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Different Expression Patterns of the Promoters of the Ng*rolB* and Ng*rolC* Genes during the Development of Tobacco Genetic Tumors

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Hybrids (F1) between *Nicotiana glauca* and *N. langsdorffii* are prone to develop tumorous tissues on normal-type F1 tissues, namely, genetic tumors. To investigate the patterns of expression of Ng*rol* genes during the development of genetic tumors, we performed an analysis of transgenic genetic tumors that harbored the promoters of the Ng*rolB* and Ng*rolC* genes fused to a reporter gene for β-glucuronidase (GUS) using a tumorization-redifferentiation system derived from F1 plants in vitro.

Histochemical analysis of the expression of Ng*rolB*-GUS in normal-type F1 transgenic plants revealed GUS activity in meristematic zones, while in Ng*rolC*-GUS transformed plants the activity was detected mainly in the vascular systems of various organs. Tumorous tissues, which arose spontaneously as a consequence of aging or were induced by cutting, showed high levels of GUS expression under the control of promoters of both the Ng*rolB* and the Ng*rolC* gene. Time course analysis during tumorization that followed cutting of leaves of normal-type F1 plants showed clearly that Ng*rolB*-GUS was expressed in all dividing cells in the cut region after 3 days. By contrast, the expression of Ng*rolC*-GUS was detected in organized tissues, such as procambium in teratomatous tumors, 7–10 days after cutting treatment. During redifferentiation from genetic tumors to normal-type plants, the expression of GUS under control of both Ng*rol* promoters decreased and expression resembled that in normal-type tissues. These results suggest the possibility that the Ng*rol* genes might be involved in formation of genetic tumors and, moreover, that the expression of Ng*rolB* might be linked to mitosis and while that of Ng*rolC* might be related to differentiation of tissues, such as the vascular system, in F1 plants.

Key words: Differentiation — Genetic tumor — β-Glucuronidase — *Nicotiana* — *rol* — Tumorization.

Many tumorous outgrowths on plants develop as a consequence of infection by insects, microorganisms and viruses. For example, crown gall tumors are caused by the soil bacterium *Agrobacterium tumefaciens*. Another agents that induce the formation of tumors are external mutagenic factors, such as irradiation by UV light, X-rays or γ-rays. However, spontaneous tumors have been reported to occur in the absence of any obvious external cause in a number of species of higher plants, and these tumors are known as genetic tumors. Such genetic tumors are probably the result of aberrant gene regulation, rather than direct mutation of a gene, as occurs in the formation of crown galls.

The genetic tumors in *Nicotiana*, have been well studied since they were first discovered in 1930 by Kostoff (Ichikawa and Syōno 1991, Kehr 1965, Kostoff 1930, Kung 1989, Sekine et al. 1993, Syōno and Fujita 1994, Smith 1988). Näf (1958) proposed that the species involved in the production of tumorous interspecific hybrids of *Nicotiana* can be divided into two groups, designated arbitrarily the plus group and the minus group. A cross between a plus species and a minus species results in a tumorous hybrid and a cross within the same group results in a non-tumorous hybrid. The hybrid of *N. glauca* (minus group) and *N. langsdorffii* (plus group) develops tumors at high frequency. In this paper, we refer to hybrids of this cross as F1 plants.

The genetic tumors of F1 plants usually develop on various parts of the plant at the end stage of vegetative growth, an indication that aging is probably one of the factors that induces the formation of genetic tumors. F1 hybrids are also prone to develop tumors under stress conditions, in particular after wounding. Considering this feature, Ichikawa and Syōno (1988) developed a tumorization-redifferentiation system in vitro for studies of the mechanism of conversion from normal growth to aberrant growth. Segments from all parts of tissues on F1 hybrids after cutting treatment produce tumorous tissues on hormone-free MS medium, although the parental F1 plants grown under the same conditions do not form tumors. Furthermore, the genetic tumors in tissue culture exhibit remarkable hormonal independence, growing without added auxins and/or cytokinins or other growth factors. They continue to grow into unorganized masses and become partly organized as teratomas, under continuous high-intensity light (more than 50 $\mu E\ s^{-1}\ m^{-2}$) but no roots or elongated shoots are formed. However, normal etiolated shoots are frequently induced from teratoma tissues in continuous

Abbreviations: GUS, β-glucuronidase; MS, Murashige and Skoog; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-glucuronide.

darkness in the absence of plant hormones, and such etiolated shoots regenerate into whole plants with a normal appearance when put in the light. This tumorization-redifferentiation system has provided morphological details of the division and proliferation of cells in tumorous tissues, and it has also been used for qualitative and quantitative studies of auxins and proteins after the induction of genetic tumors (Ichikawa et al. 1989, Fujita et al. 1991). Fujita et al. (1993, 1994) isolated cDNAs for tumor-related genes by a subtractive hybridization procedure. A total of 17 distinct cDNA clones was isolated and the corresponding genes were shown to be specifically expressed during the formation of genetic tumors.

