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Agrobacterium-Mediated Transformation of Large DNA Fragments Using a BIBAC Vector System in Rice

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Abstract Large DNA fragments were transferred to rice (*Oryza sativa* L.) by an *Agrobacterium*-mediated transformation protocol using the binary bacterial artificial chromosome (BIBAC) vector system. Calli derived from mature embryos of japonica rice cultivar H1493 were used as target tissues. LBA4404 with the pCH32 helper plasmid carrying *virE* and *virG* was found to be the most efficient strain for the transfer of large DNA fragment into the rice genome. One notable difference between *Agrobacterium*-mediated transformation using standard binary vectors and that reported herein was that transformation using the BIBAC system required *Agrobacterium tumefaciens* carrying the virulence helper plasmid with *virG*/*virE*. Polymerase chain reaction, Southern blot, and progeny analyses confirmed the integration and inheritance of the insert fragment and marker genes carried by BIBAC in the T_0, T_1, and T_2 generations of transgenic events. To our knowledge, this represents the first report in which fertile, stable transgenic rice has been produced using the BIBAC vector system. The transformation system developed here would be useful for transferring large DNA fragments and for cloning and functional analysis of genes in rice.

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Introduction

DNA transfer to plants has been accomplished by many methods, including *Agrobacterium*-mediated transformation, biolistic transformation (particle bombardment), electroporation, and microinjection. Whereas plant transformation with DNA fragments below 20 kb is routine, success in stable plant transformation with DNA fragments larger than 50 kb is limited (Ercolano et al. 2004). The technologies of cloning and transferring large DNA fragments in plants are important for the high-efficiency identification of new genes and the study of gene functions. A reliable system for transforming large fragments of DNA into plants makes it feasible to introduce a natural gene cluster or a series of previously unlinked foreign genes into a single locus. Thus, several diseases and/or pest resistance genes or genes encoding the enzymes of metabolic pathways could be simultaneously introduced in one transformation step. Large insert transformation would make it feasible to study the expression of plant genes or gene clusters in their native genomic context and might eliminate integration site-dependent gene expression, which can be a serious problem in plant transformation experiments.

Two new vector systems, i.e., binary bacterial artificial chromosome (BIBAC; Hamilton 1997) and transformation-competent artificial chromosome (Liu et al. 1999), were developed to clone large DNA fragments and transform plants directly by *Agrobacterium*-mediated methods. Genomic libraries constructed with these vectors (Hamilton et

al. 1999; McCubbin et al. 2000; Bentolila and Hanson 2001; Liu et al. 2002; Chang et al. 2003; He et al. 2003; Wang et al. 2010) can be used for positional cloning of genes, and the candidate clones of target genes can be used directly to transform plants for genetic complementation. Therefore, the use of these vector systems can simplify and increase the efficiency of positional cloning of genes in plants. The BIBAC system has been shown to be efficient for transferring large DNA into tobacco (Hamilton et al. 1996), *Brassica napus* (Cui et al. 2000), and tomato (Frary and Hamilton 2001) and for complementation of mutants (Cui et al. 2000). The introduced T-DNA was stably maintained and inherited through several generations and no gene silencing was observed (Frary and Hamilton 2001).

Rice (*Oryza sativa* L.), a monocotyledonous plant, is the staple food for almost two thirds of the world's population. In addition, its relatively small genome makes it one of the most important models for genome research and breeding of other cereal crops. *Agrobacterium*-mediated transformation of rice was widely reported (Hiei et al. 1994; Dong et al. 2001; Cheng et al. 1998; Ye et al. 2000). However, introduced DNA fragments by the above transformation methods were generally not larger than 25 kb. We developed a BIBAC-based protocol (He et al. 2006) and, in this study, we report the updated results with transforming large fragments of DNA into rice by the BIBAC system.

Materials and Methods

Plant Material, Bacterial Strains, and Vector Plasmids

Seeds of the japonica rice cultivar H1493 was used in this study.

