Improved 'golden' indica rice and post-transgeneration enhancement of metabolic target products of carotenoids (β-carotene) in transgenic elite cultivars (IR64 and BR29)

Karabi Datta[1,4], Mayank Rai[1,2], Vilas Parkhi[1,3], Norman Oliva[1], Jing Tan[1] and Swapan K. Datta[1,4,*]

[1]International Rice Research Institute, Plant Tissue Culture and Genetic Engineering Lab, Manila, Philippines
[2]Plant Molecular Biology Lab, Cornell University, USA
[3]Institute of Plant Genomics and Biotechnology, Texas A&M University, USA
[4]Botany Department, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, India

Transgene stability and post-translational expression levels of genes are of tremendous interest for developing value-added transgenic crops. Transgenic high-yielding indica rice cultivars (IR64 and BR29) with enhanced level of carotenoid accumulation have been developed by *Agrobacterium*-mediated transformation. Genetic transformation was done using non-antibiotic Positech™ marker system. Selectable marker gene, phosphomannose isomerase (*pmi*), and two carotenogenic pathway genes, phytoene synthase (*psy*) and phytoene desaturase (*crt*I) were introduced in two popular Asian rice cultivars, IR64 and BR29. The highest level of total carotenoids obtained in progenies of trangenic BR29 was 9.34 μg/g and β-carotene level alone reached to 3.92 μg/g in polished grains. Whereas the highest accumulation of total carotenoids obtained in transgenic progenies of IR64 was 2.32 μg/g in polished grains. T2 seeds showed higher carotenoid content than the original parental line which might be attributed to post-transgeneration effect.

Keywords: *Agrobacterium* transformation, carotenoids, post-transgeneration effect, transgenic rice.

RICE is the most important cereal staple food for more than half the world population. Malnutrition and hidden hunger due to deficiency of micronutrients is becoming a severe problem in the world, especially in developing countries. There is a growing concern about the nutritional quality of our daily diet. Vitamin A deficiency is one of the major outcomes of malnutrition. Worldwide, nearly 100 to 140 million children are vitamin A-deficient and an estimated 250 to 500 thousand vitamin A-deficient children become blind every year. Though rice is an important source of food energy and calories for 50% of the total world population predominantly in developing countries, milled rice is deficient in many essential micronutrients like iron, zinc, vitamin E and vitamin A[1,2]. This could be one of the main reasons for high prevalence of vitamin A deficiency (VAD) in developing countries.

Genetic engineering for biofortification of rice could be an important approach to improve the nutritional quality of rice. Rice plants possess carotenoids in photosynthetic tissues but not in the endosperm, the edible part. Genes for two key enzymes in β-carotene (provitamin A) biosynthesis pathway, phytoene synthase (*psy*) and phytoene desaturase (*crt*I) were isolated and characterized from daffodil (*Narcissus pseudonarcissus*) and the plant pathogenic bacteria (*Erwinia uredovora*) respectively[3,4]. Genetic engineering of the metabolic pathway for biosynthesis of β-carotene (1.6 μg/g total carotenoids) in the endosperm of japonica-type rice cultivar was demonstrated[5]. Later, it was demonstrated in indica-type cultivated rice cultivars[6,7].

There is concern among consumers and environmentalists over the use of antibiotic selectable marker gene for development of transgenic plants, although there is no strong evidence against the antibiotic markers. Considering the public concern, we have developed transgenic rice using a non-antibiotic positech™ selection system with phosphomannose isomerase (*pmi*) as an alternative to antibiotic resistance or herbicide tolerance marker system for selection. We have introduced two key genes, *psy* and *crt*I, of carotenogenic pathway in two indica-type rice cultivars, BR29 (a popular high-yielding variety of Bangladesh) and IR64 (important IRRI-bred line popularly grown in Asia), effectively to synthesize β-carotene in the target endosperm tissue. The binary plasmid pCaCar was obtained from the University of Friburg, Germany. The following genes are present in the T-DNA of the pCaCar plasmid: the selectable marker gene *pmi* under the control of the CaMV 35S promoter, *psy* under the control of the endosperm-specific *Glutelin* promoter and *crt*I fused to the open reading frame for the Rubisco transit peptide sequence under the control of the CaMV 35S promoter (Figure 1). Two *Agrobacterium* strains, LBA4404 and EHA101, were transformed with the binary vector pCacar using freeze–thaw transformation method[8], applying chloramphenicol (15 mg/l) as the selection agent to confirm the presence of the plasmid in the strain. For *Agrobacterium*-mediated transformation, embryogenic calli derived from the scutellum of immature embryos were used as explants. Calli were generated on MS medium[9] supplemented with 2.0 mg/l 2,4-D and 3% (w/v) sucrose or maltose. The embryogenic calli (3–4 sq. mm, 3–4 weeks old) of indica rice varieties BR29 and IR64 were incubated for 30 min in *Agrobacterium* (LBA4404/pCaCar or EHA101/pCaCar) culture (OD600 = 0.8–1.0). Calli were then transferred to co-cultivation medium (MS medium with 2 mg/l 2,4-D and 200 μM acetosyringone) and incubated in the dark at 28°C for 3 days. This was followed by three successive selection cycles of 2 weeks each. The selection medium consists of MS basal medium with 2 mg/l 2,4-D, 250 mg/l ceffotaxim and mannose and sucrose combination (15/20, 20/15, 25/10 g/l mannose/

