农杆菌介导的转谷氨酰胺合成酶基因小麦 的抗除草剂特性研究

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摘 要 谷氨酰胺合成酶(Glutamine synthetase, GS, E.C. 6.3.1.2)是植物氮同化过程中的关键酶,对植物的氮素吸收和代谢起着至关重要的作用。谷氨酰胺合成酶还是除草剂草胺膦(Phosphinothricin (PPT)或 Basta)的靶标酶。前期工作已从我国特有的豌豆(*Pisum sativum*)品种中克隆了细胞质型谷氨酰胺合成酶(GS1)cDNA 和叶绿体型谷氨酰胺合成酶(GS2)cDNA。为了验证谷氨酰胺合成酶的功能,构建了同时含有 GS1 cDNA 和 GS2 cDNA 的植物表达载体 p2GS。以该表达载体通过农杆菌介导法,转化小麦(*Triticum aestivum*)的未成熟胚愈伤组织,经 PPT 筛选及分化再生培养,获得了抗 PPT 的转基因小麦植株 41 株。PCR 和基因组 Southern 杂交分析证实了 GS1 和 GS2 基因已经整合到转基因小麦的基因组。用除草剂草胺膦 Basta 溶液涂抹转 p2GS 小麦叶片,结果证明 GS 转基因植株可以抗高达 0.3% 的 Basta 溶液,而对照植株叶片逐渐变黄直至枯死。转基因小麦植株能正常结实。上述实验结果表明:1) GS 基因在小麦植株中获得了有效表达,从而赋予小麦植株抗 PPT 特性;2) GS 基因能够作为研究小麦遗传转化的筛选标记基因。

关键词 谷氨酰胺合成酶 农杆菌介导的遗传转化 转基因小麦 除草剂抗性

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSGENIC WHEAT PLANTS WITH GLUTAMINE SYNTHETASES CONFER TOLERANCE TO HERBICIDE

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Abstract Cloning of cytosolic (GS1) cDNA and chloroplast glutamine synthetase (GS2) cDNA from *Pisum sativum* was carried out previously in our laboratory. To identify the functions of the GS genes, we first constructed a plant expression vector, p2GS, harboring two different isoenzymes, GS1 and GS2 cDNAs, under the control of two constitutive promoters of rice, Actin1 (Act1) and maize Ubiquitin (Ubi) genes. Then, using an *Agrobacterium tumefaciens*-mediated transformation method, we introduced GS1 and GS2 genes into wheat (*Triticum aestivum*) plants, producing 2GS-transgenic wheat plants using immature embryos as the explants. Presence of the transgenes GS1 and GS2 in wheat plants was confirmed by PCR and Southern blot hybridization analyses. Forty-one independent transgenic wheat plants with tolerance to an herbicide (Phosphinothricin, PPT) were generated. Results from Basta tests showed that the 2GS-transgenic wheat plants were endowed with herbicide-tolerant properties. Almost all of the transgenic plants were normal in morphology, and seed production was similar to that of the control wheat plants. Our study suggest that PPT resistance is conferred by effective expression of glutamine synthetases in transformed wheat plants, and glutamine synthetase genes can serve as a selective marker gene of wheat transformation system in our study.

Key words Glutamine synthetases, *Agrobacterium tumefaciens*-mediated transformation, Transgenic wheat, Herbicide-tolerance

Glutamine synthetase (GS, E.C.6.3.1.2) plays an important role in the flow of nitrogen into nitrogenous or-

ganic compounds of plant cells by catalyzing the formation of glutamine from glutamate and ammonium. GS is repre-

