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THE ISOLATION AND CHARACTERIZATION OF THE FIRST AUXIN
CONJUGATE AMIDO-HYDROLASE FROM *PHYSCOMITRELLA PATENS*

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

RICHARD SYLVESTER SKIBITSKI

Montclair State University

Montclair, NJ

2013

Thesis Signature Page

MONTCLAIR STATE UNIVERSITY

“The isolation and characterization of the first auxin conjugate
amido-hydrolase from *Physcomitrella patens*”

by

Richard S. Skibitski

A Master’s Thesis Submitted to the Faculty of

Montclair State University

In Partial Fullfillment of the Requirements

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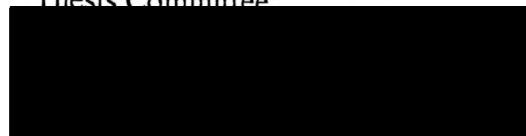
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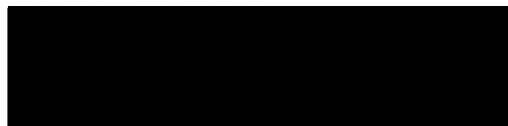


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(January 2017)

Abstract

Auxin is arguably the most important phytohormone found in the plant kingdom. The hormone is required for a multitude of growth control functions, including gravitropism and phototropism. Auxin homeostatic control is achieved in plants by a process of conjugation in which auxin is inactivated by being bound to another molecule, such as an amino acid or sugar. Auxin can be kept in a large, inactive pool by this method of chemical “conjugation” and the amide or ester bonds hydrolyzed as needed to provide “free” active hormone to the plant. The IAR3 gene family is highly conserved in Plantae and function as auxin conjugate hydrolases. We have investigated the substrate recognition and activity of a new homologue to the IAR3 family, PpIAR31, which we isolated from a species of moss, *Physcomitrella patens*. This is the first such enzyme isolated from moss and may allude to how the gene family originally evolved. We found that PpIAR31 is able to recognize and hydrolyze several forms of auxin conjugates as substrates (e.g. IAA-Alanine, IBA-Alanine, and IPA-Alanine), but was less efficient at cutting bonds on auxins with larger, more hydrophobic amino acids (e.g. IAA-leucine, and IAA-Phenylalanine). The genetic distances of the four hydrolases (PpIAR31, -32, -33, and -34) detected in the moss genome positioned them structurally closer to bacterial than plant hydrolases. Evidence from codon usage and Principal Coordinate Analyses provides support that these enzymes may have originated in Plantae by at least one Horizontal Gene Transfer event from soil bacteria into early moss.

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Introduction

The process by which land plants evolved is well documented in the fossil record, though not greatly understood (Stewart and Rothwell, 2010). The time-frame and constituent species in the invasion of the land by terrestrial plants has been debated for decades (Bhattacharya and Medlin, 1998). Ambiguities in the exact phylogenetic divergence of terrestrial plants from aquatic plants have been debated as well (Qiu *et al.* 1998), although it is generally accepted that land plants evolved from a shared algal ancestor (Friedman 2004). The exact ancestral algal species is currently unidentified (Bhattacharya and Medlin 1998), although it has been suggested that the ancestry links back to streptophyte algae (Wodniok *et al.* 2011).

Liverworts and mosses were the earliest plants to adapt to land conditions around 475 million years ago (Heckman *et al.* 2001; Stewart and Rothwell, 2010). Bryophytes and tracheophytes eventually further diverged, resulting in the appearance of gymnosperms and angiosperms in the range of 360-400 Million years ago (Kenrick 1997; Beerling *et al.* 2001; Heckman *et al.* 2001; Nishiyama *et al.* 2003).

Over further evolutionary time various plant species not only became more divergent from their predecessors, but also became more morphologically complex. While plants have conserved an array of enzymatic functions that regulate growth, they have also developed more intricate developmental pathways as morphology became more complex. All multicellular eukaryotes have developmental stages that are induced through the production of specific compounds expressed at the appropriate times, places, and concentrations. These pathways required more precise regulation, as well as more

coordinated functionality. This coordination and regulation was, and still is, maintained in part through the use of phytohormones.

Phytohormones are used by plants to regulate all aspects of development, and, continued growth. The expression and control of phytohormones in *Plantae* is complex and multi-faceted, with multiple layers of regulation, and interactive regulation amongst the various phytohormones (Bjorklund 2007; Stamm and Kumar 2010). The four best understood phytohormones are ethylene, gibberellin, cytokinin, and auxin (Fig. 1). Auxins are the most well-known and characterized of the phytohormones, and their characterization goes back to Darwin and Darwin (1897).

Darwin and Darwin (1897) recognized auxin's ability to affect development in plants while studying coleoptiles and their response to shifting light sources, though at the time he could not have stated that it was auxin specifically. The *in vitro* application of exogenous auxin to modify root growth, and even seed germination are excellent additional illustrations of its regulatory abilities (Thimann 1937, Cohen *et al.* 1992, 2002). Equally important endogenous auxin, is vital to the proper molecular development of roots (Sachdev 2009), introducing another level of complexity to its regulation and impact (Eliasson 1989, Le Floch 2003, Tabatabaei *et al.* 2016). Auxin also appears in some instances to work in conjunction with regulatory molecules, while exhibiting regulatory properties in regards to other compounds. For example, auxin has been shown to work both cooperatively and antagonistically with both gibberellin and ethylene in varying degrees of specificity (Eliasson 1989; Bjorklund 2007). Because of this interactivity, as well as ability to become toxic (Thimann 1939) auxin is an incredibly potent regulatory molecule. These traits along with the ability to induce major

developmental processes at low concentrations caused Plantae to evolve a system of storage and activation for auxin.

Auxins utilize mechanisms of storage and activation similar to the hormones found in animal systems. Auxins can be physically attached, i.e. conjugated, and inactivated with amino acids by amide bonds, sugars by ester bonds, or even short peptides (Cohen *et al* 2002). The most prevalent endogenous form of auxin is Indole-3-Acetic Acid (IAA). Plant IAA, as well as less common forms of auxin, is primarily found *in vivo* in the form of chemically inactive conjugates. Conjugate types vary by plant species (Davies *et al.* 1999; Schuller and Ludwig-Müller 2006; Teale *et al* 2006; Campanella *et al* 2003, 2004, 2007). Indole-3-Acetic Acid (IAA) concentrations are tightly controlled by this conjugation mechanism which is both highly efficient and specific. It is unclear why there are a variety of conjugated forms of auxin, but presumably they are used in various developmental and regulatory pathways.

Much work has been done to uncover the mechanisms of how plants utilize auxin (Fig. 2). The regulation of active auxin is controlled by auxin conjugate hydrolases. Auxin conjugates are cleaved by hydrolases as needed to provide active hormone to maintain levels of functioning IAA *in vivo* (Cohen *et al* 1992; Bartel and Fink 1995; Lasswell *et al* 2000; Campanella 2003; Ludwig-Muller 2006). These hydrolases were first isolated and characterized in *A. thaliana* (Bartel and Fink 1995). It is now known that there is an entire superfamily of auxin conjugate hydrolases found throughout the plant kingdom. A great number of the known hydrolase homologues have been identified and characterized. Orthologues have previously been identified in a number of different angiosperm and gymnosperm species including *Arabidopsis thaliana*, *Triticum aestivum*,

Pinus taeda, *Picea sitchensis*, and *Medicago truncatula* (Bartel *et al.* 2004, Campanella 2003, 2004, 2010). Yet, it appears unlikely that auxin and auxin conjugates could have spontaneously appeared in vascular plant species, and so must have existed in earlier land plants (Osborne *et al.* 1996; Sztein *et al.* 1999). Somehow, even with all of the work being done on vascular plants, there is little prior evidence for conjugation and hydrolysis in earlier land plants.

Sztein *et al.* (1999) shows that one of the oldest land plants, liverworts, do not utilize conjugation pathways efficiently and does not appear to possess conjugate hydrolase. Thus, conjugation may be unidirectional in liverworts, if it occurs at all.

Our own search for IAR3 homologues among an entire series of algal species (red algae, green algae, blue-green algae, etc....) identified no orthologues. However, there is evidence that suggests that auxins are ancient signaling molecules used by bacteria, fungi, and algae (Stirk *et al.* 2013). Taking this into consideration, we proposed to determine the most likely evolutionary ancestry for the auxin conjugation pathway and therefore the evolutionary origin of auxin conjugate hydrolases.

Moss appeared to be the most feasible ancestor for auxin conjugation and hydrolysis after liverworts. These species were under consideration because non-vascular plants such as liverworts, hornworts, and mosses are the predecessors to vascular plants (Friedman *et al.* 2004; Qiu *et al.* 1998). If auxin and a conjugate amidohydrolase homologue were present in one of these species, then we could rule out any species that developed later than these bryophytes as the evolutionary point of origin. If a hydrolase homologue was not present, then the pathways developed after the evolution of vascularity.

Physcomitrella patens was our most likely candidate for examination, because it is one of the few bryophytes whose genome has been completely sequenced. Ludwig-Mueller (2008) investigated *P. patens* and its homeostatic maintenance of auxin, suggesting at least in some instances an endogenous pool of auxin is available to the moss. Because our goal was to better understand the presence of auxin regulation and amido-hydrolases in the predecessors of modern vascular plants, we focused on this single moss species.

In the *P. patens* genome we detected four identifiable homologue sequences for AtIAR3 (Fig. 3). With the discovery of these sequences, we endeavored to isolate, identify and characterize the activity of the IAR3 moss orthologues in order to better understand their function in moss and their evolutionary origin prior to vascular development. We subsequently performed genomic analyses, phylogenetic comparisons, principal coordinate analyses, and enzymatic hydrolase assays with PpIAR31, the first of these auxin conjugate sequences isolated in *Physcomitrella patens*.

Materials and Methods

Polymerase Chain Reaction

It was necessary to search the latest version of the *P. patens* genome (v3.3) in order to find any hydrolase sequences. The putative IAR3 orthologue sequence of PpIAR31 was obtained through a BLAST search of the United States Department of Energy's Phytozome webpage: www.phytozome.jgi.doe.gov using the AtIAR3 DNA sequence. PpIAR31 was synthesized by NeoScientific Labs (Cambridge, Massachusetts) into pUC57 at a concentration of 400ng DNA /ul. We then performed PCR to subclone the gene into an expression plasmid. Primers were designed for PpIAR31 utilizing Primer 3 (biotools.umassmed.edu/bioapps/primer3.www.cgi) and were synthesized by Life Technologies (Thermo-Fisher Scientific, Waltham, Massachusetts). Primer sequences were: Forward primer 5'-TACCAGTCATAAAGGAGCGAATC-3 and Reverse primer 5'-CCGGTTACGTGTTTCGATCC-3'.

