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Production of transgenic sweetpotato plants resistant to stem nematodes using *oryzacystatin*-I gene

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ABSTRACT

Stem nematode (*Ditylenchus destructor* Thorne) is one of most serious diseases limiting sweetpotato (*Ipomoea batatas* (L.) Lam.) production, and it is urgent to develop sweetpotato varieties resistant to this disease. In present study, we have developed transgenic sweetpotato (cv. Xushu 18) plants resistant to stem nematodes using *oryzacystatin*-I (*OC*I) gene with *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* strain EHA105 harbors a binary vector pCAMBIA1301 with *OC*I gene, *uidA* gene and *hpt*II gene. Selection culture was conducted using 7 mg/l hygromycin. A total of 2119 plants were produced from the inoculated 1710 cell aggregates of Xushu 18 via somatic embryogenesis. GUS assay and PCR analysis of the regenerated plants randomly sampled showed that 92.8% of the regenerated plants were transgenic plants. Transgenic plants exhibited enhanced resistance to stem nematodes compared to the untransformed control plants by the field evaluation and the inoculation test with stem nematodes and stem nematode-resistant plants were selected from the transgenic plants. Stable integration of the *OC*I gene into the genome of resistant transgenic plants was confirmed by Southern blot analysis, and the copy number of integrated *OC*I gene ranged from 1 to 3. High level of *OC*I gene expression in the resistant transgenic plants was demonstrated by real-time quantitative PCR analysis. This study provides a way for improving stem nematode resistance of sweetpotato.

1. Introduction

Sweetpotato, *Ipomoea batatas* (L.) Lam., is an important food and industrial material crop in the world. It is also an alternative source of bio-energy as a raw material for fuel production (Zang et al., 2009). The improvement of this crop by conventional hybridization is limited because of its high male sterility, incompatibility and the hexaploid nature (Dhir et al., 1998). Gene engineering offers great potential for the improvement of sweetpotato. There have been several reports on this subject in the literature. Transgenic plants expressing cowpea trypsin inhibitor (*CpTI*), snowdrop lectin, delta-endotoxin, soybean kunitz trypsin inhibitor (*SKTI*-4), sweetpotato feathery mottle virus (SPFMV-S) coat protein, granule-bound starch synthaseI (*GBSS*I), tobacco microsomal ω-3 fatty acid desaturase (*NtFAD*3), starch branching enzyme II (*IbSBE*II) or *bar* gene have been produced (Newell et al., 1995; Morán et al., 1998; Cipriani et al., 1999; Okada et al., 2001; Kimura et al., 2001; Wakita et al., 2001; Shimada et al., 2006; Otani et al., 2003; Yi et al., 2007; Choi et al., 2007; Zang et al., 2009). But, in most cases only a low transformation efficiency was obtained, which limits the successful application of genetic engineering in sweetpotato improvement.

Stem nematode (*Ditylenchus destructor* Thorne) is one of most serious diseases limiting sweetpotato production. This disease usually decreases sweetpotato yield by 20–50%, and even no yield in the field if seriously infected by stem nematodes in China (Xie et al., 2004). Thus, the breeding of sweetpotato varieties resistant to stem nematodes has become especially important. A significant negative correlation between stem nematode resistance and important quality traits (starch content, soluble sugar content, etc.) was observed, which limits the improvement of these important traits for sweetpotato by conventional hybridization (Ma et al., 1997). The use of transgenic plants expressing stem nematode resistance gene will be an alternative approach to improve the nematode resistance of sweetpotato.

Transformation of plants with proteinase inhibitor genes has great potential to enhance resistance against pathogens and insects (Masoud et al., 1993). Protein inhibitors mainly exist in storage organs of plants. The content of protein inhibitors can be as much

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as 1–10% of total proteins in seeds and bulbs of plants (S.Y. Lu et al., 1998; X.F. Lu et al., 1998). Proteinase inhibitors accumulate when the leaves of plants suffer from mechanical injury or chemical treatment. Up to date, more than 90 kinds of plants have been found to produce protease inhibitors.

Oryzacystatin-I (OCI) protein is one member of proteinase inhibitors, which can inhibit the proteinase activity in insects' intestinal canal, finally prevent the assimilation of proteins (Murdock et al., 1988). *OC*I gene with a length of 1.4 kb, cloned from rice cDNA library, is composed of three exons and two introns. The gene encodes 102 amino acid residues, and has a typical conserved sequence as Gln-Val-Val-Ala-Gly (Abe et al., 1987), which is necessary for its inhibiting effect (Meng et al., 2000). Oryzacystatin plays a role in inhibiting cysteine proteinase and may play an important role in biodefense in rice seed (Abe et al., 1987, 1988; Kondo et al., 1989).

It has been found that the *OC*I gene confers the improved resistance to plant nematodes such as *Meloidogyne incognita* and *Globodera pallida*, and nematode-resistant transgenic rice and tomato plants have been produced (Vain et al., 1998; Atkinson et al., 1996). Cipriani et al. (2001) and Jiang et al. (2004) reported the regeneration of transgenic sweetpotato plants with *OC*I gene at a low frequency, and the nematode resistance of transgenic plants was not evaluated.

