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Rapid Embryogenesis and Plant Regeneration of Target Tissue Derived from Juvenile Shoots for Transformation of Oat by Microprojectile Bombardment

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The successful genetic transformation of plants using tissue culture procedures is strongly dependent on the regeneration capacity of target tissues. In the case of cereal plants, embryogenic cell suspension, immature embryos, and regenerable calli derived from immature embryos have been shown to be suitable targets for microprojectile bombardment [1-3]. In some cereals where stable transformation has been achieved, long culture periods have been required to obtain the embryogenic calli [4-7]. Such long-term cultures are at risk for undesirable somaclonal variations, reduced regeneration capability, and reduced fertility of regenerated plants [8]. Furthermore, available regenerable systems are often highly specific to certain cultivars [9]. These problems are critical in oat, for which an efficient system for diverse oat cultivars is needed for rapid production of embryogenic calli suitable for transformation. A technique of using stem segments cut from the base of young shoots offers a promising tool to produce such calli [10, 11]. However, gene transfer that used these culture systems has not been attempted yet. We describe here a simple and short-term culture for obtaining embryogenic calli and regenerated plants, and its application to gene introduction by microprojectile bombardment, using juvenile shoots of oats.

Source of embryogenic cultures

Seeds of oats (*Avena sativa* L. 'Starter' and 'GAF/Park') were hulled by hand and then sterilized with 70% ethanol for 5 min followed by 2% sodium hypochlorite solution containing Tween 20 (one drop per 200 m*l*) for 20 min. Surface-sterilized seeds were washed three times with sterile distilled water and germinated on filter paper moistened with Murashige & Skoog (MS) [12] medium which was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin and adjusted to pH 5.8 with 0.1 N NaOH before autoclaving. The cultures were incubated at 27°C under continuous illumination of 31-45 μmol/cm^2/sec with fluorescent light. Two cultivars, 'Starter' and 'GAF/Park' were used in this study. They have been used for the cytogenetic analysis of plants regenerated from tissue cultures [8]and also for the genome mapping.

In a primary experiment, seeds of two cultivars were cultured with thirty-six combinations of different concentrations of 2,4-D (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/*l*) and kinetin (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/*l*). Germination of seeds and shoot formation were observed in all of these combinations, but regenerable calli were induced only when seeds were germinated on MS medium supplemented with 3.0 mg/*l* 2,4-D and 0.1 mg/*l* kinetin (A medium). This contrasts with an optimum of 2 mg/*l* 2,4-D reported by Chen *et al.* [10], possibly indicating differences in hormone requirements among cultivars. Growth regulators applied 3-5 days after the start of germination appeared ineffective. Small nodular calli were produced in some combinations of the growth regulators, but these calli remained non-embryogenic during the entire period of culture. The regenerable calli were jelly-like and friable. They were produced at the basal regions of shoot tissues by 5 days after incubation (**Fig. 2-A**).

In subsequent experiments using 3.0 mg/*l* 2,4-D and 0.1 mg/*l* kinetin, the percentage of germinating seeds that produced primary calli was 56% for Starter and 80% for 'GAF/Park' (**Fig. 1-A**). If primary calli were detached from shoot tissues at 5 days after the start of experiments (**Fig. 1-B**), subsequent growth of

