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Live Cell Imaging Provides Novel Insights into Fungicide Mode of Action

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ABSTRACT

Over the past two decades, the use of fluorescent proteins has changed our understanding of living cells. Fused to proteins of interest, green and red proteins enable a quantitative assessment of cellular dynamics. In recent times, fluorescent protein-based live cell imaging was successfully established in various plant pathogenic fungi. Such investigation provided unique insights into the molecular mechanisms of plant invasion. Moreover, when fused to organelle- or cytoskeleton-specific proteins, these fluorescent proteins become markers that enable observation of defined cellular compartments and structures. Here, we highlight the potential of fluorescent protein markers (FPMs) to better understand the mode of action of fungicides.

INTRODUCTION

Over the past twenty years, the use of fluorescent proteins has revolutionized our understanding of cellular organization and behaviour. Enhanced green-fluorescent protein (eGFP; Yang et *al.* 1996) or red-fluorescent proteins, including monomeric red-fluorescent protein (mRFP; Campbell et *al.* 2002) or monomeric Cherry (mCherry; Shaner et *al.* 2004) have been widely-used in a broad range of organisms. These include numerous plant pathogenic fungi, such as *Ustilago maydis* and *Zymoseptoria tritici* (overview in Kilaru et *al.* 2015b; Schuster et *al.* 2015a). When fused to a protein of interest, cells usually target the fluorescent fusion proteins to specific structures or organelles. This enables the study of protein localization or dynamics in living cells (Chalfie et *al.* 1994). Moreover, when fused to organelle- or structure-specific proteins, such fluorescent fusion proteins become cellular markers (fluorescent protein markers= FPMs) for their respective compartments or cellular structures. A good example is tubulin, which is the building subunit of microtubules. Fused to GFP, the modified tubulin incorporates into microtubules, which, when visualized by fluorescent microscopy, show dynamic behaviour that was initially only recognized for purified tubulin in cell-free *in vitr*o assays. In recent years, our group extended this approach to plant pathogenic fungi. Over the past 17 years, the use of FPMs in the corn smut fungus *Ustilago maydis* ("Maisbeulenbrand") has provided novel and important insights into the mechanisms underpinning plant infection (e.g. Bielska et *al.* 2014; Fuchs et *al.* 2006; Treitschke et *al.* 2010; Weber et *al.* 2006; Weber et *al.*

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2003) and fundamental principles of fungal cell organisation and growth (e.g. Guimaraes et *al.* 2015; Lin et *al.* 2016; Schuster et *al.* 2016). Moreover, FPM expressing *U. maydis* cell lines (=strains) have also proven to be powerful tools in understanding the mechanism of fungicide delivery by liposome-like carriers (Steinberg 2012). This overview article highlights the potential use of live cell imaging of FPMs in gaining a better understanding of the mode of action (MoA) of fungicides. This area of research is novel, and thus the results summarised in this article provide only a "snap-shot" of our ongoing research in this arena.

LIVE CELL IMAGING OF FPMS IN FUNGICIDE MODE OF ACTION STUDIES

Example 1: MBC-fungicides

Methyl benzimidazole carbamate fungicides (MBC-fungicides) were introduced in the 1960's – 1970's and have been widely used as a protective and eradicant anti-fungal to protect cereals, vines, fruit, rice and vegetables (Oliver & Hewitt 2014). The MoA of MBC-fungicides in fungi is well understood. Biochemical and molecular biology studies have shown that these fungicides target the β–tubulin subunit of the tubulin dimer (overview in Davidse 1986). Binding of the MBC-fungicide to tubulin prevents the addition of the GTP-bound tubulin dimer to the elongating plus-end of the microtubule (Figure 1A). In these non-growing microtubules, the intrinsic hydrolytic activity of tubulin itself cleaves the bound GTP and thus leaves only GDP-tubulin dimers in the microtubule (Figure 1A). The loss of the GTP-cap results into microtubule instability and a switch to "catastrophy", followed by rapid depolymerisation of the microtubule (Desai & Mitchison 1997). As a consequence, mitotic spindles and interphase microtubules disappear. This results in cell cycle arrest, growth defects and, ultimately, in cell death.

