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Cyanobacterial GR6 glutamate-1-semialdehyde aminotransferase: a novel enzyme-based selectable marker for plant transformation

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Abstract The mutant glutamate-1-semialdehyde aminotransferase (GSA-AT) enzyme encoded by the *hem*L gene of the gabaculine-resistant cyanobacterium *Synechococcus* PCC6301 strain GR6 was expressed in tobacco following Agrobacterium-mediated transformation of leaf discs. When targeted to plastids, the GR6 *hem*L gene product conveyed gabaculine resistance to transgenic plants. Selection using 50 and 100 µ*M* gabaculine was shown to produce two distinct explant phenotypes: 'greens' and 'whites'. T_1 progeny displayed Mendelian segregation ratios, and PCR analysis demonstrated the 'green' phenotype corresponded with the presence of the GR6 *hem*L gene. Furthermore, 'whites' could be rescued after 9 days growth on solid media containing between 5 µ*M* and 25 µ*M* gabaculine, allowing the potential use of this system for the isolation of gabaculine-sensitive transformants in mutagenesis screening. The use of GR6 *hem*L as a selectable marker gene provides a novel enzyme-based method for the selection of transgenic regenerants without the need for antibiotic-resistance markers.

Keywords Glutamate-1-semialdehyde aminotransferase · Plant transformation · Gabaculine

Abbreviations *ALA:* Aminolaevulinic acid · *CaMV:* Cauliflower mosaic virus · *GSA-AT:* Glutamate-1-semialdehyde aminotransferase · *EPSP:* 5-enol pyruvylshikimate 3-phosphate

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Introduction

Selectable marker genes encode proteins that render transformed plants resistant to phytotoxic agents and have been invaluable in the development of methods for the genetic manipulation of plants. Among the most widely used selectable marker genes are those that confer tolerance to antibiotics (Bevan 1984; Van den Elzen et al. 1985) or to herbicides (Shah et al. 1986; De Block et al. 1987). Resistance to herbicides is conferred by two principal modes of action. In the first, the marker gene encodes an enzyme that detoxifies the herbicide, which is exemplified by resistance to phosphinothricin (basta) conferred by the bacterial gene for phosphinothricin acetyl transferase (De Block et al. 1987). The second mode involves the expression of a mutated form of the herbicide target that is resistant to the herbicide but is still biologically active, as demonstrated by glyphosate resistance conferred by mutant forms of 5-enol pyruvylshikimate 3-phosphate (EPSP) synthase (Shah et al. 1986).

The purpose of the investigation reported here was to develop a novel enzyme-based system involving a mutant form of cyanobacterial glutamate-1-semialdehyde aminotransferase (GSA-AT) that confers resistance to the phytotoxin gabaculine (3-amino-2, 3-dihydrobenzoic acid; Kobayashi et al. 1976). The enzyme GSA-AT is found in a wide range of organisms, including cyanobacteria and higher plants, where it is involved in the C5 pathway and catalyses the conversion of glutamate-1-semialdehyde into aminolaevulinic acid (ALA). The enzyme is encoded by the *hem*L gene and is sensitive to gabaculine, an irreversible inhibitor of a wide range of pyridoxal-5-phosphate-linked aminotransferases (Rando 1977).

Several gabaculine-resistant mutants have been isolated (Kahn and Kannangara 1987; Houghton et al. 1988), one of which is a strain of *Synechococcus* PCC6301, designated GR6 (Grimm et al. 1991b). This mutant harbours a *hem*L gene coding for an enzyme with two changes in its primary structure compared to the wild type: a deletion of the amino acids serine-proline-phe-

nylalanine from positions 5, 6 and 7, respectively, and a methionine to isoleucine substitution at position 248 (Grimm et al. 1991b). Furthermore, studies involving the in vivo characterization of the GR6 enzyme have demonstrated conclusively that both mutations are necessary for resistance to high concentrations of gabaculine (Allison et al. 1997) and that the replacement of the wild-type cyanobacterial *hem*L gene with that of GR6 results in transformants which display tolerance to high levels of gabaculine.