A 1 ul aliquot of plasmid (stock 400 ng/ ul) was diluted into 99 ul of sterile deionized water resulting in a concentration of 4 ng DNA/ul. This was employed for PCR. The reactions utilized Hotstart Taq Polymerase (Denville Scientific, Denville, New Jersey), which required an incubation for 10 min at 95°C to activate. The denaturing phase ran for 45 sec at 95°, followed with an annealing phase for 45 sec at 55°C. The elongation phase was 60 sec at 72°C, these steps were repeated for 35 cycles.

Ligation and Transformation

We purified the PpIAR31 PCR insert before ligation by mixing 5ul of insert DNA with 2ul of EXOSAP-IT (Affymetrix Corp, Santa Clara, California). The insert and

enzyme were then incubated for 15 min at 37°C. In order to stop the reaction and kill the enzyme activity, the mix was incubated at 80°C for 15 min.

The purified PpIAR31 PCR product was blunt-end ligated into the pETBlue-2 expression vector (EMD, Gibbstown, New Jersey). We employed 330 ng of DNA in the blunt-end ligation process into the EcoRV cloning site of pETBlue-2. A mixture of 1 ul insert, 4 ul sterilized water, and 5 ul “conversion mix” was incubated for 15 min at 26°C and then for 5 min at 75°C. This was followed by a 5 min incubation on ice. We then added 1ul of “blunted” pETBLUE-2 vector and 1ul of T4 DNA Ligase to the conversion mix giving a total volume of 12 ul and incubated overnight at 26°C.

The putative PpIAR31 was transformed into Nova Blue *E. coli* cells (EMD, Gibbstown, New Jersey) using heat shock (Sambrook *et al* 1989). The Nova Blue cells required for the transformation were stored at -80 °C. The cells were melted by incubation on ice for 5 min followed by thorough resuspension. A 5 ul sample of the ligation mix was added to the cell, followed by a 5 min incubation on ice, 45 second incubation at 42°C, and finally 2 min on ice. 250 ul of sterile Super Optimal Broth (SOC) (Hanahan 1983) media was then added prior to a 37°C incubation for 30 min.

The 300 ul transformation mix was plated into three aliquots of 30 ul, 30 ul, and 240 ul. Two LB plates containing 50 ug/ml of ampicillin were spread plated with the 30 ul aliquots of transformation mix atop 30 ul of SOC. We spread a third LB Amp50 plate with the remaining 240 ul of cells. All petri dishes were then incubated overnight at 37°C. Following incubation, colonies were blue-white selected based on Sambrook *et al.* (1989).

White colonies were selected from the LB plates containing XGAL and IPTG, and re-streaked onto new LBamp50 plates. From these single colony plates, cultures were grown in 5-10 ml liquid LB with 50 ug/ml Ampicillin. Liquid cultures were placed on a shaker and incubated overnight at 37°C at 220 rpm.

DNA Extraction/ Clean-up

We extracted plasmid DNA from overnight liquid cultures employing Qiagen Spin-prep mini-prep kit (Qiagen Corp., Hilden, Germany). Approximately 3 ml of cells were centrifuged at 13000 rpms for 2 minutes. The supernatant was removed and cells were resuspended in 250 ul of "P1" buffer (50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), and 0.5 ul of RNase. This mixture was inverted six times for mixing purposes and then 250 ul of "P2" Buffer (200 mM NaOH, 1% SDS (w/v)) was added. The total volume (500 ul) was inverted six more times. A 350 ul volume of the "N3" Buffer (proprietary composition) was added and inverted a third time. The 850 ul total mix was centrifuged for 10 min at 13,000 rpm. We removed the supernatant and applied it to a Qiagen mini-prep column. This was subsequently centrifuged at 13,000 rpm for 60 sec. The flow through was discarded. The "PE" wash buffer was then applied to the top of the DNA binding column at a volume of 750 ul. The "PE" buffer, and column were placed in a 2 ml centrifuge tube and spun for 60 sec at 13,000 rpm. The flow through was discarded and the centrifugation repeated once more. Finally, the column was removed from its original collection tube and placed in a sterile 1.5 ml Eppendorf tube. 50 ul of sterile deionized water was applied to the top of the column and incubated for 1 min at

room temperature. The column was centrifuged for 1 min at 13000 rpm to obtain the final DNA elution.

The purity and concentration of each extract was determined using a NanoDrop ND-1000 UV spectrophotometer (Thermo-Fisher Scientific, Wilmington, Delaware).

Digestion and analysis of putative transformants

In order to determine if the PpIAR31 insert was present, we performed endonuclease digestions on all putative transformants. A 3 ul sample of DNA [614.4 ng/ul] was added to 14ul of Deionized water. We then mixed 2 ul of Buffer 2 (New England Biochemical, Ipswich, Massachusetts) and 1 ul of HindIII (New England Biochemical, Ipswich, Massachusetts) was added. This final digestion solution was incubated overnight at 37°C.

Endonuclease digested samples were analyzed for correct plasmid size and insert orientation through agarose gel electrophoresis. Samples were loaded on ~ 75 ml premade 1% agarose gel ("*Ready Pouch Agarose Gel*" - Thermo-Fisher). Gel was mixed with 7 ul of Ethidium Bromide [10mg/ml]. Electrophoresis was performed for 32 min at 150 volts. Hi-Lo marker (Bionexus, Oakland, California) was employed as a size standard. DNA fragment analysis was performed on a trans-illuminator with Panasonic CCD camera (Ultra-Lum Inc., Claremont, California). Images were captured using the scion imager program (Scion Corp, Frederick, Maryland). Images of the gel were then used to approximate the band sizes.

PpIAR31 has an internal cutting site for HindIII approximately 164 bases from the 3' end of it sequence. The vector also has a cutting site approximately 77 bases

upstream from the 5' end of the insertion site. We calculated the expected fragment sizes for both correct and incorrect orientation and performed the digest. The correct orientation fragment sizes due to the HindIII cutting sites were calculated to be 3740 bases and 1146 bases. Incorrect fragment sizes were expected to be 241 bases and 4645 bases. For further confirmation, the insert was sequenced on an ABI 3730 DNA Analyzer, following manufacturer's protocol using the Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, California).

Hydrolase Assays

We grew a liquid culture of PpIAR31 in 10 ml LB medium with 50 ug/ml Ampicillin. This culture was placed on an incubating shaker set to 250 rpm at 37°C and left overnight. A 5 ml sample of the culture was then added to 50 ml LB and 50 ul of Ampicillin. This was shaken for two hours at 37°C with a cycle of 250 rpms. Induction was produced by adding 1 ml 1 mM IPTG and shaking for four hours with the following conditions: 250 rpm and 37°C. This protocol was performed as previously described by Campanella *et al* (2004, 2007, and 2010).

The substrates utilized in this study were conjugates of Indole-3 Acetic Acid, Indole-3-Propionic Acid, and Indole-3 Butyric Acid. The substrates studied were: IAA-alanine, IAA-Aspartate, IAA- Glycine, IAA-Isoleucine, IAA-Leucine, IAA-Phenylalanine, IPA-Alanine, and IBA-Alanine (Sigma Aldrich, St. Louis MO).

The enzyme assay was performed in 500 ul reactions. All reactions were comprised of 200 ul of bacterial extract with varying combinations of assay buffer and conjugate based on stock concentrations. The assay buffer's final concentration is

comprised of 100 mM Tris, pH 8.0, 10 mM MgCl₂, 100 uM MnCl₂, 50 mM KCl, 100 uM PMSF, 1 mM DTT, and 10% sucrose (Campanella *et al.* 2007). Assay buffer and conjugate stock volumes were adjusted accordingly to maintain a uniform total volume and protein concentration. Assays of IBA-Alanine were comprised of the bacterial stock, 15 ul of 6.6 mM conjugate stock, and 285 ul of assay buffer. IPA- Alanine [5 mM] and IAA- Isoleucine [5mM] assays included 20 ul conjugate stock, and 280 ul of assay buffer with the 200 ul bacterial extract. Assays with 10 mM conjugate stocks (IAA-Gly, IAA-Ala, IAA- Asp, IAA- Phe) utilized 10 ul of stock, 290 ul assay buffer and 200 ul bacterial extract. For a negative control and background hydrolysis, untransformed Nova Blue cells (EMD, Gibbstown, New Jersey) were employed in the same process.

Assays were incubated at 40°C for 60 minutes each, the reactions were stopped with 100 ul 1N HCl. The aqueous phase was extracted using 600 ul of C₄H₈O₂, followed by a 5 min incubation at RT°C and a 60s 13,000 rpm centrifugation. Using a centrifugal evaporator comprised of a SC110 centrifuge, RT100 condensation trap, and VP190 vacuum pump (Thermo-Fisher Scientific, Waltham Mass.), for 20 min at a medium setting, all of the remaining moisture was removed from the organic phase. The dried samples were suspended in 200 ul running buffers: 50% methanol with 1% acetic acid, or just 50% methanol. IAA-Ala, IAA-Asp, and all IBA and IPA conjugates were resuspended in 50% methanol. IAA-Leu, IAA-Iso, IAA-Phe, and IAA-Gly were suspended in 1% acetic acid and 50% methanol. After the dried sample pellets were resuspended, all were incubated at 40°C (5 min). After incubation samples were centrifuged for 60 sec at 13,000 rpm in preparation for HPLC analysis.

Conjugate Analysis

High Pressure Liquid Chromatography equipment was comprised of a Waters Millipore 510 HPLC pump (Waters Inc, Milford, Massachusetts), a C18 reverse phase column (Phenomenex Inc, Torrance, California), and LDC Analytical SpectroMonitor 3200 Variable Wavelength Detector (Thermo-Fisher Scientific, Waltham, Massachusetts).

The HPLC system was initially washed in 100% methanol for 20 min at a rate of 2 ml/min, followed by 1 ml/min for 10 min. Following the wash step, a 10 min equilibration was performed utilizing the appropriate running buffer at a rate of 2 ml/min. The pump run rate was slowed to 1ml/min, and allowed to further equilibrate at this rate for 5-10 min in an unloaded state. An HPLC syringe was cleaned in 100% methanol prior to use, and was loaded with a single sample. Data collection software, WinDaq (DATAQ Inc., Akron, Ohio), was activated manually at the same time as injection. Sample injections were allowed to run for 10-20 minutes each in an effort to ensure complete analysis.

The peaks produced during data collection were measured using the WinDaQ software . Using WinDaQ, the area under each peak was recorded and retained for further analysis. The area under each peak was compared to a series of previously compiled standard curves for each IAA, IBA, and IPA. This process was used for each conjugate analysis in order to convert the area into a value indicative of the concentration of any conjugate present.

Codon Usage/ Principal Coordinate Analysis

Codon usage analysis is an analytical method that provides statistical frequency as evidence of a particular codon being responsible for a particular amino acid in a given sequence (Guoy and Gautner 1982). We performed comparative codon usage analysis with each PpIAR3 sequence and a series of other auxin amido-hydrolase producing species, including eubacteria, vascular plants, and archaea bacteria. The orthologue nucleotide sequences employed were obtained from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) and were used in conjunction with the European Molecular Biology Software Suite (EMBOSS) program CUSP (Rice *et al.* 2000) in determining the codon frequencies. The resulting codon frequencies were applied as the relative abundance values of each codon.

