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Influence of Phytohormones on the Promoter Activity of T-*cyt* Gene from *Agrobacterium*.

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Influence of phytohormones on the expression of T-*cyt* gene of the Ti plasmid, which codes for cytokinin biosynthesis, was examined in transgenic tobacco plants with a fusion gene between the T-*cyt* promoter and β-glucuronidase reporter gene (P*tmr*-GUS). The expression of P*tmr*-GUS was not largely influenced by any hormones tested, maintaining a significant level of expression.

Keywords: T-cyt gene; promoter activity; phytohormone response.

The molecular mechanism for the crown gall disease which is caused on various dicotyledonous plant species by *Agrobacterium tumefaciens* has been revealed for these two decades (see reviews).[1, 2] Upon infection the bacterium transfers a particular DNA segment (T-DNA) of its harboring tumor-inducing (Ti) plasmid to the host plant cell, then the genetically transformed plant cell comes to overproduce phytohormones to form tumorous outgrowth. Two genetic loci, *tms* and *tmr*, on the T-DNA code for the synthesis of auxin and cytokinin, respectively.[3-6]

Although auxin and cytokinin contents are elevated in the tumor tissue due to the introduced hormone synthesis genes, the resultant balance between the hormones is somewhat contradictory. Ishikawa *et al.*[7] measured endogenous auxin and cytokinin levels in tobacco and carrot crown galls as well as those tumors induced with mutant Ti plasmids which were deficient in auxin or cytokinin production (*aux*⁻ and *cyt*⁻ mutants, respectively). The results with tobacco tumors showed that the endogenous level of either hormone was much higher in the mutant tumor with the functional corresponding hormone synthesis gene than in the wild-type tumor. This suggests a possibility that the expression of either hormone synthesis gene might be regulated by the other gene through the resultant hormone.

So far, there are several works concerning the effect of phytohormones on the expression of T-*cyt* gene, which is also called as *tmr* or *ipt* gene and encodes the enzyme isopentenyltransferase (IPT). Song *et al.*[8] first demonstrated that exogenous auxin reduced the cytokinin level in a bean crown gall as well as the T-*cyt* mRNA level in the tissue. Zhang *et al.*[9, 10] reported a similar effect of auxin on the T-*cyt* expression in tobacco transformed with T-*cyt* gene alone, demonstrating that the endogenous cytokinin, T-*cyt* mRNA and IPT protein levels were all reduced by exogenously supplied auxin, naphthaleneacetic acid (NAA). These results seem to be consistent with Ishikawa's observation on the hormone levels in wild-type and mutant tumors. In this study, we examined the influences of auxin as well as several other phytohormones on the expression of GUS reporter gene connected to a 980 bp (-688/+292) promoter fragment of T-*cyt* gene in transgenic tobacco.

We utilized a 980 bp *Bam* HI fragment, which contained 688 bp of the 5' non-coding sequence and 292 bp of the coding sequence of T-*cyt* gene, from the octopine-type Ti plasmid pTiAch5 as the promoter fragment to drive the β-glucuronidase (GUS) reporter gene in the binary vector pBI101.[11] The resulting fusion gene referred to as P*tmr*-GUS was then introduced into tobacco (*Nicotiana tabacam* cv. Petit Havana SR1). Leaf disks 5 mm square were excised from the P*tmr*-GUS tobacco and incubated at room temperature for 24 hours in liquid MS medium supplemented with various phytohormones. Control leaf disks were incubated with a hormone-free MS medium. Leaf disks were then subjected to fluorometric assay of the GUS activity with 4-MUG as the substrate.[11] The activities of the hormone-treated leaf disks were expressed as the relative values against the control values.