Another approach revealed that Ng*rol* genes are expressed in conjunction with the formation of genetic tumors (Ichikawa et al. 1990, Nagata et al. 1995). Ng*rol* genes were found in the nuclear DNA of *N. glauca*. They have a high degree of sequence homology (more than 80%) to *rol* genes of the Ri plasmid of *Agrobacterium rhizogenes* (Aoki et al. 1994, Furner et al. 1986). The *rol* genes of the Ri plasmid are designated as Ri*rol* genes in this paper. Ri*rol* genes have been shown to promote formation of hairy roots (White et al. 1985). The mechanism of action of the products of Ri*rol* genes is unknown, but physiologically pleiotropic alterations have been observed in transgenic plants that express Ri*rol* genes (Oono et al. 1987, Schmülling et al. 1988, Spena et al. 1987). It has been suggested that the products of Ri*rol* genes change the levels of plant hormones or the sensitivity of plant tissues to such hormones (Shen et al. 1988, Maurel et al. 1991, Walden et al. 1993). Ng*rolB* and Ng*rolC* genes are transcribed in cultured tissues of genetic tumors, but not in *N. glauca* plants (Ichikawa et al. 1990). We previously compared the patterns of expression of Ng*rol* and Ri*rol* genes using transgenic genetic tumors of F1 that contained a reporter gene for β-glucuronidase (GUS) fused to the respective promoters (Nagata et al. 1995). The regulatory functions of the Ng*rolB* and Ng*rolC* promoters appeared to be basically similar to those of Ri*rolB* and Ri*rolC* promoters. Furthermore, we found that GUS activity of the transgenic Ng*rolB*-GUS and Ng*rolC*-GUS plants was high in tumorous tissues eventhough the activity was low in various organs of normal-type F1 plants. However, the earlier works did not describe the tissue specificity of the expression of the Ng*rolB* and Ng*rolC* genes in sufficient detail. Therefore, in this study, we investigated in detail the patterns of the expression of promoters of Ng*rol* genes during the development of genetic tumors in order to examine the possible involvement of Ng*rol* genes in the formation of genetic tumors. We present here an analysis of transgenic F1 plants that harbored the Ng*rolB* or the Ng*rolC* promoter fused to the GUS reporter gene during the conversion of normal tissues to aberrant tissues, as well as during the reverse process, using a tumorization-redifferentiation system in vitro.

Materials and Methods

GUS constructs and transgenic plants—The Ng*rolB*-GUS and Ng*rolC*-GUS fusion genes shown in Figure 1 were prepared as described previously (Nagata et al. 1995). The Ng*rolB*-GUS fusion was constructed as a long version (1,077 bp) and a short version (515 bp) from the clone λNg31 (White et al. 1983), while the Ng*rolC*-GUS fusion was constructed as a long version (1,152 bp) only from the cosmid clone pLJ-1 (Jouanin 1984). NgB1077-GUS, NgB515-GUS and NgC1152-GUS transgenic genetic tumors were obtained from normal-type F1 plants (*N. glauca* × *N. langsdorffii*) by the leaf disc transformation method, as described previously (Nagata et al. 1995).

Cultivation of plants—Transgenic plants were cultivated according to the tumorization-redifferentiation system described previously (Ichikawa et al. 1988). Transformed tissues in culture were routinely grown in vitro at 26°C under continuous light (more than 50 $\mu E\ s^{-1}\ m^{-2}$) or in darkness on MS solid medium (Murashige and Skoog 1962) supplemented with 50 mg $liter^{-1}$ kanamycin but without plant hormones. Three to four transgenic F1 normal-state plants for each construct were redifferentiated from

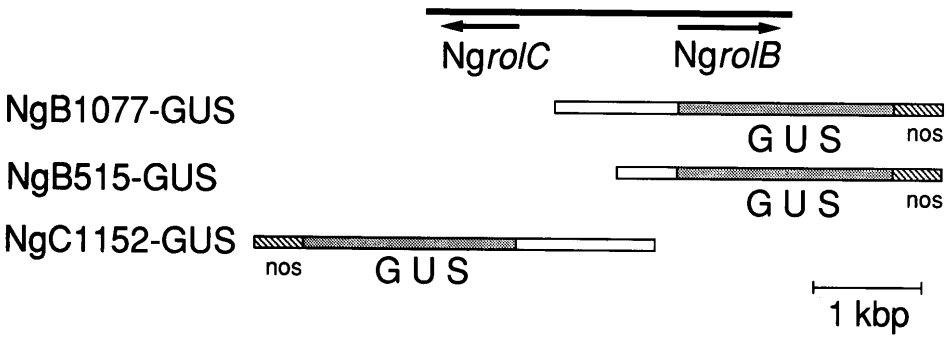

Fig. 1 Schematic representations of the fusion constructs. Fusions of the promoter regions of Ng*rolB* and Ng*rolC* with the GUS coding sequence are shown. The Ng*rolB* and Ng*rolC* coding regions are shown by arrows. The truncated fragments were amplified from clone λNg31 or pLJ-1, with a pair of appropriate primers (see Nagata et al. 1995), by the polymerase chain reaction. Each promoter was fused to the β-glucuronidase (GUS) coding region of pBI101, giving rise to pNgB1077-GUS, pNgB515-GUS and pNgC1152-GUS, respectively. The GUS coding region is represented by gray bars and the nopaline synthase (nos) terminator by hatched bars.

tumorous tissue by controlling light conditions. Regenerated transgenic F1 plants of the normal type were grown in a naturally lit greenhouse for more than 3 months. They developed spontaneous genetic tumors on their stems and roots as a result of aging. Tumorization was also induced directly and synchronously by cutting treatment. Newly expanded leaves of normal-type F1 plants were cut into pieces of about 1 cm^3 and cultured on solid, hormone-free MS medium under continuous light at 26°C.

Fluorometric assay—The GUS activity of each transformant was measured by a fluorometric assay with 4-methyl umbelliferyl glucuronide as substrate, as described previously (Nagata et al. 1995). Fluorometric assays were performed using 100–500 mg fresh weight of each sample.

Histochemical staining—Histochemical staining was performed as described by Jefferson et al. (1987). Plant materials or small segments obtained by hand manual sectioning were fixed in 0.3% formaldehyde for 1 h at room temperature and they incubated in 50 mM sodium phosphate buffer (pH 7.0) that contained 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) for 4 h at 37°C. Green of chlorophyll was removed by incubation in 70–100% alcohol at room temperature. Tissues were examined under a light microscope.

For preparation of thin sections, samples after incubation with X-Gluc were dehydrated by transfer through an alcohol series (30, 50, 70, 95 and 100% absolute ethanol and absolute propylene oxide). They were then embedded in Spurr's Epoxy resin and cut into 1- to 2-μm sections with a glass knife on a microtome. Sections were transferred to glass slides, flattened by drying and examined under a light microscope without counter-staining.