The relative abundance values obtained from CUSP were imported into a series of programs based in the R language (J. Smalley, unpublished method, 2016). Using *vegan* (Oksanen *et al.* 2016), a dissimilarity matrix (Bray and Curtis 1957) was constructed from the codon usage abundance data. The results from the Bray-Curtis dissimilarity matrix were exported to *cmdscale* (R core team 2014), which was used to perform Principal Coordinate Analysis (Gower 2015). This method allowed the construction of two- and three-dimensional visualizations of the relationships between IAR3 homologue producing groups. Two-dimensional plots were constructed using *ggplot2* (Wickham 2009). The three-dimensional plots were created with CAR (Fox and Weisberg 2011) and RGL (Adler *et al.* 2015). These visualizations allowed us to analyze the data in a multivariate manner.

Additional Computational Methods

All hydrolase homologues used in alignments and cladogram construction were obtained through the National Institute of Health's online database GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and Clustal X version 1.8 (Thompson *et al.* 1997) was utilized to generate cladograms using 1000 bootstraps (Felsenstein 1985). The unrooted tree was constructed using FigTree v 1.3.1 (Rambaut and Drummond 2009). The program TREEVIEW (Page 1996) was used to visualize phylogenetic trees. Similarity and identity matrices of the four paralogues was generated using MatGAT (Campanella *et al.* 2003b).

Results

Ligation/Digestion

The HindIII digested PpIAR31 insert size was found to be ~1233 bp (Figs. 4a, b). The pETBlue2 vector size of 3563bp, and the total correct insertion should have provided an uncut length of 4886bp. Fig. 4a provides an illustration of the vector pETBLUE2 (EMD, Gibbstown, NJ). This illustration highlights in particular the insert region and the cutting site of HindIII including base pair distances. Agarose gel electrophoresis ensured us that the transformed pETBLUE2 vector included the putative PpIAR31 insert in the correct orientation (Fig. 1c).

Analysis of auxin conjugate hydrolysis

The moss PpIAR31 enzyme demonstrates specific hydrolytic activity against a series of auxin amido-conjugates (Table 1, Fig. 5). The IAA conjugates IAA-Ala (2883.3 nmol/ml/min) and IPA-Ala (2726.1 nmol/ml/min) appear to be the best substrates for hydrolysis in this enzymatic system. This hydrolysis provides support that moss *can* hydrolyze auxin conjugates, but also suggests that PpIAR31 can recognize “longer” auxins such as IPA and IBA. The Indole Butyric Acid conjugate, IBA-Ala, is also hydrolyzed by PpIAR31, but at a lower level of substrate specificity (102.2 nmol/ml/min) (Table 1).

Auxin conjugates with larger amino acid conjugates (IAA-Asp, IAA-isoleu, IAA-Leu, and IAA-Phe) were hydrolyzed at a lower efficiencies. Since IAA-Asp appears to be the most common auxin conjugate in plants (Ostin *et al.* 1998) we were surprised at its relatively low level of substrate specificity (~271.6 nmol/ml/min – adjusted for

background hydrolysis). We observed an almost complete lack of IAA-Isoleu hydrolysis (<1 nmol/ml/min), as well as low level of substrate hydrolysis in IAA-Leu (1.6 nmol/ml/min) and IAA-Phe (14 nmol/ml/min).

Negative background bacterial controls for hydrolysis (Table 1) displayed no activity at all, except with IAA-Asp as a substrate. Even the background activity against the IAA-Asp substrate in the bacteria was relatively low (~73.3 nmol/ml/min). It should also be known, that the moss enzyme showed a decrease in function or lack of substrate specificity when the conjugate chemistry was more hydrophobic. This was made evident through the hydrolysis of IBA-Ala. The alanine conjugates were hydrolyzed strongly (Table 1), with the exception of IBA-Ala. Also, the amino conjugates IAA-leu, IAA-Iso, and IAA-Phe were barely hydrolyzed suggesting that there may be a chemical factor in substrate specificity.

Phylogenetic Analysis of hydrolases

The least similar amino sequences are PpIAR33 and PpIAR34 having only 40.2% similarity (Table 2, Fig. 3). PpIAR32 and PpIAR33 present a 78.9% similarity, and are in the same clade (Table 2, Fig. 3). The same paralogues, PpIAR32 and PpIAR33, demonstrate a slightly stronger homology to the amido-hydrolase sequence in soil bacteria *Brevibacillus borstelensis*, 79% and 79.5%, respectively, than they do to each other (Table 2). PpIAR34 shows low similarity to all vascular plant sequences, with a low value of 29.4% against the sitka spruce orthologue PsIAR32. However, the PpIAR34 sequence manifests its highest homology against *Bacillus niacin* (78.2%), and

Psychrobacillus sp. FJAT-21693(78.7%) (Table 2). All of the PpIAR3 sequences appear to be more homologous to the soil bacteria, than to each other or vascular plants.

The range of similarity values of moss hydrolases to those of vascular plants is wide. The values ranged from 29.4 which was lowest (PpIAR34 vs PtIAR32, or PsIAR32), to the greatest similarity of 52.2, between the tobacco hydrolase sequence and PpIAR31 (Table 2). The average similarity of all four paralogue sequences for soil bacteria sequences is 58.4%, while the average similarity to vascular plant sequences is 44.5%. The similarity matrix is paralleled by the cladogram (Fig. 6) employing amino sequences. The angiosperm and gymnosperm nodes and clade locations support that the PpIAR3 enzyme could be a molecular ancestor. The clades group into clear ancestral relationships. The non-soil bacteria *Campylobacter jejuni*, used as outgroup, is completely separate from all other clades.

The IAR3 family of genes group into three separate clades (Fig. 6). The moss and gymnosperms diverge early in the tree, and the gymnosperms diverge again between Sitka spruce (PsIAR) and loblolly pine (PtIAR). The angiosperms are found diverging from a later node and illustrating a further separation between monocots and eudicots. The high bootstrap values above 700 are found at all major nodes and indicate this proposed tree is reliable in its dendritic structure, and furthermore that the groupings are statistically significant.

Evidence for Horizontal Gene Transfer

The PpIAR3 family similarity matrix (Table 2) and cladogram analysis suggest that PpIAR3 may have originated in bacteria, and was introduced through horizontal gene

transfer. With this hypothesis, we further investigated the nucleic and amino acid similarities between moss, vascular plants, soil bacteria, and archaea bacteria, and whether or not the data would support horizontal gene transfer.

We constructed cladograms from nucleic and amino sequences in order to more immediately visualize the genetic distances between homologues being compared. When compared with bacterial sequences and plant sequences the PpIAR3 group divides itself amongst the bacterial sequences. This occurs in both nucleic and amino sequence analyses (Fig. 7, Fig.8). PpIAR34 is most closely linked to *Psychrobacillus*. PpIAR31 is most closely related to *Bacillus sp.* Soil768D1. PpIAR32 and PpIAR33 are both closely related to *B. borstelensis* and *B. parabrevis*, respectively (Fig. 7, Fig. 8).

The gymnosperm homologs of the PpIAR3 DNA sequences are grouped together on two clades within the central region of the tree (Fig. 2). Within the gymnosperm sequences there is a close relationship between loblolly and sitka sequences, PtIAR31 and PsIAR 31, as well as their IAR32 counterparts (Fig. 7, Fig. 8). The gymnosperm sequences remained in a single clade branch amongst themselves, and present no bacterial sequence interruption at all. Nor did any of the angiosperm species appear on separate clades (Fig. 8). The *P. patens* sequences are genetically closer to sequences of bacteria than they are to the most recently evolved of our IAR3- carrying species (i.e. corn, wheat, etc.) (Fig. 7, Fig. 8, Figs.9 a, b).

We then employed abundance frequencies from a codon usage analysis to perform PCoA. Principal Coordinate Analyses can construct visualizations of statistical data, and that is specifically the reason it was employed in this study. The PCoA using codon frequency highlights the presence of a gap between the bryophyte species and the

angiosperm species (Figs. 9a, b). The monocot and eudicot sequences are both equilaterally divergent from the bryophyte sequences (Fig. 9a, b) and the same bryophyte sequences correlate strongly with the eubacteria (Fig. 9b). The relationship between bryophyte and gymnosperm groups though present a correlation somewhere in between the other vascular plant groups and bryophytes (Fig. 9a). We observed an evolutionary separation between the bryophyte species hydrolase sequences and the orthologous sequences from vascular plants.

Discussion

Discovery of Hydrolase Sequences

This investigation has been focused on the identification and characterization of PpIAR31's enzymatic activity, and the implications on the evolution of auxin conjugate amido-hydrolases. Initially, the most challenging portion of this project was cloning the PpIAR3 homologues. Due to early versions (v1.1 from BASF Corp., Ludwigshafen, Germany from 2007/2008) of the *P. patens* genome containing bacterial contamination, our first priority was to ensure that this version of the genome (v3.3 from Phytozome, 2014) was "untainted".

We detected contiguous sequences upstream and downstream of the v3.3 PpIAR3 genes, which supported the hypothesis that these orthologue sequences are not contaminants. There is some controversy however surrounding the existence of these hydrolases. Ludwig-Muller *et al.* (2009) was able to identify candidate genes for ester and amide conjugases in *P. patens*, yet was unable to identify any auxin conjugate hydrolase sequences in version 1.1 of the genome (BASF Corp., Ludwigshafen, Germany). Ludwig-Muller *et al.* (2009), based on this lack of homologues in moss, suggested that auxin conjugation in *P. patens* was unidirectional and excess free auxin was regulated by degradation.

The updated and more complete Phytozome v3.3 of the *P. patens* genome allowed us to identify several auxin conjugate amido-hydrolase sequences. We have observed that there is at least one fully functional hydrolase sequence in *P. patens*, and the concept of "unidirectional conjugation without hydrolysis" now seems less likely. We then began investigating the substrate specificity of the new hydrolase sequences.

Hydrolase Activity and Specificity

Previous research has studied substrate specificity in the activity of amido-hydrolases (Campanella *et al.* 2004, Ludwig-Muller *et al.* 2008). Campanella *et al.* (20011) reported that the size of the substrate conjugate side-chain plays a role in hydrolysis by looking at the effects of truncation on MtIAR3 enzymes and their hydrolase functions. It was found that truncating the protein head domain caused the active site to be “opened”, allowing for greater substrate recognition and increased activity. The results from our own hydrolase assays with PpIAR31 suggest that the overall size of the substrate molecules do indeed impact PpIAR31’s ability to execute its function. PpIAR31 seems to cleave auxin molecules with smaller amino acid conjugates (Table 1). PpIAR31 hydrolyzed IAA-Ala, IPA-Ala, and to a lesser extent, IBA-Ala. The hydrolytic activity of PpIAR31 was severely reduced in the presence of larger conjugating amino side chains. IAA-Asp was recognized with lesser efficiency than either IAA-Ala or IPA-Ala, and the Aspartate amino side chain is larger than an alanine conjugate. Auxin conjugates such as IAA-Leu, IAA-Phe and IAA-Isoleu are hydrolyzed (Table 1), yet they are hydrolyzed with little efficiency. In the case of IAA-Isoleu, hydrolysis is almost indiscernible. This suggests that early hydrolases may not have bound conjugates with large amino acids, or physiologically did not need to bind larger conjugates.