Agrobacterium tumefaciens strains LBA4404 (Ooms et al. 1982), EHA105 (Hood et al. 1993), and AGL1 (Lazo et al. 1991) were used in this work. The virulence helper plasmid pCH32 carrying *virG* from the supervirulent *A. tumefaciens* strain Bo542 and *virE* locus containing *virE1* and *virE2* from *A. tumefaciens* strain A6 (Hamilton 1997) was introduced into the three *A. tumefaciens* strains to be used in the experiments. A BIBAC clone, 134J9 (60 kb), was selected from the BIBAC library of wild rice *Oryza officinalis* (He et al. 2003). The plasmid DNA was isolated by the alkaline lysis method (Sambrook and Russell 2001) and was introduced into the three *A. tumefaciens* strains with the virulence helper plasmid pCH32 and the three strains without the plasmid pCH32, respectively. Structural stability of the clone in *A. tumefaciens* strains was checked as described previously (He et al. 2003).

Rice Transformation and Regeneration Procedure

Callus Induction, Subculture, and Precultivation

Mature fresh seeds were selected and dehusked manually and soaked in 70% ethyl alcohol for 1 min, surface sterilized with 1.5% (*w*/*v*) NaClO for 30 min followed by rinsing thoroughly (at least four changes) with sterile distilled water in a sterile hood. Seeds were blotted dry on a sterile filter paper and placed on the surface of 25 ml solidified induction medium N6I (N6 [N6 major salts, N6 minor salts, and N6 vitamins; Chu et al. 1975], 1.0 g/L proline, 0.4 g/L enzymatic casein hydrolysate [CH], 2.0 g/L 2,4-dichlorophenoxyacetic acid [2,4-D], 45 g/L sucrose, 3 g/L phytagel, pH 5.9) in 9-cm plates, with 10–12 seeds in every plate. The plates were wrapped with Parafilm and placed inside an unlit growth chamber at 26±2°C for 2 weeks. After 2 weeks, the light yellow, compact, and relatively dry calli derived from scutella were dissected out and subcultured on the same N6I medium for another 2 weeks. The actively growing, healthy looking, embryogenic calli were selected and inoculated onto precultivation medium N6P (N6, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, 3 g/L phytagel, pH 5.6) for 4 days.

Agrobacterium Preparation, Infection, and Cocultivation

Agrobacterium strains (harboring the BIBAC clone 134J9) were grown for 2–3 days at 28°C on solid Luria–Bertani medium supplemented with appropriate antibiotics (50 mg/L kanamycin [Km] and 5 mg/L tetracycline [Te] for LBA4404 with pCH32; 50 mg/L Km and 10 mg/L Te for EHA105 with pCH32 and AGL1 with pCH32; 50 mg/L Km for LBA4404, AGL1, and EHA105). Bacterial cells were resuspended in liquid medium N6A (N6, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, pH 5.6, with 100 μM acetosyringone added before using) with shaking (150 rpm) at 28°C for 2 h to an OD_{600}=1.0. Freshly precultured (for 4 days) embryogenic calli (2–4 mm in diameter) were immersed in bacterial suspension for 15 min with gently shaking (80 rpm). The excess bacteria were removed by decanting the liquid. The calli were transferred to sterile filter papers for blot-drying and then placed on cocultivation medium N6C (N6A supplemented with 3 g/L phytagel) to cocultivate in the dark at 24°C for 3 days.

Selection and Regeneration

After coculture of the infected calli, they were washed (thoroughly and gently) in sterile water several times until water became clear. Then, they were washed twice (15 min each time) with sterile water containing 400 mg/L of

cefotaxime with gentle shaking (80 rpm). They were blot-dried on filter papers and placed on resting medium N6R (N6, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.8, with 400 mg/L cefotaxime added before using) at 26±2°C for 1 week. Next, the explants were transferred to selection medium N6S (N6, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.9, with 250 mg/L cefotaxime and 50 mg/L hygromycin added before using) and were subcultured every 2 weeks. The cultures were kept in the dark at 26±2°C for 6–8 weeks until resistant calli proliferated. The hygromycin-resistant calli selected by 50 mg/L hygromycin were moved to preregeneration medium MSP (MS [MS major salts, MS minor salts, and MS vitamins; Murashige and Skoog 1962], 2 g/L CH, 2 mg/L KT, 0.2 mg/L 1-naphthaleneacetic acid [NAA], 30 g/L maltose, 3 g/L phytagel, pH 5.8) for 1 week. Afterwards, the growing calli were cultured on regeneration medium MSR (MS, 3 mg/L KT, 0.5 mg/L NAA, 30 g/L maltose, 3 g/L phytagel, pH 5.8) for 2–4 weeks. When the shoots developed into 2- to 4-cm plantlets, they were transferred to 30×200 mm tubes containing root-growing medium MSG (1/2 MS, 0.2 mg/L NAA, 10 g/L sucrose, 2.5 g/L phytagel, pH 5.8, with 50 mg/L hygromycin added before using) and were grown under the same condition for 2–3 weeks. Well-rooted plants were then transferred to soil in pots and grown in a greenhouse.

