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Original Contribution

β-GLUCURONIDASE REPORTER GENE TRANSFORMATION TO *Papaver bracteatum* Lindl. AS A WELL-DEFINED MODEL SYSTEM IN TRANSFORMATION OF EFFECTIVE GENES IN BENZYLISOQUINOLINE ALKALOIDS BIOSYNTHESIS

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ABSTRACT

An efficient protocol for the establishment of transgenic Persian poppy (*Papaver bracteatum* Lindl.) using β-glucuronidase (*GUS*) transformation is reported. In this research, callus was induced on *P. bracteatum* Lindl. seeds. The optimised callus induction media consisted of the basic Murashige and Skoog (MS) salt mixtures and vitamin solutions solidified with 1% agar. The callus induction medium was supplemented with 1.0 mg l^{-1} 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 15 mg l^{-1} ascorbic acid at 25°C in the dark. The calli were co-cultivated with the *Agrobacterium tumefaciens* strain GV3101 carrying the pBI121 binary vector. Except for the co-cultivation medium, all formulations included 40 mg l^{-1} paromomycin to select for transformants and 200 mg l^{-1} cefotaxime to eliminate the Agrobacterium. Shoot regeneration of transformed calli in vitro was achieved on ¾ MS medium containing 0.5 mg l^{-1} N6-benzyladenine (BA) and1.0 mg l^{-1} a-naphthaleneacetic acid (NAA). Detection of *GUS* gene and GUS histochemical localization confirmed the integrative transformation. Transgenic plants of Persian poppy are well-defined model systems in transformation of effective genes in benzylisoquinoline alkaloids biosynthesis, and for evaluating the rate of increase of benzylisoquinolines alkaloids of these important medicinal plants.

Key Words: *Papaver bracteatum*; β-glucuronidase; Transgenic plants; Alkaloids

INTRODUCTION

Benzylisoquinoline alkaloids are a diverse group of nitrogenous compounds found in about 20% of plant species such as Papaveraceae The potent biological activity of some alkaloids has also led to their widespread use as pharmaceuticals. The benzylisoquinoline alkaloid class, in particular, includes several important medicinal compounds such as analgesics, morphine and codeine, papaverine, berberine, the muscle relaxant (+)-tubocurarine, and the antibiotic sanguinarine.

Numerous reports can be found of the productions of the morphinans thebaine, codeine, and morphine, from cell cultures of *Papaver somniferum* and *P. bracteatum*, although the yields are low compared to the high yields of the plants (1). **Figure 1** shows the morphinan alkaloid pathway in the opium poppy (2). Plant metabolic engineering is a relatively new field of research with the potential to create new opportunities for the improvement of plant metabolic, cellular, and physiological processes. In recent years, a number of impressive strategies for the genetic modification of several important plant metabolic pathways have been reported. These include the elevation of the *α*-tocopherol (vitamin E) content of Arabidopsis seeds (3), and the introduction of the entire *β*-carotene (provitamin A) biosynthetic pathway into rice endosperm cells (4). The first application of metabolic engineering to a plant alkaloid pathway involved the transformation

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of *Atropa belladonna*, which normally accumulates hyoscyamine, with the gene encoding hyoscyamine 6β-hydroxylase (H6H) from *Hyoscyamus muticus* (5). Plants expressing the H6H transgene accumulated high levels of scopolamine, the H6H reaction product, demonstrating that alkaloid metabolism can be altered in transgenic plants. However, the metabolic engineering of plant alkaloid pathways has generally been restricted by the limited availability of cloned biosynthetic genes and the inability to genetically transform many alkaloid-producing species. Recently, several new genes encoding enzymes involved in benzylisoquinoline alkaloid biosynthesis have been reported (6) and protocols for the genetic transformation of the benzylisoquinoline alkaloid producing species, opium poppy (*P. somniferum*) and California poppy have been established (7,8). These developments have created the opportunity to metabolically engineer benzylisoquinoline alkaloid pathways in plants.

