ABSTRACT

Dunaliella bardawil is a unicellular marine microalga which is well known for its halotolerant nature and for producing highest amount of β-carotene. However, a stable transformation methodology is lacking in this alga which is the major hurdle for its genetic improvement and regulation of carotenoid pathway through metabolic engineering.

For the standardisation of Agrobacterium mediated transformation in D. bardawil, preliminary studies like, sensitivity of algae for different antibiotics, media selection for the co-cultivation for both algae and the bacteria were carried out. The various selection antibiotics tested against D. bardawil proved to be insensitive even in higher concentrations in growth medium. The sensitivity studies showed that the D. bardawil was able to tolerate cefotaxime and potassium clavulanate up to 1000 mgL⁻¹ and 1500 mgL⁻¹ of antibiotics while minimum concentration of these antibiotics that arrested growth of A. tumefaciens was determined to be 500 mgL⁻¹ cefotaxime and 300 mgL⁻¹ potassium clavulanate respectively. Co-cultivation and selection was carried out in solid TAP medium containing 0.2 M NaCl. The selection antibiotic hygromycin was found to be effective in completely killing D. bardawil cells at a concentration of 100 mgL⁻¹. Hygromycin sensitivity of D. bardawil was found to be inversely proportional to the NaCl concentration in the medium. The transformation frequency observed with the addition of 100 µM acetosyringone was found to be 42.0 ± 3 per 10⁶ cells plated. Addition of acetosyringone during co-cultivation has no effect on transformation frequency. Transformation was confirmed by GUS, GFP expression, PCR for hpt gene, southern and western blotting. The site of integration of trans-gene in the transformants was analysed by adaptor ligated genome walking method and the sequences showed that integration has occurred in non-coding regions in the genomic DNA of transformants. Pigment and growth profile of transformants were similar to that of wild D. bardawil. The transformants obtained through the standardised protocol were found to be stable even in the absence of selection antibiotic in the growth medium for a period of nearly 4 years. Molecular characterisation of Dunaliella spp used in the present study using 18S rDNA showed that the alga belongs to the category Dunaliella salina var teod, a hyper β- carotene accumulating strain of Dunaliella salina.
In order to increase the hygromycin sensitivity, glucose which is a known inducer of \( \text{H}^+ \)-ATPase was added at 10 mM in the selection medium. A decrease in hygromycin tolerance level from 100 to 25 mg L\(^{-1}\) was noted for HRC. Control cells were sensitive to hygromycin levels as low as 12 mg L\(^{-1}\). \( \text{H}^+ \)-ATPase activation by glucose was confirmed by medium acidification and \( \text{H}^+ \)-ATPase assay. The \( \text{H}^+ \)-ATPase assay values indicated maximum activity of 18 \( \mu \)mol Pi \( \text{hour}^{-1} \text{mg}^{-1} \) protein at 10 mM concentration of glucose. Vanadate at 10 \( \mu \)M and DES at 15 \( \mu \)M completely inhibited the activation by glucose which confirmed that \( \text{H}^+ \)-ATPase is involved in medium acidification and that it is activated by glucose. There was slight increase in the transformation frequency of treatments with 10 mM glucose. The transformants were confirmed with PCR amplification of \( hpt \) from genomic DNA. Identification of the phenolic compounds present in spent medium of \textit{D. bardawil} which may induce \textit{vir} gene so as to facilitate gene transfer without acetosyringone was carried out using HPLC and HPTLC methods. Major phenolics identified from extracts from spent medium at basic pH were acetosyringone, vanillic acid, vanillin and proto-catechuic acid out of which acetosyringone was observed as the major phenolic compound. The compounds were confirmed with mass spectrometry (ESI negative mode). The identified phenolic compounds were checked for their transforming efficiency by incorporating them at 100 \( \mu \)M concentration in the co-cultivation medium. A maximum cell count of 95±8 per \( 10^6 \) cells was obtained by addition of vanillin in the co-cultivation medium.

A binary vector harbouring \( bkt \) from \textit{H. pluvialis} which is driven by Rubisco smaller subunit promoter with its transit peptide was constructed. The genomic clone of \( bkt2 \) was used in vector construction. The RBCS2 promoter region along with the transit peptide was amplified from \textit{D. bardawil} using adaptor ligated genomic walking method. The 1.4 kb amplicon was confirmed by sequencing and was shown to have 99% similarity with other Rubisco sequences. The promoter obtained was analysed for the presence of transcription factors and was shown to have several light responsive elements. The presence of chloroplast targeted signal peptide was also analysed. The amplicon was subsequently used for constructing two binary vector constructs; p1304-RBB-BKT with 1.4 kb and p1304-RBS-BKT with 0.6 kb promoter regions.

\textit{Agrobacterium} mediated transformation of \textit{D. bardawil} was carried out using
the standardised procedure using 4 binary vector constructs viz; p1304, p1304-CaMV-BKT, p1304-RBB-BKT and p1304-RBS-BKT. The selected transformants were confirmed using GFP & GUS expression, PCR and southern blot analysis. The expression of various carotenogenic genes PSY, PDS, LCY, BKT and CHY were carried out in MAS100, De Walnes and MAS100 with ¼ strength phosphate and nitrate media. The expression of BKT and CHY was higher in transformants with RBCS promoter contracts, particularly with the deletion construct (p1304-RBS-BKT). There is an upregulation in the endogenous hydroxylase level of transformants where the BKT expression was higher. The expression of all genes was higher in nutrient limiting medium. The analysis of carotenoids extracted from wild and transformant Dunaliella were carried out using spectrophotometry, TLC, HPLC and MS (APCI). Astaxanthin and canthaxanthin were detectable in p1304-RBB-BKT and p1304-RBS-BKT transformants in HPLC and MS analysis. There was a decrease in β-carotene and lutein content in p1304-RBB-BKT and p1304-RBS-BKT. Maximum content of astaxanthin observed in p1304-RBS-BKT transformant is 3.5 µgg\(^{-1}\) DW. The cDNA clone of \(bkt\) was expressed in \(E. coli\) which showed an additional protein band of 33 KDa size in SDS-PAGE analysis. The presence of 33 KDa band was also observed in p1304-CaMV-BKT, p1304-RBB-BKT and p1304-RBS-BKT transformants.

The full potential of a stable genetic transformation has not been realized for Dunaliella, which is a commercially important microalga valued for its suitability as a perfect candidate for molecular farming. Agrobacterium mediated genetic transformation method described in the present study is a highly efficient technique for developing stable transformants of Dunaliella which is most recommended for production in outdoor conditions due to its halotolerant nature. The standardised transformation methodology may be used for production of foreign proteins in Dunaliella spp. which are relevant to food, pharmaceutical and nutraceutical industries. The present study is the first report in genetic manipulation of carotenoid biosynthetic pathway in \(D. bardawil\) for the production of keto-carotenoids including astaxanthin. This robust transformation method would thus pave the way for utilisation of \(D. bardawil\) for the production of valuable carotenoids through metabolic engineering of carotenoid biosynthetic pathway.