Gus Assay, PCR, and Southern Blot Analysis

Calli and seeds were assayed for the expression of the *gus* gene following the histochemical procedure (He et al. 2003). Genomic DNA was isolated from young leaves of the control, T_0, T_1 (from selfed seeds of T_0 transformants), and T_2 (from selfed seeds of T_1 transformants) transgenic rice plants (McCouch et al. 1988). Regenerated plants were analyzed by polymerase chain reaction (PCR), using three sets of primers specific to the *gus* sequence (5′-TCGCGAAAACTGTGGAATTGATC-3′, 5′-AGCCGACAGCAGCAGTTTCAT-3′), the *hpt* sequence (5′-GATGTAGGAGGGCGTGGATATGTC-3′, 5′-CTTCTACACAGCCATCGGTCCAGA-3′), and the *npt*II sequence (5′-TCGGCTATGACTGGGCACAACAGA-3′, 5′-AAGAAGGCGATAGAAGGCGATGCG-3′), respectively. The PCR reaction were run using the following reaction conditions with a final volume of 25 µl: (1) 10 mM primers (R+F); (2) 20 ng genomic DNA; (3) 10× buffer; (4) 0.5 U Taq. PCR was carried out with the following program: 94°C for 5 min for one cycle; 94°C for 50 s, 55°C for 50 s, and 72°C for 80 s for 34 cycles; 72°C for 10 min for one cycle. PCR products were analyzed by gel electrophoresis on 1% agarose gels. Genomic DNA (5 µg) was digested with *Eco*RV + *Hin*dIII. The PCR-amplified fragment of the *hpt*/*npt*II gene was ^{32}P-labeled by a Prime-a-Gene® labeling system (Promega, USA) and used as the radioactive probe for Southern blot analysis (Sambrook and Russell 2001).

Results

The Transformation Effect of Different *A. tumefaciens* Strains

Different *A. tumefaciens* strains (AGL1, EHA105, and LBA4404) were investigated for transformation efficiency. Table 1 summarizes the results of three *Agrobacterium* strains with additional *vir* genes on rice transformation. Among the different strains of *Agrobacterium* tested, only the strains with additional pCH32 could successfully produce transformants. A significant enhancement of transformation efficiency was observed when the strain LBA4404 with pCH32 contained additional *vir* genes (Table 1). The three strains which produced transformants also contained the virulence helper plasmid pCH32, which overexpresses the VirG and VirE virulence proteins involved in the transcriptional activation of *vir* genes and the protection of the T-DNA from degradation by endonuclease, respectively (Hamilton 1997). But the same strains without additional *vir* genes cannot transform large DNA fragment clones. The results suggest that there are different transfor-

Table 1 The effect of *Agrobacterium* strains and *vir* genes on transformation events of BIBAC clone (134J9) in rice cultivar H1493

Agrobacterium strains + *vir* genes	Antibiotic selection (mg/L)	No. of calli cocultivated (A)	No. of resistant calli[a]	No. of transformants (B)	Transformation efficiency (B/A, %)
LBA4404 + pCH32	Km 50 + Te 5	436	193	12	2.8
LBA4404	Km 50	458	41	0	0
EHA105 + pCH32	Km 50 + Te 10	427	203	5	1.2
EHA105	Km 50	432	65	0	0
AGL1 + pCH32	Km 50 + Te 10	419	164	2	0.5
AGL1	Km 50	415	19	0	0

[a] Hygromycin-resistant calli selected by 50 mg/L hygromycin

mation efficiencies for different strains and that, for strains to produce transformants, they need to contain additional *vir* genes. Additional *vir* genes as well as the *Agrobacterium* genetic background are very important for the transformation of large DNA fragments in rice.