Here, we describe the use of the GR6 *hem*L gene as a selectable marker gene in plant transformations. The GR6 GSA-AT was expressed from the T-DNA of the binary vector pCAMBIA 1300 following *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf discs. Expression from the *hem*L gene conveyed gabaculine resistance to transgenic tobacco plants resulting in two distinct explant phenotypes: 'greens' and 'whites'. The presence of the GR6 *hem*L gene in T_1 progeny 'greens' was confirmed by polymerase chain reaction (PCR) analyses, demonstrating that this system provides a novel enzyme-based method for the selection of transgenic regenerants without the need for antibiotic resistance markers.

Materials and methods

Micro-organisms and plasmids

Escherichia coli TG1 [*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r_K^- m_K^-) (F′ *traD36 porAB lacI^{q}ZΔM15*)] cells were from Strategene (Cambridge, UK). The expression cassette from pJIT117 (Guerineau et al. 1988) was used along with the binary vector pCAMBIA 1300 (CAMBIA, Canberra, Australia). *Agrobacterium tumefaciens* strain C58 was used. The GR6 GSA-AT gene (GR6 *hem*L) cloned into the expression vector pMalp2 (New England Biolabs, Hitchin, UK) between the restriction sites for *Xmn*I and *Hin*dIII was kindly provided by Dr. Arnold Smith (Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion, UK).

Molecular biology techniques

Protocols for DNA manipulation were taken from Sambrook et al. (1989) or were those recommended by the manufacturers. The GR6 *hem*L gene situated in the expression vector pMalp2 was amplified by PCR using the primers PstGR6 (5′-ACATCTGCAGGGGTCACGTCTAAAACC-3′) and EcoGR6 (5′-ACTCGAATTCTGTCATTGCGGCTTACA-3′). The PCR product was cloned into the *Pst*I and *Eco*RI sites of pJIT117, and the expression cassette containing the GR6 *hem*L gene was sub-cloned into the binary vector pCAMBIA 1300 using the restriction enzyme site for *Kpn*I (Fig. 1). Genomic DNA was isolated from *Nicotiana tabacum* (cv. Samsun) plants using the DNAeasy plant midi kit from QIAGEN (Crawley, Sussex, UK). For diagnostic purposes, the cyanobacterial GR6 *hem*L gene was amplified using the primers 6301-forward (5′-GTCACGTCTAAAACCATC-3′) and 6301-reverse (5′-CGCACTCATCACCGTACG-3′). These primers show 100% homology to the GR6 *hem*L gene (Grimm et al. 1991a, b) but only 22% and 27% homology, respectively, to the tobacco *hem*L gene (Hofgen et al. 1994), resulting in the specific amplification of the cyanobacterial gene sequence.

The GR6 *hem*L gene within pCAMBIA 1300 was sequenced by dye termination with Amplitaq DNA polymerase F3 on a 377 ABI automated DNA sequencer using the following internal cyanobacterial *hem*L primers for the sense (S) and antisense (X) strands (the start nucleotide is shown):

- S2 (180) 5′-GACTACGTCGGCACCTGG-3′
- S3 (435) 5′-CACGCTGACATGTTCTTG-3′
- S4 (693) 5′-GAAATCACGCTGGAGCAT-3′
- S5 (1008) 5′-CGCCTGAGTGATGGTTTG-3′
- X2 (1038) 5′-CGCATGACCGGTTTCTTG-3′
- X3 (767) 5′-GCCAAACTTTTCTTGCAC-3′
- X4 (480) 5′-GGTCGCCACCCCAGACCC-3′
- X5 (156) 5′-GCGATTGCCGTCCACATC-3′

Plant transformations

Agrobacterium tumefaciens C58 was transformed with the GR6 *hem*L pCAMBIA 1300 construct (Fig. 1) and the transformants selected with kanamycin. These were used to transform *N. tabacum* (cv. Samsun) leaf discs in a standard protocol as described by Horsch et al. (1985) and Klee et al. (1987). Transformants were selected on MS medium (Murashige and Skoog 1962) containing gabaculine (Sigma-Aldrich, Poole, UK) at concentrations up to 100 μ*M*.

