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Gene cloning, nucleotide analysis, and overexpression in *Escherichia coli* of a substrate-specific indole-3-acetyl-L-alanine hydrolase from *Arthrobacter ilicis*

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ABSTRACT. Indole-3-acetic acid (IAA) is an essential plant hormone and plays many important roles in plant growth and development. In plants IAA is found mostly as conjugates, thus studies of IAA-conjugated hydrolases may provide clues on the function and metabolism of IAA during plant growth and development. We studied an alanine-conjugated IAA hydrolase in bacteria *Arthrobacter ilicis* in order to clone IAA conjugate hydrolase genes., and a gene coding for an IAA-Ala hydrolase was successfully cloned and sequenced without the need of protein preparation and analysis. The procedure involved the design of universal degenerate PCR primers for IAA amidohydrolases with similar sequence alignment to IAA amidohydrolases found in the GenBank database. Then, more pairs of specific PCR primers were generated based on degenerate PCR products. Real time PCR was performed to determine which PCR products were inducible under specific conditions. A colony screening procedure was performed later to screen a partial genomic DNA library. Based on this study, a gene with an ORF of 1218 nucleotides was found and then overexpressed in *E. coli*. The enzyme activity assay confirmed this gene as IAA-Ala hydrolase gene.

Keywords: Amidohydrolase; Arthrobacter; Auxin; Gene cloning; Hydrolase; IAA; IAA conjugate; Plant growth regulator; Rhizobacteria.

INTRODUCTION

Indole-3-acetic acid (IAA) is the most studied auxin and plays many important roles in plant physiology. In addition to its regular function in plant growth and development, IAA may work in concert with growth regulators such as cytokinins and gibberellins during plant-microbe interactions. The best-known example is the the crown-gall disease in plants, which is thought to be caused by the overproduction of IAA and cytokinins by invading bacteria such as *Pseudomonas*, or by DNA-transformation of the host by *Agrobacterium*. Plant tissues infected with the crown-gall disease suffer changes in their morphogenesis that lead to the growth of tumor-like crown-galls. Many rhizobacteria are also believed to elicit changes in root metabolism through the involvement of plant growth regulators.

Recent studies have concluded that IAA conjugation and IAA-conjugate hydrolysis may play important roles in aspects related to IAA physiology and metabolism. IAA conjugation (both amide-linked and ester-linked) may be involved in functions such as homeostatic control of free IAA levels (Bandurski, 1980), storage and subsequent reuse of IAA (Cohen and Bandurski, 1982), protection of IAA from other oxidase attack (Cohen and Bandurski, 1978), transport of IAA (Norwacki and Bandurski, 1980), IAA non-decarboxylative oxidation (Tuominen et al., 1994), and adaptation to high temperature environment (Oetiker and Aeschbacter, 1997). Because of the wide range of processes involving conjugation and hydrolysis, the study of IAA conjugate metabolism and its impact on IAA homeostatic control is important for the understanding of the biochemistry and physiology of IAA in plants.

Earlier studies on IAA conjugate biochemistry include the discovery of IAA conjugate synthetases such as the *N*-indole-3-L-ε-lysine (IAA-Lys) synthetase and its gene *iaaL* from *Pseudomonas savastanoi* (Glass and Kosuge, 1996; 1998; Hutzinger and Kosuge, 1968a; 1968b), the 1-*O*-indole-3-acetyl-β-D-glucose (IAA-Glc) synthetase and its gene *iaglu* from maize (Kowalczyk and Bandurski, 1991; Szerszen et al., 1994), and the *GH3* of *Arabidopsis* in IAA-Asp formation (Staswick et al., 2005). In addition, many hydrolases have been found to target IAA-conjugates for hydrolysis, such as a large protein complex of 200 kDa from carrot (*Daucus carota*) that can hydrolyze several IAA- amide conjugates (Kuleck and Cohen, 1992), an IAA amidohydrolase gene family from *Arabidopsis thaliana*

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(Bartel and Fink, 1995; Davies et al., 1999; LeClere et al., 2002), and a substrate-specific indole-3-acetyl-L-aspartic acid hydrolase and its gene *iaaspH* from *Enterobacter agglomerans* (Chou et al., 1996; 1998). Most recently, a series of Mt IAR3 genes from *Medicago truncatula* were reported to carry hydrolase activity against IAA-Asp, IAA-Ala, IAA-Gly, IAA-Ile, and IAA-Glc (Campanella et al., 2008).

To facilitate studies on IAA metabolism, several bacteria carrying IAA-related activities have been studied since bacterial materials are much easier to handle and prepare (Chou et al., 1996). The IAA-Asp hydrolase from *E. agglomerans* was the first bacterial IAA amidohydrolase reported and yielded the first purified IAA-amidohydrolase specific for the hydrolysis of IAA-Asp (Chou et al., 1998) . This IAA-amidohydrolase has the potential to become a molecular tool for plant studies because IAA-Asp has been found to play many important physiological and biochemical roles in plants. For instance, IAA-Asp levels can increase dramatically in plants when high doses of active auxins are applied (Andreae and Good, 1955). In most plants IAA-Asp is also reported as the last intermediate that retains the indole ring in the IAA non-decarboxylative oxidation pathway (Normanly, 1997; Tuominen et al., 1994). In henbane cell cultures IAA-Asp has been reported to be the main IAA conjugate to accumulate in high temperature-resistant lines (Oetiker and Aeschbacher, 1997).

