

# RNA Processing Bodies, Peroxisomes, Golgi Bodies, Mitochondria, and Endoplasmic Reticulum Tubule Junctions Frequently Pause at Cortical Microtubules

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Organelle motility, essential for cellular function, is driven by the cytoskeleton. In plants, actin filaments sustain the long-distance transport of many types of organelles, and microtubules typically fine-tune the motile behavior. In shoot epidermal cells of *Arabidopsis thaliana* seedlings, we show here that a type of RNA granule, the RNA processing body (P-body), is transported by actin filaments and pauses at cortical microtubules. Interestingly, removal of microtubules does not change the frequency of P-body pausing. Similarly, we show that Golgi bodies, peroxisomes, and mitochondria all pause at microtubules, and again the frequency of pauses is not appreciably changed after microtubules are depolymerized. To understand the basis for pausing, we examined the endoplasmic reticulum (ER), whose overall architecture depends on actin filaments. By the dual observation of ER and microtubules, we find that stable junctions of tubular ER occur mainly at microtubules. Removal of microtubules reduces the number of stable ER tubule junctions, but those remaining are maintained without microtubules. The results indicate that pausing on microtubules is a common attribute of motile organelles but that microtubules are not required for pausing. We suggest that pausing on microtubules facilitates interactions between the ER and otherwise translocating organelles in the cell cortex.

**Keywords:** ER tubule junction • Golgi body • Microtubule • Mitochondrion • Peroxisome • Processing body.

**Abbreviations:** ER, endoplasmic reticulum; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; P-body, RNA processing body; PTS1, peroxisomal targeting signal 1; SP, signal peptide.

## Introduction

A fundamental attribute of life is polarity. Cells are polarized, and cellular polarity forms the base supporting the polarity of the organ and organism. Among the many features that contribute to polarizing cells, the cytoskeleton is paramount. Both microtubules and actin filaments are themselves polar, being polymerized from asymmetric subunits so that the polymer retains asymmetry, and hence is polar (Li and Gundersen 2008). The cytoskeleton anchors and also transports organelles (Hirokawa et al. 2009). By virtue of cytoskeletal filaments being polarized, the cell controls this flow of material and consequently of information. Therefore, cytoskeletal-driven motility, more than overcoming the inefficiency of diffusion over tens of micrometers, reinforces and even establishes the polarity of cells.

In animal cells, microtubules are involved principally in organelle transport, whereas actin filaments are concerned with cell shape and cell contacts, being enriched at the cell periphery (Hirokawa et al. 2009). In plant cells, these roles are reversed, with actin supporting most organelle transport (Shimmen and Yokota 2004) and microtubules being involved in expansion and enriched at the cell periphery (Sedbrook and Kaloriti 2008).

The pivotal role of cortical microtubules in controlling plant expansion was suggested in the first publication to image a microtubule (Ledbetter and Porter 1963) and since then has been amply confirmed with both chemical inhibitors and genetics (Sedbrook and Kaloriti 2008). The role of actin filaments in supporting organelle motility in plants was first observed with respect to cytoplasmic streaming (Kamitsubo 1966, Nagai and Rebhun 1966). Indeed, powering cytoplasmic streaming in plants was among the first roles documented for actin and myosin in any non-muscle cell type (Shimmen 2007). Subsequently, actin filaments in plants were demonstrated to

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support the transport of various organelles, including the Golgi apparatus, peroxisomes, mitochondria, plastids, and endoplasmic reticulum (ER) (Boevink *et al.* 1998, Nebenführ *et al.* 1999, Mathur *et al.* 2002, Van Gestel *et al.* 2002, Ueda *et al.* 2010). Interestingly, although actin filaments sustain long-distance transport of plant organelles, it has recently been observed that microtubules influence short-distance behavior, causing pauses for both peroxisomes (Chuong *et al.* 2005) and the Golgi (Crowell *et al.* 2009, Gutierrez *et al.* 2009).

In addition to deploying membrane-bound organelles, the cytoskeleton deploys other kinds, including those related to RNA metabolism. The most familiar of these is the ribosome, but cells contain several types of large RNA–protein complex, collectively termed RNA granules. In animal cells, RNA granules—including the RNA-processing body (P-body), stress granule, neuronal granule, and germ cell granule—are transported on microtubules (Hirokawa *et al.* 2009). In plants, RNA granules homologous to the stress granule and the P-body have been described (Bailey-Serres *et al.* 2009), but the involvement of the cytoskeleton in their transport remains for the most part uncharacterized.