Gabaculine powder was handled in a fume hood and 50 m*M* stocks made up in water, filter-sterilized through a 0.2-μm filter and stored at –20°C. Gabaculine containing MS or Lehle (Lehle Seeds, Round Rock, Tex., USA) media was prepared by cooling the melted media to approximately 40°–50°C before the addition of a 50 m*M* gabaculine stock to produce the desired final concentration. Where gabaculine was applied as a spray, a 50 m*M* gabaculine stock was diluted in distilled water to 1.14 m*M*, and application was carried out in a fume hood with seedlings contained in clear plastic bags.

Growth of plants

N. tabacum (cv. Samsun) seeds were surface-sterilized in 30% (v/v) bleach containing 0.02% (v/v) Tween 20 for 10 min and then washed five times with sterile water. Explants and seedlings were plated out on MS and Lehle media, respectively, and grown in a tissue culture room at 22°C under a 16/8-h (light/dark) photoperiod. Plants were grown in greenhouses under a 16/8-h (light/dark) photoperiod. Seedlings sprayed with gabaculine solution were sealed within clear plastic bags and grown in a tissue culture room at 22°C under a 16/8-h (light/dark) photoperiod.

Results and discussion

The GR6 *hem*L gene was amplified by PCR and cloned into the multiple cloning site of the expression vector pJIT117 (Fig. 1) using the restriction enzymes *Pst*I and *Eco*RI. This produced an expression cassette consisting of two cauliflower mosaic virus (CaMV) 35S promoters followed by a chloroplast targeting signal sequence from the small sub-unit of ribulose-bisphosphate carboxylase (Anderson and Smith 1986), the GR6 *hem*L gene and a polyadenylation sequence. This expression cassette was sub-cloned into the T-DNA region of the binary vector pCAMBIA 1300 using the *Kpn*I restriction enzyme site (Fig. 1). Sequencing of the GR6 *hem*L gene confirmed the cloning strategy and that the cyanobacterial gene contained no PCR errors.

Wild-type *N. tabacum* leaf discs were grown on MS medium containing 0–250 μ*M* gabaculine, demonstrating

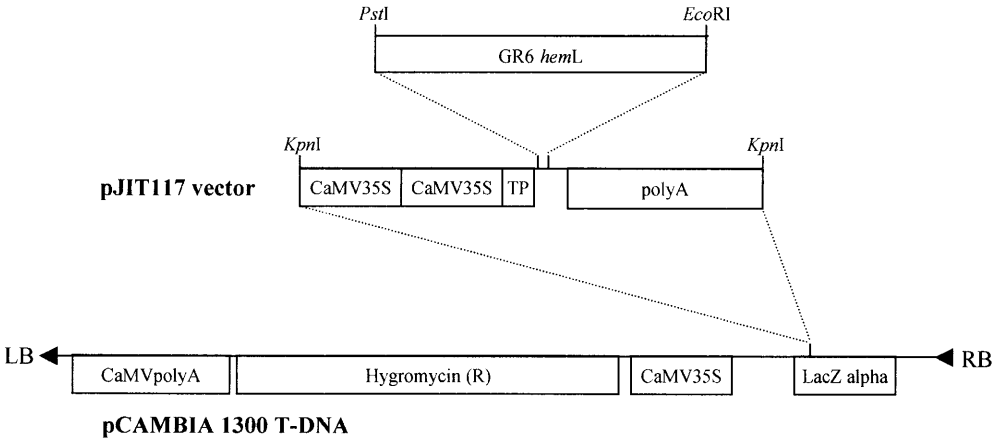

Fig. 1 T-DNA construct used in this study. The GR6 *hem*L gene was amplified by PCR, thereby introducing the restriction enzyme sites for *Pst*I and *Eco*RI, and the cut product was ligated into the *Pst*I/*Eco*RI-digested pJIT117 vector. The resulting expression cassette consisted of two cauliflower mosaic virus (*CaMV*) 35S promoters followed by a chloroplast targeting signal sequence (*TP*) from the small sub-unit of ribulose-bisphosphate carboxylase (Anderson and Smith 1986), the GR6 *hem*L gene and a polyadenylation sequence (*polyA*). This expression cassette was excised using *Kpn*I and cloned into the multiple cloning site of the binary vector pCAMBIA 1300