IAA-Ala is another well characterized IAA amide conjugate (Hangarter et al., 1980) . In earlier reports, IAA-Ala was considered a storage form of IAA in tomato, tobacco, and pea and was proposed to function as a slow-release agent for IAA through regulated enzymatic hydrolysis (Hangarter and Good, 1981; Hangarter et al., 1980). Additional *in vivo* studies have provided evidence of IAA-Ala hydrolysis in a variety of plants including bean (*Vicia faba*) (Bialek et al., 1983), *A. thaliana* (Campanella et al., 1996), and *Lemna gibba* (Slovin, 1997). A novel amidohydrolase from wheat was also found to cleave to IBA-Ala instead of to IAA-Ala (Campanella et al., 2004). The enzyme catalyzing IAA-Ala hydrolysis has been identified in *A. thaliana* (LeClere et al., 2002), and a gene homolog to *ILR1*, *ILL2* (which has strong IAA-Ala hydrolase activity) has been described. However, as in many other plant IAA hydrolases, this enzyme is not highly specific for IAA-Ala. For instance, it was observed that in *E. coli* GST-*ILL2* overexpression lines *ILL2* can efficiently hydrolyze IAA conjugates such as IAA-Lys, IAA-Met, IAA-Pro, IAA-Phe, IAA-Ser, IAA-Thr, IAA-Tyr and IAA-Val, with hydrolase activities higher than 100 nmol IAA per mg of crude extract per minute. Therefore it was important to search for a substrate-specific enzyme for studies on IAA-Ala hydrolysis.

Previously we found that bacterial amidohydrolases exhibited a more restricted substrate requirement and this specificity suggested a fundamental difference between the better-studied plant enzymes and their microbial counterparts (Chou et al., 1996; Chou et al., 2004). Based on these results, we set to use bacteria as a promising source of highly substrate-specific amidohydrolases.

In an earlier report, we successfully developed an inexpensive and efficient method to screen rhizobacteria for IAA-amino acid hydrolase activities (Chou and Huang, 2005). By using N-acetyl-L-alanine (a common peptide synthesis precursor) as an inducer we reported for the first time IAA-Ala hydrolase activity in *Arthrobacter ilicis*. Since IAA amidohydrolases are not expressed in regular bacterial growth conditions, the molecular analyses of these bacterial IAA amidohydrolases remained difficult. The standard approach for cloning a gene such as amidohydrolase requires the purication of the enzyme, obtention of partial amino acid sequences, analysis of partial DNA sequences based on PCR and southern blotting, and finally, DNA sequencing of the entire gene (Chou et al., 1998; Lin et al., 2007). The first two steps involving protein purification and sequence analysis are the most difficult and time-consuming since the enzyme is inducible and may not be stable. In this paper, we adopted a molecular approach in order to clone IAA amidohydrolase genes without the protein preparation steps mentioned above. By employing this approach, we successfully identified and cloned the bacterial IAA-Ala hydrolase from *A. ilicis*.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *A. ilicis* strain D-50 and the conditions for its culture have been described previously (Chou and Huang, 2005). The *E. coli* strain NovaBlue was used as host for plasmid constructs derived from pRSET-C (Invitrogen, Carisbad, California, United States), pGEM-T (Promega, Madison, Wisconsin, United States), and pETBlue-1 based DNA cloning (Novagen, Darmstadt, Germany). The *E. coli* strain KRX (Promega, Madison, Wisconsin, United States) was used as the host for pETBlue-1-iaalaH-His for protein expression.

Genomic DNA extraction

Genomic DNA of *A. ilicis* D-50 was purified based on a modified miniprep protocol described by Wilson (1989). Bacteria from a single colony of *A. ilicis* D-50 were transferred to a 5 ml LB medium and cultured at 37°C with aeration for 16 h. A 1.5 ml aliquot of bacterial cells was pelleted in a microcentrifuge. The pellet was then resuspended in 570 µl of TNE buffer (10 mM Tris-HCl at pH8 with 10 mM NaCl and 10 mM EDTA), 20 µl of 20% sodium dodecyl sulphate (SDS), and 20 µl lysozyme (30 mg ml^{-1}) and incubated at 37°C for 1 h. After 1 h incubation for 1 h, 160 µl of 3 M NaCl was added, followed by 80 µl of CTAB (cetyl trimethyl ammonium brombide) in 0.7 M NaCl solution. The sample was incubated at 65°C for 10 min to lyse the cells. The genomic DNA was extracted with an equal volume of phenol. The

solution was mixed by gentle vortexing and centrifuged at 9,400 ×*g* for 5 min in a microcentrifuge at room temperature to separate the aqueous and organic phases. The upper aqueous phase containing the genomic DNA was transferred to a fresh tube and extracted with equal volume of phenol/chloroform (1:1, v/v) to remove all the protein contaminants and membrane debris. The solution was mixed by vortex and then spun in a microcentrifuge for 5 min. The upper aqueous phase was transferred to a fresh tube and the DNA was precipitated with 0.6 volume isopropanol at -20°C overnight. Later, the DNA was isolated by centrifugation, air-dried, and redissolved in 50 µl of TE buffer. To obtain RNA-free genomic DNA, 50 µl of genomic DNA was treated with 1 µl DNase-free RNase (500 ng μl^{-1}). The concentration and purity of the DNA sample was determined by spectrophotometric ratio assay at 260 nm and 280 nm.

Bacterial induction and total RNA extraction

Bacteria from a single colony of *A. ilicis* D-50 were transferred to 100 ml LB medium and cultured at 37°C to OD_{600}=0.8. The cells were pelleted in a microcentrifuge and resuspended in 100 ml BSM (Chou et al., 1996). The resuspended cells were divided equally into four parts. The first part of bacteria was added N-acetyl-L-alanine as an inducer to the final concentration at 10 mM. The same treatment was applied to the second and third parts of bacteria, but with different inducers, IAA-alanine and acetamide, respectively. The last part of bacteria was not treated with any inducer. The induction was performed at room temperature for 4 h and the bacteria were subjected for total RNA extraction.

