In Vitro Fertilization with Isolated, Single Gametes Results in Zygotic Embryogenesis and Fertile Maize Plants

Erhard Kranz and Horst Lörz
Institut für Allgemeine Botanik, Angewandte Molekularbiologie der Pflanzen II, Universität Hamburg, Ohnhorststrasse 18, 22609 Hamburg, Germany

We demonstrate here the possibility of regenerating phenotypically normal, fertile maize plants via in vitro fertilization of isolated, single sperm and egg cells mediated by electrofusion. The technique leads to the highly efficient formation of polar zygotes, globular structures, proembryos, and transition-phase embryos and to the formation of plants from individually cultured fusion products. Regeneration of plants occurs via embryogenesis and occasionally by polyembryony and organogenesis. Flowering plants can be obtained within 100 days of gamete fusion. Regenerated plants were studied by karyological and morphological analyses, and the segregation of kernel color was determined. The hybrid nature of the plants was confirmed.

INTRODUCTION

Sexual crossing between plant varieties with desirable characteristics has been used for generations to produce new cultivars of agricultural importance. Additional techniques such as cell and tissue culture have been developed to overcome many cases of incompatibility and thus to extend the gene pool. Kanta et al. (1962) were able for the first time to accomplish the artificial fertilization of opium poppy by the pollination of excised ovules with mature pollen. This was subsequently followed by explant culture and the in vitro development of mature seeds. Embryo rescue (Stewart, 1981) and in vitro pollination/fertilization, in which flower explants, ovaries, ovules, and mature pollen were used, have led to numerous new hybrids being produced, often between only remotely related species (for review, see Zenkteler, 1990). In vitro fertilization with isolated gametes is a logical extension of this work (Kranz et al., 1990, 1991a).

Gametes are predetermined to form zygotes and consequently to develop into embryos. These progenitor cells are therefore the most useful source for studies in embryology. Previously, however, plant egg cells and nongametic cells of the embryo sacs were not used for protoplast isolation and regeneration experiments. In higher plants, the female gametes are normally deeply enclosed in surrounding tissue and are therefore difficult to manipulate. Recently, isolation procedures for pollen protoplasts, generative cells, and male and female gametes of a wide range of species have been successfully established; potentials of these procedures were reviewed recently by Theunis et al. (1991), Dumas and Russell (1992), Huang and Russell (1992), Kranz et al. (1992), and Yang and Zhou (1992).

In vitro culture and plant regeneration from many different species and explant types of somatic and androgenic origin are now routine. Although difficulties such as cultivar specificity still remain, in the past years considerable advances have also been made with protoplasts of members of the Poaceae, and it is now possible to regenerate fertile plants from mass cultures of protoplasts of rice (Abdullah et al., 1986; Toriyama et al., 1986), barley (Jähne et al., 1991), and maize (Prioli and Söndahl, 1989; Shillito et al., 1989; Mórocz et al., 1990), for example.

Here we describe the regeneration, via zygotic embryogenesis, of single maize zygotes produced from the in vitro fusion of isolated, single gametes. The regenerated plants were studied by karyological and morphological analyses, and the inheritance of kernel color was followed. We also confirmed the hybrid nature of regenerated plants. The relevance of this work to future developments in fundamental research, genetic manipulation, and breeding are discussed.

RESULTS

Early Events of Zygote and Embryo Formation

When an alternating current field and electrical pulses were applied to egg cell protoplasts in the absence of any sperm cells, these cells never divided. Similarly, attempts to fuse sperm and egg cell protoplasts in the absence of electrical pulses failed, even when the protoplasts were held in close contact by dielectrophoretic alignment. However, after electrofusion of an egg cell protoplast and a sperm cell protoplast that were aligned as shown in Figure 1A, it was observed by following the starch grains that there was an unequal distribution of cytoplasmic components of the cell. In most of the zygotes, this was followed by an unequal first cell division. The
zygotes were bipolar, consisting of a relatively small, plasma-rich cell and a larger, vacuolized cell (Figure 1B).

