Plasmid construction for genetic modification of dicotyledonous plants with a glycolate oxidizing pathway

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ABSTRACT. There are many kinds of dicotyledonous C₃ plants, which often release CO₂ fixed by photosynthesis and consume energy in photorespiration. In Escherichia coli, glycolate can be metabolized by an oxidation pathway that has some of the same compounds as dicotyledonous photorespiration. With the bacterial glycolate metabolism pathway, photorespiration of dicotyledonous plants is genetically modified for less CO₂ release and more biomass. In this study, two plasmids involved in this modification were constructed for targeting two enzymes of the glycolate oxidizing pathway, glyoxylate carbonylase and tartronic semialdehyde reductase, and glycolate dehydrogenase in Arabidopsis thaliana mitochondria in this pathway. All three enzymes are located in chloroplast by transit peptide derived from Pisum sativum small unit of Rubisco. So far, some crops have been transformed by the two plasmids. Through transformation of the two plasmids, photosynthesis of dicotyledonous plants may be promoted more easily and release less CO₂ into the atmosphere.

Key words: Plasmid construction; Dicotyledonous plants; Genetic modification; Glycolate oxidizing pathway
INTRODUCTION

Many vegetables, fruits, economic crops, and urban greening plants are dicotyledons. The majority of them are C₃ plants with loss of energy and release of CO₂ during photorespiration, such as cabbage, tea, cotton, apple, soybean, and poplar. In C₃ plants, O₂ and CO₂ compete for the same active site of the difunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which can catalyse both the carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP) (Bowes et al., 1971). Phosphoglycerate (PGA), the product of RuBP carboxylation by Rubisco, is processed into carbohydrate in photosynthesis. However, photorespiration begins with the oxygenation of RuBP by Rubisco to form phosphoglycolate (PG) as well as PGA. PGA can enter the Calvin cycle directly, whereas PG enters the photorespiration cycle. Plants can recycle part of the carbon by photorespiration, in which PG is metabolized to glyceraldehyde, which can re-enter the Calvin cycle (Leegood et al., 1995).

Photorespiration is important for carbon recycling from PG (Keys, 1986; Leegood et al., 1995) and energy dissipation in order to prevent photoinhibition (Kozaki and Takeba, 1996), but it is a wasteful metabolic pathway in general. Oxygenation of RuBP is accompanied by consumption of ATP and reducing equivalents in the form of NAD(P)H (Maroco et al., 2000). Ammonia formed in mitochondria has to be refixed with extra-ATP in chloroplast. Furthermore, 25% of the carbon from PG is lost as CO₂ in mitochondria through the photorespiration pathway (Leegood et al., 1995).

The photorespiration pathway of C₃ plants proceeding in the chloroplast, peroxisome and mitochondria is represented in Figure 1. Many bacteria can metabolize glycolate, the primary product of the oxygenase activity of Rubisco in plant chloroplast, as the sole carbon source. *Escherichia coli* is one of the bacteria that has the glycolate oxidizing pathway (Lord, 1972; Pellicer et al., 1996). The first step is oxidation of glycolate to glyoxylate catalyzed by glycolate oxidase (GO). And the following reaction catalyzed by glyoxylate carboligase (GCL) condenses two molecules of glyoxylate to tartronic semi-aldehyde and releases CO₂. Tartronic semialdehyde is reduced to glyceraldehyde by tartronic semialdehyde reductase (TSR) in *E. coli*.

In a previous study, the *E. coli* glycolate oxidizing pathway was introduced into *Arabidopsis thaliana* chloroplast by transformation of GO, GCL and TSR for greater biomass and reduced release of CO₂ (Kebeish et al., 2007). Additionally, photosynthesis was enhanced with an increased chloroplastic CO₂ concentration in the vicinity of Rubisco while photorespiration was reduced. This was a complex task because bacterial GO is constituted from three different protein subunits, which are encoded by three different genes known as *glcD*, *glcE* and *glcF*, all of which are located in the *glc* operon of *E. coli* (Pellicer et al., 1996). In fact, they transform 5 independent expression cassettes in all containing protein coding sequences in plant chloroplast.

We replaced the GO with glycolate dehydrogenase (GDH) from *A. thaliana* mitochondria. GDH encoded by the *gdh* gene can catalyze the same reaction as GO, and it can complement any subunit of GO in *E. coli* (Bari et al., 2004). Furthermore, *gdh* encodes a single protein instead of several subunits. These features of *gdh* simplify the genetic modification of dicotyledonous plants with *gcl* and *tsr* from *E. coli* and *gdh* from *A. thaliana*. We can modify dicotyledonous plants by transformation of two vectors rather than three.
Firstly, a universal expression vector pUE was constructed, which was modified from the commercial vector pBlueScript SK+ (pSK). pUE contains the coding sequence of dicotyledonous chloroplast transit peptide (CTP) for targeting a protein into chloroplast, between the duplicated cauliflower mosaic virus (CaMV) 35S promoter (2×35S) and nopaline synthase gene (nos) polyA (PA) terminator sequences. The ctp sequence was cloned from the *Pisum sativum* small unit of Rubisco (RbcS) while 35S promoter and nos polyA sequences were cloned from binary vector pCAMBIA 1301. Secondly, the gdh, gcl and tsr sequences were inserted into pUE multiple cloning sites (MCS) between ctp and nos polyA, respectively, resulting in three individual expression vectors. Thirdly, gcl and tsr expression cassettes were inserted into one vector pCAMBIA 1301 tandemly, yielding a binary expression vector p1301DiGT. Meanwhile, the gdh expression cassette was inserted into another vector pPGN, yielding another binary expression vector pPGNG. The backbones of pCAMBIA 1301 and pPGN offer hygromycin and kanamycin resistance to transgenic plants. Genetic modification of dicotyledonous plants will be established by co-transformation of the two final binary expression vectors to receptor plants.

