Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker


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Abstract

The olive fruit fly (olive fly) *Bactrocera oleae* (Dacus), recently introduced in North America, is the most destructive pest of olives worldwide. The lack of an efficient gene transfer technology for olive fly has hampered molecular analysis, as well as development of genetic techniques for its control. We have developed a *Minos*-based transposon vector carrying a self-activating cassette which overexpresses the enhanced green fluorescent protein (EGFP). Efficient transposase-mediated integration of one to multiple copies of this vector was achieved in the germ line of *B. oleae* by coinjecting the vector along with *in vitro* synthesized *Minos* transposase mRNA into preblastoderm embryos. The self-activating gene construct combined with transposase mRNA present a system with potential for transgenesis of very diverse species.

Keywords: Olive fruit fly; *Bactrocera oleae*; germ line transformation; transgenesis marker.

Introduction

Arthropods are key pests of food and fibre crops as well as major disease vectors. Field resistance to pesticides presents an increasing challenge to pest control. Progress in molecular manipulation of insect species is an important way to further comprehension of the genetic and biochemical basis of insect biology. In addition, it will open the way for novel strategies to control insect pest populations, as, for example, through production of insects for sterile insect technique (SIT) programs.

The technologies for insect transgenesis developed so far rely on transposable elements. Manipulation of an insect genome with a transposable element was first achieved in *Drosophila* with the P element (Rubin & Spradling, 1982). However, the host range of P seems to be restricted to the drosophilids (Handler *et al*., 1993). The discovery of new elements with a broader host range has led to the development of vector systems for transformation of several non-drosophilid insect species, including the Mediterranean fruit fly *Ceratitis capitata* (Loukeris *et al*., 1995b; Handler *et al*., 1998; Michel *et al*., 2001), the mosquitoes *Aedes aegypti* (Jasinskiene *et al*., 1998; Coates *et al*., 1998), *Anopheles albimanus* (Perera *et al*., 2002), *Anopheles stephensi* (Catteruccia *et al*., 2000) and *Anopheles gambiae* (Grossman *et al*., 2001), the housefly *Musca domestica* (Hediger *et al*., 2001), the Australian sheep blowfly *Lucilia cuprina* (Heinrich *et al*., 2002), the silkworm *Bombyx mori* (Tamura *et al*., 2000), the beetle *Tribolium castaneum* (Lorenzen *et al*., 2003; Pavlopoulos *et al*., 2004) and the New World screw-worm *Cochliomyia hominivorax* (Allen *et al*., 2004).

*Bactrocera oleae* is the main pest of olives in the Mediterranean region and in California, where it has been introduced recently. Currently, control of *B. oleae* is based on the use of insecticides either in bait or in cover sprays, resulting in adverse effects on the environment and sometimes presence of unacceptably high levels of insecticides in olives and olive oil (Ferreira & Tainha, 1983). Development of olive fly control strategies leading to reduced or no use of insecticides has been attempted with mixed results, for example, ‘lure and kill’ (Haniotakis *et al*., 1991), localized bait spray (Pucci, 1990) and inundative or inoculative release of parasitoids (Tzanakakis, 1995). SIT has been used with limited success (Economopoulos *et al*., 1977), apparently because of low competitiveness of the mass-reared males compared to the wild males (Economopoulos...
Availability of a method for olive fly transgenesis is a prerequisite for genetic manipulation of this species for development of more effective control methods, such as release of insects carrying a dominant lethal allele (RIDL strategies (Heinrich & Scott, 2000; Thomas et al., 2000) and sensitization of insect populations to pro-insecticides (SIPP) (Markaki et al., 2004).

The transposon Minos from Drosophila hydei belongs to the Tc1/Mariner family of transposable elements (Franz & Savakis, 1991) which, in addition to insects, has been shown to mediate transgenesis in human cells (Klinakis et al., 2000a), in mouse somatic and germ cells (Zagoraiou et al., 2001; Drabek et al., 2003) and in the ascidian Ciona intestinalis (Sasakura et al., 2003). Encouraged by this broad host range, we attempted germ-line transformation of B. oleae with a Minos vector carrying an enhanced green fluorescent protein (EGFP) gene. EGFP is a universal marker for insect transgenesis (Berghammer et al., 1999).

