estimates involve some degree of uncertainty. The uncertainty depends chiefly on the amount of reliable geologic and engineering data available at the time of the estimate and the interpretation of these data. The relative degree of uncertainty may be conveyed by placing reserves into one of two principal classifications, either proved or unproved. Unproved reserves are less certain to be recovered than proved reserves and may be further sub-classified as probable and possible reserves to denote progressively increasing uncertainty in their recoverability.

Proved reserves are those quantities of petroleum which, by analysis of geological and engineering data, can be estimated with reasonable certainty to be commercially recoverable, from a given date forward, from known reservoirs and under current economic conditions, operating methods, and government regulations. Proved reserves can be categorized as developed or undeveloped.

If deterministic methods are used, the term reasonable certainty is intended to express a high degree of confidence that the quantities will be recovered. If probabilistic methods are used, there should be at least a 90% probability (P10) that the quantities actually recovered will equal or exceed the estimate.

Unproved reserves are based on geologic and/or engineering data similar to those used in estimates of proved reserves – but technical, contractual, economic or regulatory uncertainties preclude such reserves being classified as proved. Unproved reserves may be further classified as probable reserves and possible reserves.

Probable reserves are those unproved reserves which analysis of geological and engineering data suggests are more likely than not to be recoverable. In this context, when probabilistic methods are used, there should be at least a 50% probability (P50) that the quantities actually recovered will equal or exceed the sum of estimated proved plus probable reserves.

Possible reserves are those unproved reserves which analysis of geological and engineering data suggests are less likely to be recoverable than probable reserves. In this context, when probabilistic methods are used, there should be at least a 10% probability (P90) that the quantities actually recovered will equal or exceed the sum of estimated proved plus probable plus possible reserves.

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Agrobacterium tumefaciens-mediated transformation of Brahmi [*Bacopa monniera* (L.) Wettst.], a popular medicinal herb of India

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***Agrobacterium*-mediated genetic transformation of Brahmi [*Bacopa monniera* (L.) Wettst.] was standardized using the *Agrobacterium tumefaciens* strain EHA105 that harboured the binary vector pBE2113 containing genes for β-glucuronidase (GUS) and neomycin phosphotransferase. Putative transformants were selected by the ability of the leaf explants to produce kanamycin-resistant calluses that regenerated into kanamycin-resistant plantlets. Successful transformation was confirmed by histochemical assay for GUS activity, PCR analysis and RT–PCR. The frequency of transformation from the leaf explants was more than 60% and a period of nearly two months was required for the regeneration of transgenic plantlets from the explants. The morphology of the transformed plants resembled that of the parent. The development of an efficient transformation protocol for Brahmi can lead to the genetic improvement of the plant for secondary metabolite content in future.**

METABOLIC engineering is emerging as one of the important approaches to improve and modify secondary metabolite contents of medicinal and aromatic plants. Recently, some examples of successful genetic manipulation of secondary metabolite pathway through metabolic engineering for increased metabolite content[1–5] and exploitation of the plants as bioreactors for the production of natural or recombinant secondary metabolites of commercial interest[6] have been presented. The rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable *Agrobacterium*-mediated transformation protocols for different crop species[7]. Similar success can be achieved in medicinal plants by developing efficient regeneration and *Agrobacterium*-mediated transformation protocols, which in turn could be used for the enhancement of their secondary metabolite content. Brahmi [*Bacopa monniera* (L.) Wettst.] is an important ayurvedic herb used in India for more than 3000 years. The plant is a member of Scrophulariaceae family and is quite widespread throughout the tropics, where it is found on the banks of slow-flowing rivers and lakes. The herb is primarily used for

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its ability to enhance memory capacity, improve intellectual and cognitive functions, reduce stress-induced anxiety, and increase concentration[8]. The active constituents of the plant are brahmine, herpestine, alkaloids and saponins[9]. The memory-enhancing effects have been attributed to the saponins, bacosides A and B[10]. Till date, no report has been published regarding the genetic transformation of the plant, though experiments have been done to develop reliable protocols for shoot regeneration and somatic embryogenesis[11].

Here, we report the successful genetic transformation of Brahmi using *Agrobacterium tumefaciens* strain EHA105 (ref. 12) harbouring the binary vector pBE2113 (ref. 13; gift from Dr Ichiro Mitsuhara, National Institute of Agrobiological Resources, Japan).