Results

Expression of GUS in normal-type F1 transgenic plants—For each construct, three to four transgenic F1 plants were analyzed during tumorization and redifferentiation from tumors. All of them exhibited similar localization of GUS activity. Here, it was shown one representative transgenic line.

The normal-type F1 transgenic plants that harbored NgB1077-GUS had high GUS activity in the meristematic zones of roots, including lateral root primordia (Fig. 2A, B). Furthermore, the shoot apex was intensely stained (data not shown). Lower activity was detected around vascular tissues of stems (Fig. 2C). In leaves, the expression of GUS was scarcely detectable (Fig. 2D). By contrast, staining of the normal-type F1 transgenic plants that harbored NgC1152-GUS was detected mainly in phloem cells of various organs. GUS expression in roots of these plants was evident in the vascular tissues of both the main and the lateral roots (Fig. 2E), and GUS activity in stem sections was localized in phloem cells (Fig. 2F). Figure 2G shows the staining in the vascular system of leaves of a NgC1152-GUS transformant.

Expression of GUS in genetic tumors induced by aging—With time, the normal-type F1 plants spontaneously developed genetic tumors on their stems, roots and other tissues. The genetic tumors appeared most frequently as protuberances on leaf nodes and leaf scars when leaves of F1 plants were dying, after about 3 months in the greenhouse. Histochemical analysis of stem sections that contained genetic tumors that had developed on leaf nodes of the NgB1077-GUS transgenic normal-type plants is shown in Figure 3A. Very intense staining was detected in all of the tumorous region and weak staining was seen in vascular tissues of stems. A similar tendency was found in the corresponding NgC1152-GUS transformed plants (Fig. 3B).

High GUS activity was found in genetic tumors on roots of both NgB1077-GUS and NgC1152-GUS transgenic plants. However, a difference in the pattern of expression of GUS in genetic tumor tissues was noted between Ng*rolB*-GUS and Ng*rolC*-GUS. The GUS activity due to the Ng*rolB*-GUS gene was observed throughout all tumorous tissues throughout development, from newly developed tumors (diameter of 100–300 μm; Fig. 3C) to mature tumors (diameter of more than 500 μm; Fig. 3D), with high-level expression in proliferating cells. In NgC1152-GUS transgenic plants, however, no GUS activity was observed in newly developed tumors (Fig. 3E). At later stages, GUS expression under control of the Ng*rolC* promoter was found in the center of developing tumors on roots (Fig. 3F).

Expression of GUS in genetic tumors formed as a consequence of wounding—Genetic tumors developed on all parts of F1 plants when they were exposed to stress conditions, and the most severe and effective stress was wounding, such as cutting treatment. Figure 4 shows the time course of changes in activity of GUS, determined by a fluorometric assay, during induction of tumors by cutting of leaves of NgB1077-GUS, NgB515-GUS and NgC1152-GUS transformants. The GUS activity increased during tumorization in each case and reached a maximum 15 days after cutting treatment. The high activity remained steady when genetic tumors were maintained under continuous light. In particular, the GUS activity under control of the Ng*rolB* promoter in leaves before cutting treatment was extremely low but it increased immediately and very considerably after cutting. The intact leaves of Ng*rolC*-GUS plants had considerable GUS activity and the activity kept constant after 5 days. This activity increased gradually after cutting but no further increase in GUS activity was observed after 15 days.

Histochemical localization of GUS activity in leaves of NgB1077-GUS transformed plants revealed staining specific for GUS in the cut regions of leaves 3 days after cutting treatment (Fig. 5B) eventhough no GUS activity was detected on day 0 (Fig. 5A) or after 2 days (data not shown). No change in the morphological appearance of cut regions after 3 days was visible with the naked eye. However, further observations under the light microscope of thin perpendicular sections of NgB1077-GUS transgenic leaves revealed that some dividing cells, in which GUS activity was located, were visible in a few layers of cut regions

