Development of Laboratory Resistance to Metalaxyl in *Phytophthora citricola*

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ABSTRACT

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Isolates of *Phytophthora citricola* resistant to the systemic fungicide metalaxyl were obtained by treating zoospores with ultraviolet radiation or the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Both methods produced similar numbers of resistant mutant isolates from a total of $\sim 8.9 \times 10^7$ zoospores. In vitro resistance was exhibited by 22 isolates that had growth rates indistinguishable from the parent isolate, and five of these isolates were also resistant in vivo. The stability of metalaxyl resistance was tested by using single-zoospore isolates of the resistant strains cultured for >20 generations in the absence of the fungicide. Three single-spore isolates proved to be extremely stable, both in vitro and in vivo. In vivo resistance to metalaxyl was associated with cross-resistance to four acylanilide fungicides: benalaxyl, RE 26745, cyprofuram, and oxadixyl. However, these resistant isolates remained sensitive to the chemically unrelated fungicide fosetyl-Al. The competitive fitness in vivo of one resistant mutant isolate was high as only that isolate was recovered from stem lesions that developed on seedlings of *Persea indica* previously inoculated with zoospores in the ratio of 1:1 of the parent and mutant isolates.

The fungicide metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl]alanine methyl ester, Ridomil®) is widely used as a systemic fungicide to control many soilborne pathogenic fungi of the taxonomic order Peronosporales (14). Since metalaxyl may persist in soil for several months (1,12), there is the possibility that resistant fungal strains may develop due to the duration of the selection pressure (15). In fact, the occurrence of metalaxyl-resistant field isolates of the foliar pathogen *Phytophthora infestans* (Mont.) de Bary has been reported in Holland (8), Ireland (10), and Israel (5). Metalaxyl-resistant strains of *P. capsici* Leonian and *P. megasperma* Drechsler have also been generated in the laboratory by utilizing either mass selection (2), ultraviolet (UV) irradiation (3), or chemical mutagenesis treatments (7). The UV and chemical mutagenesis procedures probably cause increases in the frequency of genetic damage and DNA replication errors, which are also the basis of spontaneous mutations, thus making such laboratory systems important in investigating the problem of fungicide resistance (7).

In this study, *P. citricola* Sawada, a species known to be relatively resistant to metalaxyl in vitro (4), was exposed to either UV irradiation or chemical mutagenesis treatments to determine the degree of metalaxyl resistance that would develop. Mutant isolates of *P. citricola* were then tested for the stability of their metalaxyl-resistance, their cross-resistance to other acylanilide fungicides, and for their fitness in competition with the wild-type isolate.

MATERIALS AND METHODS

Fungicides. The following fungicides were utilized: metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl]alanine methyl ester, technical grade and Ridomil 25WP), supplied by CIBA-Geigy Corp., Greensboro, NC; benalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[phenylacetyl]alanine methyl ester, Galben® 2E), provided by Gruppo Montedison, Milano, Italy; RE 26745 (2-methoxy-*N*-[2,6-

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dimethylphenyl]-*N*-[tetrahydro-2-oxo-3-furanyl]-acetamide, 50WP), supplied by the Chevron Chemical Co., Richmond, CA; oxadixyl (2-methoxy-*N*-[2-oxo-1,3-oxazolidin-3-yl]-acet-2′,6′-xylidine, 75WP), supplied by Sandoz Inc., San Diego, CA; cyprofuram (3-chloro-*N*-[2-oxoperhydro-3-furyl-cyclopropane-carboxanilide; Vinicur® 25WP), supplied by NOR-AM Agricultural Products, Naperville, IL; and fosetyl-Al (aluminum tris-*O*-ethyl phosphonate; Aliette® 80WP), supplied by Rhône-Poulenc Chemicals, Monmouth Junction, NJ.

Chemical mutagen. *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) was supplied by Aldrich Chemical Co., Milwaukee, WI.

