donated by Ming-Bo Wang, CSIRO PI, Canberra) was isolated using two specific primers (RB19_35S_F: 5′ ACGAAGCTT-CGTCAACATGGTGGAGCA 3′ and RB19_35S_R: 5′ GACGGATCCGTC-CTCTCCAAATGAAAT 3′) designed to contain sites for *Hin*dIII and *Bam*HI. PCR product of 429 bp and pRR21 were cut with *Hin*dIII + *Bam*HI and ligated, and the product was transferred to *Escherichia coli* DH5α to get pRR20 (Figure 1 *b*).

Recombinant promoter-probe vector (pRR20) containing CaMV 35S when restricted with *Hin*dIII + *Bam*HI gave a fragment of 429 bp, indicating the presence of complete expression cassette (CaMV 35S promoter + *SgfpS65T* + *Nos*-ter). pRR21 and pRR20 were transferred to *Agrobacterium tumefaciens* for tobacco leaf disc transformation by following the standard protocol[10]. Co-cultivated leaf discs, calli and plants regenerated on hygromycin selection medium were observed for SgfpS65T expression under stereo-microscope (Olympus, SZX-16) at the National Centre for Biological Sciences. Images of SgfpS65T-expressing tissues were captured. Of the 30 leaf discs co-cultivated with pRR20, 19 showed SgfpS65T expression (Figure 2 *a*). However, leaf discs co-cultivated with pRR21 failed to show green fluorescence (Figure 2 *b*). In addition, calli formed from all SgfpS65T-positive leaf discs also showed fluorescence. Putative transgenic plants (for pRR20) which survived hygromycin selection were confirmed by *SgfpS65T*-specific PCR. Eleven plants produced an amplicon of expected size upon *SgfpS65T*-specific PCR (data not shown) and all 11 transgenic plants showed SgfpS65T expression.

Though GUS reporter was used for validating the pathogen-inducible synthetic promoters[11], the GFP has advantages such as (i) it is a non-destructive method, (ii) permits both qualitative and quantitative assays[12,13] and (iii) does not need any substrate or cofactors. In the present study, an improved version (*SgfpS65T*) resulting from chromophore mutation, S65T in the wild *gfp* was used, which is known to result in 35-fold increased fluorescence[14] compared to wild-type when excited at 488 nm. The *SgfpS65T* gene has been successfully used as a visual marker for *iAc* in pKU352NA for use in rice functional genomics[15]. Because pRR21 is a binary vector with pCAMBIA1305.1 background, it is expected to work in many plant systems for probing novel promoters.

1. Yoshida, K. and Shinmyo, A., *J. Biosci. Bioeng.*, 2000, **90**, 353–362.
2. Lessard, P. A., Kulaveerasingam, H., York, G. M., Strong, A. and Sinskey, A. J., *Metab. Eng.*, 2002, **4**, 67–79.
3. Yamaguchi-Shinozaki, K. and Shinozaki, K., *Plant Cell*, 1994, **6**, 251–264.
4. Seki, M. *et al.*, *Plant Cell*, 2001, **13**, 61–72.
5. Rabbani, M. A. *et al.*, *Plant Physiol.*, 2003, **133**, 1755–1767.
6. Ohler, U. and Niemann, H., *Trends Genet.*, 2001, **17**, 56–60.
7. Casadaban, M. J. and Cohen, S. N., *J. Mol. Biol.*, 1980, **138**, 179–207.
8. Silhavy, T. J. and Beckwith, J. R., *Microbiol. Rev.*, 1985, **49**, 398–418.
9. Simons, R. W., Houman, F. and Kleckner, N., *Gene*, 1987, **53**, 85–96.
10. Hooykaas, P. J. J. and Schilperoort, R. A., *Plant Mol. Biol.*, 1992, **19**, 15–38.
11. Rushton, P. J., Reinstadler, A., Lipka, V., Lippok, B. and Somssich, I. E., *Plant Cell*, 2002, **14**, 749–762.
12. Albano, C. R., Randers-Eichhon, L., Chang, Q., Bentley, W. E. and Rao, G., *Biotechnol. Tech.*, 1996, **10**, 953–958.
13. Scholz, O., Thiel, A., Hillen, W. and Niederweis, M., *Eur. J. Biochem.*, 2000, **267**, 1565–1570.
14. Cormack, B. P., Valdivia, R. H. and Falkow, S., *Gene*, 1996, **173**, 33–38.
15. Upadhyaya, N. M. *et al.*, *Theor. Appl. Genet.*, 2006, **112**, 1326–1341.