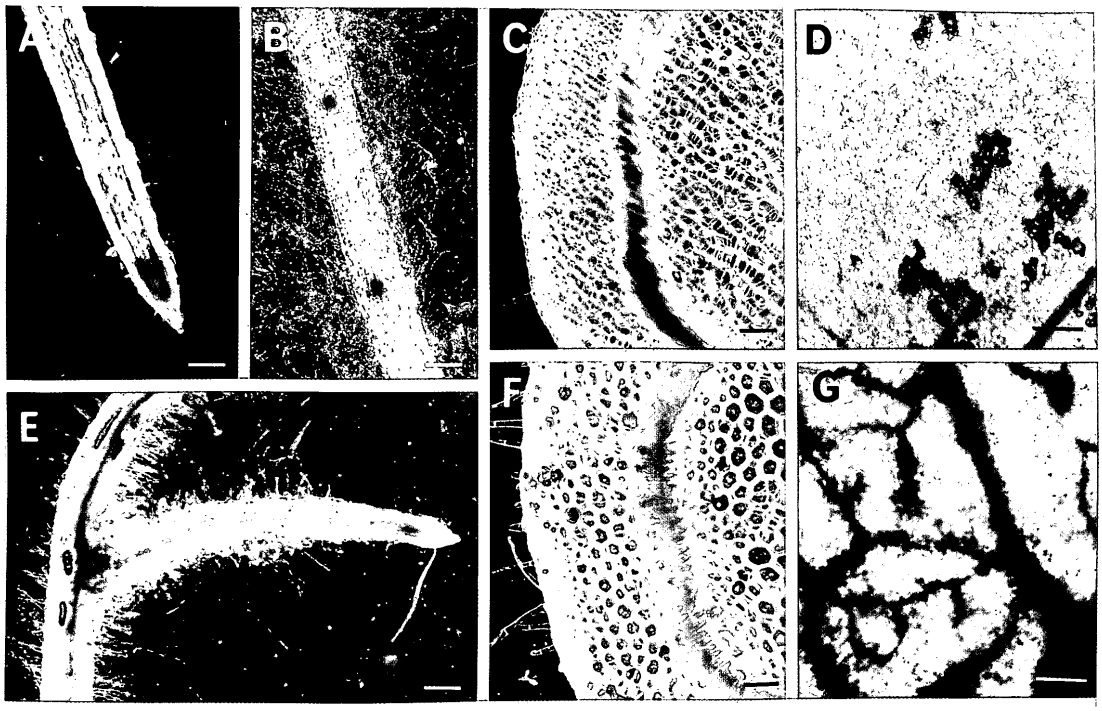

Fig. 2 Histochemical staining for GUS activity in roots, stems and leaves of transgenic normal-type F1 plants. The expression is shown of NgB1077-GUS in (A) and (B) roots, (C) stem sections and (D) leaves. The expression is also shown of NgC1152-GUS in (E) roots, (F) stem sections and (G) leaves. Bars=200 μm.

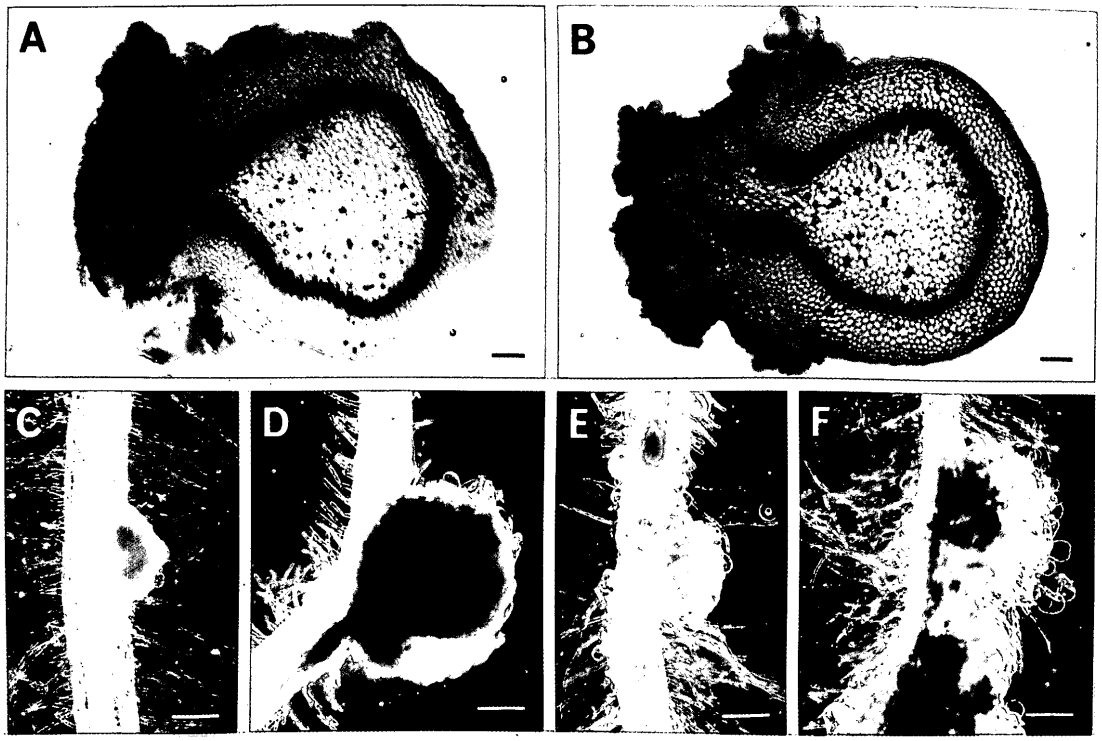

Fig. 3 Histochemical staining for GUS activity in genetic tumors that developed spontaneously on stems and roots with aging. Staining for GUS activity in stem sections with developing genetic tumors on leaf nodes: (A) NgB1077-GUS and (B) NgC1152-GUS. Staining for GUS activity in roots with developing genetic tumors: (C) and (D) NgB1077-GUS; (E) and (F) NgC1152-GUS. Bars=1 mm (A and B); 200 μm (C, D, E and F).

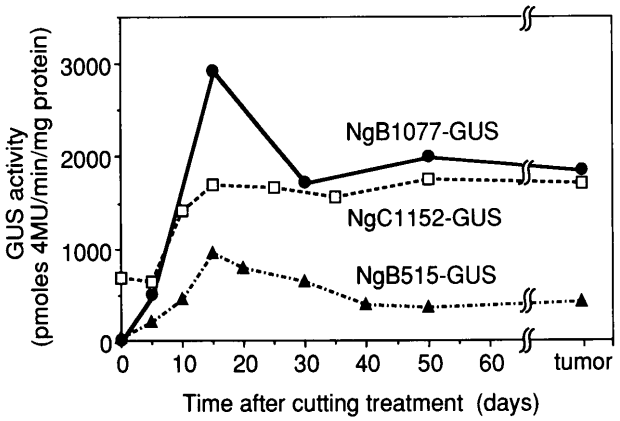

Fig. 4 Time course of changes in GUS activity during induction of tumors by cutting of leaves of Ng*rolB*-GUS and Ng*rolC*-GUS transgenic plants. Fluorometric assays were performed using extracts of (—●—) NgB1077-GUS, (-·-▲-·-) NgB515-GUS and (----□----) NgC1152-GUS transformed tissues. Tumor: transgenic genetic tumor that had been maintained for about 12 months by transfer to fresh medium once a month.

(Fig. 5M). Five days after cutting, the regions of dividing cells that expressed GUS were larger (Fig. 5C, N). After about 10 days, the histochemical staining of new proliferating cells that had been induced by tumorization became more intense as time passed (Fig. 5G, H). By contrast, activity of the Ng*rolC* promoter in proliferating cells in the cut regions was not detected until 5 days after cutting treatment (Fig. 5D, E, F). In spite of the fact that expression of NgC1152-GUS was detectable in the vascular system of normal-type F1 leaves (Fig. 2G), staining of veins was reduced 3 days and 5 days after cutting treatment. About 7 days to 10 days after cutting treatment, when organized tissues, such as bud primordia and procambia, began to develop in unorganized masses of cells, histochemical staining of GUS in the NgC1152-GUS transformants was confined to the organized tissues (Fig. 5J, K, O).

