatment with 0.1 nM, 1 nM, or 10 nM isoxaben, 38%, 42%, and 80% of the hypocotyls, respectively, exhibited amorphous clusters of cells (hyperplasia), showing that cell division continues but
cell differentiation is hampered. More roots formed after 6 d, suggesting that in some cases differentiation was inhibited but not abolished.

To further examine the effect of MTs on the walls of AR primordia cells, polarized light retardation was determined and quantified using an LC PolScope (Abraham and Elbaum, 2013). It was first determined that the signal obtained by the polarized light shows parallel arrays in stage V primordium epidermal cells similar to that of MTs (Fig. S6). Next, serial sections of single primordia were examined to determine whether xylem typical cell wall thickenings, rich in crystal-line cellulose, are present in stage V primordia. Fig. S7 shows high light retardation by the xylem of the hypocotyl but no such signal in the AR primordia. Light retardation was then compared in transverse sections of stage V primordium or cell clusters of similar size. In wild-type plants, a higher retardation was observed in the outer face of epidermal cells and a circumferential gradient was found from the epidermis inward, showing reduced retardation at the outer face of cells in inner layers (Fig. 4). This pattern indicates specific polarity of cell wall architecture at the cellular level along with a specific gradient and radial symmetry at the tissue level, which are characteristic for differentiated root primordia. This pattern was hampered in wild-type plants treated with oryzalin and in mor1-1 plants at 29°C. As a control, anyl plants, in which cellulose organization is changed and crystallinity is reduced, were used. A dramatic reduction in light retardation was observed in anyl AR primordial cells (Fig. 4). It is concluded that MTs are important for optimal cell wall formation during AR differentiation, but when MTs arrays and dynamics are intact, normal AR primordia are formed even in the presence of slight perturbations to the cell wall.

The amorphous clusters of cells exhibit non-polarized PIN1, lack of auxin maxima, and failure of epidermis differentiation

To address the question of how auxin accumulates in the amorphous clusters of cells formed when MTs are perturbed, DR5pro:venus (Heisler et al., 2005) was introduced to
mor1-1 plants, which were then compared to wild-type plants (Laskowski et al., 2008). In addition, localization of PIN1 was determined by immunofluorescence. At 22°C, PIN1 was localized in a polar manner to the cell membrane facing the root growth axis, and auxin activity was greatest at the tip of root primordia in mor1-1/DR5\_pro:venus as in control plants (Fig. 5). At 29°C, however, polar localization of neither PIN1 nor auxin maxima was observed in the amorphous clusters of dividing cells in mor1-1/DR5\_pro:venus plants (Fig. 5). By contrast, polar localization of PIN1 and auxin gradients eventually developed in wild-type plants expressing DR5\_pro:venus, although the formation of root primordia was slightly inhibited by the high temperature (Fig. 5). In the presence of oryzalin, PIN1 lost polarization, and was often distributed to two to four cell sides. In the presence of isoxaben, PIN1 lacked coherent distribution between cells, and within individual cells could be found at more than one face, in the cytoplasm, or, rarely, focused at three-way cell junctions (Fig. 5 and Fig. S8). This is reminiscent of previous findings showing that oryzalin and isoxaben change the distribution of PIN1 in the membrane of shoot apical meristem cells (Hamant et al., 2011), and in line with a previous suggestion that MTs contribute indirectly to PIN1 localization (Boutte et al., 2006; Heisler et al., 2010; Kleine-Vehn et al., 2008). Concomitantly, no auxin maxima were observed in cell clusters developed under slight MT perturbation.