One exception where cytoskeletal involvement has been characterized is the movement of viral RNA. Many plant virions spread systemically by moving from cell to cell through plasmodesmata. To reach plasmodesmata, the viral RNA is often transported on actin filaments, although microtubules might be required for efficient systemic spread (Niehl and Heinlein 2011). In another example, pertaining to rice endosperm, actin filaments support the motility of an ER-associated, prolamine mRNA particle (Hamada *et al.* 2003, Wang *et al.* 2008).

Here, we studied the movement of the P-body. This organelle is a cytoplasmic aggregation of protein and RNA, without a membrane. The P-body functions to degrade mRNA and to produce small RNAs. In animal cells, P-bodies undergo directed motility based on microtubules and pause around actin filaments (Aizer *et al.* 2008). In plants, although P-bodies have been reported (Xu *et al.* 2006, Iwasaki *et al.* 2007), their movements have been little studied.

We report that P-bodies undergo long-distance transport on actin but pause at cortical microtubules. Interestingly, the pattern of pauses is apparently unaffected by the loss of microtubules. Likewise, we find that pauses in the transport of mitochondria, peroxisomes, and Golgi, as well as the maintenance of stable branches of tubular ER, occur near microtubules but do not require the microtubules. We suggest that microtubules are associated with sites in the cortex that promote interactions among the various organelles as they are transported through the large plant cell.

## Results

### P-bodies are transported by actin filaments and pause at cortical microtubules

To image the P-body, we used plants in which one of the major P-body components, the mRNA de-capping enzyme, DCP2, was

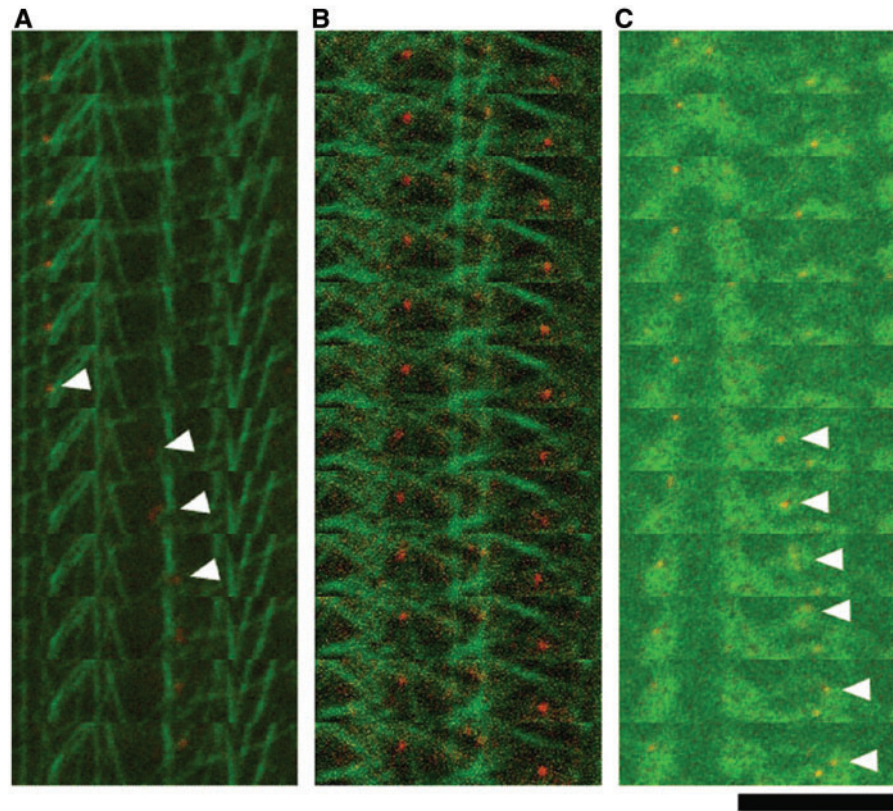
tagged with green fluorescent protein (GFP). We transformed plants harboring a putative null mutation in this enzyme with DCP2–GFP and obtained lines indistinguishable from the wild type, which we used for imaging. A similar approach has been used by others to image P-bodies (Xu *et al.* 2006, Iwasaki *et al.* 2007). P-bodies underwent directed motility, and in addition frequently paused (**Fig. 1A, Supplementary Video S1**).