Fifteen days after cutting treatment, the GUS activity of the NgB1077-GUS transformed tissues appeared to be confined to the clusters of meristematic cells in the organized tissues of teratomatous growths (Fig, 5I). The pattern of expression of the NgC1152-GUS fusion was similar to that obtained with the NgB1077-GUS fusion (Fig. 5L).

Expression of GUS during redifferentiation from genetic tumors to normal-type plants—The genetic tumors induced by cutting treatment grew rapidly and for long periods of time on hormone-free MS medium under continuous high-intensity light (more than 50 μE s^{-1} m^{-2}), without formation of roots or elongated shoots. Figure 6A shows the GUS-specific staining of transgenic genetic tumors that harboured NgB1077-GUS in the meristematic cells of bud primordia and the procambium. The expression of GUS under control of the NgC1152-GUS fusion in genetic tumors in the light was evident throughout the procambium (Fig. 6B). The patterns of expression of GUS within tumor tissues of the NgC1152-GUS transformants did, however, exhibit some resemblance to those of the NgB1077-GUS transformants, in which expression of GUS was detected in bud primordia (Fig. 6C, D). In genetic tumors that were maintained for more than one year under continuous light by transfer to fresh medium once a month, the Ng*rolB* and Ng*rolC* promoters retained high-level and analogous patterns of activity.

Normal, etiolated and elongated shoots can frequently be induced from tumorous tissues by the transfer of tumors from continuous light to continuous darkness. In the etiolated and proliferating tumorous tissues at the base of elongated shoots in continuous darkness, the GUS activity of both NgB1077-GUS and NgC1152-GUS transformants was localized mainly in the meristematic cells of bud primordia and procambium, resembling the GUS activity in tumorous tissues under continuous light (Fig. 6E, F). However, the patterns of GUS expression under control of the Ng*rolB* and the Ng*rolC* promoters became clearly different during the redifferentiation from genetic tumors to normal-type plant. Intense blue staining of normal shoots of NgB1077-GUS transformants was detected on shoot apices and axial buds, but little staining was seen on vascular tissues (Fig. 7A, B). By contrast, high-level expression of GUS was detected in shoots of the NgC1152-GUS transformants in vascular tissues (Fig. 7C).

Regenerated F1 plants developed genetic tumors after cutting treatment and these tumors again regenerated normal-type F1 plants. We performed the tumorization and redifferentiation of F1 for two entire cycles and examined the expression of GUS under control of the NgB1077 and NgC1152 promoters by histochemical staining. We confirmed the same tendencies towards higher GUS activity in tumors than in normal-type tissues and found no changes in respective patterns of expression of each stage (data not shown).

Discussion

There have been few studies of the genes that are involved in the transition of cells in the normal state to genetic tumors. If a certain gene(s) is expressed specifically only in tumorous tissues, it is likely that the gene(s) has a role in the formation of genetic tumors. In this study, we showed that the levels of expression of the Ng*rolB* and Ng*rolC* promoters increased specifically during tumorization. Moreover, we obtained full details of the different patterns of expression of the Ng*rolB* and Ng*rolC* promoters which shown help us to understand the mechanism of tumorization.

The expression of the Ng*rolB* and Ng*rolC* promoters increased considerably during formation of genetic tumors

