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CROSS-PROTECTION AND SELECTABLE MARKER GENES IN PLANT TRANSFORMATION

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SUMMARY

Selectable marker genes play an important role in plant transformation. The level of selection pressure is generally established by generating a kill curve for the selectable marker. In most cases, the lowest concentration which kills all explants is used. This study examined two selectable marker genes, phosphinothricin acetyl transferase (PAT) and hygromycin phosphotransferase (HPT), in transformation of tobacco leaf disks. Experiments to determine the lethal level of the herbicide, glufosinate-ammonium (phosphinothricin) (PPT) using a leaf-disk regeneration assay established that no shoots regenerated at 2 to 4 mg PPT per l. Likewise with the antibiotic, hygromycin (HYG), no plants regenerated at 50 mg hygromycin per l. In contrast, after cocultivation of the leaf disks with *Agrobacterium tumefaciens* containing either the PAT or HPT gene in combination with a Bt gene for insect resistance, plants were successfully regenerated from leaf disks at 2 to 4 mg PPT per l and 50 mg hygromycin per l. However, most plants regenerated at 2 and 3 mg PPT per l were found to be nontransformed (95–100% escapes) by i) Southern-blot analysis, ii) herbicide application test, and iii) insect feeding bioassay. On the other hand, plants that regenerated on 50 mg hygromycin per l and 4 mg PPT per l were transgenic as determined by Southern analysis, leaf assay for PPT or HYG resistance, and death of tobacco budworms feeding on these leaves. This study showed a significant level of cross-protection and/or transient expression of the PAT selectable marker gene allowing escapes (95–100%) at selection levels of 2 and 3 mg PPT per l which completely kill controls. On the other hand, the HPT gene at 50 mg is efficient in selecting for T-DNA integration.

Key words: tobacco; *Agrobacterium tumefaciens*; phosphinothricin; hygromycin; selection pressure; cross-protection.

INTRODUCTION

Selectable marker genes play an important role in plant transformation. Effective selection can lead to a substantial reduction in the number of untransformed regenerants. One of the major problems encountered when using selective agents is protection of untransformed tissue by the detoxifying activity of surrounding transformed cells (herein referred to as "cross-protection"). Such cross-protection allows regeneration of untransformed cells, leading to many nontransformant escapes. The most widely applied selective agents for use in plant transformation are hygromycin (Hiei et al., 1994; Ortiz et al., 1996), kanamycin (Deblaere et al., 1987), and phosphinothricin (Cao et al., 1992; Wan et al., 1994). Dekeyser et al. (1989) evaluated a number of selectable markers for rice transformation. Their results indicated that hygromycin (HYG) and phosphinothricin (PPT) were very effective as selective agents. However, Christou et al. (1991) cautioned, when using selective agents (HGY or PPT) for rice transformation, that nontransformed rice tissue had the same regeneration capacity as transformed tissue, due to the inactivation of the selective agents by surrounding transformed cells. Park et al. (1996) used PPT and similarly found chimeric rice plants with both transformed and untransformed proliferating cells. To overcome the rescuing ability of transformed cells, the most effective concentration of the selective agent must be determined in tissue culture experiments for each crop and for each type of explant. Usually, a concentration is chosen that stops growth of untransformed tissue, but allows proliferation of transformed cells, without causing too much cell death. Dying cells release toxic products that inhibit the regeneration of transformed cells (Klee et al., 1987). In order to obtain the maximum number of transformants, it is important to determine the lowest level of selective agent needed to prevent growth of untransformed tissue. However, care must be taken to control the number of escapes.

A general approach in transformation studies is to establish a kill curve for the selective agent and use the lowest level of selective agent which inhibits 100% of the control growth. For HPT, 50 mg/l has been used for tobacco leaf disk selection (Ingelbrecht et al., 1994) and 0.5 to 50 mg PPT per l depending on the explant size (DeBlock et al., 1987). However, with this approach, cross-protection and/or transient expression can result in a high level of escapes. This paper examines the effectiveness of two selectable marker genes, phosphinothricin acetyl transferase (PAT) and hygromycin phosphotransferase (HPT) using tobacco leaf disk transformation to evaluate the appropriate level to achieve effective selection but at the same time minimize escapes.

