Proliferative Somatic Embryogenesis from Zygotic Embryos of Grapevine

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Additional index words. Vitis vinifera, Vitis longii, plant tissue culture

Abstract. Zygotic embryos isolated from mature berries of Vitis longii Prince and V. vinifera L. cvs. Chardonnay, French Colombard, Grenache, and White Riesling produced somatic embryos when cultured on solid Nitsch and Nitsch medium. Explants of V. vinifera cv. Pinot noir did not produce somatic embryos. The highest incidence of somatic embryogenesis occurred on medium containing (all in mg-liter⁻¹) 1.0 NOA plus 0.2 BAP, except in ‘White Riesling’, where 1.0 NOA plus 1.0 BAP was optimal. Somatic embryos developed asynchronously from a mass of white proembryos and passed through globular, heart, and torpedo-shaped stages. Embryos proliferated by budding at the surface of globular embryos. Explants washed in sterile distilled water eventually failed to germinate by 10 days after the last subculture. Explants cultured on medium containing 1.0 NOA plus 0.2 BAP produced seedlings at a frequency 5-10 times that obtained with basal medium and, in some cases, severely reduced germination. Plants were established in the greenhouse and no morphological abnormalities were observed. Chemical names used: 6-benzylaminopurine (BAP); gibberellic acid (GA₃); 2-(1-naphthyl)acetic acid (NAA); 2-naphthoxyacetic acid (NOA).

Grapevine (Vitis vinifera L.) is an ancient crop in which important cultivars have been maintained for centuries by clonal propagation. The development of new cultivars by conventional breeding methods has not only been limited by the large size and long generation time of grapevines, but also by the genetic heterozygosity of the species, such that the complex, and often ethereal, nature of valuable cultivars is unavoidably disrupted through sexual crosses. Cellular and molecular strategies for genetic modification warrant consideration because they offer possibilities for directed and specific genetic changes that may not otherwise disturb the genotype of an established cultivar. Successful application of cellular and molecular approaches requires suitable regeneration systems that allow novel genotypes identified or engineered at the cellular level to be preserved at the whole-plant level. Such systems should be efficient and should be applicable to a wide range of economically important cultivars.

In vitro plant regeneration in grapevine can occur via both adventive caulogenesis and somatic embryogenesis. Adventitious shoots have been observed in cultures derived from anthers (8), fragmented shoot apices (1, 2), internode segments (18), and leaves (4). Somatic embryogenesis has been reported from anther (3, 5, 11, 17, 19), leaf (7), ovary (5), ovule (12, 21), and stem (9) cultures. Success has been achieved with several economically important V. vinifera cultivars and interspecific hybrids, but, of the wide range of procedures used, none was applicable to more than a few genotypes. We recently reported culture procedures that promoted somatic embryogenesis from both anther and leaf cultures of several economically important cultivars (23), but optimal regeneration conditions varied with cultivar, explant type, and explant source. In an effort to further our understanding of in vitro regeneration in grapevine and to develop a culture procedure suitable for many cultivars, we have investigated the in vitro response of zygotic embryos. Because of the heterozygous nature of grapevines, zygotic embryos differ genotypically from the parental cultivar from which they were obtained and are thus unsuitable for direct use in cellular and molecular genetic improvement programs that aim to preserve cultivar integrity. However, since zygotic embryos have proven to be morphogenically highly competent in many species, they may serve as a useful model for regeneration studies with clonal tissues.

This constitutes the first report of somatic embryogenesis from zygotic embryos of V. longii Prince, a wild species, and four economically important cultivars of V. vinifera, thus adding to the number of grapevine tissue types known to be morphogenically competent. Furthermore, we describe procedures for germination of both somatic and zygotic embryos and for proliferation of somatic embryos and maintenance of the morphogenically competent state.

Materials and Methods

Plant material and sterilization. Mature berries of five V. vinifera cultivars (Chardonnay, French Colombard, Grenache, Pinot noir, and White Riesling) and V. longii were collected from the Univ. of California, Davis, vineyard and stored in the dark at 5°C in the presence of SO₂-generating pads. Seeds were removed from the berries within 3 to 4 months of cold storage, cleaned, sterilized for 5 min in 45% (v/v) commercial bleach (2.4% w/v NaOCl) containing Tween 20 (a wetting agent), and washed in sterile distilled water until all visible traces of detriment were removed. Seeds were soaked in sterile distilled water for 72 hr at room temperature and then re-sterilized with 45% bleach before entire zygotic embryos were excised with the aid of a stereomicroscope. Zygotic embryos were white, 0.5 to 1.5 mm long, and consisted of a root axis, hypocotyl, and two cotyledons up to 0.5 mm long.