In this report, we describe a self-stimulating transcription system for high levels of regulatable expression of marker genes in diverse species. Using this system in combination with a Minos transposon, we demonstrate stable and efficient transgenesis of B. oleae.

Results

A potentially universal EGFP-based genetic marker

To achieve high levels of EGFP expression, we used a self-activating and self-sustained positive feedback loop based on the tetracycline-sensitive transcriptional activator tTA (Gossen & Bujard, 1992), which is based on concomitant expression of EGFP and tTA from a bi-directional promoter driven by tTA. Two versions of the expression cassette were generated. The first version was derived from a commercially available cassette consisting of a 7-mer of the tetracycline operator (tetO) (Gossen & Bujard, 1992) flanked by two divergent copies of a cytomegalovirus (CMV) minimal promoter (the ‘mammalian version’). The second was constructed for use in insects and consisted of a 14-mer of the tetO element flanked by two divergent copies of the minimal promoter of the Drosophila melanogaster hsp70 gene (the ‘insect version’). A 14-mer was used in the insect version in order to maximize expression levels (Fig. 1, top).

The insect cassette was tested in D. melanogaster and in the medfly Ceratitis capitata by P- and Minos-based germ line transformation, respectively. In D. melanogaster, most of the transgenic lines exhibited strong, ubiquitous and constitutive expression of EGFP throughout development (Fig. 1a–d), while only some lines showed tissue-specific expression patterns (not shown). Fluorescence was less intense in medfly transformants, which were characterized by specific patterns of expression during development (Fig. 1e–f).

Figure 1. Top: the tTA/enhanced green fluorescent protein (EGFP) self-stimulating expression cassette. Bottom: EGFP expression. (a–d) transformed Drosophila melanogaster adults, embryos, third instar larva and pupae; red arrowheads point to non-transformed individuals. (e–f) Transformed medfly pupa and adult. (g–h) Human HeLa cells stably transfected with insect and mammalian cassette, respectively. (i–j) Nicotiana tabacum leaf transiently transfected with insect and mammalian cassette, respectively. (k) Transgenic Caenorhabditis elegans animal harbouring a plasmid vector carrying the insect cassette.
The versatility of the bidirectional loop was further explored in somatic cells of animals and plants. HeLa cells were transiently and stably transfected with both the ‘mammalian’ and the ‘insect’ construct; fluorescence was detectable 24 h post transfection (Fig. 1g–h) and peaked 48 h post-transfection. The same constructs were also introduced into tobacco leaves (Nicotiana tabacum and N. bentamiana) via gold particle ‘bombardment’; examination 48 h post transfection showed strong EGFP expression in certain cells (Fig. 1i–j). The ‘insect’ vector was also introduced into the nematode Caenorhabditis elegans, leading to strong and uniform EGFP expression (Fig. 1k).

Experiments performed in Drosophila and in HeLa cells carrying stable, active integrations of the tTA-containing cassette showed that EGFP expression is reversibly suppressed by the teracycline analogue doxycycline at concentrations known to inhibit tTA binding to the promoter (data not shown). Furthermore, high levels of EGFP expression were dependent on the presence of the bidirectional loop because no fluorescence was obtained from control constructs containing the bidirectional promoter without the tTA gene (data not shown).

Olive fly transformation with a Minos element
Preblastoderm embryos of the B. oleae Demokritos strain were coinjected with the Minos-based ‘insect’ construct and Minos transposase mRNA, a method that has been previously shown to result in high transformation frequencies (Kapetanaki et al., 2002). Transient EGFP expression was observed in about 60% of the injected embryos examined (Fig. 2, panels A to C), indicating that the genetic marker is functional in the olive fly.

From the 3833 injected G0 embryos, 641 larvae hatched, from which 151 male and 174 female flies developed. The male G0 flies were mated in a single cage to 506 non-injected female flies, and the female G0 flies were mated in another cage to 250 wild-type male flies. The G1 progeny from the two crosses was screened for EGFP fluorescence at the third instar larval stage. A total of 8824 third instar larvae was screened; 67 of them exhibited green fluorescence in various tissues and were therefore considered transformant.

To establish transformed lines, 13 individual G1 flies were back-crossed to wild-type flies and the resulting G2
third instar larvae were again screened for EGFP expression. Four of the crosses did not give any progeny. Where numbers allowed assessment, the proportion of progeny expressing the EGFP marker was consistent with segregation of a single allele (Fig. 3).