The isolate of *Phytophthora*. *P. citricola* isolate P 1273 was originally isolated in 1981 from a trunk canker on an avocado tree located in a grove at Vista, San Diego County, CA. Zoospores were obtained by growing the organism on cleared V8-juice agar (V8C) at 24 C for 3–4 days in 90-mm-diameter petri plates. A 5-mm-diameter cork borer was used to cut disks from each plate which were placed (20 per 90-mm plate) in 15 ml of 20%-cleared V8 broth and grown for 1 day at 24 C. The mycelial mats were rinsed three times, placed in 15 ml of sterile soil extract (10 g of soil per liter of water), and incubated 2–3 days at 24 C to induce sporangium formation. To initiate zoospore release, the mats were placed in sterile demineralized water and chilled at 4 C for 20 min. The zoospore suspension was collected by filtration through a 15-cm-diameter, sterile, fluted filter paper (grade 515; Eaton-Dikeman, Mount Holly Springs, PA), that had been fastened over a sterile 400-ml beaker.

Mutagenesis procedures. Chemical mutagenesis of isolate P 1273 was induced with a 15-min exposure to 30 μg of MNNG per milliliter carried out in closed 90-mm-diameter petri plates according to the method of Davidse (7). UV mutagenesis was performed with a portable UV lamp (George W. Gates and Co., Franklin Square, Long Island, NY) at a wavelength of 254 nm by irradiating suspensions of cysts in open 90-mm-diameter petri plates for 30 sec at a distance of 20 cm. Cysts in the UV mutation were overlaid with V8C agar amended with 100 μg of metalaxyl (technical grade) per milliliter; with MNNG, 500 μg of metalaxyl per milliliter was used. As a control, cysts that were not exposed to a mutagen were also overlaid with V8C agar containing metalaxyl. With both mutagenesis methods, 100 μg/ml vancomycin and 5 μg/ml rifampicin were added to the media to control possible bacterial contamination. Plates were placed at 24 C to allow for the development of colonies on the agar surface. At this time, the plates were overlaid with ~10 ml of cooled, molten V8C agar amended with 1,000 μg of metalaxyl per milliliter. A 5-mm-diameter plug was taken from each fungal colony that grew to the surface of the agar and transferred to fresh V8C agar containing 1,000 μg of metalaxyl per milliliter and grown for 2 wk at 24 C. Finally, the radial growth of all transferred isolates was measured. The most vigorously growing isolates had a radial growth of at least 28 mm, identical to that of the parent isolate P 1273, and were saved for in vivo testing. The remaining isolates were discarded.

Screening for pathogenicity and in vivo resistance. The pathogenicity and in vivo resistance to metalaxyl of the mutant isolates of *P. citricola* were determined by inoculating 2-mo-old, bare-rooted seedlings of *Persea indica* L. with a zoospore suspension. For each isolate, two 355-ml Styrofoam cups, one containing 200 ml of demineralized water and the other 200 ml of an aqueous solution of 10 μg a.i. of metalaxyl (Ridomil 25WP) per milliliter, were each inoculated with a suspension of ~10^6 zoospores, and three seedlings of *P. indica* were placed in each cup. After 1 wk, the plants were examined for the presence of stem cankers caused by *P. citricola*.

Characterization of resistant isolates. The degree of in vitro resistance of the mutant isolates was determined by measuring the radial growth of the mycelium on cornmeal agar containing 10 and 100 μg/ml of metalaxyl. Resistance in vivo was determined using a concentration series of 10, 100, and 250 μg a.i. of metalaxyl (Ridomil 25WP) per milliliter. Two hundred milliliters of each fungicide solution were placed in styrofoam cups each containing three seedlings of *P. indica*. Each cup was inoculated with ~10^5 zoospores, and the plants were examined after 10 days for the presence of stem cankers.