Conversely, there are a number of extant species possessing hydrolases that do cleave conjugates with larger amino acids. Various homologues of IAR3 are known to exist in both angiosperm and gymnosperm species and the substrate specificity of the IAR3 gene family seems to vary amongst taxa. Gymnosperms show strong specificity for a larger auxin conjugate, yet also show diversity in substrate recognition (Campanella *et al.*

2015). Angiosperms like *Brassica rapa* have shown an observable affinity for larger IBA conjugates (Ludwig-Muller 2011), and less diverse recognition than gymnosperms or moss. PpIAR31 on the other hand, appears able to cleave multiple auxin conjugates. Even so, in the presence of IAA-Ala, there are certain hydrolytic similarities among the moss, angiosperm, and gymnosperm genes. This similarity suggests that alanine conjugation and hydrolysis appeared early in the evolutionary history of Plantae. PpIAR31 is able to hydrolyze various sized substrates, but falls short of more recently evolved plants in terms of hydrolyzing larger conjugate. This could suggest that general conjugate size increased after the development of gymnosperm species, or perhaps, is due to some other environmental pressure that we are unaware of at present. This overarching theme, this similarity in activity to both angiosperm and gymnosperm hydrolases, piqued our curiosity in terms of the evolutionary origin for auxin conjugate hydrolases.

Auxin Conjugate Hydrolase Evolution

We began by investigating the presence of hydrolases in different extant plant species, and then compared their amino acid sequences for similarities. The most recently evolved IAR3 families in angiosperms contain numerous genes (*A. thaliana* has nine), while gymnosperms contain fewer (*P. sitchensis* contains four copies, *P. taeda* has three) (Campanella *et al.* 2015). *Physcomitrella patens* appears at the present time, to contain four sequences. Some angiosperm species such as soybean and grape contain between twelve and fifteen paralogue sequences (Campanella *et al.* 2015). The evolution of these extra genes, or additional gene copies could indicate a need for increased production of enzyme, or perhaps the need to segregate the functions. This could also be the result of

multiple gene copying events, but given the alignment of the moss sequences and their dissimilarity to each other (Fig. 3), this seems unlikely. Had the sequences been copied as most paralogues are duplicated, it is safe to conclude that the sequences would show more homology to each other (Fig. 3, Table 2).

Furthermore, many angiosperm (e.g. corn, potato, tomato, tobacco) hydrolase sequences have been observed containing an amino terminus sequence that targets transport to the endoplasmic reticulum (ER) (Bartel and Fink 1995, Campanella 2003, 2004). These localization sequences do not appear to be present in the gymnosperm hydrolases studied (Campanella *et al.* 2015). These targeting sequences are also missing from the PpIAR3 protein sequences, suggesting that the ER targeting is an angiosperm development. Even still, the relation between these homologues is undeniable. Nishiyama *et al.* (2003) found that between *A. thaliana* and *P. patens* genomes there is up to 90% homology, which suggests more conservation than we are observing in our own system.

We observed relationships that are conserved among species regardless of genetic sequence used to examine phylogenetic relationship (Figs. 6,8). Each plant taxa separated into its own clade, but the hydrolase gene and protein sequences of *P. patens* segregate among soil bacteria (Figs. 6, 8). Even in alignment to each other, the various PpIAR3 gene sequences present more homology to bacteria than to Plantae (Table 2, Figs. 7, 8). Our cladogram analyses in conjunction with the enzymatic studies further support a larger and, perhaps, a far deeper molecular ancestry.

Auxin conjugate hydrolase genes in Plantae have been traced back through evolutionary time as far as the earliest vascular plants, ferns and possibly lycophytes (Sztein *et al.* 1999, Nishiyama 2003). Furthermore, there are reports of auxin being

produced in seaweeds and in microalgal species (Stirk *et al.* 2013). Also, there is evidence that the colonization of land by plants is the result of a symbiotic relationship between fungi and an early aquatic autotroph, such as algae (Heckman *et al.* 2001). Root elongation was hypothetically antagonized by fungal by-products, but neither auxin nor any conjugates were detected (Le Floch *et al.* 2003). Of course, it can be presumed that auxin production and conjugation were inherited through evolutionary predecessors. But, there have also been contradictory reports as to whether or not algae produce auxin. It has been suggested that algae related bacteria are responsible for the auxin found in algal species (Yue *et al.* 2014). Therefore, the origin of auxin biosynthesis is still debatable (Yue *et al.* 2014).

We can say with some level of certainty that the enzymes used in auxin conjugate hydrolysis did not derive evolutionarily from algae. There is literature that supports this point (Sztein *et al.* 1999, Stirk *et al.* 2013), as well as our own BLAST analyses for algal hydrolase homologues. This would imply that either moss is the progenitor of auxin hydrolysis, or there is some other evolutionary source for the introduction of auxin conjugate hydrolases to Plantae.

Horizontal Gene Transfer

If the conjugation and conjugate hydrolase system did not come from algae, where could it have come from? Before that question is answered, we can ask what the original source for auxin may have been. Bacteria have been shown to produce various types of auxin (Egorshina *et al.* 2012, Ali 2015, Tabatabaei *et al.* 2016). It has also been documented that plant-associated bacteria are able to not only produce auxin, but to

conjugate active auxin and even hydrolyze auxin conjugates (Costacurta and Vanderleyden 2008). Furthermore, it has been noted that IAR3 orthologues in *A. thaliana* share homology with the “M40 class of bacterial carboxypeptidases”, that cleave small molecules such as IAA conjugates (Rampey *et al.* 2004).

It has been suggested that up to 80% of rhizobacteria produce auxin (Patten and Glick 1996). The plant-associated bacterium have also been shown to induce various responses from plants including seed dormancy from exogenously administered auxin (Li *et al.* 2016), and increased lateral root growth, when roots are inoculated with auxin producing *Azospirillum brasilense* (Spapen *et al.* 2013).

The impact of exogenous auxin on plant growth and development can be either stimulatory or inhibitory, depending on the tissues involved and the plant’s sensitivity to auxin concentrations (Remans *et al.* 2007). Some of bacterial auxin producers are symbiotic and some are pathogenic. It is possible that a bacterial infection played a role in the moss’s ability to hydrolyze auxin conjugates.

Nielsen *et al.* (1998) suggest a high likelihood of horizontal gene transfer occurring among various bacterial species in areas containing high soil bacteria activity. These observations, as well as our own results (Figs. 6,7,8, Table 2), suggest at least one horizontal gene transfer event from soil-bacteria to moss may be responsible for the amidohydrolase(s) found in *P. patens*.

Our cladistic analyses highlight a stronger correlation between the bacterial sequences and the *P. patens* IAR3 sequences (Table 2, Figs. 6, 7, 8), than between the vascular plant sequences and the PpIAR3 genes. In some cases the differences in homology of PpIAR31-to-bacteria exceed the PpIAR31-to-plant homology by a margin 20% (Table 2).

There is evidence supporting the feasibility of transgenic horizontal transfer in the literature (Bergthorsson 2003), and in fact Liu *et al.* (2011) propose that ferns obtained hydrolase sequences from viral infection.

Our own work and other published research are suggestive that at least one transfer event could have occurred from soil bacteria to *P. patens*. We propose a hypothetical model (Fig. 10) for the origin of auxin conjugate hydrolysis in Plantae.

Though not the first organism to utilize auxin signaling or conjugation, we hypothesize that *P. patens* was the first land plant species to utilize reversible conjugation as a form of homeostatic control (Fig. 10). We suggest that this is due to increased concentrations of endogenous auxin, as well as exogenous auxin from bacteria and fungi that resulted in an internal environment that was toxic for the plants. Only the organisms that produced a conjugase enzyme similar to the Indole-3-Acetic Acid Synthetase GH3, were able to develop and reproduce, resulting in an increased presence of these enzymes in the population (Fig. 10).

The increase in GH3 production by *P. patens* caused a physiological reduction in active auxin, allowing the moss to regulate its growth and protect itself from toxicity to some extent. We propose that either a bacterial infection or uptake of bacterial pathogenic material during root interception occurred, resulting in the horizontal transfer of genetic material including the gene(s) for auxin conjugate hydrolysis. These enzymes allowed *P. patens* to regulate endogenous and exogenous levels of auxin and thereby control homeostatic balance much better. The organisms whose genomes contained the conjugate hydrolase were more environmentally fit, better able to regulate their own growth, and thus able to populate their habitat to a greater extent (Fig. 10).

As the fitness in these organisms increased over evolutionary time, they could further interact with other potential gene donors in their environment. Because it is clear that there are multiple hydrolase gene sequences present in the v3.3 *P. patens* genome, we propose that these paralogues are the results of multiple horizontal gene transfer events, or possibly one event involving multiple auxin conjugate hydrolase producing species. To fully appreciate this hypothesis, one must examine the history of auxin prior to Plantae terrestrial invasion and after the development of vascularity.

Algae use auxin as a signaling molecule (Stirk *et al.* 2013). Liverworts conjugate auxin in order to control its concentrations and to begin the degradation process (Sztein *et al.* 1999), but the *P. patens* genome contains sequences for auxin conjugate hydrolysis. Furthermore, more recently evolved vascular plant species contain homologous sequences for this hydrolytic gene, PpIAR31. It is clear that auxin conjugation and hydrolysis pre-dates tracheophytes and even lycophytes. It is equally obvious that this reversible conjugation system predates plant vascularity completely. Thanks to the work of others, it is also clear that auxin hydrolysis is not present in older autotrophic species such as algae. It can then be concluded, based on current knowledge, that the appearance of auxin conjugate hydrolases in Plantae most probably first occurred in a moss species such as *P. patens*.

Summation/Conclusions

We have shown that the moss genome does in fact contain at least one active auxin amidoconjugate hydrolase. The implications of this discovery present the unique opportunity to suggest an evolutionary path followed by this particular enzyme. It

appears that from an evolutionary standpoint, the hydrolase enzymes needed to recognize auxin conjugates of varying and increasing sizes as time progressed. It is possible that the change in enzyme activity was a result of environmental pressures.