Axenic cultures of *B. monniera* (Brahmi) were obtained from the Plant Tissue Culture Laboratory, Plant Molecular Biology and Biotechnology Centre, Kerala Agriculture University, Vellayani, Kerala, India. The cultures were maintained in MS medium[14] supplemented with 30 g/l sucrose and 6.0 g/l phytagar (Gibco BRL, Grand Island, NY USA). The pH of the medium was adjusted to 5.8 ± 0.1 and sterilized for 15 min at 121°C and 15 lb. The cultures were incubated at 25°C under cool white fluorescent light ($30\ \mu E\ m^{-2}\ s^{-1}$, 16 h photoperiod). Routine sub-culturing was carried out every 2–3 weeks. The kanamycin sensitivity of the plant was studied and kanamycin at a concentration of 15 mg/l inhibited callus regeneration and rooting response (data not shown).

The *A. tumefaciens* strain EHA105 mobilized with binary plasmid pBE2113 was used for transformation studies. The plasmid pBE2113 has genes for neomycin phosphotransferase (*npt*II) and β-glucuronidase (*uid*A) reporter gene under the control of chimeric combined promoter cassette consisting of enhancer of cauliflower mosaic virus (CaMV) 35S RNA promoter, and 5′-untranslated region (Ω) of tobacco mosaic virus for enhanced expression (Figure 1).

The general protocol followed for transformation after optimizing the conditions was as follows. The leaf segments from axenic cultures were preincubated for two days in shoot regeneration medium, which comprised of basal MS medium supplemented with 0.1 mg/l naphthaleneacetic acid (NAA), 1.5 mg/l benzylaminopurine (BA) and 0.1 mg/l gibberellic acid (GA_3). *A. tumefaciens* EHA105 (pBE2113) was grown overnight at 28°C in liquid Luria–Bertani medium containing 50 mg/l kanamycin. Preincubated leaf segments were infected with the overnight culture of *Agrobacterium* diluted to 5×10^8 cells/ml (as measured by OD at 600 nm) for 15 min. The infected leaf explants were blotted-dry using sterile Whatman No. 1 filter paper and co-cultivated on the same regeneration medium for 48 h. Following co-cultivation, the explants were washed with sterile water several times, blotted using Whatman No. 1 filter paper and transferred to the selection medium. The selection medium comprised of shoot regeneration medium supplemented with 15 mg/l kanamycin and 300 mg/l cefotaxime. Four weeks after bacterial infection, co-cultivated explants showed callusing on the selection medium, while the control uninfected explants inoculated onto the selection medium showed browning and senescence. The kanamycin-resistant cal-

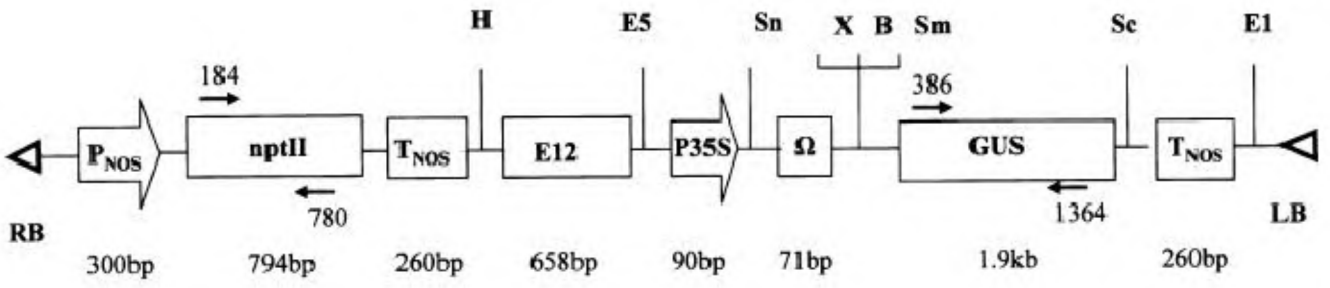

Figure 1. Schematic map of the T-DNA region of pBE2113 (RB, Right border; LB, Left border; P_{NOS}, Nopaline synthase promoter; *npt*II, Gene for neomycin phosphotransferase; T_{NOS}, Nopaline synthase terminator; E12, 5′-upstream sequence of CaMV 35S promoter; P35S, 5′-upstream sequence of CaMV 35S promoter; Ω, 5′-untranslated sequence of TMV; GUS, Gene for β-glucuronidase; *H*, *Hin*dIII; E5, *Eco*RV; Sn, *Sna*BI; X, *Xba*I; B, *Bam*HI; Sm, *Sma*I; Sc, *Sac*I; E1, *Eco*RI). Bold arrows above the boxes indicate the position of primers used for PCR analysis.