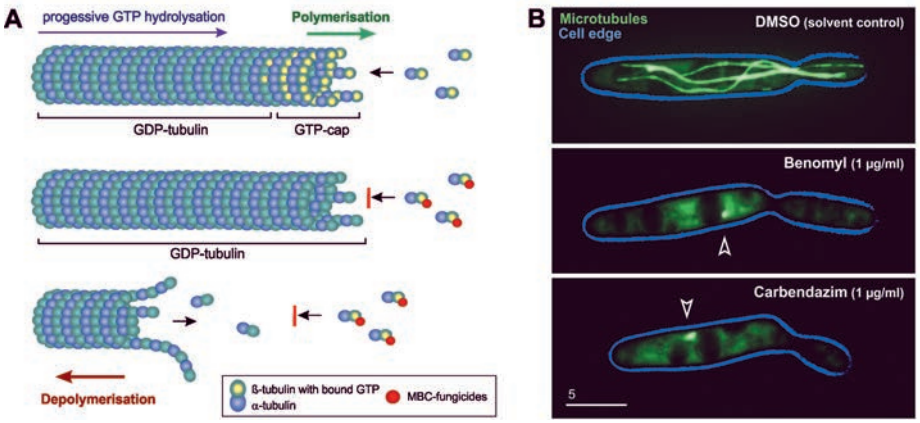

Figure 1 MoA of MBC-fungicides. (A) Schematic drawing of the effect of MBC-fungicides on microtubule dynamics. (B) Fluorescent microtubules in *U. maydis* cells, treated with the solvent dimethyl-sulfoxide (DMSO), 1 µg/ml benomyl and 1 µg/ml carbendazim for 30-45 minutes. Open arrowheads indicate short microtubule fragments. Scale bar is given in micrometres.

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Strikingly, live cell imaging of fluorescent tubulin reveals the MoA of MBC-fungicides in a single experiment. GFP-αtubulin expressing *U. maydis* cells, treated with the solvent dimethyl sulfoxide, contain long microtubules (Figure 1B, DMSO). After 30 minute exposure to 1 µg/ml benomyl or carbendazim, these microtubules disappear, and only short fragments remain in the cytoplasm (arrowhead, Figure 1B). It should be noted that the depolymerisation of microtubules is only one of several microtubule-related phenotypes possible. Depending on the nature of the anti-fungal compound, more stable and longer microtubules, as well as microtubule bundles may be seen. Thus, live cell imaging of fluorescent microtubules provides a rapid way of gaining insight into the MoA of microtubule-affecting fungicides.

Example 2: Dodine

Secondly, we chose to further illustrate the power of live cell imaging of FPMs by investigating the MoA of the fungicide 1-dodecylguanidinium acetate (dodine, FRAC code U12). This protectant fungicide is used to control black spot on apples, pears and roses, and leaf curl in nectarines and peaches. Dodine is a surfactant that likely inserts into membranes. Indeed, early reports in fungi (Brown & Sisler 1960; Somers & Fisher 1967; Somers & Pring 1966), including *U. maydis* (Solel & Siegel 1984), suggested that dodine perforates the plasma membrane and thus affects the integrity of the cell. On the other hand, an effect on metabolic and other fungal enzymes was suggested (Brown & Sisler 1960; Solel & Siegel 1984), suggesting that dodine enters the fungal cytoplasm. Indeed, work in *Neurospora crassa* concluded "enzyme inhibition or intracellular reaction appear to be the most probable hypotheses to explain the fungitoxicity of dodine" (citation taken from Somers & Fisher 1967). The FRAC code list 2016 reports the MoA of dodine as "not known" (http://www.frac.info/publications/), which reflects the seemingly contradictory results in these publications.

