Reports

Direct Embryogenesis from Mesophyll Cells of Orchardgrass

Abstract. *Segments taken from the basal 15 to 20 millimeters of the two innermost leaves of an orchardgrass (*Dactylis glomerata *L.) genotype produced somatic (nonzygotic) embryos directly from mesophyll cells without an intervening callus when cultured on an agar medium with 30 micromolar 3,6-dichloro-o-anisic acid (dicamba). This demonstration of high-frequency embryogenesis from mesophyll cells in Gramineae is strong evidence for totipotency of the cells.*

Although whole plants can be regenerated from tissues, single cells, and even protoplasts in several plant species, particularly those in the family Solanaceae, progress has been much slower for most of our important cereals and grasses (*1*). We previously reported somatic embryogenesis from cultured leaf segments in orchardgrass (*Dactylis glomerata* L.) (*2*). While most of the plantlet regeneration was from embryogenic calli, embryos also formed directly on the leaf surface in rare instances. We now report embryogenesis in high frequency and a differential response of segments along the leaf to produce embryogenic calli or embryos directly without an intervening callus. Histological examination of leaf cross sections shows that embryos arise directly from mesophyll cells.

Plants used in this study were regenerated by somatic embryogenesis from a highly embryogenic genotype (*2*). The plants had been maintained in the greenhouse for approximately 15 months. Individual tillers were selected in which the innermost emerged leaf protruded approximately 4 to 6 cm beyond the sheath-blade junction of the third leaf outward. This leaf was about 15 cm long and the next leaf outward about 30 cm long. The extreme basal portions of the two leaves were used. If a smaller nonemerged leaf was present it was discarded. Since the leaf sheaths of orchard grass are flattened with united edges rather than convolute, individual leaves can be separated easily from each other and from the apical meristem.

Leaves were split longitudinally along the midrib and cut into segments 3 to 4 mm long. The first six sections (from the basal portion upward) of both halves of each leaf were serially plated onto Schenk and Hildebrandt (SH) medium (*3*) containing 30 μM 3,6-dichloro-*o*-anisic acid (dicamba) and 0.8 percent agar. The complete medium had been sterilized by autoclaving for 15 minutes at 121°C. Results of experiments conducted over several months have shown that the response of leaf segments to culture is the same regardless of which surface (abaxial or adaxial) is placed in contact with the medium. The leaf sections were incubated in the dark at 25°C.

For scanning electron microscopy, embryo-bearing leaf tissue was fixed in 3 percent glutaraldehyde adjusted to *p*H 6.8 in 0.1*M* phosphate buffer for 24 hours at 4°C. The specimens were rinsed in buffer, postfixed for 2 hours in similarly buffered osmium tetroxide, and dehydrated in a graded ethanol series. After solvent exchange with liquid CO_2 the specimens were subjected to critical point drying, mounted on stubs, and sputtered with gold-palladium. For light microscopy, leaf tissues were fixed for 24 hours in 2.5 percent glutaraldehyde, dehydrated in a *t*-butanol series, embedded in paraffin, sectioned, and stained by the procedure described by McDaniel *et al.* (*4*).

Between 4 and 6 weeks after plating, cultured leaf segments exhibited embryogenic calli, direct embryo formation, or a combination of both depending on the original position of the segment in the leaf. Segments from the most basal portion formed highly embryogenic calli. The callusing response decreased and the incidence of direct embryo formation increased in segments taken at progressively more distal positions from the base. On the older of the two leaves, direct embryo formation began nearer the basal segment. A typical response is shown in Fig. 1A. Segments of the innermost leaf are shown in the top row and those from the second leaf in the bottom row. The basal segment is at the extreme

