Expression Analysis of the NgORF13 Promoter during the Development of Tobacco Genetic Tumors

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We investigated the expression pattern of the promoter of Nicotiana glauca (Ng) ORF13 in the hybrids between N. glauca and N. langsdorffii harboring the NgORF13-β-glucuronidase (GUS) chimeric gene. The promoter of NgORF13 of N. glauca had lower activities than the promoter of RiORF13 of Agrobacterium rhizogenes agropine-type root-inducing (Ri) plasmid. However, the localization of GUS activity in the NgORF13 transgenic plants was similar to that in the RiORF13 transgenic plants. The GUS activity of NgORF13-GUS was high in genetic tumors cultured in vitro or developed spontaneously on F1 plants with aging or by wounding. The GUS activity in tumors was observed in bud primordia, vascular bundles and leaves in the buds. While the activity was lower than in tumors, NgORF13-GUS was also expressed in vascular bundles and the parenchymatous tissues in plants regenerated from tumors. Furthermore, the promoter activity of NgORF13 was induced by wounding and activated by exogenous application of methyl jasmonate. During tumorization, NgORF13 was induced at an early stage and showed expression patterns similar to both NgrolB and NgrolC whose expression were investigated by Nagata et al. (1996) Plant Cell Physiol. 37: 489–498. It is thought that Ngrol genes might be involved in the formation of genetic tumors, and, moreover, NgORF13 might work in cooperation with NgrolB and NgrolC.

Keywords: Agrobacterium rhizogenes — Genetic tumor — β-glucuronidase — Nicotiana — ORF13 — Transgenic plant.

Abbreviations: ORF, open reading frame; GUS, β-glucuronidase; 4MU, 4-methylumbelliferone; X-Glc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide; NAA, α-naphthaleneacetic acid; MeJA, methyl jasmonate.

The nucleotide sequences reported in this paper has been submitted to EMBL Data Library under accession numbers D16559 for NgORF13, NCBI GenBank under accession numbers K03313 for RiORF13 of Agrobacterium rhizogenes pRiA4 (agropine-type) and M60490 for RiORF13 of A. rhizogenes pRI8196 (mannopine-type).

Introduction

Agrobacterium rhizogenes carrying a root-inducing (Ri) plasmid infects plants at the wound sites and induces hairy root formation by insertion of a well-defined T-DNA region of the plasmid into the plant genome. The agropine-type Ri plasmid has two segments of T-DNA, TL-DNA and TR-DNA (Huffman et al. 1984, Jouanin 1984). TL-DNA is known to be essential for the induction of hairy root formation. Transformed roots containing TL-DNA regenerate to whole plants which show characteristic morphological alterations. Among the 18 ORFs on TL-DNA, Rirol genes (rol genes derived from Ri plasmids) containing rolA, B, C, and D, which correspond to ORF10, 11, 12, and 15, respectively, have been considered to play key roles in hairy root induction. Comparison of the frequency of root formation by various combinations of Rirol genes revealed the synergistic function of Rirol genes. Furthermore, the fact that RirolB alone induced hairy root formation on leaf segments of tobacco and Kalanchoe suggested the pivotal role of RirolB on hairy root formation (Spena et al. 1987, Schmülling et al. 1988, Capone et al. 1989, Aoki and Syono 1999b).

Other ORFs on T-DNA such as RiORF13 and RiORF14 were also shown to function in root induction. Rooting capability of RirolB on carrot disks was induced only by the concomitant presence of RiORF13 and RiORF14 (Capone et al. 1989). In tobacco leaf segments, however, Aoki and Syono (1999b) showed that induction of roots by the single gene RirolB was promoted by RirolC, RiORF13 and RiORF14 independently. Among these genes RiORF13 promoted rooting most effectively, although RiORF13 itself had no ability to induce roots. Thus, RiORF13 functions to assist the role of RirolB on hairy root formation.