Clearly detectable EGFP fluorescence was observed in second and third instar larvae, pupae and adult flies in all transformed lines examined. Nine independent transformed lines were established (lines 2, 5, 6, 8, 9, 10, 11, 12 and 13). The pattern of EGFP expression differed considerably from line to line (Fig. 2). Furthermore, some lines showed variability of EGFP expression (variegation) between siblings, although the overall pattern was characteristic (lines 9, 11 and 13; data not shown). With one exception (line 9), patterns of expression did not change through the generations. At least two different expression patterns were detected and were segregating in line 9, one of which, highly localized, is shown in Fig. 2(j–l). Because line 9 contained multiple insertions, it is likely that two active insertions (i.e. insertions expressing EGFP) existed in this line, each with a distinct pattern of expression. Transmission of the EGFP marker has been maintained in all lines for at least eight generations, demonstrating that the marker is stably inherited, as expected in the absence of transposase.

Molecular analysis of the transformed lines

Insertion of the Minos element into the B. oleae genome was verified by Southern blot analysis on EcoRI-digested genomic DNA with an EGFP probe. Any single, transposase-dependent insertion of the transposon into genomic DNA is expected to give a band of at least 1300 bp in size. Based on this criterion, the presence of a single insertion was detected in progeny of transgenic flies 2, 5 and 6, while progeny of transgenics 8, 9, 10, 11, 12 and 13 contained multiple insertions (Fig. 4). Of the nine cases analysed, only two, 5 and 6, were found to yield a band of the same size (Fig. 4). Additionally, these two showed the same pattern of EGFP expression, suggesting that they represent the same transformation event. The others were regarded as independent transformation events. Southern blot analysis performed in individual flies from lines 13 and 11 at subsequent generations showed that some of the bands were lost, clearly suggesting that individual bands represent independent, unlinked integrations of the element (data not shown).

To verify that the insertions are indeed transposase-mediated, integration sites were amplified by inverse PCR (Triglia et al., 1988) from four of the lines. The PCR products were cloned and sequenced. As shown in Fig. 5, each of the four integration sites contains the Minos inverted terminal repeat followed by a TA dinucleotide and a sequence not present in the original plasmid. We therefore conclude that the Minos construct inserts through transposition and not by recombination.

The sequences adjacent to the insertion sites were subjected to BLAST analysis against GenBank. In two of the
sites analysed, the retrieved flanking sequences were too short to allow a meaningful BLAST analysis (Fig. 5). One of the two other sequences (line 10) showed a 91% identity (E-value: 2e-11) to a region within the large intron of the corkscrew gene of Drosophila (see Supplementary Material).

Discussion

We have constructed a versatile marker cassette, in which the EGFP gene is expressed from a bidirectional promoter controlled by the synthetic transcriptional activator tTA (Gossen & Bujard, 1992); the promoter also drives the expression of tTA. This results in self-sustaining expression of the tTA activator. This positive-feedback system should be constitutively active once transcription is initiated, for example from an enhancer near the site of insertion.

The marker cassette presents several interesting features. Firstly, strong EGFP expression in most stages of development, allowing easy selection of transformants. Secondly, the marker is active in diverse organisms, because it depends on the versatile tTA/tetO system rather than a host-derived promoter. Thirdly, transient expression of the marker in embryos injected with DNA, allowing early assessment of injection efficiency and functionality of the marker. This can be a highly desirable property, especially for transformation of species that have a long generation time or are difficult to breed. Fourthly, the EGFP expression patterns differ widely between different insertions in some species such as in medfly and in olive fly, a feature that can be used to distinguish easily between different transgenic lines. Additionally, the system can be used as a base for conditional expression of exogenous genes in diverse species. The results presented here suggest that the system may also be useful as a sensitive enhancer-trap (Bellen et al., 1989; Wilson et al., 1989) for identification of tissue- or stage-specific genes in diverse species.