In some of the experiments, the fusion products began to divide within 40 hr after gamete fusion, although division generally did not occur until ~60 hr after fusion. Compared to the size of the in vitro-produced zygote (mean diameter 65 μm, day 1 in culture), the cells of the multicellular structures developing from the fusion zygotes became much smaller (mean diameter 12.5 μm, 6 days in culture). The mean frequency at which a first cell division and microcallus development took place was 85%, based on ~2000 cultured fusion products.

During the development of multicellular structures, the planes of cell division appeared to be irregular, as demonstrated in Figure 1C. Within 10 to 12 days after gamete fusion, a bilateral symmetry was observed again. The globular structures developed into oblong structures and subsequently into transition-phase embryos at a frequency of 43%, as shown in Figure 1E. The meristematic part further enlarged, as is characteristic for the coleoptilar stage. The stages of embryo development are based on the classification schemes of Abbe and Stein (1954) and Sheridan and Clark (1993). An area of these polarized structures consisted of small cytoplasmic-dense cells, while at the other end, larger, highly vacuolized cells were present. These larger cells subsequently formed an elongated part, the suspensor (Figure 1E). A distinct outer cell layer, protoderm-like, of the meristematic region was often observed (Figure 1D).

Most of the 2000 fusion products developed only to microcalli, up to a size of 180 μm. However, sustained growth of the multicellular structures was achieved reproducibly in subsequent experiments using actively growing feeder cells that were cytoplastically rich and lacked large vacuoles. Using this improved culture system, 13 independent experiments were conducted, in which a total of 170 fusion products were created. We observed 157 first cell divisions and obtained 154 microcalli up to a size of 90 μm. From these, 114 microcalli grew to a size of 180 μm. These yielded 39 large globular structures and proembryos (average diameter 0.4 to 0.7 mm) and 30 transition-phase embryos with an enlarged scutellum and an average size of 0.4 x 0.5 mm. Thus, of the 170 fusion products, a total of 41% were capable of further growth. These were transferred onto solid media for plant regeneration experiments (Figures 1D and 1E).

Plant Regeneration and Fertility

When transferred onto solid media (approximately 10 to 12 days after gamete fusion), proembryos, transition-phase embryos, and those with an enlarged scutellum enlarged further and developed structures from which white compact callus arose. Subsequently, coleoptiles developed from these, as occasionally did some leaflike structures, as shown in Figure 1F. Plant regeneration was observed on media containing no growth hormones as well as on media containing 0.1 or 0.5 mg/L 2,4-D or a combination of 1.0 mg/L naphthalene acetic acid and 1.0 mg/L benzyladenine during the first passage onto solid regeneration medium. In some experiments, polyembryony occurred, with more than one plant developing from a cultured proembryo. The bipolar embryoids developed quickly into plantlets so that within 5 to 6 weeks of gamete fusion, well-developed green plants were produced that were capable of growth in soil after another 10 days (Figures 1G and 1H). In two cases, rooting of shoots was delayed; however, rooting could be promoted by the application of 5.0 mg/L naphthalene acetic acid, 2.0 mg/L benzyladenine, and 0.1% activated charcoal.

Although several different regeneration media and subculture times could be used to regenerate fertile plants from in vitro-produced proembryos and transition-phase embryos, the following example illustrates one efficient procedure for regeneration of a fast-growing fertile plant. A single transition-phase embryo measuring 0.38 x 0.45 mm was transferred 15 days after gamete fusion into a 3.5-cm-diameter plastic dish filled with 1.5 mL of solid MS medium (Murashige and Skoog, 1962) without hormones and 60 g/L sucrose for a first passage of 13 days. A 7.0-mm structure of white compact callus with a coleoptile and roots was formed. The callus was subcultured for another 7 days on 1.5 mL of the same medium but with 40 g/L sucrose. At the end of this period, a plantlet with green leaves had developed. The largest leaf was 4.0 cm long. The plantlet was transferred into a glass jar containing 50 mL of MS medium (macro- and microsalts half concentrated) solidified with 4 g/L agarose and 10 g/L sucrose for another 9 days. This subculture resulted in a plant with leaf lengths of 15 and 20 cm and roots (up to 15 cm long). This plant was capable of growth in soil in the greenhouse and flowered 99 days after gamete fusion (Figure 1I). Similar results were obtained if the first passage was on solid media with 2,4-D alone or a combination of naphthalene acetic acid and benzyladinoine. Variations in the stepwise reduction of the sucrose concentrations also had no observable influence on plant regeneration.