For total RNA extraction, a 1.5 ml bacterial culture was centrifuged at 8,000 rpm, 4°C for 5 min to collect the pellet. A 250 µl protoplasting buffer (15 mM Tris-HCl at pH8 with 0.45 M sucrose and 8 mM EDTA) was added to the pellet and completely mixed by pipetting. Another 500 µl protoplasting buffer and 6 µl lysozyme (50 mg ml^{-1}) were then added. The sample was mixed well, put in ice for 15 min, and then centrifuged at 7,000 rpm, 4ºC for 5 min to collect the pellet. A 38 µl lysing buffer (10 mM Tris-HCl at pH 8 with 10 mM NaCl, 1 mM sodium citrate, and 1.5% SDS) was added to resuspend the pellet with pipeting. A 1.2 µl DEPC (diethylpyrocarbonate) was then added and mixed gently. The sample was centrifuged for a few seconds and incubated at 37°C for 5 min. After incubation, the sample was transferred to the ice for 5 min. Another 19 µl saturated NaCl was added and continued to incubate on ice for 10 min. The sample was centrifuged at 13,000 rpm, 4°C for 10 min; then, the supernatant was transferred to a new tube. A 150 µl 100% EtOH was added to the sample and the sample was, then, stored at -20°C overnight. After the overnight cold treatment, the sample was centrifuged at 14,000 rpm, 4°C for 15 min to collect the pellet. The pellet was washed with 150 µl of 70% EtOH, centrifuged at 13,000 rpm, 4°C for 5 min, and air-dried before being redissolved in 15 µl of DEPC-treated H_2O. The concentration and purity of the RNA sample was determined by spectrophotometric ratio assay at 260 nm and 280 nm.

Protein alignment and preparation of the universal primers

The IAASPH (IAA-Asp hydrolase) amino acid sequence was used to search the NCBI GenBank database for similar IAA-amidohydrolases. A total of 26 protein sequences were chosen for protein sequence alignment based on the CLC Free Workbench software (CLC bio, Cambridge, Massachusetts, United States). Three conserved domains (domain 1 through domain 3 in the order from N-terminus to C-terminus) were chosen to design a degenerate primer pair for PCR. The forward primer (IAALA-F1) was designed based on the domain 1 sequence and the reverse primer (IAALA-R1) was designed based on the domain 3 sequence. The domain 2 was used to confirm the PCR products.

Degenerate PCR and PCR product analysis

The two degenerate oligonucleotides, IAALA-F1 (5′-MGN GYN GAY ATG GAY GCN YT-3′) and IAALA-R1 (5′-CCY T CY TCN GCN GGY TGR AA-3′), were used as PCR primers and the genomic DNA of *A. ilicis* D-50 as the PCR template to synthesize the DNA fragments. The PCR temperature profile was one cycle at 94°C (5 min) followed by 30 cycles of 55°C (30 s): 72°C (15 s): 95°C (30 s). DNA products of approximately 200 to 230 bp were expected for this PCR experiment. DNA products obtained from the degenerate PCR were cloned into the DNA cloning vector pGEM-T, maintained in the *E. coli* NovaBlue strain, and extracted later for DNA sequencing (Seeing Bioscience Co., Ltd, Taipei, Taiwan). Based on the DNA sequencing information, two pairs of non-degenerate PCR primers (IAALA-F3/IAALA-R3 and IAALA-F4/IAALA-R4) were successfully designed based on two PCR products for reverse transcription and real time PCR experiments.

Reverse-transcription experiments

Based on the study of degenerate PCR primers, two PCR products were found and sequenced. To obtain the first strand of cDNA for the following real time PCR experiments the following sequences were used: IAALA-R3 (5′-GGT TGG AAC ATG AGT ACG AC-3′) based on the first DNA product sequence, IAALA-R4 (5′-AAG ACT GCG ATC AGG GTG-3′) based on the second DNA sequence, and the 16s rRNA lower primer (5’-ACG GCT ACC TTG TTA CGA CTT-3′) as an internal control. Total RNA samples from different induction treatments were used as templates. Each experiment mix contained 2 µl of 1 µM 16s rRNA lower primer, 2 µl of 1 µM IAALA-R3 or IAALA-R4, and 8 µl DEPC-treated-H_2O to the final volume of 12 µl. The reaction mix was incubated at 70°C for 5 min and then transferred to ice for another 2 min. Another 2 µl 5 mM dNTP mix, 1 µl 100 mM DTT,

4 µl 5X first strand synthesis buffer, and 1 µl reverse-iT RTase blend (ABgene, Taipei, Taiwan) were added to the reaction mix. The reverse-transcription reaction was performed at 47°C for 50 min and then inactivated by 75°C for 10 min. The first strand cDNA samples were stored at -20°C for real time PCR experiments.

Real time PCR

Real time PCR was performed to determine whether the IAA amidohydrolase candidate DNAs were inducible under the above induction conditions or not. Each induction treatment was tested with three sets of PCR primers, including IAALA-R3/IAALA-F3 for the first DNA product, IAALA-R4/IAALA-F4 for the second DNA product, and 16s rRNA-F (5′-TCG AAC GAT GAT CCC AGC TT-3′)/16s rRNA-R (5′-TCC GGT ATT AGA CCC AGT TTC C-3′) as internal controls. The real time PCR mix contained 2 µl of the above reverse transcription product as template, 1.8 µl of each appropriate primer pair, 12.5 µl of Absolute QPCR SYBR Green Mix (ABgene, Taipei, Taiwan), and addition of H_2O to a final volume of 25 µl. The reaction condition was 1 cycle 50°C for 2 min; 1 cycle 95°C for 15 min; 40 cycles 95°C (15 sec): 65°C (1 min). In addition, the dissociation protocol was set at 60°C (ABI prism 7000 sequence detection system, Applied Biosystems, Foster City, California, United States).

IAA-Ala hydrolase gene cloning

The genomic DNA of *A. ilicis* D-50 was digested by various restriction enzymes and analyzed under a 1% agarose TAE gel electrophoresis. The DNA from three agarose gel blocks containing different DNA sizes of 1-3 kb, 3-5 kb, and 5-7 kb was gel extracted. The extracted DNA was tested to see whether or not it contained the inducible DNA of the earlier degenerate PCR by regular PCR with IAALA-R3/IAALA-F3 primer set. (The first DNA product was confirmed inducible from the real time PCR experiments). The DNA fragments with positive PCR results were cloned to a plasmid vector pRSET-C and transformed into *E. coli* NovaBlue.

The transformed *E. coli* NovaBlue colonies were colony-screened by PCR with IAALA-R3/IAALA-F3 primer set. The temperature profile used was one cycle at 94°C (5 min) followed by 30 cycles of 58°C (30 s): 72°C (15 s): 95°C (30 s). The colonies that yielded a 161 bp product were subjected to further DNA sequencing and analysis.

