Photosynthetic Declines in *Phytophthora ramorum*-Infected Plants Develop Prior to Water Stress and in Response to Exogenous Application of Elicitins

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ABSTRACT

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Phytophthora ramorum, causal agent of sudden oak death, is responsible for widespread oak mortality in California and Oregon, and has the potential to infect 100 or more species. Symptoms range from stem girdling and shoot blight to leaf spotting. In this study, we examined the physiological impacts of *P. ramorum* infection on *Rhododendron macrophyllum*. In stem-inoculated plants, photosynthetic capacity (V_{cmax}) significantly declined by $\approx 21\%$ 3 weeks after inoculation in visibly asymptomatic leaves. By 4 weeks, after the development of significant stem lesions and loss in water transport capacity, water stress led to stomatal

closure and additional declines in photosynthetic capacity. We also report the isolation, characterization, and biological activity of two *P. ramorum* elicitors. Both elicitors were capable of inducing a hypersensitive-like response in one incompatible (*Nicotiana tabacum* SR1) and three compatible hosts (*R. macrophyllum*, *Lithocarpus densiflorus*, and *Umbellularia californica*). Infiltration of leaves from all three compatible hosts with both *P. ramorum* elicitors caused significant declines in chlorophyll fluorescence (F_v/F_m). For all four species, the loss of photosynthetic capacity was directly proportional to H^+ uptake and ethylene production, two common components of the hypersensitive response. This is the first report of elicitors causing photosynthetic declines in compatible hosts independent of plant water stress.

Additional keywords: photosynthesis, toxin.

Sudden oak death (SOD), caused by the oomycete *Phytophthora ramorum*, was first reported in trees of North America in 1995, and threatens to radically alter the composition of North American forests (39). SOD has caused significant mortality in natural stands of several tree species, including *Quercus agrifolia* (coast live oak), *Q. kelloggii* (California black oak), *Q. parvula* (coast oak), and *Lithocarpus densiflorus* (tanoak) from central California to southern Oregon (40). However, this disease is not limited to forest situations, because a variety of ornamental species have been found to be suitable hosts from 12 plant families, including Aceraceae, Anacardiaceae, Betulaceae, Caprifoliaceae, Cupressaceae, Ericaceae, Fagaceae, Hippocastanaceae, Lauraceae, Pinaceae, Rhamnaceae, and Rosaceae (17), and the list keeps growing.

Symptoms of *P. ramorum* infection fall into three separate categories: (i) bole cankers that often lead to death, (ii) shoot tip dieback, and (iii) leaf spots or blight (20). Depending upon the host species, *P. ramorum* infection may develop one or more of these symptoms. For example, *L. densiflorus* (tanoak) exhibits all three symptoms, *Rhododendron macrophyllum* (rhododendron) develops shoot tip dieback or leaf blight, and *Umbellularia californica* (myrtle) is typified by leaf blight. The distribution of *P. ramorum* within host tissues normally is associated only with visibly symptomatic tissues and may be confined to particular tissues (stem or leaves) (21). SOD derives its name from the rapid transition from

green, nonsymptomatic crowns to brown, dead crowns in a period of weeks (18,33). However, despite its name, mortality associated with SOD in natural stands may take several years to develop, is largely limited to the red oak and tanoak, and is typified by a consistent sequence of signs and symptoms beginning with the development of bleeding bole cankers, followed by bark or ambrosia beetle infestation, and *Hypoxylon thouarsianum* infection (32). In the absence of the latter two pests, McPherson et al. (32) estimate that it may take 10 years or more to reach a 50% mortality rate, or just 3 years in their presence.

Infection of compatible plants by *Phytophthora* spp. often leads to a decline in stomatal conductance and photosynthesis (8,9,15,16,25,29,30,44), although the mechanistic basis for such declines is not completely understood. In many cases, declines in leaf gas exchange rates have been linked to losses in water supply capacity associated with root or xylem necrosis (8,9,15,16,41,42). However, the reductions in gas exchange may not be proportional to changes in hydraulic capacity (9,29), and may be observed in noninvaded regions (11), suggesting the presence of a toxin, or host-derived signal, that is responsible for some of the physiological impairment.

