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EFFECTS OF CHLORAMPHENICOL ON PLANT CELLS: POTENTIAL AS A SELECTABLE MARKER FOR TRANSFORMATION STUDIES

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The growth of tobacco and carrot cells in suspension cultures and on solidified medium was strongly inhibited by the antibiotic chloramphenicol. This effect was irreversible. The drug had no effect on [^{3}H]-uridine incorporation but inhibited protein synthesis and induced striking morphological alterations in intoxicated cells. O_2 consumption was also reduced in treated cells. Tobacco and carrot cells were unable to inactivate chloramphenicol by enzymatic acetylation.

INTRODUCTION

The possibility to introduce foreign genes into plant cells and protoplasts through Agrobacterium tumefaciens infection (1,2) and fusion with DNA-loaded liposomes (3-9) or A. tumefaciens spheroplasts (10) has generated interest in the search for genetic markers selectable in plant cells. Auxotrophic mutants in plants are extremely rare, and among these, only the tobacco and barley nitrate reductase-deficient mutants have been well characterized, both genetically and biochemically (11,12). On the other hand, prokaryotic genes linked to suitable promoters have been shown to be expressed in mammalian cells (13) and in yeast (14-16). Interestingly, it has been shown recently that the E. coli gene coding for chloramphenicol acetyltransferase can be expressed in mammalian cells when coupled to an early SV 40 promoter (C. Gorman, personal communication). Therefore, it is important to determine whether antibiotics in general and chloramphenicol in particular are toxic to plant cells. If such were the case, certain antibiotics could be used in transformation experiments involving bacterial transposons known to code for the resistance to these antibiotics,

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coupled to promoters which can be recognized by plant RNA polymerase II. This paper shows that chloramphenicol may be used as a selective agent in such experiments.

MATERIALS AND METHODS

Daucus carota L. and Nicotiana xanthi cells were grown in fine suspensions at 29°C as described previously (9,17). Cell growth was monitored by taking 10 ml aliquots of culture medium and measuring the packed cell volume (PCV) after centrifugation at 1,000 rpm for 10 minutes in a GLC-1 Sorvall centrifuge. Chloramphenicol was purchased from Sigma and was used without purification. Photomicrographs of control and intoxicated cells were taken in a Leitz microscope. The oxygen consumption of control and chloramphenicol-treated cells was measured with a Rank and Brothers oxygen electrode (Cambridge, England) at 23°C using a 0.4 ml PCV in 1 ml of culture medium. Tobacco cells in logarithmic phase of growth were treated for four days with 50 µg/ml chloramphenicol prior to O_2 consumption determination.

The incorporation of radioactive precursors was measured after resuspending 0.2 ml PCV of tobacco cells in 1 ml fresh medium and preincubating these cells for 30 minutes at 23°C in the presence of increasing concentrations of chloramphenicol. [^{3}H]-uridine (ICN, 50 Ci/mmole) or [^{3}H]-leucine (NEN, 152 Ci/mmole) were added to a concentration of 5 µCi/ml and aliquots were precipitated as a function of time with cold 5% trichloroacetic acid. Precipitates were harvested by centrifugation and washed four times with 5 ml cold 5% trichloroacetic acid. The pellets were then dissolved in 0.2 ml hyamine hydroxide and counted by liquid scintillation in acidified cocktail.

The fate of [^{14}C]-chloramphenicol (NEN, 43.2 mCi/mmole) in plant cell extracts was studied by thin layer chromatography. Protoplasts were prepared from tobacco and carrot suspension cells as described in Rollo et al. (5). Protoplasts were homogenized, centrifuged (15 min in Eppendorf microfuge at 4°C) and the supernatant assayed at 37°C in the presence of 2.8 µCi/ml [^{14}C]-chloramphenicol and 0.4 mM acetyl CoA for 30 min as described in Gorman et al. (18). The chromatograms were developed as described in Shaw and Bradsky (17). Chloramphenicol and its acetylated forms were detected by fluorography. Cowpea mesophyll protoplasts were used as an additional control and were prepared as in Lurquin (3). β-lactamase activity in protoplast extracts was determined by the Nitrocefin spectrophotometric assay technique (21). Nitrocefin was a gift from Glaxo.

