Production of Herbicide-Resistant Sweetpotato (*Ipomoea batatas* (L.) Lam.) Plants by *Agrobacterium tumefaciens*-mediated Transformation

Motoyasu Otani*, Yoichi Wakita¹⁾ and Takiko Shimada

Research Institute of Agricultural Resources, Ishikawa Agricultural College, 1-308 Suematsu, Nonoichi-machi, Ishikawa 921-8836, Japan

¹⁾ Present address: Hokkaido Forestry Research Institute, Higashiyama, Bibai, Hokkaido 079-0198, Japan

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Sweetpotato (*Ipomoea batatas* (L.) Lam.) is a major crop that ranks seventh among food crops in annual production in the world (FAOSTAT database, May 2001). It would be important to further improve the quality of sweetpotato by applying genetic engineering technology, in addition to the use of traditional breeding methods. Recently, transgenic sweetpotato plants with genes introduced for insect and virus disease tolerance have been reported (Newell *et al.* 1995, Murata *et al.* 1997, Morán *et al.* 1998). We developed an efficient method for the production of transgenic sweetpotato plants through *Agrobacterium tumefaciens*-mediated transformation using embryogenic calli (Otani *et al.* 1998) and produced transgenic sweetpotato plants with genes for key enzymes of the metabolic pathway such as the granule-bound starch synthase gene (*GBSSI*) to modify the amylose and amylopectin contents of starch (Kimura *et al.* 2001), and the ω -3 fatty acid desaturase gene isolated from tobacco (*NtFAD3*) to modify the fatty acid composition of sweetpotato plants (Wakita *et al.* 2001).

Recently, transgenic crop plants expressing herbicide tolerance have been commercialized, because the use of non-selective herbicides such as glufosinate and glyphosate provides economically superior weed control. The *bar* gene encoding the enzyme, phosphinothricin acetyltransferase (PAT), that confers resistance to the commercial herbicides, glufosinate and bialaphos, was isolated from the bialaphos biosynthetic pathway of *Streptomyces hygroscopicus* (Murakami *et al.* 1986). Glufosinate-resistant transgenic crops, for example, *Brassica napus* (DeBlock *et al.* 1989), corn (Gordon-Kamm *et al.* 1990), and rice (Cao *et al.* 1992), have been produced. Weed control in sweetpotato fields after planting of cut sprouts is critical for high productivity. The application of genetic manipulation techniques to sweetpotato should enable the development of herbicide-resistance

that otherwise would be difficult to achieve by the implementation of conventional breeding programs. Transgenic herbicide-resistant sweetpotato may enable to control weeds in cultivation more conveniently and more economically.

We have introduced the *bar* gene into embryogenic calli via *Agrobacterium tumefaciens* and produced transgenic sweetpotato plants. In this paper, we describe the expression of the *bar* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter or the modified CaMV35S promoter, El2 Ω promoter (Mitsuhara *et al.* 1996) and subsequent tolerance of the transgenic sweetpotato plants to the herbicides.

Transformation

Agrobacterium tumefaciens strain EHA101 and plasmids pCAM35Sbar and pCAMEl2 Ω bar contained a binary vector harboring the gene for hygromycin resistance (*hpt*) used in the present study (Fig. 1). Plasmid pCAM35Sbar, a derivative of pCAMBIA1300 (Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, England), contained the *bar* cDNA fragment from pDM302 (Cao *et al.* 1992) driven by the CaMV 35S promoter. Plasmid pCAMEl2 Ω bar, a derivative of pCAM35Sbar, contained the *bar* cDNA fragment driven by the El2 Ω promoter (Mitsuhara *et al.* 1996). The *bar* gene from *Streptomyces hygroscopicus* encodes the enzyme, PAT and confers resistance to the herbicide, bialaphos.

Embryogenic calli were induced from shoot meristems of the sweetpotato cultivar Kokei 14, on 4F1 medium, i.e., LS medium (Linsmaier and Skoog 1965) containing 1 mg/l 4-fluorophenoxyacetic acid (4FA), 3% (w/v) sucrose and 0.32% (w/v) gellan gum (Otani and Shimada 1996). Transformation was performed according to the method of Otani *et al.* (1998).