White et al. (1983) found DNA sequences (cT-DNA) in the genome of uninfected Nicotiana glauca with homology to the TL-DNA region of Ri plasmid. A high degree of homology of the four ORFs in the cT-DNA of N. glauca, to RirolB, RirolC, RiORF13 and RiORF14 was discovered and hence these genes were designated as NgrolB, NgrolC, NgORF13 and NgORF14, respectively (Furner et al. 1986, Aoki et al. 1994). Northern hybridization reveals that NgORF13 and NgORF14 genes were transcribed in hybrids (F1) between N. glauca × N. langsdorffii and its genetic tumors (Aoki et al. 1994). The combination of NgORF13 with RirolB evoked root formation more effectively than RirolB alone, although NgORF14 also showed a weak promoting effect (Aoki and Syono 1999a). Thus,
NgORF13 has a function similar to RiORF13 on root induction. Overexpression of NgORF13 gene in transgenic tobacco plants caused morphological abnormalities; reduction of longitudinal growth on leaves and flowers resulted in rounded leaves and stout flowers (Aoki and Syono 1999a).

Ngrol genes in N. glauca were introduced by horizontal gene transfer from a mikimopine-type Ri plasmid-like ancestor and conserved during the evolution of N. glauca, implying N. glauca might be a natural transgenic plant (Furner et al. 1986, Aoki and Syono 1999c, Suzuki et al. 2002). However, N. glauca plants do not exhibit characteristics of hairy-root syndrome because of the two point mutations in the NgrolB gene. NgrolB restored the capacity for root induction by site-directed mutagenesis at these two positions (Aoki and Syono 1999c).

The transcripts of NgrolB and NgrolC were also detected in F1 plants and its genetic tumors (Ichikawa et al. 1990, Aoki and Syono 1999c). The expression patterns of NgrolB and NgrolC promoters fused to a β-glucuronidase (GUS) reporter gene were investigated in detail using the tumorization-redifferentiation system, by which we can convert the morphology between the state of tumors and the state of normal F1 plants easily and reproducibly (Ichikawa and Syono 1988). As a result, promoters of NgrolB and NgrolC were regulated by similar mechanisms with RirolB and RirolC, respectively (Nagata et al. 1995). Furthermore, the expression patterns of NgrolB might be linked to mitosis while NgrolC might be related to differentiation of tissues, such as the vascular system. It was also suggested that the expression of NgrolB-GUS and NgrolC-GUS was concommittant with the formation of genetic tumors (Nagata et al. 1996).

In this study, we focused on the expression of NgORF13 in the two states of F1 plants: genetic tumors and morphologically normal plants. The chimeric gene of NgORF13-GUS obtained by inserting a 625-bp promoter region into the GUS coding region of pBI101 introduced into F1 plants. For comparison, the transgenic F1 plants that harbored a 560-bp promoter region of RiORF13 from agropine-type Ri plasmid fused to GUS gene were also constructed (Fig. 1). The synergistic function of NgORF13, NgrolB and NgrolC during tumorization is also discussed.

Results

The GUS activities in NgORF13-GUS and RiORF13-GUS transformants

Each transgenic tumors developed on the leaf disks of F1 plants was isolated separately and subcultured on the MS basal medium with 50 mg liter⁻¹ kanamycin. The obtained lines of NgORF13-GUS and RiORF13-GUS transgenic tumors were assayed fluorometrically. Furthermore, we compared the promoter activities between the genetic tumors and the normal-type F1 transgenic plants of NgORF13-GUS and RiORF13-GUS using tumorization-redifferentiation system (Ichikawa and Syono 1988). As shown in Fig. 2, tumorous tissues showed higher GUS activity than various organs with a normal appearance in the regenerants of both constructs. Among the organs of normal-type F1 transgenic plants, the higher GUS activities were detected in aerial parts than in the roots in both cases. The promoter activity of NgORF13 was always lower than that of RiORF13 in any tissues.
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Histochemical analysis of tissue-specific expression

Under continuous high intensity light, genetic tumors showed teratoma structures with rudimentary bud primordia-like structures termed bud primordia here in and partially developed buds with vascular bundles and leaves (Fig. 3A). Histochemical staining revealed that the GUS activity of NgORF13-GUS in genetic tumors was localized in bud primordia, and vascular bundles and leaves in the buds (Fig. 3C). RiORF13-GUS transgenic tumors showed similar expression patterns with NgORF13-GUS transgenics (data not shown).

