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Tumorization-Redifferentiation System of Tobacco Genetic Tumor

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Genetic tumor was easily and rapidly induced by cutting treatment of tobacco (*Nicotiana glauca* × *N. langsdorffii*) F_1 seedlings in a simplified system in vitro. When this tumor was cultured under weakened light intensity normal shoots developed frequently. After these shoots were maintained under appropriate light conditions (ca. 2–3 W/m^2) they regenerated into whole plants with normal appearance. The regenerated plants were identical to those developed after germination with respect to their response to cutting treatment. Using this system we are able to switch the morphology between the tumorous state and the normal state rapidly and with ease. The significance and the implications of the availability of this system for studies of the mechanism of conversion between these two states at the molecular level are discussed.

Key words: Cutting treatment — Genetic tumor (tobacco) — Light condition — Redifferentiation.

In general it is believed that the causes of tumor formation derived from the mutation of genes or aberrant gene regulation (Melchers 1971). The occurrence of teratomas in interspecific hybrids obtained by crosses between *Nicotiana glauca* and *N. langsdorffii* is probably the result of aberrant gene regulation. Thus, there is a possibility that we can identify the gene regulatory mechanism and gain clues towards the understanding of the morphogenesis of the higher plant itself by studying of genetic tumors.

White (1939) showed that whole plants can be regenerated from tumorous tissue. The same type of differentiation was also observed by Skoog (1944), Kehr and Smith (1953), Hagen (1962) and Kehr (1965) in studies of genetic tumors. The conditions for differentiation in these early experiments, however, are complicated, for example, tumor tissue must be cultured on a medium with a large number of complex components or submerged in liquid medium and cultured at low temperature and in weak light. Moreover, the differentiation takes a greater deal of time. As the most of the material hitherto used to study the nature of genetic tumors has been grown in the green house (Bayer 1982), there are many problems. In the system in vivo it is hard to generate tumor tissue, and it remains possible that microorganisms may influence the formation of tumor. Thus, it is indispensable that we establish an appropriate system in vitro in order to study the nature of genetic tumors at the molecular level. The purpose of the present study was to establish a system in vitro under simplified conditions in which two types of differentiation can easily and reproducibly be induced: differentiation from morphologically normal tissue to tumor tissue and reversion from the tumor tissue to normal tissue.

Abbreviations: MS, Murashige-Skoog; NAA, α-naphthaleneacetic acid; RZ, ribosylzeatin.

Materials and Methods

Induction of tumors from morphologically normal F_1 tissues—Seeds of an F_1 hybrid were obtained by pollinating *N. glauca* with *N. langsdorffii*. The seeds were sterilized for 5 min in a 3% solution of sodium hypochlorite, then rinsed thoroughly with sterilized distilled water. They were germinated aseptically on MS basal medium (Murashige and Skoog 1962) which was solidified with 1.0% (w/v) agar in 100-ml Erlenmeyer flasks. Seedlings were grown in a controlled environment room at ca. 27°C under ca. 3.3 W/m^2 illumination. Internodes of F_1 hybrid plants, about 5 cm in height, were aseptically cut into pieces approximately 3 mm in length. These internode tissues were cultured on MS basal agar medium at ca. 27°C under cool white fluorescent lamps (ca. 10 W/m^2) in growth chamber.

Reversion to morphologically normal tissues from tumor tissues—F_1 hybrid tumors induced by the above method were successively subcultured at monthly intervals on the same basal medium and in the same controlled environment as used in the case of tumor induction. These tumor tissues were transferred onto versions of MS solidified medium, in which levels of components such as Ca^{2+}, Mg^{2+}, Fe^{2+}, vitamins, and/or sucrose, were cut down to 50% or zero. Other samples of tumor tissues were cultured on basal MS solidified medium in a controlled environment chamber at various temperatures, (17°C, 22°C, 27°C, 32°C, 38°C, 40°C) under ca. 10 W/m^2 illumination, or at 27°C under illumination of various intensities (10 W/m^2, 0.099 W/m^2, 0.027 W/m^2, 0.010 W/m^2), or in complete darkness. The degree of redifferentiation from tumor tissues was expressed as the sum of the length of shoots which were morphologically normal and were more than 5 mm in height.

Regeneration of whole plants—After cutting at a boundary region between the redifferentiated shoot and the tumor, the shoot was stuck into MS solidified basal medium and cultured at 27°C under various intensities of illumination (12 W/m^2, 2.8 W/m^2, 0.24 W/m^2, and complete darkness). The degree of regeneration of whole plant was expressed by the percentage of plants that exhibited normal stems and leaves and regained apical dominance.