Gus Assay, PCR, and Southern Blot Analyses

The transformed calli growing on selection medium and the T_1 seeds were tested for GUS activity. About 80% of the calli (infected by LBA4404 with the virulence helper plasmid pCH32) and the transformed T_1 seeds were GUS positive. No GUS expression was observed in untransformed calli and seeds (Fig. 1).

Putatively transformed T_0 plants were analyzed by PCR with primers specific to plants' selectable marker genes *gus*, *npt*II, and *hpt*; 82% (18 out of 22) of the plants tested positive by PCR for *gus*, *npt*II, and *hpt* (Fig. 2).

The transformed T_0 plants were confirmed by Southern hybridization with the *npt*II and *hpt* probes (Fig. 3). Genomic DNA from transformed plants was digested with *Eco*RV and *Hin*dIII and allowed to hybridize with the *hpt* and *npt*II probes. The number of hybridizing bands indicates the *hpt*/*npt* gene copy number. The copy number of the 18 transgenic plants ranged from one to five copies, and most (83%) of the plants (15 out of 18) contained two to four copies. Only 11% (two out of 18) of the transformants had single-copy integration. Most of bands

Fig. 1 GUS expression in the transformed callus and T_1 seeds; the controls are on the *left*

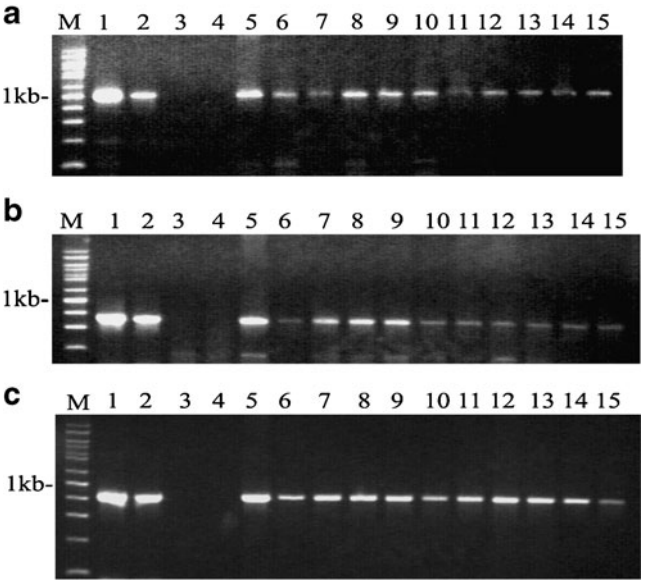

Fig. 2 PCR analysis of genomic DNA to detect the presence of the *gus*-coding (**a**), *npt*II-coding (**b**), and *hpt*-coding (**c**) regions in transformed plants. *Lane M* molecular weight markers, *lanes 1* and *2* positive control BIBAC2, *lanes 3* and *4* untransformed control plant, *lanes 5–15* rice plants regenerated from 11 independent, hygromycin-resistant calli, showing amplification of the predicted specific sequences 998 bp for *gus*, 722 bp for *npt*II, and 852 bp for *hpt*

detected with the *hpt* or *npt* probes had the fragment size of more than 3 or 2 kb, respectively, which are the minimum sizes of hybridizing fragments expected from the map of BIBAC2. Plants that regenerated from a given colony of cells gave an identical pattern (lanes 3 and 6 in Fig. 3), indicating that these plants were clonal. Otherwise, the mobility of the bands differed from plant to plant, indicating independent event and random integration. Some of the fragments were smaller than 3 kb probed by *hpt* (lanes 3, 6, and 7 in Fig. 3a). In some transformants, the number of fragments probed by *hpt*-specific right border (lane 1 in Fig. 3a) was more than that of the ones probed by *npt*-specific left border (lane 1 in Fig. 3b). It was suggested that irregular integration patterns of transgenes could exist in the transformation system using the BIBAC clone.