We could hypothesize that, as plants began their journey away from coastal areas, the soil chemistry changed. This change in geochemistry in turn caused the exogenous (and by extension endogenous) auxin and auxin conjugates to vary due to hydrophobicity or some other variable. In a natural succession, this caused a shift in the plant invaders levels of fitness due to auxin toxicity. The resulting soil toxicity benefits the plants with ability to hydrolyze the auxin conjugates, and they are able to more successfully reproduce, thus increasing the expression of the hydrolase in the population. However, there is little evidence to suggest that this was the case.

Future investigations should be performed in order to better understand PpIAR31's ability to recognize additional conjugates. Our study analyzed only a small portion of the available and existing auxin conjugates, and it remains to be seen what information the entire series of auxin conjugates could yield. In particular, auxin that is conjugated to highly hydrophobic compounds should be examined. The sequence of the PpIAR31 gene was discovered from genomic scaffolding and was artificially synthesized. With this knowledge, further investigations into the extent to which the PpIAR3 gene family is present *in vivo* should be performed. Finally, we have hypothesized that the presence of PpIAR3 may be the result of an ancient horizontal gene transfer. A study of the entire moss genome could be considered in order to uncover further instances of horizontal gene transfer, and perhaps uncover the impact these transferences may have had on the evolution of auxin conjugate hydrolases.

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TablesTable 1- Auxin conjugate hydrolase in *Physcomitrella patens*

Conjugates	Activity measured in nmol/ml/min			Adjusted Value
	Δ PIAR31	Negative Controls		
IAA- Ala	2883.333 \pm 183.584	0		2883.333 \pm 183.584
IAA- Gly	1166.000 \pm 618.155	0		1166.000 \pm 618.155
IAA- Asp	345.000 \pm 43.524	73.333 \pm 23.570		271.667 \pm 33.547
IPA- Ala	2726.111 \pm 487.895	0		2726.111 \pm 487.895
IBA- Ala	102.222 \pm 19.300	0		102.222 \pm 19.300
IAA- Isoleu	< 1	0		< 1
IAA- Leu	1.611 \pm 0.874	0		1.611 \pm 0.874
IAA- Phe	14.275 \pm 10.403	0		14.275 \pm 10.403

Figure Legends

Figure 1. A) Molecular structures of Indole Acetic Acid, Indole Butyric Acid, and Indole Propionic Acid. B) Structure of auxin conjugates IAA-Aspartate, and IAA-Glutamate as compared to Indole Acetic Acid before conjugation

Figure 2. An illustration of the various regulatory pathways followed by auxin, including conjugation, hydrolysis, and beta-oxidation.

Figure 3. CLUSTAL sequence alignment of PpIAR3 DNA sequences. Asterisks indicate complete residue conservation.

Figure 4. A) An illustration of the pETBLUE2 Vector, insertion location, and endonuclease cutting sites. B) Agarose electrophoretic image of pPIAR31 insert after amplification. C) Agarose electrophoretic image HindIII digest of pPIAR31 inserted in the correct orientation in the pETBLUE2 vector.

Figure 5. Images of HPLC tracings demonstrating the hydrolytic activity of PpIAR31. A) Induced PpIAR31 transformed cells with IAA-Ala peak at ~176 seconds retention time. B) Control for background hydrolysis, uninduced, empty NovaBlue cells with IAA-Ala at ~176 seconds retention time.

Figure 6. Protein cladogram examining genetic distance of the PpIAR3 gene family against other Plantae species. 1000 bootstrap iterations were employed for the analysis in CLUSTAL.

Figure 7 Nucleotide cladogram examining the genetic relationships between the IAR3 gene family members in moss, Plantae, eubacteria and archaeobacteria. 1000 bootstrap iterations were employed for the analysis in CLUSTAL.

Figure 8 Protein Cladogram of PpIAR3 family with Plantae, but including eubacteria, and archaeobacteria. 1000 bootstrap iterations were employed for the analysis in CLUSTAL.

Figure 9 A) Principle Coordinate Analysis performed on PpIAR3 family and including Plantae, eubacteria, and archaeobacterial in two dimensions. B) Principle Coordinate Analysis performed on PpIAR3 family and including Plantae, eubacteria, and archaeobacterial in three dimensions.

Figure 10 Proposed hypothetical evolutionary pathway suggesting the evolutionary source of auxin conjugates hydrolases.

Fig. 1

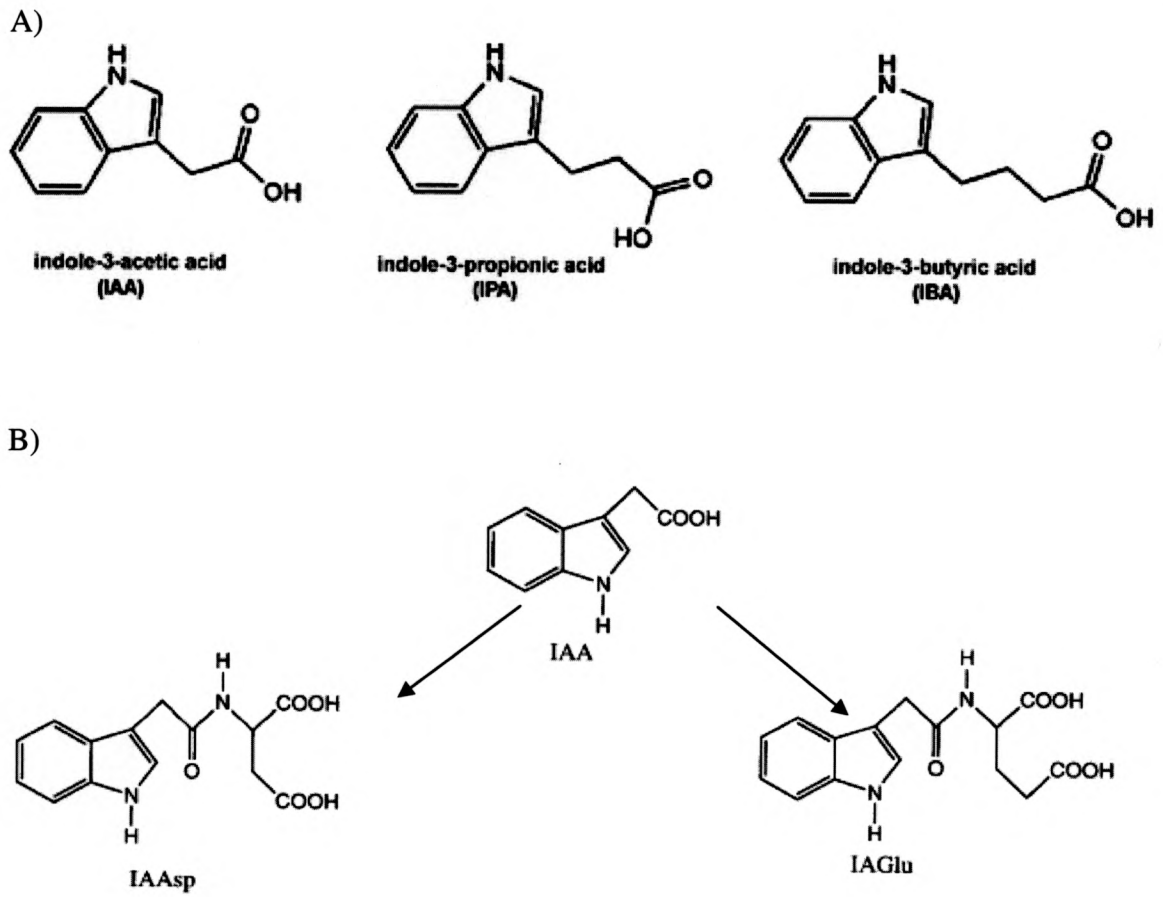
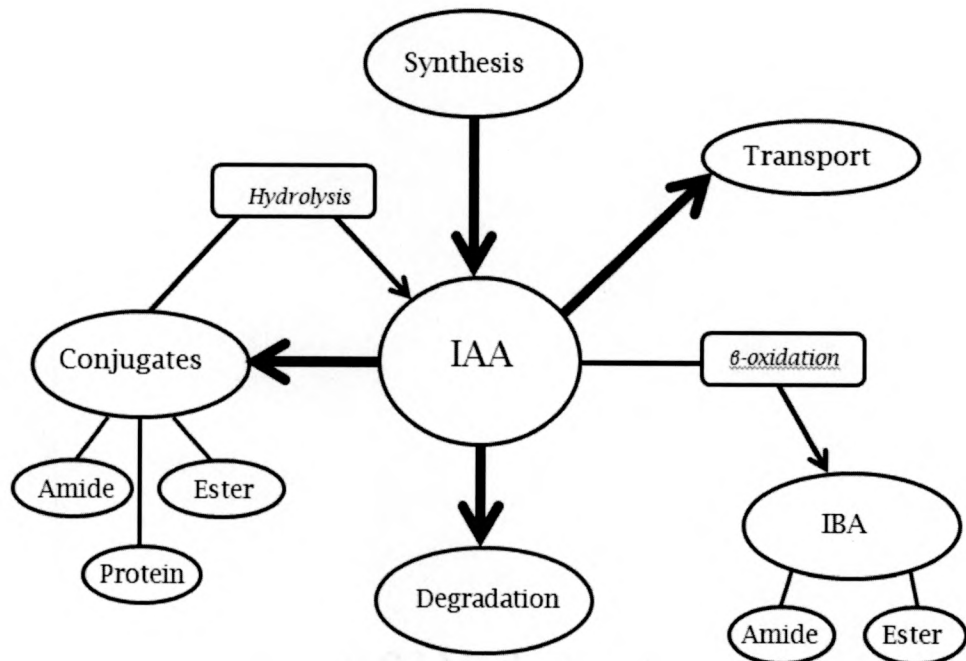