First, we ascertained whether long-distance P-body movement depended on actin. In seedlings treated with 2  $\mu$ M latrunculin B, long-distance P-body movements were suppressed if not abolished (**Fig. 1B, Supplementary Video S2**). Next, we checked the relationship between microtubules and P-body motility, in plants that co-expressed mCherry–TUB6 and DCP2–GFP. P-bodies appeared to pause at or near cortical microtubules (**Fig. 1A, Supplementary Video S1**). To assess pausing quantitatively, we examined the probability of a pause happening at a position that is coincident with a microtubule. The numbers of P-bodies whose surface touched, or did not touch, the tubulin fluorescence were counted. The microtubule area was widened manually to create strips whose radius is equal to the diameter of the largest P-body and the total microtubule area was obtained. Were pausing behavior independent of microtubules then the frequency of pausing within the microtubule area should be the same as the ratio of the microtubule area to the total image area. On the contrary, pausing was significantly more likely to occur within the microtubule area (**Table 1**).

### Pausing behavior of P-bodies in the absence of microtubules

To elucidate the role of cortical microtubules in the pausing behavior of P-bodies, we examined their motility in seedlings treated with 50  $\mu$ M oryzalin, sufficient to remove nearly all visible microtubules. Surprisingly, despite the complete absence of microtubules, the P-bodies still paused (**Fig. 1C, Supplementary Video S3**).

To determine whether P-body pausing in the absence of microtubules was changed quantitatively, we measured the distances moved by a P-body between consecutive frames of time-lapse sequences. Note that in the image sequences, because long-distance transport is rapid and actin filaments occupy focal planes different from those occupied by microtubules, P-bodies moving on actin are effectively excluded from the analysis. For about three-quarters of the time spent in these sequences, P-bodies moved by less than half a micron, which we equate with the paused state (**Fig. 2A**). The frequency of larger displacements decreased with distance, as expected for diffusion-based movement. Note that the largest step-size category includes all movements greater than 2  $\mu$ m. Surprisingly, the extent of pausing was apparently unaffected by microtubule depolymerization. Although microtubules substantially overlap the position of pause sites, this analysis shows that microtubules are not required for pausing.



**Fig. 1** Movements of P-bodies. Confocal time series of a hypocotyl expressing DCP2-GFP, which labels P-bodies (red), and mCherry-TUB6 (green). Image panels are separated by 5 s. (A) Control. One P-body jumps between microtubules and stops around a microtubule. (B) Latrunculin B treatment ( $2\ \mu\text{M}$ ) to depolymerize actin filaments. Long-distance movements of P-bodies are not observed. (C) Oryzalin treatment ( $50\ \mu\text{M}$ ) to depolymerize microtubules. Both pausing and moving P-bodies are seen (arrowheads show moving P-bodies). Still images correspond to [Supplementary Videos S1–S3](#). Bar =  $20\ \mu\text{m}$ .

**Table 1** Correlation of paused organelles with cortical microtubules

Organelle	Microtubule area, % of image total	Overlapping organelles, % of total	Replication
P-bodies	$50 \pm 6.2$	$94 \pm 0.7$	$n = 1,137$
Golgi bodies	$60 \pm 5.0$	$90 \pm 1.7$	$n = 1,420$
Peroxisomes	$26 \pm 5.3$	$95 \pm 2.5$	$n = 1,215$
Mitochondria	$36 \pm 2.3$	$95 \pm 4.4$	$n = 2,105$
ER stable junctions	$50 \pm 15.1$	$84 \pm 6.5$	$n = 1,296$

Data are the mean  $\pm$  SD, with  $n$  representing the number of scored organelles. The microtubule area for peroxisomes and mitochondria is less than for Golgi bodies and P-bodies because plants were treated with subsaturating oryzalin ( $5\ \mu\text{M}$ ) to decrease microtubule density. Note that the stable ER junctions are far less motile than the other tabulated organelles.