Received 11 July 2008; revised accepted 19 March 2009

G. M. RAVEENDRA
R. S. BHAT*
SUMANGALA BHAT
M. S. KURUVINASHETTI

Department of Biotechnology,
University of Agricultural Sciences,
Dharwad 580 005, India
**For correspondence.*
e-mail: bhatramesh12@rediffmail.com

Calcium deprivation markedly enhances guggulsterone accumulation in cell cultures of *Commiphora wightii*

Guggulsterone (Gs; two isomers -E and -Z, GsE and GsZ) is an effective anti-hyperlipidemic agent obtained from the gum resin of guggul tree, *Commiphora wightii* (Arnott.) Bhandari (syn. *C. mukul*)[1]. The recent reports explain the mechanism of its lipid lowering properties[2] and its potential role in cancer therapy, making it an attractive molecule for production through biotechnological methods using this species.

Production of metabolites in cell cultures and development of scale-up technology require standardization of various growth parameters for optimal yield[3]. To overcome the low secondary metabolites production in cell cultures, some strategies have been proposed to increase the productivity. Manipulation of culture medium constituents and culture conditions is the first step to increase the production of secondary metabolites[4,5]. There is sufficient evidence that Ca^{2+} serves as a secondary messenger in many growth and metabolic activities such as cell division and differentiation[6], cell polarity, cell elongation, photomorphogenesis, and biotic and abiotic stress responses[7]. Ca^{2+} influx is necessary for alkaloid accumulation in suspension cultures of *Catharanthus roseus* in response to the addition of cytokinin and cytokinin-enhanced alkaloid accumulation relies on the calcium–calmodulin-dependent process[8]. Ca^{2+} increases the accumulation of different sets of secondary metabolites, such as benzophenanthridine alkaloids in cell suspension of *Sanguinaria canadensis*[9], while Ca^{2+} deprivation enhances the alkaloid release in the medium in *C. roseus* cell cultures[10]. Earlier, we reported Gs accumulation in cell cultures grown in the growth medium[11] and production medium[5]. In our efforts to improve the production system, this report describes the marked influence of Ca^{2+} deprivation on the production of Gs in cell cultures of *C. wightii*.

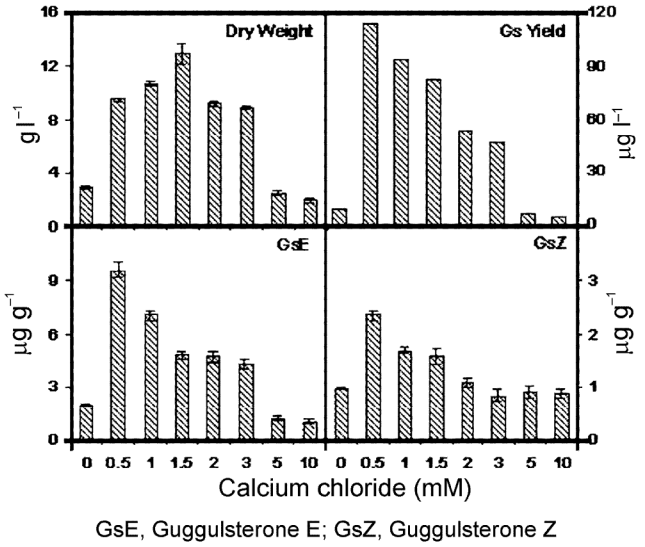

Figure 1. Effect of calcium chloride on growth and guggulsterone (Gs) production in cell cultures of *Commiphora wightii* grown in 250 ml flasks containing 100 ml MS medium. The cultures were harvested at 15 days. Bars represent standard deviation.

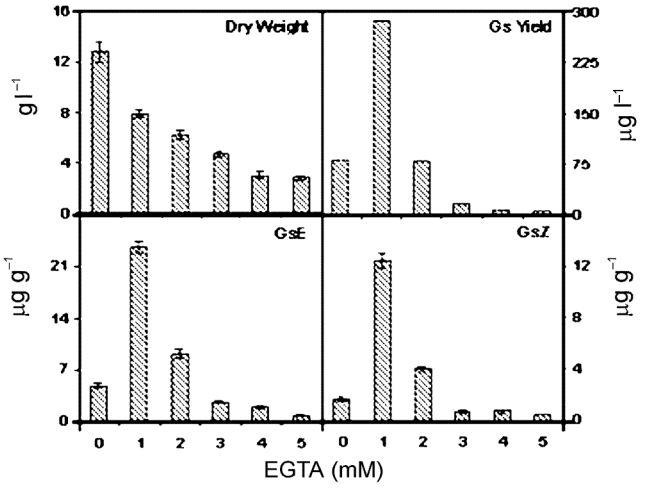

Figure 2. Effect of EGTA on growth and Gs production in cell cultures of *C. wightii* grown in 250 ml flasks containing 100 ml MS medium. The cultures were harvested at 15 days.