*For correspondence. (e-mail: swpndatta@yahoo.com)

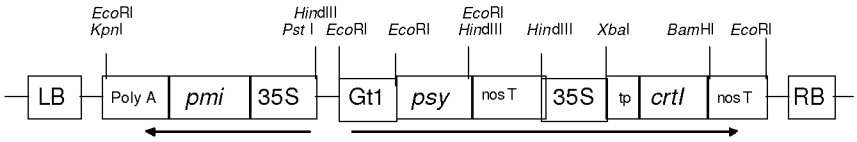

Figure 1. Partial diagram of the binary vector pCaCar, *pmi* gene driven by 35S CaMV promoter, *psy* driven by endosperm-specific gluteline promoter and *crt*I with 35S CaMV promoter and Rubisco transit peptide sequence (tp). LB, Left border; RB, Right border.

Table 1. 'Golden' indica rice obtained by *Agrobacterium*-mediated transformation

Cultivar	Agro-strain used	Vector	No. of plants analysed	No. of Southern-positive plants
BR29	EHA101	pCaCar	304	221
	LBA4404	pCaCar	263	57
IR64	LBA4404	pCaCar	100	10

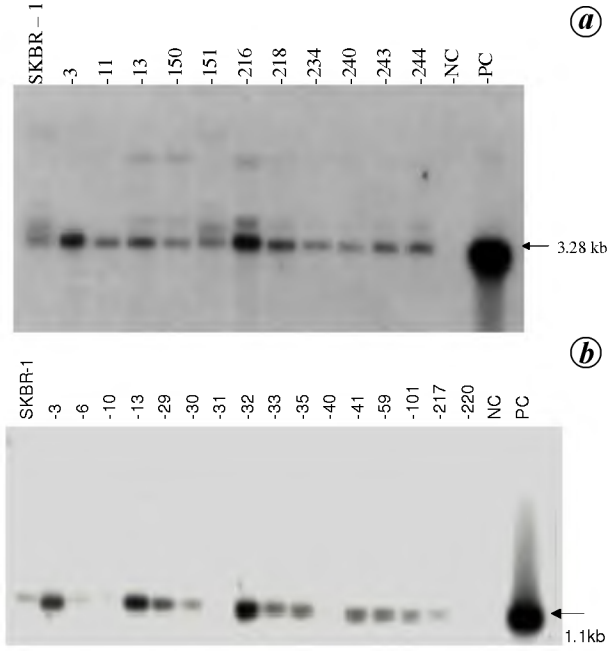

Figure 2. Southern blot analysis of primary transgenics of BR29 showing stable integration of (*a*) *crt*I gene (3.28 kb) and (*b*) *pmi* gene (1.1 kb) in the genome. PC, Positive control; NC, Negative control.

sucrose for first, second and third selection respectively). The selected calli were transferred to regeneration medium as described earlier[10,11]. The regenerated rice plants were grown in a special greenhouse with containment facilities made for transgenic plants. Stable integration of genes and their expression in edible endosperm tissue was evidenced from molecular analysis. High performance liquid chromatography (HPLC) analysis provided the carotenoid profile in the polished grains.

