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Effects of Some Auxins on IAA-Amino Acid Conjugate Formation in Carrot Tissues Transformed with Ti Plamids

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IAA-conjugate formation in carrot tissues transformed with aux$^-$ Ti plasmids was induced by exogenously applied IAA. When the tissues were treated with NAA or 2, 4-D, IAA-conjugate formation was also induced. This result suggests that IAA-conjugate formation may be generally induced by substances having auxin activity.

On the other hand, 2, 4-D was not metabolized to conjugate form even when the transformed carrot tissues were treated with IAA. This suggests that the effect of 2, 4-D is different from that of IAA in carrot.

Introduction

When plant cells are infected with *Agrobacterium tumefaciens* harboring Ti plasmids, a certain part of the Ti plasmid, called T-DNA, is integrated into the plant genomic DNA. Genes for the synthesis of auxin and cytokinin encoded in the T-DNA are expressed in transformed plant cells. As a result, tumors are induced at the sites of infection[1].

It was previously reported that endogenous levels of indole-3-acetic acid(IAA) were similar among carrot tissues transformed with 3 different plasmids, namely, wild-type, aux$^-$ Ti (a mutant Ti plasmid with transposon insertion at *tms* locus) and cyt$^-$ Ti (a mutant Ti plasmid with transposon insertion at *tmr* locus) plasmids[2], though the tissues transformed with wild-type or cyt$^-$ Ti plasmids exhibited a higher rate of biosynthesis of IAA than the tissues transformed with aux$^-$ Ti plasmids[3]. One of the reasons for maintenace of free IAA at a same level in the tissues transformed with the three different Ti plasmids is that the synthesized IAA was metabolized to amino acid conjugate form[4]. The tissues transformed with aux$^-$ Ti plasmids had only a low activity of IAA-conjugate formation, because the rate of biosynthesis of IAA was so low that free IAA did not accumulate. High IAA-conjugate formation activity was induced by the treatment with exogenously applied IAA[4]. These results suggested that the formation of conjugated IAA, which is induced by endogenous IAA, may have an important role in maintaining free IAA at a low level. Furthermore, we reported that this mechanism (conversion of free IAA to IAA-conjugate form) needed enzymatic reaction(s) and was regulated at the transcriptional level[5].

It has been reported that carrot somatic embryogenesis was induced by some auxins, and that 2, 4-dichlorophenoxyacetic acid (2, 4-D) had stronger activity in inducing somatic embryogenesis than some other auxins such as IAA and 1-naphthaleneacetic acid (NAA)[6]. It is thought that IAA and NAA have only a weak effect on the induction of somatic embryogenesis, because they are inactivated more easily than 2, 4-D. To examine this possibility, we investigated the effects of 2, 4

-D, NAA and IAA on IAA inducible IAA-conjugate formation which is one of the IAA inactivating mechanisms and the metabolism of 2, 4-D in carrot crown gall.

In the present study, we report that IAA-conjugate formation in carrot tissues transformed with aux$^-$ Ti plasmids was also induced by treatment with substances having auxin activity and that enzyme(s) for IAA conjugate formation utilized IAA but did not utilize 2, 4-D as substrates. Also we discuss the relationship between auxin inactivation and the effects of auxin on somatic embryogenesis in carrot cells.

Materials and Methods

1. *Carrot tumor culture*

Hypocotyl segments of carrot seedlings (*Daucus carota* L. cv. US-Harumakigosun) were inoculated with *Agrobacterium tumefaciens* (C58C1) harboring pGV2250 (a mutant of pTiB6S3 with a transposon insertion at the *tms* locus; R. Deblaere, unpublished: aux$^-$ Ti plasmids). To establish axenic cultures of the tumors, they were cut out and cultured on Murashige and Skoog's (MS) solid medium supplemented with Carbenicillin (500 mg/liter). After being subcultured several times tumors were transferred and subcultured monthly on MS solid medium without antibiotics and phytohormones[2]. Tumor tissues which proliferated rapidly after ten days of subculture were utilized throughout the experiments.

