IAA PRODUCTION BY *STREPTOMYCES SCABIES* AND ITS ROLE IN PLANT MICROBE INTERACTION

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ABSTRACT

Streptomyces scabies is the cause of the economically important disease potato scab, and a pathogen of tap and fibrous roots. A previous study indicated that *Streptomyces* can synthesize indole-3-acetic acid (IAA) through the Indole-3-acetamide (IAM) pathway (Manulis et al., 1994). Two enzymes, tryptophan monoxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH), participate in IAA synthesis via the IAM pathway. Based on homology to other IAM and IAH amino acid sequences we identified candidate *iaaM* (SCAB75511) and *iaaH* (SCAB75501) genes in the completely sequenced genome of *S. scabies* 87-22. The function of the candidate genes in the IAA biosynthetic pathway was evaluated by creating the deletion mutants Δ*iaaH*, Δ*iaaM5* and Δ*iaaM8*. IAA production by all three mutant strains was lower than production by the wild type strain (87-22). When inoculated onto radish seedlings, all three mutant strains were reduced in virulence relative to the wild type strain, as measured by root necrosis. Genetic complementation of Δ*iaaM5* and Δ*iaaM8* deletion strains partially restored both IAA production and virulence on radish seedlings. These results suggest that the *iaaM* (SCAB75511) and *iaaH* (SCAB75501) genes are IAA biosynthetic genes and that they contribute to virulence of *S. scabies* through the synthesis of IAA as a virulence factor.

BIOGRAPHICAL SKETCH

Shih-Yung Hsu was born in Taipei, Taiwan. Her father was an insurance agent and her mother was a high school mathematics teacher. At the age of five, her mother gave her a sister, Shih-Cheng, a delight of her life. As a family of four, her parents paid a lot of attention to these two young girls and trained them to think independently.

Shih-Yung first showed her love of science in primary school. She enjoyed the observations of microorganisms under the microscope, plant growth and astronomy. She also volunteered to help the teacher in science classes. During high school, she participated in many scientific competitions and earned awards. These experiences and her interest in science led her to study Crop Science as an undergraduate at National Taiwan University in Taipei, Taiwan. After four years of academic training, she decided to continue her studies and obtained a Master degree in Agronomy. In 2000, she passed national qualification exams and worked for various federal governmental organizations. Then, in 2004, she was awarded a 3-year scholarship from the Taiwan government. She decided to leave her family and continue her research under the guidance of Dr. Rosemary Loria and Dr. Donna Gibson at Cornell University.

Shih-Yung is married to her best friend, Stanley. Now they live in San Diego, one of their favorite cities in the world.

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Finally, I want to commemorate my grandmother, her braveness and kindness will always be in my heart.

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INTRODUCTION

Plant hormones are chemicals produced by plants that regulate plant development and metabolism at very low concentrations. Hormones are synthesized in specific tissues in the plant, and then are transported to other target tissues where they serve as signal molecules. These signals stimulate specific physiological responses in the target tissues (Went and Thimann, 1937). Plant hormone activity was first discovered by Charles Darwin in 1890. He found that if the tips of coleoptiles were removed in Canary grass (*Phalaris carnariensis*), the plants would lose their growth response toward light, known as phototropism (Darwin, 1880). We now know that the family of compounds responsible for the loss in phototropic response is the auxins, of which indole-3-acetic acid (IAA) is the major auxin in plants and is composed of an indole and a carbon side chain (Figure 1).

Auxins are responsible for phototropism, as well as gravitropism, the growth response to gravitational fields. In fact, auxins have many regulatory functions in plants, including stimulating cell enlargement, cambium cell division, differentiation of phloem and xylem, root initiation, and lateral root formation. Auxin produced in the apical bud represses the growth of lateral buds. Depending on the timing of the plant's life cycle and the relative developmental position on a plant, auxins may inhibit or promote leaf and fruit abscission. Finally, auxins have many functions in regulating flowering and fruiting (Davies, 2004).

Auxin is also important in plant-microbe interactions. Many studies have demonstrated that, in addition to plants, some plant-associated fungi and bacteria also produce IAA (Ludwig-Müller, 2004). The plant growth-promoting bacteria (PGPB), *Azospirillum*, *Agrobacterium*, *Pseudomonas* and *Rhizobium*, can stimulate plant growth regulators, IAA or cytokinins or both, to increase host growth; some of these

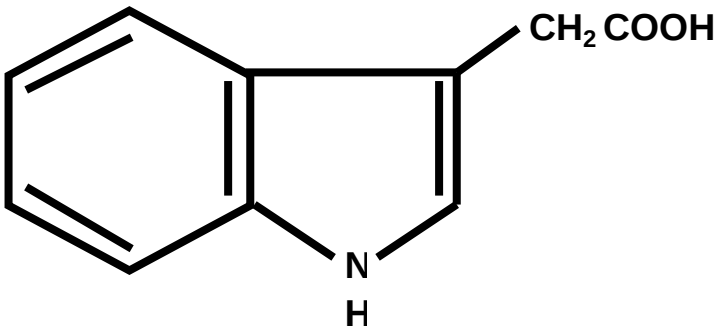


Figure 1 Chemical structure of the auxin, indole-3-acetic acid.

bacteria can be used for biocontrol of plant diseases (Costacurta and Vanderleyden, 1995). Another example is *Erwinia herbicola*, a common colonist on plant surfaces such as leaves and buds. *E. herbicola* produces IAA through tryptophan-independent pathways. IAA can increase colonization of plant surfaces by this epiphyte (Brandl and Lindow, 1996; Lindow and Brandl, 2003).

Production of IAA can proceed through several biosynthetic pathways in plants and microbes. The indole-3-acetamide (IAM) pathway is used by many plant pathogens such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* (Comai and Kosuge, 1982; Yamada et al., 1985). *A. tumefaciens* is a serious pathogen in many high-value crops such as sugar beets, grape vines and peaches, on which it causes a crown gall disease in roots, stems and branches. *P. savastanoi* produces a gall disease of olive branches, called olive knot.

There are two enzymes involved in the IAM pathway, tryptophan monoxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH) (Figure 2, left). IaaM will oxidize tryptophan to indole-3-acetamide after which IaaH will convert indole-3-acetamide to IAA by hydroxylation. Interestingly, these IaaM and IaaH enzymes are only found in microorganisms (Davies, 2004; Taiz and Zeiger, 1998).

The indole-3-acetonitrile (IAN) pathway converts tryptophan to indole-3-acetaldoxime which is then converted to indole-3-acetonitrile (Figure 2, second from left), although the enzymes have not been described. The enzyme, nitrilase, can convert indole-3-acetonitrile (IAN) to IAA. All plants and some bacteria, including *Agrobacterium* and *Rhizobium*, can synthesize IAA through this pathway (Bartling et al., 1994; Kobayashi et al., 1995).

The indole-3-pyruvic acid (IPA) pathway (Figure 2, middle) is one of the major tryptophan-dependent IAA synthetic pathways in plants (Schneider, 1974) and in the plant growth-promoting bacteria *Azospirillum* sp., *Agrobacterium rhizogenes*

and *Pseudomonas savastanoi* (Costacurta and Vanderleyden, 1995). Tryptophan transaminase deaminates tryptophan to produce indole-3-pyruvic acid (IPA) and IPA is then decarboxylated by the action of IPA decarboxylase to produce indole-3-acetaldehyde (IALd). IALd is then oxidized to IAA by IAld dehydrogenase (Davies, 2004; Taiz and Zeiger, 1998).

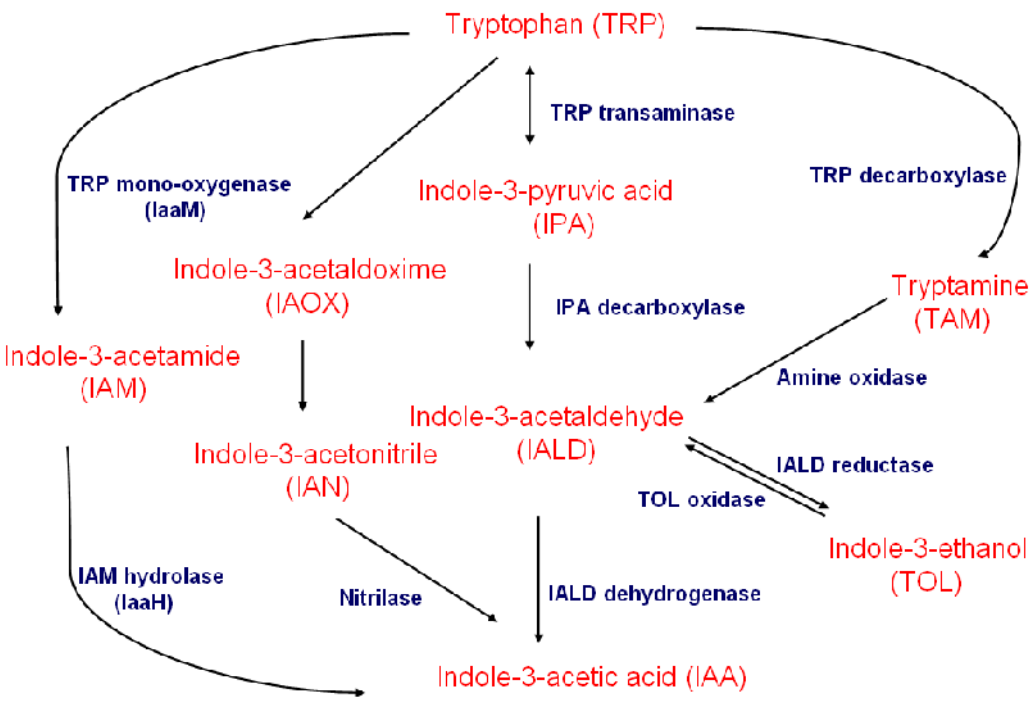
The tryptamine (TAM) pathway (Figure 2, right) is a common IAA-synthesizing pathway in plants and some fungi (Patten and Glick, 1996). The TAM pathway is similar to the IPA pathway; the only difference between these two pathways is the order of the deamination and decarboxylation reactions.

There are many factors such as light, temperature and pathogen infections that will perturb IAA synthesis through tryptophan-dependent synthesis pathways in plants (Davies, 2004). In this study, the questions are concentrated on how microorganisms synthesize IAA through tryptophan and how these pathogen-derived auxins are involved in the disease development in host plants, using *Streptomyces scabies* (strain 87-22) as the plant pathogen.

IAA production in bacteria

Many bacteria synthesize auxins in order to perturb host physiological processes for their own benefit. Here we discuss five bacteria, *Pseudomonas syringae*, *Pseudomonas savastanoi, Agrobacterium tumefaciens, Erwinia herbicola* and *Rhodococcus fascians* as examples of IAA production in bacteria.

Pseudomonas syringae is a Gram-negative bacterium. Most of the strains (pathovars) in this species are plant pathogens that have host-specific interactions. *P. syringae* causes lesions in leaves, buds and fruits. IAA production via the IAM pathway has been detected in most strains of *P. syringae* and associated with the



(Taiz and Zeiger, 1998)

Figure 2 Tryptophan-dependent pathways of IAA biosynthesis in plants and bacteria. Enzymes are indicated in blue while substrates, intermediates and the final product of the pathways are indicated in red. The IAM pathway is from left, and the TAM pathway is on the right. The IAN pathway is second to the left and IPA pathway is in the middle of the diagram.

epiphytic survival of the bacteria (Costacurta and Vanderleyden, 1995; Mazzola and White, 1994; Patten and Glick, 1996).

Pseudomonas savastanoi can synthesize auxins and cytokinins, which leads to the formation of galls on olive and oleander. *P. savastanoi* produces IAA through the IAM pathway; the *iaaM* and *iaaH* genes are located on the chromosome and encode proteins that have significant homology to proteins encoded by the *tms1* and *tms2* genes responsible for IAA biosynthesis in *Agrobacterium tumefaciens* (Yamada, 1993; Yamada et al., 1985). An IAA deficient mutant of *P. savastanoi*, EW 2009-3, does not produce galls on host plants. Interestingly, if the IAA deficient mutant of *P. savastanoi* is transformed with a vector containing the *iaaM* and *iaaH* genes, the ability to produce galls is recovered (Glickmann et al., 1998; Surico et al., 1984; Yamada, 1993), confirming the importance of IAA in the infection process.

Agrobacterium tumefaciens is a rod-shaped, Gram-negative bacterium that causes crown gall of many plant species. The virulent strains of *A. tumefaciens* carry T-DNA (transfer DNA) on the Ti plasmid, which is introduced into host cells during infection. The T-DNA encodes genes for the biosynthesis of auxins and cytokinins, which induce abnormal cell division in infected tissue. As a consequence, infected tissue produces opines, which is an energy source for the bacterium. The virulence region of T-DNA carries the *tms-1* and *tms-2* genes, which encode proteins that functionally correspond to two auxin synthesis enzymes, tryptophan monooxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH), respectively (Yamada, 1993).

Erwinia herbicola is a Gram-negative bacterium that colonizes plant surfaces, especially leaves and buds. Pathogenic strains induce gall diseases on hosts. Both pathogenic and non-pathogenic strains synthesize IAA through the IPA pathway. Interestingly, only virulent strains synthesize IAA through the IAM pathway (Manulis

et al., 1998). Deactivating the IAM pathway by disrupting either the *iaaM* or i*aaH* genes reduced the virulence of *E. herbicola pv. gypsohhilae* (Clark et al., 1993).

