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PROLIFERATION OF AND PLANT REGENERATION FROM THE EPIBLAST OF TRITICUM AESTIVUM (WHEAT; GRAMINEAE) EMBRYOS[1]

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ABSTRACT

Cultured immature embryos of wheat, approximately 1 mm in length, often showed proliferation of the epiblast. Murashige and Skoog's medium with 0.2 or 0.4 mg/l 2,4-dichlorophenoxyacetic acid and 0.2 mg/l benzylaminopurine supported epiblast proliferation in 20–90% of the embryos depending upon their orientation. Epiblast growth occurred more frequently when the scutellum of the embryo was placed in contact with the medium. The compact tissue derived from the epiblast resembled scutellar callus morphologically and in its developmental potential. Differentiation took place by the formation of a leafy structure along one side of the epiblast proliferation, followed by the initiation of shoot primordia. The pathway through which rooted plants were formed has been interpreted as precocious germination of somatic embryos. The relevance of these observations to the interpretation of the homology of the epiblast is discussed.

SOMATIC EMBRYOS or embryo-like structures may arise from a variety of plant tissues in culture (Vasil and Vasil, 1972). In the Gramineae, leaves (Wernicke and Brettell, 1980; Lu and Vasil, 1981; Haydu and Vasil, 1981; Wernicke, et al., 1981), inflorescences (Brettell, Wernicke and Thomas, 1980; Vasil and Vasil, 1981; Lu and Vasil, 1982; Wang and Vasil, 1982), and immature embryos (Dale, 1980; Vasil and Vasil, 1981; Lu and Vasil, 1982; Ozias-Akins and Vasil, 1982; Lu, Vasil and Ozias-Akins, 1982) are capable of producing embryogenic callus. In cultures of immature embryos of many grass species, the scutellum most frequently gives rise to compact callus and, subsequently, to embryoids and plants. In this study we report the proliferation of the epiblast in cultured immature wheat embryos and the formation of plants from the proliferated epiblast.

MATERIALS AND METHODS—Immature caryopses from four *Triticum aestivum* cultivars (Arthur 71, Coker 747, McNair 3069, Oasis) were sterilized sequentially with 0.1% mercuric chloride (60 sec), 70% ethanol (60 sec), and 20% Clorox (11 min). The caryopses were rinsed after each step with sterile water. Immature embryos (900, approximately 1 mm in length) were cultured in the dark with either the shoot-root axis or the scutellum in contact with the agar medium. Murashige and Skoog's (1962) inorganic constituents were supplemented with inositol (100 mg/l), casein hydrolysate (100 mg/l), sucrose (2, 4, 6%) and 2,4-dichlorophenoxyacetic acid (2,4-D; 0.2, 0.4, 2.0, 4.0 mg/l). One experiment included benzylaminopurine (BAP; 0.2 mg/l) with all concentrations of sucrose and 2,4-D. The media were adjusted to pH 5.75 with NaOH, solidified with 1% agar, and autoclaved at 15 psi, 121 C, for 15 min.

Paraffin sections (7–10 μm) were cut from tissues fixed in formalin-acetic acid-ethanol, dehydrated in a tertiary butanol series, and embedded in Paraplast. The sections were stained with Heidenhain's hematoxylin and safranin.

Scanning electron microscopy was performed on tissues fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO_4, dehydrated in ethanol, critical point dried, and coated with gold.

RESULTS—On media containing 2,4-D, the epiblast began to proliferate and became compact, yellowish, and shiny. Four concentrations of 2,4-D in combination with three concentrations of sucrose were tested to determine which combination best promoted proliferation of the epiblast in the presence or absence of BAP (Table 1). The embryos were cultured with the shoot-root axis in contact with the medium. A low 2,4-D concentration (0.2 and 0.4 mg/l) was markedly better for epiblast proliferation than a high 2,4-D concentration (2

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TABLE 1. *Percentage of embryos that showed proliferation of the epiblast when embryos from cv. Oasis were cultured with the shoot-root axis in contact with the medium (25–30 replicates per medium)*[a]

Sucrose concentration (%)	2,4-D concentration (mg/l)			
	0.2	0.4	2.0	4.0
2	20/16	24/16	4/0	0/4
4	36/0	37/17	4/0	0/0
6	28/0	36/12	16/0	4/0

[a] Shown as % proliferation of epiblasts of embryos cultured on media containing BAP vs. not containing BAP, +BAP/−BAP.