RESULTS AND DISCUSSION

The growth of both tobacco and carrot cells was already strongly inhibited at 10 µg/ml chloramphenicol and was totally inhibited at 100 µg/ml (Fig. 1). Cells treated with 100 and 200 µg/ml chloramphenicol were kept in culture for up to 60 days without noticeable increase of PCV. The effect of the drug was irreversible since aliquots of cells treated with 200 µg/ml chloramphenicol for 16 days failed to resume division after transfer to fresh medium devoid of drug. Cells plated on agar medium containing 100 µg/ml chloramphenicol also failed to grow. However, on plates inoculated with the equivalent of 0.3 ml PCV a

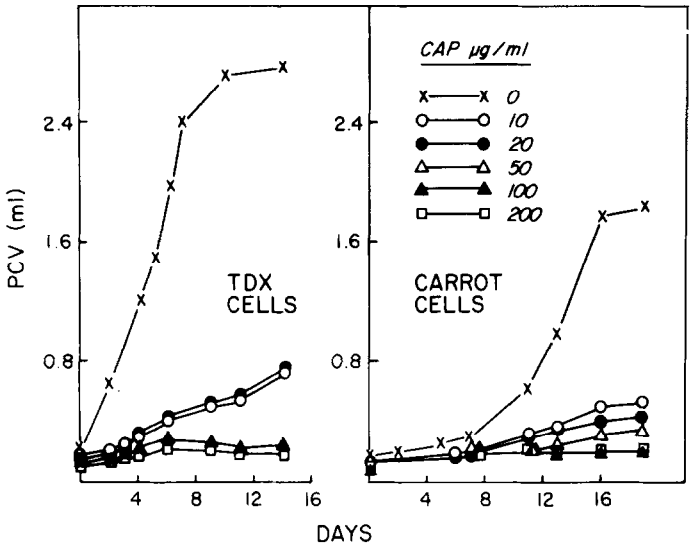

Fig. 1. Growth curves of tobacco and carrot cells without chloramphenicol, X-X and in the presence of 10 µg/ml, O-O; 20 µg/ml, ●-●; 50 µg/ml, △-△; 100 µg/ml, ▲-▲ and 200 µg/ml, □-□; chloramphenicol.

few pinpoint size colonies appeared after two weeks of incubation. Due to large size of the inoculum, it was not clear whether these cells represented chloramphenicol-resistant variants or cells partially shielded from the medium by dead cells. Figure 2 shows the morphological effects of 200 µg/ml chloramphenicol on tobacco cells after 15 days in culture. Control cells contained a large central vacuole, a clearly distinguishable nucleus and a thin cytoplasmic layer. Intoxicated cells displayed a shrunken cytoplasmic mass pushed away from the cell wall, no central vacuole and no distinct nucleus. In addition, the yellow pigmentation typical of healthy cells was absent from chloramphenicol treated cultures.

Figure 3 shows that short term (30 minutes) incubation with a lethal dose of chloramphenicol did not affect the incorporation of [^{3}H]-uridine into TCA-precipitable compounds but had an appreciable effect on the incorporation of [^{3}H]-leucine. Chloramphenicol blocks protein synthesis in prokaryotes by binding to the ribosome. Plant cells in suspension cultures do not possess fully formed chloroplasts and therefore have to rely on mitochondrial functions to generate most of their ATP. It is conceivable that chloramphenicol might interfere with plant mitochondrial ribosomes and shut off protein synthesis in