It has been shown recently that a construct expressing tTA from a tetO promoter causes tetracycline-repressible lethality in transgenic medfly (Gong et al., 2005); toxicity of overexpressed tTA was presumably the cause of lethality. Such lethals would not have been recovered in our screen for transformants, which was performed in the absence of tetracycline and was based on EGFP expression. We observed, nevertheless, tetracycline-repressible lethality in a number of Drosophila transformants, only in the homozygous form. As we did not perform a systematic screen for dominant tet-repressible lethals, we cannot estimate the frequency of tTA-induced lethal transgenes carrying our bidirectional tTA/EGFP construct. It is also possible that the strong lethality observed by Gong et al. (2005) may be caused by higher levels of tTA, as a tTA variant was used which is optimized for expression in D. melanogaster.

Using the tTA/EGFP self-sustaining cassette, we have shown that the transposable element Minos from D. hydei can mediate stable germ-line transformation of B. oleae, thus extending the number of species that have been transformed with this element. Transformation frequency, expressed as the fraction of G0 individuals producing transformed progeny, could not be estimated from these experiments due to the fact that G0 flies were bred in groups. However, the overall number of phenotypically detectable transformation events (67 EGFP expressing G1 progeny from 325 G0 adults) is sufficient to produce several independent transformants from a single experiment, as thousands of embryos can be injected and hundreds of G0 adults can be obtained within the productive 10-day period of egg laying.

Of the nine transformed lines analysed, three lines had a single copy of the transgene, line 11 carried two copies, lines 10 and 12 three and lines 8, 9 and 13 contained more than three copies, contrary to other Minos-based transformation experiments (Loukeris et al., 1995a,b) where most of the individuals analysed contained a single copy of the transgene. This difference may be a function of the species transformed. Alternatively, it may be the result of high levels of transposase in embryos injected with mRNA, rather than with a transposase-expressing plasmid (Kapetanaki et al., 2002). The presence of multiple Minos insertions in lines such as line 13, which show Mendelian inheritance of EGFP expression, suggests that all insertions except one are silent, that is, they do not express EGFP. This is supported by the observation that in subsequent generations...
some of the insertions are lost without apparent changes in EGFP expression patterns. A possible explanation for the existence of silent insertions is discussed below.

Multiple integrations, although useful for enhanced transgene expression, may increase the potential for lethal or semilethal mutations, decreasing the viability of transgenic lines and furthermore, may complicate applied use of transgenic strains. This, however, could be avoided by using transposase-expressing helper plasmids or lower concentrations of transposase mRNA in embryo injections.

Patterns of EGFP fluorescence varied dramatically among different transgenic lines of olive fly (Fig. 2) and medfly (Fig. 1); in these species EGFP expression was characteristically tissue-specific and variegated. In contrast, the majority of Drosophila transformants carrying single insertions of the self-stimulating cassette exhibited strong and ubiquitous expression of EGFP (Fig. 1). Expression of the EGFP gene from the self-activating construct used in these experiments depends on initial activation (triggering) of the minimal Hsp70 promoter. It is known that the complete Drosophila Hsp70 promoter exhibits low activity in transgenic non-Drosophila species (Berger et al., 1985; our own unpublished results from medfly). It is possible that the minimal Drosophila Hsp70 promoter is also less active in medfly and olive fly, failing to ‘trigger’ the self-sustaining loop in these species, unless the transposon has inserted near an active enhancer. This could explain both tissue-specific, variegated expression from some insertions and lack of expression from others.

This report describes what appears to be a universal genetic marker for detection of transgenic organisms and its use for transgenesis of the major olive pest B. oleae by means of Minos-mediated germline transformation. With this system, the olive fly is now amenable to transgenesis. Availability of a transformation system for B. oleae should now make it possible to use genetic engineering for development of genetic strategies for control of this important insect pest.

**Experimental procedures**

**Fly strains and rearing**

The olive fruit flies used in this work originated from the Democritos Laboratories (Athens, Greece) B. oleae stock. Flies were bred locally at 25 °C, 45–50% relative humidity, under a 13 h light/11 h dark cycle and were fed on a diet consisting of 100 g yeast hydrolysate, 400 g sugar, 30 g egg yolk and 250 mg streptomycin. Newly hatched larvae were transferred on to the surface of larval medium consisting of 550 ml distilled water, 30 g soy hydrolysate, 0.5 g potassium sorbate, 2 g Nipagin, 20 g sugar, 75 g brewer’s yeast, 30 ml concentrated HCl, 275 g cellulose powder, 20 ml olive oil and 7.5 ml Tween-80 (Tzanakakis, 1989). Larvae burrowed into the food and emerged again when ready to pupate. At this stage they were transferred into small plastic boxes for pupation. Pupae were kept at 25 °C until adult emergence.