To date, a wide variety of secreted, translocatable effectors—pathogen molecules that manipulate host cell structure and function affecting pathogenicity and colonization—have been identified in a variety of *Phytophthora* spp. (24). Effectors may either facilitate infection (virulence factors or toxins) or limit infection (avirulence factors or elicitors), and differences in the timing or degree of host responses may cause the same effector to be a virulence factor in one host or an avirulence factor in another host (10). In *P. ramorum*, numerous effector genes (e.g., NPPI, PcF, Crn, elicitors, and so on) homologous to those found in other *Phytophthora* spp. have been identified in its genome (45).

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In the current study, we first conducted a series of experiments to determine whether toxins secreted by *P. ramorum* are likely contributors to physiological injury in the host by examining the temporal changes in photosynthesis, stomatal conductance, and hydraulic conductivity of *R. macrophyllum* artificially inoculated with *P. ramorum*. Second, we tested the ability of culture filtrates and purified, recombinant *P. ramorum* elicitors (i.e., the major protein secreted in *P. ramorum* grown in vitro) to induce physiological changes in incompatible (*Nicotiana tabacum*) and compatible (*L. densiflorus*, *R. macrophyllum*, and *U. californica*) host species.

MATERIALS AND METHODS

Plant material. All artificial inoculations assays were conducted on 3-year-old *R. macrophyllum* plants grown in 7.6-liter pots. The biological activity of purified *P. ramorum* elicitors was assessed on four plant species (*N. tabacum* SR1, *L. densiflorus*, *R. macrophyllum*, and *U. californica*). *N. tabacum* plants were ≈ 1 month old and grown in a greenhouse under ambient conditions, *L. densiflorus* plants were 2 years old and grown under ambient conditions in a coldframe at Oregon State University (Corvallis), and *U. californica* leaf tissue was harvested from an ornamental planting on the campus of Oregon State University.

Physiological response of *R. macrophyllum* to artificial inoculation. To determine whether toxins secreted from *P. ramorum* contribute to physiological injury in the host, two stems (≈ 2.5 cm in diameter) from each of 12 3-year-old *R. macrophyllum* plants were artificially inoculated with a 5-mm-diameter hyphal plug cut from ≈ 2 week old *P. ramorum* starter cultures (2% cornmeal agar) or uninoculated control plates, which was secured under the bark, ≈ 15 cm below the lowest leaf, using dH₂O-saturated gauze. The *P. ramorum* isolate used for inoculation was a North American mating type (A2) obtained from infected native plants growing in Curry County, OR. On a weekly basis (1 to 4 weeks after inoculation), A/C_i curves (net CO₂ assimilation over a range of CO₂ concentrations), stem-specific hydraulic conductivity, and stem lesion lengths were monitored.

A/C_i curves were measured on one leaf from each control and inoculated stem ($n = 6$) using an LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE) following the procedures outlined in Manter and Kerrigan (27). The A/C_i curves were used to estimate one of the biochemical components (the maximum rate of carboxylation limited by the amount, activity, and kinetics of rubisco [V_{max}]) contributing to photosynthetic capacity using the equations of Farquhar et al. (13). In addition, stomatal conductance (g_s) of water vapor at ambient CO₂ (40 Pa) also is reported.

Stem-specific hydraulic conductivity (K_s), a measure of water transport capacity, was measured on a 3-cm segment cut from a nonsymptomatic region immediately above the inoculation site of each control and inoculated stem ($n = 3$) using the procedures outlined in Manter and Kavanagh (26). The same seedlings ($n = 3$) destructively sampled for K_s were used for measurements of lesion lengths (in centimeters) by recording discolored phloem tissue after removal of bark tissue.

Verification of *P. ramorum* infection within the tissues of the artificially inoculated *R. macrophyllum* plants was determined by direct plating on a *Phytophthora* spp.-selective medium (pimaricin-ampicillin-rifampicin-pentachloronitrobenzene agar, [PARP]) (12). On each sample date, 1-cm² pieces ($n > 3$) from symptomatic (lesion margin) and nonsymptomatic phloem tissues and the leaves used for gas exchange analysis were plated on PARP media, incubated in the dark at 20 to 22°C, and examined daily over the course of 3 weeks for *P. ramorum* emergence.