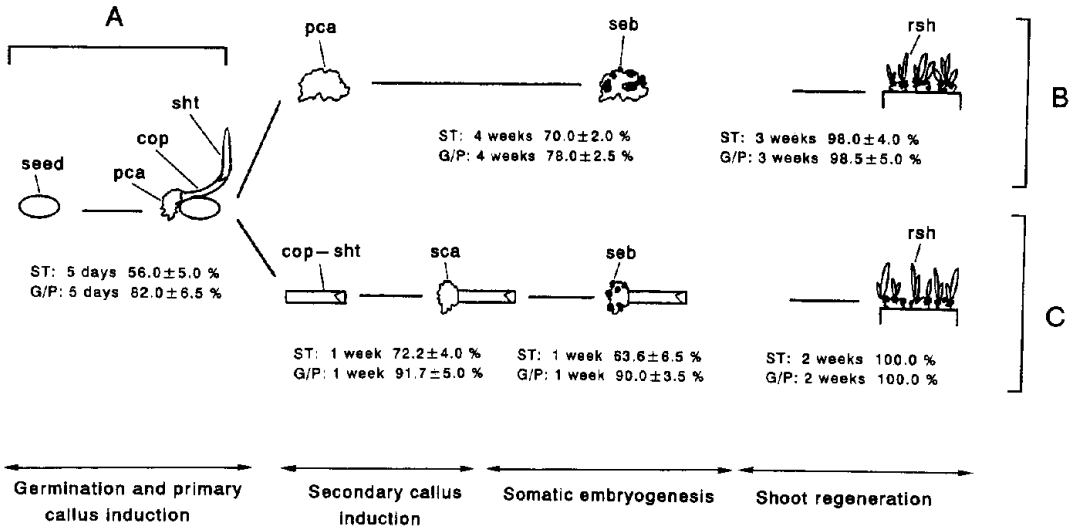

Fig. 1 Diagram for embryogenic callus induction and plant regeneration of oat cultivars 'Starter' (ST) and 'GAF/Park' (G/P). Surface-sterilized seeds (sed) were incubated on MS medium with 3.0 mg/*l* 2,4-D and 0.1 mg/*l* kinetin for germination and shoot formation. Primary callus (pca) and detached coleoptile (cop)-shoot (sht) tissue were used for induction of secondary callus (sca) or somatic embryo (seb) and regenerated shoot (rsh). The incubation periods for each stage are given as days or weeks and the frequencies for callus induction and shoot formation are represented as means of percentages from three separate experiments.

the calli was conspicuously promoted. The detached calli produced several green protuberances (**Fig. 2-B**) after 2 weeks, and developed small and abnormal leaflets (**Fig. 2-C**) from protuberances after 4 weeks of incubation, and these leaflets developed no further. However, at regions different from those of protuberances, the calli began to produce numerous somatic embryos in clusters within 4 weeks after the start of incubation (**Fig. 2-C**). The difference in the rate of embryogenic callus formation between the two cultivars was not significant (**Fig. 1-B**). The embryo clusters were formed at more than 20 sites on callus clumps originating from single shoot. Within 7 weeks after the start of experiments, each cluster developed 20-30 shoots (**Fig. 1-B**). Developed shoots (approximately 1 cm long) were separated from each other and transferred to B medium (MS medium without growth regulators) for root initiation. In both cultivars, nearly all of these shoots produced roots within 10 days after transfer and developed normal plants after transfer to soil.

In other experiments, juvenile shoots were detached from primary calli and placed to determine the capability of the shoots to produce embryogenic calli (**Fig. 1-C**). Each shoot, including the surrounding coleoptile, was detached at the base (**Fig. 2-A**) 5 days after incubation and continuously incubated on A medium. As a result, the shoots produced undifferentiated calli (secondary calli) at their cut ends within 1 week after excision (sca, **Fig. 1-C**). The calli rapidly grew to be present over the entire cut-surface of the shoots and extended about 1 cm up the outside surface of the shoots within 3-4 days. Green protuberances and somatic embryos originated on these secondary calli by 2 weeks after excision (**Fig. 2-D**). Embryogenic calli (seb, **Fig. 2-C**) were induced in 90% of detached shoots of the cultivar 'GAF/Park' and 64% of detached shoots of 'Starter'. The embryos of both cultivars produced new shoots within 4 weeks after the original shoots were detached from germinating seeds (**Fig. 1-C, Fig. 2-E**). Roots were produced after the shoots were transferred to B medium. After acclimation, the regenerated plants showed normal growth in soil in a plant growth chamber.

Because shoots were produced more rapidly from basal portions of detached shoots (in 4 weeks) than from primary callus (in 7 weeks), we further investigated embryogenic potential of detached shoot tissue. Five days after germination, three pieces of 2 mm segments (I, II, III) were excised from the base of each shoot generally followed by Chen *et al.* [10, 11] (**Fig. 2-F**). As shown in **Fig. 3**, embryogenic calli were produced in about 80% of segments from the most basal position (segment I). The rates were the same for 'GAF/Park' and 'Starter'. For both cultivars, rates were much lower for segments from other positions, segments II and III (**Fig. 3**).