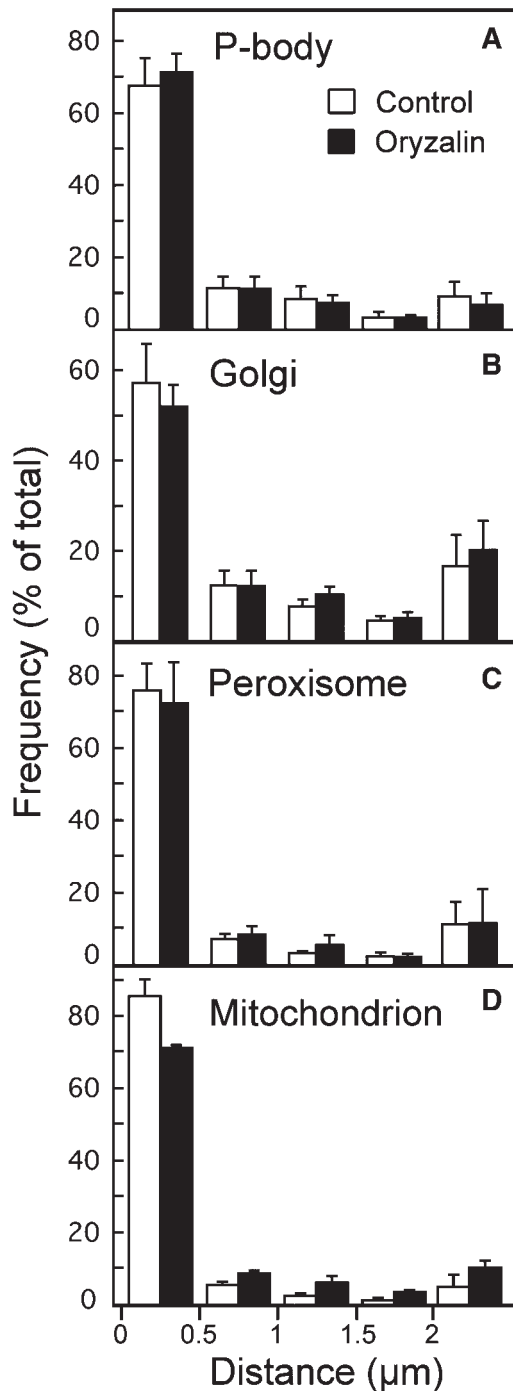
### Golgi, peroxisomes, and mitochondria also pause in the absence of microtubules

Because it is surprising that complete removal of cortical microtubules does not affect the frequency of P-body pauses, we checked whether removal of microtubules affected the pausing behavior of other organelles. Previously, Golgi have been

reported to pause at cortical microtubules (Crowell et al. 2009, Gutierrez et al. 2009). To label Golgi, we used a *trans*-Golgi network marker, monomeric red fluorescent protein (mRFP)–SYP43 (Ebine et al. 2008). Consistent with previous reports, we observed that Golgi bodies frequently stopped at or near a microtubule, sometimes jumping to a neighboring microtubule ([Fig. 3A](#), [Supplementary Video S4](#)), and the co-localization of paused Golgi with microtubules was significant ([Table 1](#)). Without microtubules, Golgi bodies also paused frequently ([Fig. 3B](#), [Supplementary Video S5](#)), and similarly to the P-body, the removal of microtubules had little if any effect on the frequency of pausing ([Fig. 2B](#)).

Likewise, in onion epidermal cells, peroxisomes have also been reported to pause on microtubules (Chuong et al. 2005). That report labeled peroxisomes with GFP-tagged multifunctional protein, which also labeled microtubules non-specifically; while the double labeling was convenient for imaging, the pausing of peroxisomes might reflect a non-physiological interaction. Therefore, we examined peroxisomes labeled with a peroxisomal signal peptide construct, peroxisomal targeting signal 1 (PTS1)–GFP (Mano et al. 2002), in a line also expressing mCherry–TUB6. Although in control plants,





**Fig. 2** Frequency distribution of displacement distances. The distance between the centroid of an organelle in two adjacent frames ('step size') was measured in at least four independent videos with 61 frames each. From five to 16 organelles were measured, per video. Bars indicate mean frequency  $\pm$  SD. Time between frames was 5 s for the P-body (A), Golgi body (B) and mitochondrion (D), and 2 s for peroxisome (C). Bars on the far right pool all steps over 2  $\mu$ m.

peroxisomes appeared to pause at or near cortical microtubules (Supplementary Video S6), the density of cortical microtubules obscured the relationship. To better resolve the