Table 1. Transformation efficiency in Brahmi transformed by *Agrobacterium* strain EHA105 (pBE2113)

Batch no.	No. of explants co-cultivated	No. of kanamycin-resistant calluses	Percentage of explants showing callus induction	No. of regenerated shoots showing GUS activity	Transformation frequency (%)
1	30	20	66.67	19	63.33
2	26	19	73.08	16	61.54
3	32	24	75.00	21	65.63
Mean			71.58		63.50

luses were transferred to a fresh selection medium where they proliferated into shoots within two weeks. The frequency of regeneration of transgenic plants from the kanamycin-resistant calluses was more than 60% (Table 1). These shoots subsequently formed roots on the selection medium within a week (Figure 2). The transformed plants were hardened for a week and were transferred to pots kept under enclosure and the leaf samples were harvested for subsequent analysis.

Histochemical assay to detect the β-glucuronidase (GUS) activity in the putative transformants was performed on the leaves of kanamycin-resistant plants. Four putative

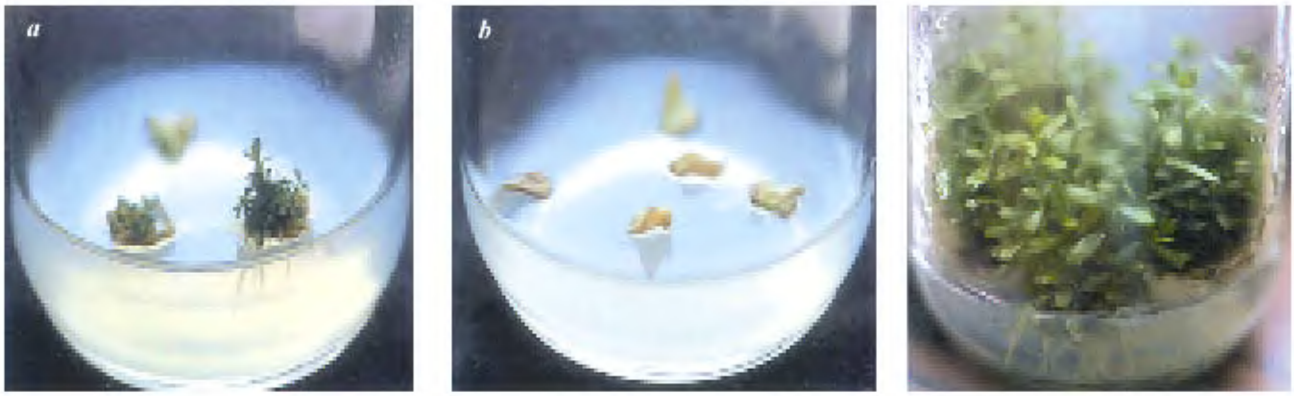

Figure 2. Genetic transformation of *Bacopa monniera* using *Agrobacterium tumefaciens*. ***a***, Infected explants on kanamycin medium; ***b***, Control uninfected explants on kanamycin medium; ***c***, Infected explants in regeneration medium without kanamycin.

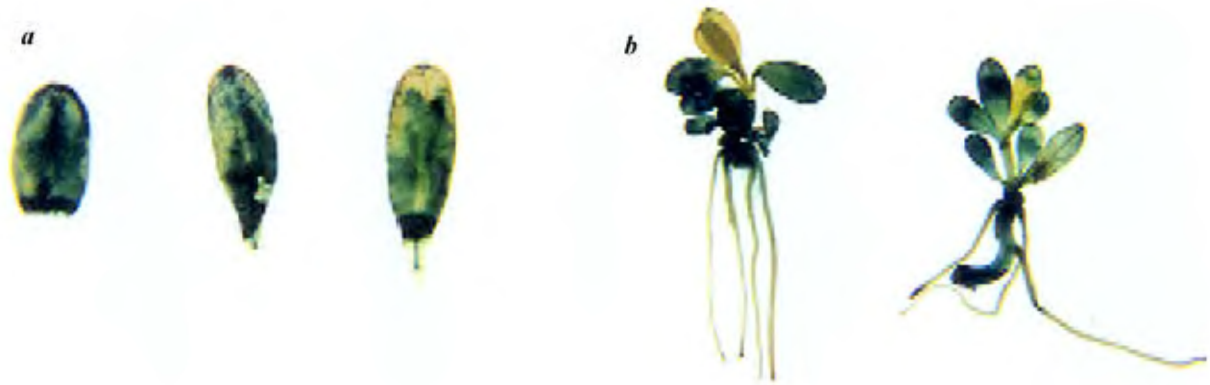

Figure 3. Histochemical GUS assay of transformed Brahmi. ***a***, Leaf explants showing varying degrees of expression of GUS gene; ***b***, Kanamycin-resistant plantlets showing GUS expression.