TABLE 2. *Percentage of embryos that showed proliferation of the epiblast when embryos from cv. Coker 747 were cultured with the scutellum in contact with the medium (10 replicates per medium)*[a]

Sucrose concentration (%)	2,4-D concentration (mg/l)		
	0.2	0.4	2.0
2	60/80	90/90	70/10
4	80/60	80/50	50/0
6	90/100	80/40	50/10

[a] Shown as % proliferation of epiblasts of embryos cultured on media containing BAP vs. not containing BAP, +BAP/−BAP.

or 4 mg/l). No proliferation of the epiblast occurred on media without 2,4-D. Epiblast growth was initiated at all three sucrose concentrations, although 4% and 6% sucrose caused delayed organization of the tissues and increased root formation. The addition of BAP was not essential, but it appeared to enhance epiblast proliferation in certain media (Tables 1 and 2).

The manner of placement of the embryo on the medium also had an effect on epiblast proliferation. When embryos of cv. Arthur 71 were cultured in two orientations, 65% (13/20) of those with the scutellum touching the medium and 30% (7/23) of those with the axis in contact with the medium showed proliferation of the epiblast. All of the cultivars used gave a positive response for epiblast proliferation. The epiblast of embryos nearing maturity (2–3 mm in length) did not proliferate.

The epiblast is evident in wheat embryos approximately 1 mm in length (Fig. 1, 8). Within 6 days of culture on low sucrose, low 2,4-D media, the epiblast became compact and enlarged due to cell divisions (Fig. 2, 9), and then rapidly began to differentiate. Plant regeneration occurred most rapidly after transfer of the epiblast proliferation to MS medium with 2% sucrose and 0.2 mg/l 2,4-D. Embryos placed with the shoot-root axis touching the medium often showed scutellar proliferation as well (Fig. 3, 11). The initial morphology of the epiblast and scutellar proliferations was identical. Therefore, the cultures had to be observed every 2 or 3 days to prevent misinterpretation of the diverse origins of the compact tissues when roots and soft callus began to form. The proliferated epiblast could most easily be located by its position relative to the coleoptile and suspensor of the in vivo embryo.

The cells of the outer portion of the epiblast proliferation were small and densely cytoplasmic, whereas those nearer to the coleorhiza were larger and more vacuolated (Fig. 10). The first sign of differentiation in most epiblast proliferations was the formation of a notch along one side of the developing structure (Fig. 4, 11). The notch delineated a broad meristematic area from a narrower zone of tissue which began to elongate and assume leafy characteristics (Fig. 5, 6, 12, 13). The cells between the epiblast proliferation and the coleoptile and coleorhiza elongated, and the epiblast could be removed easily at this stage from the remainder of the embryo. The compact proliferation continued to differentiate whether attached to the original embryo or excised and subcultured on the same medium. The vascular tissue of the first-formed leafy structure occasionally could be seen in connection with a well-defined root primordium (Fig. 12). The broad meristematic area produced shoots which gave rise to plants (Fig. 7). No attempt was made to grow the plants to maturity.

DISCUSSION—Although *Hordeum* embryos typically lack an epiblast, young embryos (0.5 mm) cultured on White's medium plus coconut milk frequently developed an epiblast (Norstog, 1961). Embryoid formation from the epiblast region of young *Hordeum* embryos (0.5 mm) in the presence of 0.1 mg/l kinetin has been reported by Norstog (1970). These embryoids consisted of a shoot-root axis but a scutellum did not develop. Norstog did not consider these embryoids to have originated from coleorhizal tissues, but rather to have been formed at the scutellar node.