The present study demonstrated that juvenile shoot tissue of oat was a useful potential source of highly regenrable calli. However, these calli were not induced by shoot tissues obtained from seeds germinat-

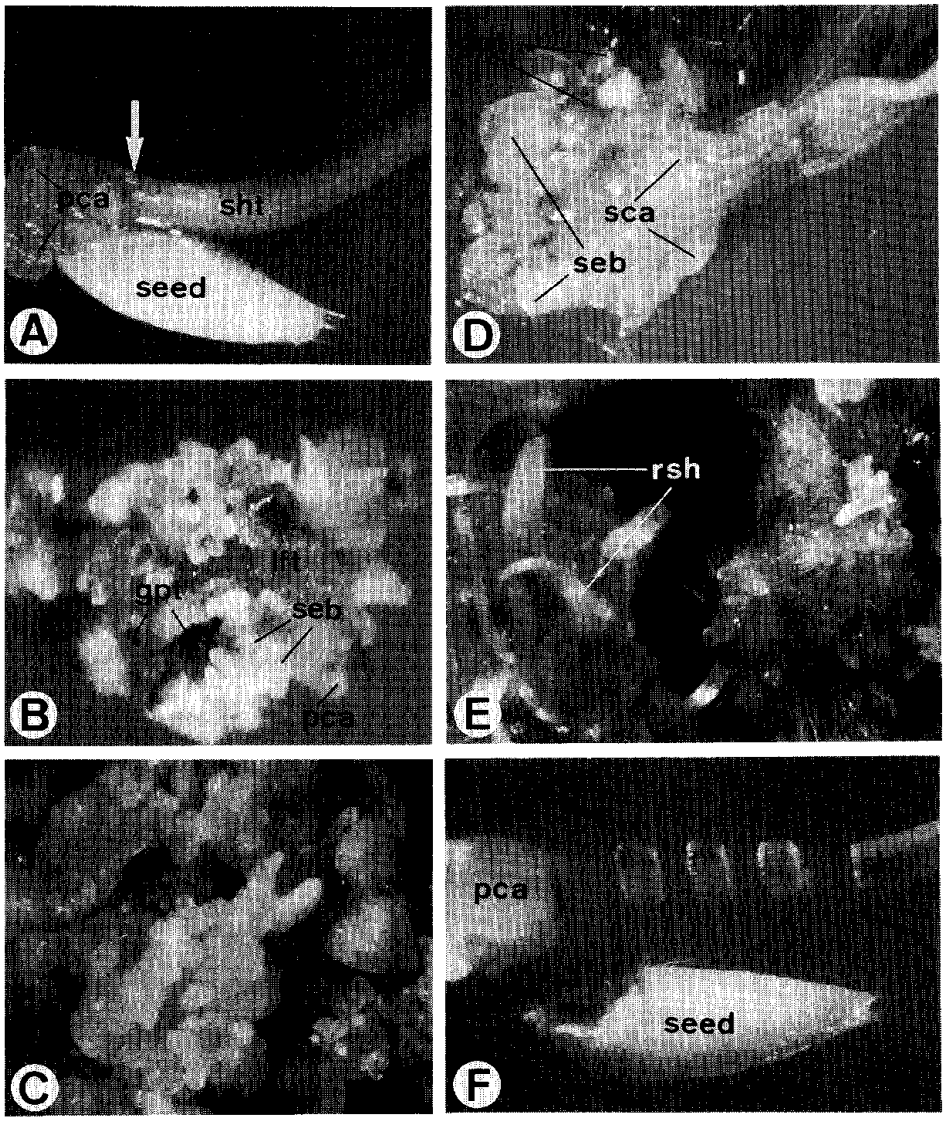

Fig. 2 Somatic embryogenesis in juvenile shoots of oat (*A. sativa 'Starter'*). A : Primary callus primarily induced at the basal region of juvenile shoot of seed germinated on MS containing 3.0 mg/*l* 2,4-D and 0.1 mg/*l* kinetin, 5 days after incubation (×10). Arrow represents the site for detaching shoots in some experiments. B : Green protuberances and small leaflets produced on primary callus excised from shoot tissue, 2 weeks after incubation (×25). C : Clustered somatic embryos on primary callus, 4 weeks after incubation (×100). D : Green protuberances and somatic embryos produced on the secondary callus which was formed on the cut end of shoot tissue, 2 weeks after incubation (×20). E : Shoots developed from somatic embryos on callus, 4 weeks after incubation (×5). F : Three segments (I, II and III) sectioned at the base of shoot tissue detached from seed (SD), 5 days after incubation (×10). Pca, primary callus ; sht, juvenile shoot ; gpt, green protuberance ; lft, leaflet ; seb, somatic embryo ; sca, secondary callus ; rsh, regenerated shoot

ed in the absence of 2,4-D and kinetin, or even when these growth regulators were given at different concentrations. The results suggest that this potential could be activated by the addition of 3.0 mg/*l* 2,4-D and 0.1 mg/*l* kinetin during the stage of germination (for initial 5 days). Chen *et al.* [10]also pointed out the embryogenic potentiality of the basal portion of juvenile shoot tissues of some oat cultivars ('Coolabah', 'Cooba', 'Blackbutt', 'Mortlock', 'Victorgram' and 'HVR') cultured on a medium supplemented with 2.0 mg/*l* of 2,4-D. Thus, the present study provided the short-term embryogenic system for 'Starter' and 'GAF/Park' and enabled us to conduct transformation of these two cultivars.