2. *Induction of IAA-conjugate formation*

Tissues (0.2 g fr wt) were incubated for 1 h in 0.2 m*l* of MS liquid medium containing 20 μM unlabelled auxins (IAA, NAA and 2, 4-D). After incubation, they were rinsed 3 times with 1 m*l* of MS liquid medium and cultured in 0.2 m*l* of MS liquid medium containing [1-^{14}C] IAA (37 kBq/m*l*). After incubation with ^{14}C-IAA, they were rinsed 5 times with 1 m*l* of MS liquid medium and extracted in 10 m*l* of 80% acetone with pestle and mortar.

The extracts were concentrated *in vacuo* and dissolved in 0.2 m*l* of methanol. An aliquot (50 μ*l*) of the methanol solution was applied to a thin-layer chromatography on an aluminium sheet silica gel 60 (without fluorescent indicator; Art. 5553; Merk & Co., Inc.) and developed with *iso*-butanol: acetic acid: water (4 : 1 : 1, v/v/v)[4,5]. After development (10 cm) the radioactivity

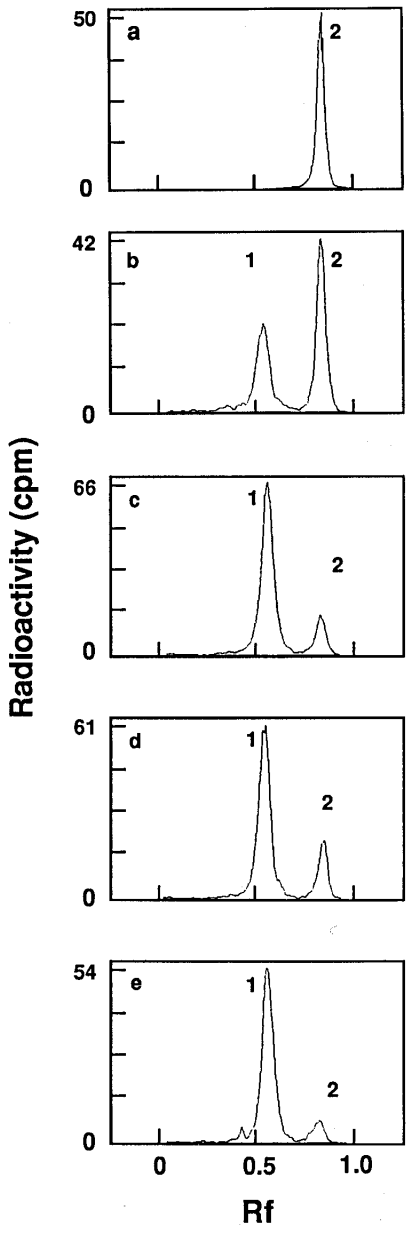

Fig. 1 Distribution of radioacitivity in chromatograms of authentic IAA (a), extracts of control carrot tissues (b) and extracts of carrot tissues treated with IAA (c), NAA (d) or 2, 4-D (e). Tissues which were pre-cultured with or without auxins for 1 h, were cultured in MS medium containing ^{14}C-IAA with auxins. After 90 minutes culture the radioactivities on TLC plates were measured. 1 : conjugated IAA, 2 : free IAA.