Integration of foreign DNA into the genome of sweet potato plants

We obtained 110 independent hygromycin-resistant lines, 67 transgenic lines transformed with pCAM35Sbar-harboring CaMV35S promoter-*bar* and 43 transgenic lines with pCAMEl2 Ω bar-harboring El2 Ω promoter-*bar*. Randomly selected six lines (3 lines with CaMV35S promoter-*bar*, 35S-3, 35S-5, 35S-8, and three lines with El2 Ω promoter-*bar*,

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*Corresponding author (e-mail: otani@ishikawa-c.ac.jp)

Fig. 1. Schematic representation of the plasmids used in the experiment.

RB: right border, LB: left border, B: *Bam*HI site, S: *Sac*I site, 35S-P: CaMV35S promoter, Nos-T: Nos terminator, 35S-T: CaMV35S terminator, El2: 5'-upstream sequence of CaMV35S promoter (−419 to −90)×2, P35S: 5'-upstream sequence of CaMV35S promoter (−90 to −1), Ω: 5'-upstream sequence of TMV.

El2Ω-2, El2Ω-5, El2Ω-35) were investigated for the integration of the transgenes by Southern-blot analysis. The total DNAs were prepared from leaf tissues harvested from transgenic sweetpotato plants by using the MagExtractor[®]-Plant Genome- DNA extraction kit (TOYOBO Biochemicals, Osaka, Japan). Five micrograms of DNAs were digested with the restriction endonucleases, *Bam*HI and *Sac*I, and then fractionated by electrophoresis in a 0.8% (w/v) agarose gel. The fractionated DNA segments were transferred to nylon membranes (Biodyne[®] Plus, Pall Ultrafine Filtration, New York, USA), and hybridized with a PCR-labeled probe. Either the 615 bp product of the *hpt* gene or the 402 bp product of the *bar* gene after PCR amplification was used as a probe. Southern-blot analysis was performed as described in the protocol of AlkPhos Detect system (Amersham Pharmacia Biotech, New Jersey, USA) (Wakita *et al.* 2001). Fig. 2 shows the results of Southern-blot analysis of DNAs after digestion with *Bam*HI and *Sac*I from the untransformed control plants and hygromycin-resistant plants. All the hygromycin-resistant lines showed the hybridizing bands for the *bar* gene, i.e., 0.6 kb cDNA of *bar* (Fig. 2). As expected, no bands were observed in any of the non-transformed control plants. These results confirmed the presence of the *bar* gene in all the hygromycin-resistant plants.

Expression of foreign DNA in the transgenic plants

Six transgenic lines were examined for the expression of the transgene by Northern-blot analysis. The RNAs were prepared from leaf tissues using ISOGEN (Nippon Gene, Tokyo, Japan). Twenty micrograms of total RNAs were denatured and fractionated by electrophoresis in a denaturing formaldehyde-1.2% (w/v) agarose gel. RNAs were transferred to positively charged nylon membranes (Roche Diagnostics, Tokyo, Japan), and hybridized with a PCR-labeled probe. The major fragments were identified by Northern hybridization against probes of the *hpt* or *bar* genes using the DIG system (Roche Diagnostics, Tokyo, Japan) (Wakita *et al.* 2001). The band of *hpt* mRNA was observed in all the transgenic lines which harbored the *hpt* gene driven by the

Fig. 2. Southern-blot hybridization of DNAs after digestion with *Bam*HI and *Sac*I from the transgenic sweetpotato plants, using the 402 bp product of the *bar* gene after PCR amplification as probe.

P1: pCAM35Sbar as positive control, N: non-transformant as negative control, 1-3: transgenic lines with CaMV35S promoter-*bar* (35S-3, 5, 8), P2: pCAMEl2Ωbar as positive control, 4-6: transgenic lines with El2Ω promoter-*bar* (El2Ω-2, 5, 35).