Construction of plasmid DNA with IAA-Ala hydrolase (IAALAH) gene

To clone the IAA-Ala hydrolase gene (*iaalaH*) into a protein expression vector, the IAALAH-U-ATG primer (5′-ATG ACC ATC GCC GCT GAC GC-3′) and IAALAH-L-SpeI primer (5′-ACT AGT GTT GGC GGC GAG GG-3′) were used as gene cloning primers to amplify *iaalaH* from *A. ilicis* D-50 genome by PCR. The PCR product was cloned into the pETBlue-1 vector and transformed into *E. coli* NovaBlue. For fast protein purification, a His-tag linker was generated by annealing of His-linker-U/SpeI-EcoRI (5′-CTA GTC ATC ACC ATC ATC ACC ACT-3′) and His-linker-L/SpeI-EcoRI (5′-AAT TAG TGG TGA TGA TGG TGA TGA-3′) primers and was incorporated into the EcoRI and SpeI treated pETBlue-1-iaalaH by ligation. The plasmid was transformed into *E. coli* KRX for protein expression. All cloned DNA were subjected to DNA sequencing analysis.

Protein expression and enzyme activity assay

In order to confirm whether or not the cloned gene is the IAA-Ala hydrolase gene, the *E. coli* KRX containing pETBlue-1-iaalaH-His was cultured in LB medium and induced based on the manufactural protocol (Promega, Madison, Wisconsin, United States). Qualitative enzyme assays were performed by incubating 95 µl of crude extract with 5 µl of 20 mM IAA-Ala at 30°C for 30 min. The reaction was terminated by the addition of 20 µl 85% H_3PO_4, and extracted by 200 µl EtOAc. The EtOAc extract was analyzed by silica gel 60-F_{254} thin-layer chromatography (TLC) using a solvent system of chloroform:methanol:H_2O (85:14:1, v/v/v) (Chou and Huang, 2005). The TLC plate was expressed by immersion in Ehmann's reagent (Ehmann, 1977) for about 5 s and was incubated at 100°C for 1 min. Both IAA and IAA-Ala were identified by their bright-blue color.

RESULTS

The universal PCR primers for IAA amidohydrolase gene cloning

Based on the three highly conserved domains of 26 representative IAASPH homologs, a pair of degenerate PCR primers, IAALA-F1 and IAALA-R1, were generated (Figure 1). The IAALA-F1 (5′-MGN GYN GAY ATG GAY GCN YT-3′) was derived from the conserved domain 1, Arg-Ala/Val-Asp-Met-Asp-Ala-Leu, and IAALA-R1 (5′-CCY TCY TCN GCN GGY TGR AA-3′) was derived from conserved domain 3, Phe-Gln-Pro-Ala-Glu-Glu-Gly (M represent A and C; N represent A, C, T and G; Y represent C and T; R represent A and G).

Two DNA fragments were found based on a degenerate PCR

A degenerate PCR was performed with IAALA-F1/IAALA-R1 as PCR primers and genomic DNA of *A. ilicis* as template. Two PCR products located around 200 bp and 230 bp were found and named as DNAa and DNAb, respectively (Figure 2A). Both DNAs were subjected for DNA sequencing and found both containing the conserved domain 2, Met-His-Ala-Cys-Gly-His-Asp, which was used for confirming the DNAs as candidates of IAA amidohydrolase genes (Figure 2B). Two pairs of non-degenerate primers were designed according to the sequence information of DNAa and DNAb and labeled as IAALA-F3 (5′-CCC GTC CAG GAA ACA ACC-3′) /