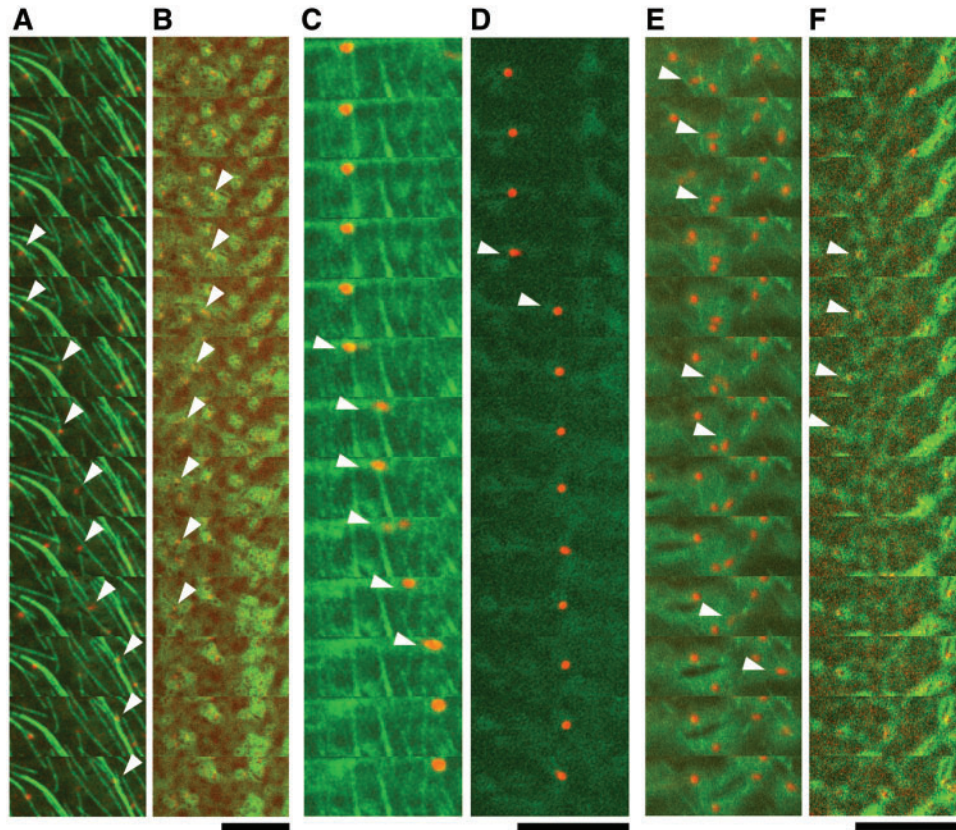
movement, we observed peroxisomes in plants treated with 5  $\mu$ M oryzalin to reduce microtubule density. In this condition, peroxisomes still appeared to pause at or near the remaining cortical microtubules (Fig. 3C, Supplementary Video S7) and the co-localization was significant (Table 1). Peroxisomes also paused in the absence of microtubules (Fig. 3D, Supplementary Video S8) and the frequency of pausing was not decreased (Fig. 2C).

Finally, mitochondria have also been reported to pause at microtubules, both in tobacco BY-2 cells (Van Gestel *et al.* 2002) and in characean internodal cells (Foissner 2004). We labeled mitochondria with mitotracker orange in a line expressing GFP-TUB6. As for the other organelles studied here, mitochondria paused at or near cortical microtubules (Supplementary Video S9) and the relationship was clearer in plants treated with 5  $\mu$ M oryzalin (Fig. 3E, Supplementary Video S10, Table 1). In plants treated to remove nearly all microtubules, mitochondria still paused (Fig. 3F, Supplementary Video S11). Interestingly, without microtubules, mitochondria pausing frequency did decrease slightly but was nevertheless substantial (Fig. 2D).

### Microtubules associate with branches of the tubular ER

Not only the long-distance transport of the ER, but also its specific structure depends on the cytoskeleton and, in plants and animals, the roles for microtubules and actin appear to be reversed. The motility and organization of the ER network depend strongly on microtubules in animal cells (Vedrenne and Hauri 2006) but on actin filaments in plant cells (Sparkes *et al.* 2009a). However, plant microtubules have been inferred to interact with the ER meshwork based on microtubule destabilization, which can change the structure of the ER (Foissner *et al.* 2009, Langhans *et al.* 2009, Sparkes *et al.* 2009b).

To analyze the relationship between ER structure and microtubules, we co-expressed GFP-TUB6 and an ER marker (SP-TagRFP-HDEL), created by adding a signal peptide to the N-terminus of RFP as well as an ER retention signal to the C-terminus. This marker was modified from the sGFP-based marker published previously (Mitsuhashi *et al.* 2000). Stable and dynamic regions of tubular ER were distinguished by merging three successive images, separated by 25 s, with each image pseudo-colored red, blue, or green: stable regions emerge as white (Fig. 4A, Supplementary Video S12). Stable junctions were appreciably correlated with microtubules (Fig. 4C-E). In this figure, stable junctions located on microtubules are shown as yellow dots and non-overlapping junctions are shown as red dots (unstable junctions, i.e. those that moved over the 50 s imaging interval, are not dotted). To assess co-localization quantitatively, we followed a similar strategy to that used for the other organelles, where the likelihood of finding ER stable junctions within the area occupied by microtubules was compared with the area of microtubules present within the image. Stable ER junctions overlapped extensively