To determine the differentiation status of these clusters, plants expressing the GL2\_pro:GFP reporter gene were used. This line expresses GFP under the regulation of the promoter of GLABRA2, a transcription factor specific for root epidermal cells (Lin and Schiefelbein, 2001) and therefore an excellent marker of root epidermal identity. It was first confirmed that the epidermis of the primary root preserves the expression of GL2\_pro:GFP under the experimental conditions (Fig. 6). GFP expression was observed in the epidermis of the primary root following incubation for 3 d in K-IBA in the absence or presence of oryzalin or isoxaben. When etiolated hypocotyls of the GL2\_pro:GFP plants were induced to form ARs with K-IBA, cells of the outer cell layer expressed the GFP at early stages (IV–VI) in 90% of primordia observed
RFL functions in rice axillary meristem development

In the presence of 10 nM isoxaben, 70% of the cell clusters did not express GFP; in 30% of the cases only some cells at the outer layer expressed the GFP (Fig. 6 and Fig. S9). In the presence of 100 nM oryzalin, only very seldom (1% of the samples) did one or two cells show GFP expression (Fig. 6 and Fig. S9). This confirmed that proper MT organization is critical for root epidermal differentiation during AR formation.

Discussion

In 1978 Oppenooirth noticed that colchicine, an MT-disrupting drug, inhibited cell de-differentiation and differentiation during AR formation in herbaceous cuttings (petioles) of Phaseolus vulgaris (Oppenooirth, 1978). By contrast, in mature woody recalcitrant cuttings of Eucalyptus grandis, MT perturbation with low concentrations of trifluralin, another MT-disrupting drug, resulted in increased AR formation (Abu-Abied et al., 2014). This suggests that optimal organization and dynamics of MTs are required for proper AR organogenesis. Therefore, intervening with MTs that might not be in an optimal form for AR formation in recalcitrant plants could increase rooting (Abu-Abied et al., 2014), but intervening with MTs in easy-to-root plants may inhibit rooting (Oppenooirth, 1978).

There is a difference in the pattern of differentiation of lateral organs from roots versus shoots. In roots there is de-differentiation before organogenesis, whereas in shoots there is no dedifferentiation before organogenesis; in roots it is the inner layers driving organogenesis, whereas in shoots it is the outer layers (Oppenooirth, 1978; Murray et al., 2012). Despite these differences, it seems that the crosstalk between MTs, cell walls, and auxin signalling play a role in the induction of lateral outgrowth in both tissues. Local application of oryzalin...
on *pin1-1* shoot apical meristem promoted outgrowth similar to the effect of local application of indoleacetic acid (*Sassi et al.*, 2014). In another case, Hernandez and Green reported that mechanical signals affect organogenesis. They showed that ectopic pressure changed the organogenesis of *Helianthus annuus* (sunflower) dyad florets, resulting in the formation of one big bract instead of a flower and a bract (*Hernandez and Green, 1993*). Preferential lateral root formation at the outer edge of root bends (*Ditengou et al.*, 2008; *Laskowski et al.*, 2008; *Richter et al.*, 2009) also suggest the participation of mechanical signals in lateral root induction.

In line with the above, the data presented here suggest that MT organization is important for proper AR induction in *Arabidopsis* plants. While the forces were not measured, the results consolidate the idea that crosstalk between MTs, cell wall properties, and auxin transport is important for the shift from cell division to cell differentiation during AR formation in *Arabidopsis*. It is difficult to define the status of the cells in the clusters that formed when AR were induced in a background of MT perturbations. On the one hand, these clusters are reminiscent of callus formed in recalcitrant plants, but on the other hand it has previously been shown that callus cells express root markers (*Sugimoto et al.*, 2010), but the clusters described here did not express the root epidermal marker *GL2pro:GFP*. Therefore, it is not clear which particular stage of AR organogenesis is hampered when MTs are perturbed. Oppenorth proposed that, in the presence of colchicine, clusters of fewer than 30 meristematic cells could not continue to form root primordia (*Oppenorth, 1978*). It is proposed that the amorphous clusters found here are in a more or less similar status; these are meristematic cells that do not form root primordia.

