INDUCTION OF SOMATIC EMBRYOGENESIS AND GENETIC TRANSFORMATION OF OHIO BUCKEYE (AESCULUS GLABRA WILD.)

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SUMMARY

Mature embryo axes of the Ohio buckeye were germinated on a medium containing 1 mg gibberellic acid (GA) per l. Three wk following germination, stem, petiole, and leaf blade tissues were excised and placed on media containing either 1 mg (4.5 µM) 2,4-dichlorophenoxy acetic acid (2,4-D) or 1 mg (4.7 µM) kinetin per l. 1 mg of both 2,4-D (4.5 µM) and kinetin (4.7 µM) per l, or 2 mg of both 2,4-D (9.1 µM) and kinetin (9.3 µM) per l. Embryogenic tissue was formed only from stem segments after 2-3 mn. of culture on media containing both 2,4-D and kinetin. Embryogenic tissue could be either maintained on solid medium for proliferation of embryogenic callus or placed in liquid medium for proliferation of embryogenic suspension cultures. For transformation of suspension cultures, tissues were inoculated with Agrobacterium EHA105 containing the binary plasmid pVecOS, briefly sonicated, and cultured in the presence of 100 µg acetylsucrose for 2 d. To eliminate Agrobacterium, tissues were washed and placed in liquid proliferation medium containing either 300 mg Colchicine per l or 400 µg timentin® per l. Selection on 20 mg hygromycin per l was initiated 3 wk after inoculation, and after an additional 10 wk, hygromycin-resistant tissue was isolated and separately cultured. Although some hygromycin-resistant clones were recovered with no sonication treatment, four to five times more clones were obtained following sonication. Putative transformed clones were confirmed to be transgenic via both biochemical β-glucuronidase (GUS) assay and southern hybridization analyses. Development of transgenic embryos occurred on a growth-regulator-free medium containing 3% sucrose. After 2 mn. of embry development, the embryos were transferred to fresh medium for germination.

Key words: 2,4-D; Agrobacterium; hygromycin resistance; regeneration; SAAT; sonication.

INTRODUCTION

The Ohio buckeye (Aesculus glabra Wild) is an understory tree, native to riverbanks and low-lying wetlands in much of the midwest and south-central United States. The buckeye is used primarily as a landscape plant and is recognized for its spiny fruit and large nut. Genetic improvement of the Ohio buckeye as well as other woody ornamentals has been limited due to the long generation time of these plants. Improvement of this plant by genetic transformation could tremendously reduce the time necessary for introduction of useful traits.

Although transformation of ornamental tree has been reported (Sullivan and Lagrimini, 1999; Wilde and Yerkiche, 1994), transformation of Aesculus species has been previously described. However, somatic embryos have been obtained from leaf (Damer et al., 1986) and stem (Gaetano et al., 1999) tissues of seedlings as well as from shoots of 30-yr-old trees (Bourgain et al., 1996). Somatic embryos can be used for both micropropagation (Denich and Atamanis, 1995; Chen, 1996) and as a target tissue for gene transfer (Bournoumis et al., 1990; Gao et al., 1994; Cheng et al., 1996). Agrobacterium-mediated transformation of embryos and embryogenic cultures has received much attention recently due to the simplicity of direct embryo transformation (Meadows et al., 1988; Hiei et al., 1994; Ishida et al., 1996) and the low copy integration events that often result from the use of Agrobacterium (Tingland and Hohn, 1995). Agrobacterium was initially not used for transformation of embryos and embryogenic tissues because of perceived incompatibilities between embryogenic tissue and this biological vector. When the more virulent Agrobacterium strains (Hood et al., 1990; Totakuy et al., 1997) are used and the vir genes are induced (Stack et al., 1995; Komari, 1999; Hansen et al., 1994), Agrobacterium becomes much more effective for transformation of embryogenic tissues. In addition, more efficient delivery of Agrobacterium to the target cells with sonication-assisted Agrobacterium-mediated transformation (SAAT) (Trick and Finer, 1997) can result in tremendous enhancements of transformation rates. Using a combination of SAAT and a virulent Agrobacterium strain, we developed a method for the successful transformation of embryogenic tissue of buckeye.