MATERIALS AND METHODS

Plant materials. Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) seeds were surface-sterilized in 95% ethanol for 1 min, then in 20% Clorox bleach for 25 min, and rinsed three times with autoclaved water. The seeds were placed on 1% (wt/vol) agar-solidified medium, pH 5.7, containing MS salts medium (Murashige and Skoog, 1962), to germinate. After germination, the seedlings were transferred to MS medium as above plus 0.5 mg nicotinic acid

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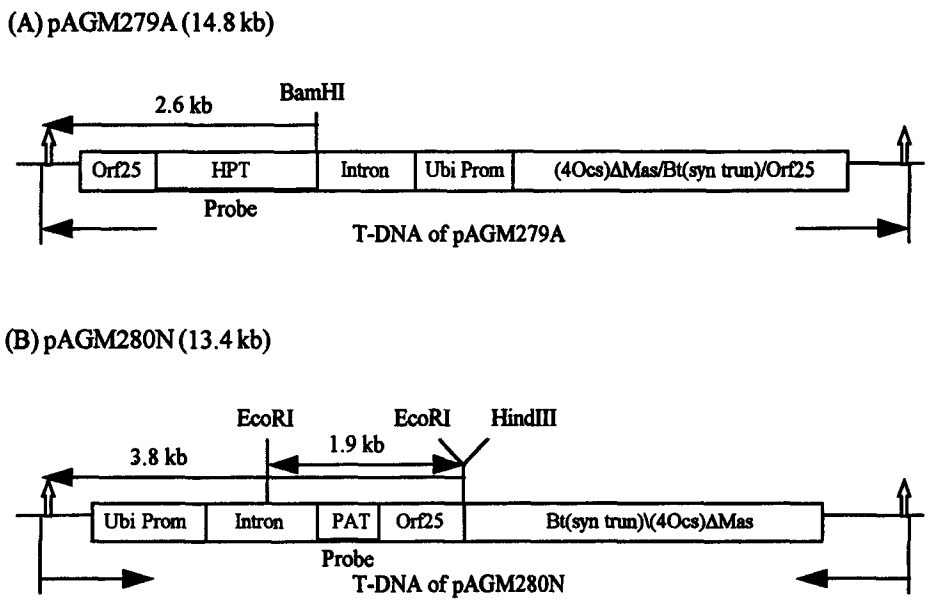

FIG. 1. Plasmid constructs, (*A*) pAGM279A and (*B*) pAGM280N, used to determine the effectiveness of selectable marker genes on tobacco transformation.

per l, 0.5 mg pyridoxine-HCl per l, and 0.1 mg thiamine-HCl per l in magenta boxes and incubated at 25° C for 16 h under 60–80 μE $m^{-2}s^{-1}$ light. Young leaves from 4–5-wk-old tobacco plants were removed and cut into explants.

Bacterial strain and plasmids. An octopine strain, LBA4404 was used. The plasmid constructs used were pAGM279A containing *orf25\hpt\ubi::(4Ocs)Δmas/bt (syn trun)/orf25* and pAGM280N containing *ubi/pat/orf25\bt (syn trun)\(4Ocs)Δmas* chimeric expression cassette (Fig. 1 *A,B*) obtained from Mycogen Plant Sciences (San Diego, CA).

Plant transformation. Tobacco transformation was performed as described by Horsch et al. (1985). Leaf explants were initially cultured on the same MS inorganic salts medium on which germinated seedlings were grown plus 1 mg N^6-benzyladenine (BA) per l, 0.1 mg α-naphthalene acetic acid (NAA) per l, 3% (wt/vol) sucrose, and 0.8% (wt/vol) TC agar at pH 5.7 for 1–2 d. Adventitious shoot regeneration rates were established on the media containing four concentrations (0, 2, 3, and 4 mg/l) of glufosinate-ammonium (PPT) or three concentrations (0, 35, and 50 mg/l) of hygromycin (HYG). Cultures were maintained at 25° C for 16 h under 60–80 μE $m^{-2}s^{-1}$ light. Leaf explants were inoculated with *A. tumefaciens* cultured on YEP medium (10 mg yeast extract per l, 10 mg trypton per l, 5 mg NaCl per l) containing the appropriate antibiotics for 2 min and then cocultivated on a complete MS medium for 2–3 d. They were then transferred to selection medium containing 250 mg clavamox per l and the established concentrations of PPT (2, 3, and 4 mg/l) and HYG (50 mg/l). After 4–5 wk, regenerated shoots were transferred to rooting medium containing 250 mg clavamox per l for 3 more wk.