Rhodococcus fascians is a Gram-positive bacterium that can infect angiosperms and gymnosperms. The symptoms of infected plants include leaf deformation, growth inhibition, fasciations and leafy galls. The features of leafy-galls are vascular tissue differentiation, cell enlargement and inhibition of bud outgrowth. From IAA precursor analysis, the IPA pathway seems to be the major IAA biosynthetic pathway in *R. fascians* (Vereecke et al., 1997).

IAA production in *Streptomyces scabies*

Streptomyces is a genus within the actinobacteria that is primarily saprophytic. They are well known for their ability to produce structurally diverse secondary metabolites with pharmaceutical uses. However, some species cause diseases in economically important plants; *S. scabies* is the best known of these species. *S. scabies* is a necrogenic pathogen that causes root rot and scab symptoms on expanded plant tissue such as potato tubers and radish tap roots (Loria et al., 2006). *S. scabies* has a filamentous growth habit in which the mycelium is branched and has very few cross walls. Cylindrical spores are formed on specialized aerial hyphae. *S. scabies* spores can spread through water and wind and in infected plant tissue. Infection of plants occurs through expanding, immature plant tissue, either directly or through natural openings. The life cycle of *S. scabies* is shown in Figure 3. *S. scabies* produces the phytotoxin, thaxtomin, which is a non-host specific toxin. Thaxtomin can inhibit cellulose synthesis and cause necrosis and scab in higher plants (Healy et al., 2000; King et al., 1992).

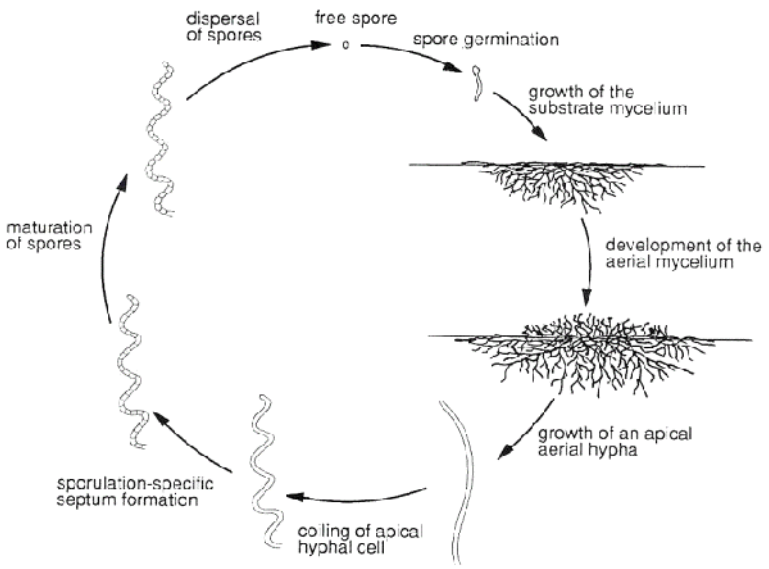


Figure 3 The life cycle of *Streptomyces*.

In 1994 Manulis et al. used HPLC and CG-MS to identify IAA biosynthetic pathways in several species of *Streptomyces,* including *S. violaceus, S. scabies, S. griseus, S. exfoliatus, S. coelicolor* and *S. lividans*. After feeding cultures with L-tryptophan, they found that all of the strains synthesized IAA from tryptophan. Interestingly, among those strains, only *S. scabies* is a plant pathogen. Data to support involvement of the IAM pathway was only obtained for *S. violaceus* and *S. exfoliates* The IAA biosynthetic pathway used by *S. scabies* to produce IAA is still unclear (Manulis et al., 1994).

IAA and disease development

Many plant-associated microorganisms have been found that can synthesize IAA causing overproduction of root hairs and lateral roots in plants. Possible functions of IAA in disease development have been hypothesized.

Increased pathogen fitness

IAA has been proposed to stimulate the release of saccharides from plant cell walls which can provide a source of nutrition for microorganisms and facilitate bacterial colonization of plant surfaces (Bender et al., 1999). For example in *Pantoea agglomerans,* IAA-producing strains had a two fold population increase relative to IAA-deficient strains in pear flowers and bean plants (Brandl and Lindow, 1996).

Pathogen colonization ability

For disease development, the first step is to infect the plant host and obtain nutrients to support the pathogen's growth and survival. In *E. herbicola,* the presence of IAA increases the ability of the bacterium to colonize on plant surfaces (Brandl and

Lindow, 1996) and the loss of IAA production decreases the colony size and population growth (Lindow and Brandl, 2003).

Plant immune system

Recent research found flagellin (flg22), a characterized pathogen-associated molecular pattern (PAMP) can induce resistance responses in plants by repressing mRNA levels of transport inhibitor response 1 protein (TIR1) (Abramovitch et al., 2006). TIR, the first auxin receptor, is part of a molecular complex that can target proteins for degradation by attachment of ubiquitin (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b). Aux/IAA proteins are transcription factors that can regulate downstream auxin responses. When auxins bind to TIR1, it facilitates breakdown of the ubiquitinate- Aux/IAA protein complex. Ubiquitinate- Aux/IAA proteins degradation will turn on the transcription of some auxin-related genes via derepression of the auxin response pathway (Laskowski, 2006).

In 2006, Navarro found that flg22 increases the transcription levels of miRNA (miR393) which will target F-box mRNAs (TIR1, AFB2 and AFB3), that then causes down regulation of auxin receptor proteins and increases *Arabidopsis* resistance to *P. syringae*. These data indicate that repression of auxin receptors will increase plant resistance to diseases (Navarro et al., 2006).

Research goal

The purpose of this study was to investigate IAA production by *S. scabies* 87-22. This study was facilitated by the availability of the genome sequence of this strain, allowing the opportunity to use genomic and molecular genetic approaches. A secondary goal of this study was to evaluate the role of IAA in plant-microbe interactions, including disease development.

METHOD AND MATERIALS

Bacterial strains and growth conditions

Escherichia coli stains were cultured on Luria-Bertani (LB) agar medium, LB broth and SOB-$MgSO_4$ buffer (20g tryptone, 5g yeast extract, 0.5g NaCl, 2.4g $MgSO_4$, 0.186g KCl per liter, final pH=7.0 at 25°C)(Gust et al., 2002). *Streptomyces scabies* 87-22 and its derivatives were grown on International *Streptomyces* Project 4 (ISP4) medium, oat bran broth (OBB) and tryptone soya broth (TBS) at 28-30^{o}C for 5-7 days. For DNA extraction, cultures were grown in 25 ml CRM buffer, which is composed of the following components (g/liter): 10g glucose, 103g sucrose, 10.2g $MgCl_2$*6 H_2O, 15g tryptic soy broth and 5g yeast extract, overnight in 250 ml flasks. All liquid cultures were shaken between 150 – 250 rpm. When required for selection, antibiotics were added as follows: apramycin 100 μg/ml, kanamycin 50 μg/ml, chloramphenicol 25 μg/ml, ampicillin 100 μg/ml, or nalidixic acid 25 μg/ml.

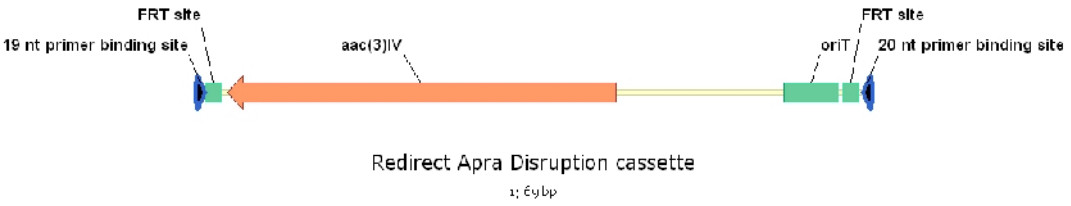
Bioinformatic analysis

The Putative ORFs were identified by database similarity searching on the National Center for Biotechnology website. Protein sequences were mapped and aligned using Vector NTI Suite 8.0 (InforMax, Frederick, MD, U.S.A.) in order to get the consensus sequence. Used the genome viewer software, Artemis, to get the best hit in *S. scabies* 87-22 genome (*S. scabies* 87-22 genome is available at the Sanger Center website (http://www.sanger.ac.uk /projects/Sscabies/). The protein sequence of candidate gene was presented and aligned with other IaaM protein sequences by using Vector NTI 8.0.

Disruption of *iaaH* and *iaaM* genes in *Streptomyces scabies*

The *S. scabie*s 87-22 IAA deficient strains, *ΔiaaH, ΔiaaM*5 and *ΔiaaM8,* were created base on the λRED-based PCR-targeting Redirect technology (Gust et al., 2002). This strategy for maker exchange mutagenesis of these genes was modified from the PCR targeting system developed in *Streptomyces coelicolor* A3(2) and is illustrated in Figure 2-2. A gene disruption cassette, which included *oriT* and the apramycin resistance gene *aac(3) IV,* was generated by PCR amplification from pIJ773. The plasmid pIJ773 (containing the apramycin- resistance gene *aac(3)IV* and *oriT*) was used as a template for the PCR amplification of the apramycin resistance cassette (Figure 4).The inclusion of *oriT* in the disruption cassettes allows for conjugative transfer of the cosmid DNA into *S. scabies* 87-22. For each gene disruption, two long PCR primers (58 nt and 59 nt) are required. Each has at the 5' end 39 nt matching the *S. scabies* sequence adjacent to the *iaaH* (SCAB75501) or *iaaM* (SCAB75511) and a 3' sequence (19 nt or 20 nt) matching the right or left end of the disruption cassette (Figure 5). The size of the PCR product was analyzed by gel electrophoresis of the PCR reaction; the expected size was 1460 bp. The *S. scabies* 87-22 cosmid (22g09) contained *iaaH* (SCAB75501) and *iaaM* (SCAB75511) was introduced into *E. coli* BW25113/ pIJ790 using electroporation as described. Plasmid pIJ790 contains the λ RED gene and a resistance marker *cat* (chloramphenicol resistance). It has a temperature sensitive origin of replication and requires 30°C for replication. Transformants were screened by antibiotic and colony PCR. After the *E. coli* BW25113/pIJ790 that contained the *S. scabies* 87-22 genomic library cosmid 22g09 cells was isolated, the apramycin resistance cassette from plasmid pIJ773 was electro-transformed to obtain mutant cosmids for knocking out *S. scabies* 87-22 through conjugation. Afterwards, mutant cosmids were moved into a non-methylating *E. coli* host: *E. coli* ET12567 (the resistance marker is chloramphenicol), which

20 nt: 5’ ATT CCG GGG ATC CGT CGA CC 3’
19 nt: 5’ TGT AGG CTG GAG CTG CTT C 3’



(Gust et al., 2002)

Figure 4 The Redirect apramycin disruption cassette, *aac(3)IV*: apramycin resistance gene, *oriT*: origin of transfer from RK2, FRT: FLP recognition target.

A

Sy-iaaHF

GCCCCCGACCCCCCTTCGTCCCGGTGAGGAACACGCATGATTCCGGGGATC

CGTCGACC

Sy-iaaHR

CCTAGCGGAAAAAGGGGTGTGCGGGCGGGGCGGGTCTCATGTAGGCTGGA

GCTGCTTC

B

Wen-IAAF

CCGCACACCCCTTTTTCCGCTAGGAGTTCGTACCCCATGATTCCGGGGATCC

GTCGACC

Wen-IAAR

GTCGTGGTCGGCCCACAAGGACTGGAGCTGCGCCTAGTCTGTAGGCTGGA

GCTGCTTC

C

Sy-iaaHF

GCCCCCGACCCCCCTTCGTCCCGGTGAGGAACACGCATGATTCCGGGGATC

CGTCGACC

Wen-IAAR

GTCGTGGTCGGCCCACAAGGACTGGAGCTGCGCCTAGTCTGTAGGCTGGA

GCTGCTTC

Figure 5 Primer sequences used for construction of gene knock out cassettes (5'- 3'). A) Primers for *iaaH* gene knock out cassette, B) Primers for *iaaM* gene knock out cassette, C) Primers for *iaaMH* gene knock out cassette.

contains the RP4 derivative pUZ8002 (the resistance marker is kanamycin). Since *S. scabies* carries a methyl-sensing restriction system, it is necessary to pass the mutant 22g09 cosmid through this non-methylating *E. coli* host. The cosmid was transferred to *Streptomyces* via intergeneric conjugation. Transconjugants were screened for apramycin (100 µg/ml) and confirmed by Southern blot hybridization 20 and 19 nt sequences are identical in all cassettes.

Southern blot hybridization

Genomic DNA of *S. scabies* 87-22 and mutant strains was extracted from overnight CRM buffer-grown cultures. One microgram of genomic DNA was digested with *Eco*RI overnight and electrophoresed on a 1% agarose gel for 8 hours at 60 volts. DNA was denatured with denaturing solution (0.5M NaOH, 1M NaCl) twice for 15 min then transferred to a nylon membrane (Whatman) with 20X SSC (20X SSC: 3M NaCl, 0.3M sodium citrate; pH=7.0). DNA was UV cross-linked to the membrane by using the BioRad Gene Linker (Program 3). The membrane was probed with DNA fragments from *iaaM* (SCAB75511), intergenic region between *iaaM* (SCAB75511) and *iaaH* (SCAB75501) and apramycin cassette (Figure 6) that were labeled with dioxigenin-11-dUTP (Roche). Hybridization was performed for 12 hours at 42°C with pre-heat (42°C) DIG Easy Hyb solution (3.5 ml/100 cm^2 filter). Processing of the membrane from this point forward was performed according to the manufacturer's instructions (Roche).

