The Effects of Expression of Procaryotic-type *iaa* Genes in *Agrobacterium tumefaciens* on Plant Tumorigenicity^{*,**}

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Pseudomonas syringae pv. *savastanoi*, a causal agent of olive and oleander knot, produces the auxin indole-3-acetic acid (IAA) from L-tryptophan via indole-3-acetamide (IAM) as an intermediate¹⁻³. The so-called olive knot or tumor, is considered to be the result of abnormal cell multiplication at infection sites as a host response to the large amount of IAA produced by this pathogen. The genes involved in IAA biosynthesis in this organism are *iaaM* that encodes tryptophan monooxygenase and *iaaH* that encodes indole acetamide-hydrolase⁴. *iaaM* and *iaaH* form one transcriptional unit, tentatively named the *iaa* operon, having a typical procaryotic-type gene structure^{5,6}.

Agrobacterium tumefaciens, the causal agent of crown gall in many species of dicotyledonous plants, produces IAA through the same pathway⁷⁻⁹. Comparable genes involved in IAA biosynthesis are *tms-1* and *tms-2*, encoding tryptophan monooxygenase and indoleacetamide hydrolase^{3,8,10}, respectively, having a eukaryotic-type structure in contrast to *iaa* operon in *P. savastanoi*. We have shown that *P. savastanoi-iaaM* and *-iaaH* show strong nucleotide sequence homology to that *A. tumefaciens-tms-1* and *tms-2*, respectively¹¹. The expression of *tms-1* and *tms-2* genes in *A. tumefaciens* is activated upon the establishment of infection, accompanied by T-DNA integration into the host plant genome¹². The *iaa* genes in *P. savastanoi*, however, appear to be constitutively expressed in the absence of the host plant⁹.

Isolates of *P. syringae* pv. *pisi* and *P. syringae* pv. *syringae* also contain *iaa* genes nearly identical to those of *P. savastanoi*.¹³ Genes encoding tryptophan monooxygenase and indoleacetamide hydrolase are also present in

plant symbiotic bacteria, including some *Rhizobium* and *Bradyrhizobium* species, which produce and release IAA^{14,15}. On the other hand, in *Azospirillum* species¹⁶ and *Enterobacter cloacae*¹⁷ isolated from the rhizosphere of Gramineae species and cucumber, respectively, IAA is synthesized via indole-3-pyruvate (IPyA) as an intermediate. At present, however, little is known about the function of *iaaM* and *iaaH* genes which encode the enzymes synthesizing IAA via indoleacetamide in these non-tumorigenic bacteria, although the production of IAA in *P. savastanoi* and *A. tumefaciens* seems to be essential for virulence, such as in formation of tumors in host. As an initial step toward understanding the role of these genes in plant-associated bacteria, we have investigated the effect of expression of procaryotic-type *iaaM* and *iaaH* genes on *A. tumefaciens* virulence in plant tumorigenicity.

To direct the expression of *P. savastanoi-iaa* genes in *Agrobacterium*, a DNA fragment spanning the *iaa* promoter region, *iaaM* and *iaaH*^{6,18} was subcloned into the broad host-range vector pRK415²⁰ (Table 1) which carries the tetracycline resistance gene. This plasmid was designated pTET40²⁰ (Table 1). Subsequently, the hygromycin phosphotransferase gene (*hph*) from pHPI00 was inserted into the *Dra* I site of the *iaa* promoter region in pTET40. This plasmid was designated pTOY1. pTOY1 was mobilized into a wild type *A. tumefaciens* A208 or avirulent *A. tumefaciens* LBA4301 by tri-mating methods as described¹⁹. The desired trans-conjugant, *A. tumefaciens* (pTOY1) or *A. tumefaciens* LBA4301 (pTOY1), was selected by resistance to tetracycline (30 µg/ml) or to both tetracycline (30 µg/