Culture filtrate protein isolation. *P. ramorum* culture filtrates (CFs) were collected by vacuum filtration (0.2 μm PES membrane vacuum filter; VWR, West Chester, PA) from three North American (mating type A2) isolates obtained from infected native plants

growing in Curry County, OR, and three European (mating type A1) isolates obtained from infected ornamental nursery plants in Clackamas, OR, grown in glucose-yeast-peptone (GYE) broth for 30 days. Proteins contained in the filtrate were concentrated and washed with 3 volumes of double-distilled (dd)H₂O using a Viva-cell 200 filtration column with a 5,000 MWCO PES membrane (Sartorius, Edgewood, NY). CF proteins were separated by ion exchange spin columns (Vivapure S mini; Sartorius) using a Tris-Cl (pH 6.0) buffer system and analyzed on a 12% Tris-glycine gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All six *P. ramorum* isolates secreted two major proteins (≈ 8 and 10 kDa) that were purified by ion chromatography (Fig. 1). The two proteins were excised from the SDS-PAGE gels, digested with trypsin, analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry MALDI TOF/TOF, and tentatively identified by the Mascot homology search engine (Macromolecular Resources, Colorado State University, Fort Collins). In all six isolates, the 8-kDa protein exhibited high homology to ubiquitin and the 10-kDa protein was similar to the elicitor of *P. cinnamomi* (Table 1). Similarly, the presence of ubiquitin and an elicitor in the CF of *P. infestans* also was observed by Huet et al. (22).

Cloning and expression of *P. ramorum* elicitors. A search of the *P. ramorum* genome project (DOE Joint Genome Project, United States Department of Energy, Walnut Creek, CA) revealed five sequences coding for recognizable elicitor proteins (protein

Fig. 1. Proteins present in the culture filtrate of a typical *Phytophthora ramorum* isolate grown in glucose-yeast-peptone media. The two major proteins of the culture filtrate were separated by ion affinity chromatography (Vivaspin S column) and analyzed on a 12% Tris-glycine gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1: size standard, lane 2: fraction containing the 10-kDa elicitor protein, and lane 3: fraction containing the 8-kDa ubiquitin protein.

Chlorophyll fluorescence. The effect of elicitors on photosynthetic performance was assessed by measuring chlorophyll fluorescence (F_v/F_m) on dark-adapted leaves to determine the efficiency of open PSII centers, which is a sensitive measure of photosynthetic performance (31). Cut stems from all four test species were placed in test tubes containing 10 ml of a 100-nM elicitor solution or a control and incubated in a growth chamber set to maintain air temperatures of 25 and 18°C (day and night, respectively) and a 16-h photoperiod. After a 48-h incubation period, chlorophyll fluorescence was measured using an OS1-FL fluorometer (Optosciences, Tyngsboro, MA) from one leaf per stem following a 30-min dark adaptation.

Statistical analyses. Significant differences between species and *P. ramorum* elicitors were tested by analysis of variance and Holm-Sidak post-hoc tests (SigmaStat 3.1). The Holm-Sidak post-hoc test applies a “step-down” critical *P* value approach in determining significance to maximize statistical power without compromising the risk of making a type-I error (19). For comparison, the control in the CF-elicitor assays was uninoculated GYP

media subjected to the same purification procedures outlined above (see “Culture filtrate protein isolation”). In the assays involving the recombinant elicitors (ram- α 1 and ram- α 2), the control was a 100 nM solution of the SUMO-CAT fusion protein (Invitrogen) generated using the same purification procedures outlined above. All experiment–assay combinations were replicated twice with similar results. All reported values are the arithmetic mean and individual standard errors from the first replication of each experiment.

RESULTS

All artificial inoculations of *R. macrophyllum* were successful, resulting in an average lesion length of 6.9 ± 0.9 cm by the end of the 4-week study. Reisolation of *P. ramorum* was 100% successful from all symptomatic stem tissues, but not from any of the asymptomatic stem or leaf tissues (data not shown). Physiological changes developed rapidly in leaves of the inoculated stems, despite the lack of visible symptoms in the leaves or petiole. Three weeks after inoculation, when stem lesion lengths were 4.4 ± 0.6 cm, V_{cmax} was reduced by $\approx 21\%$ (Fig. 3A). Additional declines occurred during the fourth week, after the development of significant impacts on plant–water relations.