Culture medium and incubation conditions. Explants were cultured on medium containing the salts and vitamins of Nitsch and Nitsch (14), but with 5.57 g FeSO₄·7H₂O/liter (15) (hereafter designated NN medium), and further supplemented with (2,4 dichlorophenoxy)acetic acid (2,4-D), NOA, or BAP, either singly or in combination. The medium was supplemented with 20 g sucrose/liter, solidified with 0.7 g Difco Bacto-agar/liter, adjusted to pH 5.7 before being autoclaved at 103 kPa for 24 min, and dispensed at 20 ml per 9-cm plastic petri dish.

Zygotic embryos were placed on the surface of the medium and

Received for publication 1 Mar. 1988. We gratefully acknowledge support from the Winegrowers of California. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

incubated in the dark at 25° ± 1°C for 28 days, after which they were transferred to light (16-hr photoperiod, 40 μmol·s⁻¹·m⁻²) at the same temperature. Results were assessed after 10 weeks of incubation. Standard errors are given for those treatments repeated in at least two experiments.

**Plant development.** Individual 2- to 4-mm-long somatic embryos isolated from embryogenic cultures of ‘Grenache’ were transferred to solid NN medium or revised Murashige and Skoog medium (13) with salts at half-strength and supplemented with 20 g sucrose/liter (hereafter designated ½MS). Media were supplemented with BAP, GA3, or NAA, either singly or in combination. Somatic embryos were incubated in the light at 25°C and, to 3 or 4 weeks after shoot and root initiation, the plants were transferred individually to 4.5 × 10 cm glass jars containing 40 ml solid ½MS medium with 1.0 1H-indole-3-acetic acid (IAA)/liter. (Both GA₃ and IAA were filter-sterilized and added after autoclaving.) Within 8 weeks plants were transplanted to sterile soil in 10-cm-diameter pots. Plants were initially covered with plastic bags that were loosened during hardening-off. Two weeks after transfer to soil, plants were moved to the greenhouse at 24° to 28° and with natural light.

**Results**

**Somatic embryogenesis.** Zygotic embryos cultured on growth regulator-free medium, medium supplemented with BAP alone or with 0.1 mg NOA/liter plus 0.1 mg BAP/liter did not produce somatic embryos (results not shown). Instead, explants became swollen after 5 days and underwent hypocotyl and cotyledon expansion and greening, followed by shoot and root axis extension, which is characteristic of zygotic embryogenesis. In one experiment, after 21 days, 17 of 20 embryos of ‘Grenache’ on medium containing 0.1 mg NOA/liter plus 0.1 mg BAP/liter and 10 of 10 on basal NN medium developed into plants with healthy roots and leafy shoots up to 30 mm long. These results were typical for all cultivars on these media.

Zygotic embryos cultured on those media that did result in somatic embryogenesis (Table 1) also underwent expansion within 5 days. The embryos continued to swell, and cream-yellow, unorganized friable callus often developed from the explant. Sometimes, after 20 to 30 days of culture, small areas of very compact, white tissue developed exclusively from the zygotic embryo cotyledon and shoot apex region. This tissue did not look like callus, but was instead a mass of tiny individual organized units that were easily teased apart. Within 10 days after the appearance of this tissue, globular somatic embryos became evident throughout the white tissue (Fig. 1). The globular embryos either continued to develop, passing through heart and torpedo stages, or underwent budding at their surface to give rise to additional globular embryos. Somatic embryos developed asynchronously, and established cultures usually consisted of cream-yellow friable callus and white embryogenic tissue that was composed of hundreds of somatic embryos at various developmental stages. After 3 to 4 weeks, adventitious roots commonly developed from the original zygotic embry explant tissue. After 5 weeks, somatic embryos remained white and often possessed cotyledons 2 mm in diameter and had well-defined root and shoot axes and hypocotyls.

The results of many experiments in which zygotic embryos of five *Vitis* genotypes were cultured for 10 weeks on various media are summarized in Table 1. Explants of ‘Pinot noir’ did not produce somatic embryos and results are not shown. Not all medium-genotype combinations were tested, but, in all instances, growth regulators were required for somatic embryogenesis, either singly or in combination. The embryogenic response was influenced by both growth regulators and genotype. Although some genotypes were cultured on a wider range of media, the optimal embryogenic response in all, except ‘White Riesling’, occurred with 1.0 mg NOA/liter plus 0.2 mg BAP/liter. Somatic embryogenesis most often occurred on media containing both cytokinin and auxin, although 8% of ‘White Riesling’ explants initiated somatic embryos on NN medium with 1.0 mg NOA/liter alone. NOA/BAP combinations were generally superior to 2,4-D/BAP combinations. Explants that did not initiate somatic embryos on these media commonly developed friable callus and, sometimes, adventitious roots.