By conversion of a high light intensity to a lower light intensity or dark, tumors with developing green teratoma to tumorous tissues developing normal, etiolated and elongated shoots (Fig. 3B). The proliferating tumorous tissues at the base of elongated shoots showed the GUS staining of NgORF13-GUS in bud primordia, partially developed buds, and vascular bundles and leaves in the shoot (Fig. 3D). In prolonged shoots, the gradient patterns of GUS expression was found that strong GUS activity in upper part of the shoots weakened at the base of the tumorous tissues. The GUS staining of RiORF13-GUS transgenic tumors grown under dark condition was similar to that of NgORF13 (data not shown).

When transferred to a high light condition, the etiolated shoots gradually grew to green shoots with developing normal
leaves and finally regenerated whole plants which showed a normal appearance. The localization of NgORF13 promoter activity in normal-type F1 plant was examined in leaves, stems and roots. By contrast to non-transformant F1 leaves (Fig. 3E), the GUS expression was observed predominantly in vein and mesophyll cells in NgORF13-GUS transgenic leaves (Fig. 3F). The cross-sections of leaves harboring NgORF13-GUS revealed staining in mesophyll cells but not in epidermal cells (Fig. 3G). In stems, the GUS activity of NgORF13-GUS was found mainly in lateral buds and vascular systems. Weaker activity was also found in the parenchymatous tissues around vascular bundles (Fig. 3H, I). The GUS expression patterns of RiORF13-GUS in leaves and stems were similar to those of NgORF13-GUS (data not shown).

In NgORF13-GUS harboring transgenic roots, the expression was below the histochemical detection threshold (data not shown). GUS staining of RiORF13-GUS transgenic roots was detected in the vascular system and cortex but not in the epidermis (Fig. 3J). No GUS staining was detectable in the apex of main and lateral roots (data not shown).

**The expression of NgORF13 promoter during tumorization**

Wild-type F1 plants have the potential ability to develop tumors. With time, tumors spontaneously occur on stems, roots
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and the other tissues of NgORF13-GUS transgenic F1 plants (Fig. 4A, C). Fig. 4B shows the stem section developing tumors on leaf nodes of NgORF13 transgenic plants. The expression of the NgORF13-GUS was found in tumors, but not in stems.

In aged NgORF13-GUS transgenic roots with tumors, GUS-staining was observed only in tumorous regions (Fig. 4D). The GUS staining was also found in the regions partly differentiated of the tumor (Fig. 4E). The GUS expression of NgORF13-GUS transgenic F1 plants was found in tumorous tissues regardless of the region where the tumors developed. However, the expression of NgORF13-GUS in normal-type tissues on which the tumors developed decreased with aging. These expression patterns were also found in RiORF13-GUS transgenics (data not shown).

Wounding induced tumors on various parts of the F1 plants regardless of the growth period. Fig. 5A shows the schematic illustration of tumorization on a leaf tissue by cutting treatment. The effect of wounding on the promoter activity of NgORF13 was determined in leaves by histochemical analysis. Leaves of transgenic plants were wounded by cutting treatment and cultured for 10 d. The GUS activity in NgORF13-GUS transgenic leaves was found in the vein and mesophyll cells on 0 d (Fig. 5B). Three d after wounding treatment, the staining was also detected in cut regions in addition to the vein and mesophyll cells (Fig. 5C). The intensity of staining gradually increased for 5 d (Fig. 5D). Ten d after treatment, the tumors developed large enough to be seen with the naked eye. Fig. 5E shows the cross-sections of tumors developed on cut regions of transgenic leaves that harbored NgORF13-GUS. The GUS staining was observed throughout the dividing cells of developing tumors. When the tumors were differentiated into teratoma, the GUS activity was confined to the region which was partly organized (Fig. 5F). The expression patterns of RiORF13 promoter showed similar wound-inducibility with NgORF13, but the GUS staining was stronger in leaves of RiORF13-GUS transgenic leaves than in leaves of NgORF13-GUS transgenics (data not shown).