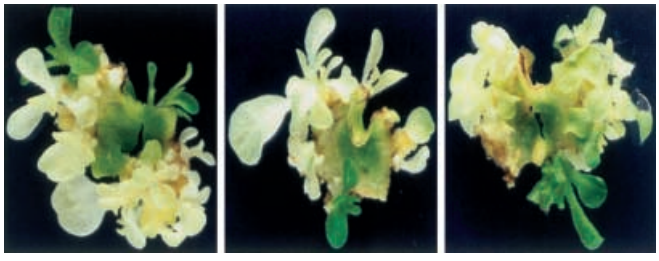

Fig. 2 Regeneration of gabaculine-tolerant tobacco shoots. Leaf discs from tobacco were transformed with *A. tumefaciens* C58 harbouring the GR6 *hem*L pCAMBIA 1300 construct. The plant material was grown on MS medium containing 50 µ*M* gabaculine for 3 weeks and then transferred to fresh MS medium containing 100 µ*M* gabaculine and grown for a further 2 weeks. Distinct 'green' and 'white' phenotypes can be seen

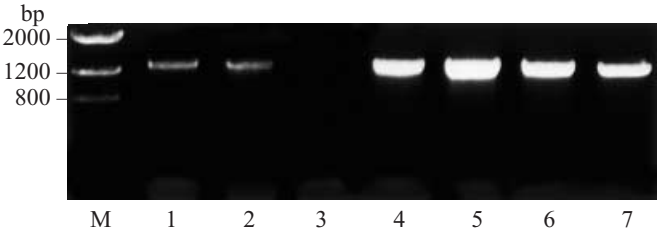

Fig. 3 PCR screening of the T_0- and T_1-generation GR6 *hem*L transformants. GR6 *hem*L-specific primers were used to screen T_0- and T_1-generation transformants for the presence of the transgene. Analysis of genomic DNA from transgenic lines T_0-4 (*lane 1*), T_1-4 [isolates 2 (*lane 4*) and 7 (*lane 5*)] and T_1-6 [isolates 3 (*lane 6*) and 11 (*lane 7*)] along with wild-type genomic DNA (*lane 3*) and the pCAMBIA 1300-GR6 *hem*L construct vector DNA (*lane 2*) are shown

that 50 µ*M* inhibited the regeneration of 'green' explants (data not shown). Leaf discs were transformed with *A. tumefaciens* harbouring the GR6 *hem*L pCAMBIA 1300 construct and grown on MS medium containing 50 µ*M* gabaculine for 3 weeks. The leaf discs were then transferred to fresh MS medium containing 100 µ*M* gabaculine and grown for a further 2 weeks. At this point two distinct phenotypes – 'greens' and 'whites' – were observed (Fig. 2). It is likely that the 'white' phenotypes are chlorotic due to gabaculine inhibition of the C5 pathway at the step catalysed by GSA-AT. The resulting lack of ALA leads to a deficiency in tetrapyrrole compounds, including chlorophyll. Ten of these T_0 generation 'greens' were isolated and grown on in soil under greenhouse conditions and T_1 generation seeds harvested. PCR analysis of the T_0-generation 'greens' showed that all ten contained the GR6 *hem*L gene (Fig. 3). The expression of GR6 GSA-AT from this transgene appears to overcome explant sensitivity to gabaculine as a consequence of the mutant form of GSA-AT displaying resistance to the inhibitor in vivo. Furthermore, it is unlikely that the introduction of an additional GSA-AT gene will result in any phototoxic effects due to the over-production of the enzyme and, consequently, increased ALA and porphyrin accumulation because of the well-characterized feedback regulation of ALA synthesis (Beale and Weinstein 1990).

In order to determine segregation ratios and conditions under which gabaculine could be used to select for T_1-generation transformants, plates of 49 seeds from transformed and wild-type *N. tabacum* were plated out on Lehle medium containing between 0 µ*M* and 250 µ*M* gabaculine. The inhibitor was lethal to the majority of wild-type seedlings at concentrations as low as 10 µ*M* and was lethal to all of the wild-type seedlings when present at levels of 25 µ*M* and above (Fig. 4). In contrast, the majority of seedlings from the transformed plants displayed resistance to the inhibitor (Figs. 4, 5) even at concentrations as high as 125 µ*M*.