We succeeded in developing an efficient system of embryogenic suspension cultures for a wide range of sweetpotato genotypes especially for commercial cultivars (Liu et al., 2001). Using embryogenic suspension cultures of sweetpotato, we have also established an efficient *Agrobacterium tumefaciens*-mediated transformation system (Yu et al., 2007) and obtained transgenic plants exhibiting complete herbicide resistance with the *bar* gene (Zang et al., 2009). In this study, we have developed transgenic sweetpotato plants resistant to stem nematodes using the *OC*I gene.

2. Materials and methods

2.1. Plant material

Sweetpotato cv. Xushu18 used in this study is one of most important commercial cultivars widely planted in China, but it is susceptible to sweetpotato stem nematodes. Embryogenic suspension cultures of Xushu 18 were prepared according to the method of Liu et al. (2001). Sixteen weeks after initiation, cell aggregates 0.7–1.3 mm in size from embryogenic suspension cultures of 3 days after subculture were employed for the transformation.

2.2. Bacterial strain and plasmid

The *A. tumefaciens* strain EHA 105 harboring a binary vector, plasmid pCAMBIA1301 was employed in this study. This binary vector contains the *OC*I, *uidA* and hygromycin phosphotransferase II (*hpt*II) genes driven by a CaMV 35S promoter, respectively (Fig. 1). *Eco*RI has a unique cleavage site in the T-DNA region in the vector.

2.3. Sensitivity of cell aggregates to hygromycin

The sensitivity of uninoculated cell aggregates to hygromycin (Hyg) added to the medium was tested according to the method of Yu et al. (2007) using 0, 3, 5, 7, 9, 12, 15, 20, 25, and 30 mg/l Hyg in order to determine the optimal concentration of this antibiotic in the selective medium. The experiments were repeated three times with 50 cell aggregates per treatment. The data were analyzed using SAS V8.02 (SAS Institute Inc., Caly, NC, USA) and the differences between the means were compared by Duncan's (1955) multiple range tests at the 0.05 level.

2.4. Transformation, selection and plant regeneration

Transformation and selection of the cell aggregates were conducted as described by Zang et al. (2009). The *Agrobacterium* single colony was cultured in 25 ml Luria–Bertani (LB) liquid medium containing 50 mg/l kanamycin and 50 mg/l rifampycin on a reciprocal shaker (200 rpm) at 28 °C for 16–18 h until $OD_{600\,nm} = 0.5$ was reached. The bacteria were collected by centrifugation at 5000 rpm for 5 min, washed with LB liquid medium and further with Murashige and Skoog (1962) (MS) medium containing 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and then were resuspended in 25 ml MS medium containing 2.0 mg/l 2,4-D for the inoculation. Cell aggregates were infected for 5 min in the bacteria suspension at room temperature. Following inoculation, the cell aggregates were blotted on sterile filter paper and placed on filter paper in a Petri dish with 25 ml MS solid medium with 2.0 mg/l 2,4-D and 30 mg/l acetosyringone (AS) for the cocultivation. The cocultivation was conducted for 3 days in the dark at 27 ± 1 °C. After cocultivation, the cell aggregates were washed twice with MS liquid medium containing 2.0 mg/l 2,4-D and 500 mg/l carbenicillin (Carb) and maintained in MS liquid medium with 2.0 mg/l 2,4-D and 100 mg/l Carb on a reciprocal shaker (100 rpm) at 27 ± 1 °C under 13 h of cool-white fluorescent light at 10 μmol/(m^2 s) for 1 week, and then were cultured at 2-week interval on MS solid medium supplemented with 2.0 mg/l 2,4-D, 100 mg/l Carb and 7 mg/l Hyg for the selection culture in the dark at 27 ± 1 °C.

Eight weeks after selection, the obtained Hyg-resistant embryogenic calluses were transferred to MS solid medium supplemented with 1.0 mg/l abscisic acid (ABA), 100 mg/l Carb and 7 mg/l Hyg to induce formation of somatic embryos and regeneration of plantlets at 27 ± 1 °C under 13 h of cool-white fluorescent light at 54 μmol/(m^2 s). The regenerated plantlets were further transferred to the basal medium for the development of whole plants under 13 h of cool-white fluorescent light at 54 μmol/(m^2 s).

2.5. GUS assay

The Hyg-resistant calluses and leaves, stems and roots of transgenic plants were tested for GUS expression using histochemical GUS assay according to the method of Jefferson et al. (1987). These tissues were incubated in GUS assay buffer at 37 °C for 12 h. Blue staining of the cells or tissues denoted positive reaction.

2.6. PCR analysis

Genomic DNA was extracted from fresh leaf tissues of in vitro-grown GUS-positive/-negative transgenic plants and untransformed control plants by the cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984). Equal amounts of 200 ng of total DNA were amplified in 50 μl reactions using specific primers for the *OC*I gene: 5′-ATG TCG AGC GAC GGA GGG-3′ and 5′-TTA GGC ATT TGC CGA GGC ATC-3′. These primers were expected to give products of 311 bp. PCR amplifications were performed with an initial denaturation at 95 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1% (w/v) agarose gel.

2.7. Assay for stem nematode resistance

The transgenic plants and the untransformed control plants were transplanted to pots with a mixture of soil and vermiculite (1:1) in a greenhouse for the domestication. These plants were then planted in a field infected by stem nematodes at a density of 300 nematodes per 100 g soil and their stem nematode resistance was evaluated for 2 years according to the method of S.Y. Lu et al. (1998)