From 28 fusion products representing four independent experiments, 11 plants were regenerated, a frequency of 48%, as shown in Table 1. The regenerated plants were pollinated 99 to 171 days after gamete fusion, as shown in Figure 1J. All regenerated plants were fertile and set seed. The cobs with mature seeds were harvested 148 to 215 days after fusion, as shown in Figure 1K and Table 1. The seeds were normal, with well-developed embryos and endosperm. They germinated and developed into phenotypically normal F2 plants.

Karyological and Morphological Analysis of the Regenerated Plants

Karyological analysis of root tip cells of all regenerated plants showed that all contained the diploid set of 2n = 20 chromosomes, as illustrated in Figure 2A. Morphological analysis
Figure 1. Development of Zygotes after In Vitro Fusion of Isolated, Single Egg and Sperm Cells of Maize. 

(A) Alignment of an egg cell protoplast with a sperm cell protoplast on one of the electrodes before electrofusion. E designates the egg cell protoplast; the arrow indicates the sperm cell protoplast (S). Bar = 50 μm.

(B) First polar cell divisions 42 hr after fusion. Bar = 50 μm.

(C) Multicellular structures 5 days after fusion. Bar = 100 μm.

(D) Polarized multicellular structure with an outer cell layer at one pole and vacuolized cells at the other end 12 days after fusion. Bar = 200 μm.

(E) Transition-phase embryo 14 days after fusion. Bar = 200 μm.

(F) Structure with compact white and green tissue. The arrow indicates the coleoptile (Co) 30 days after fusion. Bar = 4 mm.

(G) Plantlet 35 days after fusion. Bar = 2 cm.

(H) Plant 39 days after fusion. Bar = 6 cm.

(I) Flowering plant 99 days after fusion. Bar = 50 cm.

(J) Self-pollination. One hour after pollen grain deposition, the pollen tube penetrated the red-colored trichome and the grain contents are moving into the style. Bar = 100 μm.

(K) Cob 148 days after fusion. Bar = 4 cm.
Table 1. Summary of Four Independent Experiments for the Regeneration of Embryogenic Structures and Plants

<table>
<thead>
<tr>
<th>Regenerated Structures and Plant Development</th>
<th>Number</th>
<th>Time after In Vitro Fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion products</td>
<td>28</td>
<td>0 hr</td>
</tr>
<tr>
<td>First divisions</td>
<td>20</td>
<td>40-60 hr</td>
</tr>
<tr>
<td>Multicellular structures up to 90 μm</td>
<td>17</td>
<td>64-86 hr</td>
</tr>
<tr>
<td>Globular structures 380 to 700 μm, including transition-phase embryos, 380 x 450 μm</td>
<td>9 6</td>
<td>8-11 days 10-12 days</td>
</tr>
<tr>
<td>Embryos, coleoptiles, and shoots</td>
<td>11</td>
<td>22-34 days</td>
</tr>
<tr>
<td>Plants</td>
<td>11</td>
<td>31-86 days</td>
</tr>
<tr>
<td>Plants transferred to soil</td>
<td>11</td>
<td>45-109 days</td>
</tr>
<tr>
<td>Self pollinations</td>
<td>11</td>
<td>99-171 days</td>
</tr>
<tr>
<td>Mature seeds, cob harvest</td>
<td>11</td>
<td>148-215 days</td>
</tr>
</tbody>
</table>

Segregation Ratios

Segregation analysis confirmed the hybrid nature of the fusion plants. The endosperm color of the line Pirat parent is dark yellow and that of the inbred line A188 parent is white. Kernels produced by selfing the fusion plants showed color segregation, e.g., dark yellow, light yellow, and white kernels, as shown in Figure 1K. We observed a 3:1 segregation ratio in seven of eight selfed fusion plants tested, as demonstrated in Table 2.