DNA isolation and Southern hybridization analysis. Tobacco genomic DNA was extracted from leaf tissue according to the modified mini-prep extraction procedure of Dellaporta et al. (1983). Isolated DNA was digested with three restriction enzymes, *Bam*HI, *Eco*RI or *Hin*dIII, separated in a 1.0% agarose gel by electrophoresis and blotted onto a nylon membrane (Zeta-probe GT membrane, BIO-RAD Laboratories, Hercules, CA) according to manufacturer's instruction. DNA was fixed to the membrane by baking at 80° C for 30 min and hybridized to DNA fragments labeled with ^{32}P-dCTP with a random primers DNA labeling system (GIBCO BRL Life Technologies, Burlington, ON, Canada). The probe for HPT was made from a *Eco*RI and *Sac*I restriction fragment of the pAGM103 plasmid (1.2-kb fragment) and the probe for PAT from a *Sal*I restriction fragment of the pAGM102 plasmid (558-bp fragment). The membranes were prehybridized for at least 4 h at 65° C in 7% sodium dodecyl sulfate (SDS) and 0.25 *M* Na_2HPO_4 and hybridized overnight at 65° C in the same solution. Membranes were briefly washed twice for 5 min each with 20 m*M* Na_2HPO_4/5% SDS at 65° C, then washed twice for 30 min each with 20 m*M* Na_2HPO_4/5% SDS, and finally washed twice for 30 min each with 20 m*M* Na_2HPO_4/1% SDS at 65° C. Filters were exposed to X-ray film at 70° C.

Selective agents application test. Tobacco leaves from regenerated plants were cut into small pieces (at least 5 mm in diameter). The leaf pieces were transferred to media containing 9.5 mg PPT per l or 40 mg HYG per l in order to identify transformed plants. At these concentrations, necrotic symptoms were apparent on untransformed leaf tissues (unpublished data established these levels lethal to untransformed leaf tissue). Changes in the pigmentation of leaf pieces were monitored for 2 wk.

Insect feeding bioassay. Leaf disks from T_0 plants were cut from recently expanded tobacco leaves (2 disks/each leaf cut into 0.5-inch-diameter disks) and were placed on a moistened filter paper in a 60 × 15-mm petri dish. Five tobacco budworm (*Heliothis virescens*) larvae were allowed to feed for 4 d. This test was maintained at 25–26° C with a 16-h photoperiod (60–80 μE $m^{-2}s^{-1}$).

RESULTS AND DISCUSSION

Selection of putative transformed shoots. Experiments to determine the lethal level of the herbicide PPT on shoot regeneration established that no shoots regenerated at 2, 3, or 4 mg PPT per l (Fig. 2 *A*). Likewise, with HYG no shoots regenerated at 50 mg HYG per l. Therefore, 2, 3, and 4 mg PPT per l and 50 mg HYG per l were chosen for selection. After leaf disk cocultivation with *Agrobacterium* containing either the pAGM279A or pAGM280N plasmid constructs, plants regenerated from leaf disks at 2, 3, and 4 mg PPT per l and 50 mg HYG per l. PPT-resistant shoots regenerated on 2 mg PPT per l (Fig. 2 *B*) and HYG-resistant shoots on 50 mg/l (Fig. 2 *C*) are shown. HYG at 35 mg/l without cocultivation resulted in callus proliferation with some pale, vitrified shoot development; occasionally a shoot would green up and elongate. These shoots were not continued in this study. Selection pressure was not used during rooting, as all surviving shoots were analyzed for phenotype expression (leaf painting and insect resistance) as well as T-DNA integration by molecular analysis. According to the literature, some researchers do selection for rooting and some do not. In crop plants, some of the selective agents appear to interfere with rooting.