CaMV35S promoter (data not shown). The expression of the introduced *bar* gene was also confirmed in all the transgenic lines (Fig. 3). However, the expression level of the *bar* gene under the control of the El2Ω promoter seemed to be lower than that under the control of the CaMV35S promoter based on the Northern-blot analysis. On the contrary, in our previous study related to Northern analysis in transgenic sweetpotato plants, the expression level of the *NtFAD3* gene driven by the El2Ω promoter was estimated to be about 3 times higher than that of the gene driven by the CaMV35S promoter (Wakita *et al.* 2001). For the *bar* gene expression in sweetpotato plants, the CaMV35S promoter may be more suitable than the improved promoter, El2Ω promoter.

Fig. 3. Northern-blot hybridization of mRNA from transgenic sweetpotato plants, using the 402 bp product of the *bar* gene after PCR amplification as probe. N: non-transformant as negative control, 1-3: transgenic lines with CaMV35S promoter-*bar* (35S-3, 5, 8), 4-6: transgenic lines with EI2Ω promoter-*bar* (EI2Ω-2, 5, 35).

Herbicide resistance in transgenic plants and their second vegetatively propagated progenies

Transgenic plants were transplanted to pots containing a mixture of vermiculite and perlite (3:1) and maintained at 26°C under a 16-photoperiod in a growth chamber for 14 days. The 20 transgenic plants, 10 lines with CaMV35S promoter-*bar* and 10 lines with EI2Ω promoter-*bar* were grown in a greenhouse. One month later, the surface of the fully expanded leaves of each transgenic plant and untrans-

formed control plant was wetted with a 1000 mg/l bialaphos solution. Four days later, the leaves of all the transgenic lines failed to show visible damage, although those of the untransformed control plants treated with bialaphos exhibited severe necrosis (Fig. 4). We did not observe any differences in herbicide resistance at the concentration of 1000 mg/l between the transgenic lines. Different levels of resistance to herbicides were observed in the transformants of *Brassica napus* (DeBlock 1989), alfalfa (D'Hallium *et al.* 1990) and *Picea abies* (Bishop-Hurley *et al.* 2001). In these plants, the expression of the *bar* gene analyzed based on the PAT activity was mostly in agreement with the scoring of resistance to the herbicide treatment, that is a low-level of *bar* gene expression reduced the tolerance to the herbicide. In our study, the results of Northern blotting failed to reveal any differences in the expression of the *bar* gene among the transformant lines with CaMV35S-*bar*, while the gene expression level of the transgenic lines with the EI2Ω promoter-*bar* varied and was lower than that of the transgenic lines with CaMV35S-*bar*. However, there were no differences in the bialaphos resistance between them. Since even plants expressing PAT at a level of 0.001% of total leaf protein could survive herbicide application (Hinchee *et al.* 1993), the transgenic lines with EI2Ω promoter-*bar* might display an adequate herbicide tolerance, although the PAT activity of the lines was not analyzed in this study.

These transgenic sweet potato plants grew normally and formed storage roots after 3 months in a greenhouse. No morphological differences were observed between the untransformed control plants and the transgenic plants. Leaves of freshly sprouting shoots from harvested storage roots of the transgenic plants also showed bialaphos resistance, suggesting that the *bar* gene was transmitted to their vegetatively

Fig. 4. Leaf test for the resistance of transgenic sweetpotato plants to bialaphos. C: untransformed control plant, 35S: transgenic lines with CaMV35S promoter-*bar*, EI2Ω: transgenic lines with EI2Ω promoter-*bar*.

propagated progenies through storage roots. Since sweet-potato is commonly propagated using storage roots, the use of genetically engineered sweetpotato may be suitable for the practical breeding of this plant species.

We have succeeded in developing genetically engineered herbicide-resistant sweetpotato plants. We will select a few transformants and evaluate the expression of herbicide resistance under experimental field conditions. Genetic engineering of herbicide tolerance in sweetpotato may enable to achieve a much simpler and more cost-effective weed control in cultivation.

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