Insertion of genes in the genome of the regenerated putative transgenic rice plants was primarily checked by polymerase chain reaction (PCR) analysis using the standard method[12] with gene-specific primers (data not shown). PCR-positive plants were further confirmed by Southern blot analysis. For this, genomic DNA was extracted using the procedure described earlier[6,13]. Genomic DNA was digested with *Eco*RI and run in 1% TAE-agarose gel. Southern hybridization and exposure was carried out using standard method[14]. Table 1 shows that 278 independent transgenic BR29 (221 from EHA101/pCaCar transformation and 57 from LBA4404/pCaCar transformation) and ten independent transgenic IR64 (from LBA4404/pCaCar transformation) were obtained. Presence of the 3.28 kb fragment band in Southern analysis of a few selected PCR-positive first generation (T0) BR29 plants confirmed the integration of *crt*I gene in the genome (Figure 2 *a*). Both simple and rearranged types of gene-integration patterns were observed. Similarly, integration of *pmi* (Figure 2 *b*) and *psy* genes was also checked by Southern blot analysis.

Mature seeds (T1) from individual transgenic lines were polished to confirm the visible expression level of the integrated genes by yellow colour. Clear segregation of genes due to meiotic division was noted in colour expression (Figure 3). Variation in the yellow colour intensity of the endosperm of individual lines seemed to indicate variation of the level of carotenoid accumulation among them. Polished seeds from individual lines were analysed quantitatively by spectrophotometer and qualitatively for β-carotene and other carotenoids by HPLC. Carotenoids from individual samples were extracted and absorbance was measured at 450 nm in a spectrophotometer. HPLC analysis was performed using Waters Alliance 2690 separation Module (Waters Corporation, Milford, MA, USA) equipped with a Waters 996 photodiode array detector, Waters 474 scanning fluorescence detector and Waters Millennium 32 Chromatography Manager. The column was developed with a solvent solution of acetonitrile–tetrahydrofuran–water (10 : 4 : 6) for the first 3 min; then a linear gradient to another solvent solution of acetonitrile–

Table 2. Variation of carotenoid content in different progenies (selected based on higher content of total carotenoids)

Parent no.	T0 (μg/g)	Progenies T1 (μg/g)				
		1	2	3	4	5
SKBR29-3	1.33	2.56	2.9	4.68	nd	nd
SKBR29-11	1.80	1.73	3.21	2.2	4.05	nd
SKBR29-13	1.60	2.04	2.63	4.21	4.46	4.49
SKBR29-216	1.254	2.24	3.05	3.15	3.63	4.12
SKBR29-217	2.560	4.56	4.98	6.43	6.59	7.55
SKBR29-218	1.312	2.38	3.91	4.56	6.08	nd
SKBR29-234	2.03	3.43	4.1	4.57	nd	nd
SKBR29-240	1.434	4.62	5.12	5.79	nd	nd
SKBR29-241	1.584	3.37	3.44	4.31	nd	nd
SKBR29-244	1.004	4.3	4.54	4.59	6.77	9.34
SK6429-561	0.592	1.03	1.47	2.32	1.16	1.38
SK6429-562	0.948	1.05	1.94	nd	nd	nd
SK6429-560	0.748	1.08	1.32	1.25	1.08	0.76

nd, Not done.

Figure 3. Polished seeds of primary transgenics (T1) showing yellow colour of endosperm due to expression of integrated carotenoid pathway genes in the genome. White (normal) seeds represent the post-meiotic segregation.

tetrahydrofuran–water (10:8.8:1.2) was applied over a period of 7 min, and the second solvent solution was pumped through the column for 20 min. Peak identification was based on retention time, the main absorption maxima, and spectrum shape comparing with the corresponding standards[15]. Transgenic lines showing appreciable level of expression based on yellow colour and HPLC analysis data were advanced to the next generation.