A	
iaaM southern probe-For	CGACTTCTCCGACATGAACC
iaaM southern probe-Rev	AGTCGTCACAGGCGATCTTC
B	
IAAMH- southern(F)	GGCTCCTGATCTGCTACGAC
IAAMH- southern(R)	GCCAGGTCGATGTAGTGCTG
C	
Apra-F	CATTCTTCGCATCCCGCC
Apra-R	CTCCTTCCGTAGCGTCCG

Figure 6 Primers sequences used for DIG-labeled DNA probe (5'- 3').
A) Primers for *iaaM* southern probe, B) Primers for *iaaMH* southern probe, C) Primers for apramycin disruption cassette.

Thaxtomin extraction and analysis

Streptomyces scabies strains were grown in 5 ml of oat bran broth (OBB) (Shirling and Gottlieb, 1966) with 10^5 spores for 5 days in the dark at room temperature. Following centrifugation to pellet cells, each culture broth (5 ml) was passed through preconditioned Alltech (DeerWeld, IL) Extract-Clean solid phase extraction cartridges (C18; 200 mg), rinsed with 5 ml of 100% methanol and 5 ml deionized water, then with a 5 ml mixture of methanol: water (25:75). Thaxtomin A was eluted with a 3 ml mixture of methanol: water (50:50). 100 µl of each elute was collected then analyzed on C18 reverse column (0.5 cm x25 cm, 5 µm particle size, 100 Å pore size) with a premixed mobile phase of acetonitrile:water (40:60) 1 ml/min. Thaxtomin A was detected via absorbance at 215 nm and 380 nm and quantified by absorbance 380 nm, using pure thaxtomin A as a standard.

Indole groups extraction and high performance liquid chromatography analysis

Streptomyces scabies spores ($1x10^5$ spores) were inoculated in 25 ml TSB medium with 1 µg/ml L-tryptophan at room temperature for 5 days in the dark. Centrifuged culture cells and acidified the supernatant to PH=2.5 and extracted the supernatant twice with equal volume of ethyl acetate. Each extract was pooled and flash-evaporated at 30^oC. The dry residue was weighed and dissolved in 1 ml methanol of which 100 µl was filtered through 0.2 µm filter and used for chromatography on C18 reverse phase column (0.5 cm x25 cm, 5 µm particle size, 100 Å pore size) with acetonitrile:water:acetic acid 35:65:1, 1 ml/min flow rate, detected at 280 nm.

Plant production and bioassays

Radish seeds (cv. Burpee White) were surface sterilized with 70% ethanol plus

1% Tween 20 for 20 min. Seeds were soaked in water for 24 hours in darkness at room temperature, then transferred to sand or a synthetic soil (Cornell Mix) and watered with 25% Murashige and Skoog solution (Murashige and Skoog, 1962). Plants were grown at 24±2°C with a 16 hour day-length for 5 days. Then, each 5-day-old seedling root was inoculated with $1x10^5$ of *S. scabies* 87-22 spores and grown for another 10 days. 15-day-old radish roots were harvested and a section (50 mm measured from the cotyledon) was excised for further experiments.

Malondialdehyde (MDA) Extraction

Malondialdehyde (MDA), which is routinely used to quantify of lipid peroxidation, which is an indication of plant stress (Heath and Packer, 1968). Roots were weighted and extracted with 2 ml of trichloroacetic acid (5%, w/v). Following centrifugation, 0.5 ml supernatant was transferred to a clean tube then added 2 ml thiobarbituric acid solution (0.5%, w/v in 20% trichloroacetic acid). Incubated for 30 min in 95°C water bath then put onto ice for 5 min to terminate reactions. 0.5 ml of each reaction was analyzed in spectrophotometer (Ultrospec 3100 pro UV/ Visible Spectrophotometer) at 532 nm. The trichloroacetic acid (5%, w/v) was used as the blank.

RESULTS

Location of putative IaaM and IaaH encoding genes in *S. scabies* 87-22

An ORF encoding a putative IaaM protein in the genome of *S. scabies* 87-22 was identified by database similarity searching using the *iaaM* sequence from *Agrobacterium tumefaciens* Ag57 (accession number: Z18270), *Pseudomonas syringae* pv. *Syringae* strain 3023 (accession number: AY530536), and *Agrobacterium vitis* (accession number: AF061780) as search queries. The best hit in the *S. scabies* genome was SCAB75511, which is located between 8349372 and 8351069 on the chromosome. Identified SCAB75511 protein in Pfam protein families' database (http://pfam.sanger.ac.uk/) and result indicated SCAB75511 protein encodes an amino oxidase (PF01593) (Figure 7). For clarity, this protein will be referred to as ssIaaM. An alignment of the 565 aa protein ssIaaM with characterized IaaM proteins from *S. coelicolor* A3 (2) (accession number: NP_625735), *A. tumefaciens* (accession number: AAF77123), *P. syringae* pv. *syringae* (accession number: AAR06971) and amino oxidase (conserved domain, PF01593) is shown in Figure 8. Identities in SCAB75511 vs. *S. coelicolor* A3 (2), SCAB75511 vs. *A. tumefaciens* and SCAB75511 vs. *P. syringae* pv. *syringae* are 95%, 28% and 31%, respectively (Table 1).

Since the IAA biosynthetic pathway requires an additional protein, IaaH, we examined the region of the *S. scabies* genome surrounding *ssiaaM*, using the genome viewer software Artemis (http://www.sanger.ac.uk/Software/Artemis/). We found a gene (SCAB75501) encoding a putative hydrolase protein upstream and adjacent to the gene encoding *ssiaaM*, which we designated *ssiaaH* (Figure 9). The amino acid sequence of the putative *iaaH* gene is 262 aa in length and shown in (Figure 10). This gene encodes a carbon-nitrogen hydrolase protein (PF00795). An alignment of the

MTSTVPNAIEHADEQQPPITMFGPDFPYAYDDFLAHPAGLGQIPATEHGAEVAV
IGGGLSGIVAAYELMKMGLKPVVYEADRIGGRLRTVGFDGCDPSLTAEMGAM
RFPPSSTALQHYIDLAGLETRPFPNPLAEATPSTVVDLKGESHYAETIDDLPQVY
RDVAAAWNACLEGGADFSDMNRALRERDVPRIREIWAKLVERLDNQTFYGFL
CDSEAFKSFRHREIFGQVGFGTGGWDTDFPNSILEILRVVYTEADDHHRGIVG
GSQQLPLRLWEREPEKIVHWPYGTSLRSLHVDGEPRPAVTRLHRTAGNRITVT
DANGDIRTYRAAIFTAQSWMLLSKIACDDSLFPIDHWTAIERTHYMESSKLFVP
VDRPFWLDKDEHTGRDVMSMTLTDRMTRGTYLLDDGPDKPAVICLSYTWCD
DSLKWLPLSANERMEVMLKSLGEIYPNVDIRSHIIGSPVTVSWENEPYFMGAF
KANLPGHYRYQRRLFTHFMQDRLPEDKRGIFLAGDDISWTAGWAEGAIQTAL
NAVWGVMHHFGGTTDTTNPGPGDVYDAIAPVELPE

Figure 7 Protein sequence of the putative IaaM protein in *S. scabies* 87-22.

Figure 8 Alignment of the protein sequence of tryptophan monoxygenase from *S. scabies* 87-22 (SCAB 75511) in *S. coelicolor* A3 (2) (NP_625735), *A. tumefaciens* (AAF77123), *P. syringae* pv. *syringae* (AAR06971) and amino oxidase (PF 01593). Identical: Red text on yellow background; Weakly similar: Dark green text on a white background; Block of Similar: Black on a light green background; Conservative: Dark blue on a light blue background; Non-similar: Black on a white background.