**AR induction in *rid5*, *mor1-1*, *bot1-1*, *RIC1-OX3*, and any1 plants**

Intact *mor1-1* plants, when stimulated to produce ARs by transfer from dark to light, generated fewer ARs at the restrictive temperature of 29°C but, interestingly, also at the permissive temperature. This finding is intriguing because although *mor1-1* MT organization patterns and plant growth are indistinguishable from those of wild-type plants grown at the same 'permissive' temperature, there are subtle reductions in *mor1-1* MT growth and shrinkage rates (*Kawamura and Wasteneys, 2008*). This suggests that sustained MT dynamics optimize AR induction and that the failure of ARs to form is not simply a consequence of disrupted MT arrays or altered division patterns. The relationship between MT dynamics and AR induction, however, is complex. On the one hand, a reduction was found in the rate of AR induction at a high temperature (29°C) compared to 22°C, and it has been shown that MT growth and shrinkage rates increase dramatically at higher temperatures (*Kawamura and Wasteneys, 2008*). On the other hand, higher activity of the auxin-responsive *DR5* promoter was found in *Arabidopsis* seedlings exposed to 29°C (*Stavang et al.*, 2009) but PIN1 density in the plasma membrane of cells at the shoot apical meristem decreased at 37°C (*Nakayama et al.*, 2012). Taken together, it cannot be ruled out that the lower efficiency in AR induction in both wild-type and *mor1-1* plants at the restrictive temperature involves factors unrelated to MT integrity. The level of auxin is likely to be important because the application of K-IBA to cut etiolated hypocotyls led to somewhat similar AR primordia formation in *mor1-1* or *rid5* compared to wild-type plants at 22°C. This is in agreement with the observation that the *rid5* mutant requires higher concentrations of auxin.

![Fig. 6. Differentiation of primordia epidermis in control or in oryzalin- or isoxaben-treated plants. Etiolated hypocotyls of *GL2pro:GFP* plants were induced to form ARs with auxin in the presence of oryzalin or isoxaben. From each treatment, 50–70 primordia were scored for the presence of GFP in the epidermis. (A–C) As a control, primary roots were followed under the same conditions. (D–F) *GL2pro:GFP* expression in AR primordia under the different treatments. Scale bars in A–C 50 μm and in D–F 20 μm.](image-url)
than wild-type plants in order to make roots (Konishi and Sugiyama, 2003).

Plants with katanin loss-of-function mutations also made fewer ARs than wild-type plants. Katanin is a severing protein that is essential for cortical MT remodelling (Lindeboom et al., 2013; Zhang et al., 2013) and the disappearance of peri-nuclear MTs after cell division (Burk et al., 2001). However, it is not clear yet at which step AR formation is inhibited when katanin is not functioning. Interestingly, while a katanin mutant bot1-1 produced fewer AR than wild-type plants, another katanin mutant, bot1-7, produced more flowers than wild-type plants when auxin transport was blocked (Sassi et al., 2014). In addition, while overexpression of RIC1 promoted more AR formation than in wild-type plants, ric1 plants produced more flowers than wild-type plants under auxin transport inhibition (Sassi et al., 2014). This suggests that MT perturbations can have differential consequences on organogenesis depending on different contexts of auxin and mechanical signalling and on tissue-, cell layer-, and development-specific gene expression.

The increased induction of ARs in RICI-OX3 plants is consistent with RICI’s involvement in katanin activation (Lin et al., 2013). RICI is a target of the plant Rho-GTPases ROP1 and ROP2/4. ROP2/4 activity inhibits RICI binding to MTs and, in turn, RICI promotes the formation of MT bundles to inhibit ROP2 from interacting with its effector RIC4 (Fu et al., 2005). RIC4, via the accumulation of cortical actin microfilaments, inhibits PIN1 endocytosis (Nagawa et al., 2012). These findings, along with the observation from this study that the RICI-OX3 line is more resistant to oryzalin inhibition of AR formation, suggest that RICI promotes AR formation through its effects on MT polymer formation. RICI might also be situated in a road junction between MTs and auxin—it is an effector of the GTPase ROP6, and together they are involved in PIN1 and PIN2 internalization (Chen et al., 2012; Lin et al., 2012) and, in turn, are activated by auxin (Xu et al., 2014). Of note, it was previously shown that the crystallinity in cell walls of RICI-OX3 plants was lower than that of wild-type plants at 22°C, whereas wall crystallinity in morl-1 at 29°C was higher than that of wild-type plants (Fujita et al., 2011). This suggests a causative correlation between low crystallinity and high AR induction. In line with this, anyl plants, which have reduced cellulose crystallinity (Fujita et al., 2013), made more AR. Nevertheless, cellulose crystallinity remains to be determined in the specific tissue giving rise to ARs.