sented by two classes of proteins, GS1 being restricted to the cytosol of the phloem companion cells, GS2 being located in the stroma matrix of the chloroplasts. GS1 would synthesize glutamine from nitrogen imported in roots and phloem cells, whereas GS2 is indispensable for the re-assimilation of ammonium released from the photorespiratory nitrogen cycle (Oliveira *et al.*, 2002; Obara *et al.*, 2000; Giuseppe, 2000). Moreover, GS is the targeting enzyme of a herbicide phosphinothricin (PPT) or Basta (Li *et al.*, 1993; Sobanha & Ann, 1996; Oliveira *et al.*, 1997; Cánovas *et al.*, 1998). Up to now, many studies on GS-related abiotic stress have been reported. Overexpression of alfalfa GS2 conferred the transgenic tobacco 20 times of higher PPT resistance as compared to non-transgenic control plants (Peter *et al.*, 1988). Overexpression of GS can confer transgenic rice PPT resistance. Herbicidal pyridyl derivatives of aminomethylene-bisphosphonic acid inhibit plant glutamine synthetase (Obojska *et al.*, 2004). Water stress stimulates tomato GS1 (Cytosolic glutamine synthetase) gene expression (Bauer *et al.*, 1997). Glutamine synthetase plays a major role in regulating proline production consistent with the function of proline as a key osmoprotectant synthesized in response to water stress. Proline accumulation has been correlated with tolerance to drought and salinity stresses in plants, overproduction of proline results in the increased tolerance to osmotic stress in plants (Brugière *et al.*, 1999; Liu & Zhu, 1997; Yoshida *et al.*, 1997; Igarashi *et al.*, 2000). In addition, GS can improve nitrogen utilization of crops and decrease toxicity of ammonia (PPT can result in an accumulation of free ammonia) to plant cells (Downs *et al.*, 1994).

In this study, to enhance the function of GS, we simultaneously introduced 2GS (GS1 and GS2) genes into the wheat (*Triticum aestivum*) plants. To date, even though GS-transformed experiments have been done with different species including crop plants (Gallardo *et al.*, 1999; Harrison *et al.*, 2000; Habash *et al.*, 2001), no plants transformed with 2GS genes have been reported.

Genetic transformation of monocots, particularly in wheat, continues to be important challenge facing us. In our study, we confirm that wheat is suitable for *Agrobacterium tumefaciens*-mediated gene transfer. In addition, our result reveals that GS gene can serve as a selectable marker gene of wheat transformation system replace of other potential-harm marker genes, such as antibiotic-resistance gene and herbicide-resistance gene. It has been argued that the antibiotic-resistance gene could be transferred from plant to pathogenic microbes (Smalla *et al.*,

2000; Puchta, 2000). Moreover, herbicide-resistance gene might be transferred to weeds (Puchta, 2000). However, antibiotic-resistance gene and herbicide-resistance gene may be harmful to health.

Weeds in crop fields severely affect the growth and development of crops. Therefore, development of herbicide resistant transgenic plants is one of the most important goals of crop breeding. This can be achieved, at least in part, by glutamine synthetase gene (Targeting enzyme genes of PPT) transfer and its regulation for effective expression of GS in transgenic plants.

1 Materials and methods

1.1 Materials

1.1.1 Strains and plasmids

Strains used were *Escherichia coli* DH5- α and *Agrobacterium tumefaciens* LBA4404. Plasmids pAN, pUBI100, pGS1, pGS2 were constructed or collected in our lab. GS1 and GS2 cDNAs were cloned from *Pisum sativum* by RT-PCR in our lab (Su *et al.*, 2000).

1.1.2 Wheat (*Triticum aestivum*) used were 96G25 and Bobwhite.

1.2 Methods

1.2.1 Construction of p2GS: Act1(P)/GS1CN cDNA/Nos3'//Ubi(P)/GS2CN cDNA/Pin2 3'

The backbone used is pUBI100. Digestion of pUBI100 was performed with SpeI and blunted with klenow enzyme, pGS2 was digested by EcoRI following filled with klenow enzyme, then GS2 fragment was cloned at pUBI100 to form pUBI100-GS2. Act1(P)/GS1 cDNA/Nos3' (T) was isolated from pAN-GS1 by XhoI and SacII digestion, then blunted with klenow enzyme. pUBI100-GS2 was digested by HindIII digestion and filled with klenow enzyme. Act1(P)/GS1 cDNA/Nos3' (T) was cloned at HindIII site of pUBI100-GS2 to create p2GS.

