REGENERATION OF TOBACCO PLANTS FROM CROWN GALL TUMORS

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SUMMARY

Tissue culture methods have been developed for regeneration of normal appearing tobacco plants from bacteria-free crown gall strains incited by Agrobacterium tumefaciens C58, II BV7, B6, CGIC, A6NC, 27, and AT4. Regenerants fall into two categories depending on the properties of tissues from these plants. The first type of regenerant was obtained from tumors incited by A. tumefaciens C58 and it retained the potential for expression of tumor characteristics such as a nonrequirement for phytohormones (auxin and cytokinin) by explants in vitro and the presence of detectable concentrations of nopaline. Normal appearing plants obtained from C58 tumors had much lower concentrations of nopaline than the corresponding tumor tissue (130 versus 1700 μg per g dry wt) indicating a parallel repression of abnormal growth and nopaline concentrations in regenerants. The second type of regenerant was obtained from tumors incited by the other A. tumefaciens strains and was characterized by requirements for phytohormones by explants in vitro and the apparent lack of octopine or nopaline in regenerant tissues.

Key words: regeneration; crown gall tumor; auxin; cytokinin; octopine; nopaline.

INTRODUCTION

Evidence to date suggests that the transformation causing plant crown gall tumors involves incorporation of genetic information from a Ti plasmid, contained in an Agrobacterium tumefaciens strain, into the plant cell followed by replication and expression of this DNA as the tumor grows (1). Presumably, as a consequence of these processes, crown gall tissues express unique characteristics such as the nonrequirement in vitro for the phytohormones auxin and cytokinin and, in most cases, the production of large quantities of the arginine derivatives, octopine or nopaline (2).

Two laboratories have reported on the regeneration of normal appearing tobacco plants from crown gall tumors using tissue strains originally cloned from single cells. Braun and co-workers (3,4), using a tobacco tissue strain incited by A. tumefaciens strain T37, obtained results that were interpreted as evidence for the retention of tumor characteristics in regenerated plants. Sacristan and Melchers (5,6), on the other hand, who worked with tobacco tissue strains transformed by A. tumefaciens strain 542, found no evidence for tumor characteristics either in regenerated plants or in tissue obtained from these plants and cultured in vitro.

The question of whether normal appearing plants derived from crown gall tumors can retain and express implanted DNA is relevant not only to an understanding of the biochemistry of the disease but also to possible uses of crown gall as a tool for plant genetic manipulation (1). In this paper, we report on methods developed for regenerating normal appearing tobacco plants from crown gall tumors and on results of analyses of tissues from these plants for phytohormone requirements in vitro and for concentrations of octopine and nopaline. Our results show that plants regenerated from crown galls are of two types characterized by expression or nonexpression of tumor characteristics. The properties of tissues from regenerated plants appear to be dependent on the strain of A. tumefaciens that incites the original tumor.
MATERIALS AND METHODS

Sucrose and the organic growth factors myo-inositol, thiamine·HCl, indole-3-acetic acid (IAA) and kinetin were all obtained from Sigma Chemical Company as was octopine. Murashige and Skoog (7) salt mixture and Phytagar were obtained from Grand Island Biological Company. Phenanthrenequinone was obtained from ICN Pharmaceuticals, Inc. The chemically synthesized nopaline (8) was a gift from E. W. Nester, University of Washington.

Crown galls were induced on young tobacco (Nicotiana tabacum cv. Wisconsin 38) plants in the greenhouse by inoculation of an A. tumefaciens strain through a wound made on the stem with a scalpel. After 2 to 4 weeks, tumors were excised from host plants and surface cleaned by vigorous washing in dishware detergent followed by a cold water rinse. Tumor tissue was then infiltrated with a solution containing 150 mg per l citric acid and 100 mg per l ascorbic acid as antioxidants. Subsequent sterilization was by soaking in 0.5% sodium hypochlorite for 10 min and a brief dip in 95% ethanol followed by flaming. Tissue pieces were then cut from the inner part of the tumor and planted in tubes with 20 ml of an agar basal medium for crown gall tissues that contained inorganic nutrients as specified by Murashige and Skoog (7): 30 g per l sucrose, 100 mg per l myo-inositol, and 0.4 mg per l thiamine·HCl, pH 5.6 (9). Cultures were incubated in the dark at 27° C. Tissue from the small percentage (approximately 5%) of cultures free of bacterial and fungal contamination were subcultured to the same basal medium for 1 month and then maintained as stocks on this medium with monthly subculture. The collection of tobacco tumor strains categorized according to the inciting A. tumefaciens strain is listed in Table 1 (10).

To regenerate plants from crown gall tumors, tissues were transferred to tubes with basal medium plus 2 mg per l kinetin and incubated for 6-12 weeks in the dark. Calli forming compact, knob-like shoot primordia were transferred to 50 ml of basal liquid medium in flasks that were incubated in the dark on a New Brunswick G10 gyratory shaker (150 rpm). Primordia grew out rapidly during 2 weeks to form elongated shoot axes, which, in most cases, were rootless. These etiolated shoots were then transferred to fresh basal medium under illumination and cultures were aerated with bubbling air. In the cases of tumors incited by all A. tumefaciens strains except strain C58, green rooted plants could be produced in 1 week. These were transplanted to pots of soil in the greenhouse where the plants grew normally, flowered, and set viable seed within 4 months. We have successfully grown plants to seed from tumors incited by A. tumefaciens strains HVB7 (2 plants), B6 (13 plants), CGIC (7 plants), A6NC (9 plants), 27 (5 plants), and AT4 (1 plant). Tumors incited by A. tumefaciens C58 could not be grown as normal plants beyond the etiolated shoot stage.

Tissues from regenerated plants at the etiolated shoot stage (internodal stem segments, 2 mm thick) and at the greenhouse plant stage (pith sections, 2 mm thick) were analyzed for in vitro requirements for auxin and cytokinin by transferring tissue to basal agar medium in tubes with or without 0.1 #M kinetin and/or 11.4 #M IAA. Fresh weight yields were determined after a 5-week growth period in the dark at 27° C. For studies of the requirements of subcultured tissue, callus pieces (10 mg) were used.

For octopine and nopaline determinations 1 g of tissue was mixed with 1 ml of water and 50 mg activated charcoal in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 5° C for 20 min at 10,000 ×g. After decantation the supernatant was spotted on Whatman 1M paper (15 cm × 23 cm) and electrophoresed in 0.05 N formic acid, pH 3.6 at 5 mA for 2 hr. Standard octopine and nopaline samples were run parallel to the tissue extracts. Octopine and nopaline were visualized under short UV illumination after staining with phenanthrenequinone reagent (11). The limit of detection of octopine and nopaline was 0.1 µg; i.e. 50 µg per g dry weight.

RESULTS

Regeneration of tobacco plants. All tumor strains grew as undifferentiated callus on the