When pieces of tissue 2 to 5 mm in diameter consisting of proembryos and globular somatic embryos were subcultured on the same media that promoted the de novo somatic embryogenesis from zygotic embryo explants, further budding occurred, resulting in proliferation and doubling of the inoculum size in 4 to 5 weeks (Fig. 2). With regular transfers at 4- to 6-week intervals, embryogenic tissues proliferated and remained healthy for up to 1 year.

**Plant development.** Somatic embryo greening and root and shoot extension rarely occurred in the parental zygotic embryo.

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**Table 1. Media promoting somatic embryogenesis from zygotic embryos of grapevine.**

<table>
<thead>
<tr>
<th>Growth regulators (mg-liter⁻¹)</th>
<th>Chardonnay</th>
<th>French Colombard</th>
<th>Grenache</th>
<th>White Riesling</th>
<th>Vitis longiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D NoA BAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 0 0</td>
<td>0 (45)⁺</td>
<td>0 (30)</td>
<td>0 (56)</td>
<td>0 (12)</td>
<td>0 (50)</td>
</tr>
<tr>
<td>0 0 1.0</td>
<td></td>
<td></td>
<td>0 (50)</td>
<td>0 (12)</td>
<td>0 (49)</td>
</tr>
<tr>
<td>0 1.0 0</td>
<td></td>
<td></td>
<td>8 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 2.0 0</td>
<td></td>
<td></td>
<td>0 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 4.0 0</td>
<td></td>
<td></td>
<td>0 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 1.0 0.2</td>
<td>16 ± 12 (43)</td>
<td>13 ± 7 (24)</td>
<td>32 ± 12 (57)</td>
<td>0 (12)</td>
<td>14 ± 1 (128)</td>
</tr>
<tr>
<td>0 1.0 0.4</td>
<td>0 (25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 1.0 1.0</td>
<td>0 (36)</td>
<td></td>
<td>32 ± 16 (34)</td>
<td>8 (12)</td>
<td>0 (24)</td>
</tr>
<tr>
<td>0 1.5 0.2</td>
<td>5 ± 5 (22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 1.5 0.4</td>
<td>9 ± 7 (22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 0 0.2</td>
<td>0 (36)</td>
<td></td>
<td>3 ± 3 (34)</td>
<td>0 (12)</td>
<td>8 ± 8 (24)</td>
</tr>
<tr>
<td>1.0 0 1.0</td>
<td>0 (36)</td>
<td></td>
<td>3 ± 3 (29)</td>
<td>0 (12)</td>
<td>0 (24)</td>
</tr>
</tbody>
</table>

⁺Number of zygotic embryos cultured.
*Not tested.*
Fig. 1. Zygotic embryo explant of *Vitis vinifera* cv. Grenache after 6 weeks of incubation. White embryogenic tissue is evident in the lower right area of the explant, while non-embryogenic friable cream-yellow callus has developed over other areas.

Fig. 2. Proliferating embryogenic tissue of ‘Grenache’ consisting of somatic embryos at all stages.

cultures. To encourage plant development, somatic embryos from both parental cultures and those maintained in a state of embryogenic proliferation were transferred onto several solid media. Plants developed from somatic embryos of all genotypes and both sources, although the incidence of germination varied, was dependent on the culture medium and did not exceed 40%. Further studies were undertaken only with somatic embryos of ‘Grenache’ (Table 2). Results were assessed after 10 weeks, although somatic embryo germination was asynchronous and occurred anywhere from 2 to 15 weeks after transfer.

Freshly transferred somatic embryos expanded and became green within 5 to 10 days. Soon thereafter, adventitious root initiation and root axis extension were observed, followed by further expansion and swelling of the somatic embryo. On growth regulator-free medium, the shoot axis usually developed without extensive friable callus formation or cotyledon swelling and the resulting plants (Fig. 3) resembled germinating zygotic embryos. Two to 3 weeks after the initiation of shoot extension, plants were up to 30 mm long, with several nodes, healthy leaves, and extensive root systems. On media containing GA₃, the cotyledons of somatic embryos often became misshapen prior to shoot growth, while embryos cultured on media with NAA and/or BAP frequently initiated friable callus. Such development was common from somatic embryos on most media containing growth regulators, but did not apparently affect germination.