We applied various concentrations of dodine, solved in methanol (MetOH), to FPM-expressing strains of *U. maydis*. In a first set of experiments, we attempted to see an effect of dodine on the plasma membrane in a strain that expresses a fluorescent plasma membrane syntaxin Sso1 (Treitschke et *al.* 2010). In untreated cells, and in the MetOH solvent controls, this FPM localizes to the periphery of the cell (Figure 2A, 2B). With increasing concentrations of dodine, applied to shaking liquid cultures for 30-45 minutes at 28°C, the plasma membrane marker mis-localizes. This begins with the appearance of a spherical structure (Figure 2B, open arrowhead) that most likely represents the nuclear envelope, but ends in large deposits of the FPM in the cytoplasm at higher concentrations (Figure 2B, closed arrowhead, Figure 2C; both 50 µg/ml dodine). As the plasma membrane marker protein is an integral membrane protein, its concentration in the cytoplasm is likely accompanied by membrane accumulation. This indicates a failure of Sso1 targeting of Sso1-carrying vesicles that may fail to fuse with the plasma membrane.

An effect of dodine on the plasma membrane is also indicated by observation of a FBM that labels filamentous actin (F-actin; Schuster et *al.* 2012). This FPM localizes to actin patches,

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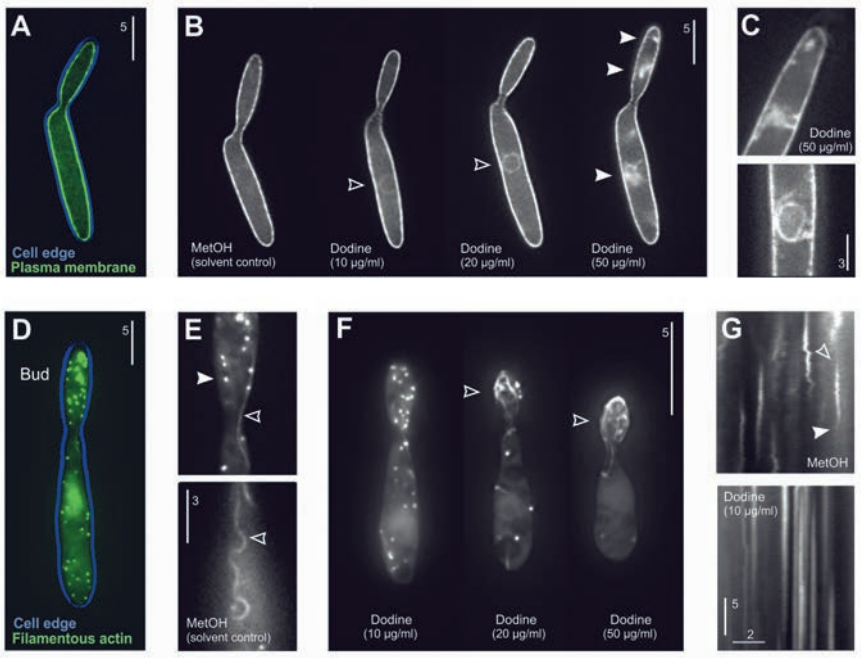

Figure 2 The effect of dodine on plasma membrane and F-actin dynamics and organization. (A) In untreated cells, a fluorescent marker for the plasma membrane localises to the cell periphery. (B, C) Localisation of the plasma membrane FPM in cells that were treated with the solvent methanol (MetOH) or increasing concentrations of dodine (treated for 30-45 minutes). Open arrowhead indicates the appearance of a spherical structure, closed arrowheads indicate large aggregates of the marker protein. (D) In untreated cells, a fluorescent marker for filamentous actin concentrates at the growing bud ("Bud"). (E) Higher magnification reveals that the FPM stains actin patches (closed arrowhead) and actin cables (open arrowheads), here shown in a methanol-treated cell (MetOH). Note that cables are located at the cell periphery. (F) Effect of increasing concentrations of dodine on actin organization in *U. maydis* cells. Note that actin patches disappear and actin cables collapse into the bud (open arrowheads). (G) Kymographs showing actin patch dynamics in methanol- (MetOH) and dodine-treated cells. In control cells, actin patches appear (open arrowhead) and disappears after a few seconds (closed arrowhead). This reflects the formation of endocytic vesicles. Dodine inhibits this dynamic behaviour, suggesting that it blocks endocytic uptake at the plasma membrane. Scale bars in A-F are given in micrometres. In G, horizontal bar indicates micrometres and vertical bar indicates seconds.