The stability of the resistant trait was examined over 20 generations of transfer on agar medium utilizing two single-spore isolates of each resistant strain and the parent isolate P 1273. Each generation was grown at 24 C on cornmeal agar in the absence of metalaxyl. The extent of in vitro growth inhibition was estimated at generations 0 and 20 by measuring the radial growth of the isolate on cornmeal agar amended with 10 and 100 μg of metalaxyl per milliliter after 4 days. Isolates from generations 0 and 20 were examined in vivo for their ability to infect 2-mo-old seedlings of *P. indica*. Five 355-ml Styrofoam cups, each containing three seedlings of *P. indica*, 200 ml of water, and ~4×10^5 zoospores were used for each single-spore isolate. After 24 hr, metalaxyl (Ridomil 25WP) was added to give a concentration series of 0, 10, 100, and 250 μg a.i./ml. Eleven days after inoculation, the stem of each seedling of *P. indica* was sliced into 15 1-cm segments and plated on PARP medium (11) modified by substituting 125 μg of ampicillin trihydrate per milliliter for ampicillin sodium. The percentage of segments infected with *P. citricola* was determined after 2 days of incubation at 24 C.

Cross-resistance. Cross-resistance was tested to four acylanilide fungicides: benalaxyl, RE 26745, cyprofuram, and oxadixyl, and to the ethyl phosphonate fungicide fosetyl-Al. Seedlings of *P. indica* in Styrofoam cups containing either 10 μg a.i. of metalaxyl per milliliter; 50 μg a.i. of RE 26745, benalaxyl, and cyprofuram per milliliter; 100 μg a.i. of oxadixyl per milliliter; or 500 μg a.i. of fosetyl-Al per milliliter were inoculated with ~10^6 zoospores of either the parent isolate, or one of three mutant isolates. After 9 days, the stem of each seedling was sliced into 15 1-cm segments which were plated on a PARP medium. The percentage of stem segments infected with *P. citricola* was determined after 2 days of incubation at 24 C.

Fitness of a metalaxyl-resistant isolate. The competitive fitness of a resistant isolate was investigated by testing strain M1. Seven 355-ml Styrofoam cups each containing three seedlings of *P. indica* in 200 ml of demineralized water were inoculated with ~1.3×10^5 zoospores in ratios of 1:0, 1:9, 1:3, 1:1, 3:1, 9:1, 0:1 of the parent isolate P 1273 and strain M1, respectively. Two weeks after inoculation, the seedlings were cut into 1-cm segments and plated on PARP medium. After 3 days of incubation at 24 C, five 8-mm-diameter disks were randomly cut from the recovered colonies and arranged in a small circle on V8C agar. After 4 days of incubation at 24 C, an ~8-cm-diameter mixed colony was obtained. Twenty 5-mm-diameter disks were cut from the margin of each mixed colony and placed on a 90-mm-diameter petri disk with 15 ml of 1/5 diluted V8C broth. After incubation overnight at 24 C, the disks were rinsed in sterile water, placed in 15 ml of sterile 1% soil extract and incubated for 3 days at 24 C to induce the formation of sporangia. Zoospores were released by chilling the disks at 4 C for 30 min. Ten single-zoospore colonies were obtained from each zoospore ratio by streaking 1 ml of 10^3 spores per milliliter across a V8C plate and removing colonies after 12 hr by using a transfer needle and dissecting microscope. Single-spore colonies were grown at 24 C for 4 days. Two 5-mm-diameter plugs were then cut from the margin of each culture and transferred to V8C agar amended with 100 μg of metalaxyl per milliliter. Radial growth was measured after 4 days to determine the recovered ratio of P 1273 to Ml colonies based on their in vitro response to metalaxyl.

RESULTS

Development of resistant isolates. The parent isolate P 1273 grew poorly on V8C agar amended with 100 μg of metalaxyl per milliliter and failed to grow at all on V8C agar containing 500 μg/ml. No mutant isolates were obtained in three experiments by mass selection in the absence of a mutagenic agent. Both methods of mutation, UV and MNNG, produced approximately the same number of resistant isolates (Table 1). Mutant isolates that grew through V8C agar amended with 1,000 μg of metalaxyl per milliliter were transferred to the same agar medium. After 2 wk, the majority of the isolates had a radial growth of 15–27 mm; ~17% of the isolates had grown $<$ 14 mm; and ~7% of the isolates had grown

≥28 mm (Table 1). The latter, most vigorous, group was tested further for in vivo resistance. Five isolates proved to be resistant in vivo and were saved for further study. Two of the MNNG-induced isolates were eventually excluded; one isolate lost its in vivo resistance, while the other lost its pathogenicity after 4 mo of usage.