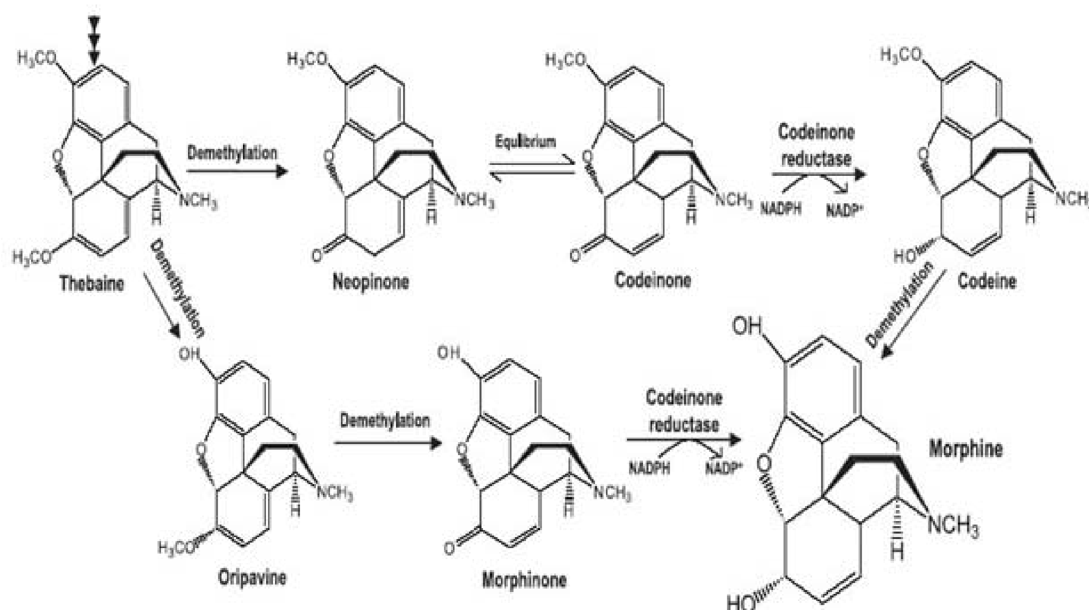

Figure 1. *Benzophenanthridine alkaloid biosynthesis. The reduction of codeinone to codeine and the reduction of morphinone to morphine by codeinone reductase results in the production of morphine*

In this paper, the optimised callus induction medium consisted of the basic MS salt mixtures and vitamin supplemented with 1.0 mg l^{-1} 2,4-D and 15 mg l^{-1} ascorbic acid at 25°C in the dark. The calli were co-cultivated with the *Agrobacterium tumefaciens* strain GV3101 carrying the pBI121 binary vector. Shoot regeneration of transformed Calli *in vitro* was achieved on ¾ MS medium containing 0.5 mg l^{-1} BA, 1.0 mg l^{-1} NAA, 40 mg l^{-1} paromomycin and 200 mg l^{-1} cefotaxime. The main aim of this study was to find a well-defined model system in the transformation of effective genes in benzylisoquinoline alkaloids biosynthesis. The findings of this study should help enable the production of alkaloids of biological origin under controlled conditions. In other words, this is a starting point for the isolation of secondary metabolites in transgenic plants of *P. bracteatum* Lindl.

MATERIALS AND METHODS

Seed sterilization and germination

Seeds of Persian poppy (*P. bracteatum* Lindl.) were surface-sterilized with 70% (v/v) ethanol for 1 min and 5% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water.

Media for callus induction

The surface sterilized seeds were placed on 25 ml of agar-solidified culture medium. The basal medium consisted of MS salts and vitamins (9) solidified with 0.8% (w/v) agar-agar (Merck). The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121°C for 20 min.

All media were supplemented with 3% sucrose, various concentrations of 2,4-D (0.0, 0.1, 1.0, 2.0 and 3.0 mg l^{-1}) and various concentrations of ascorbic acid (0.0, 5.0, 10.0, 15.0 and 20.0 mg l^{-1}) for the induction of callus at 20°C and 25°C in the dark. Ten seeds

were placed on 25 ml of agar-solidified culture medium in Petri dishes.