Transformation of embryogenic calli by microprojectile bombardment

Transformation was attempted using segments excised from the most basal portion (segments I), since

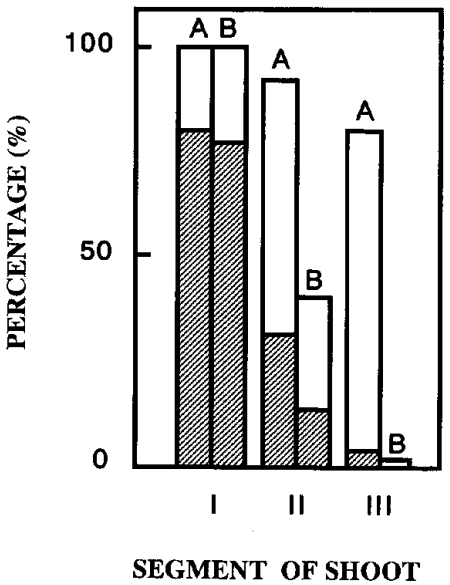

Fig. 3 Induction of undifferentiated (open) and subsequent embryogenic calli (shaded column) on the apical cut surface of three segments (I, II and III as shown in **Fig. 2-F**) of basal shoot tissues of oat cultivars 'GAF/Park' (A) and 'Starter' (B) (after two weeks of incubation).

the explant had produced embryogenic calli at the highest rate (**Fig. 4-G**). The segments from position I produced friable calli on both basal and apical cut-ends (**Fig. 4-H**) and differentiated somatic embryos from these calli (**Fig. 4-I**) 1 and 2 weeks after incubation, respectively. Furthermore, the cut surface of the segments offered a flat surface for uniform disposition of DNA-coated microparticles. Basal excised segments were placed on A medium, incubated until undifferentiated callus first began to form after 3-5 days (**Fig. 4-G**), and subjected to microparticle bombardment. The vector used was pNGI [13, 14] which contained the neomycin phosphotransferase gene (*npt II*) and the β-glucuronidase gene (*gus*) flanked by CaMV 35S promoter and the Adh I promoter with the Adh I intron sequence, respectively. Tungusten particles (1.5 μm in diameter) were coated with 1 mg/m*l* of plasmid DNA and shot into the basal segments by means of the Biolistic PDS-1000 Particle Delivery System (DuPont Co., CA, USA) according to the methods described by Somers *et al.* [6]. The segments were bombarded in groups of 15-20, with the basal surface exposed for bombardment. In an initial experiment, tissues were incubated for 3 days after bombardment, then assayed for transient expression of GUS gene. The segments were histochemically