In vitro resistance to metalaxyl. An original isolate U1, plus the single-spore isolates Ml-1 from a MNNG-induced mutant M1 and U2-1 from a UV-induced mutant U2, exhibited similar in vitro mycelial growth responses to lower concentrations of metalaxyl. Their responses, however, were different at higher concentrations. The EC_{50} values in micrograms per milliliter (actual ingredient) for growth inhibition due to metalaxyl for isolates U1, M1-1, and U2-1 were 505 ± 26, 252 ± 57, and 231 ± 89, respectively (Fig. 1, Table 2). Isolate U2-1 had the highest EC_{90} value of 26,182 ± 12,147 μg/ml (Table 2).

Stability of resistance. Two single-spore isolates of the parent strain and each of the selected mutant isolates were examined after 0 and 20 transfers in vitro for differences in their in vitro and in vivo resistance. An analysis of variance showed that while the two single-spore isolates of each UV mutant reacted similarly to 100 μg of metalaxyl per milliliter in vitro at generation 0, they had diverged significantly by generation 20; the single-spore isolates of the MNNG mutant had not (Table 3). Both single-spore isolates from mutant M1 and one single-spore isolate U2-2 maintained their levels of in vivo resistance to metalaxyl, while the other UV-induced single-spore isolates exhibited a loss of resistance over 20 generations of culturing in vitro on cornmeal agar (Table 4).

Cross-resistance. Resistance to metalaxyl of the mutant isolates was associated with resistance to four other acylanilide fungicides that were tested: benalaxyl, RE 26745, cyprofuram, and oxadixyl (Table 5). The exception was isolate U1 which did not exhibit cross-resistance to benalaxyl. All three resistant isolates were controlled by 500 μg of fosetyl-Al per milliliter (Table 5).

Competitive fitness of a resistant isolate. When the initial inoculum ratio was 1:1, only the resistant isolate M1 was recovered from stem cankers on seedlings of *P. indica* (Table 6). However, the metalaxyl-resistant isolate M1 was not recovered at a ratio of 9:1 of the parent to the mutant isolate (Table 6).

TABLE 1. Mutation rate of *Phytophthora citricola* obtained in three different experiments utilizing either UV radiation or the chemical mutagen MNNG[a]

Mutagen	Total zoospores used (no.)	In vitro resistant isolates recovered (no.)[b] Radial growth 5–14 mm	Radial growth 15–27 mm	Radial growth >28 mm	In vivo resistant isolates recovered (no.)[c]
UV	2.7×10^7	19	104	6	2
MNNG	3.6×10^7	17	70	8	1
MNNG	2.6×10^7	20	77	8	2

[a] *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.
[b] Isolates that grew through cleared V8-juice agar amended with 1,000 μg/ml metalaxyl were transferred onto the same medium and radial growths were measured after 2 wk of incubation at 24 C.
[c] Isolates that grew >28 mm were tested for in vivo resistance.

TABLE 2. In vitro resistance to metalaxyl of three mutant isolates of *Phytophthora citricola* compared to the parent isolate P 1273

Isolate	EC_{50}(μg/ml)[a]	EC_{90}(μg/ml)[a]
P 1273-1[b]	0.26 ± 0.16	25.5 ± 6.0
U1[c]	505 ± 26	26,182 ± 12,147
U2-1[d]	230 ± 89	7,816 ± 6,940
M1-1[e]	252 ± 57	2,979 ± 1,292

[a] EC_{50} and EC_{90} values represent means ± standard deviation of the means based on linear regressions of the response (percent mycelial growth inhibition) plotted against the dosage (log concentration metalaxyl) for the individual isolates. Correlation coefficients ranged from 0.89 to 0.95.
[b] Single-spore isolate of the parent isolate of *P. citricola*.
[c] Original UV-induced mutant isolate U1.
[d] Single-spore isolate of UV-induced mutant isolate U2.
[e] Single-spore isolate of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced mutant M1.

