Transformed progeny can be recovered from chimeric plants regenerated from *Agrobacterium tumefaciens* treated embryonic axes of sunflower

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Abstract

Stable transformants have been recovered from *Agrobacterium tumefaciens* treated split embryonic axes of sunflower. Sunflower seeds were imbibed in water for 1 hour and the embryonic axes were isolated by removal of the cotyledons and root radical. The explant was bisected through the meristem, wounded by microprojectile bombardment and incubated with *A. tumefaciens* strain EHA101 carrying a 35S driven neomycin phosphotransferase (NPTII) gene within the T-DNA. The explants were transferred to kanamycin selective medium and regenerating shoots expressing NPTII were excised and recovered to the greenhouse. Each Ro plant was mapped for the presence of NPTII activity in leaf tissue and in the corollas of the flower disc. Uniformly transformed progeny from Ro plants chimeric for NPTII activity have been recovered from apical and side branch heads. Transformation has been confirmed by NPTII enzymatic assay and southern hybridization analysis.

Introduction

The first published report describing the recovery of transformed shoots and progeny of sunflower was by Everett et al. (1). The system utilized hypocotyl callus regeneration protocols combined with transformation mediated by *Agrobacterium tumefaciens*. We investigated this transformation system but found it to be difficult to recover good quality transgenic shoots. We also considered other culture systems including immature embryo (3), hypocotyl protoplast (4) and cotyledon (5) cultures. We found that each system presented problems similar to those we encountered with the hypocotyl callus method.

One explant, the embryonic axis, appeared to have potential use in a direct transformation protocol for sunflower. Pre culture of the explant isolated from germinating seeds on cytokinin, auxin and gibberellic acid containing medium readily exposed the meristem and promoted meristematic development (6). Bombardment of meristems with tungsten microprojectiles (1.8 μm average diameter) coated with a construct containing the b-glucuronidase gene produced high levels of transient expression within the meristematic dome (6). The transient responses, however, did not extend into stable transformation events in plantlets recovered from the bombarded meristems. Further transformation experiments with meristem explants using *Agrobacterium tumefaciens* did not result in stable events. The combination of the 2 technologies, particle bombardment of the meristem to generate wounds followed by *Agrobacterium* to mediate transformation, resulted in significant stable transformation events (6). A modified split embryonic axis protocol combined with the particle wounding prior to *Agrobacterium* application has resulted in the transformation method we are currently utilizing. This paper gives a brief description of the procedures and results.

Materials and Methods

Mature sunflower seeds (*Helianthus annuus* L.) were dehulled and surface sterilized for 20 minutes in a 20% Chlorox bleach solution with a drop of Tween 20. The seeds were rinsed two times with sterile distilled water and then imbibed in sterile water for an additional hour. The cotyledons and root tip were removed and the explants were bisected longitudinally between the primary leaves (2). The two halves were placed exposed surface upward on a solid medium (M1) containing MS mineral elements, 3% sucrose, 0.5 mg/l BAP, 0.25 mg/l IAA and 0.1 mg/l GA. Explants were bombarded 2 times in a PDS 1000 particle acceleration device with 1.8 μm diameter tungsten particles. A 150 μm nytex screen was placed above the plate of material being bombarded.

Disarmed *Agrobacterium tumefaciens* strain EHA101 was used in conjunction with binary vector pPHI158 (6). The vector contained a Kan gene that confers resistance to kanamycin sulfate when expressed in plants. The *Agrobacterium* cells were grown overnight at 28°C in YEP medium plus antibiotics. Bombarded explants were incubated for 30 minutes with a mid-log phase of the bacterial

suspension in inoculation medium. The split embryonic axes were then transferred to M1 medium and cocultured for an additional three days at 25°C at which time the explants were then transferred to M2 medium (M1 without plant growth hormones) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants remained on selection for 2 weeks and were then transferred to fresh M2 medium lacking kanamycin sulfate.

Leaf samples from small, green shoots emerging from the explants were tested for NPTII activity (7). Those that were enzyme positive were recovered to the greenhouse for further testing. Once established, the plants were mapped for NPTII activity. Self pollinated seeds were harvested from each T_0 transgenic plant, and the T_1 plants were characterized for the presence of the transgene.

Results

1. No transformed progeny have been recovered from nonbombarded, split embryonic axes. The wounding induced by particle bombardment is a prerequisite for the generation of T_0 plants capable of producing transgenic seed.

2. Most transformants are chimeric for the expression of the selectable marker. Analysis of greenhouse grown T_0 plants for NPTII activity in leaves and floral structures has shown that sectors of transformation extend vertically up the plant and into the apical flower. Branches that develop from nodal meristems associated with transformed sections of the plant will also be transformed. The converse is also true, branches arising from untransformed regions of the plant will not exhibit NPTII activity. The numbers and locations of transgenic seed within main or side branch heads are not predictable. No useful inheritance information can be obtained from the characterized populations of seed recovered from the primary transformants.

3. Transformants conform to normal rules of genetic inheritance from the T_1 generation forward. The transformed progeny recovered from primary transformants are not chimeras.

4. Southern hybridization analysis has verified the presence of the Kan gene in T_1 progeny.

5. Transgenic progeny can be recovered approximately 5 months following the initiation of a transformation experiment.

6. Transformation frequencies (mapped T_0's that produced at least 1 transgenic seed) have ranged from 0.2% to 2% depending on the binary used.

7. Stable transgenics have been recovered for 7 different promoter/gene constructs from 9 different binarys, each relying on the NPTII selectable marker.

8. We are now conducting our second field evaluation of transgenic sunflower at the Pioneer Sunflower Breeding station, Woodland, California. The expression of the methionine rich storage protein gene from Brazil nut (8) and cotyledonary accumulation of the 2S gene product in sunflower embryos are being evaluated.

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