The T_1-generation seedlings from six of the ten 'green' explants isolated from the initial transformations were grown on Lehle medium containing gabaculine at a concentration of 25 µ*M*. After 27 days the numbers of the two phenotypes were counted and clearly showed

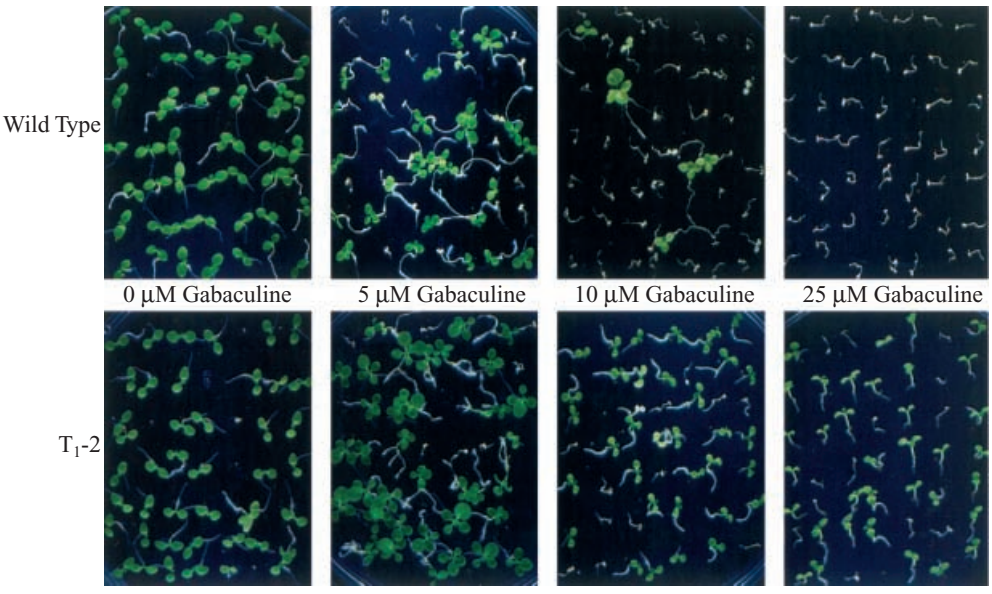

Fig. 4 Effect of gabaculine on wild-type and T_1-generation GR6 *hem*L transformants. Seeds from wild-type tobacco and transgenic line T_1-2 were plated out on Lehle medium containing 0, 5, 10 and 25 µ*M* gabaculine and grown for 34 days

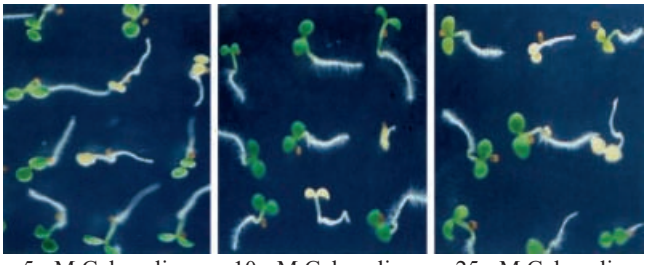

Fig. 5 Phenotypic effect of gabaculine on T_1-generation GR6 *hem*L transformants. Seeds from transgenic line T_1-4 were plated out on Lehle medium containing 5, 10 and 25 µ*M* gabaculine and grown for 9 days

Table 1 Phenotypic segregation of T_1-generation transgenic tobacco

Transgenic line	Greens	Whites	Ratio 'greens': 'whites'	Number of 'greens' which were GR6 *hem*L PCR-positive
T_1-1	95	9	11:1	–
T_1-2	89	19	5:1	–
T_1-4	323	94	3:1	15 out of 15
T_1-6	346	24	14:1	15 out of 15
T_1-7	67	7	10:1	–
T_1-9	70	32	2:1	–

Mendelian segregation ratios of approximately 3:1 or 15:1 (Table 1). To further demonstrate that the 'green' phenotype was due to the presence of the GR6 *hem*L gene, we analysed 15 of the 'greens' from each of the T_1-4 and T_1-6 transgenic lines by PCR (Fig. 3), and all 30 isolates were shown to contain the selectable marker gene.