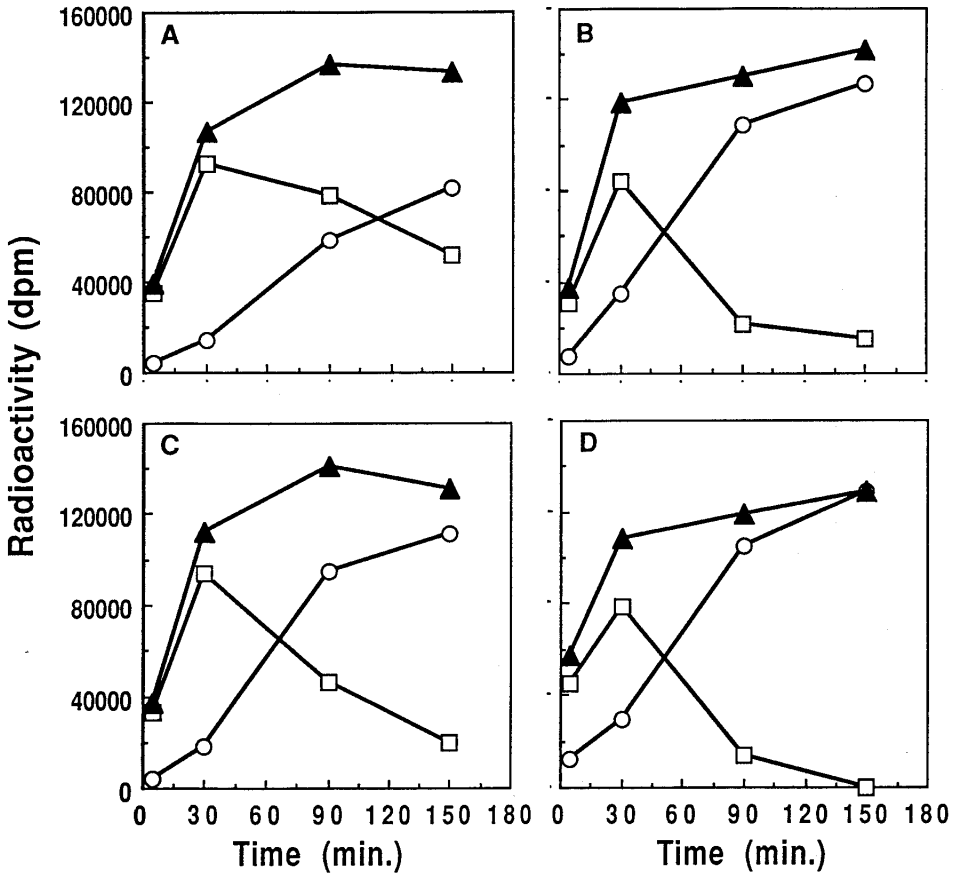

Fig. 2 Changes in the total radioactivity (▲) and distribution of radioactivity incorporated into free IAA (□) and conjugated IAA (○) fractions in carrot tissues transformed with aux⁻ Ti plasmids. Tissues which were pre-cultured with or without auxins for 1 h, were cultured in MS medium containing ^{14}C-IAA. After culture, the radioactivities of free IAA and conjugated IAA were measured. A : pre-cultured without any auxins, B : pre-cultured with IAA, C : pre-cultured with NAA, D : pre-cultured with 2, 4-D.

on the chromatogram was counted by an automatic TLC linear analyzer (TLC-Multi-Tracemaster LB 285; Laboratorium Prof. Dr. Berthold)[5]. All experiments were repeated thrice.

3. *Determination of metabolites of 2, 4-D*

Tissues (0.2 g fr wt) were cultured in 0.2 m*l* of MS liquid medium containig [1-^{14}C] 2, 4-D (37 kBq/m*l*) with or without IAA (20 μM). After incubation with ^{14}C-2, 4-D for 3 hrs, they were rinsed 5 times with 1 m*l* of MS liquid medium and extracted in 10 m*l* of 80% acetone with pestle and mortar.

The extraction, separation and detection of ^{14}C-2, 4-D were carried out in a similar way to the method described above for ^{14}C-IAA.

Results and Discussion

1. *Induction of IAA-conjugate formation*

IAA-conjugate in the extracts of carrot crown gall treated with IAA, NAA or 2, 4-D, was detected in the position whose Rf value was 0.6 (**Fig. 1**).

Total amounts of radioactivity incorporated into control tissues without any auxin-treatments

increased rapidly until 90 min. and reached a steady level after 90 min.. Radioactivity incorporated into conjugated IAA increased gradually (**Fig. 2-A**). This increase of IAA-conjugate formation is thought to be induced by ^{14}C-IAA utilized as a tracer. Pre-treatment with IAA, NAA or 2, 4-D did not alter the total amounts of radioactivity incorporated into the tissues, but the conversion rates of IAA to conjugated IAA was much greater than those of the controls (**Fig. 2-B, C** and **D**). Similar tendencies were observed in all three experiments.

IAA, NAA and 2, 4-D had the same effects on induction of IAA-conjugate formation. This suggested that substances having auxin activity can induce IAA-conjugate formation in carrot tissues. These results are consistent with previous reports indicating that indole-3-acetylaspartate (IAAsp) formation was induced by several auxins (IAA, NAA and 2, 4-D) in pea[7-9].