In the present study of young wheat embryos, it is clearly the epiblast which proliferates and subsequently organizes a leafy structure and shoot primordia. The morphological nature of the epiblast proliferation and its differentiation are similar to the proliferation and differentiation of the wheat immature scutellum (Ozias-Akins and Vasil, 1982). Therefore, it seems reasonable to interpret the structures arising from the epiblast as atypical embryoids in which

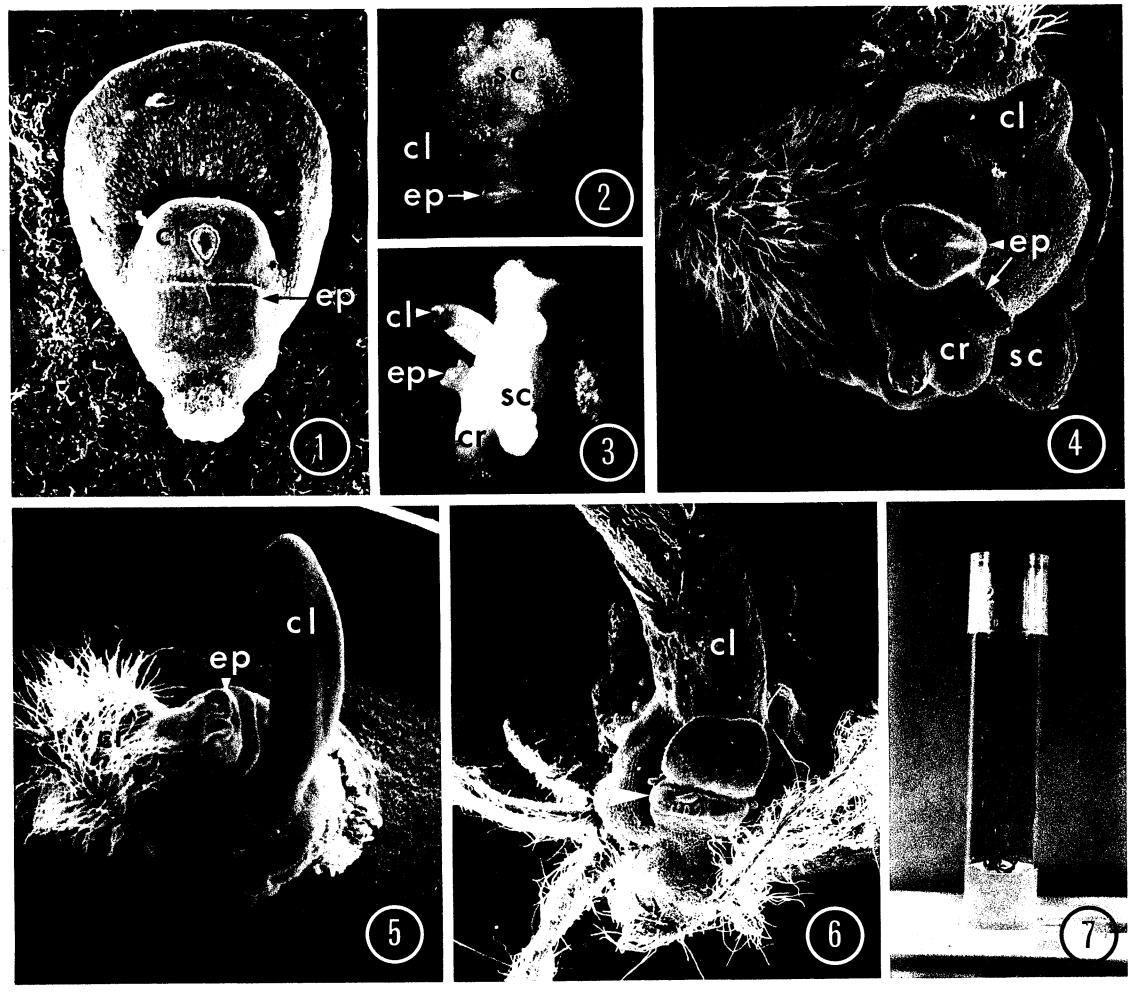

Fig. 1–7. Proliferation and plant regeneration from epiblast of wheat. cl, coleoptile; cr, coleorhiza; ep, epiblast; sc, scutellum. **1.** Immature embryo of wheat at the time of culture. ×60. **2.** Immature embryo cultured for four days showing a ridge of compact tissue which is the proliferated epiblast. ×18. **3.** The epiblast proliferation 14 days after culture is beginning to differentiate by the formation of a notch. The scutellum has also proliferated in this cultured embryo. ×14. **4.** Scanning electron micrograph of a cultured embryo in which only a portion of the epiblast proliferated and has formed a notch. The prominent root is arising from the scutellum. ×30. **5.** The notch formed on the epiblast proliferation is clearly defined and the meristematic region has begun to differentiate. ×20. **6.** The epiblast proliferation (arrowhead) has differentiated into a meristematic area and an elongated leafy structure. ×16. **7.** Plantlet formed from an epiblast proliferation.