Results

Spontaneous formation of tumors in vitro—Under normal field conditions and in the greenhouse, tumors appear ordinarily at the end of vegetative growth and rarely appear at the early or the middle phase of vegetative growth. By contrast, in the in vitro system described in "Materials and Methods" the spontaneous formation of tumor was frequently observed without difficulty on all parts of the F_1 plantlets within a month, most frequently at the upper axillary nodes, leaf scars, and on areas subjected to stress, such as tissues that touched the surface of the glass culture flask (Fig. 1) and wounded tissues. Some seeds gave rise to tumor just after germination, and some gave rise to tumors around the border line between the stem and agar medium (Fig. 2).

Tumor induction—As mentioned above, the F_1 hybrid was apt to develop tumor under stress conditions. Therefore, several kinds of stress (see "Materials and Methods") were tested for induction of tumors from normal F_1 plantlets. Light conditions, high and low temperatures and absence of some components from MS medium had no effect on the formation of tumor. However, we were able to induce tumor tissues from normal F_1 tissues when they were cut or injured. Fig. 3 shows the typical response of normal F_1 tissues after explantation of cuttings of stem internodes on MS basal medium. The tumors first appeared from a cut end as white small protuberances and gradually grew into teratomas with rudimentary buds but rarely exhibited evidence of root formation. The same types of tumor also developed from the

Fig. 1 F_1 seedlings cultured for about one month after germination. Arrow indicates the tumor tissues that arose from the stem that had touched the surface of the glass culture flask.

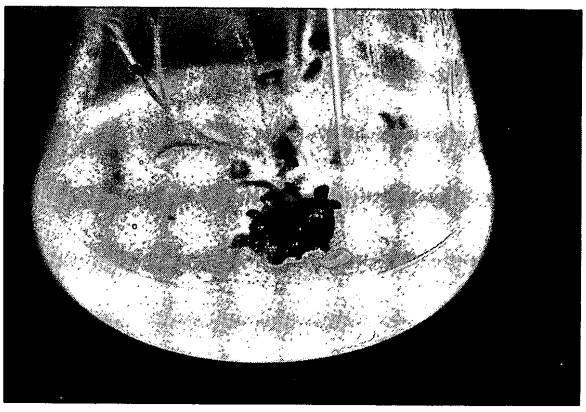

Fig. 2 Tumors that developed spontaneously at the transitional region between shoot and root. 4 weeks after germination.