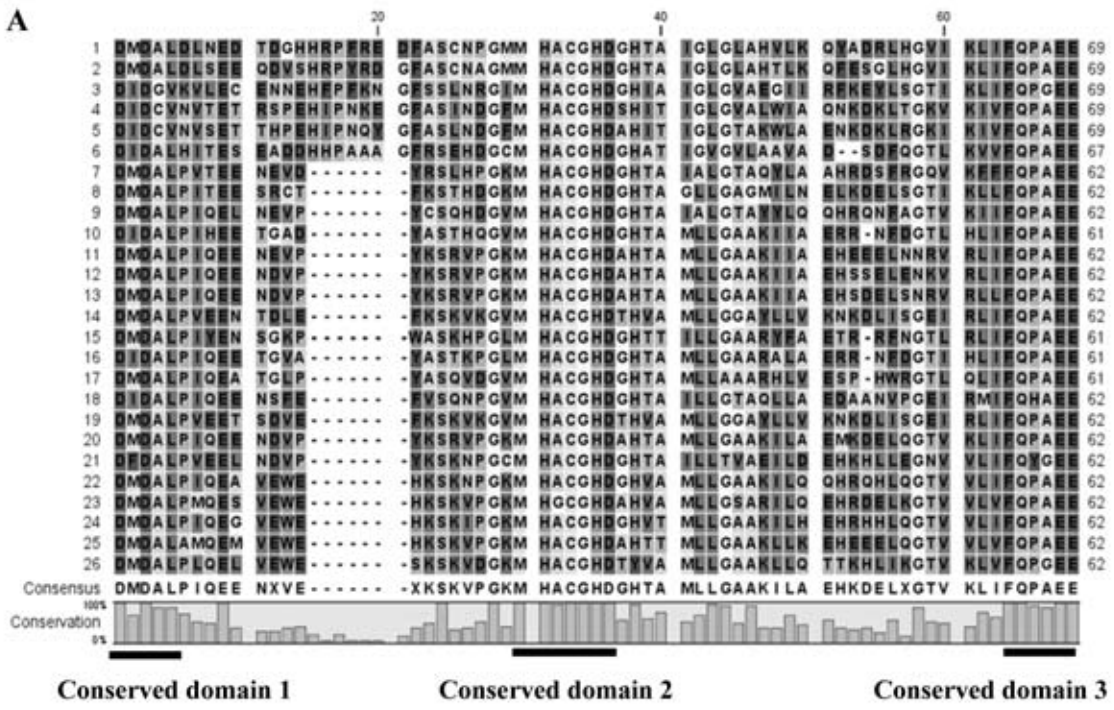

Figure 1. Partial alignment of 26 selected IAA-Asp hydrolase homologs (A) and strategy for generation of universal degenerate PCR primers for IAA amidohydrolase genes based on the conserved domains 1 and 3 (B). The protein assignments, organism names and accession numbers for the 26 protein sequences are listed as follow: 1, IAASPH (IAA-Asp hydrolase) from *E. agglomerans* (AF006687); 2, a putative amidohydrolase from *E. coli* (AE000231); 3, a probable amino acid amidohydrolase from *Clostridium perfringens* (AP3189); 4, a putative hydrolase from *Haemophilus influenzae* (U32740); 5, an unknown protein from *Pasteurella multocida* (AE006168); 6, a hypothetical protein from *Halobacterium* sp. (AE004999); 7, a theoretical *N*-acyl-L-amino acid amidohydrolase from *Synechocystis* sp. (D90917); 8, a theoretical *N*-acyl-L-amino acid amidohydrolase from *Fusobacterium nucleatum* (AE010570); 9, a theoretical *N*-acyl-L-amino acid amidohydrolase from *Nostoc* sp. (AP003598); 10, a putative hippurate hydrolase from *Agrobacterium tumefaciens* (AE009220); 11, an IAA-amino acid hydrolase homolog from *Pyrococcus furiosus* (AE010182); 12, a hypothetical amino acid amidohydrolase from *Pyrococcus horikoshii* (AP000003); 13, an amino acid hydrolase from *Pyrococcus abyssi* (AJ248287); 14, a thermostable carboxypeptidase from *Sulfolobus solfataricus* (AE006750); 15, a putative amino acid amidohydrolase from *E. coli* (AP002567); 16, a putative hippurate hydrolase from *Sinorhizobium meliloti* (AL591784); 17, a probable hydrolase from *Pseudomonas aeruginosa* (AE004718); 18, a putative *N*-acyl-L-amino acid amidohydrolase from *Deinococcus radiodurans* (AE001894); 19, a thermostable carboxypeptidase from *S. solfataricus* (AE006803); 20, a hypothetical amidohydrolase from *P. horikoshii* (AP000004); 21, an *N*-acyl-L-amino acid amidohydrolase from *Staphylococcus aureus* (AP003359); 22, ILL1 from *A. thaliana* (U23795); 23, a putative IAA-Ala hydrolase from *Oryza sativa* (AP003924); 24, ILL2 from *A. thaliana* (U23796); 25, an IAA-amino acid hydrolase from *A. thaliana* (AF081067); 26, an IAA-amino acid hydrolase from *A. thaliana* (U23794).

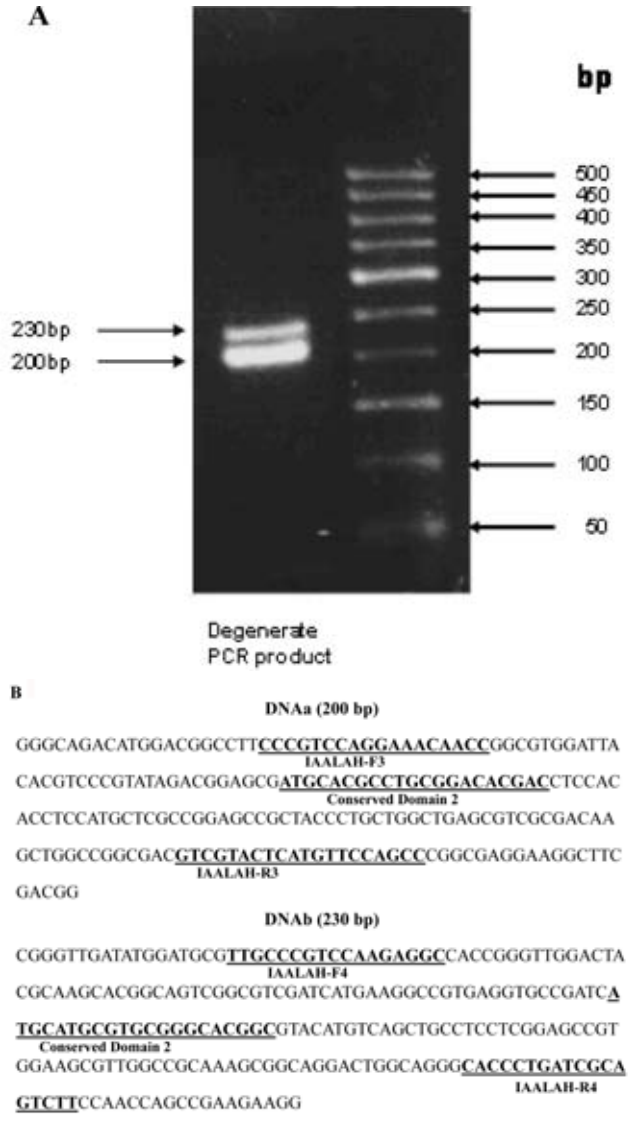

Figure 2. The degenerate PCR results analyzed on a 2% TAE agarose gel electrophoresis (A) and the DNA sequencing results of the PCR products (B). Two PCR products at 200 bp (DNAa) and 230 bp (DNAb) were identified and sequenced, respectively. The specific primer pairs of IAALA-F3/IAALA-R3 and IAALA-F4/IAALA-R4 were designed and generated for further real time PCR and colony screening based on DNAa and DNAb sequences.

IAALA-R3 (5′-GGT TGG AAC ATG AGT ACG AC-3′) and IAALA-F4 (5′-TTG CCC GTC CAA GAG GC-3′) / IAALA-R4 (5′-AAG ACT GCG ATC AGG GTG-3′). The IAALA-R3 and IAALA-R4 were also used to generate first strand cDNA as the templates for real time PCR experiments.