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source and reference
<i>E. coli</i>		
DH5	<i>supE44 hsdS20(r_B⁻m_B⁻)recA13 ara-14 proA2/lacY1 galK2 rpsL20 xyl-5-mtl-1</i>	
<i>A. tumefaciens</i>		
A208	pTi ⁺	Follin <i>et al.</i> 1985
LBA4301	pTi ⁻	Follin <i>et al.</i> 1985
Plasmids		
pRK415	broad host-range vector ; Tc ^r	Ditta <i>et al.</i> 1985
pRK2013	helper plasmid for mating	Ditta <i>et al.</i> 1985
pLUC12	5 kb <i>Pst</i> I- <i>Eco</i> RI fragment, containing <i>iaaM</i> and <i>iaaH</i> , in pBR328 ; Tc ^r , Amp ^r	Comai & Kosuge, 1982
pTET40	5 kb <i>Pst</i> I- <i>Eco</i> RI fragment, containing <i>iaaM</i> and <i>iaaH</i> , in pRK415 ; Tc ^r	This study
pTOY1	<i>hph</i> gene between <i>iaa</i> promoter and <i>iaaM</i> in pTET40 ; Tc ^r , Hm ^r	This study

ml) and hygromycin (30 µg/ml).

The level of IAA accumulation in cultures of *A. tumefaciens* A208, A208 (pTOY1), LBA4301 and LBA4301 (pTOY1) supplemented with L-tryptophan (100 µg/ml) was determined by HPLC analysis as described²⁰⁾ with respect to the duration of incubation. To avoid the loss of the recombinant plasmid carried by *A. tumefaciens*, tetracycline (30 µg/ml) and hygromycin (30 µg/ml) was added to the culture supplemented with L-tryptophan. Very little IAA accumulated in the culture filtrates of *A. tumefaciens* A208 and LBA4301, whereas the level of IAA accumulation increased along with growth. Approximately 90% of supplemented L-tryptophan was converted to IAA in the culture filtrates of *A. tumefaciens* A208 (pTOY1) and LBA4301 (pTOY1) at the stationary phase (Fig. 1).

To examine the effect of the expression of the *P. savastanoi-iaa* operon in *A. tumefaciens*, 15 µl of a cell suspension (10⁸ cells/ml) of the virulent strain *A. tumefaciens* A208, the transconjugant *A. tumefaciens* A208 (pTOY1), the avirulent strain *A. tumefaciens* LBA4301 or the transconjugant *A. tumefaciens* LBA4301 (pTOY1) were injected into tomato (*Lycopersicon esculentum* Mill., cv. TVR-2) stems or pipetted onto sterilized potato (*Solanum tuberosum* L. May Queen) tuber discs (8 mm diameter × 5 mm length). In tomato stems, small galls were initiated about 2 weeks after inoculation with *A. tumefaciens* A208 and A208 (pTOY1) and continued to grow for several months. After a month, a distinct difference in the size of galls was observed after inoculation with the recombinant *A. tumefaciens* carrying pTOY1, compared to the wild type (Fig. 2). On the other hand, galls did not appear on tomatoes inoculated with *A. tumefaciens* LBA4301 or LBA4301 (pTOY1) (Fig. 2). In the case of inoculation of potato tuber discs, distinct differences in callus inducing abilities were observed. By 15 day after inoculation with *A. tumefaciens* A208 (pTOY1), many small calli had been initiated. They gradually enlarged each day (Fig. 2). After inoculation with the parental *A. tumefaciens* A208, a few calli of relatively larger size formed. However, no calli formed after inoculation with *A. tumefaciens* LBA4301 or

Fig. 1. Growth and IAA accumulation in culture filtrates of parental strains and transconjugants of *A. tumefaciens*. (A) Growth of *A. tumefaciens* A208 (---○---), A208 (pTOY1) (—●—), LBA4301 (---△---) and LBA4301 (pTOY1) (—▲—). (B) IAA accumulation in culture filtrates of *A. tumefaciens*. Each point represents the mean value determined in two separate experiments.

LBA4301 (pTOY1) (Fig. 2). In these crown gall tissues, *A. tumefaciens* A208 and *A. tumefaciens* A208 (pTOY1) proliferated at the same rate (data not shown). These results suggested that the expression of *P. savastanoi-iaa* genes in *Agrobacterium* alone was not enough to induce tumor formation in an avirulent strain, although it did enhance virulence in the virulent *Agrobacterium* strain.