The functionality of *P. ramorum* infected stems to supply water to host leaves and maintain photosynthetic rates was assessed from K_s and g_s measurements. Four weeks after inoculation, but not before, both measures declined; g_s , a measure of stomatal openness, declined by 36% (Fig. 3B), and K_s , a measure of xylem water supply capacity, declined by 64% (Fig. 3C). A CF-derived elicitor from *P. ramorum* was purified and tested for its ability to influence leaf processes. Similar to the artificial inoculation experiment, the CF-elicitor caused a significant decline of 23.4% in photosynthetic capacity (V_{cmax}) and 14.8% in the efficiency of open PSII centers (F_v/F_m) (Table 2). Two components often associated with the hypersensitive response (HR), H^+ uptake and ethylene production, also were influenced by elicitor uptake, changing by 78.8 and 92.4%, respectively (Table 2).

The genome database of *P. ramorum* contains five gene models that encode for mature 10-kDa elicitor protein (protein ID: 47376, 47381, 47386, 40636, and 78569). However, it is unknown which one or more of these elicitors are secreted into the CF. Therefore, we used a polymerase chain reaction (PCR)-based approach to isolate and clone full-length *P. ramorum* elicitor genes, which then could be tested for biological activity. Each primer set amplified one unique product (GenBank accession no. DQ680026 and DQ680027) that was identical for all six isolates tested (data not shown). The predicted amino acid sequences are shown in Figure 2. Although there are five different elicitor gene models in the *P. ramorum* genome database, based on our results and the high homology between sequences, it is likely that only two class-I

Fig. 3. A, Maximum rate of carboxylation limited by the amount, activity, and kinetics of rubisco (V_{cmax}), B, stomatal conductance (g_s), and C, stem-specific hydraulic conductivity (K_s) of *Rhododendron macrophyllum* artificially inoculated with *Phytophthora ramorum*. Each observation is the mean and standard deviation of six plants, from which one control and one inoculated leaf were measured.

TABLE 2. Influence of a *Phytophthora ramorum* culture filtrate elicitor (CF-elicitor) on various leaf physiological parameters in *Rhododendron macrophyllum*

Parameter ^a	Treatment ^b		Change (%) ^c
	Control	CF-elicitor	
V_{cmax}	48.1 ± 1.8	36.8 ± 1.7	–23.4*
F_v/F_m	0.813 ± 0.003	0.693 ± 0.005	–14.8*
H^+ uptake	2.8 ± 1.3	13.2 ± 2.5	78.8*
Ethylene	11.2 ± 1.7	148.2 ± 5.9	92.4*

^a Maximum rate of carboxylation limited by the amount, activity, and kinetics of rubisco (V_{cmax} ; $\mu\text{mol m}^{-2} \text{s}^{-1}$) and chlorophyll fluorescence (F_v/F_m) were assayed on cuttings (≈ 1 cm in diameter), whereas H^+ uptake ($\mu\text{mol m}^{-2} \text{h}^{-1}$) and ethylene production (ppb) were assayed on leaf discs immersed in a CF-elicitor solution (100 nM) or a control (uninoculated glucose-yeast-peptone media).

^b Mean $\pm$ standard error for six plants per treatment.

^c Asterisk denotes significant difference (Holm-Sidak post-hoc test, $P < 0.05$).

Fig. 4. A, Dark-adapted chlorophyll fluorescence (F_v/F_m), B, H^+ uptake, and C, ethylene production in leaves of four plant species exposed to 100 nM *Phytophthora ramorum* recombinant elicitors (ram- $\alpha 1$ and ram- $\alpha 2$) or a control (100 nM SUMO-CAT fusion protein). Each observation is the mean and standard error ($n = 6$). Tobacco = *Nicotiana tabacum*; Tanoak = *Lithocarpus densiflorus*; Myrtle = *Umbellularia californica*; Rhode = *Rhododendron macrophyllum*. Bars with the same letter are not significantly different using a Holm-Sidak post-hoc test ($P > 0.05$).

