Time Course of Hormonal Control of the First Mitosis in Tobacco Mesophyll Protoplasts Cultivated In Vitro

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Abstract. The presence of auxin and cytokinin is necessary for the induction of mitosis in tobacco mesophyll protoplasts cultivated in vitro. In their absence, the protoplasts firstly accumulate inhibitors of mitosis in the culture medium, possibly because of non-coordinated cell-wall synthesis, and secondly evolve a non-mitotic and degenerative metabolism. By changing the intoxified medium, it is possible to show that auxin is necessary from the beginning of culture, while cytokinin is only required later to allow a step in the development of the mitotic apparatus.

Key words: Mitosis — Nicotiana — Phytohormones — Protoplasts.

Introduction

Tobacco mesophyll protoplasts are obtained from cells which last divided during the growth phase of the leaf, this being 4 to 6 weeks before isolation of the protoplasts. During this time the mesophyll cells show an active but non-mitotic metabolism. During the first two days of in vitro culture the protoplasts undergo considerable cytological changes, leading to mitosis (Nagata and Takebe, 1970). This development is dependent on the presence of auxin and cytokinin in the culture medium. Thus, this experimental system is particularly well suited to the study of mitosis and its hormonal control in a higher plant. The goal of the present study is to determine in which processes and at which times the hormones are involved in the development leading to mitosis.

Materials and Methods

Tobacco plants (Nicotiana tabacum var. Maryland) were grown in a greenhouse and used just before flowering. Protoplasts were isolated by the method previously described (Meyer and Abel, 1975a; b), except that cellulase was purified by dialysis (Capesius and Meyer, 1977). All experiments were carried out in medium WO.6 (Meyer and Abel, 1975b), with or without hormones. Dichlobenil (Serva, Heidelberg, F.R.G.) was added in some experiments in order to inhibit wall formation (Meyer and Herth, 1978). Nuclei were stained with orcein after glutaraldehyde fixation of protoplasts (Capesius and Meyer, 1977).

Determination of mitotic frequency: Freshly isolated protoplasts constitute a population of free cells. The proportion of cells having undergone their first mitosis was estimated from the percentage of cells, or groups of cells, which were polynucleate. This figure is a slight underestimate toward the end of the culture, because some cells become detached from the groups and are counted as non-divided.

Results

Variability in the Development of Different Protoplast Batches

Our method of isolation of protoplasts produces protoplast batches showing very little contamination with cell debris. The frequency of dicaryotic protoplasts is always less than 1:1000, due to the use of mature tobacco leaves. During culture less than 20% of protoplasts burst in medium WO.6 and at least 75% of the remainder have undergone mitosis after 120 h. However, in complete medium, the gradients of the mitotic curves show small variations, but these
variations become much greater in deficient medium. Taking this into account, each experiment was repeated at least four times.

**Determination of Optimal Hormone Concentrations**

Optimal development, as estimated from the slopes of the mitotic curves, was found for a 2,4-D concentration between 0.5 and 2 mg l\(^{-1}\) (2-8 \(10^{-3}\) mM), and a 6-BA concentration between 0.1 and 10 mg l\(^{-1}\) (0.4-40 \(10^{-3}\) mM). These values are in agreement with those obtained previously by measurement of cell proliferation after 10 days of culture (Meyer and Abel, 1975b). In addition, in contrast to soybean and tobacco cells cultivated in vitro (Witham, 1968; Peaud-Le Noel, 1971), a cytokinin deficiency cannot be compensated for by superoptimal doses of auxin. The remainder of the experiments (unless otherwise stated in the text) were carried out at a concentration of 1 mg l\(^{-1}\) (4 \(10^{-3}\) mM) of each hormone. We have also carried out similar experiments using NAA and IAA with analogous results.

**Effect of Deferred Addition of Hormones**

In this group of experiments, cells were cultivated for varying times in medium deficient in auxin, cytokinin, or in both hormones. At the end of deficient culture, the absent hormone was added as a concentrated solution of 10 mg l\(^{-1}\) (40 \(10^{-3}\) mM) in the culture medium. Controls showed that this addition of fresh medium during culture does not affect development in complete medium. When culture in the medium deficient in both hormones continues for 24 h, the mitotic curve is shifted by a similar time and is much shallower (Fig. 1). After 48 h deficiency, addition of hormones can no longer induce mitosis. However, there is a wide variation in this development between protoplast batches; certain batches can not be induced to undergo mitosis after a 24 h preculture in deficient medium, whereas others can be partially reversed up to 72 h. It was observed that protoplast batches which rapidly regenerate a wall in complete medium are those which are the most sensible to culture in deficient medium.

If the medium is deficient solely in auxin, the subsequent development is very similar to that obtained with a deficiency of both hormones. However, experiments carried out with the same batch of protoplasts show that presence of cytokinin slightly increases the effects of the deficiency.

The effect of a deficiency solely in cytokinin (Fig. 2) is different. Not only is reversion possible after 72 h of deficiency, but the time between cytokinin addition and the first mitosis also shortens with an increasing duration of deficiency.

**Effect of Changing the Medium after Deficiency**

In this group of experiments protoplasts were cultivated in a medium deficient in one or both hormones, then recovered by centrifugation and replaced in cul-