Plant regeneration from protoplasts of wheat

(Triticum aestivum cv. Hartog)

D. G. He, Y. M. Yang, and K. J. Scott

Department of Biochemistry, The University of Queensland, Brisbane, Qld. 4072, Australia

Received October 31, 1991/Revised version received November 6, 1991 -- Communicated by H. Lörz

ABSTRACT

Morphologically normal green plants have reproducibly been regenerated from protoplasts of an Australian wheat (Triticum aestivum cv. Hartog). The protoplasts were isolated from fine embryogenic suspension cultures which were initiated from embryogenic callus. Protoplasts were incubated in a modified liquid MS medium containing half strength of the macroelements, 5 μM 2,4-D and 0.6 M glucose. Colonies were formed at frequencies ranging from 0.1% to 5%. The frequency of colonies forming fully developed plants varied between 1% and 25%. More than eighty green plants with morphologically normal shoots and roots have been obtained and there was no difficulty in establishing these plants in soil. A cytological study of several randomly selected regenerated plants showed the normal chromosome complement for wheat (2n = 42).

Key words: Embryogenesis - Plant regeneration - Protoplast - Suspension cultures - Wheat

INTRODUCTION

Protoplasts provide one of the most promising avenues for obtaining transgenic cereals and transgenic plants have been successfully obtained from protoplasts of rice and maize through direct gene transfer (Potrykus 1990). Protoplasts are readily transformed by exogenous DNA, and plants derived from transformed single protoplasts are homogeneously transformed. However, protoplast culture of the economically important cereals has been very difficult to achieve (Vasil 1987, Lörz et al. 1988, Potrykus 1990).

Due to the world-wide economic importance of wheat, many attempts have been made to achieve plant regeneration from wheat protoplasts. However, wheat is one of the most recalcitrant cereal species for protoplast culture (Maddock 1987). Recently there have been a few successful reports of plant regeneration from wheat protoplasts (Harris et al. 1989, Ren et al. 1990, Wang et al. 1990, Vasil et al. 1990, Chang et al. 1991). However, in most cases the experiments were not reproducible (Harris et al. 1989, Ren et al. 1990, Wang et al. 1990, Guo et al. 1991). In only two instances were protoplast-derived plants successfully transferred to soil (Vasil et al. 1990, Chang et al. 1991) and in one the plants were probably not fertile due to chromosomal deletion (Chang et al. 1991).

We now report the highly reproducible regeneration of morphologically normal green plants from protoplasts of an Australian wheat (Triticum aestivum cv. Hartog). These plants are readily established in soil and have the normal chromosomal complement of wheat (2n = 42).

MATERIALS AND METHODS

Cell cultures. In this paper, liquid cultures refer to cultures maintained in a liquid medium and consisting of callus clumps larger than 1 mm in diameter; suspension cultures refer to finely dispersed cultures consisting mainly of small cell clusters, containing less than a few hundred cells. The establishment and subculture of embryogenic liquid cultures and suspension cultures of wheat cv. Hartog were as previously reported (Yang et al. 1991). The non-embryogenic suspension cultures used in this study were initiated from embryo-derived primary callus of cv. Oxley and cv. Timmo; and from protoplast-derived callus of cv. Timmo (He et al. 1990).

Isolation and culture of protoplasts. Cell clusters were collected from either liquid cultures or suspension cultures by filtering through a stainless steel mesh (Swiss Screen), usually 53 μm for fine suspension cultures and 200 μm for liquid cultures. The cells retained on the mesh were transferred to 5 cm Petri dishes (Disposable Products, South Australia) to which a solution containing 2% Cellulase RS (Yakult, Tokyo), 0.2% Pectolyase Y23 (Seishin, Tokyo), 0.6M mannitol was added (solution : cells = 5-10 : 1, v/v). The mixture was maintained on a rotary shaker (ca 50 rpm) for 3-5 hours and then left stationary for another 2-5 hours. The protoplasts were purified by filtering through 53 μm stainless steel mesh once and 38 μm mesh twice followed by 3 washes in a solution containing 20 mM CaCl₂ and 0.6M mannitol. Purified protoplasts were resuspended in 1/2 MS medium (Yang et al. 1991) containing 5μM 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.6 M glucose at a density of 1-10 X 10⁵ protoplasts/ml. The cultures were incubated in 3 cm (Falcon, California) or 5 cm (Disposable, Australia) Petri dishes in the dark at 25 °C. Enzyme solutions, washing solutions and all media for protoplast culture were sterilized by passage through 0.22 μm filters (Millipore).