One DNA product was confirmed inducible by N-acetyl-L-alanine or IAA-L-alanine based on real time PCR analysis

For the real time PCR experiments, we used the IAALA-F3/IAALA-R3 primer set for detection and quantification of DNAa, and the IAALA-F4/IAALA-R4 primer set for DNAb. The PCR templates were prepared from four different induction treatments, including 10 mM N-acetyl-L-alanine, 10 mM IAA-L-Ala, 10 mM acetamide, and without inducer as a control. Results show that DNAa was significantly inducible by N-acetyl-L-alanine and IAA-L-Ala (Figure 3A), but DNAb was not (Figure 3B). When we set the non-induced sample as 1, we could quantify the relative induction of DNAa by acetamide, values for N-acetyl-L-Ala and IAA-L-Ala were 1.09±0.10, 1.43±0.02, and 3.30±0.46, respectively, and 1.46±0.34, 1.02±0.12, and 1.29±0.35, respectively, for DNAb. Based on these results, DNAa was concluded to be an inducible gene and a candidate for an IAA-Ala hydrolase gene.

Gene cloning and nucleotide sequence analysis of IAA-Ala hydrolase from *A. ilicis*

To clone and analyze the full-length sequence of gene containing DNAa, we made a partial DNA library which contained 1~3 kb DNA fragments derived from the BamHI/KpnI double digestion of *A. ilicis* genomic DNA. The DNA fragments were tested positive by PCR with IAALA-F3/IAALA-R3 as primers indicating these DNA fragments contained DNAa. The DNA fragments were ligated to pRSET-C treated with BamHI/KpnI double digestion. A PCR-based colony screening using IAALA-F3/IAALA-R3 as primers was performed to the subgenomic library. One of 96 colonies was tested positive and picked up for further analysis for IAA-Ala hydrolase gene. From sequence analysis of the plasmid from this colony we found a partial open reading frame (Figure 4).

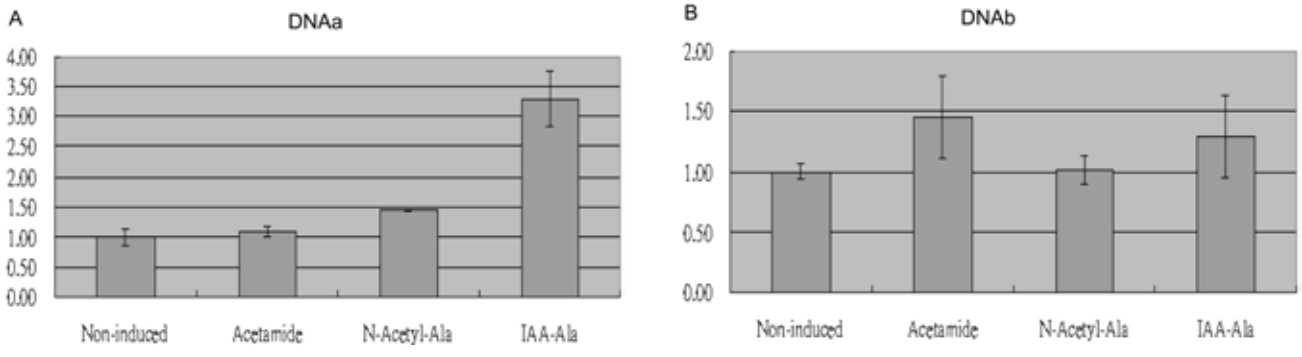

Figure 3. Expression of genes containing DNAa (A) and DNAb (B) under various induction conditions based on real time PCR analysis with IAALA-F3/IAALA-R3 for detection of DNAa and IAALA-F4/IAALA-R4 for DNAb, respectively.

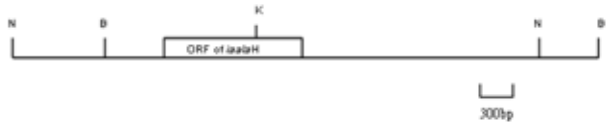

Figure 4. The restriction endonuclease map of a 4.4 kb DNA containing *iaalaH* ORF from *A. ilicis* genomic DNA. The "B", "K", and "N" denote *Bam*HI, *Kpn*I, and *Nco*I, respectively.

Therefore, another subgenomic library was made from a BamHI/NcoI double digestion of *A. ilicis* genomic DNA. Based on the same screening procedure, we obtained the rest of the DNA sequence of the gene containing DNAa. The complete open reading frame contains 1218 nucleotides and encodes a protein of 405 amino acids (Figure 5) with the calculated molecular weight of 43044.69. The complete DNA sequence of this gene was deposited to GenBank database under the accession number "EU400596" and named as "indole-3-acetyl-L-alanine hydrolase gene".

Overexpression and enzyme activity assay of bacterial IAA-Ala hydrolase

To confirm that the gene product was capable of hydrolyzing IAA-Ala, we cloned this gene into a protein expression vector pETBlue-1 as pETBlue-1-iaalaH-His which contains the full ORF of this gene and a His-tag for easy protein purification under the control of a T7 promoter (Figure 6). The protein was over-expressed in *E. coli* KRX strain under the induction of 0.1% rhamnose and purified through His-bind nickel column (Novagen, Darmstadt, Germany). The purified protein was then subjected for the enzyme activity assay. The results showed that IAA-Ala was the major subtrate while IAA-Phe and IAA-Asp were weakly hydrolyzed by this protein (Figure 7). The enzyme activity profile completely agreed with our earlier report (Chou and Huang, 2005) and strongly supported that the gene containing DNAa is the gene of IAA-Ala hydrolase.

DISCUSSION

As part of efforts to better understand the metabolism of IAA-conjugates we have isolated the gene encoding a bacterial IAA-Ala hydrolase from *A. ilicis,* strain D-50. The gene was identified by DNA sequence analysis and confirmed by the assay of enzyme activity, that is IAA-Ala hydrolysis in cell-free extracts of *E. coli* transformed with pETBlue-1-iaalaH-His.