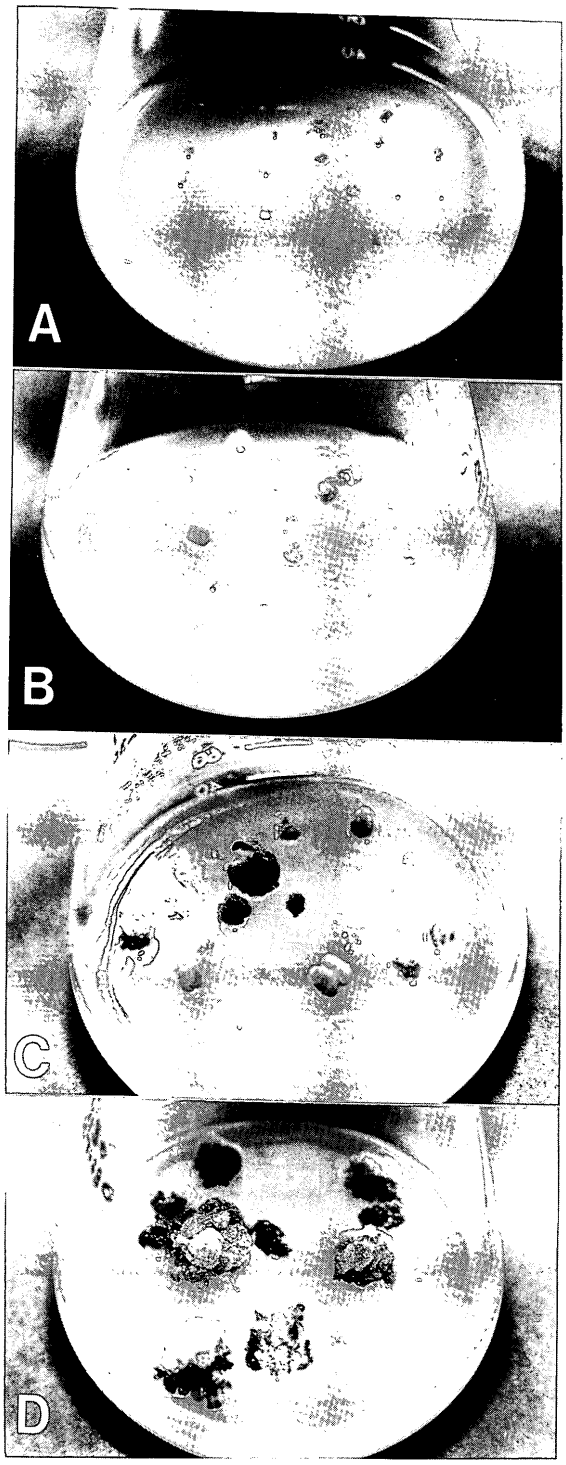

Fig. 3 The response of F_1 tissue after cutting treatment. (A) Normal F_1 stems were cut into pieces of 2 mm in length, then placed on MS basal medium. (B) After 5 days of culture. The stem became thicker and the small green protuberance appeared from the cut end. However, with the naked eye these changes were not very conspicuous. (C) After 15 days of culture. Partially organized teratomas with rudimentary buds appeared from the cut end and the stem became thicker. (D) After 22 days of the culture.

cutting end in the decapitated region of normal F_1 seedlings.

Induction of normal shoots—As in the case of tumor induction, various culture conditions, including stress, were applied to induce normal shoots from tumor tissues. Adding of NAA (from 0.001 to 100 ppm) or RZ (from 0.04 to 10 ppm) and removal of some of the components of MS medium had no effects on shoot formation. However, as shown in Fig. 4, light intensity, temperature and prolonged culture period affected the formation of shoots. Similar results were obtained reproducibly in several identical experiments. Consequently, we concluded that the weaker the light intensity, the more frequent the occurrence of normal shoots. This result implies that there is no threshold light intensity for the normal induction of shoots. However, these causes of shoot formation were divided into two patterns: the formation of long shoots of which a few appeared in a given culture flask, and the formation of short shoots of which several dozens or more appeared in a given culture flask. These two patterns of tumor formation never arose at the same time in the same culture flask. These results were obtained when we used a tumor line that had been subcultured for more than a year, that is, normal shoots were induced within a month by culturing the tumor at 37°C in complete darkness. By contrast, when we used tumors which were transferred just after tumor formation

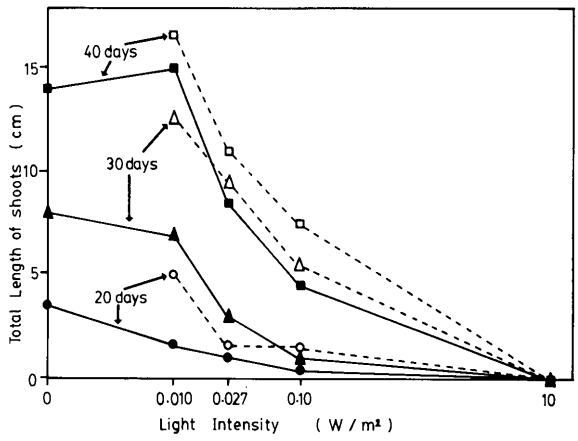

Fig. 4 Effects of light intensity and temperature on the regeneration of morphologically normal shoots from the F_1 tumor line. A piece of tumor subcultured for more than a year at 27°C under continuous light (ca. 10 W/m^2) was transplanted onto fresh medium and cultured for 20, 30 and 40 days under various conditions of temperature and light intensity: temperature of 27°C (—) or 37°C (----); and light intensities of 10 W/m^2, 0.099 W/m^2, 0.027 W/m^2, 0.010 W/m^2 and complete darkness. The heights of all of the shoots which exhibited normal morphology and were more than 5 mm in height were added together. Values on the ordinate represent the mean of total shoot lengths per culture flask. These data are taken from one of three representative experiments.