which are sites of endocytosis (Berepiki et *al.* 2011); Figure 2D, fluorescent "dots" in the growing bud; Figure 2E, closed arrowhead) and to filamentous actin cables (Figure 2E, open arrowhead), which support membrane trafficking driven by myosin-5 motors (Steinberg 2011). At low concentration of dodine (10 µg/ml), actin patches and cables are still visible (Figure 2F). However, these actin patches lose their dynamic behaviour, which is best visible in kymographs (Figure 2G). In this graphical representations, time is shown in vertical direction, whereas distance is indicated in horizontal direction. In MetOH-treated control cells, actin patches appear at the plasma membrane (Figure 2G, upper panel, open arrowhead). Here,

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they remain during the formation of an endocytic vesicle, but disappear after this vesicle has "pinched off" the plasma membrane and it moves into the interior of the cell (Figure 2G, closed arrowhead). In the presence of 10 µg/ml dodine, this dynamic behaviour is strongly impaired (Figure 2G, lower panel), indicating that initial endocytosis is blocked by the fungicide. This process occurs at the plasma membrane, suggesting that dodine affects the functionality of membrane-associated processes. Surprisingly, higher concentrations of dodine result in a collapse of actin cables into the growing bud of the FPM-expressing cells (Figure 2F, open arrows). Such reorganization of F-actin could also be due a defect at the plasma membrane, as membrane-associated proteins are known to anchor F-actin to the plasma membrane (Ponuwei 2016). However, further studies are needed to clarify if dodine affects the localization of such actin-binding and anchoring proteins at the plasma membrane.

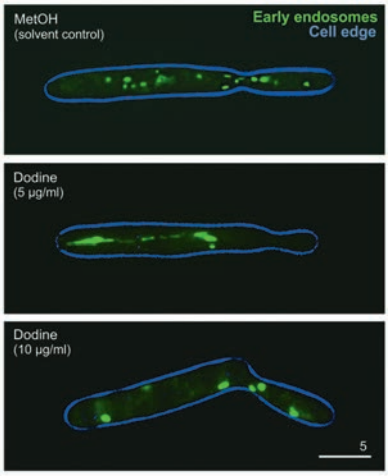

Figure 3 The effect of dodine on organelle organization. A fluorescent marker for early endosomes labels small punctuate organelles in methanol-treated control cells (MetOH). These early endosomes aggregate in the presence of low concentrations of dodine (treated for 30-45 minutes). Scale bars are given in micrometers.

An alternative explanation for the defect in actin cable organization could be that dodine interact with the actin cables directly. However, this would require that the charged dodine molecule passes through plasma membrane barrier and enters the fungal cytoplasm. Indeed, the observation of organelle-specific FPMs supports such a view. An effect on organelle organization is evident in dodine treated cells that express an early endosome-specific FPM. Here, low concentration of dodine induce clustering of the small organelles (Figure 3), suggesting that dodine enters the fungal cell. How the amphipathic dodine molecule crosses the plasma membrane is not evident from these experiments, but the suggested disruptive effect on the plasmas membrane may allow entry into the cell (Somers &

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Pring 1966). Clearly, more studies are needed to elucidate the MoA of dodine. However, these results illustrate the power of live cell imaging of FPMs as an important new approach to understand MoA of fungicides.