The results shown in Table 2 indicate that in NN medium GA₃ promoted somatic embryo germination at 1.0 mg-liter⁻¹, but inhibited the process at lower concentrations or when in combination with BAP. MS-based media containing NAA alone, or in combination with BAP, also supported plant development, but at a frequency no greater than that observed on growth regulator-free NN medium.

Further root and shoot development occurred after the plants were transferred to glass jars. After being transferred to greenhouse conditions, plants grew quickly and appeared morphologically similar to clonal material of the same cultivar. No abnormal plants were observed.

**Discussion**

Zygotic embryos isolated from mature berries of *V. longii* and *V. vinifera* cvs. Chardonnay, French Colombard, Grenache, and White Riesling produced somatic embryos when incubated on growth regulator-supplemented media (Table 1). Under the test conditions, optimal somatic embryogenesis from all cultivars except ‘White Riesling’ occurred on medium with 1.0 mg

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**Table 2. Media promoting plant development from somatic embryos of grapevine.**

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Growth regulator (mg-liter⁻¹)</th>
<th>No. embryos cultured</th>
<th>Embryos forming plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal GA₃</td>
<td>NAA</td>
<td>BAP</td>
<td></td>
</tr>
<tr>
<td>NN 0</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>NN 0.5</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>NN 1.0</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>NN 0.5</td>
<td>0</td>
<td>0.5</td>
<td>18</td>
</tr>
<tr>
<td>NN 1.0</td>
<td>0</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>½MS 0</td>
<td>0.01</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>½MS 0.1</td>
<td>0.01</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>½MS 0.01</td>
<td>0.01</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>½MS 0.1</td>
<td>0.01</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

NOA/liter plus 0.2 mg BAP/liter. This medium is similar to those used successfully in other studies with grapevine for the induction of somatic embryogenesis from anthers (3, 17), ovules (21), and leaves (7) and is identical to that found to be optimal for embryogenesis from young leaves and anthers in our own earlier work (23).

We have observed no evidence of any intervening callus in this system. At all times, the white embryogenic tissue we saw was composed entirely of distinct individual proembryos and globular embryos; there was no unorganized tissue. The friable cream–yellow callus that does form from other areas of explant does not give rise to somatic embryos. While we have not directly observed the initial development of isolated single somatic embryos on the surface of the explant, we presume that they do arise directly from explant tissues but do not become evident until after further proliferation has produced a larger mass of proembryos and globular embryos. This direct somatic embryogenesis, with subsequent proliferation, that we observed from zygotic embryo explants is identical to that which we saw with leaf and anther explants (23).

In the present study, embryogenesis occurred on several media, and in some cultivars more than one medium produced an optimal response. That somatic embryogenesis can occur on a range of media is an indication of the level of morphogenic competence of zygotic embryo explants. Morphogenic competence has often been associated with immature tissues and it is commonly believed that as plants become more differentiated they lose “regenerative capacity” (20). The morphogenic competence of zygotic embryos has been noted in other species, including cassava (Manihot esculenta Crantz) (22), guineagrass (Panicum maximum Jacq.) (10), cacao (Theobroma cacao L.) (16), maize (Zea mays L.) (6), and walnut (Juglans spp.) (24). Grapevine zygotic embryos from immature berries are also morphogenically competent (unpublished results) and embryos taken from berries or seeds stored at low humidity at 5°C for up to 1 year will also produce embryogenic tissue and somatic embryos.

Embryogenic cultures were maintained in a state of proliferation by regular subculture to fresh medium. Evidence of the morphogenic competence of these tissues is provided by the germination of somatic embryos isolated from them. Embryogenic tissue proliferation has been observed previously in grapevine cultures derived from anthers (5, 11, 17, 23), leaves (23), ovaries (5), and ovules (12).

Plant development from somatic embryos of ‘Grenache’ occurred on basal NN medium as well as NN or ½MS medium supplemented with particular growth regulator combinations (Table 2). None of the growth regulator treatments resulted in any substantial increase in plant development over basal NN medium. Three of the four treatments that included GA_3 resulted in a considerable reduction in germination, but the fourth (NN medium with 1.0 mg GA_3/liter) was comparable to NN basal medium. All of the other growth regulator combinations produced results similar to NN basal medium. Gibberellic acid has been reported to stimulate germination from somatic embryos of ‘Cabernet Sauvignon’ (12) and Glorywine (17), and (in combination with BAP) ‘White Riesling’ (23). Gray and Mortensen (5) reported 50% germination in somatic embryos of V. longii derived from anther and ovary cultures when they were incubated on medium supplemented with BAP alone.