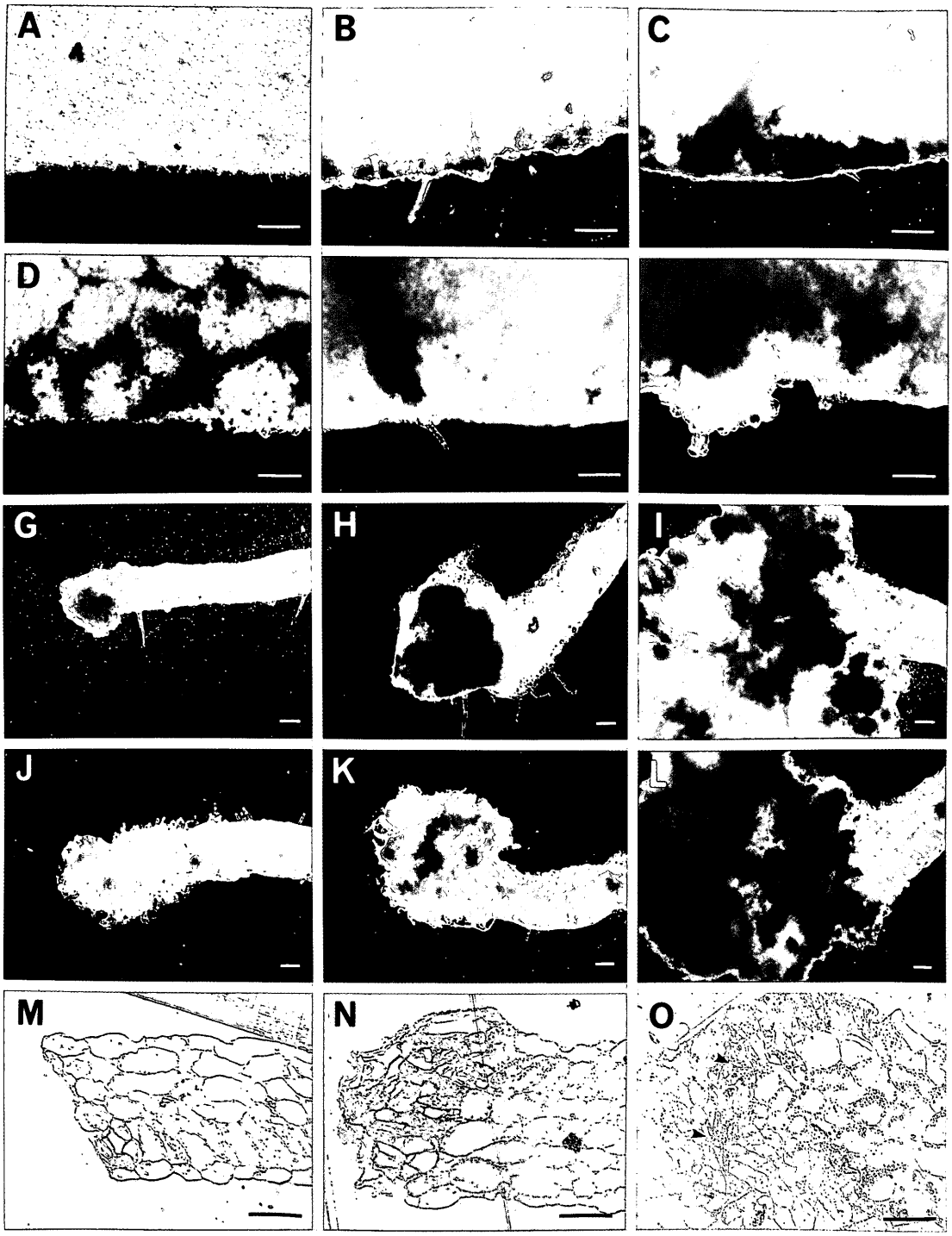

Fig. 5 Histochemical staining for GUS activity in genetic tumors that developed after cutting of F1 leaves. GUS-specific staining in leaves of NgB1077-GUS transgenic plants (A) 0 day, (B) 3 days and (C) 5 days, and in NgC1152-GUS plants (D) 0 day, (E) 3 days and (F) 5 days after cutting. Staining for GUS activity of perpendicular sections of leaves that expressed NgB1077-GUS (G) 7 days, (H) 10 days and (I) 15 days, and NgC1152-GUS (J) 7 days, (K) 10 days and (L) 15 days after cutting treatments. GUS staining of perpendicular thin sections (1–2 μm) of leaves that expressed NgB1077-GUS (M) 3 days and (N) 5 days after cutting, and of leaves that expressed NgC1152-GUS (O) 7 days after cutting. Arrow heads show locations of staining for GUS, corresponding antecedent cells of organized tissues, such as bud primordia and procambia. Bars = 200 μm.

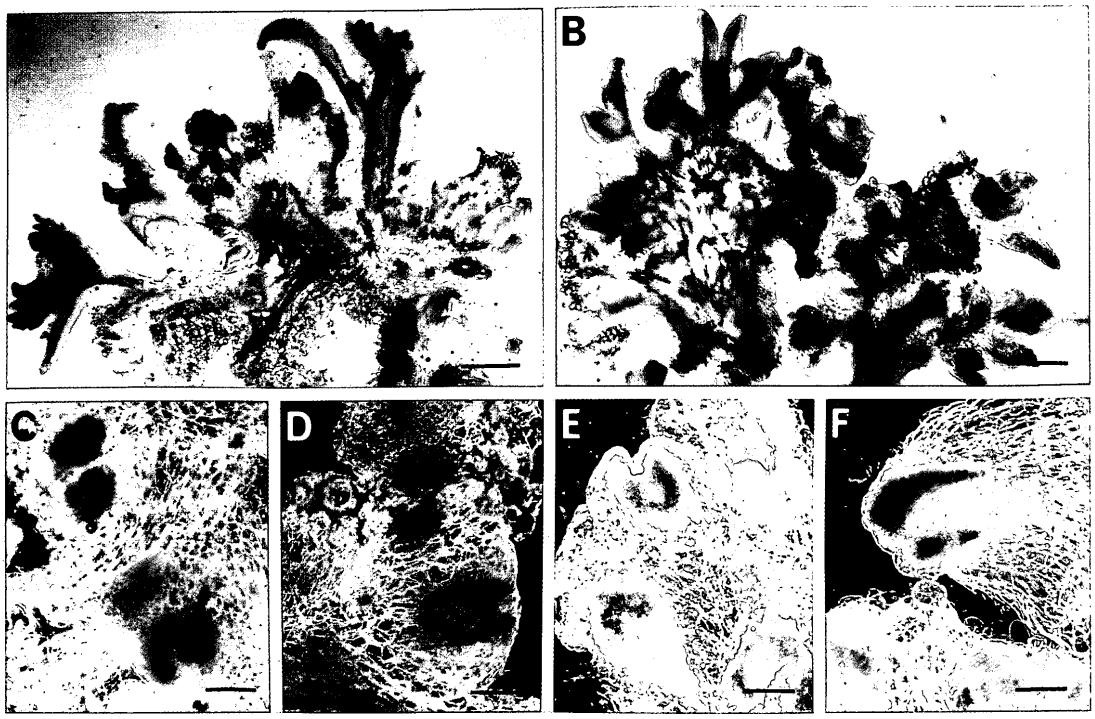

Fig. 6 Histochemical staining for GUS activity of genetic tumors that had been maintained semipermanently on hormone-free MS medium under continuous high-intensity light. Staining for GUS activity in sections in genetic tumors that had been cultured under continuous light: (A) NgB1077-GUS and (B) NgC1152-GUS. Higher-magnification view for GUS activity in tumorous tissues of genetic tumors that had been grown under continuous light: (C) NgB1077-GUS and (D) NgC1152-GUS. GUS activity of tumorous tissues of genetic tumors grown in continuous darkness: (E) NgB1077-GUS and (F) NgC1152-GUS. Bars=1 mm (A and B); 200 μm (C, D, E and F).