1.2.2 Mobilization of p2GS into *Agrobacterium tumefaciens* LBA4404

We used tri-parental mating method to mobilize the p2GS plasmid into the *A. tumefaciens* LBA4404. Cultures of *A. tumefaciens* LBA4404 were grown in YEP at 28 °C overnight with shaking 250 r·min⁻¹.

1.2.3 Preculture of wheat immature embryos

Developing wheat grains were collected at 8 – 12 days post anthesis from greenhouse-grown wheat plants. The grains were surface sterilized with 2% w/v sodium hypochlorite for 12 minutes followed by washing in sterile deionised water. The immature embryos were excised and cultured on MS medium with 2.4-D 2 mg·L⁻¹ for one week (Ozgen *et al.*, 1998).

1.2.4 *Agrobacterium tumefaciens*-mediated transformation of wheat and regeneration of GS1-GS2 transgenic wheat plants

A. tumefaciens LBA4404 harboring p2GS was grown in liquid YEP media supplemented with $50 \text{ mg} \cdot \text{L}^{-1}$ kanamycin and $50 \text{ mg} \cdot \text{L}^{-1}$ streptomycin. *A. tumefaciens* LBA4404 was collected by centrifugation when the OD600 read 0.7–0.9 and resuspended in MS liquid media for the infection. The explants (precultured wheat immature embryos) were transferred into *A. tumefaciens* LBA4404 cell suspension for infection. The inoculation was performed for about 30 min, then *A. tumefaciens* LBA4404 cells were removed, and the explants were placed on cocultivation media at 26°C in the dark for 3 days. After the cocultivation, the infected explants were cultured on callus formation media (MS with 2.4-D $2 \text{ mg} \cdot \text{L}^{-1}$) containing carbenicillin $500 \text{ mg} \cdot \text{L}^{-1}$ to eliminate the growth of *A. tumefaciens* and selection agent (L-PPT) of different concentrations ($3 \text{ mg} \cdot \text{L}^{-1}$, $5 \text{ mg} \cdot \text{L}^{-1}$) to inhibit growth of non-transformed wheat cells for 15 days (First round of selection), then the embryos were transferred to fresh media (MS with 2.4-D $2 \text{ mg} \cdot \text{L}^{-1}$) containing $7.5 \text{ mg} \cdot \text{L}^{-1}$ PPT for 10 days (Second round of selection), at this step, concentration of PPT was increased to enhance the selection pressure. The PPT-resistant calli were cultured on differential media (Kt $0.5 \text{ mg} \cdot \text{L}^{-1}$) containing $3 \text{ mg} \cdot \text{L}^{-1}$, $5 \text{ mg} \cdot \text{L}^{-1}$, $7.5 \text{ mg} \cdot \text{L}^{-1}$ L-PPT for 15 days respectively. The PPT-resistant transformants were regenerated into plantlets on regeneration medium (Kt $0.5 \text{ mg} \cdot \text{L}^{-1}$, NAA $0.5 \text{ mg} \cdot \text{L}^{-1}$) for about one month. Most of PPT-resistant and control plants were moved to soil in greenhouse.

1.2.5 PCR amplification

DNA was purified from small leaf samples of transformed wheat plants and non-transformed control plants (Frederick *et al.*, 1995). Two μl of the extract were used as template. Promega Taq polymerase was used. The coding regions of transgenes (GS1 and GS2) were amplified using simultaneously the following sets of primers: GS1 gene (Upper) 5'-ACC ATG TCT AGG CTT TCA GAT CTC ATC AAC -3' (This primer is located in 5' region of GS1 cDNA) and (Lower) 5'-AGC TTA TGG TTT CAA GAG GAT GGT AGT CTC -3' (This primer is located in 3' region of GS1 cDNA). GS2 gene (Upper) 5'-AAC ATG GCG CAG ATT TTG GCA CCT TCG ACG -3' (This primer is located in 5' region of GS2 cDNA) and (Lower) 5'-GAT TTA GAC CTT TAA TGC AAT CTT CTG AGC -3' (This primer is located in 3' region of GS2 cDNA). Cycling parameters were: an initial template denaturation at 94 °C for 3 min, 30 cycles of 94 °C 1 min,

60 °C 1 min, 72 °C 1 min, followed by elongation 72 °C 10 min. The PCR products were analyzed by 0.8% agarose gel electrophoresis.