Auxins play a significant role in the growth and development of plants and can alter the levels of plant defense enzyme, including phenylalanine ammonia-lyase

Fig. 2. Tumorous symptoms on tomato stems or potato tuber discs one month after inoculation with *A. tumefaciens* A208, A208 (pTOY1), LBA4301 or LBA4301 (pTOY1).

(PAL)^{21,22}, chalcone synthase²¹) and hydrolytic enzymes such as chitinase and β -1-3-glucanase^{23,24}). To investigate the effect of IAA produced by *A. tumefaciens* A208 (pTOY1) on plant defense response, the activity of PAL, which catalyzes the first committed step in the synthesis of antimicrobial phytoalexins and lignin, a barrier to infection in peripheral tissue, was measured in potato discs inoculated with *A. tumefaciens* LBA4301, *A. tumefaciens* A208 or *A. tumefaciens* A208 (pTOY1) as described above.

The activity of PAL reached a maximum by 32 hr after incubation, regardless of the presence or absence of *Agrobacterium* (Fig. 3). PAL activity increased

Fig. 3. Change in phenylalanine ammonia-lyase activity over time. Potato tuber discs were inoculated with water (—■—), *A. tumefaciens* A208 (---○---), A208 (pTOY1) (—●—), LBA4301 (---△---) determined by HPLC analysis. All data represent the mean PAL activity in three independent discs.

approximately eight-fold by 32 hr incubation in water-treated potatoes, whereas the increase was approximately three-fold in potatoes inoculated with virulent *Agrobacterium* (Fig. 3). Potatoes inoculated with avirulent strain *A. tumefaciens* LBA4301 exhibited higher PAL activity than potatoes inoculated with virulent *Agrobacterium* (Fig. 3), suggesting that the virulent *Agrobacterium* suppresses plant defense response. In addition, inoculation with hypervirulent *A. tumefaciens* A208 (pTOY1) resulted in lower PAL activity than did inoculation with *A. tumefaciens* A208. This result further supports the idea that IAA produced by *A. tumefaciens* A208 (pTOY1) directs further suppression of PAL activity, one of the major active defense responses in infected plant cells. All these data implicate that IAA production, particularly through the IAM intermediate, potentially facilitates the interaction between plant and pathogenic bacteria, probably by suppressing the host plant defense response. It is intriguing to ask why these plant pathogens carry strongly homologous genes *iaaM* and *iaaH* to *P. savastanoi* which encode enzymes synthesizing IAA through IAM, but not IPyA, as an intermediate.

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和 文 摘 要

村上泰弘・西野友規・平飼 良・Ahmad YUNUS・一瀬勇規・白石友紀・山田哲治：根頭癌腫病菌における原核型 *iaa* 遺伝子発現の植物腫瘍化に及ぼす影響

植物関連細菌が持つ原核型 *iaa* 遺伝子の役割を調べるために、オリーブこぶ病菌 (*Pseudomonas syringae* pv. *savastanoi*) の *iaa* オペロンを広宿主範囲ベクター pRK415 に導入した組み換え体プラスミド pTOY1 を構築し、これを根頭癌腫病菌 (*Agrobacterium tumefaciens* A208) に導入した。A. t. A208 は感染した植物細胞でのみ IAA を生産するのに対し、組み換え体 A. t. A208 (pTOY1) はそれとともに恒常的に IAA を生産する。組み換え体 A. t. A208 (pTOY1) を接種したトマト茎には A. t. A208 に比べて大きなクラウンゴールが形成され、さらにポテトディスクでもカルス誘導が促進された。一方、A. t. A208 あるいは A. t. A208 (pTOY1) をポテトディスクに接種した後、植物防御応答に関連する phenylalanine ammonia-lyase の酵素活性を経時的に調べると、A. t. A208 よりも A. t. A208 (pTOY1) 接種でポテトの PAL 活性が低下した。したがって、A. t. A208 (pTOY1) の強病原性は、A. t. A208 (pTOY1) によって生産された IAA によって感染現場で植物防御応答が抑制された結果であると考えられた。

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