**Plasmids and helper RNA**

Donor plasmid pMiBO14/GtTA2, containing the ‘insect’ self-sustaining marker expression cassette was constructed as follows: The EGFP cassette along with the hsp70 minimal promoter and the SV40 polyadenylation sequence was isolated from p3XP3-EGFP (Horn & Wimmer, 2000) as a BstBI (filled-in)/CiaI fragment and was cloned into the EcoRV and CiaI sites of plBluescript II SK (+) (Stratagene, La Jolla, CA, USA) to generate pBS/hTATA-EGFP. The tetracycline operator (tetO) sequence was obtained as an AluI fragment from PBI-L (Clontech, Mountain View, CA, USA). Two copies of this fragment were cloned into the Smal site of plBluescript II SK (+) to give a tetO-14mer (pBS/14tetO). The hsp70 minimal promoter, the I TA gene, the hsp70 polyadenylation sequence and the tetO-14mer (tetO-14) were then cloned into an EcoRI/NotI-digested pBS/hTATA-EGFP in a five-fragment ligation giving pBO14/GtTA. The hsp70 minimal promoter was on a BarnHI/KpnI fragment from plasmid p3XP3-EGFP (Horn & Wimmer, 2000), hsp70pA was on a Xbali/NotI fragment from plasmid pHS6s/LMI2 (Loukeris et al., 1995b) and tetO-14 was on a BarnHI/NotI fragment from pBS/14tetO. The I TA gene was a KpnI/Nhel fragment from plasmid pBl/Gt: Plasmid pBl/Gt was constructed as follows: the EGFP gene was cloned on a PstI/Xbali fragment from plasmid pEGFP-N1 pBl/Gt (Clontech) into plasmid pBlI-1 (Clontech), replacing the luciferase gene, resulting in plasmid pPB1-g. The I TA gene derived from pUHD15-1 (Gossen & Bujard, 1992) on an EcoRI/BamHI fragment (Klinakis et al., 2000b) to give pMiBO14-G. The rest of the cassette from pBO14/GtTA2 was subsequently moved as an Xbali/NotI fragment into pBO14-G to reconstitute the I TA gene, resulting in pMiBO14/GtTA2. The donor plasmid pMiBO7/GtTA was constructed in an analogous manner as pMiBO14/GtTA2. Minos transposase mRNA was synthesized using the linearized plasmid pBlue(SK)/MimRNA as a template (Pavlopoulos et al., 2004), according to the manufacturer’s instructions (Ambion mMessage mMACHINE T7 kit Austin, TX, USA).

**Expression from the I TA/EGFP cassette in animal and plant cells**

Germ line transformation of D. melanogaster and Ceratitis capitata was performed as described previously (Loukeris et al., 1995a,b). HeLa cells were cultured and transfected with super-coiled plasmid DNA by Ca++ coprecipitation as described previously (Klinakis et al., 2000b) and examined for EGFP fluorescence 12 h post-transfection. Transfection of Nicotiana tabacum leaf cells was carried out with a PDS-1000/ Helios Bio-Rad gene-gun (Bio-Rad, Hercules, CA, USA). DNA was loaded on 1 μm gold particles. Bombardment was performed under vacuum using 1100 psi rupture discs and the leaf-disc-containing Petri dish was placed on the top shelf of the apparatus. Leaves were cultivated for two days in Murashige and Skoog (SM) medium (Murashige & Skoog, 1962) and were subsequently examined for EGFP fluorescence. Standard procedures were followed for Caenorhabditis elegans strain maintenance, crosses and other genetic manipulations (Brenner, 1974). The nematode rearing temperature was kept at 20 °C. Supercoiled plasmid DNA was injected into the gonads of Caenorhabditis elegans N2 animals, together with plasmid pRF4 carrying the dominant transformation marker rol-6(su1006) as
described (Mello et al., 1992). Transgenic lines were obtained by screening the F2 generation progeny of injected hermaphrodites for roller animals. Individual transgenic rollers were examined for EGFP fluorescence.