The effect of cytokinin and MeJA on NgORF13 promoter activity

The wound-inducibility of NgORF13 promoter activity was further investigated fluorometrically in genetic tumors. Wounding treatment was performed by cutting the tumor tissues into small pieces. As a control, samples without the treatment were used. The wound-inducibility of NgORF13 promoter activity was investigated in the presence of cytokinin. Cytokinin caused the morphogenic alterations such as
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short and variable length of internode, poorly developed roots, small rounded leaves and short flowers in transgenic plants overexpressing RiORF13 from mannopine-type Ri plasmid (Hansen et al. 1993). As shown in Fig. 6, wounded tissues showed higher activity of GUS than unwounded tissues at all concentrations of zeatin. However, no GUS activity was activated by zeatin in both wounded and unwounded tissues. The same results were obtained with kinetin treatment (data not shown).

Time course in changes of GUS activity revealed that the NgORF13 promoter activity gradually increased and increased two fold 5 d after wounding treatment. No increase was observed in control tumors (data not shown). Jasmonate (JA) acts as a regulator in defense responses and JA synthesis upon wounding activates various genes (Titarenko et al. 1997). We monitored the relevance of MeJA to the wound-inducibility of NgORF13 promoter (Fig. 7). Although the NgORF13 promoter activity increased by MeJA treatment in both wounded and unwounded tumors, it was more effective in tumors with wounding. In wounded tumors the GUS activity increased on the first day after MeJA treatment, and reached maximum on 2 d. The high activity remained steadily for 5 d. Thus, upon wounding, the promoter activity of NgORF13 was remarkably activated by MeJA.

Discussion

We analyzed the transition of NgORF13-GUS gene expression during tumorization and redifferentiation. This approach is expected to help us understand the function of NgORF13 in genetic tumors and F1 plants.

Fluorometric and histochemical analysis revealed that the GUS activity was lower in NgORF13-GUS transgenics than in RiORF13-GUS transgenics, but there was no difference in the tissue-specific expression between the transformants. That is, the decline of NgORF13 promoter activity was due to the quantitative decrease of promoter activity. Together with Nagata et al. (1995), it seems likely that the cis-acting regions of the NgORF13 promoter that are involved in control of the tissue-specific localization were conserved as is the case for RiORF13 during the evolution of the genus Nicotiana.

In general, when the plants are wounded, various defence reactions occur at the wound site. They include the formation of wound tissue with a limited number of cell divisions to protect the wounded sites. In F1 plants, however, cells continue to divide and develop tumors at the wound site. The GUS activity of NgORF13-GUS was higher in tumors than in the organs with normal morphology (Fig. 2). It was consistent with the results of Northern hybridization that NgORF13 is expressed in genetic tumors but scarcely detected in leaf tissues (Aoki et al. 1994). These findings suggest the possibility that NgORF13 might be involved in the formation of genetic tumors.

Fig. 8A shows a schema of the expression patterns of the NgORF13 promoter during the tumorization in comparison with those of NgorB and NgorC. The expression of NgORF13 was detected at an early stage of tumorization when the cell division was uncertain (Fig. 5C, D). Thereafter, the overall tumors including dividing cells were stained as a result of GUS activity (Fig. 4D, 5E). Such an expression pattern was identical to that of NgorB-GUS (Nagata et al. 1996). However, it was different that the expression of NgORF13 was not found in meristemic zones of roots and shoot apices. The dividing cells in tumors were produced from compact and organized structures

![Fig. 6](https://example.com/fig6.png)

**Fig. 6** Effects of zeatin on the GUS activity of cultured genetic tumors transformed with NgORF13-GUS. Tumors were harvested at 5 d after transfer to medium that contained zeatin at various concentrations and cultured continuous light at 25°C. Fluorometric assays were performed using extracts of with (black circles) or without (white circles) cutting NgORF13-GUS transformed tissues. Bars indicate standard errors (n = 10).