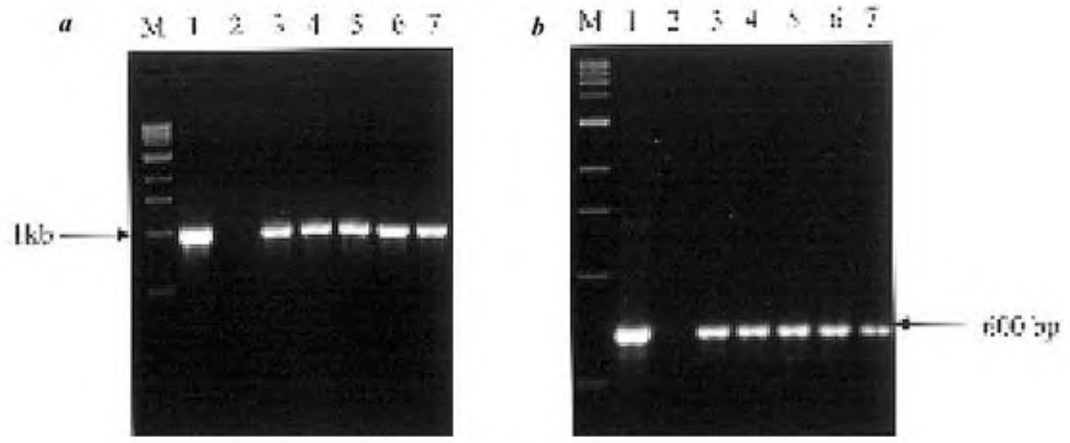

Figure 4. Brahmi genomic DNA analysis by PCR for the presence of *uid*A and *npt*II genes. ***a***, PCR using *GUS* gene-specific primers. Lane M, DNA marker 1 kb ladder; lane 1, Plasmid pBE2113 (+ve control); lane 2, uninfected plant (–ve control); lanes 3–7, putative tranformants. ***b***, PCR using *npt*II primers. Lane M, DNA marker 1 kb ladder; lane 1, plasmid pBE2113 (+ve control); lane 2, uninfected plant (–ve control); lanes 3–7, putative transformants.

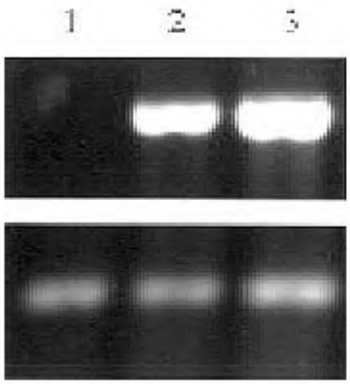

Figure 5. RT–PCR analysis of *uid*A and *Actin*1 RNA in wild type (lane 1) and transformed (lanes 2 and 3) Brahmi plants using gene-specific primers. (Top) PCR amplification with *uid*A-specific primers; (Bottom) PCR amplification with *Actin*1-specific primers.

transformants were selected at random and leaves were used for histochemical staining to detect GUS activity. The leaves were incubated overnight at 37°C in a solution of 0.1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) in 50 mM sodium phosphate buffer (pH 7.0)[15]. The distribution of GUS activity was examined after the removal of chlorophyll with 70% ethanol. The leaves showed pronounced GUS activity along the midrib region (Figure 3 *a*). GUS staining of two kanamycin-resistant plants is seen in Figure 3 *b*.

The presence of *GUS* and *npt*II genes in the genomes of the transformants was studied by PCR amplification using specific primers. Genomic DNA was isolated from fresh leaf tissues of five randomly selected kanamycin-resistant plants and a control plant using Gen Elute™ Plant Genomic DNA Kit (Sigma). PCR amplification of the DNA was carried out using the primers for *npt*II and *GUS* genes separately. The reactions were performed in a thermal cycler (Biorad) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min and a final extension of 72°C for 7 min. The PCR products were separated by 1.2% agarose gel electrophoresis and visualized on UV-transilluminator. The DNA isolated from five randomly selected kanamycin-resistant plants showed amplification of 1 kb fragment with *GUS* primers and a 600 bp fragment with the *npt*II primers, while there was no amplification with the DNA of the control plant (Figure 4 *a* and *b*).

