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Identification of Conjugated IAA in Carrot Crown Gall as Indole-3-Acetylaspartic Acid (IAAsp) by LC/MS

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Abstract. In carrot crown gall cells transformed with Ti plasmids, Ti-derived IAA biosynthetic genes are transcribed and translated, followed by overproduction of IAA. However, the newly synthesized IAA is immediately metabolized to IAA–amino acid conjugate, and the content of endogenous free IAA is maintained at a low level. In this study, IAA–amino acid conjugate in carrot tissues transformed with Ti plasmids was identified as indole-3-acetylaspartic acid (IAAsp) by using frit-fast atom bombardment liquid chromatography/mass spectrometry (LC/MS).

When plant cells are infected with *Agrobacterium tumefaciens* harboring Ti (tumor-inducing) plasmids, a certain part of the Ti plasmid, called T-DNA (transferred DNA), is integrated into the plant genomic DNA. Genes for the synthesis of auxin and cytokinin encoded on the T-DNA are expressed in transformed plant cells. As a result, tumors are induced at the sites of infection (for a recent review, see Zambryski et al. 1989).

It was reported that endogenous levels of indole-3-acetic acid (IAA) were similar among carrot tissues transformed with three different Ti plasmids: wild-type, aux$^-$ Ti (a mutant Ti plasmid with transposon insertion at *tms* locus) or cyt$^-$ Ti (a mutant Ti plasmid with transposon insertion at *tmr* locus) plasmids (Ishikawa et al. 1988a), although 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 (Ishikawa et al. 1988b). One reason for maintaining free IAA at the same level in tissues transformed with three different Ti plasmids is that the synthesized IAA was metabolized to an amino acid conjugate form. 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. Treatment with exogenously applied IAA induced high IAA-conjugate formation activity in tissues transformed with aux$^-$ Ti plasmids (Ishikawa et al. 1990). These results suggested that the formation of conjugated IAA, which is induced by endogenous IAA, may have an important role for maintaining a low level of free IAA. Furthermore, we reported that the IAA-conjugate formation was induced by some chemicals having auxin activity (Sasaki et al. 1993) and that this conversion of free IAA to IAA-conjugate form requires enzymatic reaction(s) and is regulated at the transcriptional level (Sasaki et al. 1992).

Three IAA–amino acid conjugates have been reported, namely, indole-3-acetylaspartic acid (IAAsp) in *Picea sylvestris* (Andersson and Sandberg 1982), *Heracleum laciniatum* (Cohen and Ernstsen 1991), *Lycopersicon esculentum* (Catala et al. 1992), and IAA-overproducing transgenic tobacco plants (Sitbon et al. 1993); indole-3-acetylalanine (IAAla) in *Picea abies* (Östin et al. 1992); and indole-3-acetylglutamic acid (IAGlu) in cucumber (Sonner and Purves 1985). In addition, IAAsp is known as a common product in many plants treated with high levels of exogenous IAA (Monterio et al. 1988, Norcini and Heuser 1988, Nordstrom et al. 1991, Riov and Gottlieb 1980).

In this study, by using a very simple method for identification with frit-fast atom bombardment liquid chromatographymass spectrometry (LC/MS), conjugated IAA in carrot crown gall cells transformed with Ti plasmids was identified as IAAsp.

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Materials and Methods

Carrot Tumor Culture

Seedlings of *Daucus carota* L. cv. US-Harumakigosun were grown on vermiculite for 10 days at 25°C under 16-h light/8-h dark conditions. Surface-sterilized hypocotyl segments were placed on hormone-free Murashige and Skoog's (1962) (MS) solid medium (0.2%, w/v, Gelrite, Scott Laboratories, Inc.). *Agrobacterium tumefaciens* (C58C1) harboring wild-type Ti plasmids (pTiB6S3), aux$^-$ Ti plasmids (pGV 2215, a mutant of pTiB6S3 with a transposon insertion at the *tms* locus; Leemans et al. 1982), or cyt$^-$ Ti plasmids (pGV2250, a mutant of pTiB6S3 with a transposon insertion at the *tmr* locus; R. Deblaere, unpublished) were cultured for 2 days on LB medium (Bacto tryptone 10 g · l^{-1}, Bacto yeast extract 5 g · l^{-1}, NaCl 10 g · l^{-1}) solidified with 1.5% (w/v) agar and inoculated on freshly cut surfaces of the hypocotyl segments with a needle. To eliminate bacteria, inoculated hypocotyls were transferred to hormone-free MS solid medium supplemented with carbenicillin (500 mg · l^{-1}) 5 days after inoculation and subcultured every week on the same medium. After 3 weeks, crown galls were excised and subcultured every week on hormone-free fresh MS solid medium with carbenicillin (500 mg · l^{-1}) for 1 month. After elimination of bacteria, crown gall cells were transferred and subcultured monthly on hormone-free MS solid medium without antibiotics at 25°C under 16-h light/8-h dark conditions, as described earlier (Ishikawa et al. 1988a). In the following experiments, we used crown gall cells that were proliferating rapidly 10 days after transfer to fresh MS medium.