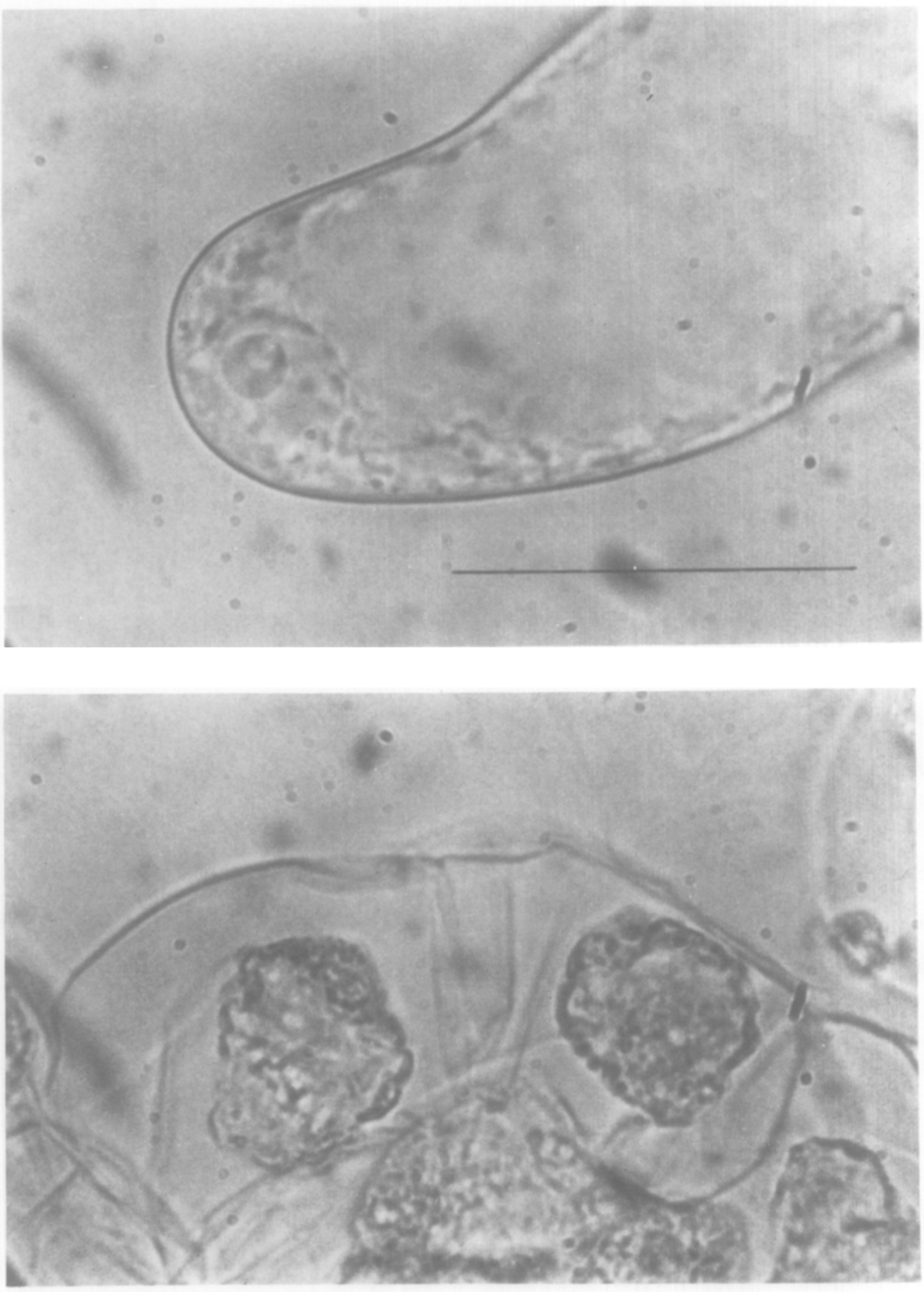

Fig. 2. Micrographs of normal tobacco cells (top) and cells treated for 15 days in the presence of 200 μg/ml chloramphenicol (bottom). Line represents 50 μ.

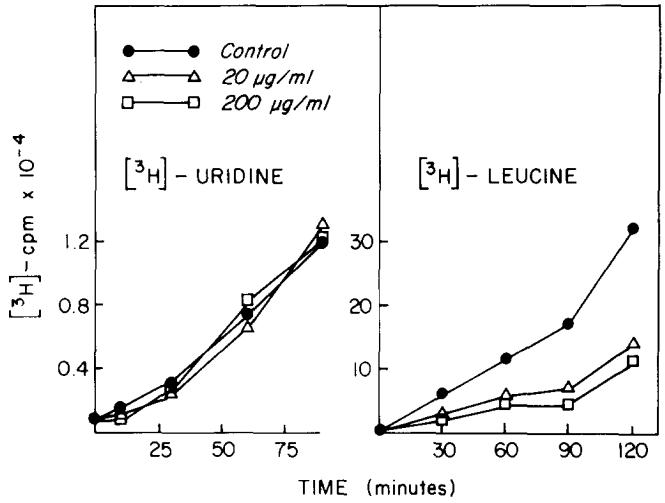

Fig. 3. Incorporation of [^{3}H]-uridine and [^{3}H]-leucine by tobacco cells in the absence of chloramphenicol, ●-● and in the presence of 20 µg/ml, △-△ and 200 µg/ml, □-□ of the drug. Cells were preincubated for 30 minutes prior to addition of the radiolabeled precursors.

those organelles. The same phenomenon might also happen in proplastids. Other effects of chloramphenicol on plant cells cannot be ruled out at the present time. For example, it has been reported that in Neurospora mutants hypersensitive to this drug, chloramphenicol is acting on the oxydase pathway (19).