**Fig. 3** Movements of organelles in the presence and absence of microtubules. Movements of Golgi bodies labeled with mRFP-SYP43 (A, B), peroxisomes labeled with PTS1-GFP (C, D) and mitochondria labeled with mitotracker orange (E, F) in the hypocotyl were observed with (A, C, E) and without (B, D, F) microtubules. Microtubules were labeled with GFP-TUB6 (A, B, E, F) and mCherry-TUB6 (C, D). In all panels, microtubules are green and the organelle is red. The diffuse green signal at B, D and F represents soluble tubulin caused by oryzalin treatment (50  $\mu$ M) and dark regions silhouette membranous organelles. Arrowheads point to organelles jumping between microtubules. Image panels are separated by 5 s (A, B, E, F) or 2 s (C, D). Still images (A–F) correspond to [Supplementary Videos S4, S5, S7, S8, S10](#) and [S11](#). Bar = 20  $\mu$ m.

with microtubules ([Table 1](#)), although the extent of the overlap was somewhat less than that of the other organelles.

To elucidate the importance of microtubules for ER mesh organization, we treated seedlings with oryzalin to depolymerize microtubules and observed ER dynamics. With oryzalin treatment, the ER still formed a mesh that included stable junctions, but their density was decreased ([Fig. 4F–J](#), [Supplementary Video S13](#)). The effect on mesh structure was evaluated by measuring the lengths of tubules linking stable junctions, which showed that oryzalin broadened the distribution of tubule length and shifted it to longer lengths ([Fig. 5](#)). We conclude that stable ER junctions typically form at microtubules in control cells, but microtubules are not required for the maintenance of stable ER tubule junctions.

## Discussion

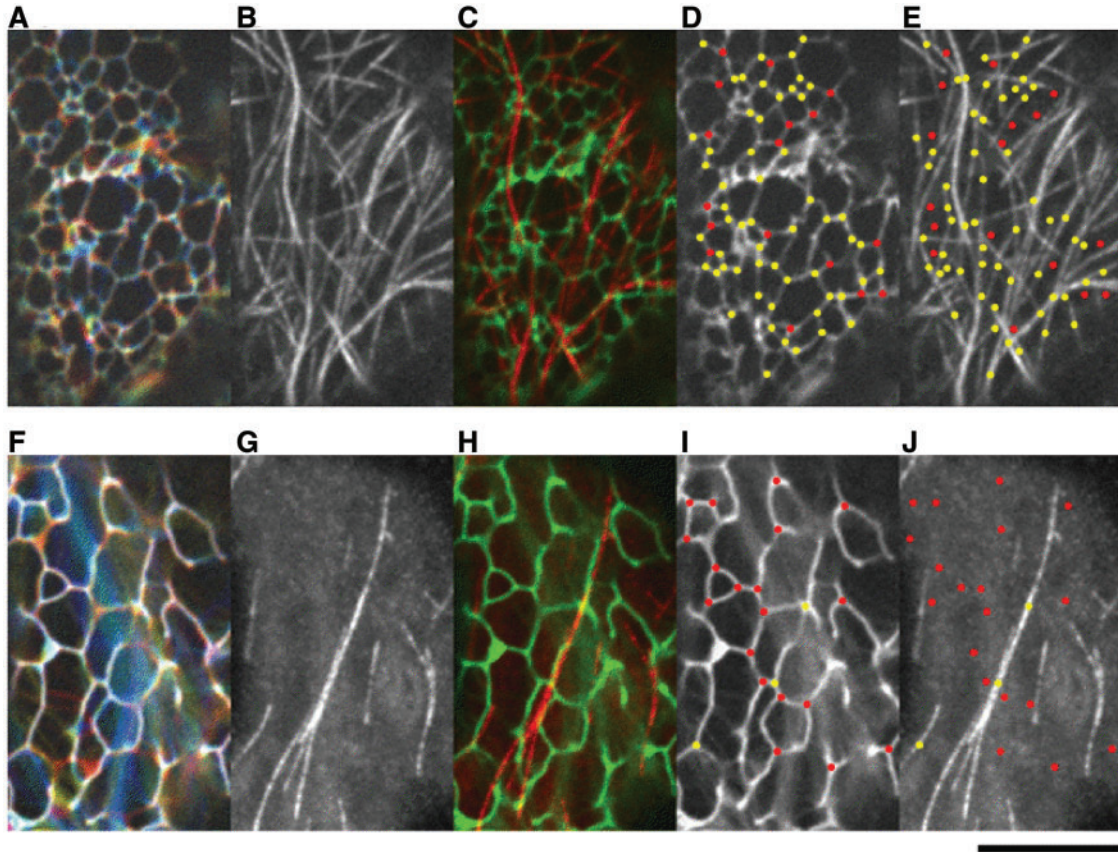
For epidermal cells of the *A. thaliana* hypocotyl and cotyledon, we report that the motility of P-bodies shares features with that of the Golgi apparatus, peroxisomes, and mitochondria,

undergoing dynamic long-distance transport on actin filaments and pausing at or near microtubules. Similarly, the ER, whose overall deployment depends on actin, positions stable tubule junctions at or near microtubules. Pausing reflects the interaction between the organelle and actin presumably being interrupted, followed by the organelle undergoing Brownian motion before becoming transiently anchored. Taken together, these results imply that the motility of plant organelles, including RNA granules, is pervasively influenced by microtubules.