From each line, which was selected for the next generation, 60 T1 progenies were grown to study the inheritance pattern of the integrated genes, and identify putative homozygous lines and their differential carotenoid expression level in the seeds (T2). PCR and Southern blot analyses of individual plants of each line showed single-locus Mendelian segregation (3 : 1) to a variable segregation pattern. This represents the possibility of transgenes insertion in one or more than one locus. Simple gene integration patterns detected in the T0 generation were maintained in T1, while rearranged patterns in T0 were resolved into a mixture of simple and rearranged gene-integration patterns. In Figure 4, Southern blot analyses showing integration of *crt*I gene (3.28 kb) in 20 individual T1 progenies of one IR64 line (SK64-560) has been presented. After harvesting, the dried seeds of individual progeny (T2) of each line were polished to assess directly by visual examination of yellow colour. Estimation of carotenoid of the polished seeds by spectrophotometer and carotenoid profiling by HPLC analysis showed wide variation in carotenoid expression in seeds (T2) of individual progenies (T1) of each line. Enhanced carotenoid levels were observed in many of the T2 seeds when compared with their respective T1 seeds. Such enhanced expression was attributed presumably to post-transgeneration positive effect on carotenoids biosynthesis in rice grains. We have also noted the enhanced chlorophyll biosynthesis in some lines. One transgenic plant, SKBR-244-26, contained total carotenoids 9.34 μg/g of polished seeds and 3.92 μg/g of β-carotene, the highest value obtained from BR29 (Table 2). Earlier studies reported the amount of carotenoid in transgenic rice as 1.6 μg/g in a japonica rice[3] and 1.05 μg/g in an indica golden rice[6]. About 4–5 μg/g carotenoids may provide more than 60% of the RDA (Recommended Daily Allowances) by the ICMR/WHO (B. Sivakumar, pers. commun.). In case of IR64, the highest value of total carotenoids obtained in T2 seeds of SK64-561-8 was 2.32 μg/g of polished seeds. However, in many lines carotenoid accumulation was much lower in the respective parents (Figure 5 and Table 2). The carotenoid profile of transgenic seeds shows the presence of lutein, β-cryptoxanthin and α-carotene.

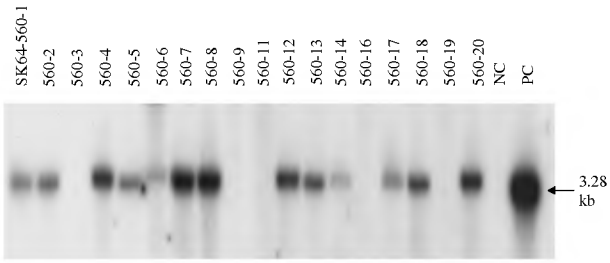

Figure 4. Southern blot analysis of T1 progenies of one line of IR64 (SK64-560) showing stable integration of *crt*I gene (3.28 kb) and its segregation (3 : 1).

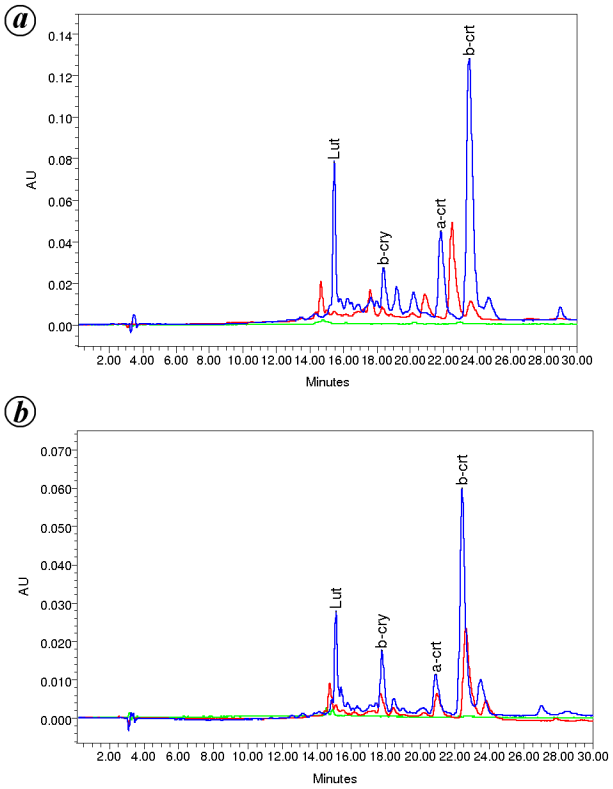

Figure 5. ***a***, HPLC chromatogram (A450) showing carotenoid profiles of T2 polished yellow seeds of SKBR29-244-26 (blue) containing 9.34 µg/g carotenoids with those of its respective parent, SKBR29-244 (red) and respective BR29 negative control (green). ***b***, HPLC chromatogram (A450) showing carotenoid profiles of T2 polished yellow seeds of SK64-561-8 (blue) containing 2.32 µg/g carotenoids with those of its respective parent, SK64-561 (red) and respective IR64 negative control (green). Lut, Lutein; b-cry, b-Cryptoxanthin; a-crt, α-Carotene; b-crt, β-Carotene.