DISCUSSION

The exposure of *P. citricola* to either the chemical mutagen MNNG or UV irradiation resulted in the development of a few isolates exhibiting high levels of both in vitro and in vivo resistance to the fungicide metalaxyl. This agrees with results of Davidse (7) who utilized *P. megasperma* and those of Bruin and Edgington (3) who worked with *P. capsici*. In our experiments, both mutation methods yielded approximately the same number of resistant isolates in vitro, and some of these were also resistant in vivo. Staub et al (13) observed that although some isolates of *P. infestans* were resistant to metalaxyl in vitro, they were still completely sensitive to the fungicide in vivo. Such was the case for some of our isolates, suggesting that some types of in vitro metalaxyl resistance result from a different mechanism than that responsible for in vivo resistance.

Investigation of the stability of the metalaxyl-resistance trait in the pathogenic mutant isolates M1, U1, and U2 revealed that after 20 generations of transfer in the absence of metalaxyl, both single-spore isolates of M1 were very stable, as was the single-spore isolate U2-2. However, by generation 20, the in vitro response to metalaxyl of both single-spore isolates of U1 and one of U2 had diverged

TABLE 3. Comparison between isolates of *Phytophthora citricola* of their radial growth on cornmeal agar amended with metalaxyl[t] over a period of 20 generations of culture in vitro on unamended cornmeal agar

Isolate	Mean radial growth (mm)[u] Generation 0	Generation 20[v]
P 1273-1[w]	3 a[x]	6 a
P 1273-2	4 a	6 a
U1-1[y]	12 b	14 b
U1-2	10 b	18 c
U2-1[y]	29 c	22 d
U2-2	28 c	43 e
M1-1[z]	58 d	59 f
M1-2	51 d	58 f

[t] Rate: 100 μg a.i./ml.
[u] Mean of three replicates.
[v] Twentieth generation of the isolate grown at 24 C on cornmeal agar.
[w] Two single-spore isolates of the parent isolate P 1273.
[x] Values in a single column followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.
[y] UV-induced mutants: two single-spore isolates of each.
[z] MNNG-induced mutant: two single-spore isolates.

TABLE 4. In vivo resistance to metalaxyl of generations 0 and 20 of UV-induced and MNNG-induced mutant isolates of *Phytophthora citricola*

Isolate[b]	Percent infection of seedlings of *Persea indica* using different metalaxyl concentrations[a]: 10 μg/ml Gen. 0[c]	10 μg/ml Gen. 20[c]	100 μg/ml Gen. 0	100 μg/ml Gen. 20	250 μg/ml Gen. 0	250 μg/ml Gen. 20
M1-1	27 ±35	51 ±4	29 ±17	44 ±4	22 ±17	33 ±23
M1-2	40 ±29	32 ±16	56 ±8	33 ±20	27 ±29	24 ±20
U1-1	24 ±19	27 ±24	9 ±8	2 ±4	4 ±8	0
U1-2	13 ±12	2 ±4	0	0	0	0
U2-1	60 ±13	2 ±4	56 ±8	0	38 ±21	0
U2-2	22 ±20	27 ±13	2 ±4	2 ±4	0	0

[a] Seedlings were inoculated with zoospores of *P. citricola*. After 24 hr, metalaxyl was added to give the appropriate concentration being tested. Eleven days after inoculation, the stem of each seedling was plated in 1-cm segments on PARP medium (11) to determine percent infection.
[b] Single-spore isolates derived from an original mutant colony.
[c] Generations zero (original mutant) and 20 of mycelial growth on cornmeal agar at 24 C.

TABLE 5. The in vivo cross-resistance of metalaxyl-resistant isolates of *Phytophthora citricola* to other acylanilide fungicides expressed as the percent infecton of *Persea indica* seedlings[a]

Isolate	Control (no fungicide)	Metalaxyl (10 μg/ml)	Benalaxyl (50 μg/ml)	RE 26745[b] (50 μg/ml)	Cyprofuram (50 μg/ml)	Oxadixyl (100 μg/ml)	Fosetyl-Al[c] (500 μg/ml)
P 1273[d]	73 ± 7	2 ± 4	0	2 ± 4	2 ± 4	2 ± 4	0
U1[e]	69 ± 21	25 ± 17	0	20 ± 0	16 ± 4	16 ± 14	0
U2[e]	67 ± 0	20 ± 0	7 ± 7	38 ± 32	18 ± 4	20 ± 7	0
M1[f]	85 ± 4	78 ± 8	20 ± 29	18 ± 8	71 ± 14	40 ± 31	0