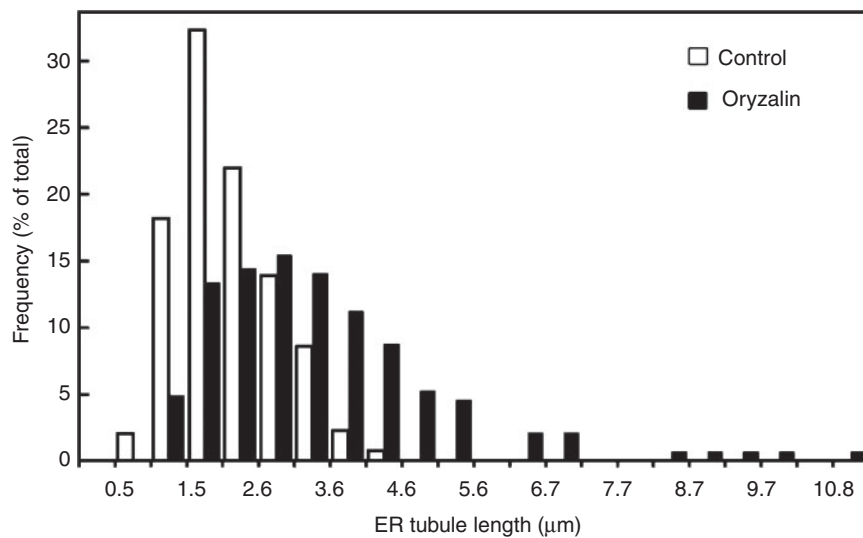
The classic influence of cortical microtubules is over the direction in which cellulose microfibrils are deposited, an influence required for anisotropic expansion and secondary wall morphogenesis. However, cortical microtubules are present in all plant cells, even those that are neither expanding nor laying down copious secondary wall. Insofar as all cells depend on the controlled movement of organelles, a common role for cortical microtubules could be providing pause sites for organelle motility.

What might be the functional significance of organelle pausing? One answer is to promote the delivery of organelle





**Fig. 4** Relationship between ER junctions and microtubules. Confocal micrographs of a leaf epidermal cell co-expressing SP-TagRFP-HDEL, which labels the ER, and GFP-TUB6. (A–E) Control. (F–J) Oryzalin (50  $\mu\text{M}$ ) for 1 h. (A, F) Overlay of the SP-TagRFP-HDEL signal from three different time points, separated by 25 s. Time 1 is colored blue, time 2 green, and time 3 red; stable regions of the ER over the imaging interval appear white. (B, G) The GFP-TUB6 signal corresponding to the ER image sequences. (C, H) Overlay of the SP-TagRFP-HDEL (green) and GFP-TUB6 (red) signals from (A, F) and (B, G). (D, E, I, J) Characterization of ER junctions. Stable (i.e. white) ER junctions co-localized (yellow circles) or not localized (red circles) with microtubules. Bar = 10  $\mu\text{m}$ .



**Fig. 5** Frequency of tubule lengths connecting stable junctions. Treatment conditions and imaging as for Fig. 4. The lengths were binned in 3 pixel increments (approximately 0.5  $\mu\text{m}$ ). Data were obtained from seven different cells for each condition. The total number of tubules measured was 396 and 284 for control and oryzalin-treated cells, respectively.

contents to the surrounding region. This idea has been supported for the pausing of Golgi bodies, which apparently deliver cellulose synthase complexes to the plasma membrane at the sites of cortical microtubules (Crowell et al. 2009, Gutierrez et al. 2009). Notwithstanding that the cargo in this case (cellulose synthase) continues to interact with the microtubule long after delivery, the ability of various organelles to deliver cargo might be enhanced by frequent and predictable stopping points. What is more, organelles often need to exchange components. The efficiency of such exchange would be increased by organelles pulling off the actin highway and parking side by side at a rest stop.

We hypothesize that microtubules establish and maintain sites within the cortical cytoplasm, sites that potentiate delivery of organelle cargo as well as interactions among organelles. Fixed, cortical sites, associated with the cytoskeleton and ER have been proposed previously (Reuzeau et al. 1997). For the sake of further discussion, we will call these sites cortical 'landmarks'. The proposed landmark sites in the cortex would allow cells to supplement chance encounters and diffusion with directed contacts, an enhancement that might be particularly valuable in large cells such as those of the hypocotyl and leaves studied here.