Construction of Transformation Vector

The binary vector pBI121 containing *GUS* reporter gene and *CaMV35S* promoter (**Figure 2**) was transformed in the armed *Agrobacterium tumefaciens* strain GV3101. Transformed *A. tumefaciens* cultures were grown to mid-log phase (A_{600}=0.5) at 28°C on a gyratory shaker at 180 rpm in liquid Luria-Bertani medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl), containing 50 mgl^{-1} kanamycin. The bacterial cells were collected by centrifugation for 10 min at 1500 rpm, and re-suspended at a cell density of A_{600}=1.0 in liquid inoculation medium (MS salts and vitamins containing 20 gl^{-1} sucrose).

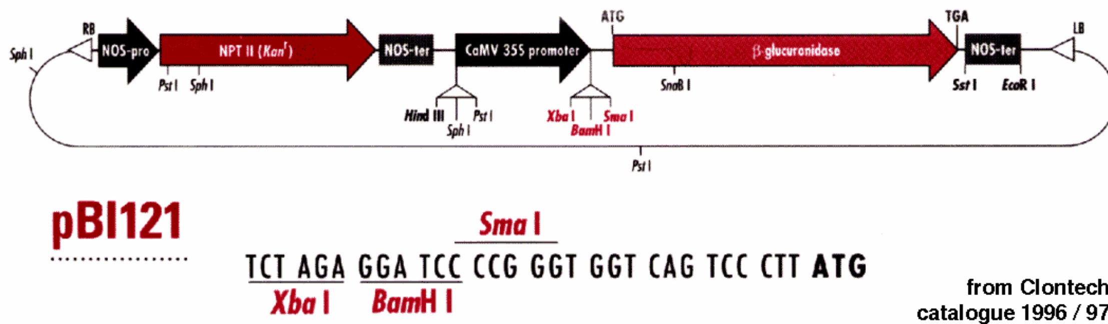

Figure 2. Construction of the binary vector pBI121 containing GUS reporter gene and CaMV35S promoter

Regeneration of transgenic plants

The calli were used as the explants material for co-cultivation with *A. tumefaciens* strain GV3101. The calli immersed in *A. tumefaciens* culture suspended in liquid inoculation medium for 5 min, blotted dry on sterile filter paper, and incubated in the dark on agar-solidified MS medium. After 2 d of co-cultivation, the calli were transformed to regeneration medium containing ¾ MS salts and vitamins, supplemented with 0.5 mg l^{-1} BA, 1.0 mg l^{-1} NAA, 40 mg l^{-1} paromomycin to select for transformants and 200 mg l^{-1} cefotaxime to eliminate the *Agrobacterium* at 25°C in a growth chamber under standard cool white fluorescent tubes with a flux rate 35 µmol s^{-1} m^{-2} and a 16 h photoperiod.

PCR analysis of transformation plants

Plant genomic DNA for polymerase chain reaction (PCR) analysis was extracted as described by Edwards *et al*. (10). Shoots (50 mg fresh weight) were homogenized in 200 µl of extraction buffer (0.5% [w/v] SDS, 250 mM NaCl, 100 mM TRIS-HCl, pH 8.0, and 25 mM EDTA) and centrifuged at 13000 rpm for 5 min; the supernatant was transferred to a new tube and equal volume of isopropanol was added. The sample was incubated on ice for 5 min and then centrifuged for 10 min at 13000rpm. The pellet was dried at 60°C for 10 min, and then re-suspended in 100 µl of TE buffer (10 mM TRIS-HCl, pH 7.4 and 1.0 mM EDTA). PCR was performed for 30 thermal cycles (denaturation at 94°C for 5 min, primer annealing at 44°C for 1 min, and primer extension at 72°C for 1 min) using primers specific to sequences found in the *CaMV35S* promoter and *GUS* gene (5?-GGTGGTCAGTCCCTTATGTTACG-3? and 5?-CCGGCATAGTTAAAGAAATCATG-3?). Amplification products were analysed on 1% (w/v) agarose gels.

β-Glucuronidase histochemical staining

Histochemical staining for GUS activity was performed as described by Jefferson (11) with modifications recommended by Kosugi *et al*. (12). Shoots were fixed in a 0.35% (v/v) formaldehyde solution containing 10 mM 2-morphino-ethanesulfonic acid (MES), pH 7.5, and 300 mM mannitol for 1 h at 20°C, rinsed three times in 50 mM sodium phosphate, pH 7.5, and subsequently incubated in 50 mM sodium phosphate, pH 7.5, 10 mM EDTA, 300 mM mannitol, pH 7.5, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt for 12 h at 37°C. Stained shoots were rinsed extensively in 70% ethanol to remove residual phenolic compounds.