Under certain conditions, zygotic embryo explants germinated and produced plants. During the course of this study, zygotic embryos were removed at different times from berries and seeds stored for up to 1 year at 5°C, but subsequent germination was not apparently affected. Shoot axis extension usually occurred within 10 days of culture and, clearly, any dormancy was overcome either before or during the isolation and culture process.

This study represents, to the best of our knowledge, the first report of somatic embryogenesis and plant development from zygotic embryos of grapevine. The procedure used was applicable to five of the six genotypes tested. That the conditions that promote somatic embryogenesis from zygotic embryos are very similar to those found to be best for anthers and leaves (23) is evidence for a common developmental process occurring in all three tissue types. Therefore, although zygotic embryos do not share the genetic identity of the parental cultivar, results obtained in studies of somatic embryogenesis from zygotic embryo explants should be relevant to other explant types and may thus contribute to the improvement of regeneration efficiency from clonal explants such as leaves.

Literature Cited

Genetic Transformation and Foreign Gene Expression in Walnut Tissue

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Abstract. A system was developed to transform walnut cultivars using the natural gene transfer system of Agrobacterium tumefaciens. We report the infection of English walnut (Juglans regia L.), northern California black walnut (Juglans hindsii), and their F₁ hybrid ‘Paradox’ with A. tumefaciens carrying various recombinant derivatives of the tumor-inducing (Ti) plasmids, pTiA6 and pTiB6S3. The three walnut species, each represented by a single micropropagated clone, were found to be equally susceptible to Agrobacterium-induced tumor formation in vitro. Stable lines were established from tumors induced on each clone, and, unlike normal stem callus, these tumor cells grew rapidly in culture media without exogenous plant hormones. High-voltage paper electrophoretic analysis revealed the presence of opines in the walnut tumor tissue. The presence of a foreign gene was demonstrated by expression of a chimeric bacterial gene that encodes resistance to the antibiotic kanamycin, and also by the presence of foreign DNA sequences in genomic DNA isolated from tumors.

The development of a method to introduce cloned genes into plants could provide a valuable tool for understanding and controlling plant gene expression and could greatly accelerate plant improvement efforts. The Ti plasmid of A. tumefaciens has long been proposed as a vector for genetic engineering of plants for several reasons: 1) it has a wide host range, infecting most dicotyledonous angiosperms and many gymnosperms (6); 2) it naturally transfers DNA into plant genomes, causing the common disease “crown gall” (1, 3, 20); 3) the genes that encode tumor-forming products can be removed from the Ti plasmid of Agrobacterium and replaced with virtually any gene, thus allowing their transfer to infectible plant tissue; 4) inoculation with such “disarmed” strains allows regeneration of morphologically normal plants that contain the novel gene (11, 12); and 5) DNA transferred by Agrobacterium is stably incorporated into genomes of plants (5, 9, 12) and seems to be inherited like any other gene in the germplasm (5, 11).

We report here the first successful introduction and expression of a foreign gene in walnut tissue. Recombinant derivatives of tumor-forming Agrobacterium containing an antibiotic resistance gene were used to infect three different walnut types. Tumor tissue obtained as a result of infection was analyzed for foreign gene expression and for the presence of novel genetic material.

Materials and Methods

Agrobacterium strains. The genotypes of the Agrobacterium strains used in our experiments are shown in Table 1. The nopaline-producing pTiB6S3 derivative, pMON200, was provided by S. Rogers (Monsanto, St. Louis). The octopine-producing derivatives of pTiA6, K12x562E, K12x167, and K12 were provided by V. Knauf (Calgene Inc., Davis, Calif).

Culture media and growth of bacteria. Agrobacterium cultures were grown at 26° to 28°C in AELB [10 g of Bacto tryptone (Difco), 5 g of yeast extract (Difco), or AB minimal media (2). Kanamycin, 100 μg·ml⁻¹, was supplemented for strains that carry the gene that encodes resistance to the antibiotic kanamycin (kan). Bacterial stocks were maintained frozen at –70°C in AELB containing 7% Me₂SO (dimethyl sulfoxide, DMSO), Plant material. The English and ‘Paradox’ walnut shoots used...