Figure 1. Schematic integration of photorespiration (black) and *Escherichia coli* glycolate metabolic pathway (red). Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP = ribulose-1,5-bisphosphate; PGP = phosphoglycerate phosphatase; PG = phosphoglycerate; PGA = phosphoglycerate; GOX = glycolate oxidase; GGAT = glyoxylate/glutamate aminotransferase; α-KG = α-ketoglutarate; Glu = glutamine; GDC/SHMT = glycine decarboxylase/serine hydroxymethyl transferase; SGAT = serine/glyoxylate aminotransferase; HPR = hydroxypyruvate reductase; OH-pyruvate = hydroxypyruvate; GK = glycerate kinase; GS = glutamine synthetase; GOGAT = glutamate/oxoglutarate aminotransferase; GDH = glycolate dehydrogenase; GCL = glyoxylate carboxylase; TSR = tartronic semialdehyde reductase.
MATERIAL AND METHODS

Strains and vectors

_E. coli_ DH5α was used as the recipient strain for recombinant plasmids. pMD® 19-T Simple Vector (TaKaRa) is a commercial T vector without MCS. pSK is a common cloning vector, which offers the backbone of the universal expression vector pUE. Both pCAMBIA 1301 and pPGN are binary expression vectors and show different antibiotic resistance in plants.

DNA and RNA manipulation

RNA was extracted from plants by TRIZOL regent (TIANGEN). Restriction enzymes were used as the conditions recommended by protocols (TaKaRa). Klenow fragment for blunting 3’ ends of DNA and alkaline phosphatase for dephosphorylation of DNA were also bought from TaKaRa.

The DNA fragments were isolated with the TIANgel Midi Purification Kit according to manufacturer instructions (TIANGEN). The plasmid DNA or ligation mixtures were transformed into DH5α competent cells (TIANGEN). All DNA fragments were sequenced by Sangon and alignments were done to identify its correctness by Nucleotide BLAST Tool in NCBI.

Polymerase chain reaction (PCR)

The primers used in this study for PCR amplification are shown in Table 1, synthesized by Sangon. PCR was performed in 20 μL mixtures as follows: plasmid DNA (1~10 ng), 1X PCR buffer, 0.2 mM of each dNTP, 200 nM of each primer and 0.25 μL TransTaq DNA Polymerase High Fidelity (TRANS). The PCR program was carried out as follows: initial denaturation at 95°C for 5 min, followed by 30~35 cycles of 95°C for 30 s, appreciate annealing temperature for 30 s, and 72°C for 2 min, and 72°C for 10 min after the cycles.

RESULTS

Clone of involved elements and genes

We amplified the full-length of the _gdh_ coding sequence from _A. thaliana_ transcriptome, _gcl_ and _tsr_ sequences from the _E. coli_ genome. Both 2×35S promoter and _nos_ PA were cloned from the pCAMBIA 1301 vector. The _ctp_ fragment was amplified from the _P. sativum_ genome. The sequences were all inserted into the T vector with designed restriction endonuclease site as shown in Table 1. Every cloned fragment, including regulatory elements and genes, was validated by sequencing.