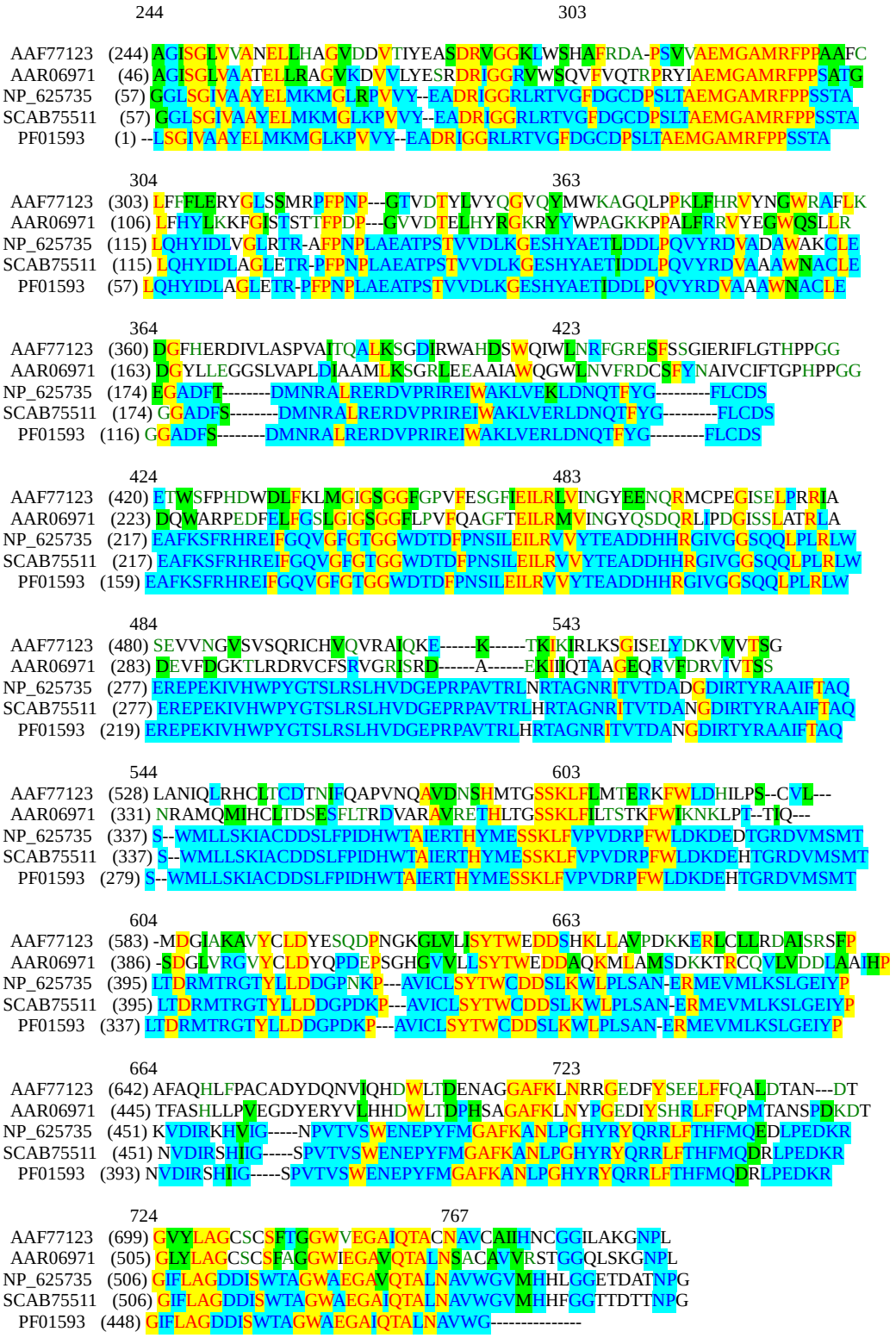

244 303
AAF77123 (244) AGISGLVVANELLHAGVDDVTIYEASDRVGGKLWSHAFRDA-PSVVAEMGAMRFPPAAFC
AAR06971 (46) AGISGLVAATELLRAGVKDVVLYESRDRIGGRVWSQVFVQTRPRYIAEMGAMRFPPSATG
NP_625735 (57) GGLSGIVAAYELMKMGLRPVVY--EADRIGGRLRTVGFDGCDPSLTAEMGAMRFPPSSTA
SCAB75511 (57) GGLSGIVAAYELMKMGLKPVVY--EADRIGGRLRTVGFDGCDPSLTAEMGAMRFPPSSTA
PF01593 (1) --LSGIVAAYELMKMGLKPVVY--EADRIGGRLRTVGFDGCDPSLTAEMGAMRFPPSSTA
304 363
AAF77123 (303) LFFFLERYGLSSMRPFPNP---GTVDTYLVYQGVQYMWKAGQLPPKLFHRVYNGWRAFLK
AAR06971 (106) LFHYLKKFGISTSTTFPDP---GVVDTELHYRGKRYYWPAGKKPPALFRRVYEGWQSLLR
NP_625735 (115) LQHYIDLVGLRTR-AFPNPLAEATPSTVVDLKGESHYAETLDDLPQVYRDVADAWAKCLE
SCAB75511 (115) LQHYIDLAGLETR-PFPNPLAEATPSTVVDLKGESHYAETIDDLPQVYRDVAAAWNACLE
PF01593 (57) LQHYIDLAGLETR-PFPNPLAEATPSTVVDLKGESHYAETIDDLPQVYRDVAAAWNACLE
364 423
AAF77123 (360) DGFHERDIVLASPVAITQALKSGDIRWAHDSWQIWLNRFGRESFSSGIERIFLGTHPPGG
AAR06971 (163) DGYLLEGGSLVAPLDIAAMLKSGRLEEAAIAWQGWLNVFRDCSFYNAIVCIFTGPHPPGG
NP_625735 (174) EGADFT--------DMNRALRERDVPRIREIWAKLVEKLDNQTFYG---------FLCDS
SCAB75511 (174) GGADFS--------DMNRALRERDVPRIREIWAKLVERLDNQTFYG---------FLCDS
PF01593 (116) GGADFS--------DMNRALRERDVPRIREIWAKLVERLDNQTFYG---------FLCDS
424 483
AAF77123 (420) ETWSFPHDWDLFKLMGIGSGGFGPVFESGFIEILRLVINGYEENQRMCPEGISELPRRIA
AAR06971 (223) DQWARPEDFELFGSLGIGSGGFLPVFQAGFTEILRMVINGYQSDQRLIPDGISSLATRLA
NP_625735 (217) EAFKSFRHREIFGQVGFGTGGWDTDFPNSILEILRVVYTEADDHHRGIVGGSQQLPLRLW
SCAB75511 (217) EAFKSFRHREIFGQVGFGTGGWDTDFPNSILEILRVVYTEADDHHRGIVGGSQQLPLRLW
PF01593 (159) EAFKSFRHREIFGQVGFGTGGWDTDFPNSILEILRVVYTEADDHHRGIVGGSQQLPLRLW
484 543
AAF77123 (480) SEVVNGVSVSQRICHVQVRAIQKE------K------TKIKIRLKSGISELYDKVVVTSG
AAR06971 (283) DEVFDGKTLRDRVCFSRVGRISRD------A------EKIIIQTAAGEQRVFDRVIVTSS
NP_625735 (277) EREPEKIVHWPYGTSLRSLHVDGEPRPAVTRLNRTAGNRITVTDADGDIRTYRAAIFTAQ
SCAB75511 (277) EREPEKIVHWPYGTSLRSLHVDGEPRPAVTRLHRTAGNRITVTDANGDIRTYRAAIFTAQ
PF01593 (219) EREPEKIVHWPYGTSLRSLHVDGEPRPAVTRLHRTAGNRITVTDANGDIRTYRAAIFTAQ
544 603
AAF77123 (528) LANIQLRHCLTCDTNIFQAPVNQAVDNSHMTGSSKLFLMTERKFWLDHILPS--CVL---
AAR06971 (331) NRAMQMIHCLTDSESFLTRDVARAVRETHLTGSSKLFILTSTKFWIKNKLPT--TIQ---
NP_625735 (337) S--WMLLSKIACDDSLFPIDHWTAIERTHYMESSKLFVPVDRPFWLDKDEDTGRDVMSMT
SCAB75511 (337) S--WMLLSKIACDDSLFPIDHWTAIERTHYMESSKLFVPVDRPFWLDKDEHTGRDVMSMT
PF01593 (279) S--WMLLSKIACDDSLFPIDHWTAIERTHYMESSKLFVPVDRPFWLDKDEHTGRDVMSMT
604 663
AAF77123 (583) -MDGIAKAVYCLDYESQDPNGKGLVLISYTWEDDSHKLLAVPDKKERLCLLLRDAISRSFP
AAR06971 (386) -SDGLVRGVYCLDYQPDEPSGHGVVLLSYTWEDDAQKMLAMSDKKTRCQVLVDDLAAIHP
NP_625735 (395) LTDRMTRGTYLLDDGPNKP---AVICLSYTWCDDSLKWLPLSAN-ERMEVMLKSLGEIYP
SCAB75511 (395) LTDRMTRGTYLLDDGPDKP---AVICLSYTWCDDSLKWLPLSAN-ERMEVMLKSLGEIYP
PF01593 (337) LTDRMTRGTYLLDDGPDKP---AVICLSYTWCDDSLKWLPLSAN-ERMEVMLKSLGEIYP
664 723
AAF77123 (642) AFAQHLFPACADYDQNVIQHDWLTDENAGGAFKLNRRGEDFYSEELFFQALDTAN---DT
AAR06971 (445) TFASHLLPVEGDYERYVLHHDWLTDPHSAGAFKLNYPGEDIYSHRLFFQPMTANSPDKDT
NP_625735 (451) KVDIRKHVIG-----NPVTVSWENEPYFMGAFKANLPGHYRYQRRLFTHFMQEDLPEDKR
SCAB75511 (451) NVDIRSHIIG-----SPVTVSWENEPYFMGAFKANLPGHYRYQRRLFTHFMQDRLPEDKR
PF01593 (393) NVDIRSHIIG-----SPVTVSWENEPYFMGAFKANLPGHYRYQRRLFTHFMQDRLPEDKR
724 767
AAF77123 (699) GVYLAGCSCSFTGGWVEGAIQTACNAVCAIIHNCGGILAKGNPL
AAR06971 (505) GLYLAGCSCSFAGGWIEGAVQTALNSACAVVRSTGGQLSKGNPL
NP_625735 (506) GIFLAGDDISWTAGWAEGAVQTALNAVWGVMHHLGGETDATNPG
SCAB75511 (506) GIFLAGDDISWTAGWAEGAIQTALNAVWGVMHHFGGTTDTTNPG
PF01593 (448) GIFLAGDDISWTAGWAEGAIQTALNAVWG----------

Table 1 The identity (similarity in red) table of tryptophan monoxygenase from *S. scabies* 87-22 (SCAB 75511), *S. coelicolor* A3 (2) (NP_625735), *A. tumefaciens* (AAF77123), *P. syringae* pv. *syringae* (AAR06971) and amino oxidase (PF 01593).

	P. syringae pv. *syringae*	*S. coelicolor* A3 (2)	*S. scabies* 87-22	amino oxidase
A. tumefaciens	50	27	28 (32)	29
P. syringae pv. *syringae*	100	31	31 (34)	32
S. coelicolor A3 (2)	31	100	95 (95)	95
S. scabies 87-22	31 (34)	95 (95)	100	100

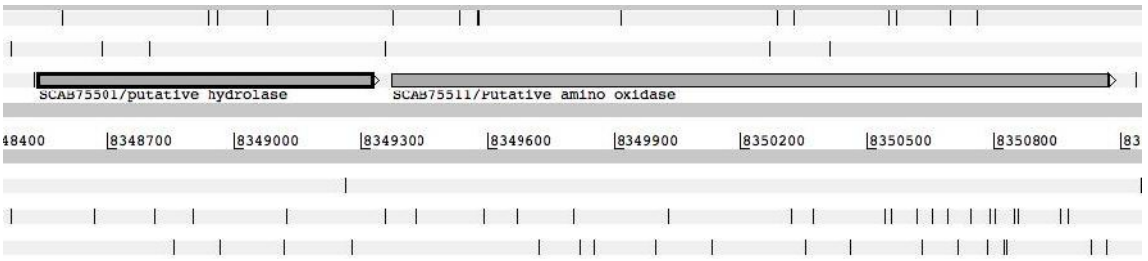


Figure 9 Genetic organization of putative *iaaH* (SCAB75501) and *iaaM* (SCAB75511) locus in *S. scabies* 87-22.

MRTALLQSSG RPGSVVGNLKVLDEAAGRAAAAGAGLVVAPEMFLTG
YAIGDDIARLAETADGDSADAVAE IAGRHGVAIVYGYPERAGDVVFN
SAQLVSADGERLANYRKTHLFGCFERDHFTPGGRSVVQAELNGLRVG
LLICYDVEFPENVRAHALAGTDLLVVPTAQMHPFQFVAESMIPVRAFE
NQMYVAYVNRVGREGEFEFVGLSTLAGPDGVARTRAGREEDLVLAD
ADPAFLAASRAANPYLQDRRPALYGS LV

Figure 10 Protein sequence of the putative IaaH protein in *S. scabies* 87-22.

Figure 11 Alignment of the protein sequence of indole-3-acetamide hydrolase from *S. scabies* 87-22 (SCAB75501), *A. vitis* (AAB41868), *A. tumefaciens* (AAF77122) and CN_hydrolase (PF00795). Identical: Red text on yellow background; Weakly similar: Dark green text on a white background; Block of Similar: Black on a light green background; Conservative: Dark blue on a light blue background; Non-similar: Black on a white background.

Table 2 The identity (similarity in red) table of indole-3-acetamide hydrolase from *S. scabies* 87-22 (SCAB75501), *A. vitis* (AAB41868), *A. tumefaciens* (AAF77122) and CN_hydrolase (PF00795).

	A. tumefaciens	CN_hydrolase	*S. scabies* 87-22
A. vitis	88	18	15 (18)
A. tumefaciens	100	18	15 (18)
CN_hydrolase	18	100	100

ssIaaH protein with *A. tumefaciens* (accession number: AAF77122), *A. vitis* (accession number: AAB41868) and carbon-nitrogen hydrolase (conserved domain, PF00795) is shown in Figure 11. Both identities in *S. scabies* vs. *A. tumefaciens* and *S. scabies* vs. *A. vitis* are 15% (Table 2).

Disruption and complementation of *iaaH* and *iaaM* genes in *Streptomyces scabies*

Mutants of *S. scabies* 87-22 deleted in the genes encoding *ssiaaH*, *ssiaaM* or both genes (*ssiaaHM*) were generated by marker exchange mutagenesis using the Redirect PCR targeting system (Gust et al., 2002). One *iaaH* deletion mutant, two *iaaM* deletion mutants and three double mutants were verified using Southern analysis. Since the putative *iaaH* gene is adjacent to the *iaaM* gene, an *iaaM* southern probe was designed to verify these three mutants, Δ*iaaH*, Δ*iaaM*5 and Δ*iaaM*8 (Figure 12). The sizes of the *iaaM* and the *iaaH* genes are 1968 bp and 789 bp respectively while the intergenic region is 166 bp. The fragment in *S. scabies* 87-22 strain is therefore 2923 bp (1968+789+166=2923). The Δ*iaaH* strain will be 3503 bp: 1369 (the DNA fragment size of disruption cassette) + 1968 (the DNA fragment size of *iaaM*) + 166 (the intergenic region) = 3503 bp.

Chromosomal DNA from each strain was digested with *Eco*RI overnight and blotted with *iaaM* southern probe. Southern blotting of Δ*iaaH* chromosomal DNA with *iaaM* southern probe revealed a 3503 kb band and Δ*iaaM*5 and Δ*iaaM*8 chromosomal DNA will have no band (Figure 13). Next, we used the apramycin gene (located on the disruption cassette and DNA length is 1369 bp) as the probe to verify the disruption of *iaaH* and *iaaM* genes. Chromosomal DNA from each strain was digested with *Eco*RI overnight and blotted with Apra southern probe. As we expect, southern blotting of Δ*iaaH* has a 3.5 kb band (1369 (the DNA fragment size of disruption cassette) + 1968 (the DNA fragment size of *iaaM*) + 166 (the intergenic

region)) = 3503 bp, Δ*iaaM*5 and Δ*iaaM*8 chromosomal DNA have a 2.3 kb band 1369 (the DNA fragment size of disruption cassette) + 789 (the DNA fragment size of *iaaH*) + 166 (the intergenic region) = 2324 bp and no hybridization in wild type strain (Figure 14).

A complementation construct was designed using the *Streptomyces* expression vector pIJ10257 and verified by colony PCR of each gene. The *iaaM* gene size is 1698 bp (Figure 15). *S. scabies* 87-22 and two complementation strains , Δ*iaaM5C* and Δ *iaaM8C*, revealed a 1698 bp band and no band revealed in Δ*iaaM* mutants strains.

The putative *iaaH* gene is adjacent to the *iaaM* gene, so an *iaaMH* southern probe was designed to verify double knockout strains Δ*iaaMH1*, Δ*iaaMH2* and Δ*iaaMH3* (Figure 16). Chromosomal DNA from *S. scabies* 87-22, Δ*iaaMH1*, Δ*iaaMH2* and Δ*iaaMH3* were digested with *Eco*RI overnight and blotted with Δ*iaaMH* southern probe. Southern blotting of *S. scabies* 87-22 chromosomal DNA with *iaaMH* southern probe revealed a 2923 bp band and double knockout mutants produced no bands (Figure 17).

Indole-3-acetic acid synthesis in *S. scabies*, putative IAA deficient mutant strains and their complementary strains

To evaluate whether the putative *iaaH* and *iaaM* genes are actually involved in IAA biosynthesis, we used high performance liquid chromatography (HPLC) to analyze the indolic compounds synthesized by *S. scabies* 87-22 and the Δ*iaaH* and Δ*iaaM* mutants. The data presented in Table 3 provides experimentally determined HPLC retention time data for standards of IAA and selected biosynthetic intermediates in known IAA biosynthetic pathways (Figure 2), using the acetonitrile:water:acetic acid (35:65:1) solvent system with 1 ml/min flow rate, detection at 280 nm.

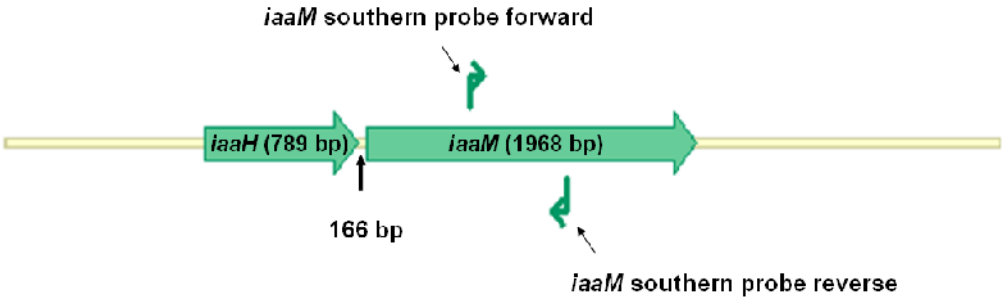


Figure 12 Genetic organization of *iaaH*, *iaaM* locus and the *iaaM* fragment used as a probe (the size of each probe is 20 bp) in southern analysis of *S. scabies* 87-22.