DISCUSSION

The goals of the work reported here were (1) the establishment of a culture system capable of sustaining growth of only a single or a few cells; (2) the determination of the optimal culture parameters so that the developing structures in individual culture were capable of regeneration into plants; and (3) the definitive characterization of artificially produced fusion zygotes and plants as true hybrids, e.g., the demonstration that both parental genomes in the fusion products were present.

Because relatively few zygotes can be created by electrofusion, a single-cell culture system had to be used. Methods have already been developed for the individual culture of protoplasts and cells (for review, see Spangenberg and Koop, 1992). However, for single fusion zygotes of maize, a “Millicell/feeder suspension” system that involved the use of suspension cells as nurse cultures had to be developed (Kranz et al., 1991a; Kranz, 1992). So far, only this method has proven to be capable of achieving sustained growth and an associated high yield of large structures, e.g., globular proembryos and transition-phase embryos.

Figure 2. Chromosomes and Phenotype of the F1 Generation.

(A) Diploid set of chromosomes (2n = 20) of a root tip cell. Bar = 10 μm.

(B) Silks. Whereas the basal parts and the trichomes of egg cell donor line A188 are green, the fusion plant silks are intermediate in that the basal parts are predominantly green while the trichomes are red (Figure 2B). A dark red color at the base of the male spikelets is a characteristic of line Pirat, whereas line A188 has green male spikelets. In fusion hybrids, a dark red color at the base of the male spikelets was observed (Figure 2C).

(C) Male spikelets. The dark red color at the base of the spikelets is a characteristic of line Pirat, whereas line A188 has green spikelets. In fusion plants, a dark red color at the base of the spikelets was observed. Left, line A188; center, fusion plant 1; right, line Pirat. Bar = 2 mm.
Table 2. Segregation Ratios of Kernel Color of the Selfed Fusion Plants

<table>
<thead>
<tr>
<th>Fusion Plant No.</th>
<th>Yellow</th>
<th>White</th>
<th>( \chi^2 ) Values</th>
<th>Follows a 3:1 Segregation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>135</td>
<td>50</td>
<td>0.405</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>106</td>
<td>33</td>
<td>0.117</td>
<td>yes</td>
</tr>
<tr>
<td>6</td>
<td>172</td>
<td>51</td>
<td>0.540</td>
<td>yes</td>
</tr>
<tr>
<td>7</td>
<td>93</td>
<td>9</td>
<td>14.236</td>
<td>no</td>
</tr>
<tr>
<td>8/1(^b)</td>
<td>77</td>
<td>24</td>
<td>0.083</td>
<td>yes</td>
</tr>
<tr>
<td>8/2(^b)</td>
<td>137</td>
<td>39</td>
<td>0.757</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>102</td>
<td>37</td>
<td>0.194</td>
<td>yes</td>
</tr>
<tr>
<td>11</td>
<td>118</td>
<td>34</td>
<td>0.561</td>
<td>yes</td>
</tr>
</tbody>
</table>

\(^a\) Significance level is \( P = 0.05 \).
\(^b\) 8/1 and 8/2 were derived from the same callus.

The isolated egg cells that were used are true protoplasts. Their organelles were ultrastructurally intact and positioned next to the nucleus (Faure et al., 1992). In the majority of these cells, the clusters of organelles were located near the periphery of the protoplasts. Isolation of embryo sacs and egg cells was further improved (Kranz, 1992) so that it was possible for an experienced researcher to routinely obtain ~15 to 20 egg cells in 2 hr. This improved isolation technique ensured that a similar number of fusion products could be obtained and cultured per day. Using the improved feeder culture system, we regenerated 11 fertile plants that grew to maturity with seed set from 28 in vitro-created zygotes in four independent experiments. Despite the limited number of zygotes, it was the high rates of cell division, sustained growth, short in vitro culture time, and rapidity of plant formation that suggested that this technique could be used efficiently for micromanipulation.