1.2.6 Southern blot analysis of wheat genomic DNA

Genomic DNA was extracted from transformed wheat plants (T0) and non-transformed control plants (Frederick *et al.*, 1995). About 25 μg of DNA was digested with HindIII (At 37 °C, overnight) and electrophoresed on 0.8% agarose gels. DNA was then transferred onto nylon membrane by vacuum blotter. Southern blot hybridization was carried out at 60 °C in a solution containing $20 \times$ SSC, $50 \times$ Denhard, 10% SDS, the probes used were GS1 and GS2 genes fragments labeled with $\alpha\text{-}^{32}\text{P}$ -dCTP via random-primed labeling system (Promega Co.) The membrane was washed twice in $2 \times$ SSC, 0.1% SDS for 10 min at room temperature, once in $0.5 \times$ SSC, 0.1% SDS for 20 min at 60 °C and finally in $0.1 \times$ SSC, 0.1% SDS for 15 min at 60 °C, then the membrane was exposed to X-ray film at -70 °C for 3 days.

1.2.7 Basta test

In order to test the efficiency of p2GS for Basta tolerance, we carried out Basta test using leaves of GS-transformed and no-transformed wheat plants. Tested leaves were painted on both sides with solution containing 0.3% Basta (v/v) and 0.05% Tween20, and then observe change of leaves painted by Basta.

2 Results

2.1 Construction of GS1-GS2 gene expression vector p2GS

For this expression plasmid construction, restriction endonuclease digestions, 2 rounds of ligations and transformations were performed to get the positive clones according to the protocols of Su *et al.* (1995) and McElroy *et al.* (1991). The positive clones were verified by DNA sequencing and endonuclease digestions (Fig. 1).

2.2 Mobilization of p2GS into *Agrobacterium* LBA4404

The *Agrobacterium* LBA4404 harboring p2GS was confirmed by PCR using 2 sets of primers based on GS1 and GS2 cDNA sequences respectively (data not shown).

2.3 Transformation of wheat with the plant expression vector p2GS and regeneration of 2GS transgenic wheat plants

The p2GS was introduced into wheat immature embryos 96G25 and Bobwhite by *Agrobacterium tumefaciens*-mediated transformation, respectively. After first and second rounds of selection, embryos had expanded in size and calli developed well. After 15 days of selection on the differential media, the development of multiple small

shoots from PPT-resistance calli were observed (Fig. 2-1), in contrast, the growth and development of non-transformed calli were inhibited (Fig. 2-1: CK). The small shoots were regenerated into plantlets on regeneration media after one month (Fig. 2-2). The Forty-one indepen-

dent GS transgenic wheat lines were obtained (Table 1). The transgenic wheat plants were normal in morphology and produced seeds (Fig. 2-6). Our study revealed that the putative PPT resistance was conferred by effective expression of GS enzymes in transformed wheat plants.

Table 1 Wheat calli selection and regeneration

Wheat cultivars	Number of transformed calli	Number of calli exhibiting PPT-resistance	Percentage of PPT-resistant calli	Number of PPT-resistant calli exhibiting shoot regeneration	Percentage of regenerated plantlets with PPT-resistance
96G25	430	83	19.30	20	24.10
Bobwhite	390	70	17.95	21	30.00
Total *	820	153	18.63 *	41	27.05 *

* Mean percentage

Fig. 1 Construction of p2GS expression vector

2.4 Identification of GS-transgenic wheat plants with PCR amplification and Southern blot hybridizations

Genomic DNA extracted from GS-transgenic wheat plants was used as the template for PCR amplification of GS1 and GS2 transgenes simultaneously in the same PCR system, the fragments amplified by PCR were the expected size: 1.1 kb for GS1, 1.3 kb for GS2 gene (Fig. 2-4). The results of Southern blot hybridization of GS-transgenic wheat plants with GS1-GS2 as probes were displayed in Fig. 2-5. T₀ wheat plants from the transgenic plants gave positive signal while the control plant did not show any signal in Southern blot hybridization. Results of PCR and Southern blot hybridization suggested that the GS1 and GS2 genes had been integrated into the genome of the transgenic wheat plants.