The main objective of this study was to develop the improved 'golden' indica rice and to find out the post-translational effect on transgene expression in transgenic rice using non-antibiotic mannose selection. Here, we have used *pmi* gene as a selectable marker in the pCaCar vector and the putative transgenic calli were selected using mannose in the culturing medium and the presence of the inserted *pmi* gene in the transformants was detected by Southern blot analysis. This has also been reported in other crops like maize and wheat[16]. We report here a variable range of accumulation of carotenoids in T2 polished seeds and the highest accumulation of total carotenoids, 9.34 µg/g, in BR29 was found in the transgenic (T2) progeny (SKBR-244-26), which contained 3.92 µg/g β-carotene. Highest accumulation of total carotenoids in IR64 was 2.32 µg/g of which β-carotene level was only 0.96 µg/g of total polished seeds. Differential expression of carotenoid levels has been reported before in rice[15] and potato[17]. However, gene integration patterns may have some effect on carotenoid levels. Enhanced carotenoid levels were observed in many T2 seeds of plants showing rearranged gene integration at T0. The dosage effect due to higher copy number to lead high expression has been reported[18]. Although not proven, a single or low copy number integration of transgene is preferable to avoid possible gene silencing and to stabilize the gene expression level. In this study, higher carotenoid levels were observed in those plants having rearranged copies and with more than one copy of the integrated gene. Accumulation of the final product, total carotenoids and β-carotene level, is perhaps dependent on the proper coordination of all the enzymes involved in the metabolic pathway. Large number of transgenic events need to be developed to select the desirable plants with the best stable high carotenoid accumulation in the endosperm with the combination of best phenotype showing superior agronomic performances. Due to the complexity of the carotenoid biosynthetic pathway, four to five generations will be needed to stabilize the expression level or anther culture could be used to develop the homozygous lines with stable gene expression. Conventional interventions, like food fortification and oral delivery of vitamin A, are possible but difficult to deliver in developing countries mainly due to the inadequate infrastructure and lack of affordability of the poor. Genetic engineering approach could be an alternative preferable solution to reduce VAD. Two genetic lines of commercial Bangladesh indica rice variety BR29 (SKBR29-3-7 and SKBR29-13-11) have been sent to Bangladesh Rice Research Institute for agronomic performance and further utilization. Biofortification of commercial high-yielding rice with improved levels of β-carotene (provitamin A) and other carotenoids could prove a useful supplement to human diet for the people who need them most. Post-transgeneration enhancement of carotenoids in any plant species including 'golden' rice may provide new insights in metabolomics.

1. Vasconcelos, M. *et al.*, Enhanced iron and zinc accumulation in transgenic rice with the *ferritin* gene. *Plant Sci.*, 2003, **64**, 371–378.
2. Tan, J. *et al.*, The screening of rice germplasm, including those transgenic rice lines which accumulate β-carotene in their polished