The expression of the transferred genes was studied by reverse transcription polymerase chain reaction (RT–PCR). Total RNA was isolated from a control plant as well as two PCR-positive plants using TRIZOL™ reagent (Gibco BRL). First strand synthesis of the cDNA was carried out using Random hexamers and MMLV-RT (Promega). The cDNA was independently amplified by PCR using the primers for GUS and a housekeeping *Actin*1 gene as control. The PCR amplification was carried out with an initial denaturation at 94°C for 5 min followed by 30 thermal cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 55°C) and extension (1 min at 72°C). Final extension was performed at 72°C for 7 min. RT–PCR using *GUS* primers showed an amplification of a 1 kb fragment in both the transformants, while the control plant showed no amplification. With *Actin*1 primers, both the transformants as well as the control plants showed the amplification of a 250 bp fragment (Figure 5). The amplification of the *GUS* transcript from the cDNA of the transformants also indicates the expression of the *GUS* gene in the transformed plants.

The mean transformation efficiency assessed based on the ability of the transformants to regenerate and root on kanamycin medium was about 63.5%. The transformation efficiency in the case of cereals varies from less than 1 to 27% (refs 16, 17) and in other systems it varies from 60 to 90% (refs 18, 19). The high transformation efficiency observed in Brahmi may be because of the efficient regeneration response, as observed in the case of model systems like tobacco and *Arabidopsis*. This is a report on successful transformation of *B. monniera*. The protocol could be used for the metabolic engineering of Brahmi to either enhance the levels of secondary metabolite(s) or to modify their structures.

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Interrelationships among reproductive traits of female lizard, *Sitana ponticeriana* (Cuvier)

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Interrelationships among maternal body size, clutch size and egg size were studied in the ground lizard, *Sitana ponticeriana*. In these lizards, clutch size varied from 7 to 19. Initial clutches were larger than the potential next clutch. Clutch size and mass were positively correlated with maternal body size (Snout–vent length, SVL), but not with maternal body condition. Maternal SVL or clutch size had no influence on egg mass and egg volume. No trade-off was evident between clutch and egg sizes. Thus, in *S. ponticeriana* egg size is essentially invariant despite a great variation in clutch size across the females of different sizes.

REPRODUCTION is an important event in the life history of all organisms. Female body size, clutch/egg and offspring body sizes are the basic components of life-history traits. Oviparous reptiles show complex interrelationships among maternal body size, clutch and egg sizes. In lizards, clutch size varies with proximate climatic factors, food availability and fat body reserves[1–5]. However, among the 456 species of reptiles inhabiting India[4], factors influencing clutch, egg and offspring sizes are studied in detail in only one species, the garden lizard, *Calotes versicolor*[6]. Such information on reproductive traits in different species of lizards is needed to understand the diversity in reproductive trade-offs, if any. *Sitana ponticeriana* (family Agamidae) is a small lizard restricted to moderately moist scrub, sandy and rocky areas with plenty of bushes and other such vegetation in India. But for some scattered information on clutch size, incubation duration and size of hatchling[7], no information is available with respect to the interrelationships among reproductive traits in this species. Hence, the present study was undertaken to investigate interrelationships among traits such as maternal size, clutch size and mass, and egg mass and size in *S. ponticeriana*.

Females of adult *S. ponticeriana* ($n = 29$) were collected during May–August 1998 from the surrounding areas of Dharwad ($n = 4$, 14, 6 and 5 in May, June, July and August, respectively). They were brought to the laboratory on the day of collection. Snout-vent length (SVL, cm) and mass (g) of these lizards were recorded. At autopsy, ovarian condition (presence/absence of vitellogenic follicles and their number), number of oviductal eggs (clutch size), if any, individual egg mass (mg), total clutch mass (g), egg length (mm) and egg width (mm) were recorded. Diameter of the largest ovarian follicles (mm) was measured using a micrometer. Follicles measuring > 2.5 mm were considered as vitellogenic[8]. An estimate of egg volume was taken as an overall measure of egg size. Egg volume was derived using mean egg length and width by a formula for prolate spheroid $4/3\pi$ (length/2) $(\text{width}/2)^2$. The number of vitellogenic follicles (diameter > 4 mm) in females having oviductal eggs was considered as their potential next clutch of the season[8].

Mean ± SEm was calculated from the untransformed data for all recorded variables. For the study of interrelationships among various reproductive traits, data were log transformed to meet the assumptions of parametric statistics and to facilitate the biological interpretation[9]. Maternal condition for each lizard was obtained by generating residuals of body mass. Residuals for body mass were generated by regressing log body mass on log SVL. Linear regression analysis was used to study the interrelationships among various reproductive traits. Variation in the number of eggs between the clutches (oviductal eggs and vitellogenic follicles) of an individual was analysed by paired sample t test. Significance level was accepted at $P < 0.05$ level. All statistical analyses were performed using SPSS software.

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