Molecular analysis of plants regenerated on PPT or HYG selection. To determine if the PPT- or HYG-resistant plants contained the T-DNA fragment, Southern analysis was performed on genomic DNA isolated from leaves of 25 plants resistant to 2 mg PPT per l, 43 plants resistant to 3 mg PPT per l, 7 plants resistant to 4 mg PPT per l, and 8 plants resistant to 50 mg HYG per l. DNA from HYG-

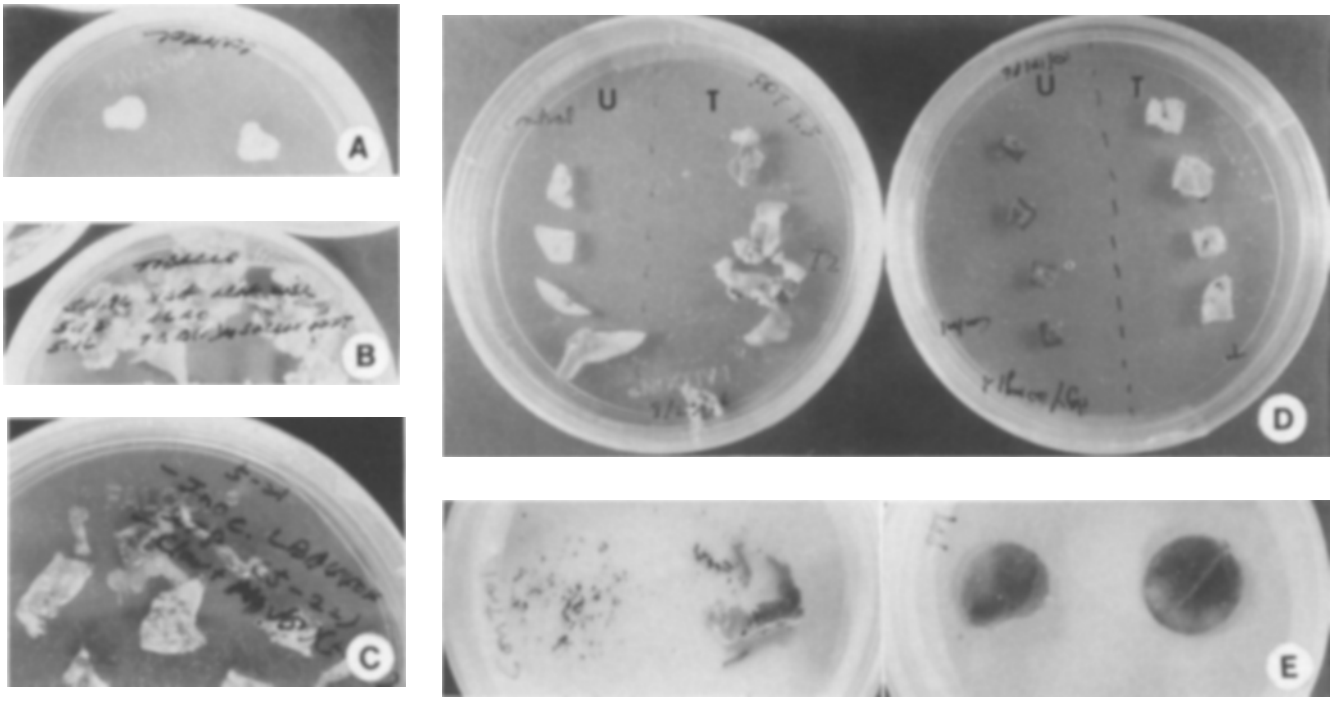

FIG. 2. Selection of putative transformed shoots and expression test. (*A*) Uninoculated control leaf disks. Production of shoots was completely inhibited on medium containing 2 mg PPT per l. (*B*) After cocultivation of the leaf disks with *Agrobacterium* containing pAGM280N, PPT-resistant shoots regenerated on medium containing 2 mg PPT per l. (*C*) After cocultivation with *Agrobacterium*-containing pAGM279A, HYG-resistant shoots regenerated on medium containing 50 mg/l. (*D*) Leaf tissue bioassays for resistance to either PPT or HYG show severe necrotic symptoms on control (U) as compared to transgenic leaf tissue (T) for 9.5 mg PPT per l (*left*) and 40 mg HYG per l (*right*). (*E*) Results of insect feeding bioassays show after 4 d that the control leaf disks were highly damaged (*left*), whereas transgenic leaf disks showed no feeding damage (*right*).