![Fig. 7](https://example.com/fig7.png)

**Fig. 7** Time course of GUS activity by MeJA treatment of genetic tumors transformed with NgORF13-GUS. NgORF13-GUS-transformed tissues with (black circles) or without (white circles) cutting treatment were treated with 10^-4 M MeJA for 0.5 h and washed by hormone-free MS liquid medium. Resulting tissues were then cultured on hormone-free MS agar medium under continuous light at 25°C. Fluorometric assays were performed using extracts prepared at the indicated times. Bars indicate standard errors (n = 10).
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(Ichikawa and Syono 1988), but we suppose they are not like those of meristems in plants in that they are not always pluripotent. Considering its effects on root formation (Aoki and Syono 1999a), the expression of NgORF13 in dividing cells of tumors is secondary and NgORF13 might have the supplementary action of NgrolB rather than acting on the cell division itself. On the other hand, torf13, a RiORF13 homolog of N. tabacum, was involved in the proliferation on carrot disks (Fründt et al. 1998). Carrot tissues might have unknown factor(s) which interact with torf13 to promote cell division. Further analysis is needed to elucidate the function of NgORF13 in cell division.

At a later stage of tumorization, the expression of NgORF13-GUS was observed only in organized primordia-like masses (Fig. 4E, 5F). This expression pattern somewhat resembled that of NgrolC (Nagata et al. 1996). These results suggest the possibility that NgORF13 acts on the maintenance of tumors synergistically with NgrolB and NgrolC.

In F1 plants, NgORF13 and RiORF13 were expressed mainly in the aerial parts including vascular systems and parenchyma tissues (Fig. 2, 3F–I) and their expression in the younger tissues gradually decreased toward the older ones. Hansen et al. (1997) reported that in transgenic plants with RiORF13 from mannopine-type Ri plasmid, the transgene expressed at higher levels at the top than at the base of the vascular tissues of stems. Accumulation of N. tabacum torf13 mRNA was high in upper leaves but low in lower leaves (Fründt et al. 1998). These results are consistent with our results. In the roots, however, the expression of NgORF13 and RiORF13 was low (Fig. 2), while that of RiORF13 from mannopine-type was reported to be high (Hansen et al. 1997). This difference remains to be solved.

Throughout normal morphological tissues of F1 plants, the expression of NgORF13 was found in vascular systems, which was similar to that of NgrolC (Nagata et al. 1995, Nagata et al. 1996). NgrolC, however, was expressed in vascular systems irrespective of the age of the tissues. The limited expression at the young tissues was unique to NgORF13. NgORF13 also showed the characteristic expression in paren-
the tumorization. This interaction needs to be studied further. Because it is possible that the suppression of NgORF13 gene processes.

As wounding has a positive effect on hairy root and genetic level of nucleotide sequences near the both ends of the region. shown in Fig. 8B, a high degree of homology was found at the with that of RiORF13 from mannopine-type promoter. As of NgORF13 and RiORF13 from agropine-type Ri plasmid.

The expression of NgORF13 in the presence of wounding (Fig. 7). Recently, Hansen et al. (1997) was amplified. Each results using the promoter of mannopine type RiORF13 by Hansen et al. (1997), the region of the promoter of NgORF13 and RiORF13 identical to that of mannopine-type RiORF13 promoter used by Hansen et al. (1997) was amplified. Each HindIII/XbaI fragment, which contains NgORF13 or RiORF13 promoter with sequences upstream from each start codon connected in frame to the β-glucuronidase (GUS) gene (Fig. 1). The NgORF13-GUS and RiORF13-GUS constructs were inserted into the binary vector pHTS6.1 separately and transferred into Agrobacterium strain LBA4404 by the triparental method as described above.