Karyogamy was demonstrated within 1 hr after electrofusion of isolated, single egg and sperm cell protoplasts (Faure et al., 1993). Subsequently, in culture, i.e., in an agitated liquid medium, unequal distribution of cell organelles preceded the first cell division of the zygote. As in vivo fertilization, in which the first cell division results in a smaller, cytoplasm-rich chalazal cell and a larger, vacuolated micropylar cell, the first cell division of most of the in vitro-created fusion products was unequal and was followed by the formation of these two types of cells. During further development of the zygotes, the cell size decreased considerably, as is found during early in vivo development of the embryos of plants such as cotton (Pollock and Jensen, 1964), barley (Norstog, 1972), and rice (Jones and Rost, 1989). Subsequent divisions did not occur in detectable, defined orientation patterns. This was also found in maize embryos formed in vivo (Randolph, 1936). Subsequently, still in an agitated liquid medium, a pronounced axis was once again detectable with the formation of oblong structures.

These transition-phase embryos are characterized by a meristematic region consisting of small, cytoplasm-rich cells at the top and highly vacuolized basal cells representing the suspensor. The meristematic part, however, broadened, forming a deltoid scutellum, as described for the coleoptilar stage (Abbe and Stein, 1954). In this respect, zygotes, proembryos, and transition-phase embryos created in vitro strongly resemble those studied after isolation from a maize plant after natural fertilization (Van Lammeren, 1981, 1986; Schel et al., 1984; Clark and Sheridan, 1991). These in vitro–produced embryos also resemble somatic embryos (Fransz and Schel, 1991), those of cultured maize ovaries (Schel and Kieft, 1986), and those of ovular explants with only a few cell layers of nucellar tissue (Möl et al., 1993) from previously fertilized plants. The process of forming proembryos and transition-phase embryos after in vitro gamete fusion must be defined as in vitro zygotic embryogenesis and is to be clearly distinguished from somatic embryogenesis. Thus, in addition to molecular and cellular approaches resulting from studies of zygotic and somatic embryogenesis (for reviews, see Dudits et al., 1991; Lindsey and Topping, 1993), this new experimental system may provide further data for understanding plant embryo formation.

The establishment of conditions for the formation of single proembryos and transition-phase embryos from in vitro–produced zygotes will facilitate the manipulation and study of many different aspects of early zygotic embryogenesis independent of the effects of surrounding cells and tissues. The development of a proembryo proceeds in vitro without an endosperm. It is now also possible to fuse a sperm cell with a central cell and to induce in vitro endosperm development (Kranz et al., 1991b; E. Kranz, unpublished results). Studies of cell-to-cell communication with these individually cultivated, highly specialized cell and tissue types are feasible.

Plant regeneration from the enlarged structures, derived from proembryos, transition-phase embryos, and those with an enlarged scutellum, might have occurred via primary or secondary embryogenesis. The late embryogenesis pathway, accompanied by an enlargement and overgrowth of the structures, was not studied histologically. Therefore, cases of plant regeneration derived directly from a possible primary shoot-forming meristem of the fusion-created embryos could not be observed. However, plant regeneration via secondary embryogenesis, which resembles somatic embryogenesis (Möl et al., 1993) from scutellum tissue, was known to occur.

The morphology and further development of the in vitro–created transition-phase embryos, the overgrowth of the scutellum, and the observation of polyembryony in some experiments resembled stages in the somatic embryogenesis pathway, as reported by Lu et al. (1982). Comparable with isolated zygotic proembryos and embryos, the cultivation of in vitro–created zygotic proembryos and transition-phase embryos on 2,4-D–containing media can result in secondary embryogenesis in which more than one plant develops. Polyembryony in maize caryopses has been recognized for many years (Kieselbach, 1926; Randolph, 1936) and may occur spontaneously in polyembryonal and apomictic genotypes. Cleavage polyembryony also can be induced by x-ray irradiation of pollen (Morgan and Rapleye, 1951) and by 2,4-D treatment of the caryopses (Erdelská and Vidovencová, 1992). The delayed development...
of later embryo stages must be performed, and the in vitro of roots in the two cases might indicate organogenesis, a third possible mechanism for plant regeneration. Histological studies of later embryo stages must be performed, and the in vitro conditions for the development of a single embryo toward its maturity after in vitro gamete fusion have to be further optimized.