the scutellum becomes green and leafy and multiple shoots are produced as a result of precocious germination. The vascular tissue of the leafy structures (= atypical scutella) originating from the wheat epiblast was occasionally found in connection with a well-defined root primordium, but a similar connection with shoot primordia was not apparent. Merry (1941), in his study of the ontogeny of barley embryos, observed that the vascular bundles of the scutellum became connected first with the primary root and later with the coleoptile bundles.

The resemblance of the epiblast and scutellar proliferations in vitro could also be used as evidence that both structures are homologous with cotyledons. Numerous theories have been expounded concerning the homologies of various structures peculiar to the grass embryo, i.e., scutellum, coleoptile, coleorhiza, epiblast, and mesocotyl (Arber, 1923; Brown, 1959, 1960, 1965; Coulter, 1915; McCall, 1934; Sargent and Arber, 1915; Weatherwax, 1920; Worsdell, 1961). Brown (1960) has compiled a comprehensive list of numerous interpretations of various organs of the grass embryo.

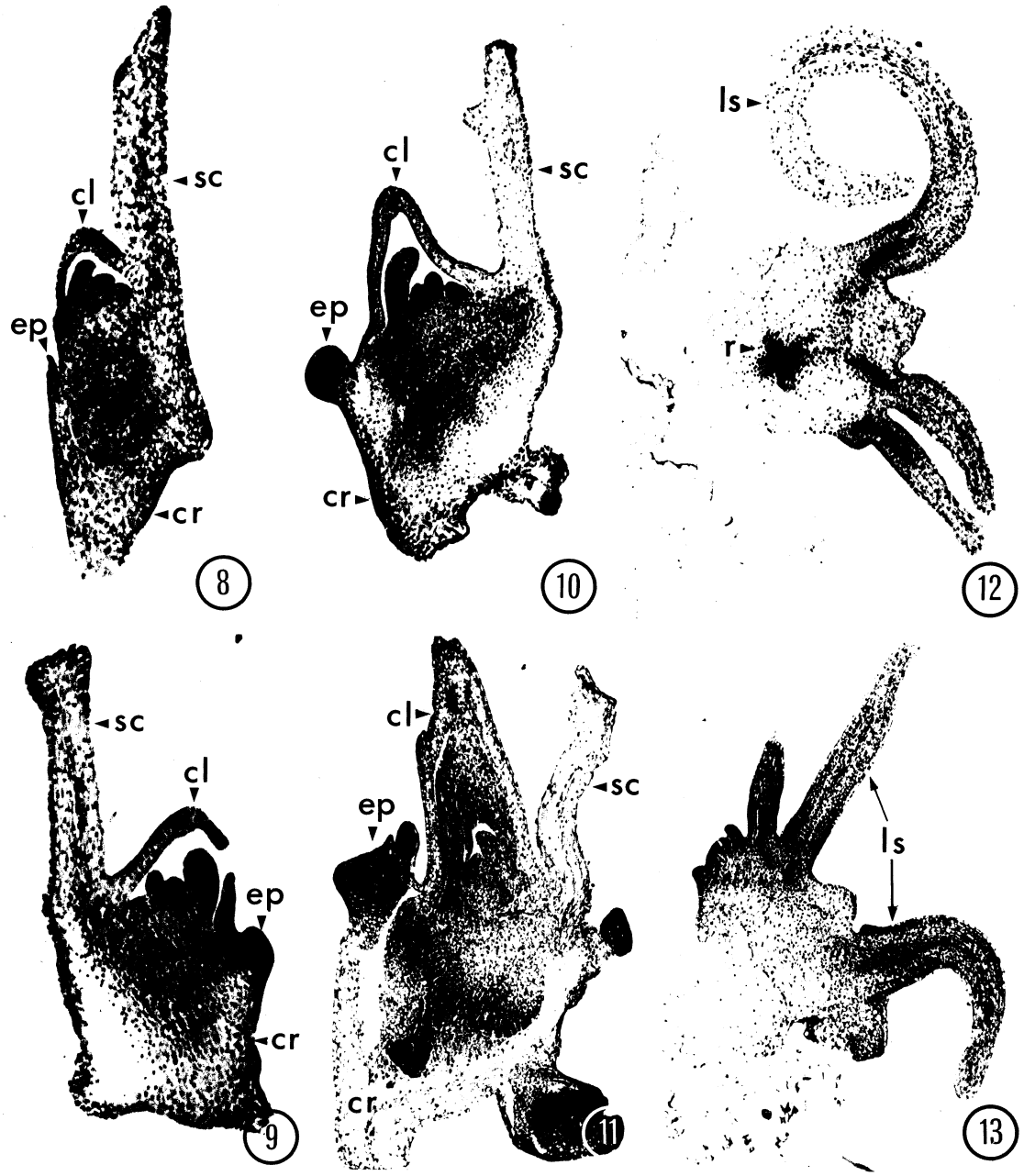

Fig. 8–13. Histology of epiblast proliferation and morphogenesis. cl, coleoptile; cr, coleorhiza; ep, epiblast; r, root; sc, scutellum; ls, leafy structure. **8.** Median longitudinal section of a wheat embryo at the time of culture. ×75. **9.** The epiblast proliferation, 6 days after culture, is composed of densely staining meristematic cells. ×75. **10.** At a slightly later stage than Fig. 9, the cells near the base of the epiblast proliferation are more vacuolated and elongated than those at the surface. ×65. **11.** Differentiation of the epiblast proliferation begins with the formation of a notch on one side. ×45. **12.** An elongated leafy structure is formed from the epiblast proliferation and root primordia occasionally were seen in association with the leafy structures. ×45. **13.** Sometimes more than one leafy structure was formed from an epiblast proliferation, and shoot primordia were also produced. ×40.

The scutellum has been interpreted most frequently as the single cotyledon of the embryo or a portion of the single cotyledon, whereas the coleoptile has been considered to be a part of the cotyledon (ligule or sheath) or the first leaf of the plumule. Various intepretations of the epiblast include it as a portion of the single cotyledon (auricle or ligule), a rudimentary or