FPMS IN THE SEPTORIA TRITICI WHEAT BLOTCH FUNGUS *ZYMOSEPTORIA TRITICI*

Our collection of FPM expressing *U. maydis* strains derives from 17 years of research (Table 1). Their successful use in better understanding plant infection strategies and fungicide delivery mechanisms (see INTRODUCTION) prompted us to extend this approach to other plant pathogenic fungi. With funding from the British Biotechnology and Biological Sciences Research Council (BBSRC), we have recently established fluorescent proteins, such as mCherry, enhanced GFP and a codon-optimized GFP, as well as numerous FPMs in the wheat blotch fungus *Zymoseptoria tritici* (=*Mycosphaerella graminicola*; "Weizen-Blattdürre"); (Guo et *al.* 2015; Kilaru et *al.* 2015a; Kilaru et *al.* 2015b; Schuster et *al.* 2015a; Schuster et *al.* 2015b; Table 1). These FPMs allow co-visualization of different *Z. tritici* strains in axenic culture or inside the infected host plant (Figure 4). Microtubule-specific FPMs, such as the plus end-binding protein ZtPeb1-GFP (Schuster et *al.* 2015b), the minus end-binding protein ZtGrc1-GFP (Schuster et *al.* 2015b) and fluorescent α-tubulin proteins GFP-ZtTub2 and mCherry-ZtTub2 (Schuster et *al.* 2015b) provide insight into the organization of the cytoskeleton in yeast-like *Z. titrici* cells (Figure 5A). Moreover, polarity markers, specifically localizing to the polarisome (GFP-ZtSpa2; Guo et *al.* 2015), the exocyst (GFP-ZtExo70; Guo et *al.* 2015) or the Spitzenkörper (GFP-ZtMlc1, GFP-ZtSec4; Guo et *al.* 2015), reveal details of the organization of the growing hyphal tip in *Z. tritici* hyphae (Figure 5B). Our current focus is to complete this FPM collection (Table 1), which will allow the use of these FPM-expressing *Z. tritici* strains in fungicide MoA studies. This opens the unique opportunity to compare fungicide induced phenotypes between the corn smut and the Septoria tritici wheat blotch fungus.

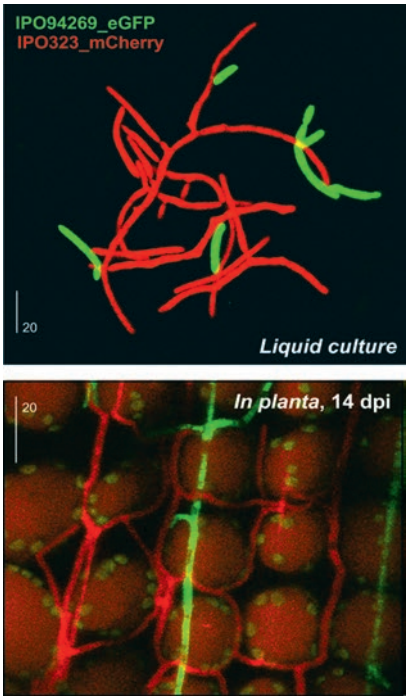

Figure 4: Co-visualisation of two strains of *Z. tritici*, both in liquid culture and inside infected plants (14 days after infection). Scale bars are given in micrometers.

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CONCLUSIONS

An important characteristic of fungicides is their MoA. This describes the biochemical, metabolic or anatomical change at the cellular level, caused by the fungicides (e.g. (Opalski et *al.* 2006), but it is also used to describe the molecular target of an antifungal (e.g. (Fernández-Ortuño et *al.* 2012). In fact, our knowledge of fungicide MoA is often restricted to either the molecular target, or the way the antifungal affects pathogen physiology. Good examples are SDHIs, which bind to subunits of succinate dehydrogenase, thereby inhibiting cellular respiration (e.g. Rehfus et *al.* 2016). Whilst this target is well-defined, the "physiological consequences on the level of the cell" (MoA) are not known. By contrast, MoA studies often provide incomplete insight, or even contradictory conclusions with certain fungicides. In this article, we summarised the MoA studies on dodine, where some studies report an effect of the fungicide on the fungal plasma membrane, resulting in increased cell permeability, whilst others report effects on enzyme activity, which are thought to underpin dodine's fungal toxicity (Brown & Sisler 1960; Solel & Siegel 1984; Somers & Fisher 1967; Somers & Pring 1966). However, these cited MoA studies were published between 1960's-1980's and were thus largely restricted by the available techniques of the time. Today, advances in genome sequencing, transciptional profiling, bioinformatics and powerful live cell imaging techniques of fluorescent markers for cellular structures in pathogenic fungi, such as *Z. tritici* open new avenues for MoA research. This promises unique and novel insights into fungicide MoA in pathogenic fungi, which will enhance our understanding of antifungal activity to inform development of sustainable disease.