These T vectors harboring involved elements and genes are named by prefixing ‘T’ to their names, as in T-gcl, T-PA, T-35S, and T-ctp.
Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Corresponding genes or elements</th>
<th>Endonuclease (underlined)</th>
<th>Sequence of primers (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>tsr-up</strong></td>
<td>sr</td>
<td><strong>BamHI</strong></td>
<td>GGATCCATGAAAATGGATTTATGG</td>
</tr>
<tr>
<td><strong>tsr-down</strong></td>
<td>sr</td>
<td><strong>PstI</strong></td>
<td>CTGCAATTCAAGGCGAGTTTAAGTTAG</td>
</tr>
<tr>
<td><strong>gcl-up</strong></td>
<td>gcl</td>
<td><strong>BamHI</strong></td>
<td>CGCCAGGCTGGCAAAATGAGAGCGTTT</td>
</tr>
<tr>
<td><strong>gcl-down</strong></td>
<td>gcl</td>
<td><strong>PstI</strong></td>
<td>TGCACCTGCACTTACATGCTGAGAGGC</td>
</tr>
<tr>
<td><strong>gdh-up</strong></td>
<td>gdh</td>
<td><strong>BamHI</strong></td>
<td>GCAGCTGCTTTGCTCAAAATT</td>
</tr>
<tr>
<td><strong>gdh-down</strong></td>
<td>gdh</td>
<td><strong>ApaI</strong></td>
<td>GCAGCGCTTTAGAACATAGAGGGGAG</td>
</tr>
<tr>
<td><strong>PA-up</strong></td>
<td>PA</td>
<td><strong>KpnI, ApaI, PstI</strong></td>
<td>GGTACCCTGTGACCCCTTGGCTTTCAAAATTTGGCAAT</td>
</tr>
<tr>
<td><strong>PA-down</strong></td>
<td>PA</td>
<td><strong>SacI, XbaI, HindIII</strong></td>
<td>GAGCTCTAGAAAGCTCCCAGACTGTTAACATAGAAT</td>
</tr>
<tr>
<td><strong>CTP-up</strong></td>
<td>ctp</td>
<td><strong>KpnI, SacI, BglII</strong></td>
<td>GGTACCGAATTCGTCGACGCTAGCTGCTCTAGTATCTCCG</td>
</tr>
<tr>
<td><strong>CTP-down</strong></td>
<td>ctp</td>
<td><strong>ApaI, Ndel, BamHI</strong></td>
<td>GGTACCCTATATGGGATCCGGCACTGATCCTGGGAACCTT</td>
</tr>
<tr>
<td><strong>35S-up</strong></td>
<td>2×35S promoter</td>
<td><strong>KpnI, EcoRI, Sall</strong></td>
<td>GGTAGCTCCCAGACTGTCGACGCTAGCTGCTGCAAC</td>
</tr>
<tr>
<td><strong>35S-down</strong></td>
<td>2×35S promoter</td>
<td><strong>BglII</strong></td>
<td>AGATCT CGGGGATCTGCGAAAGCTCG</td>
</tr>
</tbody>
</table>
Construction of universal expression vector pUE

We constructed a universal vector pUE, which can target protein under control of its promoter and terminator into dicotyledonous plant chloroplast.

pSK is the backbone of pUE, so involved elements and genes were added to it. The PA fragment digested from T-PA with KpnI and SacI was cloned to pSK MCS, which was then digested with the same enzymes, yielding pSK-PA. The ctp and 35S fragments from T-ctp and T-35S, digested with KpnI/ApaI and KpnI/BglII, respectively, were tandemly inserted into pSK-PA by the same method, yielding pUE (Figure 2A).

Construction of cassettes of exogenous genes

pUE has an MCS between ctp and PA. All the exogenous genes, gcl, tsr and gdh, were inserted into MCS of pUE, with digestion of restriction enzymes designed in the primer sequences of genes involved (Table 1) from corresponding T vectors, to form their expression cassettes (Figure 2B). The vectors containing expression cassettes of exogenous genes were named by adding the suffix “pUE” to the gene name, with their pUE origination and backbone indicated.

Figure 2. The structure of the primary vectors in this study. A. Universal expression vector pUE. B. pUE containing exogenous gene in MCS. C. Binary expression vector p1301DiGT. D. Binary expression vector pPGNG. *Exo represents the three exogenous genes involved in this study. **This endonuclease site only exists in pUE-gdh.
Construction of dicotyledonous binary expression vectors

pCAMBIA 1301 is a common commercial binary expression vector, offering hygromycin resistance when expressed in plants and an MCS for DNA insertion. The order of enzyme sites in this MCS is EcoRI-SacI-KpnI-BamHI-XbaI-Sall-PstI-HindIII.

pUE-tsr was digested by XbaI and self-ligated following 3’ end blunting and dephosphorylation. The tsr expression cassette fragment from pUE-tsr was ligated into the MCS of pCAMBIA 1301 by digestion of HindIII and Sall, and then the gcl expression cassette fragment originating from pUE-gcl was ligated to the formed vector MCS by digestion of EcoRI and XbaI, yielding p1301DiGT (Figure 2C).

pPGN is another binary expression vector, offering hygromycin resistance to plants and a KpnI-Smal-BamHI-XbaI MCS. The gdh expression cassette from pUE-gdh was ligated to pPGN MCS by digestion of KpnI and XbaI, yielding pPGNG (Figure 2D).

pPGNG and p1301DiGT were examined by PCR and restriction enzyme digestion to make sure their sequences were correct (Figure 3).

DISCUSSION

Previous study was done for genetic modification of dicotyledonous plants with a glycolate oxidizing pathway, which may improve dicotyledonous plant photosynthesis and photorespiration for greater biomass and lower CO₂ release. Our strategy of replacing GO with GDH and transforming only two vectors instead of three is much easier. Agrobacterium tumefaciens strains, which contain p1301DiGT and pPGNG, were obtained for further application on vegetables, fruits, economic crops, and urban greening plants. Besides economic benefits, application of this strategy may also reduce carbon release into the atmosphere, which is a severe ecological problem for humans.
REFERENCES