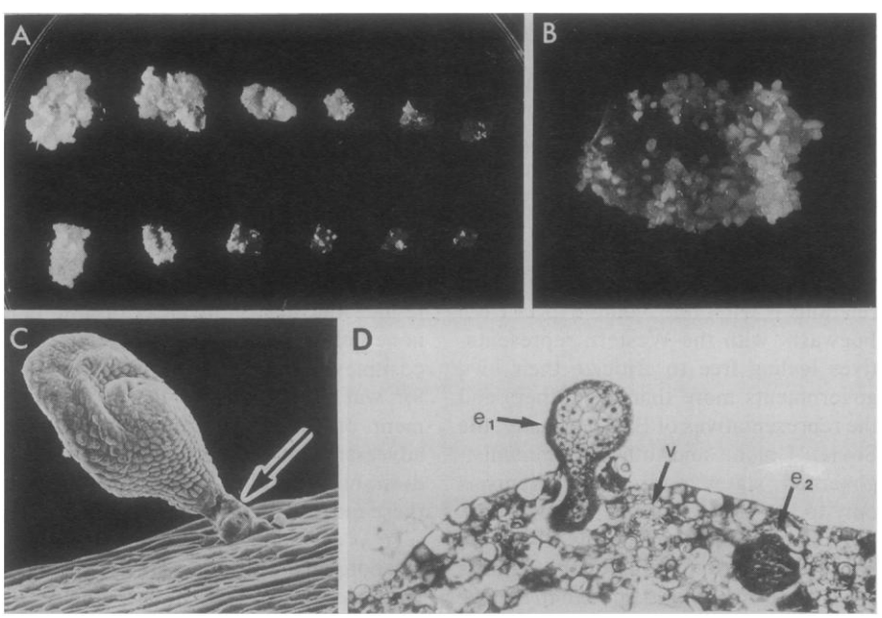

Fig. 1. Nonzygotic embryogenesis from orchardgrass (*Dactylis glomerata* L.) leaf segments cultured on SH medium (*3*) containing 30 μM dicamba. (A) Segments from the innermost emerged leaf (top row) and next leaf outward (bottom row) approximately 6 weeks after plating. Segments from the extreme basal end are shown at far left, with progressively more distal segments to the right. Note the embryogenic callusing at the basal end, which changes to a direct embryogenic response at progressively more distal segments (×0.8). (B) Leaf segment showing numerous embryos (×7). (C) Scanning electron micrograph of a well-developed embryo arising directly from a leaf segment. The embryo is supported above the undisturbed leaf surface by a suspensor (arrow) (×40). (D) Cross section of leaf (×80) showing a very young embryo (e_1) protruding from the leaf surface, with the suspensor originating from the mesophyll, and an embryo (e_2) beginning to form in the mesophyll. Note that embryogenesis does not appear to be associated with cells of the vascular bundle (*v*).

left, with progressively more distal segments to the right. A leaf segment with numerous developing somatic embryos is shown in Fig. 1B. Figure 1C shows a scanning electron micrograph of a well-developed embryo arising directly from the leaf surface. A light microscopic study of leaf cross sections showed that direct embryogenesis occurred in mesophyll tissue (Fig. 1D).

Embryos physically removed from the leaf surface and placed on SH medium without auxin germinated and formed individual plantlets. On medium with 30 μM dicamba they formed embryogenic calli from which many more embryos could be recovered.

We do not know the reason for the differential response of segments along the leaf. However, since the grass leaf grows from the base (*5*), a differentiation gradient exists, with cells at the extreme basal portion actively dividing and nondifferentiated while distal cells are less mitotically active and more differentiated. In our system there seems to be an optimum point on the differentiation gradient where direct embryogenesis is more frequent. This hypothesis would also account for the shifting of the embryogenic response toward the base of the older of the two leaves, since as leaves become older the meristematic area is restricted further toward the base. In fact, a response was not always obtained from the uppermost segments of the older leaf.

Wernicke *et al.* (*6*) observed dry, compact globules on cultured basal leaf segments of 10-day-old rice seedlings. They did not report whether these globules developed into embryos while still on the explant; only about 1 percent germinated normally when transferred to regeneration medium. Some showed a slight disorganized proliferation and multiple root and shoot formation.