HPLC Analysis

The tissues of transformed Persian poppy were frozen in liquid N_2, and extracted with methanol in a boiling water bath for 15 min.

Extracts were reduced to dryness under vacuum, dissolved in 1.0 M sodium carbonate/bicarbonate (3:2, w/w), pH 10.0, and extracted three times with ethyl acetate. Pooled ethyl acetate fractions were reduced to dryness and the residue taken up in 1 ml of methanol. Extracts were analysed using a System Gold 126 HPLC (Beckman-Coulter). Alkaloids were separated at a flow rate of 0.75 ml min^{-1} on a C18 reverse phase column (4.6×250 mm, Ultrasphere, Beckman-Coulter) using methanol:water (6:4, v/v) containing 0.1% (v/v) triethylamine. The identity of peaks was routinely analysed by comparison of UV spectra and retention times with those of identified alkaloids.

RESULTS AND DISCUSSION

Establishment of transformed plants

Induction and proliferation percentage of callus from seed explants were 100 percent on MS medium supplemented with 1.0 mg l^{-1} 2,4-D and 15 mg l^{-1} ascorbic acid. The optimum ascorbic acid concentration for *papaver* callus induction from seeds was 15 mg l^{-1} (**Table 1**). The callus was developed after 6 subcultures (**Figure 3**). Callus growth was very slow at 20°C but increased when the temperature was increased to 25°C. MS at full strength was found inhibitory for callus induction, but ¾ MS was suitable.

Except for the co-cultivation medium, all formulations used in subsequent steps included the antibiotics paromomycin for the selection of transformed calli, and cefotaxime to eliminate the *Agrobacterium* after co-culture. The ineffectiveness of kanamycin and the efficacy of paromomycin, for the selection of transformed opium poppy tissues has been reported previously (13). After 2d of co-cultivation with *A. tumefaciens* strain GV3101, calli were transferred to agar-solidified, regeneration medium. Shoots were regenerated on MS medium containing NAA (1.0 mg l^{-1}) and BA (0.5 mg l^{-1}) after 2 months (**Figure 3**).

Table 1. *Comparison of 2,4-D and ascorbic acid effects on callus induction*

2,4-D and ascorbic acid (mg l^{-1})	*No. of seeds*	*No. of seeds with callus*	*Rate of callus induction (%)*
0.1 mg l^{-1} 2,4-D + 5.0 mg l^{-1} ascorbic acid	30	0	0
0.1 mg l^{-1} 2,4-D + 10.0 mg l^{-1} ascorbic acid	30	0	0
0.1 mg l^{-1} 2,4-D + 15.0 mg l^{-1} ascorbic acid	30	0	0
0.1 mg l^{-1} 2,4-D + 20.0 mg l^{-1} ascorbic acid	30	0	0
1.0 mg l^{-1} 2,4-D + 5.0 mg l^{-1} ascorbic acid	30	14	46.6±0.21
1.0 mg l^{-1} 2,4-D + 10.0 mg l^{-1} ascorbic acid	30	19	63.3±0.15
1.0 mg l^{-1} 2,4-D + 15.0 mg l^{-1} ascorbic acid	30	30	100.0
1.0 mg l^{-1} 2,4-D + 20.0 mg l^{-1} ascorbic acid	30	20	66.6±0.19
2.0 mg l^{-1} 2,4-D + 5.0 mg l^{-1} ascorbic acid	30	4	13.3±0.14
2.0 mg l^{-1} 2,4-D + 10.0 mg l^{-1} ascorbic acid	30	5	16.6±0.19
2.0 mg l^{-1} 2,4-D + 15.0 mg l^{-1} ascorbic acid	30	9	30.0±0.11
2.0 mg l^{-1} 2,4-D + 20.0 mg l^{-1} ascorbic acid	30	7	23.3±0.24
3.0 mg l^{-1} 2,4-D + 5.0 mg l^{-1} ascorbic acid	30	0	0
3.0 mg l^{-1} 2,4-D + 10.0 mg l^{-1} ascorbic acid	30	0	0
3.0 mg l^{-1} 2,4-D + 15.0 mg l^{-1} ascorbic acid	30	0	0
3.0 mg l^{-1} 2,4-D + 20.0 mg l^{-1} ascorbic acid	30	0	0