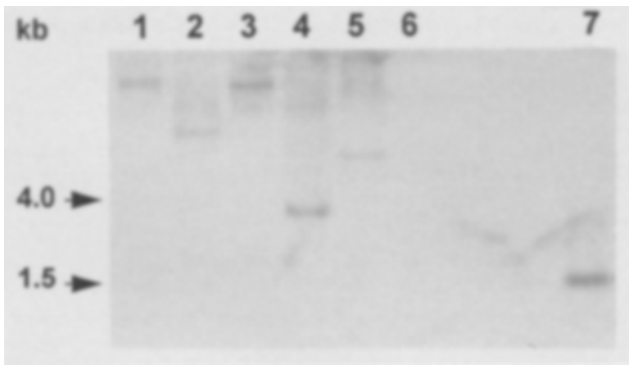

FIG. 3. Southern blot analysis of *Bam*HI-digested genomic DNA from five HYG-resistant plants. *Lanes 1* to *5*, HGY-resistant plants; *lane 6*, control plant; *lane 7*, *Eco*RI-digested pAGM103 plasmid DNA. A purified *Eco*RI-*Sac*I fragment containing the HPT from pAGM103 was used as a probe.

resistant plants was digested with *Bam*HI and hybridized with the *hpt* probe (yielding border fragments which included a portion of the inserted T-DNA and host DNA). Several different band sizes bigger than 2.6 kb were expected, corresponding to the T-DNA map of pAGM279A (Fig. 1 *A*). Fig. 3 shows the Southern blot analysis of *Bam*HI-digested genomic DNA from five of the HYG-resistant plants. The expected fragments bigger than 2.6 kb were present in *lanes 1–5*. These results reflect the copy number and random insertion of the transgenes. All plants contained a single copy of the integrated T-DNA. No hybridization was detected in DNA from the control plant (*lane 6*).

DNA from PPT-resistant plants was digested with *Eco*RI or *Hin*dIII and was hybridized with the *pat* probe. Fig. 4 *A* shows the Southern analysis of *Eco*RI-digested genomic DNA from seven of the PPT-resistant plants. The expected 1.9-kb restriction fragment was present in *lanes 4,5,* and *8* containing genomic DNA from plants resistant to 4 mg PPT per l. However, no hybridization was detected in DNA from plants resistant to 2 or 3 mg PPT per l (*lanes 1,2,3,6,* and *7*). When DNA was digested with *Hin*dIII and hybridized with the *pat* probe, fragments bigger than 3.8 kb were expected, corresponding to the T-DNA map of pAGM280N (Fig. 1 *B*). The expected fragments bigger than 3.8 kb were present in *lanes 2,4,* and *8* containing genomic DNA from plants resistant to 4 mg PPT per l (Fig. 4 *B*). Most transformed plants showed a single copy of the integrated T-DNA. No hybridization was detected in DNA from plants resistant to 2 or 3 mg PPT per l (*lanes 1,3,5,6,7,* and *9*). Neither the 19 other plants resistant to 2 mg PPT per l nor the 36 other plants resistant to 3 mg PPT per l showed any hybridization to the *pat* probe, except for two plants resistant to 3 mg PPT per l.

These results indicated that 2 or 3 mg PPT per l determined as the minimal lethal level of herbicide selection gave a high frequency of escapes (Table 1). Therefore, assessment of the minimal dose is not adequate to control the number of escapes when PPT is used as a selective agent.

TABLE 1

COMPARISON OF REGENERATED PLANTS ON GLUFOSINATE-AMMONIUM (PPT) OR HYGROMYCIN (HYG) SELECTION AND SUBSEQUENT SOUTHERN BLOTS, SELECTIVE AGENTS APPLICATION TEST, AND THE INSECT FEEDING BIOASSAY

Selective agents, mg/l	Number of explants	Number of regenerated plants	Number of tested plants	Hybridization +	HYG or PPT application test +	Insect feeding bioassays +	Escapes %
HGY, 50	16	8	8	8	8	8	0
PPT, 2	16	50<	25	0	0	0	100
PPT, 3	16	43	43	2	2	2	95
PPT, 4	16	7	7	7	7	7	0