elicitor genes are present in the *P. ramorum* genome. For the three ram- $\alpha 1$ homologs (47381, 47386, and 78569), proteins 47381 and 78569 are identical to ram- $\alpha 1$; whereas, the gene sequence of protein 47386 differs from ram- $\alpha 1$ by only one nucleotide, resulting in an amino acid substitution at position 93 (threonine [T]). For the two ram- $\alpha 2$ homologs (47376 and 71636), protein 71636 is identical to ram- $\alpha 2$; whereas, the gene sequence of pro-

tein 47376 differs from ram- $\alpha 2$ by only two nucleotides, resulting in amino acid substitutions at positions 61 (lysine [K]) and 65 (threonine [T]).

The two cloned elicitor genes were expressed using a prokaryotic expression system, and the purified recombinant elicitors were tested for biological activity in both compatible and incompatible hosts. Both recombinant elicitors produced a visible HR and developed necrotic areas when infiltrated into leaves of the incompatible host, *N. tabacum*; however, no macroscopically visible necrosis was observed in any of the three compatible hosts (data not shown). Independent of the development of visible necrosis, the recombinant elicitors significantly affected a variety of physiological characteristics of all four host species. In all species, exposure to recombinant elicitors caused a decline in the maximum efficiency of PSII centers or F_v/F_m (Fig. 4A), while enhancing H^+ uptake (Fig. 4B) and ethylene production (Fig. 4C) relative to the controls. Thus, for all treatment combinations (elicitor and host species), the decline in F_v/F_m was strongly and positively correlated to H^+ uptake (Fig. 5A) and ethylene production (Fig. 5B). As in the CF tests, tobacco exhibited the greatest responses, followed by tanoak, myrtle, and rhododendron. For all three measures (Fig. 4), ram- $\alpha 1$ triggered significantly greater responses compared with ram- $\alpha 2$, except in rhododendron, and in tanoak F_v/F_m .

DISCUSSION

Artificial inoculation of *R. macrophyllum* with *P. ramorum* led to significant declines in photosynthesis, g_s , and water supply capacity. The temporal changes in these measures suggest that physiological impacts occur in two distinct phases. The first stage occurs prior to detectable changes in water supply capacity, and the second, additional decline occurs only after a reduction in stem-specific hydraulic capacity. The second phase of impact has been shown repeatedly for a variety of *Phytophthora* spp. pathosystems and is best explained by the loss of water supply capacity associated with root or shoot necrosis (8,9,15,16,41,42). The first stage, independent of significant changes in water supply capacity, is consistent with previous studies (9,29,30) and is suggestive of the presence of a toxin or a host-derived signal.

Although toxins have been suggested to play a role in *Phytophthora* spp. pathosystems, previous efforts to document elicitor toxicity in compatible hosts have met with varying degrees of success. For example, elicitor exposure did not influence g_s in chestnut (30) or net photosynthesis in beech (15). However, ultrastructural changes in oak (5) and pepper (23) and varying degrees of necrosis or cell apoptosis have been observed in several Solanaceae plants (35,46) and the rubber tree (6). Based on these observations and those of the current study, a wide range of host responsiveness to elicitors is possible. Although the mechanistic basis for the observed photosynthetic declines was not fully explored in this study, we hypothesize that it is associated with an incomplete or hypersensitive-like response. In part, this hypothesis is based on the strong correlation between the decline in F_v/F_m and two processes typically associated with HR: H^+ uptake ($R^2 = 0.801$) and ethylene production ($R^2 = 0.884$). To date, the vast majority of work with elicitors has focused on their ability to induce the HR and systemic acquired resistance in incompatible hosts such as tobacco (2,38). Furthermore, both artificial inoculation (43) and elicitor exposure (28) result in photosynthetic declines in incompatible hosts. Part of this decline surely arises from the death of functional mesophyll cells during a successful HR. However, Scharte et al. (43) recently showed that a successful HR requires the suppression of photosynthesis, associated with callose deposition or sugar accumulation, before HR cell death can be initiated. Thus, it follows that host differences in the degree of the HR response to elicitors (i.e., highest in resistant species) could be the source of the observed photosynthetic de-