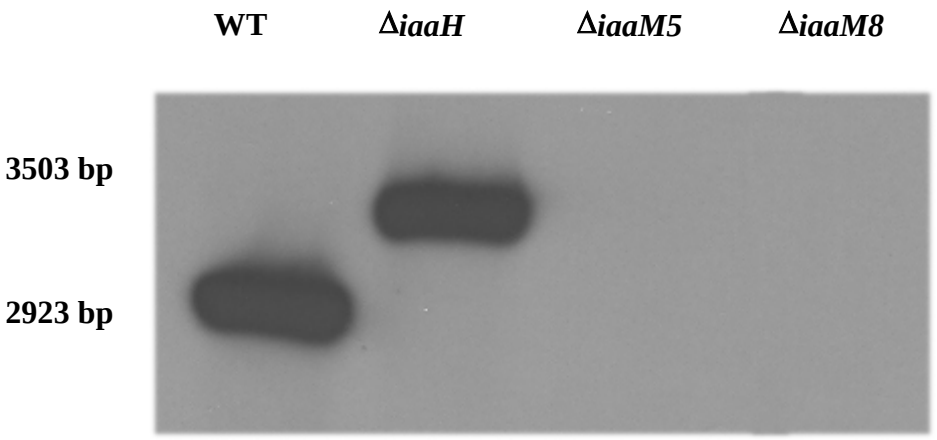


Figure 13 Southern hybridization analysis of *S. scabies* 87-22 IAA-deficient mutants. Chromosomal DNAs of *S. scabies* 87-22 (WT), Δ*iaaH*, Δ*iaaM5* and Δ*iaaM8* strains was digested with *Eco*RI and hybridized with PCR-generated dioxigenin-11-dUTP labeled fragment of *iaaM* gene.

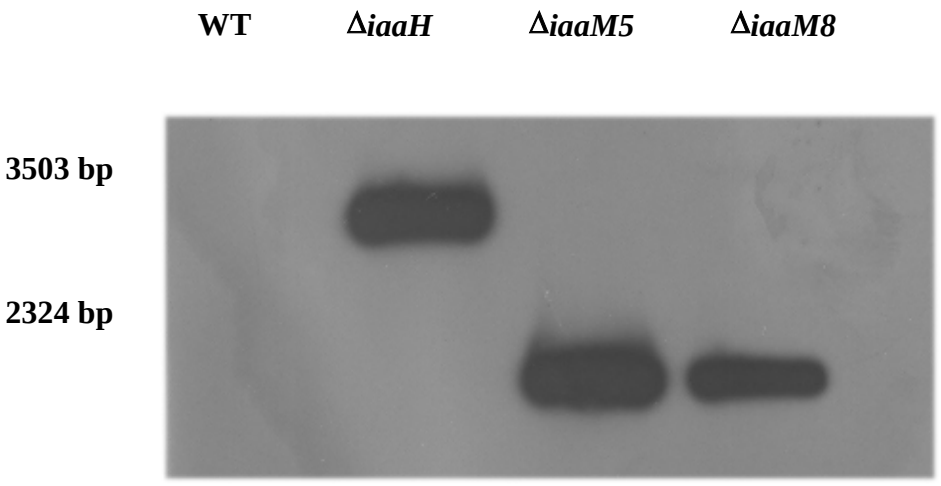


Figure 14 Southern hybridization analysis of *S. scabies* 87-22 IAA-deficient mutants. Chromosomal DNAs of *S. scabies* 87-22 (WT), Δ*iaaH*, Δ*iaaM5* and Δ*iaaM8* strains were digested with *Eco*RI and hybridized with a PCR-generated dioxigenin-11-dUTP labeled fragment of the apramycin resistance gene *aac(3) IV*.

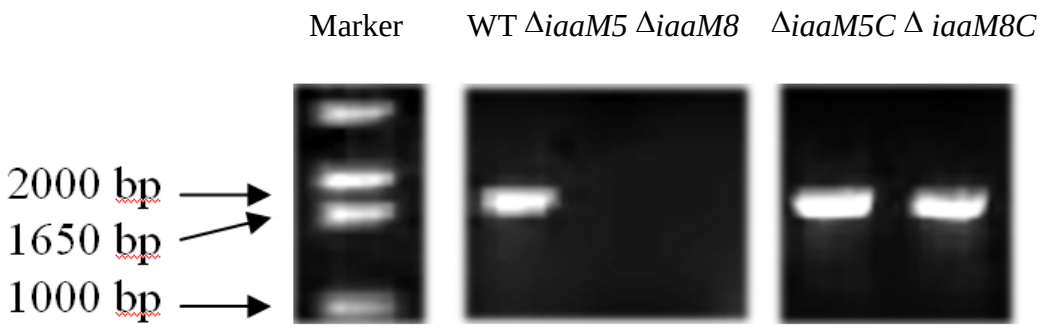


Figure 15 Gel electrophoresis of the *iaaM* gene generated by polymerase chain reaction in *S. scabies* 87-22 strains. The size of the *iaaM* gene is 1698 bp.

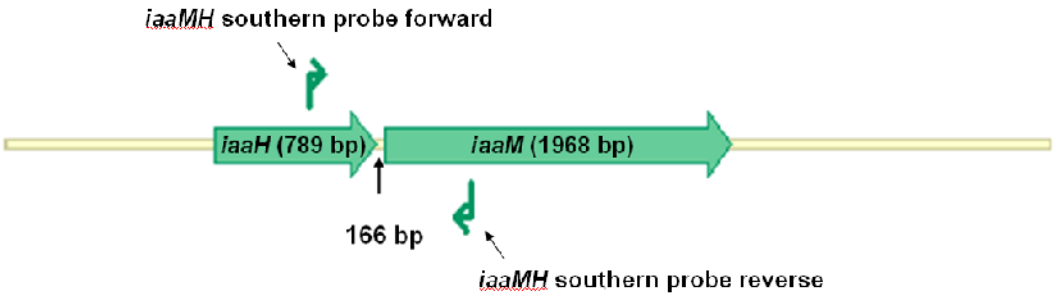


Figure 16 Genetic organization of *iaaH* and *iaaM* locus and location of the fragment used as a probe (the size of each probe is 20 bp) in southern analysis of *S. scabies* 87-22.

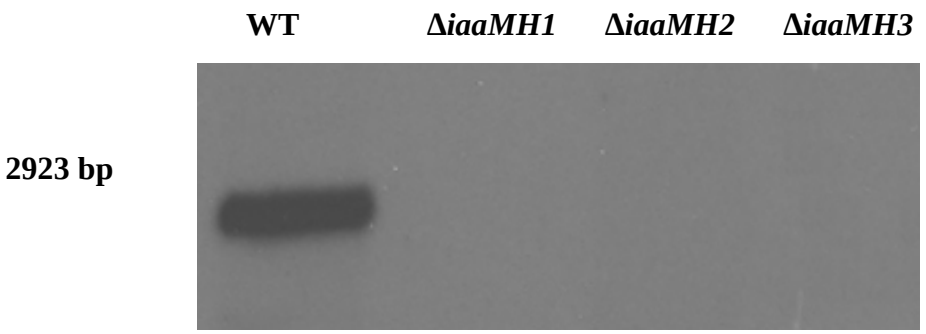


Figure 17 Southern hybridization analysis of *S. scabies*87-22 (WT), Δ*iaaMH1,* Δ*iaaMH2* and Δ*iaaMH3* strains. Genomic DNA was digested with *Eco*RI and probed with a PCR-generated dioxigenin-11-dUTP labeled fragment containing portions of the *iaaHM* gene. The size of *iaaHM* gene is 2923 bp.

Figure 18 shows the HPLC data from *S. scabies* 87-22 WT and the Δ*iaaH* and Δ*iaaM* mutant strains. Supernatants were collected from cultures grown in 25 ml TSB medium with 25 µg L-tryptophan at room temperature for 5 days in the dark and extracted with ethyl acetate. The result indicated *S. scabies* 87-22 and Δ*iaaH* strains appear to be capable of synthesizing IAA, as evidenced by a small peak at approximately 6.9 min consistent with the retention time of the IAA standard, but the relative concentration of IAA is greatly reduced in Δ*iaaH* strain. In Δ*iaaM5* and Δ*iaaM8* strains, there was no IAA peak present, indicates that these two mutant strains do not appear to synthesize IAA. This result suggests that SCAB75511 and SCAB75501 genes are involved in IAA synthesis. The relative HPLC traces of both Δ*iaaM5* and Δ*iaaM8* share some prominent peaks present in the WT strain, at approximately 5.2 min and 6.2 min but these peaks do not correspond to the retention times of available standards so their chemical identity is unknown. Compounds reported to be produced in IAA pathways include indole-3-acetaldehyde, indole-3-ethanol (breakdown product of indole-3-acetaldehyde), or indole-3-acetaldoxime.

To confirm that the putative IaaH and IaaM proteins are involved in IAA biosynthesis, we attempted to genetically complement the Δ*iaaH,* Δ*iaaM5*, Δ*iaaM8* mutants; the Δ*iaaM5* and Δ*iaaM8* mutants were successfully complemented. Cultures were grown and supernatants were collected and analyzed as described previously. Both complemented strains recovered the ability to produce IAA (Figures 19 and 20). These data support the hypothesis that the putative IaaH and IaaM proteins are involved in IAA biosynthesis.

Table 3 Retention times of indolic compounds (standards) that were separated by C18 reverse phase column (0.5 cm x25 cm) with 1 ml/min flow rate. Absorbance was monitored at 280 nm.

Compound	Retention time (min)
Indole-3-acetic acid	6.8 - 6.9
Tryptophan	2.9
Tryptamine	2.8 - 3.0
Indole-3-acetamide	4.5 - 4.7
Indole-3-pyruvic acid	9.3
Indole-3-acetonitrile	12.2

Solvent: acetonitrile:water:acetic acid 35:65:1

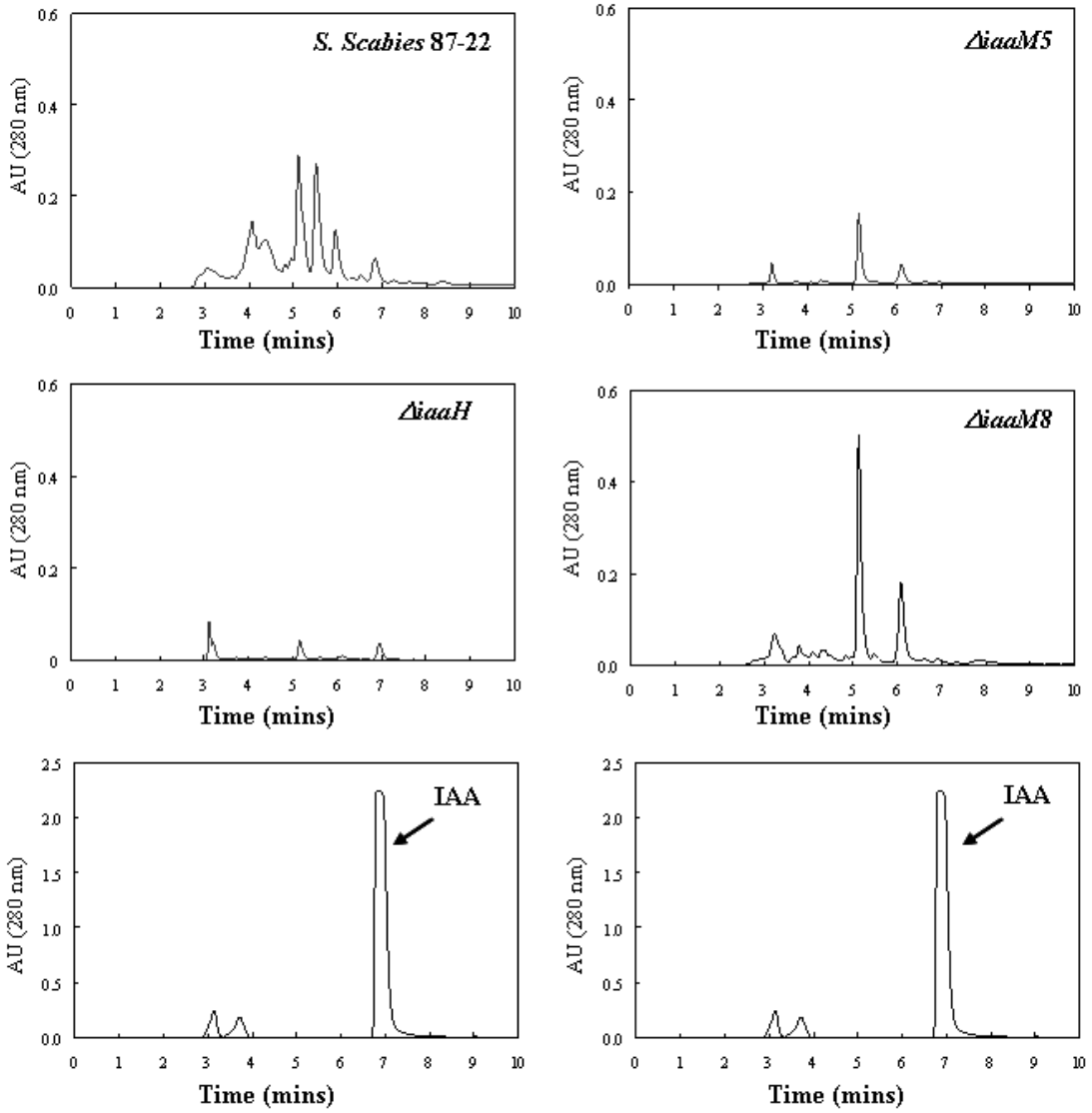


Figure 18 Chromatograms of IAA content in *S. scabies* 87-22, Δ*iaaH*, Δ*iaaM5* and Δ*iaaM8*. The retention time of IAA is between 6.8-6.9 min.