Analysis of T_1 and T_2 Plants

To check for the segregation pattern of the transgenes in the progeny, T_1 seeds randomly chosen from eight T_0 plants (eight events) were planted. The ratio of 3:1 (from T_0 self) segregation of both *hpt* and *npt*II genes of the T_1 plants was observed for five out of the eight independent events (data not shown), indicating that more than two copies of genes were integrated closely to one another at a single locus in some plants. A Southern blot showed the presence of *hpt* in the T_1 and T_2 plants (Fig. 4).

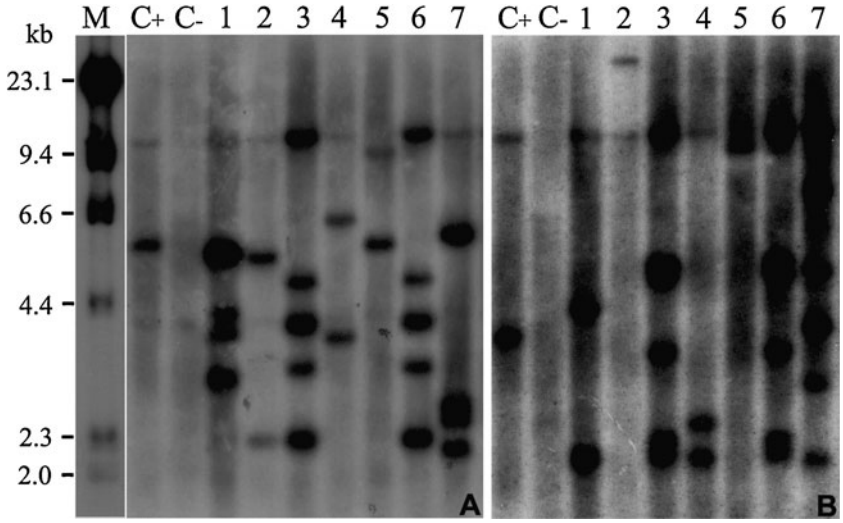

Fig. 3 Southern analysis of transgenic rice T_0 plants transformed with a 60 kb *O. officinalis* BIBAC clone (134J9). BIBAC2 in *Escherichia coli* DH10B (*C+*), genomic DNA from a nontransgenic plant (*C−*), and transgenic plants (*1–7*) were digested with *Eco*RV and *Hin*dIII; the PCR-amplified *hpt* (**a**) and *npt*II (**b**) genes were used as the probe for Southern hybridization. *M* is λDNA digested with *Hin*dIII

Discussion

Our results demonstrated that additional copies of *virG* and *virE* genes in *A. tumefaciens* obviously enhance the transformation efficiency of the large DNA fragment using the BIBAC vector in rice though different *A. tumefaciens* strains also affected the transformation. The transformation efficiency in all three *A. tumefaciens* strains is relatively low. It could be due to the large insert fragment in the BIBAC vector. Park et al. (2000) suggested that *A. tumefaciens* containing a shorter T-DNA has a higher transformation efficiency than *A. tumefaciens* containing a longer T-DNA in tobacco, cotton, and rice.

In this system, the virulence helper plasmid pCH32 has been engineered to overexpress *virG* and *virE* operon. The VirG protein is a transcriptional regulator, which activates the *vir* gene promoters and results in increased expression of all *vir* genes. The VirE1 protein mediated the export of VirE2 from *Agrobacterium* into plant cells. The larger genomic inserts were anticipated to require increased amounts of virE2 protein; this hypothesis is supported by the increased transformation efficiencies observed with