seeds, for their carotenoid profile. *Int. J. Food Sci. Technol.*, 2005, **40**, 563–569.
3. Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K., Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol.*, 1990, **172**, 6704–6712.
4. Misawa, N., Yamano, S., Linden, H., deFelipe, M. R., Lucas, M., Ikenaga, H. and Sandman, G., Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crt*I in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to bleaching herbicide norflurazon. *Plant J.*, 1993, **4**, 833–840.
5. Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I., Engineering the provitamin A (β-carotene) biosynthetic pathway (carotenoid free) rice endosperm. *Science*, 2000, **287**, 303–305.
6. Datta, K. *et al.*, Bioengineered 'golden' indica rice cultivar with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotechnol. J.*, 2003, **1**, 81–90.
7. Hoa, T. T. C., Al-Babili, S., Schaub, P., Potrykus, I. and Beyer, P., Golden indica and japonica rice lines amenable to deregulation. *Plant Physiol.*, 2003, **133**, 161–169.
8. Hellens, R., Mullineaux, P. and Klee, H., A guide to *Agrobacterium* binary Ti vectors. *Trends Plant Sci.*, 2000, **5**, 446–451.
9. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15**, 474–497.
10. Datta, S. K., Peterhans, A., Datta, K. and Potrykus, I., Genetically engineered fertile indica-rice plants recovered from protoplasts. *BioTechnology*, 1990, **8**, 736–740.
11. Datta, K., Koukolikova, N. Z., Baisakh, N., Oliva, N. and Datta, S. K., *Agrobacterium*-mediated engineering for sheath blight resistance of *indica* rice cultivars from different ecosystems. *Theor. Appl. Genet.*, 2000, **100**, 832–839.
12. Baisakh, N., Datta, K., Rai, M., Rehana, S., Beyer, P., Potrykus, I. and Datta, S. K., Development of dihaploid transgenic 'golden rice' homozygous for genes involved in the metabolic pathway of β-carotene biosynthesis. *Rice Genet. Newsl.*, 2001, **18**, 91–94.
13. Datta, S. K., Chandel, G., Tu, J., Baisakh, N. and Datta, K., Engineering of *Bt* transgenic rice for insect pest protection. *Bacillus thuringienesis: A Cornerstone of Modern Agriculture (Part 3)*, (ed. Metz, M.), Food Products, Binghamton, 2003, pp. 77–91.
14. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual* (*2nd edn.*), Cold Spring Harbor Laboratory, 1989.
15. Parkhi, V. *et al.*, Molecular characterization of marker-free transgenic lines of indica rice that accumulate carotenoids in seed endosperm. *Mol. Gen. Genomics*, 2005, **274**, 325–336.
16. Wright, M. *et al.*, Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase, *pmi* gene as the selectable marker. *Plant Cell Rep.*, 2001, **20**, 429–436.
17. Ducreux, I. J. M., Morris, W. L., Hedley, P. E., Sheperd, T., Davies, H. V., Millam, S. and Taylor, M. A., Metabolic engineering of high carotenoid potato tubers containing enhanced level of β-carotene and lutein. *J. Exp. Bot.*, 2004, **56**, 81–89.
18. Hobbs, S. L. A., Warkentin, T. D. and DeLong, C. M. O., Transgene copy number can be positively or negatively associated with transgene tobacco transformants. *Plant Mol. Biol.*, 1993, **15**, 851–864.

ACKNOWLEDGEMENTS. Financial support from USAID is acknowledged. Thanks are due to Syngenta for an international collaborative programme.

Received 5 November 2005; revised accepted 30 May 2006

Andromonoecy, insect pollination and fruiting behaviour in *Acacia caesia* (L.) Willd. (Mimosaceae) in the Eastern Ghats

A. J. Solomon Raju*, S. Purnachandra Rao and K. Henry Jonathan

Department of Environmental Sciences, Andhra University, Visakhapatnam 530 003, India

Acacia caesia **flowers during rainy season. It is an andromonoecious and obligate outcrosser. Flowers are massed into globose heads and all flowers open on the same day. They produce compound pollen grains (polyads) and nectar in traces. The plant is entomophilous, attracts different insects but only bees and butterflies effect cross-pollination. Natural pod set is below 5%. The study suggests that enhanced pod and seed set rates are possible in resource-rich habitats and in the absence of flower-feeding beetle,** ***Mylabris pustulata*****.**

Keywords: *Acacia caesia*, andromonoecy, entomophily, fruiting behaviour.

ACACIA caesia is an armed woody shrub occurring throughout the tropical and sub-tropical regions in India[1]. It is a good defensive hedge plant used for fencing agricultural fields in the Eastern Ghats. The leaf is used as a vegetable. The powdered bark is used as a substitute for soap and its decoction as a lice killer[1,2]. Woody branches are used as toothbrushes by tribal folk. The pod powder is also used as a substitute for soap (pers. obs.). The shrub is a potential source of fuel wood. With these multiple values, this species is exploited for its produce. This plant species occurs principally on hill slopes and has an important role to protect the integrity of the slopes. It is in this context that the present study was contemplated to understand the reproductive biology of this species.

Populations of *A. caesia* on the hill slopes of the Eastern Ghats forests (Lambasingi, Lotugedda and Ananthagiri) in Visakhapatnam district, Andhra Pradesh, India, were used for the study during 2004–05. Leaf-flushing, flowering and fruiting events were recorded. Fifty flowers were used to record flower morphometrics and pollen characters. The time of daily anthesis, anther dehiscence and nectar production was recorded. Pollen-grain number (polyads)/anther/flower was determined from 30 flowers distributed on different individuals following the procedure in Dafni[3]. Stigma receptivity was tested with hydrogen peroxide according to Dafni[3]. Floral sexuality was carefully observed and two flower sex types were recognized. Fifty bisexual flowers, ten each from five plants were used for each mode – autogamy, geitonogamy and xenogamy[3]. Fifty-one

*For correspondence. (e-mail: ajsraju@yahoo.com)