Also, Ilahi and Ghauri (14) reported that callus was induced on *P. bracteatum* Lindl. Seedlings were inoculated on MS medium supplemented with NAA (1.0 mg l^{-1}) and BA (0.5 mg l^{-1}). Shoots were regenerated in cultures grown on MS medium containing NAA (1.0 mg l^{-1}), BA (0.5 mg l^{-1}) and casein hydrolysate (2.0 mg l^{-1}). MS at full strength was found inhibitory for callus induction and proliferation, but ½ MS was suitable. Similarly, callus growth was very slow at 25°C but increased when the temperature was lowered to 20°C as did bud initiation (14). On the other hand, Czygan and Abou-Mondour (1986) regenerated shoots on hormone free MS (15).

Analysis of transformants

The complete and stable transformation of paromomycin-resistant transgenic plants was evaluated by determining the integration of the *GUS* gene into the plant genome; and GUS histochemical assay. PCR performed using primers specific for sequences in the *CaMV35S* promoter and *GUS* gene resulted in

the amplification of a single amplicon with the expected size of 2600 bp in transgenic plants (**Figure 4**). Histochemical staining for GUS activity was performed to determine whether *A. tumefaciens* strain GV3101 produced complete transgenic plants. The cauliflower mosaic virus *35S* promoter-*GUS* fusion contained in the pBI121 binary vector should result in constitutive GUS activity in all cell types of paromomycin-resistant tissues (**Figure 5**).

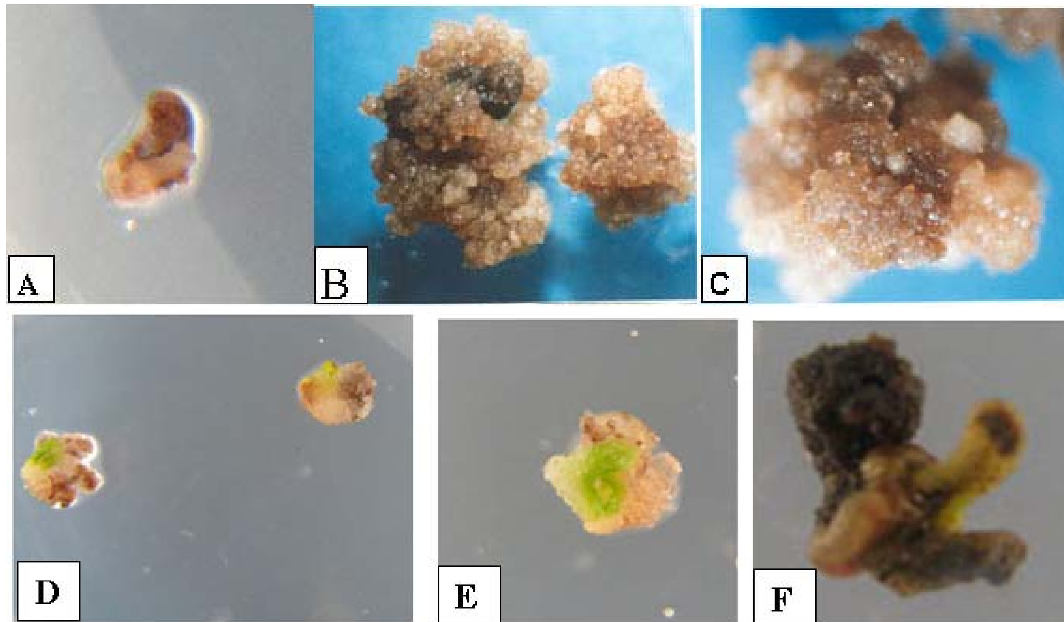

Figure 3. *Callus induction and plant regeneration of Persian poppy (P. bracteatum Lindl.); (A) callus after 1 subculture; (B) callus after 4 subcultures; (C) callus after 6 subcultures; (D,E and F) shoot regeneration*