Since the cytokinin contents in the wild-type crown galls were much lower than those in the aux-tumors,[7] we examined effects of auxin and cytokinin on the expression of the P*tmr*-GUS fusion gene first. Leaf disks were treated with NAA (1 mg/l) as an auxin, kinetin (1 mg/l) as a cytokinin, or both of them (Fig. 1). Contrary to our speculation that a high

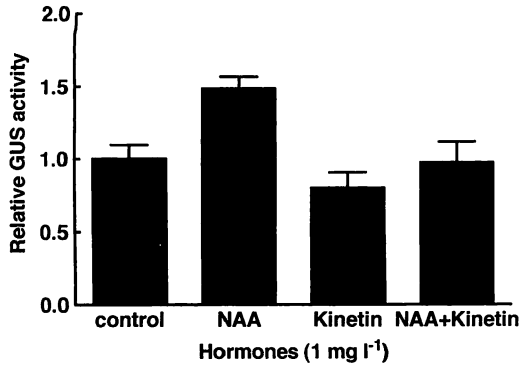

Fig. 1 Effects of auxin and cytokinin on the expression of P*tmr*-GUS.

Leaf disks taken from a transgenic tobacco were treated with NAA and/or BA for 24 hours and then subjected to GUS assays. GUS activities were expressed as relative values against the average value of the control samples. For each treatment, at least five samples were measured. Error bar: standard error of the mean.

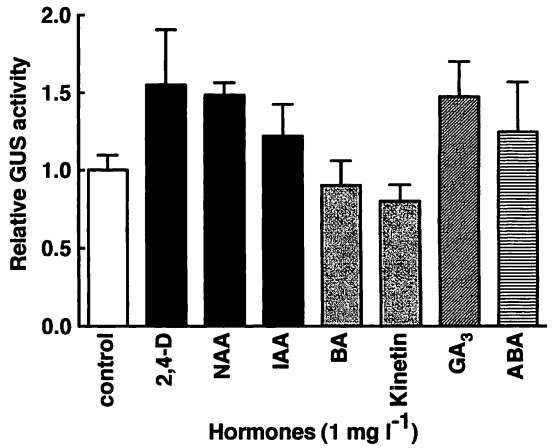

Fig. 2 Effects of various phytohormones on the expression of P*tmr*-GUS.

Leaf disks taken from a transgenic tobacco were treated with various phytohormones for 24 hours and then subjected to GUS assays. Black bars: auxins. Gray bars: cytokinins. GUS activities were expressed as relative values against the average value of the control samples. For each treatment, at least five samples were measured. Error bar: standard error of the mean.

concentration of auxin in a wild-type crown gall might reduce the cytokinin level by repressing T-*cyt* gene, NAA rather seemed to slightly promote the expression of P*tmr*-GUS. Kinetin showed virtually no effect by itself, but cancelled the stimulative effect of auxin.

As NAA showed a little stimulation on the P*tmr*-GUS expression, we examined effects of other auxins and cytokinins as well as a few other phytohormones next (Fig. 2). All hormone treatments were performed at a concentration of 1 mg/l.

Three auxins tested were all stimulative for the P*tmr*-GUS expression, and the effects differed in proportion to their so-called 'auxin activity', *i.e.* 2,4-D was most effective and IAA was least (Fig. 2). When the auxin dose-response of the P*tmr*-GUS expression was observed using the most effective 2,4-D (Fig. 3, A), the GUS expression was stimulated up to twofold as the auxin concentration increased to 3 mg/l, then reached a plateau. A similar dose-response was also observed with NAA (data not shown). These suggests that the auxin essentially has a stimulative effect on the T-*cyt* promoter activity, although the extent of the stimulation was not at a significant level. This is, however, contrary to the previous observations on T-*cyt* expression upon an auxin treatment.[8, 10] Song *et al.*[8] and Zhang *et al.*[10] reported with bean and tobacco, respectively, that the exogenously supplied auxin repressed the native T-*cyt* expression at the mRNA, protein product and/or endogenous cytokinin levels. Zhang *et al.*[10] also reported that the repression by auxin was observed even when the native promoter of T-*cyt* gene was replaced with the 5'-upstream region from Rubisco small subunit gene of Petunia. These results might mean that the regulatory region(s) responsible for the repression by auxin is located outside the promoter region, as they stated about the possibility. In the case, the hypothetical regulatory region should not exist in the 5'-part of the coding region down to +292 because the promoter fragment which was used in this study spanned from -688 to +292. Saying exclusively on the T-*cyt* promoter fragment used in this study, auxin had no suppressive effect but rather a stimulative one anyway.