vestigial second cotyledon, or more frequently, an insignificant outgrowth of the coleorhiza.

Foard and Haber (1962) observed the formation of hairs on the epiblast and coleorhiza of germinating wheat embryos that structurally and developmentally resembled root hairs. Gamma irradiation of the embryos prevented cell divisions but coleorhizal and epiblast hairs continued to be formed, whereas leaf hairs did not develop. Also the response of various plant organs to indole-3-acetic acid, gibberellic acid, and (2-chloroethyl) trimethylammonium chloride was observed. The foliage leaves always responded differently to each growth regulator than the epiblast and coleorhiza. These observations prompted Foard and Haber to conclude that the epiblast and coleorhiza were in fact a single structure. They emphasized the importance of utilizing various developmental approaches to complement classical anatomical evidence for the interpretation of morphologic relations. Brown (1965) readily accepts the observations of Foard and Haber (1962) as evidence that the epiblast is a structural extension of the coleorhiza. Other facts which support this interpretation are that neither the epiblast nor coleorhiza contain vascular tissue, and the epiblast is formed rather late in the ontogeny of the embryo (Brown, 1959). Cotyledons develop directly from the proembryo, but the epiblast cannot be distinguished until after the formation of the shoot apex. The evidence is rather strongly in favor of the epiblast as an outgrowth of the coleorhiza instead of a cotyledonary structure. Nevertheless, the similar morphogenic response in vitro of the wheat immature scutellum and epiblast to 2,4-D suggests that the epiblast may be a cotyledonary structure. Under the conditions examined to date, only the epiblast and scutellum of the immature wheat embryo, and never the coleorhiza, formed compact tissue that was capable of regenerating whole plants. In related work with another species of the Gramineae, *Zea mays,* Vasil et al. (1983) have described proliferation and plant regeneration from the nodal region of cultured immature embryos and suggested that the proliferation at the node may represent the evolutionarily extinct epiblast of the maize embryo and that it is likely cotyledonary in nature because of similarities of morphogenetic competence with the scutellar callus of maize.

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