Silks and male spikelets show a red color, a characteristic of the sperm donor plant. This is expressed also in fusion plants. Heterozygosity of the fusion plants was demonstrated by the observation of the kernel color of selfed progeny. A 3:1 segregation ratio of yellow to white was observed. This indicates that both parental genomes are present in fusion plants. These results, supported by the observations of high-frequency karyogamy in the fusion products (Faure et al., 1993) and a diploid set of chromosomes in all regenerated plants, indicate the completed in vitro fertilization at the level of isolated, single gametes.

Now that it is possible to cultivate in vitro-created, single maize zygotes as well as to regenerate plants, new possibilities occur for their genetic manipulation. Transformation is the most obvious aim, and gametes and artificially produced zygotes may well be an attractive target. Consequently, it should be possible to individually manipulate both kinds of gametes as well as the zygote before the first cell division occurs, especially in combination with such transformation procedures as electroporation and microinjection. The possibility of fusing two gametes of the opposite sex combined with subsequent individual growth of the zygote in vitro as differentiated structures rather than as disorganized calli opens new fields in the study of experimental embryology.

METHODS

Plant Material

For egg cell isolation, ears from maize (Zea mays) inbred line A188 (courtesy of A. Pryor, CSIRO, Canberra, Australia) and pollen from the commercial hybrid line Pirat (courtesy of M. Schwall, Südwestdeutsche Saatzucht, Rastatt, Germany) were used. The inbred line 1206 (courtesy of W. Schmidt, Kleinwanzlebener Saatzucht, Einbeck, Germany) was used for the establishment of a feeder cell suspension. All plants were grown in the greenhouse under standard conditions.

Pollen and Ear Collection

Pollen was collected from freshly dehisced anthers and used as previously described (Kranz and Lörrz, 1990). Ears were bagged before silk emergence, and the outer leaves of the ears were surface sterilized with ethanol (70%). Ovules were selected from the middle part of the ears (Kranz et al., 1991a). 3:1 : 1

Isolation and Selection of Sperm and Egg Cells

Sperm and egg cells were isolated and selected as described by Kranz et al. (1991a) and Kranz (1992). These were individually selected under microscopic observation (inverted microscope IM 35; Carl Zeiss, Oberkochen, Germany [equipped with Nomarski differential-interference contrast accessories]). Approximately 20 pieces of ovular tissue (containing the embryo sac) were collected in a mannitol solution (540 mosmol/kg H2O) following initial treatment in 3.5-cm-diameter plastic dishes with 1.5 mL of enzyme solution (0.75% pectinase [Serva, Heidelberg, Germany], 0.25% pectolyase Y23 [Seishin, Tokyo, Japan], 0.5% hemicellulase [Sigma], and 0.5% cellulase Onozuka RS [Yakult Honsha, Tokyo, Japan], pH 5.0, adjusted to 540 mosmol/kg H2O with mannitol). The ovular tissue was incubated at 24° ± 0.5°C for ~30 min and was followed by microdissection of the embryo sacs and the egg cells with a microglass needle. Selected egg cell protoplasts were individually transferred to a droplet (mannitol, 540 mosmol/kg H2O) by microcapillaries for washing and then transferred to another droplet for fusion. Sperm cells were released from the pollen grains after rupture by osmotic shock. The sperm cell was taken up randomly and added to the egg cell. The sperm cells could be differentiated from the vegetative nucleus by their size. The average diameter of a sperm cell of maize is 75 μm, whereas the average diameter of a vegetative nucleus is 2.5 μm.

Sperm and egg cells were transferred with a hydraulic system and a computer-controlled dispenser/diluter (Microlab-M; Hamilton, Darmstadt, Germany) (Koop and Schweiger, 1985a; Kranz et al., 1991a; Kranz, 1992).