Differentiation of plants. Protoplast-derived colonies larger than 0.5mm were transferred to a differentiation medium (1/2MS medium, either hormone free or supplemented with different combinations of phytohormones as specified in the text). After 1-4 weeks, the regenerated plants were transferred to a second medium (hormone free 1/2 MS medium containing 1% sucrose). In this paper, fully developed plants refer to those regenerants which had both normal shoots and roots and were at least 3 cm in height, whereas plantlets included both those regenerants which subsequently developed into plants and those which formed only leafy structures. All differentiation media were sterilised by autoclaving.
Cytological studies. Root tips were collected from plants grown in Petri dishes or pots and left in ice-water (0 °C) for 24 hours, 0.03% (w/v) 8-hydroxyquinoline for 3 hours at room temperature and then fixed in acetic alcohol (70% ethanol : acetic acid = 3:1). The fixed tissue was thoroughly washed and then incubated in an enzyme solution (2% Cellulase R10, 1% Macerozyme R10, 0.1% Pectolyase Y23) for 20 min at 37 °C. The cells were immediately fixed in acetic alcohol again. To observe the chromosomes, the cells were squashed in a drop of acetic carmine.

RESULTS
Isolation of protoplasts
Protoplasts were readily isolated from fine embryogenic suspension cultures of cv. Hartog (Fig. 1.A) yielding as many as 5 X 10^7 protoplasts per gram (fresh weight suspension cells). The embryogenic suspension cultures of cv. Hartog tended to grow into large clumps (Yang et al. 1991), a phenomenon also noted in other wheat cultivars (Wang et al. 1990). After 7-10 hours digestion, these large cell clusters (>1mm) were still quite solid and the yield of protoplasts was low. In one experiment, the suspension cells were separated into five different size groups by filtering the suspension cultures through stainless steel meshes of decreasing pore size (1200 to 53 μm). The yield of protoplasts from these groups varied from 0.3 to 5 x 10^7 (Fig. 2); the group (175 to 750 μm) had the highest yields; with the large clusters (>1200 μm) spontaneous fusion and rupture of protoplasts were frequently observed.

The freshly isolated protoplasts often contained large starch granules. The protoplasts containing these granules were quite fragile, resulting in their rupture during centrifugation. However, the number of cells with these granules decreased as the suspension cultures aged.

First cell division (Fig. 1.B) was observed following one week incubation. As the dividing cells usually contained dense cytoplasm and many small granules, the newly formed cell walls between two daughter cells were not always visible, and frequently the small colonies showed a irregular contour indicating the outer cell walls (Fig. 1.C). After 30-40 days incubation, colonies became macroscopically visible (Fig. 1.D). The frequency of colony formation varied from 0.1% to 5%.

Fig. 1. Plant regeneration from embryogenic protoplasts of cv. Hartog. A. Freshly isolated protoplasts from the embryogenic suspension cultures of cv. Hartog. B. First cell division of protoplast-derived cells. C. Microscopic colonies (two weeks of incubation). D. Visible colonies after 40 days of incubation. E. Formation of embryogenic callus on 2,4-D containing (5 μM) medium. F. Typical embryoid forming on protoplast-derived embryogenic callus (v. ventral scale). G. Formation of a plantlet from a bipolar structure. H. Plants with morphologically normal shoots and roots. I. A root-tip cell from protoplast-derived plant showed the normal wheat chromosome complements (2n = 42). (Bar represents 20 μm in A, B and C; 1 mm in D, E and G; 0.25 mm in F; and 1 cm in H)