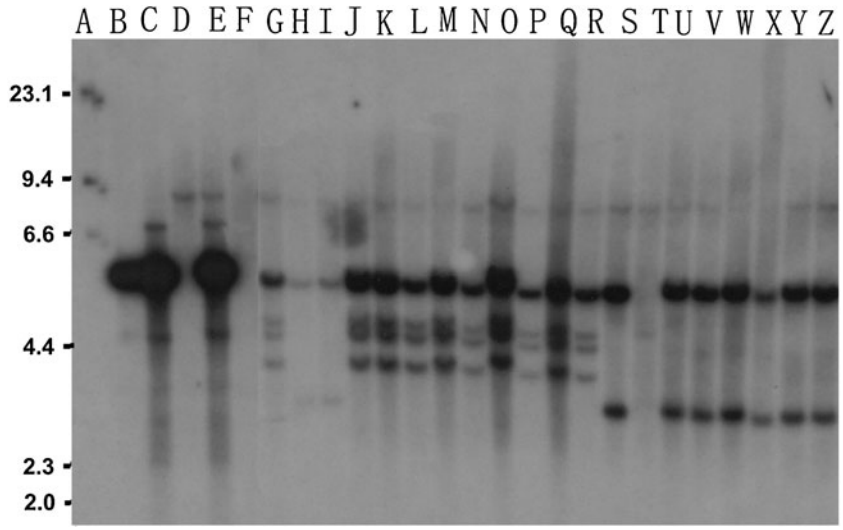

Fig. 4 Southern analysis of T_1 and T_2 plants from two T_0 plants. *Lane A* λDNA digested with *Hin*dIII, *lane B* BIBAC2 empty vector in *E. coli* DH10B, *lane C* plasmid of the clone 134J9 in *E. coli* DH10B, *lane D* the control rice (H1493) genomic DNA, *lane E* the control rice (H1493) genomic DNA spiked with plasmid of 134J9, *lane F* genomic DNA from the wild rice *O. officinalis*, *lane G* DNA from a T_0 plant, *lane H*, *I*, and *S* DNA from another T_0 plant, *lanes J–N* DNA from five T_1 plants derived from the T_0 in lane G, *lanes O–R* DNA from four T_2 plants derived from the T_1 in lane J, *lane T* DNA from *hpt*-negative T_1 plant, *lanes U–W* DNA from three T_1 plants derived from the T_0 in lane S, *lanes X–Z* DNA from three T_2 plants derived from the T_1 in lane U. DNA in lanes B–Z was digested with *Eco*RV and *Hin*dIII. The PCR-amplified *hpt* gene was used as the probe for Southern hybridization

strains overexpressing *virE*2. The *virG* or *virE* might be important factors in the *Agrobacterium*-mediated transformation process. When *virG*/*virE* was overexpressed, the transformation efficiency significantly increased (Park et al. 2000; Tang 2003; Vain et al. 2004).

Southern analysis showed different hybridization patterns among the tested transformants, indicating that T-DNAs were randomly integrated in the rice genome. The T-DNA fragments that hybridized to the *hpt* or *npt*II probe clearly did not derive from *A. tumefaciens* used in the transformation because the vectors in the strains used would have a 5.6-kb band for the *hpt* probe or a 4.2-kb band for the *npt*II probe, as in the control lanes. Based on the samples tested, only 11% of the plants have single-copy inserts. The differences of integrated transgene copy number between transformation of relative small fragment and that of the large fragment carried by BIBAC may be related, at least in part, to the T-DNA size and the additional *vir* genes. Certainly, the differences could also be due to the species or ecotype and explant type of the host plant, the *Agrobacterium* strain, and the plasmid used.

The genetic analysis of T_1 and T_2 progeny also provided strong evidence of the incorporation of T-DNA into the rice genome. Southern analysis of the T_1 and T_2 generations supported that the *hpt*/*npt*II gene was inherited to the T_1 and T_2 generations.

The BIBAC vector has been developed to permit the insertion of large fragments of DNA (up to 150 kb) in tobacco (Hamilton et al. 1996) and tomato (Frary and Hamilton 2001), making it especially useful for the simultaneous introduction of several genes, such as those encoding the enzymes of metabolic pathways (Shibata and Liu 2000). Our results demonstrate that the BIBAC vector can be used to introduce large DNA fragment into rice via *Agrobacterium*-mediated transformation. It was suggested that the transfer of large DNA fragments via the BIBAC system is likely to work not only in dicot plants but also in monocot plants. Application of this strategy could accelerate the functional analysis of single genes or complex loci and be used to streamline the positional cloning of plant-specific genes. This transformation system would also open the way for metabolic engineering in plants, which require the addition of several transgenes (Naqvi et al. 2010).

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