Degenerate oligonucleotides derived from two highly conserved peptide domains of amidohydrolases were used to generate another pair of specific DNA primers for first strand cDNA generation, real-time PCR, and colony screening of target DNA. There were no protein purification steps involved during the gene identification and cloning which greatly reduced the difficulty of

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1     ATGACCATCCCCGCTGACGCCAAGGAACTGCAGGACGACATCGTCCGCCTCCGCCACGACCTCCACCCCGAACCG
1      M  T  I  A  A  D  A  K  E  L  Q  D  D  I  V  R  L  R  H  D  L  H  R  E  P
76    GAGATCGGCCTGCAGCTCCCCCCCACCCAGGAAAAGGTGCTCAAGGCGCTGGACGGACTCCCGTACGAGATCACA
26     E  I  G  L  Q  L  P  R  T  Q  E  K  V  L  K  A  L  D  G  L  P  Y  E  I  T
151   CTCGGCAAGGACACGACGTCGGTCACCGCGGTTCTTCCGCGTGGTGCAGCTTACGCTTCGGCTGAGAAGCCGGTG
51     L  G  K  D  T  T  S  V  T  A  V  L  R  G  G  A  A  Y  A  S  A  E  K  P  V
226   GTGTTATTGCGGGCAGACATGGACGGCCTTCCCGTCCAGGAAACAACCGGCGTGGATTACACGTCCCGTATAGAC
76     V  L  L  R  A  D  M  D  G  L  P  V  Q  E  T  T  G  V  D  Y  T  S  R  I  D
301   GGAGCGATGCACGCCTGCGGACACGACCTCCACACCTCCATGCTCCCCGGAGCCCCTACCCTGCTGGCTGAGCGT
101    G  A  M  H  A  C  G  H  D  L  H  T  S  M  L  A  G  A  A  T  L  L  A  E  R
376   CCCGACAAGCTGGCCGGCGACGTCGTACTCATGTTCCAGCCCGGCGAGGAAGGCTTCGACGGCCCCAGCTACATG
126    R  D  K  L  A  G  D  V  V  L  M  F  Q  P  G  E  E  G  F  D  G  A  S  Y  M
451   ATCAACGAAGGCGTCCTGGATGCTGCCGGGGCGACGGCAGATACGGCTTACGGCATGCACGTCTTCTCCTCGCTG
151    I  N  E  G  V  L  D  A  A  G  R  R  A  D  T  A  Y  G  M  H  V  F  S  S  L
526   GAACCCCACGGCCAGTTCGTCACCAAGAACGGCGTGATGCTCAGTTCCTCCGACGGGCTCGTAGTGACCGTGCTC
176    E  P  H  G  Q  F  V  T  K  N  G  V  M  L  S  S  S  D  G  L  V  V  T  V  L
601   GGTGCCGGCGGGCATGGCTCTGCCCCACATTCAGCCAAGGACCCCGTGACCGCAGCAGCGGAAATGGTTACCGCC
201    G  A  G  G  H  G  S  A  P  H  S  A  K  D  P  V  T  A  A  A  E  M  V  T  A
676   CTTCAGGTCATGATCACGCGCCAGTTCAACATGTTCGATCCCGTAGTTCTCACCGTTGGTGTGCTGCACGCAGGT
226    L  Q  V  M  I  T  R  Q  F  N  M  F  D  P  V  V  L  T  V  G  V  L  H  A  G
751   ACCAAGCGGAATGTCATCCCTGAAACTGCCCGCATCGAAGCCACCATCAGGACCTTCTCCGAAGAGTCACGGCAG
251    T  K  R  N  V  I  P  E  T  A  R  I  E  A  T  I  R  T  F  S  E  E  S  R  Q
826   AAGATGATGGATGTAGTACCCAAGCTGCTCCAAGGCATCGCCGCAGCCCACGGTCTGGAGGTTGATGTGGACTAC
276    K  M  M  D  V  V  P  K  L  L  Q  G  I  A  A  A  H  G  L  E  V  D  V  D  Y
901   CAGCAGGAATACCCCCTCACCATCAACGACGAGGACGAAACCACCACGGCCGAGAAGGTCATCGCCGGCATGTTC
301    Q  Q  E  Y  P  L  T  I  N  D  E  D  E  T  T  T  A  E  K  V  I  A  G  M  F
976   GGCGAGTCCCGCTGACCCGATGGCAACTCCACTGAGTGGTTCCGAGGACTTCTCGCGGGTGCTGGCCGAGGTC
326    G  E  S  R  L  T  R  M  A  T  P  L  S  G  S  E  D  F  S  R  V  L  A  E  V
1051  CCGGGAACATTCGTAGGGCTCAGTGCCGTTGCCCCCGGCACCGACCACGCGACGTCGCCCTTCAACCACTCCCCT
351    P  G  T  F  V  G  L  S  A  V  A  P  G  T  D  H  A  T  S  P  F  N  H  S  P
1126  TACGCAACGTTCGACGACGGCGTCCTCACCGACGGCGCTGCCCTGTATGCAGAGCTCGCGGTATCCCGCATCGCG
376    Y  A  T  F  D  D  G  V  L  T  D  G  A  A  L  Y  A  E  L  A  V  S  R  I  A
1201  GCCCTCCCCGCCAACTAA   1218
401    A  L  A  A  N  -    405
```

Figure 5. Nucleotide sequence and deduced amino acid sequence of the *iaalaH* from *A. ilicis*. The nucleotide sequence has been deposited in the GenBank database under the accession number EU400596.

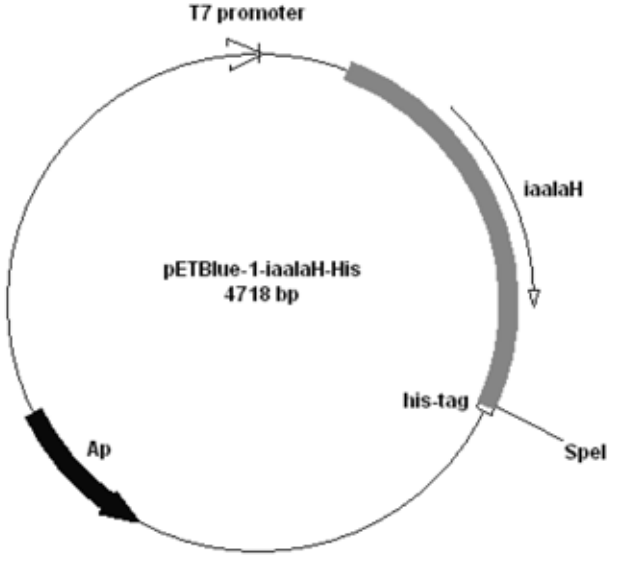

Figure 6. The pETBlue-1-iaalaH-His construct based on pETBlue-1 expression vector with *iaalaH* and His-tag sequences for overexpression in *E. coli* KRX under T7 promoter control.

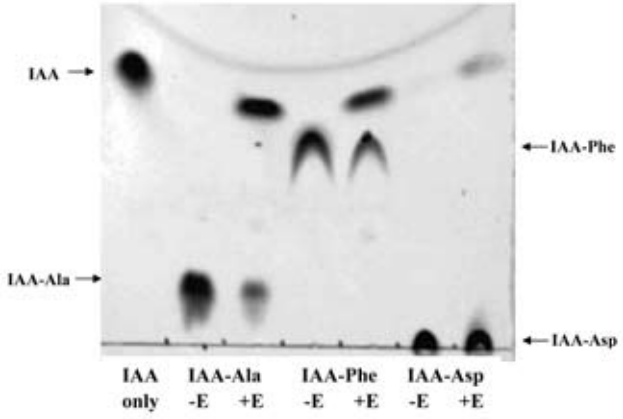

Figure 7. Enzyme activity assay of purified IAALAH with IAA-Ala, IAA-Phe, and IAA-Asp as substrates. Lane 1 was IAA standard alone, and lanes 2, 4, and 6 were reaction mixes with substrates of IAA, IAA-Ala, IAA-Phe, and IAA-Asp, respectively, but without IAALAH.