The use of GR6 *hem*L as a selectable marker gene produces tight selection for both explant and seedling transformants when the inhibitor is used in the solid growth media at concentrations of 100 µ*M* and 25 µ*M*, respectively. For transgenic seedling selection it appeared that the 'greens' displayed reduced growth on media containing over 10 µ*M* gabaculine compared to seedlings grown without inhibitor (data not shown). It is likely that this reduction in growth rate is due to gabaculine still exerting some effect upon the formation of ALA despite the presence of the GR6 GSA-AT enzyme. It was also observed that the growth rate of seedlings grown on gabaculine-containing media was restored when they were transferred to gabaculine-free media or soil after selection.

In order to determine whether the 'white' phenotype could be 'rescued' by removal from the gabaculine-containing media, seeds were germinated on Lehle medium containing 5, 10 and 25 µ*M* gabaculine. After 9 days the two phenotypes could be distinguished (Fig. 5), and approximately 30 'white' seedlings from each concentration were transferred onto half-strength (1/2) MS medium or 1/2 MS medium supplemented with 100 µ*M* ALA. After 4 weeks 86%, 74% and 41%, respectively, of seedlings from the 5, 10 and 25 µ*M* gabaculine plates recovered from the effects of gabaculine toxicity and lost their chlorotic appearance. For each of the gabaculine concentrations used for selection, three of each of the 'rescued' seedlings were grown on and analysed by PCR; only one (from the 25 µ*M* gabaculine plate) was found to contain the GR6 *hem*L gene. No differences in the number of 'rescued' seedlings were observed between medium with or without ALA. If 5 µ*M* or 10 µ*M* gabaculine selection were to be applied, it could be envisaged that the GR6 *hem*L gene under the control of a suitable promoter could be used as a negative selectable marker in mutagenesis screening. This would allow the use of a single selectable marker for the positive selection of 'greens' to establish a transgenic line and the negative selection of 'whites' after mutagenesis.

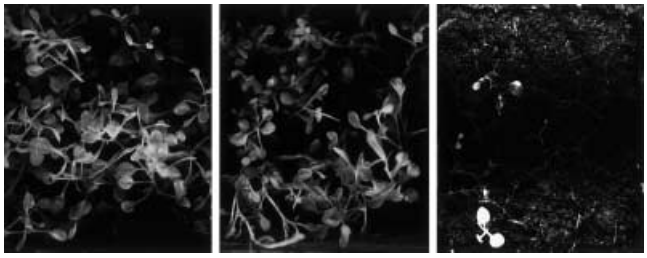

Fig. 6 Selection of gabaculine-tolerant transformants by spray application of the herbicide. Two-week-old seedlings from the wild type and transgenic lines T_1-2 and T_1-9 were grown under clear plastic in growth chambers and sprayed with 1.14 m*M* gabaculine every 4 days for 5 weeks

The use of gabaculine to select for T_1-generation transformed seedlings in solium was also assessed by spraying 2-week-old seedlings from wild-type and transformed plants with 1.14 m*M* gabaculine every 4 days for 5 weeks. Seedlings were grown under clear plastic in a tissue culture room, and after selection all of the wild-type seedlings had been killed whereas the majority of seedlings from the transformed plants were healthy (Fig. 6).

The use of cyanobacterial GR6 *hem*L as a selectable marker gene for plant transformations has many parallels with glyphosate resistance conferred by a mutant form of EPSP synthase (Shah et al. 1986). Of some importance is that it provides an alternative to the use of an antibiotic as the selective agent and, therefore, an antibiotic resistance gene as the selectable marker. Furthermore, the use of *hem*L as a selectable marker gene also offers the potential to engineer gabaculine-resistant mutant forms of GSA-AT from higher plants, thus enabling an endogenous plant gene to be used as a selectable marker.

A further use of this selection system may be in chloroplast transformation. This technique offers significant advantages over nuclear integration, such as increased levels of transgene expression (McBride et al. 1995) and in many plants the containment of foreign genes, as the transfer of the chloroplast genome is by maternal inheritance rather than pollen dispersal (Daniell et al. 1998). However, the number of selectable marker genes for use in chloroplasts is limited and has led to the development of recycling strategies (Fischer et al. 1996). The *hem*L gene from the cyanobacterium *Synechococcus* PCC6301 strain GR6 is an ideal candidate for plastid expression and, therefore, for use as a selectable marker in chloroplast transformation.

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