

Transformation of *Magnaporthe grisea* to phosphinothricin resistance using the bar gene from *Streptomyces hygroscopicus*

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Three transformation systems have been reported for the rice blast fungus *Magnaporthe grisea* (Parsons et al. 1987 Proc. Natl. Acad. Sci. USA 84:4161-4165; Daboussi et al. 1989 Curr. Genet. 15:453-456; Leung et al. 1990 Curr. Genet. 17:409-411). Among these three selection systems, only hygromycin B resistance provides a dominant selection that can be used for any wild type strain. A second dominant selection marker is needed to transform strains that are already hygromycin B resistant.

Bialaphos produced by *Streptomyces hygroscopicus* is a tripeptide consisting of two L- alanine residues and an analogue of glutamic acid called phosphinothricin (PPT). Upon cleavage of bialaphos, PPT is released and acts as an inhibitor of glutamine synthesis in plants, and hence functions as a potent herbicide. A gene (*bar*) coding for PPT acetyltransferase has been isolated from *S. hygroscopicus* and is widely used as a selective marker for the transformation of higher plants (DeBlock et al. 1987 EMBO J. 6:2513-2518) and several filamentous fungi, including *Neurospora crassa* (Avalos et al. 1989 Curr. Genet. 16:369-327; Pall, 1993 Fungal Genet. Newsl. 40:58), *Cochliobolus sativa* and *Colletotrichum species* (Straubinger et al. 1992 Fungal Genet. Newsl. 39:82-83). Here we describe a modified transformation protocol to select for phosphinothricin resistance in *M. grisea*. The strain used for transformation was Guy11 (MAT-2) and the mating strain used for genetic analysis of PPT-resistance was 2539 (MAT-1) (Leung et al. 1988 Phytopathology 78:1227- 1233). The *bar*-containing plasmid is pBARGEM7-2 (4.9 kb), the restriction map of which has been described (Pall and Brunelli, 1993 Fungal Genet. Newsl. 40:59-62). Phosphinothricin (=Ignite, =Basta) was a gift from Dr. D. Kinney (Hoeschst-Roussel Agri- Vet Company, Somerville, NJ).

Since the toxicity of phosphinothricin is presumably caused by the inhibition of glutamine synthetase, our initial task was to identify a medium with minimal complex nitrogen source in order to maximize sensitivity of *M. grisea* to PPT. The minimal salts medium described for *Cochliobolus sativa* (Leach et al. 1982 J. Gen. Microbiol. 128:1719- 1729) was used as the basis to develop a minimal medium (MM) for *M. grisea*. The MM contains (per liter) 10 g sucrose, 1 g Ca(NO₃)₂·4H₂O, 0.2 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 0.15 g NaCl, and 15 g agar. Sensitivity of Guy11 to PPT was tested on MM with varying amounts of complex nitrogen sources. Guy11 was sensitive to 50 µg/ml PPT in MM but could tolerate up to 150 µg/ml PPT in complete medium (CM, 0.6% yeast extract, 0.6% casein hydrolysate, 1% sucrose). Strong growth inhibition of strains Guy11 and 2539 was achieved in complete medium containing more than 300 µg/ml PPT. Since the fungus only grew sparingly in MM, we chose to select for PPT resistance in complete medium amended with a high concentration of PPT (400 µg/ml).

A modified transformation protocol described by Leung et al. (1990 Curr. Genet. 17:409-411) was followed. Instead of 1.2 M sorbitol, 20% sucrose was used as osmotic stabilizer. Mycelial pellets from an actively growing, overnight culture were harvested by filtering through a layer of sterile cheesecloth. The mycelium was washed with enzyme buffer (20% sucrose, 20 mM

trisodium citrate, 50 mM EDTA, pH 8.0) and then digested in 40 mg/ml of Novozyme (Novo Biolab, Wilton, CT) for 2-3 hr with gentle shaking. Protoplasts were collected, washed, and maintained in STC buffer (20% sucrose, 25 mM Tris-HCl, pH 7.5, 25 mM CaCl₂).

Approximately 1 µg of plasmid DNA was dissolved in STC and added to 250 µl of protoplast suspension. The protoplast mixture was incubated overnight in regeneration agar (CM + 20% sucrose + 1.5% agar) and 0.7% MM agar containing 600 µg/ml PPT (maintained at 45-50 C) was overlaid. Plates were incubated at 26°C for about five days. Colonies that grew through the overlay were transferred to CM agar containing 400 µg/ml of PPT. Resistance to PPT was confirmed by transferring single conidial cultures to CM + PPT medium.