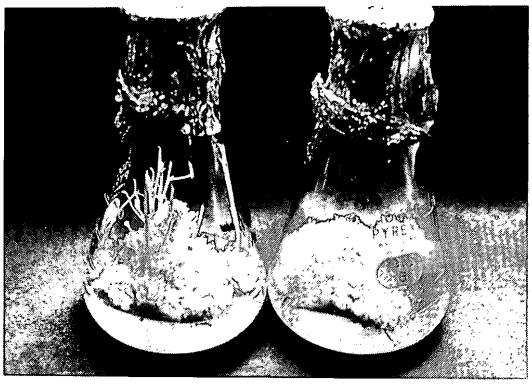

Fig. 5 Effects of temperature on normal shoot regeneration in two types of tumor line subcultured for different times. Tumor tissues from two different lines were cultured for a month at 27°C in complete darkness. The right flask contains a tumor line which was subcultured for more than a year at 27°C under continuous light conditions (ca. 10 W/m^2). The left flask contains a newly established tumor line of 21 days of age after induction at 27°C under light condition. It was transplanted onto fresh medium and transferred to the dark. The tumor line on the left differentiated normal shoots even at 27°C.

(after 21 days of culture), normal shoots were induced within a month even when tumor tissues were cultured at 27°C in complete darkness (Fig. 5). Thus, the condition of tumorous tissue (number of subculturings) affects the efficiency of induction of normal shoots at various temperatures.

Regeneration of whole plants—After normal shoots which were more than 3 cm in height were stuck into fresh medium, as described in 'Materials and Methods', they were cultivated under the light conditions. They gradually grew into green shoots within a week, then into shoots that developed normal leaves, and finally into whole plants within a month (Fig. 6). However this process was nearly completely inhibited by culturing the shoots under high-intensity light (over 10 W/m^2), which spontaneously caused teratomas at the apical or axillary meristematic regions (Fig. 7). By contrast, considerable number of the shoots exhibited a normal phenotype and maintained apical dominance after 2 weeks of growth in twilight condition. As shown in Fig. 8, light intensity of less than 2.8 W/m^2 was sufficient to maintain the normal phenotype. The weaker the illumination, the more the shoot maintained a normal appearance, although light was indispensable for differentiation of roots (Fig. 8).

Tumor induction from newly formed normal tissue—When the above newly formed normal tissues were excised and transplanted again onto new media, every tissue developed tumors in just the same manner as the induction of tumors from normal tissues grown from F_1 seeds.

Fig. 6 Regeneration of a whole plant with normal appearance from F_1 tumor tissues. A normal shoot that developed from tumor tissues was stuck into fresh medium, then cultured at 27°C, for a month, under controlled light conditions (first ca. 0.03 W/m^2, then ca. 3 W/m^2). This regenerated whole plant had roots, a thicker stem, larger leaves and rudimentary tumorous tissues at the transitional region between shoot and root.

Discussion

In the greenhouse, genetic tumors appear most frequently on mature hybrid plants during and after the flowering period, when growth of the shoot apex is reduced (Bayer 1982). Consequently, it takes a long time, usually more than 6 months, to induce tumor tissues. By contrast, in our in vitro system, spontaneous tumors developed on the plantlets more frequently and at an earlier stage. Moreover, tumors tissues could be induced synchronously on the every segment excised from morphologically normal plantlets, without any exceptions. The process was very rapid under strictly controlled conditions; active cell division started immediately and visible tumors could be observed within 5 days after the cutting treatment. This result means that we can obtain genetic tumors within a