Fig. 2



pPIAR34 GCCAGTCAGCCCGAAACGGGAAAAAATCCTTCTTTTGAATCGCCAAGGTGATCGATCGC
 pPIAR31 GGGGCTATGCCGCATGCCACGATCGACCCGTGCGTTGCAGGAAGCCAGCTGGTGTAAAC
 pPIAR32 GGTGCCATGCCGCATGATGCCGTGATCCCATCTTTATCGCGGGTCAAATCGTCAGTGGG
 pPIAR33 GGATCATCCCCGCATGAAACCGTGGATCCTACCTACGTAGCCGGACATATCTGCTCGGT
 * ** * * * * * *

pPIAR34 ATCCCCGAGCTCATCT---CGCCAAAGAATAATGAAGGCTTGGTTCTCTGCACGGTTGTT
 pPIAR31 CTGCAGCAAATCATCAGTCGGCGCATCGATACCAAATACCGGACCGTTTTGACCGTCAGC
 pPIAR32 CTGCAATCCGTCGTGAGCAGAGCGATCAGCCCTTTGCAGCCAGCTGTCTGACAGTCGGC
 pPIAR33 CTGCAGGCTATCGTCAGCCGAAAGTGAATCCTTTGCAGCCTGCGGTGATCTCGGTGGGC
 * * ** * * * * *

pPIAR34 CAGGTGAATATCGGGGAACGGGCATTGCGCATCGCGCAAGCAAAGGCGACCTGCTCTTG
 pPIAR31 TCGTTTGTGCGTGGTGGCGAAACCTACAACGTATTGAGACAAGGTCTCGTTAAAGGGA
 pPIAR32 AAGGTGCG---AAGCAGGCAGTACGTACAACATCATTCCCAACGAGGCTGTATGCTGGGT
 pPIAR33 CAGGTAC---TGCCAGGCTCCAACATAATATCATCCAAACGAGGCGATCATGACAGGA
 * * * * * * *

pPIAR34 ACCATCCGAGCGCTCTACGAGGCAGAGCTCGATAAGCTGCAGCAAAACCTGGAGACCCTG
 pPIAR31 ACCGTTGCGACCTTTGAGGAAGATGTGCGCAAAAAGATCGAGCAGGAAATGCGCTGATT
 pPIAR32 ACGACGCGCAGCCTAACCCAGAGACCAGACAGCGAATCTTTACGCAAAATGCAAAGCATC
 pPIAR33 ACGATTGCTACGTTCTCGGAAGAAACGCGGCGAGTTTATTTTGGAGAAAGTCAAAGGCTG
 ** ** * * * * * *

pPIAR34 GCACAGGCGCAAGCCGAAATCTACGGTCTGAAGGTGAGTCTCTTACAACGACGTATTC
 pPIAR31 ACCCGCACGACAGCCGAACAGGCGGGAGGAACGGCTGAATTTACTGACGCCAGGCTAC
 pPIAR32 GTGGAAGGTGTTTCTGCGAGCTTTGGCGCGTGGGCACCATCGAGTATAGCTGGGACCG
 pPIAR33 GCGGAGGGCATGAGTACGACTTTCGGTGCCGTAGGGGAAGTCTCGTACATTCTCGGCACG
 ** * * * * *

pPIAR34 CCGGAAACCGTCAATCACAAGGAAAGCTCCGACAAAATCAGAAGAGTCGCAAGGAAAAA
 pPIAR31 CCTGCCACCTGGAATCACGAAGAGGATGCCTTGCGCGTGAAAAAAGTAGCGGGGCCATC
 pPIAR32 CCTCCCTTGGTCAATGACCGGGTGCAAAGTGCATGTAGAGCGAGTCGTGTTGAGGCT
 pPIAR33 CCTCCCCTCGTGAATGACAAAGATATGAGCCGCTATGACGAAAGCGTCATTCTGTAAGC
 ** ** * * * * *

pPIAR34 GGACTCG-----CACTTGTGAAATGAAAGAAGCCTTCCGCGTTCGGAAGACTACGGA
 pPIAR31 TTTGGAGAAG---AAAAAGTACTCACCCATGAGTATGGCATGGGCGAGCAAGACTTCGCC
 pPIAR32 TTTGGTGAATCCTTACGCGGCTGACATGGAGCCGACGATGGCTGCCGAAGACTTTGCC
 pPIAR33 TACGGCCAGC---ATGTGGCCGTTGAAATCGAGCCAGTCAATGGGGCAGAGGACTTCTCC
 * * * * * * *

pPIAR34 CATTACACAAAGCTGACAAAAGGGCCATGTGCTATATCGGAA-----AC
 pPIAR31 TACTACCTCCAGCACAAAGCCCGGCATTTCTTTGTGGGGGACGCAATCCTGAGCTG
 pPIAR32 TATTATTTGGAGCATTGTCCAGGAGCGTTTGTCTTTGTGGGCATG-----GGCGGGGAG
 pPIAR33 TTCTACGGACAAAAGCGTCAAAGCACCTATCTCATGGTCGGCATG-----GGCGGGGAG
 ** * * * * * *

pPIAR34 GGGGAGGATTATCCGCATGTCCATACGTACGAGTACGATTTCCGAGACGATATTATGGAG
 pPIAR31 AATGCCATTTATCCGCACCACCATCCGAAG---TTTGATGATAGATGAGCGCTCCATGCTG
 pPIAR32 AGAAGTGCATATCTCACCATCATCCAGC---TTTGATATCGACGAAGATGCGATTCCG
 pPIAR33 AAGAGCCAGTACCCGCATCATATCCGCGT---TTTGACATCGATGAAGAAGAGATTGGC
 ** * * * * * * *

pPIAR34 ACAGCAGTAGAGGTTTTTAAAGGACTAGCTA--CCGTGTAGGTTCTGTCTGTCTGT
 pPIAR31 AACATCGGGAAGCTGTTTATCGGAATCGTACTGATTATTTGGCAGTCGAGTAG-
 pPIAR32 ACTGCGATCGAGCTGTTTATCCAGCTTGTCCGTCGCTTTTCTGA-----
 pPIAR33 ACGGCTATCGATTTGTTTTTACAGCTCGTAATTCGGTTTGAATAA-----
 * * * * * * *

Fig 4

A)