This hypothesis of landmarks is supported by the shared motility pattern observed here, as well as by finding many annotated organelle and RNA-processing proteins among microtubule-associated proteins (T. Hamada and T. Hashimoto, unpublished data). Furthermore, microtubules mediate interactions between peroxisomes and plastids that are required for chloroplast development (Albrecht et al. 2010). Nonetheless, organelles and P-bodies pause (and ER junctions persist) in the absence of microtubules. These apparently contradictory observations can be reconciled by positing that the organelles can pause at a landmark site either through associating with microtubules or through binding one or more components of the site itself.

In principle, pausing of motile organelles could be caused by direct interactions between organelle and microtubule or by indirect interactions between the organelle and some third component, which also interacts with the microtubule. That direct interactions occur is indicated by microtubule-associated proteins known to be organelle components. For example, two kinesins (microtubule-based motor proteins) are localized to Golgi bodies as well as to secretory vesicles (Lee et al. 2001, Lu et al. 2005) and a different kinesin is localized to mitochondria (Ni et al. 2005). Experiments *in vitro* have found that microtubules are bound by enoyl-CoA hydratase, which is a peroxisome protein (Chuong et al. 2002), and also by dynamin, which is localized to peroxisomes and mitochondria (Hamada et al. 2006). Although proteins mediating an interaction between microtubules and P-bodies have yet to be identified, microtubule binding *in vitro* has been reported for several proteins involved with RNA metabolism, including elongation factor 1- $\alpha$  (Durso and Cyr 1994), MPB2C (Kragler et al. 2003), THO2 (Hamada et al. 2009), and Rae1 (Lee et al. 2009).

Direct organelle–microtubule interactions are supported by certain of our observations. For mitochondria, the frequency of pausing is modestly but significantly reduced in the absence of microtubules. For ER, direct interactions seem even clearer: stable tubule junctions are localized preferentially at cortical microtubules and their removal reduces the number of such junctions (Fig. 4). This result suggests that ER tubule junctions are stabilized at microtubules. Nevertheless, that indirect interactions also contribute to pausing is indicated by the fact that removal of microtubules scarcely affects pausing frequency (Fig. 2).

We compared P-body motility not only with three kinds of relatively small and motile organelles but also with the ER. The latter comprises a complex network of tubules, sheets, and junctions that pervades the cell. The network is dynamic, with tubules extending and retracting, forming and dissolving junctions and branches. While the overall network structure depends on the actin cytoskeleton, there has been debate on whether ER motility or network structure involves microtubules (Sparkes et al. 2009a). When microtubules are depolymerized chemically, some studies have reported little if any change in ER motility (Quader et al. 1989, Knebel et al. 1990, Lichtscheidl and Hepler 1996), whereas others did observe changes (Foissner et al. 2009, Langhans et al. 2009, Sparkes et al. 2009c). Also, the reported effect of microtubule depolymerization on ER structure differs: on one hand, oryzalin treatment produced aggregations of ER termed 'nodules' in *A. thaliana* roots, tobacco BY-2 cells, and tobacco leaf protoplasts (Langhans et al. 2009); however, we were unable to observe oryzalin-induced ER nodules either in tobacco BY-2 cells or in root, hypocotyl, and leaf epidermis of *A. thaliana* (data not shown). On the other hand, in elongating internodes of *Nitella translucens*, removing microtubules increased the mesh size within the ER network (Foissner et al. 2009), which is consistent with our results.

It is still unclear how ER tubules extend and branch. The extension of tubular ER is correlated with movements of Golgi bodies, which sometimes physically interact with the ER membrane (Sparkes et al. 2009b); however, the extension of ER tubules continues more or less unchanged when the Golgi membranes are decimated by brefeldin (Sparkes et al. 2009c). Here, we show that ER tubule junctions are partially associated with microtubules. That ER tubule junctions are related to the presence of microtubules, but do not require them, is reminiscent of the pausing movements we observed for organelles and P-bodies, which occur at or near microtubules but continue when microtubules are depolymerized. This leads us to hypothesize that the positioning of ER tubule junctions shares motility characteristics with those of organelles and P-bodies, depending on actin for sustained localization but subject to local modification in the cell cortex based ultimately on the microtubule system.

Pausing, widespread among plant organelles, even while they otherwise undergo sustained long-distance transport, illustrates the fact that anchoring and stillness are as much the





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