[a] Seedlings were inoculated with zoospores of *P. citricola* in the presence of the particular acylanilide being tested.
[b] 2-methoxy-*N*-(2,6-dimethylphenyl)-*N*-(tetrahydro-2-oxo-3-furanyl)-acetamide.
[c] Aluminum ethyl-O-phosphonate, buffered to pH 6.3 with KOH.
[d] Parent isolate of *P. citricola*.
[e] UV-induced mutant isolates.
[f] MNNG-induced isolate.

TABLE 6. Competitive fitness in vivo of various zoospore mixtures of the parent isolate P 1273 and the metalaxyl-resistant mutant M1 of *Phytophthora citricola*

Initial ratio P 1273:M1	Recovered ratio[a] P 1273:M1
1:0	1:0
9:1	1:0
3:1	1:4
1:1	0:1
1:3	0:1
1:9	0:1
0:1	0:1

[a] Isolates of *P. citricola* were recovered from the stems of *Persea indica* seedlings inoculated with zoospores of isolates at indicated ratios and plated out on PARP medium. Single zoospore isolates were obtained and grown on cleared V8-juice agar amended with 100 μg of metalaxyl per milliliter to determine their identity. The ratios are based on the assessment of 10 individual single-spore isolates recovered from *Persea indica*.

significantly; isolates U1-1, U1-2, and U2-1 had also lost some degree of pathogenicity. Such instability has also been observed by Bruin and Edgington (3) with metalaxyl-resistant isolates of *Pythium ultimum* and *P. capsici*. After 12 transfers in the absence of metalaxyl, some of their isolates reverted either partially or completely to the parental level of sensitivity, while others remained stable. According to their hypothesis (3), mutant isolates require a period of time to stabilize and the variation in the behavior of the isolates may reflect different pathways along which the mutants evolve.

The cross-resistance of the metalaxyl-resistant isolates to another acylalanine fungicide, benalaxyl, supports the hypothesis that different acylalanine fungicides have the same mode of action. Resistance to two other classes of acylanilides was detected: the oxazolidinone oxadixyl and the butyrolactones cyprofuram and RE 26745, which differ somewhat in chemical structure from the acylalanines. Cross-resistance to the butyrolactone cyprofuram has also been reported with metalaxyl-resistant field isolates of both *P. infestans* and the downy mildew pathogen, *Pseudoperonospora cubensis* (6). It was also reported that some metalaxyl-resistant isolates of *P. infestans* were not controlled by the chemically unrelated ethyl phosphonate, fosetyl-Al, even though metalaxyl-sensitive isolates were controlled by this fungicide (6). Our metalaxyl-resistant isolates of *P. citricola*, however, were still sensitive to fosetyl-Al indicating that this compound could be used to control disease caused by strains of *P. citricola* insensitive to the acylanilides.

According to Dekker (9), competition experiments conducted in the laboratory can be useful in determining if there is a link between the development of fungicide resistance and a decrease in the fitness of the pathogen. Competition studies between the metalaxyl-resistant mutant M1 and the parent isolate P 1273 revealed that at ratios of up to three of the wild-type to one of the mutant, the metalaxyl-resistant isolate competed very well. Similarly, no loss of fitness was observed with metalaxyl-resistant isolates of *P. megasperma* (7). From these results it is evident that the development of metalaxyl resistance is not necessarily linked to a decrease in the fitness of the pathogen. The prediction is that once the development of significant resistance to metalaxyl occurs within populations of *P. citricola*, individual mutant isolates should be able to compete successfully with the wild-type strains. Therefore, strict precautions should be taken when using metalaxyl in a field situation to try to keep the selection pressure as low as possible, either by intermittent use at low concentrations, or possibly through its use in a mixture with another fungicide with a different mode of action. Research is urgently needed on the suitability of the use of mixtures, or sequential applications, of different fungicides for their effectiveness in lessening the risk of resistance developing to the acylanilide group of systemic fungicides.

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