+ = positive result

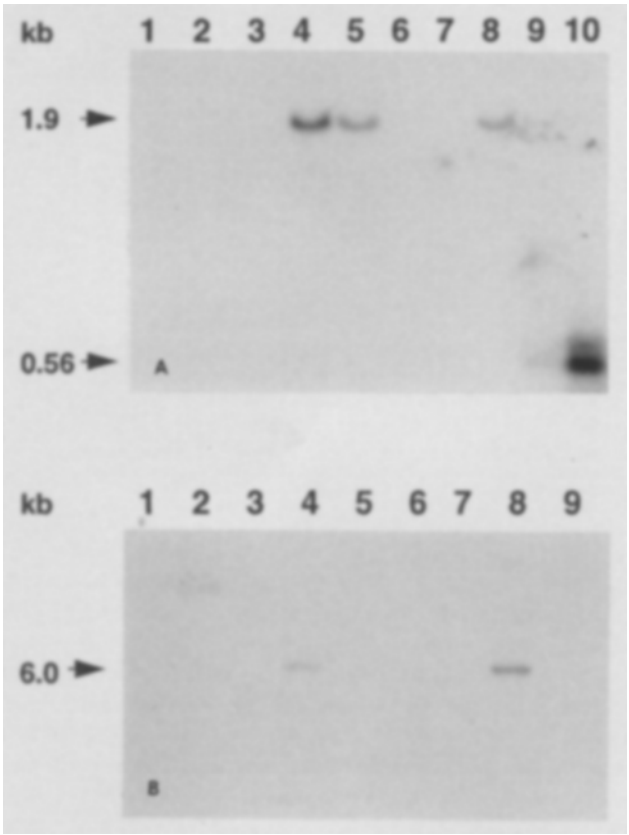

FIG. 4. Southern blot analysis of genomic DNA from 16 PPT-resistant plants. (*A*) Hybridization of *Eco*RI-digested DNA from seven PPT-resistant plants. *Lanes 4,5,* and *8*, genomic DNA from plants resistant to 4 mg PPT per l; *lanes 1,2,3,6,* and *7*, genomic DNA from plants resistant to 2 or 3 mg PPT per l; *lanes 9* and *10*, *Sal*I-digested pAGM102 plasmid DNA. (*B*) Hybridization of *Hind*III-digested DNA from nine PPT-resistant plants. *Lanes 2,4,* and *8*, genomic DNA from plants resistant to 4 mg PPT per l; *lanes 1,3,5,6,7,* and *9*, genomic DNA from plants resistant to 2 or 3 mg PPT per l. A purified *Sal*I fragment containing the PAT from pAGM102 was used as a probe.

Selective agents application test. Eighty-three plants regenerated from leaf disks at 2, 3, and 4 mg PPT per l and 50 mg HYG per l were evaluated for resistance on media containing 9.5 mg PPT per l or 40 mg HYG per l. All plants regenerated at 4 mg PPT per l and 50 mg HYG per l were resistant to selective agents, whereas all plants regenerated at 2 mg PPT per l and 41 of 43 plants regenerated at 3 mg PPT per l were sensitive to the selective agents. This result showed a perfect match between positive hybridization and resistance to the application of selective agents (Table 1). Severe necrotic symptoms on control leaf tissues and no damage on leaf tissues of transgenic plants are shown (Fig. 2 *D*).

Insect feeding bioassay. Insecticidal activity of Bt expressed in transgenic tobacco plants which showed positive results in hybridization and selective agents application tests was carried out by the insect feeding bioassay. After 4 d, control leaf disks were highly damaged, and the tobacco budworm larvae reached advanced developmental stages, whereas all transgenic leaf disks showed no feeding damage (Fig. 2 *E*).

These results have modified the approach and use of selective agents in this laboratory for transformation of agronomic plants (cotton, rice, sorghum, maize, and kenaf). The literature on transformation studies generally supports establishing the lowest lethal level of selective agent which kills all control tissue. This approach with PPT has resulted in many escapes (possibly chimeras) and the inability to confirm T-DNA integration even in shoots which also rooted on the selective agent (unpublished data). The data in this study using tobacco as a model indicate that, at least for PPT, levels of the selective agent higher than the level which kills control tissue are important to insure T-DNA integration.

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