Fig. 5. Linear relationship between the relative changes in chlorophyll fluorescence of dark-adapted leaves chlorophyll fluorescence (F_v/F_m) and **A**, H^+ uptake or **B**, ethylene production. F_v/F_m is percent change in recombinant elicitin-treated tissues (100 nM ram- α 1 or ram- α 2) relative to a control (100 nM SUMO-CAT fusion protein) and H^+ and ethylene are the absolute difference between the treated and control. Points represent the eight recombinant elicitin and host species combinations.

clines (i.e., highest in resistant species) in response to elicitin infiltration. Consistent with this hypothesis, Vleeshowers et al. (46) examined HR cell death in several *Solanum* clones and found a high degree of variation in the timing and degree of HR cell death, which was correlated with resistance to *P. infestans*. Finally, the notion of an effector triggering HR-like processes in both compatible and incompatible hosts is supported by other studies. For example, the NPP1 effector from *Phytophthora* spp. induces typical HR-associated (ethylene accumulation, callose deposition, and necrosis) and systemic-acquired resistance-associated (pathogenesis-related gene accumulation) processes in both compatible and incompatible host species (14,37).

In all species, except rhododendron, ram- α 1 consistently showed greater biological activity compared with ram- α 2. Comparisons of the biological activity of a number of other *Phytophthora* elicitins suggests a general relationship between protein charge (pI) and activity. In particular, greater activity has been observed for the basic elicitins (pI > 5) compared with the acidic elicitins (pI < 4) (35). Bourque et al. (3) suggest that this relationship may be explained by subtle differences in elicitin diffusion at physiological pH in the negatively charged cell wall.

Although neither of the two *P. ramorum* elicitins used in this study are basic elicitins (pI > 5), greater activity was observed for the relatively more basic of the two *P. ramorum* elicitins (pI of 3.84 and 3.13 for ram- α 1 and ram- α 2, respectively). Alternatively, Qutob et al. (36) compared the activity of nine elicitins from *P. sojae* and suggested that differences in elicitin activity may be related to their sterol-loading capacity. For example, elicitins with fewer amino acids interspersing the conserved cysteine residues (Fig. 2), which would result in a reduced sterol-binding tunnel (1), did not induce an HR response in tobacco. Although all known class I elicitins, including *P. ramorum*, have similar spacing between cysteine residues, subtle differences in sterol-loading capacity, perhaps related to pI, could affect their biological activity (3,4,34,47).

Although the current study shows the effect on exogenously applied elicitins, further studies are needed to explore the temporal and spatial patterns of *P. ramorum* elicitin expression during the infection cycle. For example, Colas et al. (7) showed that *P. parasitica* elicitin expression may be downregulated early on during the course of infection (3 days after infection), possibly

to avoid a successful HR that is capable of limiting colonization. However, they did not follow elicitin expression throughout the full infection process and examined only one of the potential elicitin genes found in *P. parasitica*, parA1. In another study, Brummer et al. (5) used an enzyme-linked immunosorbent assay to show that *P. quercina* produced an elicitin, quercinin, in infected oak tissues during its entire growth phase. The pathogen- or host-derived factors regulating elicitin production by *Phytophthora* spp. are unknown and deserve further research. It is possible that changes in elicitin expression or perception by the host are one of the greatest contributors to host variation in *Phytophthora* spp. resistance and pathogenicity. In our laboratory, we currently are using a reverse-transcriptase PCR assay to follow ram- α 1 and ram- α 2 expression throughout the infection process in a variety of host species.

In conclusion, we have shown that exogenous application of elicitins results in photosynthetic declines in both compatible and incompatible hosts. The mechanism responsible for the declines is unknown but arguably may be associated with quantitative differences in the timing and degree of the HR. In the compatible hosts studied here, a general correlation between *P. ramorum* colonization (21) and host responsiveness to *P. ramorum* elicitins is observed. Given the wide distribution of *P. ramorum* (more than 100 host species) and variety of disease forms, differences in elicitin expression, and perception by the host, may be one factor leading to this variation. However, because many other known effectors are contained in the *P. ramorum* genome, it is likely that host resistance and *P. ramorum* pathogenicity will be dependent upon the entire expression profile of *P. ramorum* effectors and host variations in responsivity to these effectors.

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