Cellulose microfibrils were disorganized in epidermal cells of the anyl inflorescence stems but in the cortex and pith cells no differences from wild-type plants were observed (Fujita et al., 2013). By contrast, light retardation was reduced in both epidermal and cortex cells of anyl AR primordia compared to wild-type plants. While microfibrils of morl-1 root epidermal cells remained predominantly transverse to the elongation axis after MT disruption at 29°C (Sugimoto et al., 2003), a significant decrease in light retardation was found in AR primordia in morl-1 at 29°C. A decrease in light retardation was also observed in the amorphous clusters formed when MTs were perturbed by oryzalin. This is in agreement with previous observations in which oryzalin treatment decreased microfibril uniformity in root cells (Baskin et al., 2004). Because cellulose is a birefringent material, polarized light retardation is correlated with cellulose microfibril direction and crystallinity, which in turn affect the rigidity of cell walls (Abraham and Elbaum, 2013). The increased AR formation in anyl plants is reminiscent of other observations in which softer cell walls favour organogenesis (Braybrook and Peaucelle, 2013; Kierzkowski et al., 2012; Peaucelle et al., 2011; Pien et al., 2001). The decrease in light retardation in the amorphous clusters of cells formed when MTs are perturbed suggests that MTs participate in organogenesis by optimizing cell wall properties, and that changes in cell wall properties in the background of unfavourable MT form might lead to callus-like tissue formation.

MTs during AR organogenesis

A molecular mechanism that underlies a crosstalk between MTs and auxin transport was recently revealed. CLASP, an MT-binding protein (Ambrose et al., 2007; Ambrose and Wastneyes, 2008; Kirik et al., 2007) accumulates at sharp cell edges and facilitates the interconnection of MTs on different cell faces (Ambrose et al., 2011). Through a direct interaction with the retromer component sorting Nexin 1, CLASP tethers PIN2-containing endosomes to MTs, which promotes recycling and concentration of PIN2 in cells, thereby promoting auxin polar transport (Ambrose et al., 2013). Interestingly, clasp plants exhibit auxin-related phenotypes including abundant lateral roots (Ambrose et al., 2007; Kirik et al., 2007) and the formation of callus on etiolated hypocotyls (Ambrose et al., 2013). The role of this mechanism in AR formation is still to be determined.

It is therefore concluded that induction of AR formation when MT dynamics or cell wall properties are not within a narrow optimal range results in increased formation of clusters of dividing cells but a decrease in root differentiation. This may result from disruption of formative cell division (De Smet and Beeckman, 2011) or a disruption of the mechano-sensing machinery (Besnard et al., 2011) or both. Further studies are required to reveal mechanos- and/or auxin-sensitive MT-associated proteins, the expression of which is critical for AR formation.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. The different mutant plants that were induced to form AR by the dark-to-light regime.

Figure S2. RICI-OX3 plants are less sensitive to oryzalin than wild-type plants in terms of AR formation.

Figure S3. AR formation in the presence of low concentrations of oryzalin.

Figure S4. MTs in AR primordial cells.

Figure S5. AR formation in the presence of increasing amounts of isoxaben.

Figure S6. A comparison of MT orientation and polarized light retardation pattern in epidermal cells of AR primordia.
Figure S7. Serial 10 μm sections through an AR primordia, imaged by the PoleScope.

Figure S8. Close-up on the distribution of PIN1 in the presence of oryzalin or isoxaben.

Figure S9. Various stages of AR primordia of GL2pro:GFP plants after oryzalin or isoxaben treatments.

Movie S1. MTs in stage V AR primordium of wild-type plants treated with K-IBA at 22°C.

Movie S2. MTs in stage V AR primordium of wild-type plants treated with K-IBA at 29°C.

Movie S3. MTs in stage V AR primordium of mor1-1 plants treated with K-IBA at 22°C.

Movie S4. MTs in a cell cluster of mor1-1 plants treated with IBA at 29°C.

Movie S5. MTs in a cell cluster of wild-type plants treated with IBA and oryzalin.

Movie S6. MTs in a cell cluster of wild-type plants treated with IBA and isoxaben.

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