IAA Treatment of Crown Gall Cells

Crown gall cells (0.2 g fr wt) transformed with three different Ti plasmids were cultured in 0.2 ml of MS liquid medium containing 20 μM of unlabeled IAA or 37 kBq/ml of (20 μM) [1-^{14}C]IAA for 3 h. After culturing the crown gall cells, they were rinsed five times with 1 ml of hormone-free MS liquid medium and then used for extraction of conjugated IAA.

Identification of Conjugated IAA

Extraction and purification of conjugated IAA. IAA-treated crown gall cells were extracted with 10 ml of 80% (v/v) acetone. The extracts were reduced in vacuo and dissolved in 5 ml of 1% (v/v) acetic acid. The acetic acid soluble fraction was passed through a Sep-Pak C_{18} cartridge (Waters Associated, Milford, MA) equilibrated with 1% acetic acid. After washing the cartridge with 5 ml of 1% acetic acid, the IAA metabolites were eluted with 50% (v/v) methanol. The 50%-methanol eluate was reduced to dryness in vacuo and dissolved with 100 μl of methanol, then filtered through a 0.45-μm membrane (Tosoh Co. Ltd., Tokyo, Japan). A 10-μl aliquot of the methanol solution was subjected to HPLC by using TSKgel ODS-80T$_M$CTR (4.6-mm i.d. × 10 cm; Tosoh Co. Ltd.). The HPLC solvents contained 30% (v/v) methanol and 0.7% acetic acid. The eluate was monitored with a fluorescence spectrophotometer (FS-8010, Tosoh Co. Ltd.) to detect IAA metabolites (excitation at 280 nm and emission at 365 nm) and with a radioactivity detector (RS-8020, Tosoh Co. Ltd.).

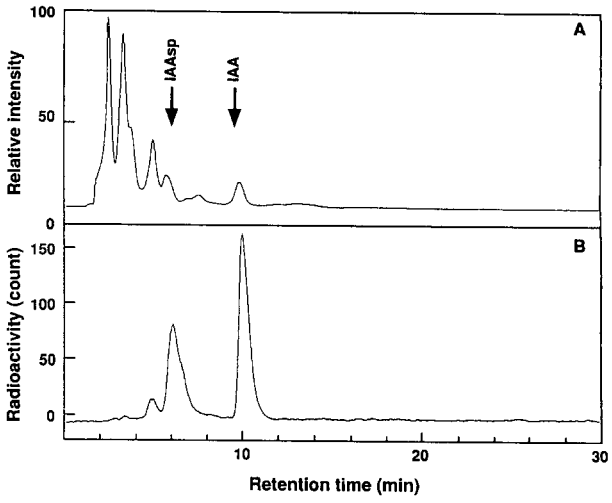

Fig. 1. Reverse-phase HPLC profile of [^{14}C]IAA metabolite extracted from crown gall cells transformed with aux$^-$ Ti plasmids following incubation with [^{14}C]IAA. Retention time of IAA and IAAsp are designated by arrows. A, fluorescent profile; B, radioactive profile.

LC-MS. The IAA metabolites were analyzed by HPLCMS. The liquid chromatograph (Beckman Instruments, Irvine, CA) consisted of a system Gold and programmable solvent module 125 with micro pump heads, which were programmed to produce a mobile phase flow rate of 200 μl · min^{-1}. The outlet was linked to a rheodyne 7725i injection valve (20-μl loop), which was coupled directly to a 4.6-mm i.d. × 10 cm HPLC column packed with 5 μm of C_{18} (ODS-80T$_M$CTR, Tosoh Co. Ltd., Tokyo, Japan). The HPLC solvent was 30% (v/v) methanol containing 0.05% (v/v) acetic acid. The HPLC eluate was mixed with methanol containing 1% glycerol matrix and sent, through a fused silica capillary tubing (1 m × 60-μm i.d.) at a rate of 5 μl · min^{-1}, to a frit-FAB-HPLC-MS interface (JEOL Ltd., Tokyo, Japan), which was attached at the ion source of a MS-LX2000 mass spectrometer (JEOL Ltd.). The ion source temperature was 30°C, and ions were generated with a beam of 6-kV xenon atoms at an emission current of 5 mA. The mass spectrometer acceleration voltage was 3 kV, and the slits were set to provide a resolution of 1500. Positive ion FAB was acquired at a rate of 5 s per scan with a cyclic time of 5 s for mass range of 50–1500 atom mass units. All data were processed by a MS-MP7000 data system (JEOL Ltd.).