protein preparation when dealing with a novel and inducible enzyme like the bacterial IAA-Ala hydrolase reported in this report. We consider this approach as a fast, and powerful for cloning genes from similar IAA amidohydrolases.

The deduced amino acid sequence of *iaalaH* from *A. ilicis* was subjected to GenBank Blastp search and similarities were found to other proteins. Most of the hits came out as hypothetical peptidases, such as metal-dependent amidases, amino hydrolases, amidohydrolases, hippurate hydrolases, carboxypeptidase, and N-acetyl-L-amino hydrolases. Surprisingly, when pairwisely aligned the IAA-Ala hydrolase (IAALAH) of *A. ilicis* with IAA-Asp hydrolase (IAASPH) of *E. agglomerans*, we found only 26% of identities and 42% of positives. The two bacterial IAA-amino acid hydrolases shared relatively small sequence similarity indicating a much complex diversity in molecular characteristics within bacterial IAA amidohydrolases. The protein sequence was also confidently predicted carrying a Pfam:Peptidase_M20 domain and a Pfam:M20_dimer domain inside the region of Pfam:Peptidase_M20 domain based on a Simple Modular Architecture Research Tool (SMART) at the website http://smart.embl-heidelberg.de/indicating that the tertiary protein structure relationship may be involved in the enzyme activity. Further analysis of this protein structure in relation with enzyme activity will be of interest to solve this issue.

The molecular size of this protein is of approximately 43 kDa and is quite different from the major induced protein at 40 kDa in our earlier report (Chou and Huang, 2005). We examined the 40 kDa protein based on an LC-MS-MS analysis (data not shown) and found this protein to be unrelated to IAA-Ala hydrolysis. This result suggests that several pathways and proteins might be induced during the induction conditions in bacteria. Thus in this particular bacterium, *A. ilicis*, the induction of IAA-Ala hydrolase might not be the only and most important induced pathway. Further analysis of the bacterial induction mechanism is underway to clarify this issue.

In this report we found that IAA-Ala is the strongest inducer for IAA-Ala hydrolase (Figure 3) and that N-acetyl-L-alanine is the second strongest one. This result agrees with the induction pattern of IAA-Asp hydrolase from *E. agglomerans* reported in our earlier paper (Chou et al., 1996) where we determined which enzyme/substrate combination was optimal for induction of activity. However, this result conflicted with another paper (Chou and Huang, 2005), which reported IAA-Ala is not an inducer for IAA-Ala hydrolase. In current study, we performed several repeats of this experiment and concluded that IAA-Ala is a strong inducer for the IAA-Ala hydrolase induction.

In conclusion, we have cloned the IAA-amino acid hydrolase gene from bacteria through simple molecular biology procedures. By using the degenerate PCR primers and real time PCR for detection of gene expression, we bypassed the examination of protein induction, enzyme assays, and protein purification and sequencing. This technique may greatly reduce the study time and increase the detection sensitivity for this kind of experiment. We expect that this experimental procedure will be applied for the isolation of similar, inducible IAA amidohydrolases and facilitate studies on IAA metabolism in plants.

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細菌 *Arthrobacter ilicis* 吲哚乙醯丙氨酸水解酵素的基因選殖、序列分析與表現

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吲哚乙酸（IAA）為一重要的植物荷爾蒙，對植物的生長與發育有非常廣泛的影響，在植物體中大部分的 IAA 是以共軛物的形式存在，而一般相信 IAA 共軛物的形成與水解對植物保持 IAA 的恆定有重要的作用。因此，為了了解 IAA 共軛物的代謝作用，IAA 與胺基酸共軛物的水解酵素及其基因便為一重要的分子生物學研究工具。本論文即在探討一種由 *Arthrobacter ilicis* 找到的 IAA-Ala 水解酵素，我們為了避免較為困難的蛋白質純化與分析步驟，採用一種 PCR 分子選殖技術成功地選殖並定序這一基因。此分子選殖技術包括了由已知的類似基因設計一對通用的非專一 PCR 引子，再由此 PCR 找出一對專一 PCR 引子，再利用定量 PCR 決定哪些基因是可被誘導的，進而選殖並定序出此基因，由此一 PCR 分子選殖技術，我們成功找到一段有 1218 核苷酸序列的基因，並且成功地在 *E. coli* 內大量表現而印證其酵素活性。

關鍵詞：醯胺共軛物水解酵素 ；Arthrobacter；植物生長激素 ； 基因選殖 ； 水解酵素 ； 吲哚乙酸 ；吲哚乙酸共軛物 ； 植物生長調節劑 ； 根際細菌。