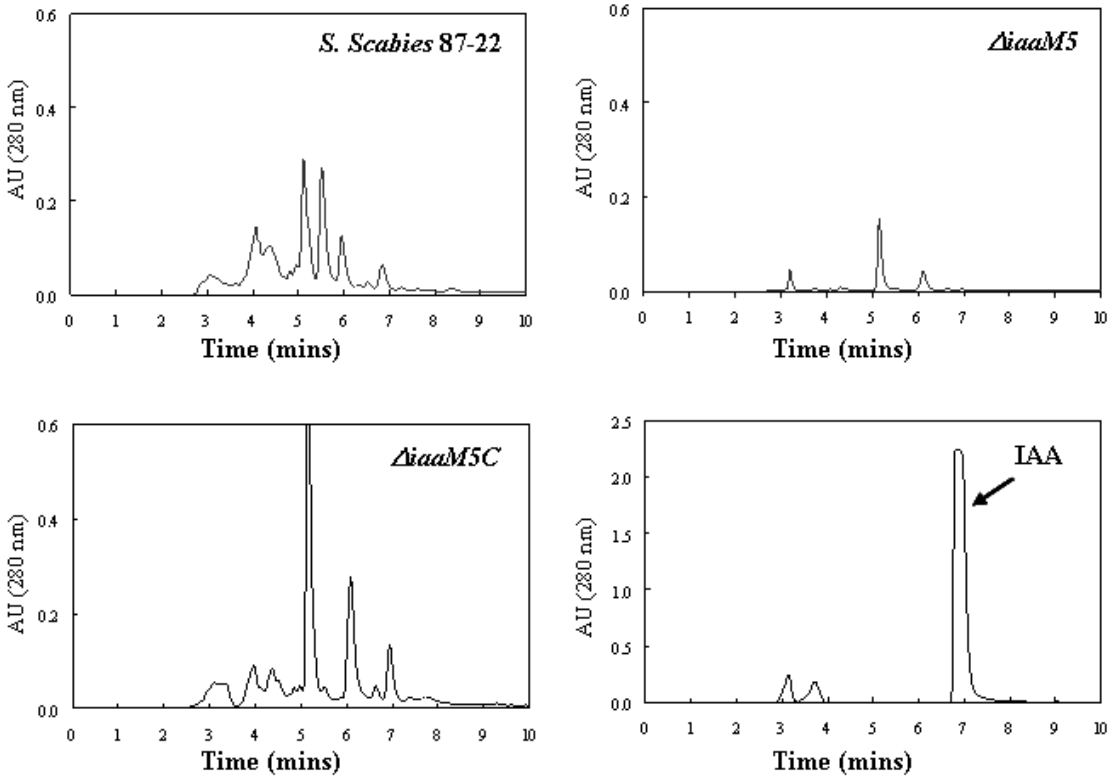


Figure 19 Chromatogram of IAA content in *S. scabies* 87-22, Δ*iaaM5* and Δ *iaaM5C*: the complementation strain of Δ*iaaM5*. The Δ*iaaM5C* strain has highest IAA content than other treatments. The retention time of IAA is between 6.8-6.9 min.

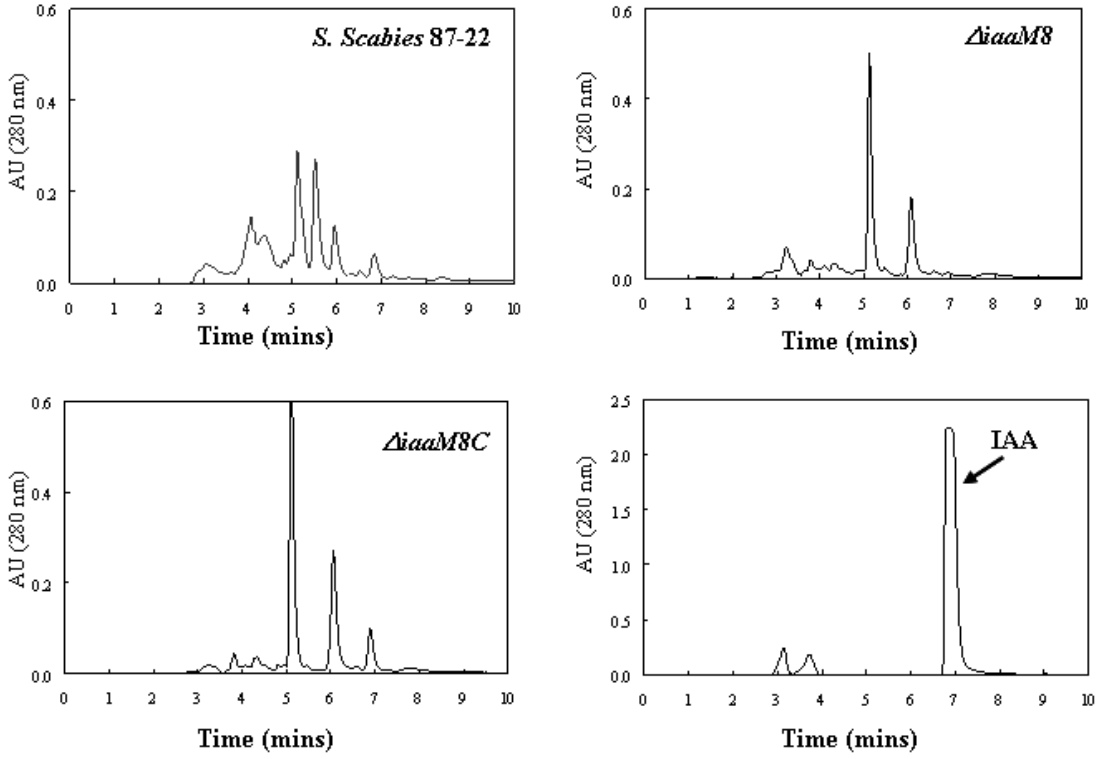


Figure 20 Chromatogram of IAA content in *S. scabies* 87-22, Δ*iaaM8* and Δ*iaaM8C*: the complementation strain of Δ*iaaM8*. The Δ*iaaM8C* strain has highest IAA content than other treatments. The retention time of IAA is between 6.8-6.9 min.

IAA production is a virulence factor in *Streptomyces scabies* 87-22

Many plant-associated microorganisms can synthesize auxins, including IAA (Costacurta and Vanderleyden, 1995; Yamada, 1993). Auxin stimulates overproduction of root hairs and lateral roots in plants and release of saccharides from plant cell walls during the elongation (Davies, 2004). Saccharides are a source of nutrients for microorganisms and can increase the colonization ability of plant-associated bacteria (Lindow and Brandl, 2003). In this study we investigated the role of microbial-produced IAA in the plant-microbe interaction of *S. scabies* and a host plant. First, we wanted to know if *S. scabies* synthesis of IAA affects lateral root formation in radish seedlings. 5-day-old radish seedling roots were inoculated with $1x10^5$ *S. scabies* 87-22 spores and the number of secondary roots per seedling was counted in each treatment, after 10 days of growth. Interestingly, plants inoculated with the *S. scabies* 87-22 strain produced more lateral roots (5.7 roots/cm) than did those plants inoculated with the IAA deficient mutant strains (3.7 roots/cm) or the noninoculated control plants (3.5 roots/cm) (Figure 21). The data also shows that the *iaaM* deletion strains that were genetically complemented (Δ*iaaM5C* and Δ*iaaM8C*) were restored in the ability to induce lateral root formation (Figure 21).

IAA production has been demonstrated to be a virulence factor in some pathogens (Yamada, 1993). To determine whether IAA production is a virulence factor in *S. scabies* 87-22, we inoculated 5-day-old radish seedling roots with $1x10^5$ of *S. scabies* 87-22 spores. After 10 days, plants inoculated with *S. scabies 87-22* had more necrotic symptoms than those inoculated with the IAA deficient mutants (*ΔiaaH, ΔiaaM*5 and *ΔiaaM8*) (Figure 22). As we predicted, the genetically complemented strains complementary strains Δ*iaaM5C* and Δ*iaaM8C* were restored in the ability to cause host cells necrosis (Figure 23, 24).

Necrosis is a process involving cell swelling, lysis, and the inflammatory

leakage of cell contents (Wyllie, 1997). Malondialdehyde (MDA), an end-product of lipid peroxidation, can react with essential macromolecules to cause cellular dysfunction at the molecular level (Esterbauer et al., 1988; Frankel, 1987). In many studies, MDA is used as an index to present cell damage. MDA concentration was measured to provide a quantitative index of tissue damage in response to infection. 5-day-old radish seedlings were inoculated with $1x10^5$ spores of *S. scabies* 87-22 and IAA deficient strains (*ΔiaaH, ΔiaaM*5 and *ΔiaaM8*) and plants were grown for 10 days, as described previously. MDA content of roots was measured for plants in each treatment. Radish seedling roots harvested from plants inoculated with the *S. scabies* 87-22 strain had higher levels of MDA than did plants inoculated with IAA deficient mutant strains. All treatments had higher MDA levels than did the noninoculated control plants. Genetic complementation of IAA production in *S. scabies* 87-22 also restored the ability of the strains to damage inoculated radish roots (Figure 25).

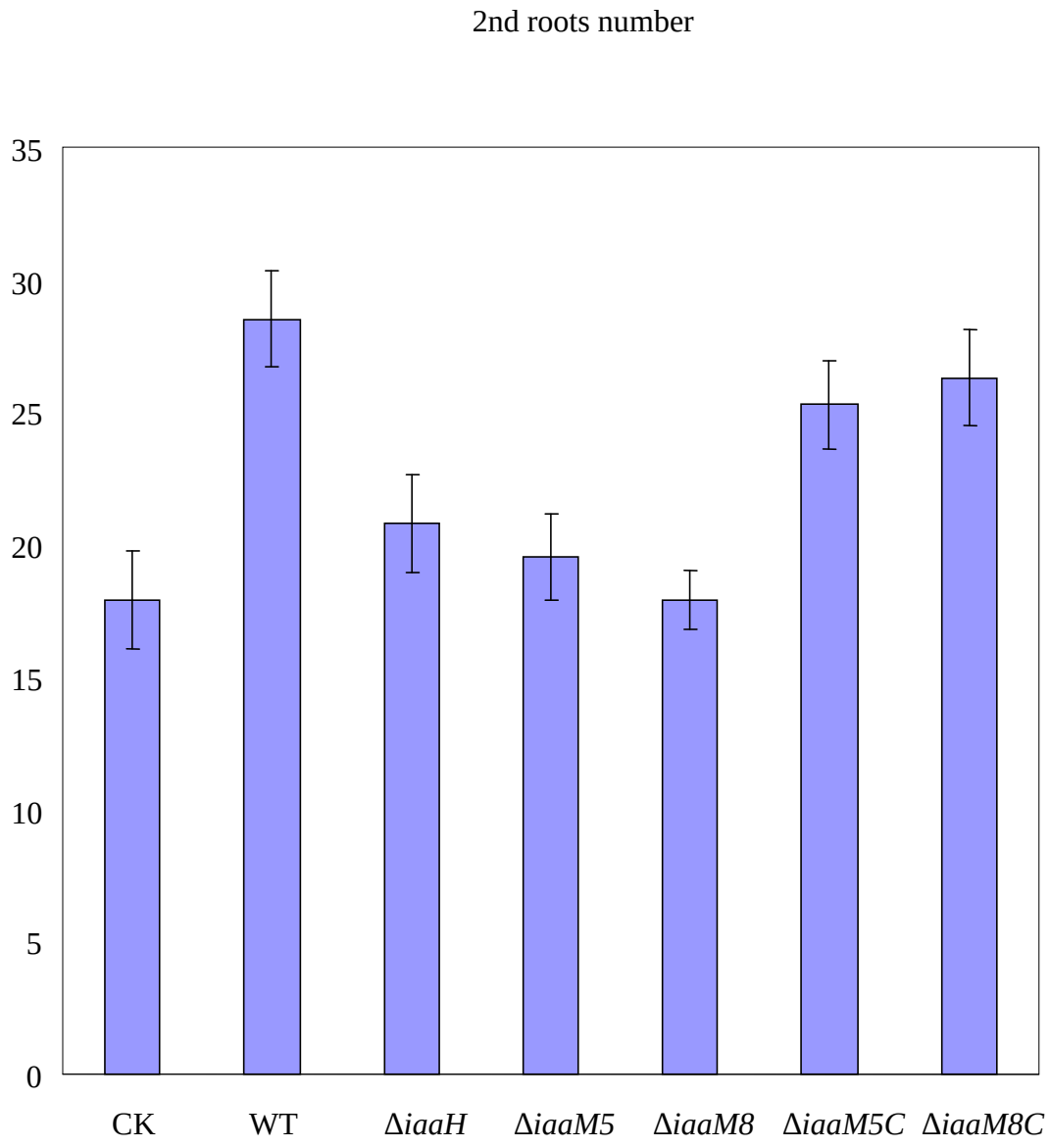


Figure 21 Secondary root production on radish roots inoculated with $1x10^5$ *S. scabies* 87-22 spores. Root number was counted at the10th day of inoculation. Treatments were: CK: water; WT: *S. scabies* 87-22; Δ*iaaH*: the *iaaH* gene knockout mutant; Δ*iaaM5* and Δ*iaaM8*: *iaaM* gene knockout mutants; Δ*iaaM5C* and Δ*iaaM8C*: the complementation strain of Δ*iaaM5* and Δ*iaaM8.* Means ± SE, n = 10.