Results and Discussion

HPLC elution of a partially purified methanolic extract from carrot crown gall cells transformed with aux$^-$ Ti plasmids and incubated with [^{14}C]IAA reveals the presence of two major metabolite peaks (Fig. 1). The peak, with a retention time of 9.70 min,

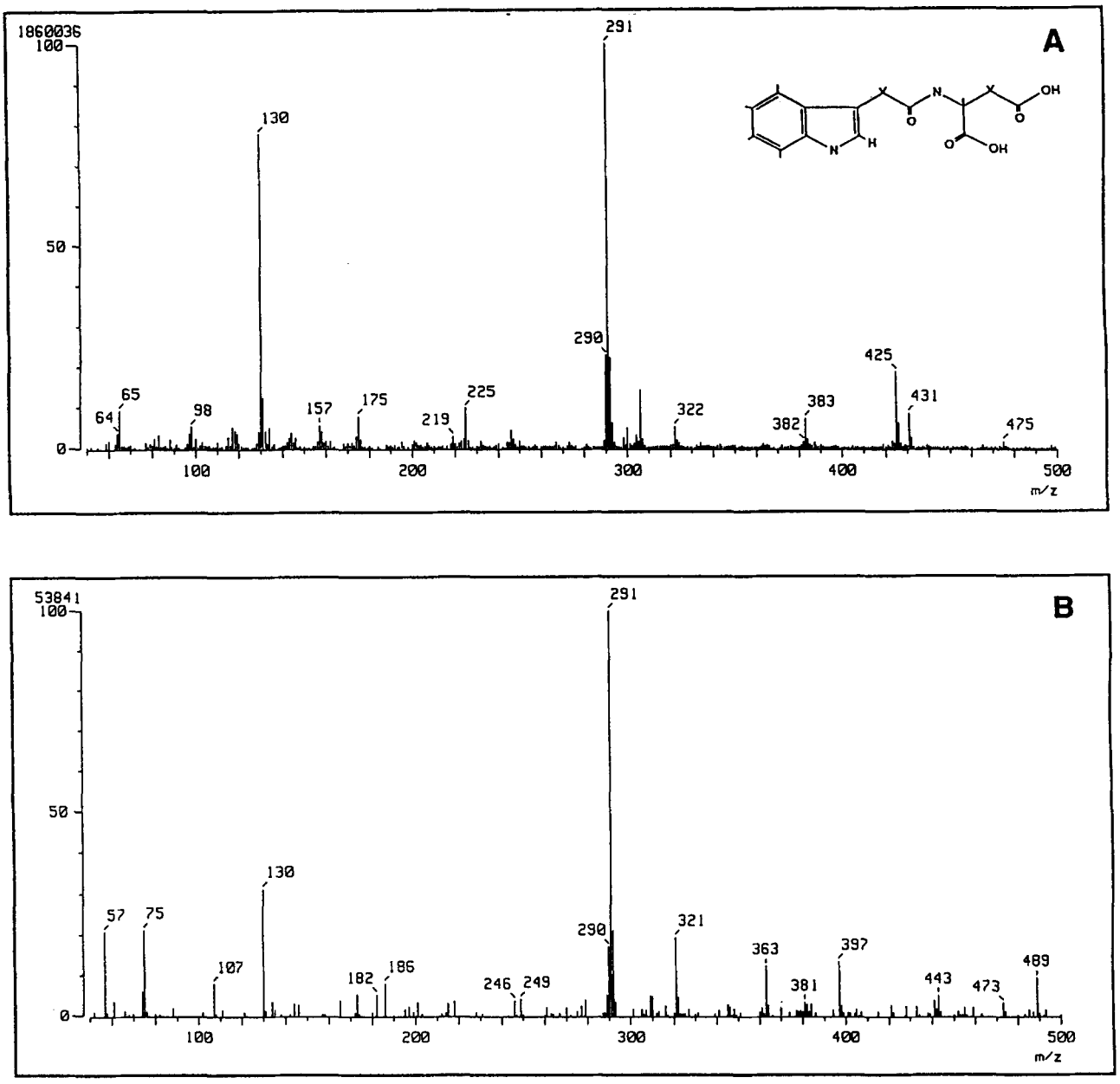

Fig. 2. Frit-FAB LCMS spectra of authentic IAAsp and IAA metabolite extracted from crown gall cells transformed with aux$^-$ Ti plasmids following incubation with unlabeled IAA. (**A**) positive ion mass spectrum of IAAsp standard; (**B**) positive ion mass spectrum obtained from the analysis of extracts from crown gall cells.

corresponded to IAA. Another peak, with a retention time of 7.41 min, had the same retention time to IAAsp. The same data was observed in the other crown gall cells transformed with wild and cyt$^-$ Ti plasmids.

The metabolite fraction extracted from crown gall cells transformed with aux$^-$ Ti plasmids incubated with unlabeled IAA was analyzed by LCMS. The peak of retention time 7.41 showed the spectrum presented in Fig. 2B, which matches the spectrum of IAAsp (Fig. 2A) with an *m/z* 291 [MH$^+$] and an *m/z* 130 quinolium ion. The conjugated IAA in carrot crown gall cells was identified as IAAsp. The same data was observed in the other crown gall cells transformed with wild-type and cyt$^-$ Ti plasmids.

These conclusions follow from these results and extend the suggestions made in other reports (Ishikawa et al. 1990, Sasaki et al. 1992, 1993). In carrot crown gall, free IAA was metabolized to

IAAsp, and the level of free IAA was maintained at a low level. IAAsp formation was induced by chemicals having auxin activity, and the induction was accompanied by enzymatic reaction(s) and was regulated at the transcriptional level.

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