We recently reported that IAA-conjugate formation in carrot tissues transformed with aux$^-$ Ti plasmids was inhibited by inhibitors of protein or RNA synthesis, and suggested that IAA-conjugate formation is regulated at the transcriptional level[5], as previously reported in pea[10]. From the results described above, namely that NAA and 2, 4-D had the same effect on the induction of IAA-conjugate formation, it is suggested that IAA-conjugate formation, which is induced by these auxins, is also regulated at the transcriptional level.

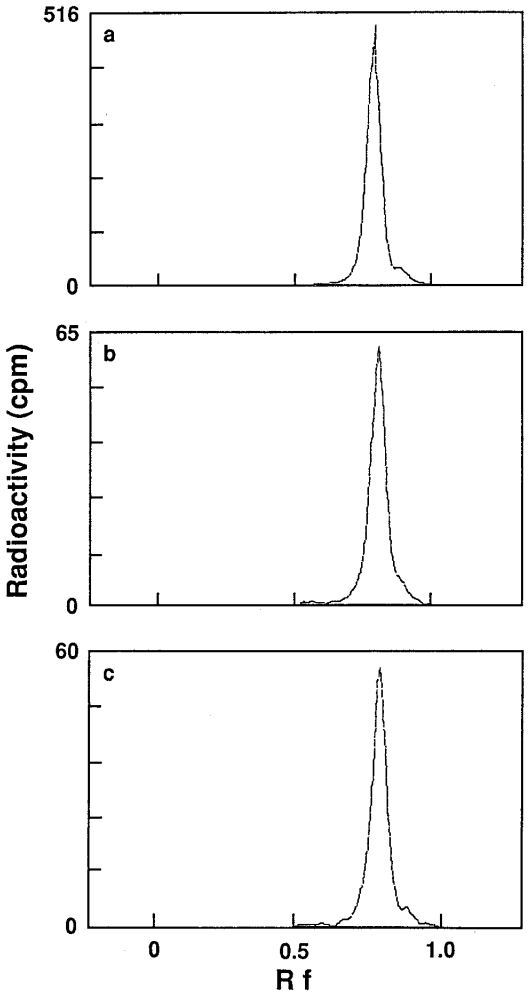

Fig. 3 Distribution of radioactivity in chromatograms of authentic 2, 4-D(a), extracts of control carrot tissues(b) and extracts of carrot tissues treated with IAA(c).
Tissues were cultured in MS medium containing ^{14}C-2, 4-D with or without IAA. After 3 hours culture the radioactivities on TLC plates were measured.

2. *Determination of metabolites of 2, 4-D*

Fig. 3-a shows that the Rf value of authentic ^{14}C-2, 4-D on TLC plate was approximately 0. 8. **Fig. 3-b** shows that no peak, except for free ^{14}C-2, 4-D, was detected in extracts of control tissues (not treated with IAA). Similarly, in extracts of IAA treated tissues, whose IAA-conjugate formation had already been induced by the IAA, only a peak of free ^{14}C-2, 4-D was observed (**Fig. 3-c**). These results suggested that 2, 4-D cannot be metabolized in carrot tissues. We previously suggested that free IAA was metabolized to conjugated IAA by enzymatic reaction(s). Therefore, it is thought that 2, 4-D cannot be recognized as a substrate by enzyme(s) catabolizing IAA-conjugate formation.

In carrot, IAA can induce somatic embryogenesis *in vitro* at a reasonably high concentration

(about 10 ppm). On the other hand 2, 4-D is efficient in inducing somatic embryogenesis at a low concentration (about 0.01 ppm)[6]. It is thought that the endogenous level of IAA is reduced by conversion to conjugated form (inactivated form), and it has only a weak effect on induction of somatic embryogenesis. In contrast, 2, 4-D is not converted to inactivated form, so it has a stronger effect on induction than IAA.

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《和文要約》

ニンジンクラウンゴールにおけるアミノ酸結合型IAA生成に対するオーキシンの効果

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A. tumefaciens の aux$^-$ 株によるニンジン形質転換細胞は，IAA処理により結合型IAA生成が誘導されるが，IAA以外でも，NAAや2, 4-D処理によっても同様に誘導されたことから，結合型IAA生成は，オーキシン活性を有する物質により誘導されると考えられる．一方，2, 4-Dは，結合型IAA生成を誘導するにもかかわらず，それ自身は結合型へは代謝されなかった．このことは，ニンジンの培養細胞における，2, 4-DとIAAの効果の違いを反映していると考えられる．