That chloramphenicol has at least an indirect effect on mitochondrial functions is shown in Figure 4. This experiment showed that tobacco cells treated for four days with 50 µg/ml chloramphenicol had a reduced rate of oxygen consumption as compared to untreated control cells.

Even though the molecular mechanisms responsible for the lethal effect of chloramphenicol on plant cells are not known, it seems that this antibiotic can be considered as a good selective agent in transformation experiments. The *E. coli* transposon Tn 9 coding for the inactivation of chloramphenicol by transacetylation could be cloned in a suitable plant vector such as the T-DNA of *Agrobacterium tumefaciens* pTi and introduced into plant cells. Selection of transformants could be performed on chloramphenicol-containing medium. Putative transformants could then be assayed for the presence of chloramphenicol acetyltransferase by thin layer chromatography. Figure 5 indicates that tobacco and carrot cells used in this study did not contain enzymes capable to acetylate

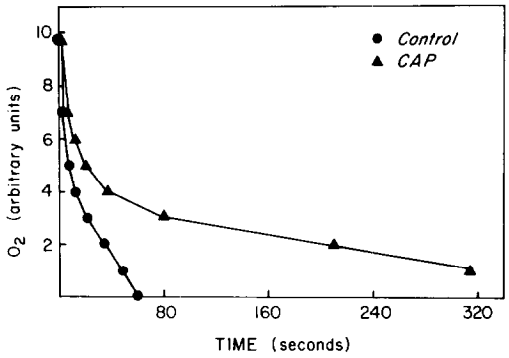

Fig. 4. Oxygen consumption of normal tobacco cells, ●-● and of cells grown for four days in the presence of 50 µg/ml chloramphenicol, ▲-▲.

chloramphenicol in the presence of acetyl CoA. Cowpea mesophyll protoplasts were also found to be devoid of chloramphenicol acetyltransferase activity.

A gentamycin derivative has been recently proposed to serve as a selective agent in plant transformation experiments (20). Our work shows that chloramphenicol is also suitable for this purpose. We have also observed that kanamycin and trimethoprim strongly inhibited the growth of the tobacco cells used in this study (Lurquin, unpublished).

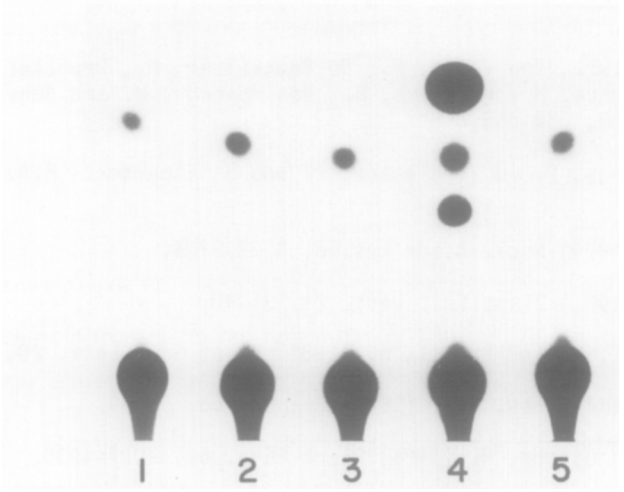

Fig. 5. Thin layer chromatography analysis of [^{14}C]-chloramphenicol incubated with protoplast extracts. Approximately 10^8 protoplasts were used per ml of buffer. 1 - cowpea, 2 - carrot, 3 - tobacco TDX, 4 - E. coli chloramphenicol acetyltransferase, 5 - blank. The spot appearing in the blank and the other preparation is presumably due to nonenzymatic breakdown of the ^{14}C-chloramphenicol during incubation.

Finally, since the gene coding for E. coli β-lactamase is often present on recombinant DNA vectors, it was of interest to check whether β-lactamase expression could be used as an unselectable marker in plant cells. We found that the sensitive assay based on the visible light spectrum modifications of Nitrocefin (21) was difficult to apply in the case of our tobacco and carrot cells. Indeed, cell extracts induced partial red shifts in the Nitrocefin spectrum after prolonged incubation periods (15 hours or more). For comparison, a single colony of Escherichia coli harboring pBR322 completely degraded (red-shifted) 0.1 μmole of Nitrocefin in about 15 minutes. Therefore, this technique will be applicable only in cases where a strong response is observed. In addition, we found that the rate of degradation of Nitrocefin by carrot and tobacco extracts was quite variable. However, no red shift was detectable between 0 and 6 hours of incubation of 0.1 μmole Nitrocefin with an extract corresponding to 10^7 protoplasts.

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