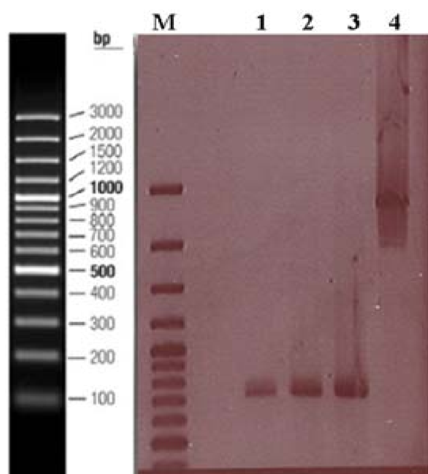

Figure 4. *PCR confirmation of GUS using specific CaMV35S+GUS primers (line 1,2 and 3 non-transgenic plants) and (line 4, approximately 2600 bp), 100 bp ladder (Fermentas) (M)*

Morphine accumulates in transgenic plants

Analysis of alkaloid extracts from Persian poppy tissues by HPLC showed that morphine accumulates in the shoots of transgenic and wild-type (non-transgenic) plants (**Figure 6**). Results showed that the content of morphine alkaloid produced by Persian poppy was identical in transformed and wild-type shoots. Numerous reports of the productions of the morphinans thebaine, codeine, and morphine, from cell cultures of *P. somniferum* and *P. bracteatum* can be found in the literature, although the yields are low compared to the high yields of the plants. Research suggests that culture conditions can be manipulated to promote morphinan alkaloid production (1).

Figure 5. *Histochemical staining for GUS activity of Persian poppy tissues transformed with the GUS gene*

In one study, numerous adventitious shoots developed from the transformed callus, but the transgenic shoots were morphologically different from wild-type shoots (16). Moreover, the relative content of morphine and codeine was altered in transgenic shoots compared to non-transformed shoots (17).

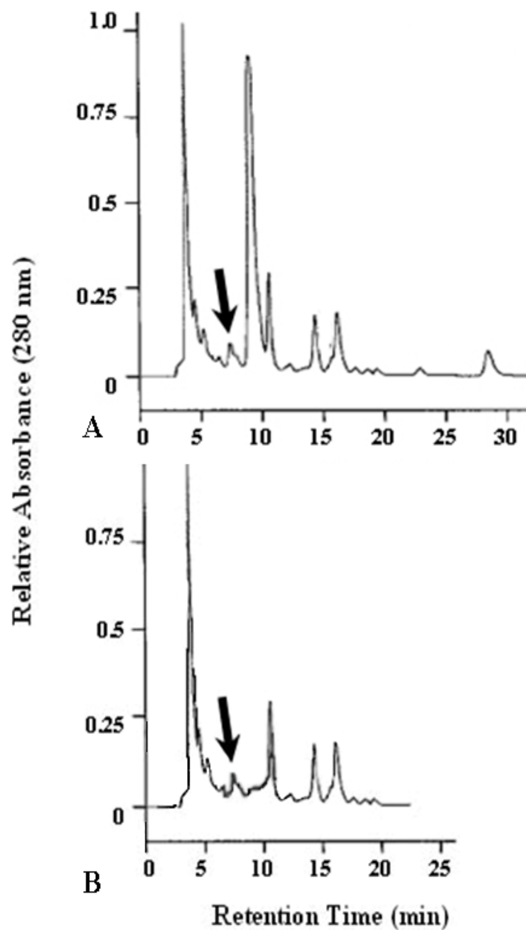

Figure 6. *HPLC elution profile of methanol extracts from transgenic tissues (A) and non-transgenic (B) of Persian poppy. Peak of morphine identified in figure exhibit UV spectra with classic benzylisoquinoline alkaloid signatures*

CONCLUSIONS

In this research, the normal morphine profile of the transgenic Persian poppy (in comparison with non-transgenic plants) shows a valuable tool for studying the regulation of genes encoding alkaloid biosynthetic enzymes, and the network architecture of the pathway via genetically engineered alternations in the activity of individual enzymes.

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