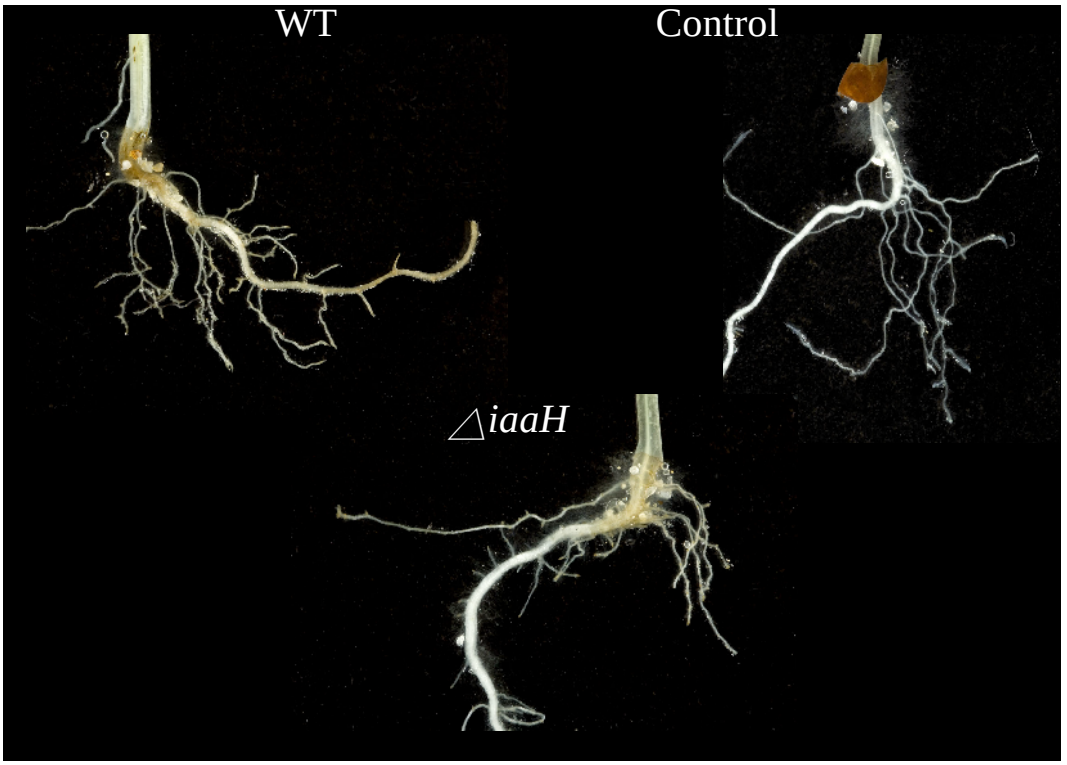


Figure 22 Root growth and necrosis on radish as affected by inoculation with *S. scabies* 87-22 wild type (WT) or mutant strains. Treatments were noninoculated (Control); *S. scabies* 87-22 (WT); and the Δ*iaaH* mutant strain.

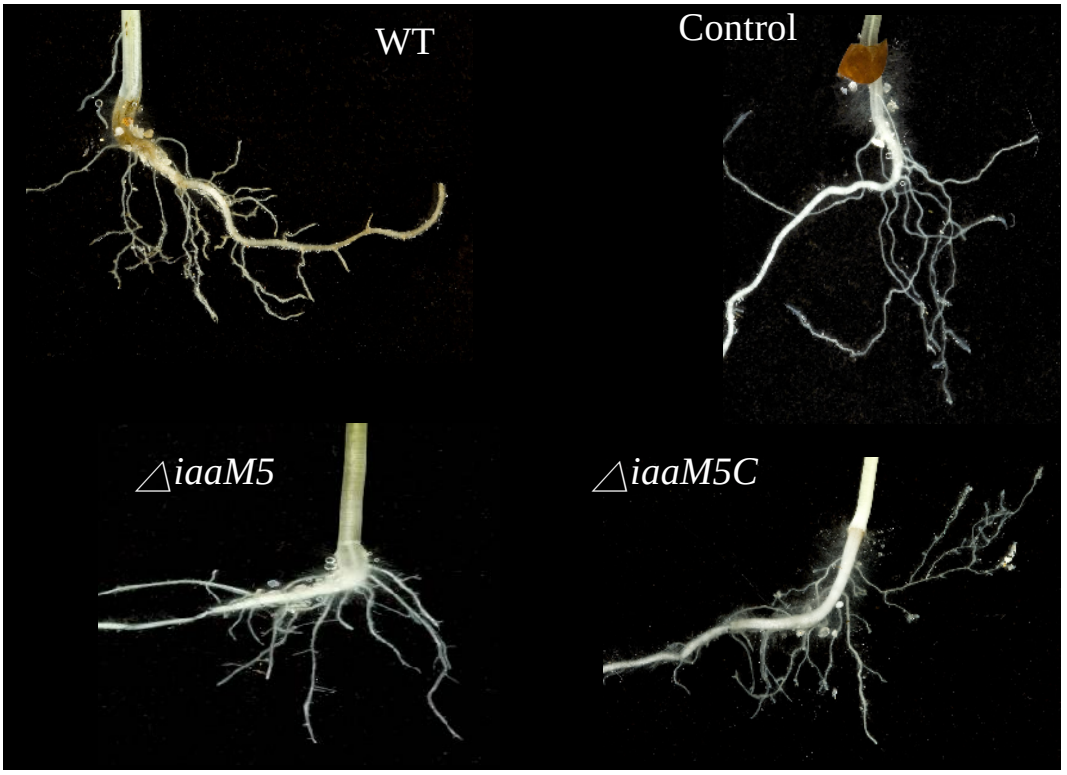


Figure 23 Root growth and necrosis on radish as affected by inoculation *S. scabies* 87-22 wild type (WT) or mutant strains. Treatments were noninoculated (Control); *S. scabies* 87-22 (WT); and the Δ*iaaM5* gene mutant strain and genetically complemented strain Δ*iaaM5C*.

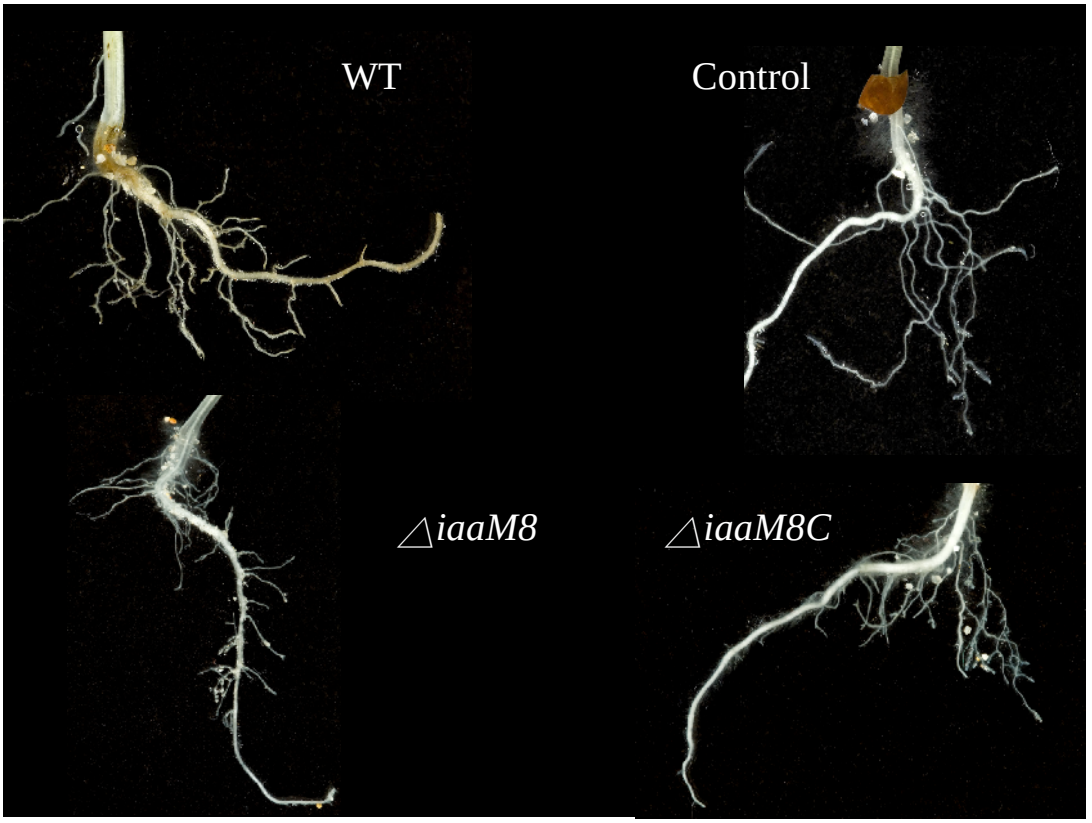


Figure 24 Root growth and necrosis on radish as affected by inoculation *S. scabies* 87-22 wild type (WT) or mutant strains. Treatments were noninoculated (Control); *S. scabies* 87-22 (WT); and the Δ*iaaM8* gene mutant strain and genetically complemented strain Δ*iaaM8C*.

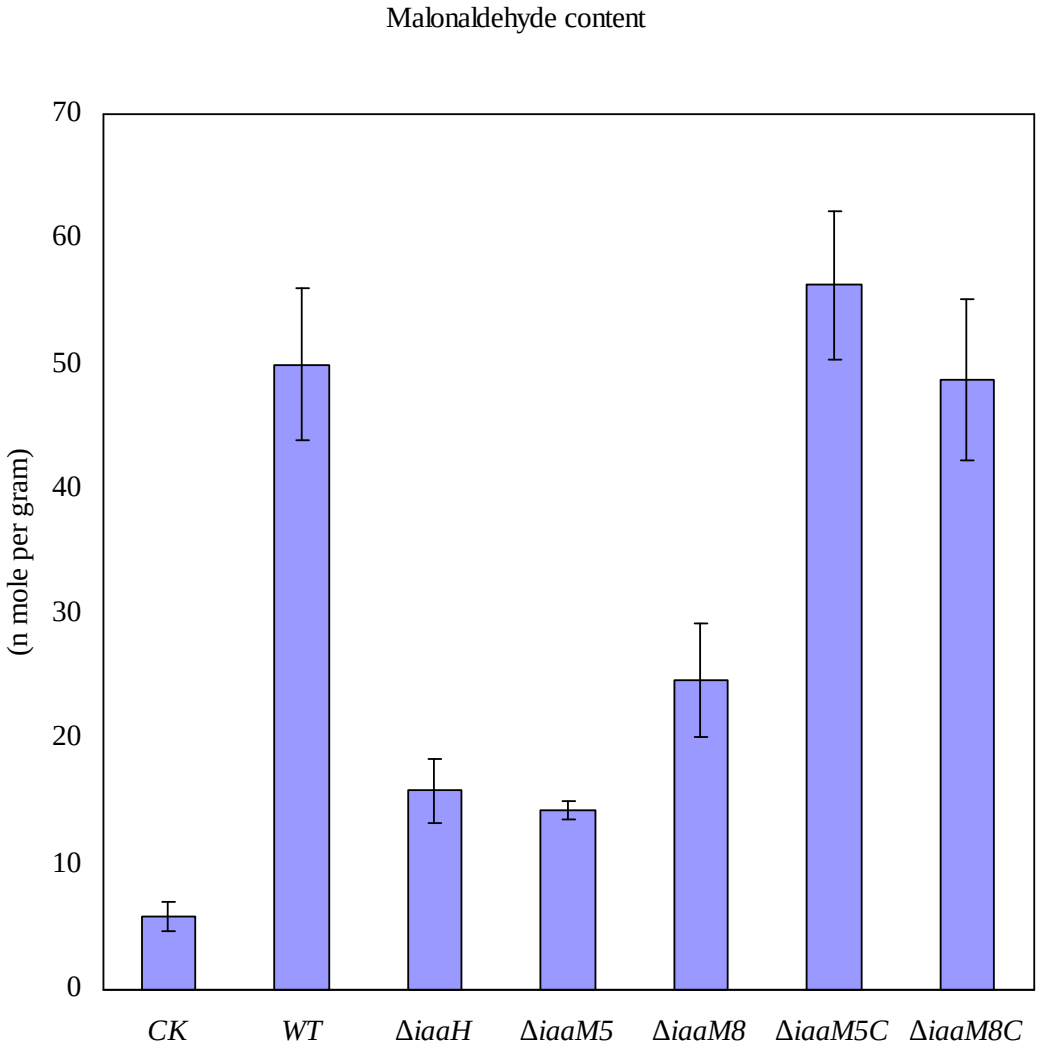


Figure 25 Changes in malonaldehyde contents in 15-day-old radish roots. Radish seedling roots (5 day) were inoculated with $1x10^5$ *S. scabies* spores then grown for 10 days. Treatments were: CK (water control); WT (*S. scabies* 87-22); Δ*iaaH*, Δ*iaaM5*, Δ*iaaM8*, Δ*iaaM5C* and Δ*iaaM8C.* Means ± SE, n = 4.

Thaxtomin A is a necrogenic phytotoxin produced exclusively by plant pathogenic *Streptomyces*, and required for pathogenicity. Thaxtomins are cyclic dipeptides containing nitro-tryptophan and phenylalanine (Healy et al., 2000). Since both IAA and thaxtomin use tryptophan as a precursor, we hypothesized that IAA and thaxtomin biosynthetic pathway will compete for tryptophan. $1x10^5$ *S. scabies* 87-22 spores were cultured in 5 ml OBB for 5 days in the dark at room temperature before extraction with 3 ml methanol:water (50:50). Thaxtomin A was detected via absorbance at 380 nm and quantified using pure thaxtomin A as a standard. All IAA deficient strains produced less thaxtomin A than wild type. Interestingly, thaxtomin production was restored in *ΔiaaM5C* but not in *ΔiaaM8C*. These results indicate that IAA and thaxtomin do not compete for tryptophan (Figure 2-26).