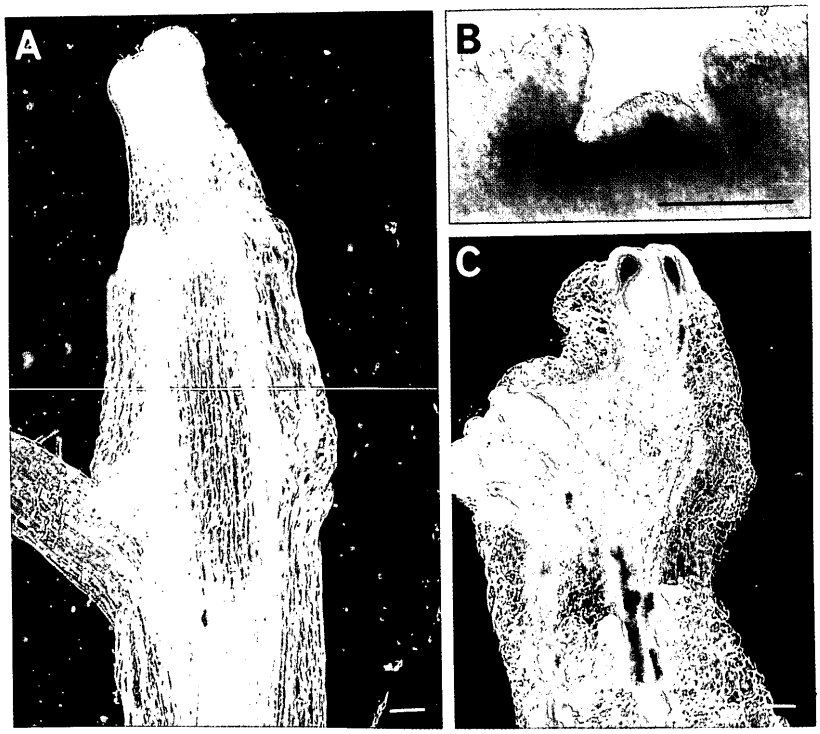

Fig. 7 Histochemical staining for GUS activity in regenerated F1 shoots by the transfer of genetic tumors from continuous light to continuous darkness. GUS staining of (A) elongated shoots and (B) a shoot apex of NgB1077-GUS tumors grown in continuous darkness, and of (C) shoots of NgC1152-GUS tumors grown in continuous darkness. Bars=200 μm.

as a consequence of aging, as well as of wounding. Histochemical analysis of both NgB1077-GUS and NgC1152-GUS transformed plants with tumorous tissues on their normal organized tissues showed that staining for GUS in tumors was more intense than that in normal organized tissues (Fig. 3, 5), in spite of the different origins of the genetic tumors, which had been induced either by aging on stems and roots or by wounding of leaves. These results suggest that aging and wounding induce the formation of genetic tumors through the same signal-transduction process. Furthermore, the specific and high-level activity of GUS in genetic tumors suggest the validity to the concept that Ng*rol* genes are involved in formation or maintenance of tumors, although the possibility that the expression merely resulted from cell proliferation can not be excluded.

The patterns of expression Ng*rolB* and Ng*rolC* promoters revealed differences in tissue-specificity. In roots, which developed tumors upon aging, the expression of GUS in NgB1077-GUS plants was detected from the early stages of genetic tumor formation (tumors with a diameter of 100–300 μm) and that in NgC1152-GUS plants was detected at a later stage (tumors with a diameter of more than 500 μm; Fig. 3). At the mature stage, genetic tumors generally consist of unorganized masses and of partly organized tissues as teratomatous growths. The GUS-specific staining of NgB1077-GUS plants was detected all over the developing genetic tumors, not only in organized teratoma tissues but also in the unorganized masses. By contrast, the GUS activity in NgC1152-GUS plants was mainly evident in organized masses only.

This tendency for different patterns of expression of the Ng*rolB* and Ng*rolC* promoters was more apparant in the experiments that involved tumorization and redifferentiation of pieces of leaves. Figure 8 shows a schematic representation of the patterns of expression of Ng*rolB* and Ng*rolC* promoters during the tumorization and redifferentiation of F1. The GUS-specific staining of NgB1077-GUS tissues coincided with the beginning of cell division (3 days after cutting treatment) and was observed throughout dividing cells (Fig. 5). As long as the active division of cells continued, high-level expression of GUS was observed in these tumorous tissues (Fig. 4, 5). In mature genetic tumors, the expression of the Ng*rolB* promoter was detected in all meristematic cells of tumorous tissues (Fig. 6). Furthermore, in normal-type F1 plants, intense staining was observed in meristematic zones of roots and shoot apices (Fig. 2, 7). Thus, the Ng*rolB* promoter was expressed in all types of meristem and in all division cells. The expression