**Olive fly embryo collection and microinjection**

Adult females were allowed to oviposit on to cerasin wax cones for 30 min. Deposited eggs were collected by rinsing with deionized water and dechorionated for 1 min in 2.5% sodium hypochlorite, followed by extensive washes with deionized water. Eggs were aligned on 24 × 50 mm cover slips using double-sided tape, the posterior pole of each egg facing the outer edge of the tape, and covered with halocarbon oil (Sigma, St Louis, MO, USA). Transposon plasmid pMIBO14/GtTA2 (400 ng/µl) and helper RNA (100 ng/µl) (Kapetanaki et al., 2002) in injection buffer (1) were coinjected into the posterior pole of eggs, 45–120 min after oviposition. Following injection, G0 embryos were kept for 2–3 days in a humidified Petri dish at 25 °C. Hatchlings were placed on larval food and grown as described above. G0 individuals were back-crossed to the parental strain. G0 males were group-mated to virgin female flies in a standard Bactrocera oleae 30 × 30 × 30 cm cage at a 1 : 4 (male : female) ratio; G0 females were group-mated to males at a 1 : 1 ratio. G1 eggs were collected daily until no more viable eggs were produced. Eggs were washed with dH2O and incubated at 25 °C for 48 h in 0.3% propionic acid for hatching. First instar larvae were transferred to 90 cm Petri dishes containing larval food. G1 individuals were allowed to develop to third larval instar and were assayed for EGFP expression.

**Mating and selection strategy**

EGFP positive G1 individuals were transferred into small plastic boxes and left to pupate as described above. Single G1s were backcrossed to wild-type flies in small cages (5 × 5 × 10 cm). G1 males were kept at a ratio of 1 : 6 with females, G1 females at a ratio 1 : 1 with males. Thin domes of cerasin wax were introduced into each cage as oviposition surfaces on the third day of adult life. Eggs were collected from the fifth day of adult life and subsequent collections occurred every other day until the 10th collection. Third instar G2 larvae were screened for EGFP expression and the EGFP-positive larvae selected and backcrossed to the parental strain as above. Three generations of backcrosses of EGFP-positive adults to the parental strain were performed. Subsequently, transformed lines were maintained by selection for EGFP positive progeny for further breeding.

**Southern blot analysis**

DNA from single adult flies was purified essentially as described previously (Laird et al., 1991). Flies were homogenized in 0.25 ml 50 mm Tris-HCl pH 8.0, 100 mm EDTA, 1% SDS, 100 mm NaCl, 330 µg/ml proteinase K and incubated at 55 °C overnight. Samples were treated with a DNAse-free RNAse solution (200 µg/ml) at 37 °C for 1 h. This incubation was followed by two phenol/chloroform extractions and one chloroform extraction. The supernatant was transferred to a fresh tube, DNA was isopropanol precipitated, washed with 70% ethanol and resuspended in 20 µl sterile ddH2O. DNA concentrations were determined by spectrophotometry at 260 nm. Five micrograms of genomic DNA were digested with EcoRI, and the resulting fragments separated on a 0.8% agarose gel. After transfer to nylon membranes, blots were hybridized with a 32P-labelled GFP probe. The probe was a 720 bp BamHI/XbaI fragment containing most of the GFP gene.

**Inverse PCR**

Genomic DNA (5 µg) of transformants was digested with isoMboI (Minotech Heraklion, Greece) and restriction fragments were circularized by overnight ligation at 16 °C at a final concentration of 200 ng/ml. PCR was performed on the circularized fragments using primers Imi01 (5′-AGAGAATAAATCTCTTTGAGACG-3′) and Imi1 (5′-CAAAATATGAGTAATTATCAACCGG-3′), followed by a second round of nested PCR with primers Imi02 (5′-GATAATAGTGTTAACATTGCACG-3′) and Imi2 (5′-GCTTAAGAGATAAAAAAGTGACC-3′) (Klipakis et al., 2000a). PCR conditions were in both cases: 94 °C for 30 s; 58 °C for 30 s; 72 °C for 2 min; 30 cycles. PCR fragments were separated on a 2% agarose gel. Purified fragments were cloned into vector pGEM-T easy (Promega, Madison, WI, USA) and sequenced using T7 and SP6 primers.

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