2.5 Basta test

0.3% Basta solution was used to scribble leaves of wheat plants. After 3 days the color of the leaves of untransformed control wheat plants turned yellow and wither, while the color of leaves of the transgenic wheat plants

endowed with herbicide-tolerate property still remained green (Fig. 2-3). The result revealed that GS effective expression conferred transgenic wheat plants herbicide-“Basta” resistance, because the GS-transgenic wheat plants were regenerated from PPT-resistant calli (PPT has the same active chemical composition as that of Basta).

3 Discussion

The objective of this study was to identify the putative functions of GS genes, carry out an effective *A. tumefaciens*-mediated method for wheat transformation, and confer transgenic wheat plants herbicide resistance by effective expression of glutamine synthetase. To achieve above goals, firstly, we have successfully completed the construction of p2GS expression vector in which two GS isoenzymes (GS1 and GS2) were involved in the same single plasmid. Selecting proper promoter is the key of GS overexpression. In our study, Rice actin1 promoter and maize ubiquitin promoter are used to drive the expression of GS1, GS2 genes respectively. These two promoters have been demonstrated to show high level expression of transgenes in monocot plants. Mar sequence can enhance the stability of transcript of the gene, increase efficiency of translation of the gene and overcome gene silence. Secondly, we developed an effective *A. tumefaciens*-mediated method for wheat transformation, advantages of an *A. tumefaciens*-mediated transformation are the stable integration of foreign genes into a plant genome, enhance frequency and stability of expression of the transgene (Hansen *et al.*, 1997). Whereas genetic engineering of wheat is usually carried out by direct method such as particle bombardment. This method is associated with DNA rearrangement and multiple integration events, which may result in inactivation of foreign gene (Meyer, 1995). PCR amplification, southern blot analyses demonstrate that the GS1-GS2 genes have integrated into wheat genome. Finally, Basta test showed that GS-transgenic wheat plants could tolerate to 0.3% Basta solution. The

Fig.2-1 Comparison of wheat calli in differential medium with PPT CK (Upper): the growth and development of control calli were inhibited in MS medium with PPT; Lower left, right: transgenic calli and shoots showing PPT resistance Fig.2-2 Regenerated transgenic wheat plantlets in regeneration medium with PPT Fig.2-3 Basta test: using 0.3% Basta solution scribble leaves of wheat plants. After 3 days the color of untransformed control wheat plants (1-3) turned yellow and wither while the color of leaves (4-10) of the transgenic wheat plants showed Basta resistance, three of them (8-10) showed strong Basta resistance Fig.2-4 PCR analysis: 1-4. 2GS-transgenics DNA showing 1.1 kb for GS1, 1.3 kb for GS2 fragments; 5. non-transgenic plant DNA (Negative control); 6. p2GS (As a positive control); M. 1 kb maker Fig.2-5 Southern blot hybridization of transgenic wheat plants. Digestion of genomic DNA transformed wheat plants (Lane 1-9) or no-transgenic wheat plants (CK as negative control) with Hind III. Lane 1, 2, 5, 8, 9, 10 (p2GS as Positive control) gave positive signal while CK and Lane 3, 4, 6, 7 did not show any signal in Southern blot hybridization Fig.2-6 Regenerated transgenic wheat plants in soil

results revealed that it was efficient to replace bar gene in the transformational system for immature embryo-derived calli of wheat, transgenic fertile wheat plants with GS1 and GS2 obtain herbicide resistance, and function of GS genes as selectable marker could be suggested. Our results suggested that it was possible to select transformants by effective expression of GS as selectable marker to avoid the use of some undesirable effects selective agents, such as herbicides or antibiotics, in plant genetic transformational system. That would be of important significance on “Biosafety”.

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