On the other hand, neither cytokinin tested influenced the GUS expression, or rather, they seemed to be somewhat suppressive (Fig. 2). When P*tmr*-GUS leaf disks were treated with kinetin in a range of concentrations, however, no significant influence on the GUS expression was observed throughout the range (Fig. 3, B), confirming that the cytokinin by itself has no effect on the T-*cyt* promoter activity. Song *et al.*[8] and Zhang *et al.*[10] also reported that cytokinin exerted no influence on the T-*cyt* expression by itself, and the latter reported that cytokinin cancelled the repression of the T-*cyt* expression by auxin. In our results, although the direction of the auxin effect on the T-*cyt* expression was opposite, *i.e.* stimulative, cytokinin cancelled the influence of auxin nevertheless. Anyway, cytokinin consistently showed an antagonistic effect against auxin on the influence for T-*cyt* expression. This seems to mean that cytokinin does not directly influence the transcription of T-*cyt* gene but antagonizes the signal transduction from auxin.

Other than the auxin and cytokinin, both gibberellin A_3 (GA_3) and abscisic acid (ABA) brought a slight increase in the P*tmr*-GUS expression, but the stimulations were not more than 1.5-fold of

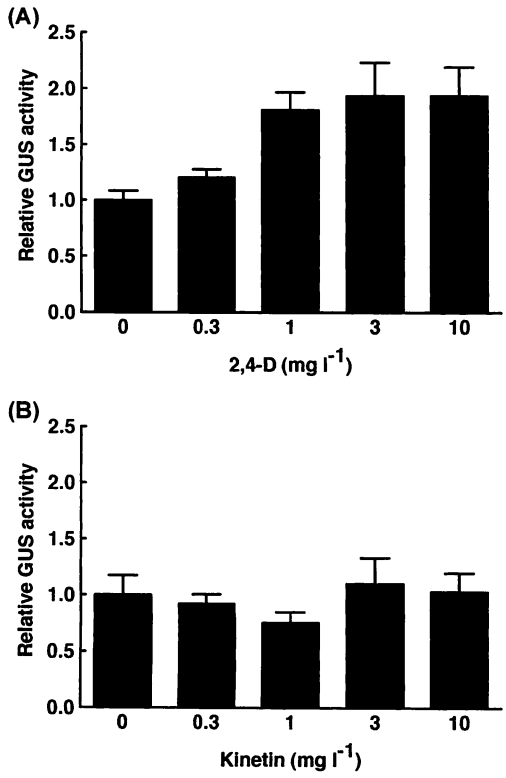

Fig. 3 Dose-response relationships for auxin and cytokinin on the expression of P*tmr*-GUS.

Leaf disks taken from a transgenic tobacco were treated with 2,4-D (A) or kinetin (B) for 24 hours and then subjected to GUS assays. GUS activities were expressed as relative values against the average value of the control (treated without phytohormone) samples. For each treatment, at least five samples were measured. Error bar: standard error of the mean.

the control level (Fig. 3). Taken all together, the promoter fragment of T-*cyt* gene used in this study conferred a constitutive expression on a connected gene in consistency with other reports.[12-14]

In conclusion, the auxin had a weak but consistently stimulative effect on the transcription from the T-*cyt* promoter fragment used in this work. The cytokinin had no effect by itself on the T-*cyt* promoter activity, but acted as an antagonist against the auxin action. Both GA_3 and ABA might have a weak stimulative effect. After all the observations, the T-*cyt* promoter was not largely influenced by any hormonal treatments. Although the fluorometric GUS reporter assay by which our results presented here were all obtained is known to be not completely reliable for quantitatively evaluating gene expressions, it is still reasonable to consider that auxin is at least not repressive to the T-*cyt* promoter as reported before because all auxins tested here were consistently not suppressive but stimulative. We are planning to confirm the effect of auxin on the T-*cyt* promoter by a quantitative RT-PCR method.

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