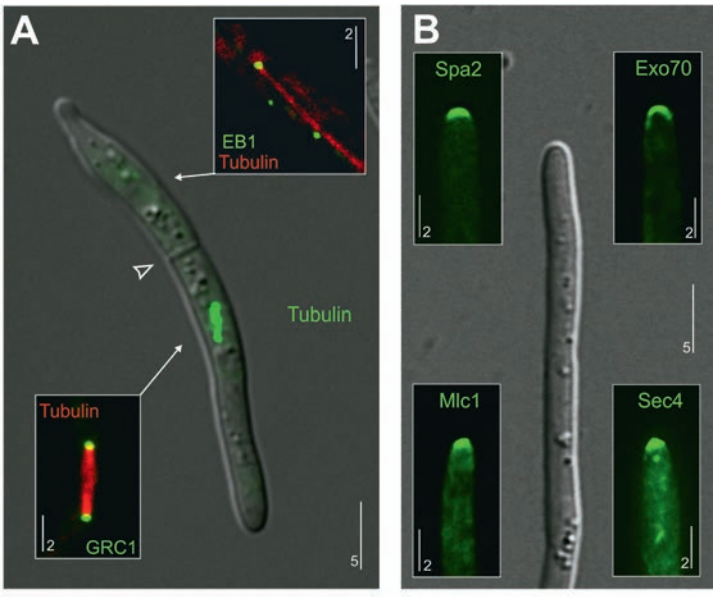

Figure 5 FPMs for visualization of microtubule structures in a yeast-like cell structure (A) and the polar growing tip of hyphae (B) in *Z. tritici*. Sale bars are given in micrometers.

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Table 1. Fluorescent protein markers for fungicide mode of action studies

Target	*U. maydis* FPMs	Reference	*Z. tritici* FPMs	Reference
1. Organelles				
Nuclear DNA	YES§	Theisen *et al.* 2008	YES	Kilaru *et al.* in prep.
Nuclear matrix	YES§	Straube *et al.* 2001	NO*	
Nuclear envelope	YES§	Theisen *et al.* 2008	NO*	
Peroxisomes	YES§	Steinberg & Schuster 2011	YES	Kilaru *et al.* in prep.
Mitochondria	YES	Steinberg & Schuster 2011	YES	Kilaru *et al.* in prep.
Vacuoles	YES§	Steinberg & Schuster 2011	NO*	
Autophagosomes	YES	unpublished	YES	Kilaru *et al.* in prep.
ER matrix	YES§	Wedlich-Söldner 2002	YES	Kilaru *et al.* in prep.
ER membranes	YES§	Adamikova *et al.*_2004	YES	unpublished
Early endosome	YES§	Fuchs *et al.* 2006	YES	Kilaru *et al.* 2015a
Late endosomes	YES	Higuchi *et al.* 2014	YES	Kilaru *et al.* 2015a
Recycling carriers	YES§	unpublished	YES	Guo *et al.* 2015
Secretory vesicles	YES§	Treitschke *et al.* 2010	YES§	Guo *et al.* 2015
Golgi apparatus	YES	Wedlich-Söldner *et al.* 2002	NO*	
Plasma membrane	YES§	Treitschke *et al.* 2010	YES§	Kilaru *et al.* in prep.
Lipid droplets	YES	Guimaraes *et al.* 2015	NO*	
Woronin body	n/a		YES	Kilaru *et al.* in prep.
2. Polarity markers				
Spitzenkörper	NO		YES	Guo *et al.* 2015
Polarisome	NO		YES	Guo *et al.* 2015
Exocyst	NO		YES	Guo *et al.* 2015
3. Cytoskeleton				
F-actin	YES§	Schuster *et al.* 2012	YES	Kilaru *et al.* in prep.
Actin patches	YES	Theisen *et al.* 2008	YES	Kilaru *et al.* 2015a
Microtubules	YES§	Steinberg *et al.* 2001	YES§	Schuster *et al.* 2015
Microtubule plus-end	YES§	Lenz *et al.* 2006	YES	Schuster *et al.* 2015
Spindle pole body	YES	Schuster *et al.* 2011	YES	Schuster *et al.* 2015
4. Miscellaneous				
Cytoplasm	YES§	Bielska *et al.* 2014	YES§	Kilaru *et al.* 2015b

§ fused to green and red fluorescent proteins; * in construction

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