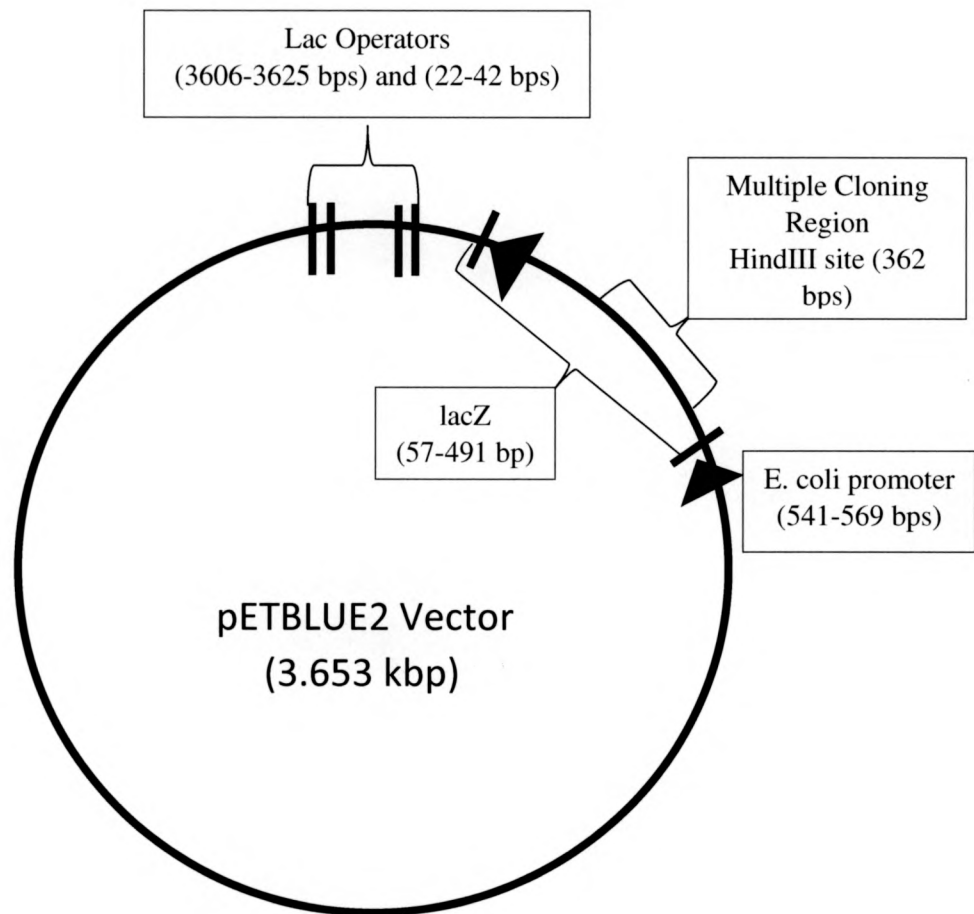


Fig. 4B

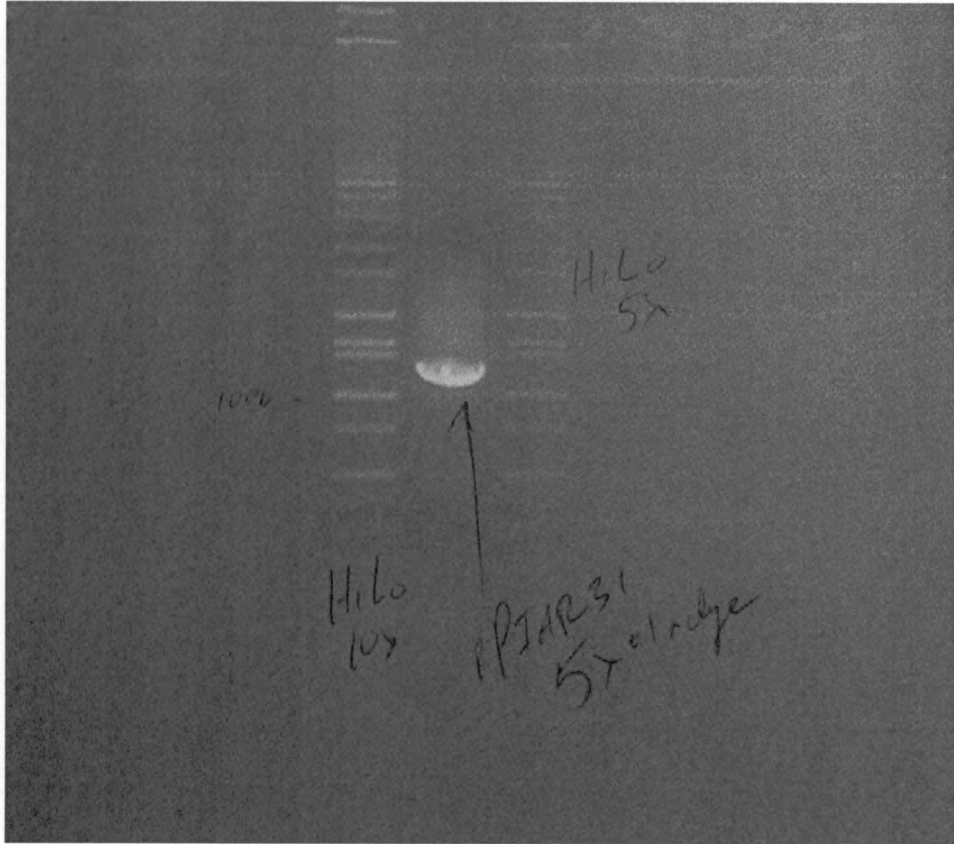


Fig. 4C

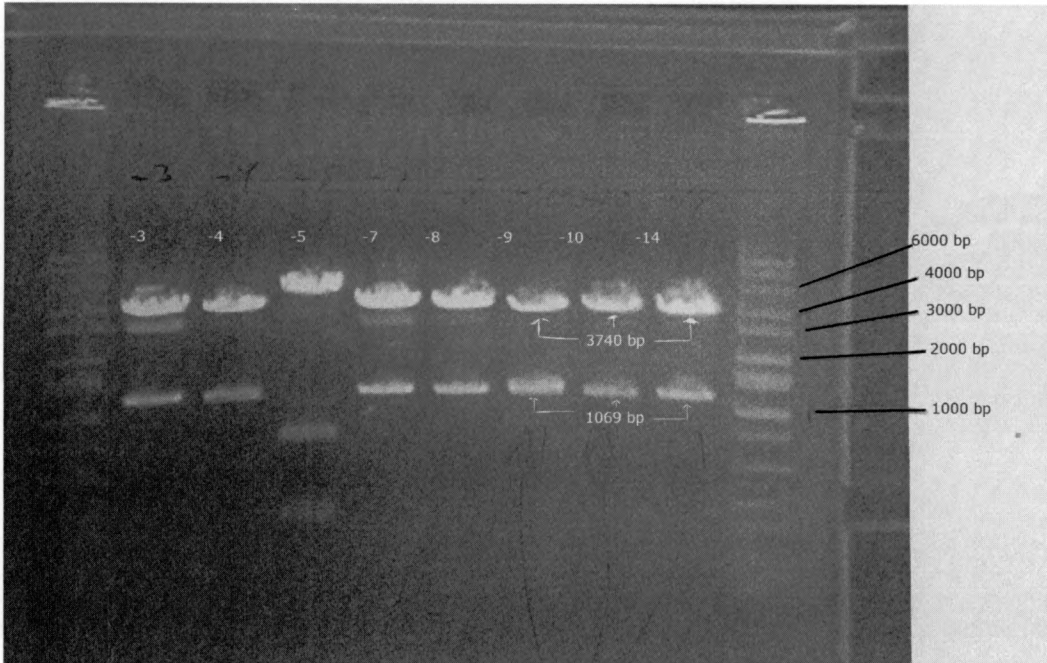
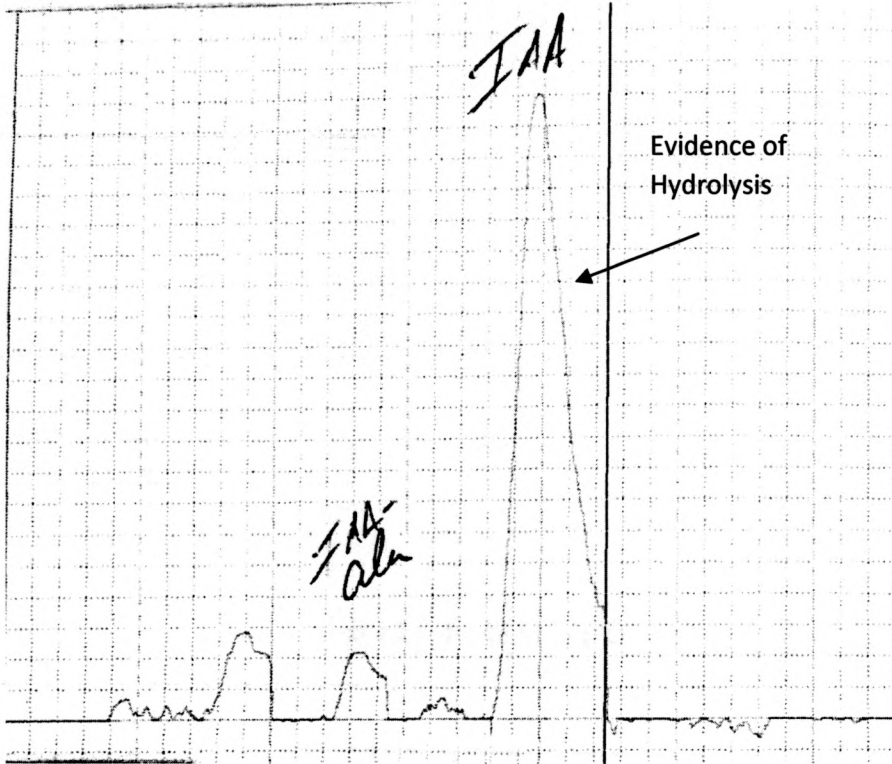


Fig. 5

A)



B)

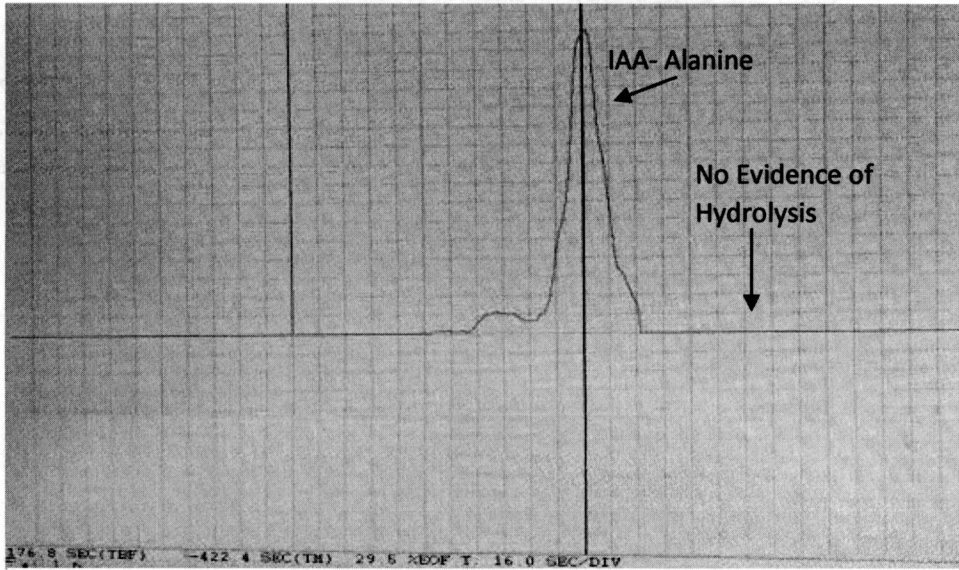
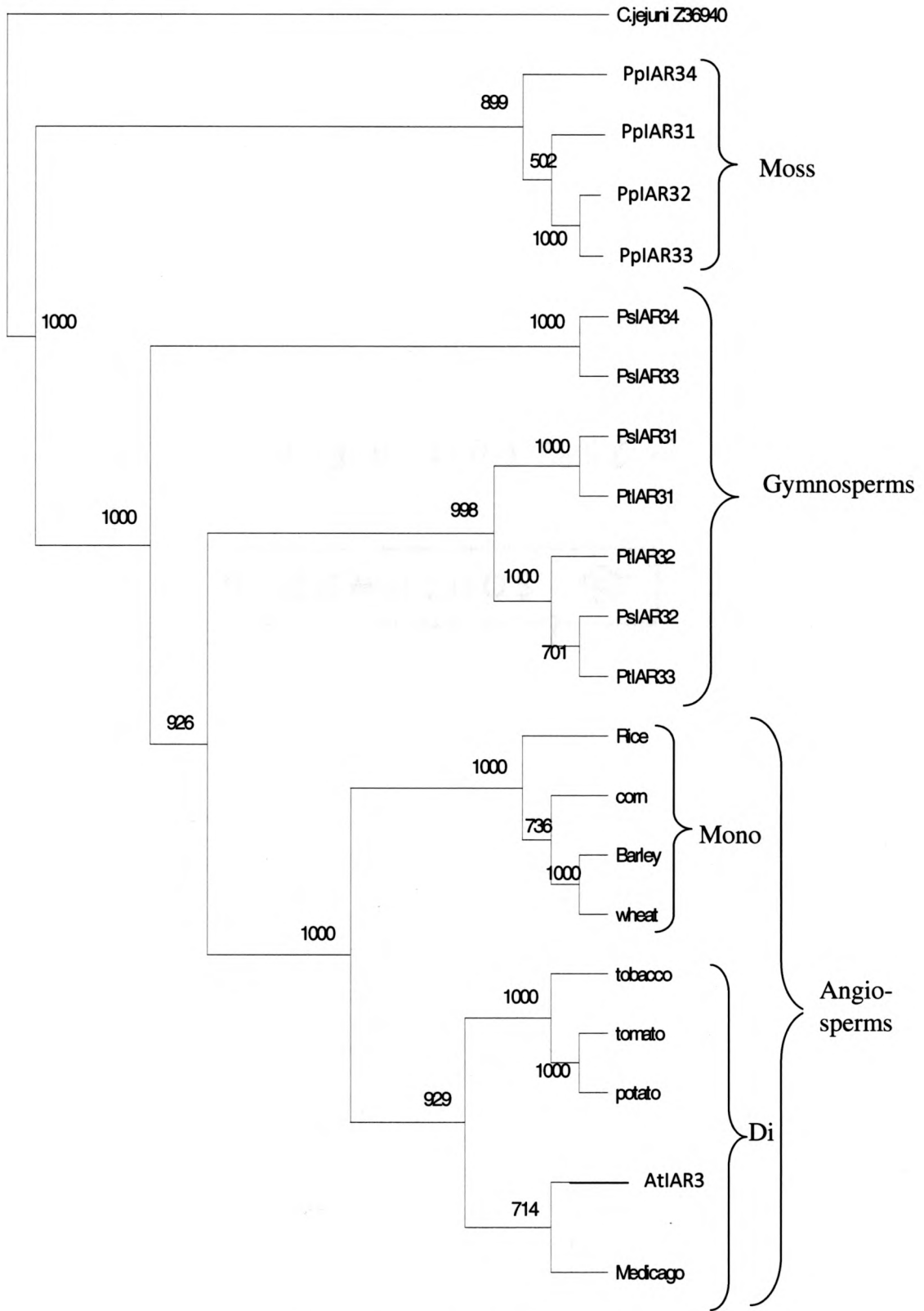
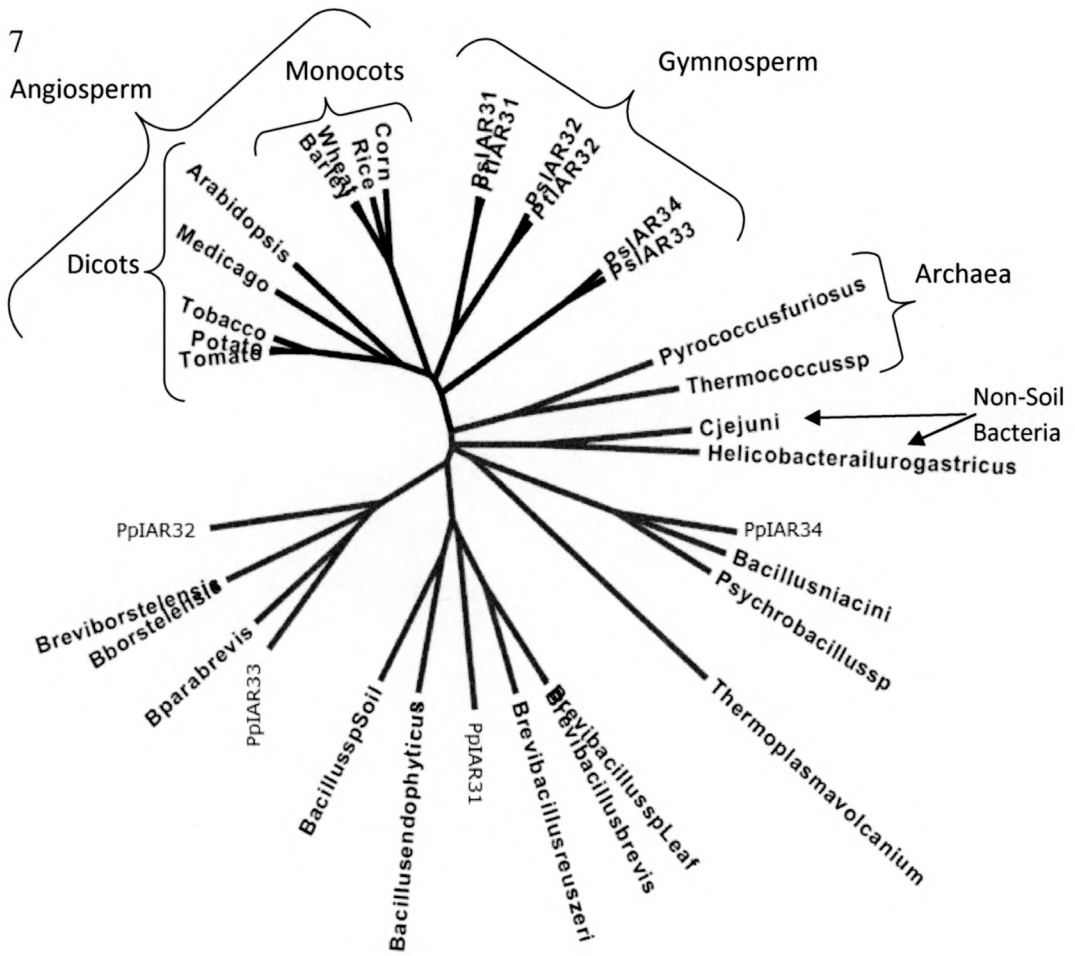