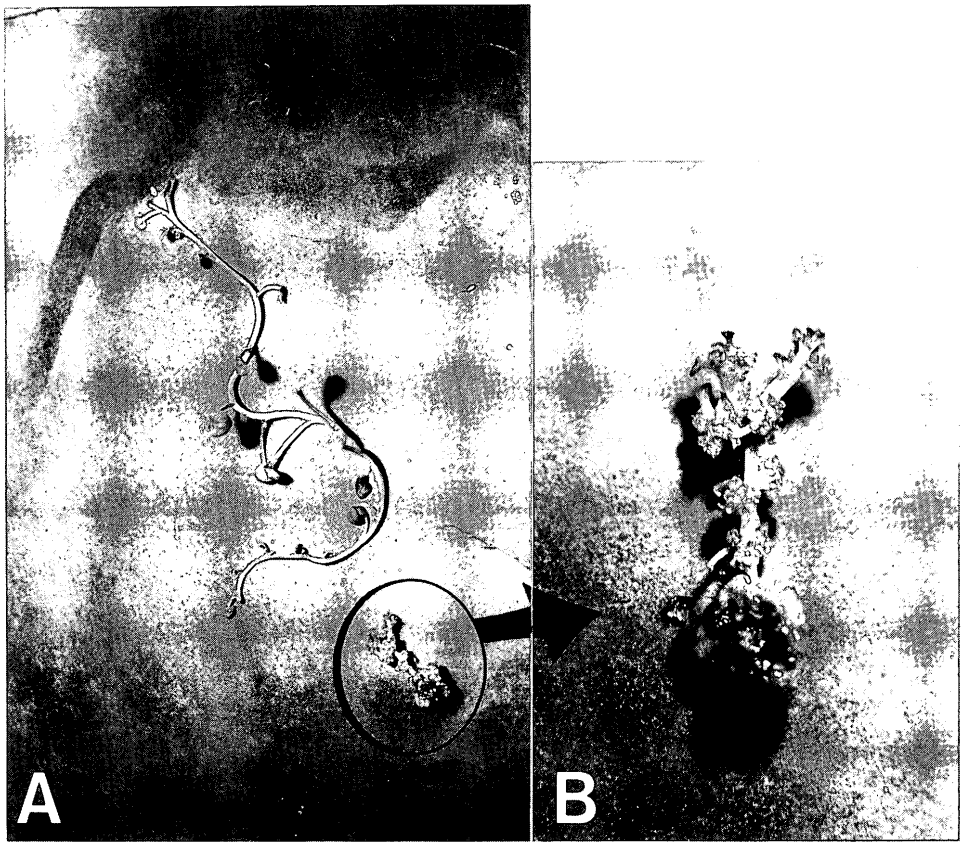

Fig. 7 Effects of light intensity on the maintenance of normal phenotype. Normal shoots, developed from tumor tissue, were transplanted and cultured at 27°C under (A) 2.8 W/m^2 light or (B) 12 W/m^2 light. Strong light inhibited the maintenance of the normal phenotype (B). A light intensity of 2.8 W/m^2 was, however, sufficient to maintain a normal phenotype (A).

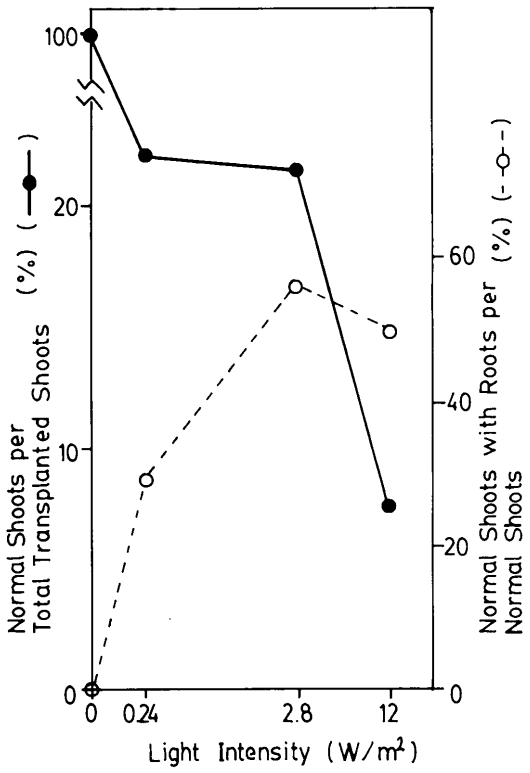

Fig. 8 Effect of light intensity on the maintenance of normal shoots and the development of roots from normal shoots. After transplantation of normal shoots, developed from tumor tissue in the dark, twilight conditions were found to be effective in maintaining a normal phenotype. The percentage of shoots which exhibited a normal appearance and developed roots was calculated (—●—). In this experiment some shoots maintained a normal phenotype, although they did not develop roots. The frequency of shoots that also differentiated roots among shoots that maintained a normal phenotype was calculated (-○-). 16 shoots were used in this experiment. Values on the left ordinate, 100 × (number of normal shoots)/(total number of transplanted shoots); values on the right ordinate, 100 × (number of normal shoots which differentiated root)/(number of normal shoots); Abscissa, light intensity.