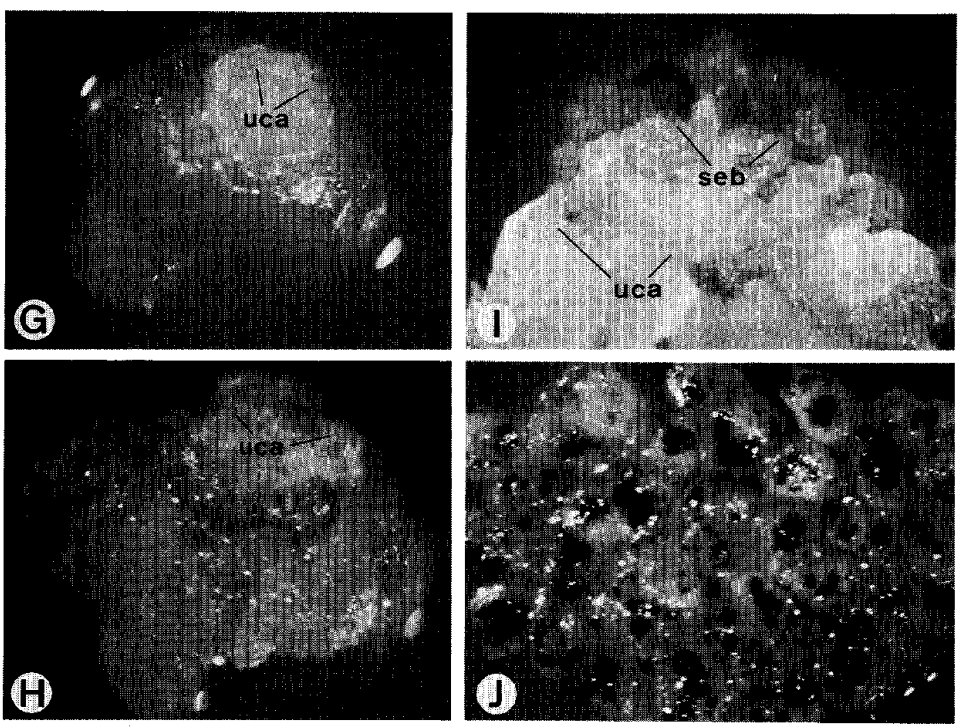

Fig. 4 Transformation of embryogenic calli by microprojectile bombardment. G : Undifferentiated callus induced on the apical cut end of the segment I, 3 days after incubation (×50). H : Somatic embryos differentiated from identical callus shown in G, 1 week after incubation (×110). I : Embryo differentiated from the callus shown in H, 2 weeks after incubation (×110). J : Positively stained GUS-spots on the apical cut surface of the segment I bombarded with pNGI-coated tungsten particles (×10). Uca ; undifferentiated callus ; seb, somatic embryo

stained with 5-bromo-6-chloro-3-indolyl β-D-glucronide by the method of Jefferson [15]. **Fig. 4-J** shows the positively stained spots on bombarded cut surfaces of the segments. The total area of blue-stained spots in each segment were 40-80% of surface area of the cut-end of the shoot tissues, indicating that the introduced GUS gene were expressing in high numbers of proliferating cells.

An important potential advantage of using basal stem tissues for transformation is the relatively short time required for production of embryogenic callus. Without selection, embryogenic calli were produced within 1 week and shoots within 2 weeks after 2 mm segments were excised from 5-day-old germinating seedlings. With selection, embryogenic calli were produced in 10-15 days and shoots within 17-22 days. This may reduce the risk of somaclonal variation. In addition, the use of mature seeds provides a uniform and ready-to-use starting material.

At the time of the experiments described here, we succeeded in developing an alternate method using mature embryos as a source of embryogenic callus for transformation experiments, discontinuing experiments with shoot explants. The mature embryos have proven to be a reliable source of embryogenic callus for transformation. However, two months are required to produce suitable calli in contrast to only 2 weeks with basal shoot tissues. Transient expression of GUS demonstrated here that shoot segments are satisfactory for the introduction of genes by particle bombardment. Since regeneration of plants from calli originating from shoots was very rapid, the use of basal shoot tissue deserves further investigation for stable transformation.

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