MATERIALS AND METHODS

Initiation of embryogenic tissue. Mature wood of the Ohio buckeye (Aesculus glabra Wild.) were collected and stored at 4 °C. Embryo axes were excised, placed in water, surface-sterilized with 20% commercial bleach for 20 min, washed four times with sterile, distilled water, and finally plated on a medium containing MS salts (Murashige and Skoog, 1962), 85 vitamines

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nificant embryo development was observed following subsequent fresh induction media. It is interesting that both 2,4-D and kinetin were required for induction of embryogenic callus of Ohio buckeye. This is in contrast to most other embryogenic systems where 2,4-D alone is sufficient for induction of embryogenic tissue (Finer, 1990). Embryogenic callus has been previously obtained from Ohio buckeye following culture of tissue of 3- to 5-leaf-containing green trees in a medium containing 2,4-D and 6-benzylaminopurine (BA) as the sole growth regulator (Bongers et al., 1996). Embryogenic callus and suspension cultures have also been obtained from leaf explants (Bintz et al., 1996) and immature embryos (Rade- jevic, 1980) of homeostatic (Aesculus hippocastanum), with media containing both auxin and cytokinin. It appears that Aesculus may be unusual in requiring cytokinin for induction of embryogenesis but not for maintenance because cytokinin was not required for proliferation of embryogenic callus in FN liquid medium which contained only the auxin 2,4-D.

Transformation. Selection and isolation of embryogenic-resistant clones was somewhat difficult as both embryogenic-resistant and hy-}
Fig. 1  Formation and transformation of embryogenic tissue of Ohio buckeye. A. Formation of embryogenic callus from a single segment cultured on medium containing 2 mg of both 2,4-D (5.4 μM) and kinetin (95.3 μM) per l. B. Immunological GUS staining in primary, transformed buckeye callus. The constitutive transgenic marker is revealed by GUS stain. C. GUS expression analysis of transgenic buckeye callus. D. GUS positive somatic embryos of Ohio buckeye. E. Derivation of embryo from transgenic buckeye callus. F. Southern blot analysis of DNA from transgenic buckeye cultures. The substrate was hybridized with the GUS coding region. Reaction with 35S probe was the initial 35S/GUS expression unit (arrow) while 35S/GUS-embryo signals were observed in the left hand of the 1DNA.
TABLE 1

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<thead>
<tr>
<th>Suspension duration (days)</th>
<th>Cell viability (%)</th>
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<tbody>
<tr>
<td>5</td>
<td>7.5 ± 5.4</td>
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<tr>
<td>15</td>
<td>10 ± 8.1</td>
</tr>
<tr>
<td>30</td>
<td>16.7 ± 12.4</td>
</tr>
<tr>
<td>60</td>
<td>43.5 ± 8.6</td>
</tr>
<tr>
<td>90</td>
<td>38.3 ± 6.8</td>
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Values represent means of three replicate experiments from each treatment (n = 10).
Ten drops of tissue were used for each replicate.

larger than 23 kb, cDNA-ligated plant DNA hybridized with the GUS coding region gave the expected signal at 3.0 kb (Fig. 1 F, arrow), which is the size of the intact GUS expression unit. The number of bands obtained from cDNA-ligated DNA (cDNA cuts the T-DNA close to the left border) indicates the number of insertion events (Fig. 1 F). Results from this digestion also verify integration of T-DNA as the various-sized bands represent T-DNA plus plant border fragments. Clone 750 apparently contained two insertion events whereas clones 380 and 600 had a single insertion. Southern hybridization analyses of additional clones probed with the GUS coding region also confirmed a low number of integration events. Although most clones exhibited one to two integration events, one clone was obtained that contained four copies of the T-DNA (data not shown). This low number of integration events is characteristic for Agrobacterium-transformed tissues (Tianland and Hohn, 1995).

We report here the successful transformation of 0h buckeye (Aesculus glabra) with somatic-assembly Agrobacterium-mediated transformation. This work verifies that SAI is an effective method for increasing transient and stable transformation of different plants with various in vitro culture requirements. We believe that this is the first report of transformation of any species in this genus of important landscape trees.

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REFERENCES