Fig. 6



Mono= monocotyledon, Di= dicotyledon

Fig. 7



*Unlabelled- Soil Bacteria and Moss

Fig. 8

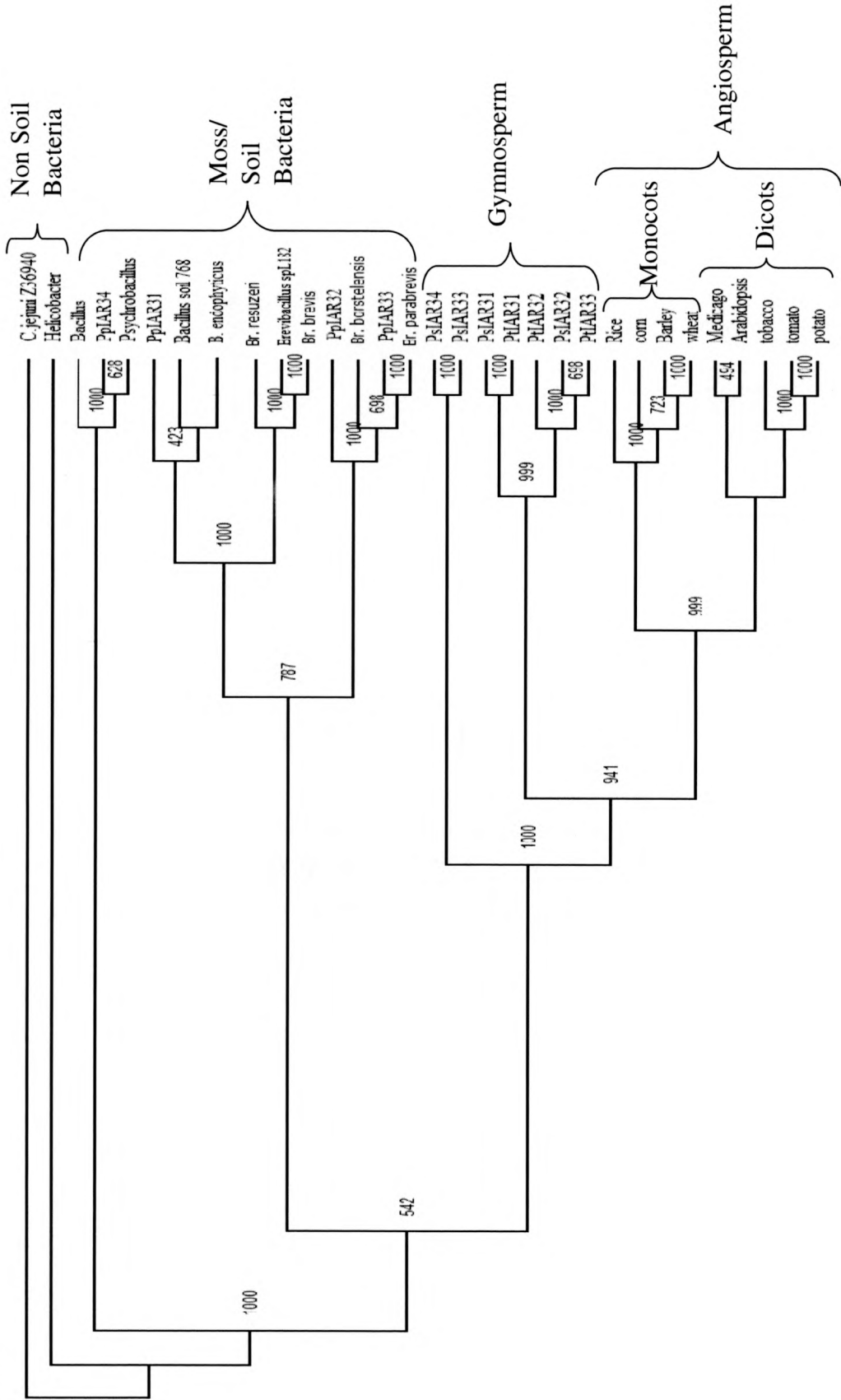
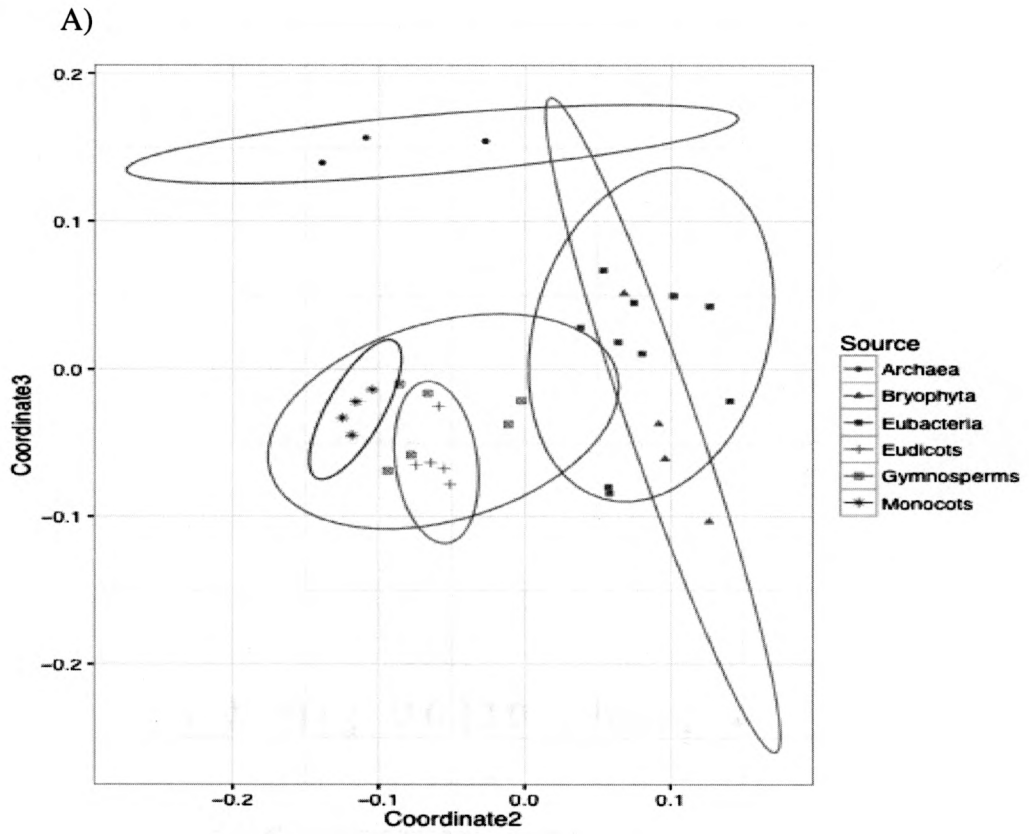


Fig. 9



B)

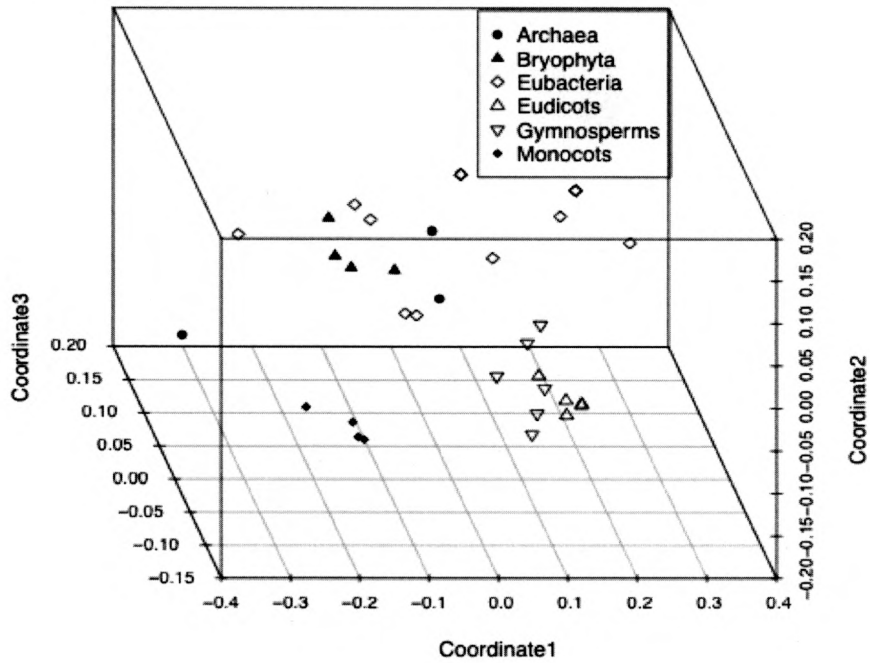


Fig. 10