DISCUSSION

Previous studies demonstrated that *S. scabies* has the ability to produce IAA, which was hypothesized to promote scab lesion formation through cell division during the development of wound periderm (Manulis et al., 1994). *S. scabies* is a world known soil-borne pathogen, which can induce scab diseases in economically important plants (Loria et al., 2006). IAA may be involved in pathogenicity of *S. scabies*, however, the IAA biosynthetic pathway had not been identified in *S. scabies*, and the disease development associated with IAA is still unclear.

The purpose of this study was to investigate IAA production in *S. scabies* and the role of IAA in *S. scabies* 87-22 interactions with its host. Since the *S. scabies* 87-22 genome has been sequenced, genomic and molecular approaches can be used to investigate IAA production in this strain. *Streptomyces violaceus* and *S. exfoliates* produce IAA through the IAM pathway. Two enzymes, IaaH and IaaM, are involved in this pathway (Manulis et al., 1994). A putative IaaM protein in *S. scabies* 87-22, SCAB75511, was indentified by using IaaM protein sequence similarity.

The SCAB75511 protein encodes an amino oxidase (PF01593), which is part of the flavin-containing amine oxidoreductase family. As the IaaM protein is included in this family (http://pfam.sanger.ac.uk//family/PF01593), we proposed that the SCAB75511 gene is an *iaaM* gene in *S.scabies*. For clarity, this 565 aa protein was aligned with the characterized IaaM proteins from *S. coelicolor* A3 (2) (accession number: NP_625735), *A. tumefaciens* (accession number: AAF77123) and *P. syringae* pv. *syringae* (accession number: AAR06971) (Figure 2-8). Amino acid identities between SCAB75511 and *S. coelicolor* A3 (2), *A. tumefaciens* and *P. syringae* pv. *syringae* are 95%, 28% and 31%, respectively. These results indicate that the

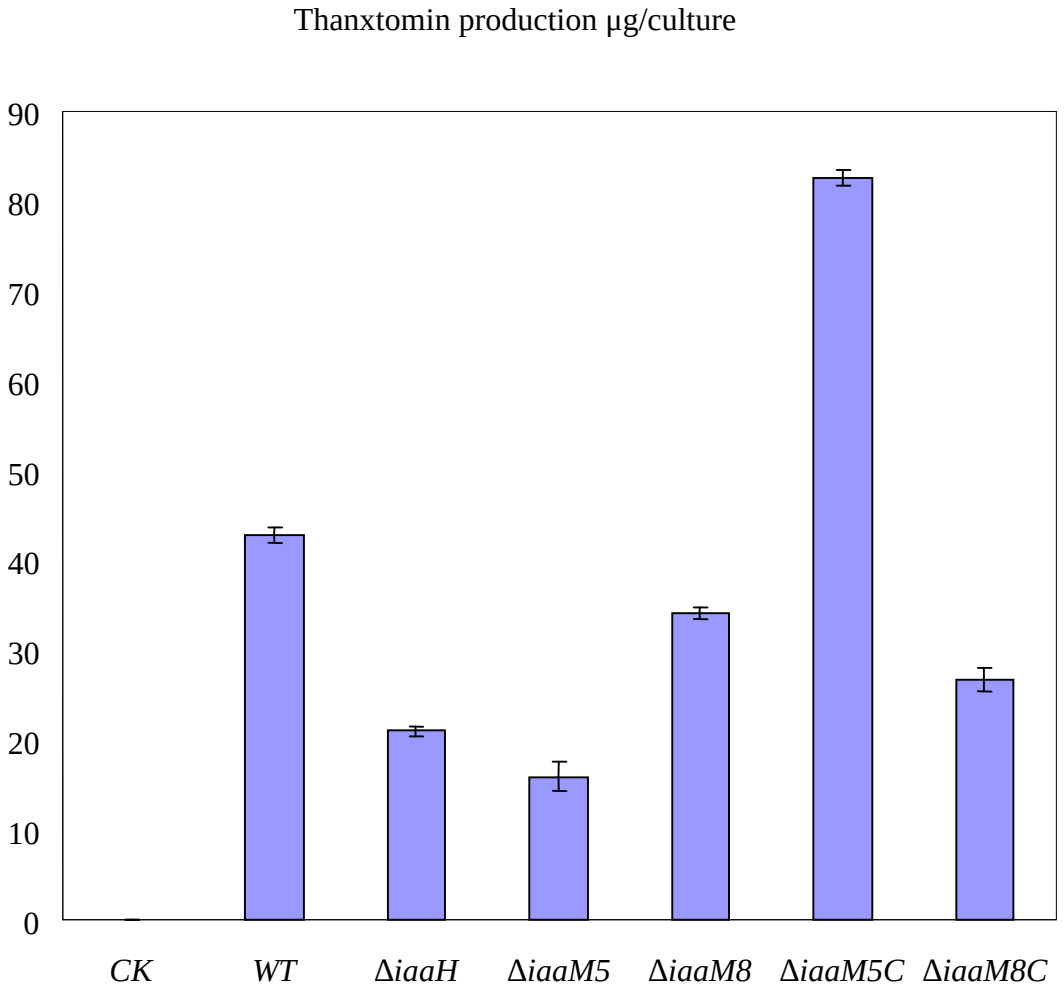


Figure 26 Thaxtomin production in 5 ml OBB culture inoculated with $1x10^5$ *S. scabies* 87-22 spores for 5 days. Treatments were CK (water control), WT (*S. scabies* 87-22), Δ*iaaH*, Δ*iaaM5*, Δ*iaaM8*, Δ*iaaM5C*, and Δ*iaaM8*. Means ± SE, N = 3.

SCAB75511 gene is a homolog of the *iaaM* genes in *S. coelicolor* A3 (2), *A. tumefaciens* and *P. syringae* pv. *syringae*.

IaaH is another enzyme required in the IAM synthesis pathway. We examined the region of the *S. scabies* genome surrounding *ssiaaM* using the genome viewer software Artemis. A gene (SCAB75501) encoding a putative hydrolase protein was found upstream and adjacent to the SCAB75511, which we designated *ssiaaH* (Figure 9). The SCAB75501 protein encodes a carbon-nitrogen hydrolase protein (PF00795). An alignment of the SCAB75501 protein with *A. tumefaciens* (accession number: AAF77122) and *A. vitis* (accession number: AAB41868) is shown in Figure 11; the identity of SCAB75501 with those proteins was very low (15%). However, given the location and putative function, SCAB75501 was considered to be a good candidate.

The Redirect target exchange system (Gust et al., 2002) was used in this study to knock out the putative *iaaH* and *iaaM* genes in *S. scabies* 87-22. Six *S. scabies* mutants were produced, including: three knockout strains (Δ*iaaH*, Δ*iaaM5* and Δ*iaaM8*) and three double knockout strains (Δ*iaaMH1*, Δ*iaaMH2* and Δ*iaaMH3*). Using HPLC, we analyzed the intermediates in the tryptophan-dependent pathways to evaluate whether the putative *iaaH* and *iaaM* genes were actually involved in IAA biosynthesis. The three knockout strains, Δ*iaaH*, Δ*iaaM*5 and Δ*iaaM8*, had lower IAA contents than *S. scabies* 87-22 (the data for the double knockout strains, Δ*iaaMH1*, Δ*iaaMH2* and Δ*iaaMH3*, are unavailable) (Figure 18). These results support the hypothesis that these putative *iaaH* and *iaaM* genes are involved in IAA synthesis. However, because we could not detect intermediates in the pathway in mutant cultures, we are not able to definitively demonstrate the function of these proteins. The HPLC traces of both Δ*iaaM5* and Δ*iaaM8* share some prominent peaks that are present in the *S. scabies* 87-22 strain, at approximately 5.2 min and 6.2 min, but these peaks do not correspond to the retention times of the available standards, therefore their chemical

identities remain unknown. Genetically complementing the IAA deficient mutants (Δ*iaaM5* and Δ*iaaM8*) confirms that the putative IaaM proteins are involved in IAA biosynthesis (Figures 19 and 20).

Although HPLC is used extensively for detection, it is limited by the availability of standards for comparison to unknowns. In this research, although we could detect the presence of IAA, the unknown indole peaks produced by mutants could not be identified. Liquid chromatography/tandem mass spectrometry (LC/MS) is a more powerful analytical technique that combines the physical separation capabilities of HPLC and the mass analysis ability of mass spectrometry with a higher sensitivity and specificity. Future studies of the unknown indole compounds produced by the mutants should incorporate LC/MS for analysis.

Auxins are major plant hormones and have many regulatory functions in plants, including: cell enlargement stimulation, cambium cell division, root formation, etc. (Davies, 2004). Many microorganisms synthesize IAA in order to perturb host physiological processes for their own benefits (Costacurta and Vanderleyden, 1995; Yamada, 1993). It has also been suggested that microorganisms synthesize IAA to stimulate the overproduction of root hairs and lateral roots in plants because plant cell walls will release saccharides during the elongation (Davies, 2004).

Saccharides are carbohydrates that can be a source of nutrients for microorganisms and increase the colonization ability of a bacterium (Lindow and Brandl, 2003). IAA also has been suggested to facilitate potato scab lesion formation through cell division during the development of wound periderm (Manulis et al., 1994). A secondary goal of this study was to evaluate the role of IAA in *S. scabies* pathogenicity during disease development. We hypothesized that IAA production in *S. scabies* 87-22 would induce more lateral root formations and necrosis symptoms in hosts. $1x10^5$ spores of wild type and IAA deficient mutant strains (Δ*iaaH*, Δ*iaaM5* and

Δ*iaaM8*) of *S. scabies* 87-22 were inoculated onto 5 day-old radish seedlings (cv. Burpee White) and were grown for 10 days. Plants inoculated with the *S. scabies* 87-22 produced more lateral roots (5.7 roots/cm) and had higher malondialdehyde (MDA) levels. MDA is the end-product of lipid peroxidation that can react with essential macromolecules and cause cellular dysfunction at the molecular level (Esterbauer et al., 1988; Frankel, 1987). In many studies, MDA is used as an index to measure cell damage. As we predicted, complementation of IAA production in *S. scabies* 87-22 restored the affects of the mutant strains on root formation and cellular damage in inoculated radish roots (Figure 21 and 25). Other evidence to support our hypothesis is shown in Figures 22, 23 and 24. Plants inoculated with *S. scabies 87-22* had more necrotic symptoms than those inoculated with the IAA deficient mutants (*ΔiaaH, ΔiaaM*5 and *ΔiaaM8*) (Figure 22), and the complemented strains, Δ*iaaM5C* and Δ*iaaM8C,* were restored in their ability to cause host cell necrosis (Figure 23, 24).

Another factor that could affect IAA synthesis in *S. scabies* 87-22 is thaxtomin A. Thaxtomin A is a necrogenic phytotoxin produced exclusively by plant pathogenic *Streptomyces* spp. and is required for pathogenicity. Thaxtomins are cyclic dipeptides containing nitro-tryptophan and phenylalanine (Healy et al., 2000). Since tryptophan is the precursor of IAA and thaxtomin A, we hypothesized that the IAA and thaxtomin A pathways would compete for tryptophan. $1x10^5$ *S. scabies* 87-22 spores were cultured in 5 ml OBB for 5 days in the dark at room temperature for thaxtomin A production. Thaxtomin A was detected via absorbance at 380 nm and quantified by using pure thaxtomin A as a standard. Interestingly, our result indicate that IAA deficient strains produce less thaxtomin A than the wild type strain, which means IAA and thaxtomin do not compete for tryptophan (Figure 26). However, since OBB contains up to 52 µg of tryptophan, there may be sufficient substrate to mask competition of the IAA and Thaxtomin A pathways.

CONCLUSION

Preliminary annotation of the *S. scabies* genome sequence conducted at the Sanger Center (http://www.sanger.ac.uk/Projects/S_scabies/) did not reveal candidate genes for tryptophan monoxygenase (IaaM) or indole-3-acetamide hydrolase (IaaH). To identify *iaaM* and *iaaH* genes in *S. scabies 87-22*, amino acid sequences of tryptophan 2-monooxygenase from *Agrobacterium tumefaciens* Ag57 (accession number: Z18270), *Pseudomonas syringae* pv. *syringae* strain 3023 (accession number: AY530536), and *Agrobacterium vitis* (accession number: AF061780) were obtained from the National Center of Biotechnology Information (NCBI) database. We used Vector NTI to align multiple IaaM protein sequences to determine the consensus sequence which was used for BLAST analysis of the *S. scabies* 87-22 genome. A candidate *iaaM* gene (SCAB75511) was located (nt8349372 and nt8351069) in the genome. Upstream and adjacent *iaaM* gene a gene encoding a hydrolase protein (SCAB75501) was identified, and hypothesized to be *ssiaaH*. *iaaM* and *iaaH* deletion mutants of *S. scabies* 87-22 were generated using the Redirect PCR targeting system (Gust et al., 2002) and evaluated for IAA production using HPLC. All three mutants were reduced in IAA relative to the wild-type strain *S. scabies* 87-22. Inoculation of radish seedlings with IAA deficient mutants strains and their respective complemented strains indicated that IAA produced by *S. scabies* affected host secondary roots production and necrosis during infection, thereby contributing to the virulence of *S. scabies*.

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