Electrofusion

A pair of a sperm and an egg cell protoplast was fused electrically and observed microscopically, using the methods described by Koop and Schweiger (1985b), Kranz et al. (1991a), and Kranz (1992). Ten 2000-nL fusion droplets, each consisting of mannitol (540 mosmol/kg H2O), were overlaid by mineral oil on a coverslip, and selected single gametes were transferred to these droplets for fusion. Controlled electrofusion was performed with a pair of platinum electrodes that were fixed to an electrode support mounted under the condenser of the microscope. The distance between the electrodes was adjusted by lowering them along the z-axis onto the coverslip; this was accomplished with a step motor and a positioning system (MCC 13 JS; Lang, Hüttenberg, Germany), which were described by Schweiger et al. (1987). Fusion was induced by single or multiple (two to three) negative D.C. pulses (50 μsec; 0.9 to 1.0 kV/cm) after electrophoretic alignment (1 MHz, 71 V/cm) on one of the electrodes for a few seconds with an electrofusion apparatus (CFA 400; Krüss, Hamburg, Germany).

Culture Procedures

Fusion products were cultured on a transparent, semipermeable membrane of 12-mm-diameter Millicell-CM dishes (Millipore, Bedford, MA) as described by Kranz et al. (1991a) and Kranz (1992). These dishes were placed in the middle of 3.5-cm-diameter plastic dishes previously filled with 1.5 mL of a maize feeder suspension. A nonmorphogenic maize cell suspension (inbred line 1206), originating from excised zygotic embryos (Kranz et al., 1991a), was used as the feeder culture. The cell suspension was precultured in the same medium as was used for the fusion products. The fusion products as well as the feeder cells were cultivated in MS medium (Murashige and Skoog, 1962), using modifications by Olsen (1967) and 2.0 mg/L 2,4-D, adjusted to 600 mosmol/kg H2O with glucose, pH 5.5, and maintained on a rotary shaker at 50 rpm. Routinely, five fusion products were cultured in one Millicell-CM dish. The culture conditions were as follows: 25° ± 1.0°C.
a light/dark cycle of 16/8 hr, and a light intensity of ~50 μmol m⁻² sec⁻¹. After 10 to 12 days, gamete fusion proembryos and transition-phase embryos were transferred individually by a Pasteur pipette from the Millscell-CM insert into 3.5-cm-diameter plastic dishes containing 1.5 mL of regeneration medium (modified MS media). The media were solidified with 4 g/L agarose (type I-A; Sigma) and contained no growth hormones, 0.1 or 0.5 mg/L 2,4-D or a combination of 1.0 mg/L naphthalene acetic acid and 1.0 mg/L benzyladenine, and 60 g/L sucrose. These were used for a first subculture of 9 to 21 days. During a second subculture of 7 to 8 days (with the exception of 2 days in one case), the media contained 90 or 40 g/L sucrose and no growth hormones. Four structures were subcultured for an additional week on the same medium, which contained 40 g/L sucrose. One structure was cultivated for a fourth subculture on 60 g/L sucrose for another 10 days. White embryos were transferred from the culture of 7 to 8 mm in diameter with roots, leaves, and shoots were transferred into 6-cm-diameter dishes containing 3.0 mL of the same medium, except that 30 g/L sucrose was added, and cultivated for 4 to 7 days. This subculture procedure was omitted for two of the structures that had already regenerated plantlets within 16 and 20 days during two subcultures onto regeneration media containing a combination of 1.0 mg/L naphthalene acetic acid and 1.0 mg/L benzyladenine or no growth hormones, respectively. Plantlets with leaf lengths of ~3 cm and occasionally compact callus with roots and shoots were transferred into glass jars for an additional 8 to 39 days. The plantlet growth medium contained 70 mL of MS medium (macro- and microsalts half concentrated) and 10 g/L sucrose and was solidified with 4 g/L agarose. After a growth period of 18 days on this medium, two structures with one shoot each were subcultured onto the same medium containing activated charcoal (0.1%) in combination with 5.0 mg/L naphthalene acetic acid, 2.0 mg/L benzyladenine, and 30 g/L sucrose. These structures were incubated for an additional 8 and 15 days for stimulation of root growth of the two shoots. From the glass jars, plants were transferred into soil for growth in the greenhouse for 45 to 108 days after gamete fusion.

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E. Kranz and H. Lorz

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