The transformation frequency with circular pBARGEM7-2 was approximately 10 transformants/µg DNA/10(6) protoplasts (based on three independent experiments). The frequency was increased by about 5-fold by restriction enzyme mediated integration (REMI, Schiestl and Petes 1991 Proc. Natl. Acad. Sci. USA 88:7585-7589; Kuspa and Loomis 1992 Proc. Natl. Acad. Sci. USA 89:8803-8807) using BamH1-linearized pBARGEM7-2. In contrast to hygromycin B selection, abundant background growth was observed at low PPT concentrations (300-400 µg/ml) in CM. The critical step in reducing background growth was to overlay the regenerated protoplasts with MM containing a high concentration (600 µg/ml) of PPT and to dilute the nitrogen source in CM by maintaining a high ratio of the overlay to the regeneration medium (about 2:1). PPT- resistant colonies (5-10 mm diameter) grew through the surface of the top agar approximately three days after overlay was applied. In a parallel transformation experiment with pAN7-2, emergence of hygromycin B resistant colonies occurred five days after overlays were applied. A similar observation was made in *N. crassa* (M. L. Pall, unpublished data), suggesting that expression of the bar gene is considerably faster than that of the hygromycin B resistance gene. To determine the tolerance of transformants to PPT, four independent transformants were tested on a range of PPT concentrations (Table 1). A significant difference in growth between transformants and the wild type parent was evident at 300 µg/ml PPT. All transformants grew normally in CM containing up to 600 µg/ml PPT.

To test the mitotic stability of PPT resistance, a transformant GT-B-1-1 was propagated by single-spore serial transfer on oatmeal agar without selection for ten generations (approximately 70 days). The resultant single-spore cultures exhibited the same level of resistance to PPT as the initial transformants. Crosses made between 10 randomly selected transformants and the wild type 2539 exhibited the same level of fertility as the crosses between the wild type parents (Guy11 x 2539). Ascospore progeny obtained from four crosses were tested for resistance to PPT and single-gene segregation was observed for all crosses. Ten PPT-resistant progeny derived from cross GT-B-1 x 2539 were pathogenic on weeping lovegrass (*Eragrostis curvula*). Single spores recovered from the lesions caused by each of the transformants also retained PPT resistance. Southern analysis of transformant DNA showed that the majority of transformants (10/12) derived from circular plasmid or REMI transformation were resulted from single-copy integrations. These results indicate that bar-transformants are mitotically and meiotically stable and do not exhibit any negative characteristics in fertility and pathogenicity.

The *bar* gene from *S. hygroscopicus* is a useful marker for *M. grisea* based on a number of criteria. First, the transformation efficiency using pBARGEM7-2 is comparable to those of integrative transformation in filamentous fungi. Second, no special medium is required; selection

for PPT resistance can be achieved by using a high concentration of PPT in CM. Third, the transformants are mitotically and meiotically stable and retain pathogenicity on plants. While spontaneous mutation to hygromycin B resistance rarely occurs in *M. grisea*, PPT selection may potentially lead to higher frequency of spontaneous PPT-resistant mutants. Based on the number of PPT-resistant colonies observed in the control (protoplasts regenerated without added plasmid), spontaneous mutation could account for 5-10% of the PPT-resistant colonies. Spontaneous PPT-resistance was apparently caused by a single-gene mutation. Thus, the effective transformation frequency using PPT selection could be less than observed. Despite this limitation, the low cost of Ignite relative to hygromycin B makes PPT resistance an effective and inexpensive alternative marker for *M. grisea*.

Table 1. Radial growth of transformants of *Magnaporthe grisea* at different concentrations of phosphinothricin in complete medium(a).

Strain(b)	Plasmid integration	Phosphinothricin(b) ($\mu\text{g/ml}$)				
		0	200	300	400	600
Guy11	none	39	35.6	17	11.3	5.6
GT-B-1	single-copy	38.6	39.6	38.6	39.3	38.0
GT-B-2	none, spontaneous mutant	39.6	40.0	40.6	38.3	38.6
GT-B-3	single-copy	38.6	38.6	41.0	40.3	40.0
GT-B-5	single-copy	39.0	37.6	39.6	39.3	38.3

a Radial growth (mm) measured 10 days after inoculation. Average of four replicates.

b Phosphinothricin in complete medium (1% sucrose, 0.6% yeast extract and 0.6% casein hydrolysate).