In our experiments embryos formed in large numbers and grew to a highly organized stage. When embryos were isolated at the stage shown in Fig. 1C or later and placed on medium without auxin, more than 95 percent germinated into single plantlets that could be established in soil.

Mesophyll has been reported to be the most suitable tissue for protoplast research because relatively uniform protoplasts can be easily isolated in large numbers (*7, 8*). However, regeneration of whole plants from mesophyll protoplasts has yet to be accomplished in grass and cereal species. Our system may be of value in research efforts toward that goal. Formation of embryos directly from mesophyll cells may also provide an opportunity for in vitro cloning not previously possible because of tissue culture–induced variability (*9*). Although we have not yet conducted cytogenetic studies, none of the many plants that we have recovered exhibit chlorophyll deficiency or other phenotypic abnormalities indicative of tissue culture–induced variability.

The single-cell origin of the embryos has not been unequivocally established. However, the thin suspensor that forms and to which the embryos are delicately attached does suggest such an origin. The extensive discussion by Haccius (*10*) on the unicellular origin of nonzygotic embryos, including those that develop directly, further supports a single-cell origin.

Direct embryogenesis, in addition to its significance for plant tissue culture, provides a model system for studying the basic developmental morphology of nonzygotic embryos and the influence of auxins and other hormones on embryo development along a gradient of leaf segments. An understanding of the factors controlling this embryogenic response should advance the status of in vitro culture of mesophyll cells and protoplasts from a wide range of grass and cereal species and genotypes.

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Bloom's Syndrome: Evidence for an Increased Mutation Frequency in vivo

Abstract. *The incidence of lymphocytes resistant to the purine analog 6-thioguanine was studied in seven patients with Bloom's syndrome. The mean frequency was* 17.3×10^{-4}. *The mean incidence in age- and sex-matched controls was* 2.1×10^{-4}, *so approximately eight times the normal number of 6-thioguanine–resistant lymphocytes were detected in Bloom's syndrome blood. The basis for this increase is unknown, but the inherent genomic instability demonstrated in the form of chromosomal aberrations is one possible explanation.*

Bloom's syndrome (BS) is a rare autosomal recessive disorder characterized clinically by deficient growth, sun-sensitivity, immunodeficiency, and, in many cases, some form of cancer (*1*). The molecular basis of the syndrome is unknown, but experimental evidence (*2, 3*) points to an impairment of DNA replication. BS cells exhibit a striking degree of genomic instability as evidenced by greatly increased numbers of sister chromatid exchanges (SCE's), chromosomal aberrations, and chromatid interchanges between homologous chromosomes (*4, 5*). Cells with specific locus mutations have also been reported to be present in abnormally great numbers in BS fibroblast cultures. These include 6-thioguanine–resistant (6-TG^r) and diphtheria toxin–resistant cells (*6, 7*). We estimated the incidence of 6-TG^r variant lymphocytes in vivo by an autoradiographic method (*8*) for detecting T lymphocytes in the circulating blood that can undergo DNA synthesis in the presence of 6-TG. A significantly increased incidence of 6-TG^r lymphocytes was found in each of the seven BS patients examined.

The basis for the detection of 6-TG^r cells in this test system is as follows. Lymphocytes capable of proliferating in short-term culture in medium containing 6-TG are of two sorts: (i) noncycling cells responsive to phytohemagglutinin (PHA) and deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) (E.C. 2.4.2.8), which catalyzes the conversion of hypoxanthine and guanine to their respective nucleotides by way of the salvage pathway, and (ii) cells that are already in a proliferating cycle in vivo and which can proceed through at least one cell cycle in vitro before succumbing to the lethal effects of 6-TG. HPRT also can convert the purine analogs 8-azaguanine (8-AG) and 6-TG