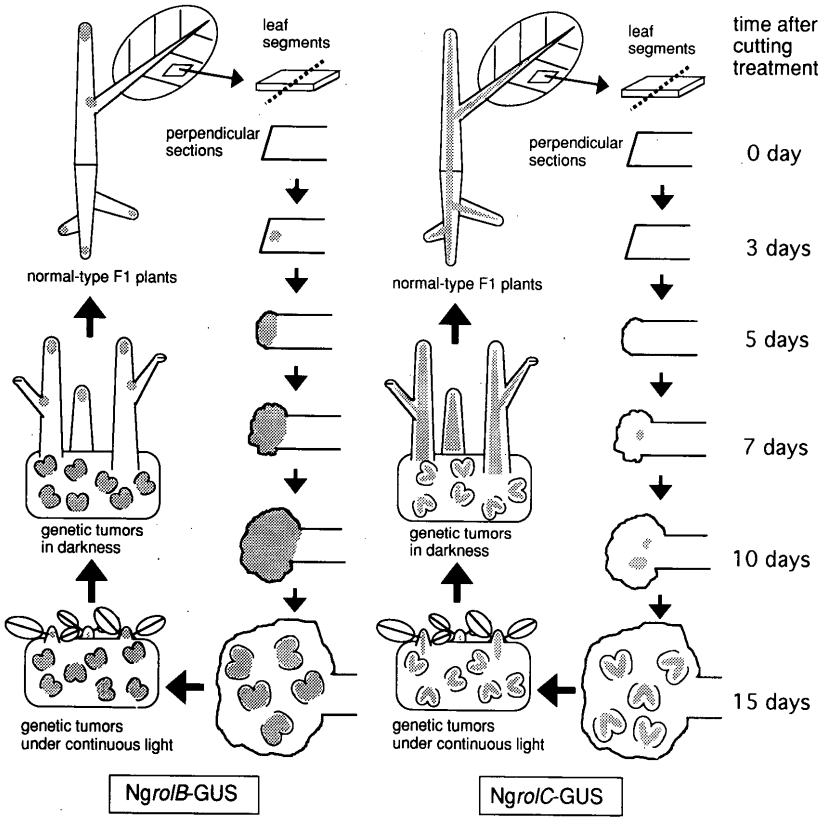

Fig. 8 Schematic illustration of the different patterns expression of the Ng*rolB* and Ng*rolC* promoters during tumorization and redifferentiation of F1 plants. The localization of the GUS-specific staining of each Ng*rol*-GUS transgenic plant or tissue is represented by shading.

of Ng*rolB* seems, therefore, to be linked to cell division or mitotic activity. By contrast, the GUS-specific staining of NgC1152-GUS tissues was detected in partly organized cells at later stages of tumorization (7–10 days after cutting treatment) and not in dividing cells (Fig. 5). In mature genetic tumors, the expression of GUS under control of the Ng*rolC* promoter was observed in procambium and meristematic cells (Fig. 6). In normal-type F1 plants, GUS was expressed in all vascular tissues (Fig. 2, 7). Judging from these observations, we can postulate that the Ng*rolC* promoter is developmentally regulated and that it is active in the differentiation of cells but not directly in cell division.

More than 15 days after cutting treatment, analogous patterns of expression of Ng*rolB* and Ng*rolC* promoters were observed in genetic tumors that had been grown semipermanently on hormone-free MS medium. However, mature genetic tumors consisted of clusters of tiny buds, with dividing cells located mainly in both the meristematic zone and procambium. It is possible that Ng*rolB* is involved in cell division and Ng*rolC* is involved in tissue and/or organ-specific differentiation, but that both are expressed at the same time since cells have to divide to form various organs.

Fujita et al. (1994) isolated 17 distinct cDNA clones for genes that are specifically or preferentially expressed in genetic tumors and they divided these genes into three groups, namely, those expressed (1) at only the early stage and transiently (2–11 days), (2) those expressed from the early stage to the late stage continuously (later than 2 days) and (3) those expessed at the late stage (later than 11 days) of the formation of genetic tumors. Thus, it is likely at least three regulatory mechanisms are operative during tumorization. They proposed that the genes in group (1) are related to defense responses, those in group (2) are related to cell proliferation and those in group (3) are related to the initiation of differentiation. It is clear that the Ng*rolB* gene corresponds to group (2), since the expression of the Ng*rolB* gene was induced immediately after cutting treatment and was maintained in subcultured genetic tumors. The Ng*rolB* gene was not involved in stress-induced expression since no GUS-specific staining was found until 2 days after cutting treatment. By contrast, the Ng*rolC* gene corresponds to group (3). In this study, we showed that the pattern of expression of the Ng*rolB* promoter coincided with the proliferation of cells and that of the Ng*rolC* promoter coincided with the initiation of differentiation of organs. Our results justified their assumption on the role of each group.

The shorter truncated Ng*rolB* promoter, NgB515-GUS, was prepared as previously described (Nagata et al. 1995). The GUS-specific expression in NgB515-GUS transgenic plants was associated with lower-level (Fig. 4) but almost analogous tissue-specific expression during tumorization (data not shown). These data confirm that the regulatory elements that control expression of Ng*rolB* in meristematic dividing cells are located downstream of position −515 bp from the initiation codon.

We have also examined the expression of the Ri*rolB* and Ri*rolC* genes, using the transgenic genetic tumors on RiB927-GUS and RiC1017-GUS plants, which correspond to NgB1077-GUS and NgC1152-GUS plants, respectively (see Nagata et al. 1995). Histochemical analysis revealed that the GUS staining of each Ri*rol*-GUS transgenic plant had a tissue-specific pattern similar to that of each Ng*rol*-GUS during each stage of tumorization and redifferentiation. It is reasonable to suppose that the roles of Ng*rol* genes are similar to those of Ri*rol* genes. There are many reports that the sensitivity to exogenous plant hormones of plants that have been transformed by Ri*rol* genes is higher than that of normal untransformed material (Shen et al. 1988, Spanò et al. 1988, Maurel et al. 1991, Schmülling et al. 1993). It has also been demonstrated that the products of Ri*rol* genes are involved in the synthesis of auxin and/or cytokinin (Estruch et al. 1991a, b, Walden et al. 1993). Recently, it was reported that RiRolB protein has tyrosine phosphatase activity so that it may play auxin signal perception/transduction (Filippini et al. 1996).

Our data suggest the possibility that the expression of Ng*rolB* is linked to mitosis while that of Ng*rolC* is related to organization of tissues during the development of genetic tumors. The differentiation into organs, such as buds and roots, is known to be controlled by the ratio of levels of auxin and cytokinin, for example. If we assume that the Ng*rolB* and Ng*rolC* genes are involved in the sensitivity to or synthesis of auxin and/or cytokinin and that the timing and localization of the expression of both genes change specifically, it seems possible that the tumorous phenotype, with rudimentary buds, is induced by effects equivalent to a partial imbalance of plant hormones in the plant tissues.

To determine whether our working hypothesis is correct or not, further work is required on the action of products of the Ng*rol* genes. The characterization of transformed tobacco with constitutive and compulsory expression of the Ng*rol* genes is in progress.

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