Cell suspension cultures of *C. wightii* were grown in modified Murashige and Skoog (MS)[12] medium (NH_4NO_3, 10 mM; KNO_3, 4.7 mM, and $CaCl_2 \cdot 2H_2O$, 1.5 mM) containing 2,4,5-trichlorophenoxyacetic acid (1 µM), kinetin (0.46 µM) and 3% (w/v) sucrose. This medium was adopted from results obtained with embryonic callus cultures[13]. A 250 ml Erlenmeyer flask containing 100 ml medium was shaken at 100 rpm at 26°C in the dark. The subculture period and inoculum size were usually 15 days and 125 mg dry wt/100 ml medium (20% v/v) respectively. Six replicate flasks were used in each treatment and experiments were conducted twice. Different concentrations of calcium chloride (0–10 mM), EGTA [ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0–5 mM] and chlorpromazine (0–100 µM) were incorporated in the medium. Calcium chloride was incorporated before autoclaving, while EGTA and chlorpromazine were added after autoclaving by filter sterilization. The medium for the latter contained 1.5 mM calcium chloride. The cell cultures were harvested after 15 days, and the lyophilized cells (1 g) were finely ground and extracted overnight with 25 ml methanol. In brief, methanol was evaporated under vacuum; the residue was extracted with ethyl acetate, from which a sample was injected in HPLC after filtration through syringe filters (0.22 µm, 4 mm nylon filter, National, USA). Separation was accomplished on a 250 × 4 mm C_{18} (5 µm) reverse phase column protected by a guard column of the same material[14], using a modified gradient at 245 nm. GsE and GsZ were obtained from Chromadex, USA, and Natural Remedies, Bangalore respectively. All the results are the average of at least two separate analyses.

C. wightii suspension cultures grown in modified MS medium containing 1.5 mM Ca^{2+}, accumulated ~13 g l^{-1} dry biomass and 82 µg l^{-1} Gs. Reduction in the medium Ca^{2+} concentration to 0.5 mM enhanced Gs yield by 66%, with slight reduction in the growth. Increased Ca^{2+} concentration was inhibitory to growth and Gs yield (Figure 1). The addition of 1 mM EGTA, a specific Ca^{2+} chelator, induced a drastic change of 250% in total Gs yield. Further increase in EGTA resulted in reduction in biomass and Gs content (Figure 2).

Chlorpromazine, a calcium–calmodulin antagonist, at its lower concentration (10 and 20 µM) enhanced Gs yield by 33%. However, increasing concentration of chlorpromazine was inhibitory to cell growth and Gs yield (Figure 3). In *C. wightii* cell cultures, GsE is usually present in higher amounts compared to GsZ, but in cells treated with chlorpromazine, GsZ was present in relatively higher amounts compared to GsE. The mechanism of inter-conversion between GsE and GsZ is not known. However, high amount of GsZ was observed with certain treatments of morphactin in callus cultures[14] and is always high in natural oleo-gum-resin exudate of the plant[4].

Marked increase in GsE, GsZ and yield l^{-1} recorded during the present work in *C. wightii* cells has not been recorded earlier in any of the treatments of plant growth regulators in the callus[14] and cell cultures grown in the growth medium[11] and production medium[5]. The present results suggest that inhibition of external Ca^{2+} flux induces an increase in Gs accumulation in *C. wightii* cells. Addition of verapamil induced no apparent change in growth and Gs accumulation in cells of *C. wightii* (data not shown).

Zhao *et al.*[15] have demonstrated that Ca^{2+} influx plays a critical role in alkaloid biosynthesis in normal and elicited *C. roseus* cell cultures. However, EGTA did not cause any apparent change in total alkaloid content in *C. roseus*, but the

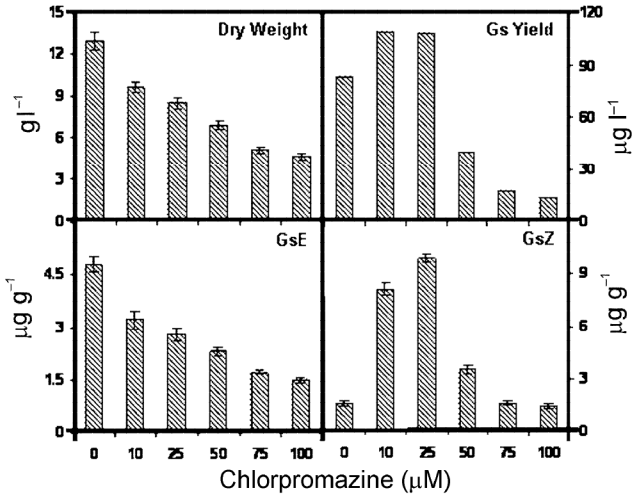

Figure 3. Effect of chlorpromazine on the growth and guggulsterone production in cell cultures of *C. wightii* grown in 250 ml flasks containing 100 ml MS medium. The cultures were harvested at 15 days.