Transgenic plants

The tobacco hybrids (F1) between N. glauca and N. langsdorfii were cultivated on hormone-free Murashige-Skoog’s medium (Murashige and Skoog 1962) in plastic culture pots under fluorescent light at 23°C. The fully developed leaves were used for transformation via Agrobacterium by the leaf disk method (Horsch et al. 1985). F1 plants that harbored NgORF13-GUS and RiORF13-GUS were grown as transformants of genetic tumors on hormone-free MS medium supplemented with 100 mg liter−1 rifampicin, 100 mg liter−1 vancomycin, 100 mg liter−1 cefotaxime and 100 mg liter−1 kanamycin under continuous light. Selected transformants were subcultured every month, on hormone-free MS medium containing 50 mg liter−1 kanamycin under continuous light condition. Tumorization-redifferentiation system (Ichikawa and Syono 1988) was used for the conversion between tumors and normal-type F1 plants.

GUS assay

The GUS fluorometric assay was performed as described by Jef-ferson et al. (1987). The supernatants of homogenized tissue was incu-

Materials and Methods

Bacterial strains

Agrobacterium tumefaciens LBA4404 strain was grown at 28°C in LB medium supplemented with streptomycin at 300 mg liter−1 and rifampicin at 100 mg liter−1. Escherichia coli DH5α strain and HB101 strain were cultivated at 37°C in LB medium as described by Sambrook et al. (1989). Recombinant plasmids derived from pHTS6.1 (Tsukaya et al. 1991) were mobilized from E. coli strain DH5α to A. tumefaciens strain LBA4404 by triparental mating with E. coli HB101 that harbored pRK2013 as a helper strain (Horsch et al. 1985). Selections were performed on solidified LB medium with kanamycin at 100 mg liter−1, rifampicin at 100 mg liter−1 and streptomycin at 300 mg liter−1.

GUS constructs

The promoters contained NgORF13 and the RiORF13 were obtained from the clone λNg31 which included the cT-DNA from the genome of N. glauca (White et al. 1983), and the cosmide clone pLJ-1 from the agropine-type pRK13 T-DNA (Jouanin 1984), respectively.

Promoter fragments of the NgORF13 and RiORF13 genes were obtained by PCR amplification using the following primers: N13-5 (5′-CCCAAGCTTACAGGGGCTGAAGGTGTTTTAC-3′) with an additional HindIII site [underlined], N13-3 (5′-GCTCTAGAACAT- AAATTATCGACATGAA-3′) and additional Xho site [underlined] and R-13-5 (5′-CCCAAGCTTACAGGGGCTGAAGGTGTTTTAC-3′) with an additional HindIII site. For comparison with the results using the promoter of mannopine type RiORF13 by Hansen et al. (1997), the region of the promoter of NgORF13 and RiORF13 identical to that of mannopine-type RiORF13 promoter used by Hansen et al. (1997) was amplified. Each HindIII/XbaI fragment, which contains NgORF13 or RiORF13 promoter with sequences upstream from each start codon connected in frame to the β-glucuronidase (GUS) gene (Fig. 1). The NgORF13-GUS and RiORF13-GUS constructs were inserted into the binary vector pHTS6.1 separately and transferred into Agrobacterium strain LBA4404 by the triparental method as described above.
bated with 4-methylumbelliferyl-β-D-glucuronide as a substrate and the amount of the reaction product, 4-methylumbelliferylone (4MU) measured fluorometrically. Protein concentrations were measured by Bradford (1976) using Bio-Rad protein assay kit. The GUS activity was determined as picomoles of 4-MU formed per minute per milligram protein.

The histochemical GUS staining was performed as described by Jefferson et al. (1987). Fixed tissues were incubated in the buffer containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glc) for an appropriate period. The reactions were stopped with 70–100% ethanol. Chlorophyll was removed from green tissues with 100% ethanol.

Acknowledgments

We wish to thank Dr. M. P. Gordon (University of Washington, U.S.A.) for the gift of a cT-DNA clone and Dr. L. Jouanin and Dr. D. Tepfer (INRA, France) for their gift of the cosmig clone. We are also grateful to Dr. H. Tsukaya (National Institute for Basic Biology) for providing the plasmids pHTS6.1. This work was supported, in part, by Grants-in-Aid for Scientific Research on Priority Areas, Grants-in-Aid for Scientific Research and The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private School of Japan to K. S. from the Ministry of Education, Science and Culture, Japan.

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(Received February 23, 2004; Accepted May 10, 2004)