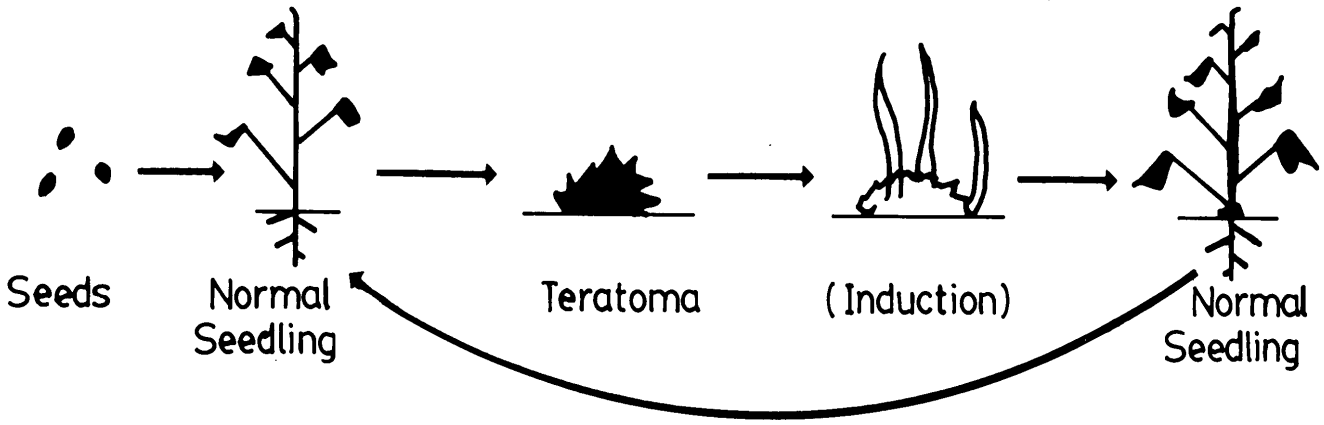

Fig. 9 Schematic illustration of the system for the differentiation and redifferentiation of tobacco genetic tumors.

few weeks after sowing seeds, if necessary. Our new system for tumor induction provides us with an appropriate experimental tool for the analysis of the various phenomena involved.

In our system, whole plants can be induced from tumor tissues under low-intensity light without use of phytohormones or grafting onto normal tissue. Appropriate temperature conditions accelerate the induction of normal shoots but the efficiency of these effects depend on the materials used in the experiments. Thus, optimal temperature conditions have not yet been an ambiguously defined. Light conditions, however, are more critical for induction of normal shoots. These light effects can be divided into two types: the effect on developing shoots of extremely faint light and the effect on developing whole plants of moderate light intensity. In order to develop leaves and roots, light is indispensable but extremely high-intensity light inhibited normal morphogenesis. Although the regenerated plant often had thicker stems and larger leaves than the plants germinated from seeds and rudimentary tumorous tissues at the transitional region between shoot and root (Fig. 6), the state of the tissues can be regarded as the same one as that of plants derived from seeds, since the regenerated plants could develop tumor tissues in just the same manner as the plants grown from seeds. Thus, it is noteworthy that in this system the germinated seedlings and the regenerated plantlets appear to be normal, although they have the potential ability to form normal organized tissues. Consequently, we can switch over from one state to the other in a short time, with only cutting treatment and appropriate light conditions in culture (Fig. 9). The importance of our results is that we can now use this system to study these processes of differentiation at a molecular level (endogenous phytohormone, regulation of gene expression and so on), since this system is controlled in vitro under simplified and strictly regulated conditions. In fact, this system has already enabled us to study the relationship between tumor formation or normal plant induction and endogenous levels of phytohormone. Our results will be published in a subsequent paper (in press).

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References

Bayer, M. H. (1982) Genetic tumours: physiological aspects of tumour formation in interspecific hybrids. *In* Molecular Biology of Plant Tumours. Edited by Kahl, G. and Shell, J. pp. 33–67. Academic Press, New York.

Hagen, G. L. (1962) Morphogenesis in a tobacco hybrid tumor. *Dev. Biol.* 4: 569–579.

Kehr, A. E. (1965) The growth and development of spontaneous plant tumors. *Encycl. Plant Physiol.* [N.S.] 15, Part 2: 184–196.

Kehr, A. E. and Smith, H. H. (1953) Genetic tumor in *Nicotiana* hybrids. *Brookhaven Symp. Biol.* 6: 55–76.

Melchers, G. (1971) Transformation or habituation to autotrophy and tumor growth and recovery. *Colloqu. Internat. C.N.R.S.* 193: 229–234.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473–497.

Skoog, F. (1944) Growth and organ formation in tobacco tissue cultures. *Amer. J. Bot.* 31: 19–24.

White, P. R. (1937) Controlled differentiation in a plant tissue culture. *Bull. Torey Bot. Club* 66: 507–513.

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