total alkaloid excretion to the culture medium increased to 90% with 1 µM EGTA[10]. In contrast, EGTA inhibited the production of phytoalexins induced by an elicitor in *Allium cepa* cells[16] and sanguinarine production in *S. canadensis*[9]. Such increase in production of a secondary metabolite (Gs) has not been recorded earlier with EGTA in cell suspension cultures. A change in cytosolic Ca^{2+} concentration represents the signal which, directly or via Ca^{2+}-binding proteins, regulates the activities of respective target enzymes[10]. It may be inferred that instead of Ca^{2+} channels, Ca^{2+}-dependent enzymes are affected by Ca^{2+} deprivation, which results in enhanced Gs accumulation.

1. Ramawat, K. G., Mathur, M., Dass, S. and Suthar, S., *Bioactive Molecules and Medicinal Plants* (eds Ramawat, K. G. and Merillon, J. M.), Springer-Verlag, Heidelberg, 2008, pp. 101–121.
2. Urizar, N. L. *et al.*, *Science*, 2002, **269**, 1703–1706.
3. Ramawat, K. G. and Mathur, M., *Biotechnology Secondary Metabolites* (eds Ramawat, K. G. and Merillon, J. M.), Science Publishers, Enfield, USA, 2007, pp. 59–101.
4. Mathur, M., Jain, A. K., Dass, S. and Ramawat, K. G., *Indian J. Biotechnol.*, 2007, **6**, 525–531.
5. Mathur, M. and Ramawat, K. G., *Plant Biotechnol. Rep.*, 2008, **2**, 133–136.
6. Suri, S. S. and Ramawat, K. G., *Ann. Bot.*, 1997, **79**, 371–374.
7. Reddy, A. S. N., *Plant Sci.*, 2001, **160**, 381–404.
8. Merillon, J. M., Liu, D., Huguet, F., Chenieux, J. C. and Rideau, M., *Plant Physiol. Biochem.*, 1991, **29**, 289–296.
9. Mahady, G. B. and Beecher, C. W. W., *Phytochemistry*, 1994, **37**, 415–419.
10. Moreno-Valenzuela, O. A., Minero-Garcia, Y. and Chan, W., *Biotechnol. Lett.*, 2003, **25**, 1345–1349.
11. Mathur, M. and Ramawat, K. G., *Biotechnol. Lett.*, 2007, **29**, 979–982.
12. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
13. Kumar, S., Suri, S. S., Sonie, K. C. and Ramawat, K. G., *Indian J. Exp. Biol.*, 2003, **41**, 69–77.
14. Tanwar, Y. S., Mathur, M. and Ramawat, K. G., *Plant Growth Regul.*, 2007, **51**, 93–98.
15. Zhao, J., Hu, Q., Guo, Y.-Q. and Zhu, W.-H., *Plant Sci.*, 2001, **161**, 423–431.
16. Dmitriev, A., Djatsok, J. and Grodzinsky, D., *Plant Cell Rep.*, 1996, **15**, 945–948.

ACKNOWLEDGEMENTS. This work was supported by financial assistance from the DBT, New Delhi, and partially supported by DST–FIST programme for infrastructure development and UGC–DRS under special assistance programme for medicinal plant research to K.G.R.

Received 28 August 2008; revised accepted 6 March 2009

SUCHISMITA DASS
K. G. RAMAWAT*

Laboratory of Bio-Molecular Technology,
Department of Botany,
M.L. Sukhadia University,
Udaipur 313 001, India
**For correspondence.*
e-mail: kg_ramawat@yahoo.com

Development of first non-lodging and high-yielding rice cultures for saline kaipad paddy tracts of Kerala, India

Explosive increase in the world population, deterioration of arable land and availability of quality irrigation water are forcing crop production into more and more marginal environments facing abiotic stresses, thus limiting the adaptation and productivity of staple food crops[1]. In future, one cannot expect a major increase in land area available for cropping. At the same time, cultivated area is declining fast in most of the developing countries due to various reasons. It is estimated that half of the world's farms have been damaged by salt[2]. It has been estimated that about one billion hectares of the world's land is affected by salt, 60% of which is cultivated[3]. In the United States, it has been estimated that 28% of the irrigated area is salinized to a greater or lesser extent. China has salinity problems on 23% of its land, Pakistan 21%, India 11% and Mexico 10%. This is not a static situation and approximately 1.5 million hectares of irrigated land is salinized each year[4]. Improving salt tolerance of major crops is considered as the best practical approach to exploit the otherwise underutilized or completely unexploited salt-affected areas. In spite of a significant amount of research on the effect of salinity