BIOCHEMICAL AND MOLECULAR STUDIES ON PHENYLALANINE AMMONIA LYASE IN THE PHYTOPATHOGENIC FUNGUS *USTILAGO MAYDIS*

by

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) is the entrypoint enzyme into phenylpropanoid metabolism in plants. Little is known about PAL in fungi. In order to explore the role PAL plays in the growth and survival of fungi, the structure and regulation of fungal PAL were investigated in the phytopathogen *Ustilago maydis*, the causal agent of corn smut.

PAL was purified from liquid-cultured cells of *U. maydis* using ion-exchange and gel filtration chromatography, and preparative PAGE. Its native molecular mass was estimated as 320 kDa and its subunit molecular mass was 80 kDa. No isoforms of the enzyme were detected, and there was no evidence of glycosylation of the protein. The enzyme was most active at pH 8.8-9.2 and 30°C and had a K_m for L-phenylalanine of 1.05 mM. The enzyme did not deaminate L-tyrosine. The synthetic PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP) strongly inhibited the enzyme, as did sulfhydryl reagents and carbonyl reagents, whereas *t*-cinnamic acid was only moderately inhibitory. *Ustilago* PAL activity had no requirement for metal ion cofactors, but was inhibited by heavy metal ions (Ag^+, Cu^{2+}, and Hg^{2+}). Polyclonal antibodies were raised against the purified PAL protein.

Using degenerate oligonucleotide primers and polymerase chain reaction, a PAL clone was isolated from a *U. maydis* genomic library and 3047 bp of its nucleotide sequence was determined. It contained 495 bp of 5' untranslated sequence, a 2172 bp open reading frame encoding 724 amino acids, and 380 bp of 3' untranslated sequence. No introns in the PAL-encoding gene were detected. In *U. maydis*, PAL was shown to be

encoded by a single gene. This is the first work on the structure of a PAL gene from a pathogenic fungus. Substantial differences in PAL gene sequence and organization were found compared to PAL genes of other species. *U. maydis* PAL showed low amino acid sequence identity with other PALs (23-26% with plant PALs, 39-40% with yeast PALs). The level of amino acid identity (25%) with bacterial histidine ammonia-lyase (HAL) suggests a possible relatedness between *U. maydis* PAL and bacterial HAL. Western blot and immunological enzyme inhibition assay confirmed that PAL and HAL enzymes are immunologically related. Overall, protein sequence analysis suggests that modern PAL and HAL genes share a common ammonia-lyase ancestor.

In *U. maydis*, PAL is constitutively produced at a low level but its regulation can be influenced by aromatic amino acids. L-tryptophan apparently induced the lyase enzyme. The inducibilty of PAL by L-tryptophan was also demonstrated in six other *U. maydis* strains and three *Ustilago* species tested. The enzyme is most readily induced during the early stationary phase of growth and the induced activity remains relatively constant during stationary stage. PAL induction was repressed by glucose but not by its reaction product *t*-cinnamic acid. Induction did not require *de novo* protein synthesis, suggesting that some form of post-translational protein modification or a metabolic effect may be the basis of the induction of *Ustilago* PAL by L-tryptophan. PAL was detected only in cell extracts and not in the growth medium.

A putative biosynthetic pathway of *Ustilago* melanin was deduced from the examination of the metabolic fate of L-phenylalanine. Overall, the pattern of regulation of PAL induction in *U. maydis* was very different from patterns known for plants and other fungi.

These results, together with evidence for genetic divergence, are consistent with a unique role for PAL in *U. maydis*. It remains to be determined whether this role is essential for survival and pathogenicity of this plant pathogen.

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5', 3'	denotes 5'-hydroxy or 3'-phosphate end of sequence
aa	amino acid
A, C. G, T	nucleotides adenosine, cytosine, guanosine, thymidine
AIP	2-aminoindan-2-phosphonic acid
AOPP	L-α-aminooxy-β-phenylpropionic acid
ASADH	aspartate semialdehyde dehydrogenase
bp	base pair
BSA	bovine serum albumin
cAMP	adenosine-3', 5'-monophosphate
cDNA	complementary deoxyribonucleic acid
C4H	cinnamic acid 4-hydroxylase (EC1.14.13.11)
CHI	chalcone isomerase
CHS	chalcone synthase (EC 2.3.1.74)
Ci	Curie
4CL	4-coumarate:CoA ligase (EC6.2.1.12)
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
Da	Dalton
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EC	enzyme classification
EDTA	ethylenediaminetetra-acetic acid
GUS	β-glucuronidase
hr	hour(s)

IEF	isoelectric-focussing
kb	kilobase
kDa	kilodalton
$K_{m,}$	the Michaelis-Menten constant
λZAPII	lambda ZAPII phagemid vector (Stratagene)
μ	micro
m	milli
M	molar
M_r	relative molecular mass
min	minute(s)
mRNA	messenger ribonucleic acid
MW	molecular weight
No.	number
nt	nucleotide
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase (EC 4.3.1.5)
PBS	phosphate buffered saline
ρ	*para*
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEG	polyethylene glycol
Phe	phenylalanine

pI	isoelectric point
PMSF	phenylmethanesulphonyl fluoride
PVDF	polyvinylidene difluoride
PVP	polyvinyl-pyrrolidone
RT	room temperature
S.D.	standard deviation
SDS	sodium dodecyl sulfate
sp	species
t	*trans*
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	tris-(hydroxymethyl)-aminoethane
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
V_{max}	maximum velocity
Vol	volume
w/w	weight by weight
w/v	weight by volume

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CHAPTER ONE

General Introduction

1.1 PHENYLALANINE METABOLISM

1.1.1 The biosynthesis of L-phenylalanine

L-phenylalanine is one of the essential amino acids which cannot be synthesized in mammals in adequate amounts to meet the requirements for protein synthesis. L-tyrosine is not an essential amino acid for mammals, since it is synthesized by the hydroxylation of phenylalanine. In mammals, phenylalanine and tyrosine are required for the synthesis of adrenaline, noradrenaline, catecholamine, and dopamine, as well as the thyroid hormones, thyroxine and triiodothyronine, and the pigment melanin.

Plants and most microorganisms are able to synthesize phenylalanine via the shikimic acid pathway (Braus, 1991; Hrazdina and Jensen, 1992; Schmid and Amrhein, 1995). The initial stages of phenylalanine biosynthesis (prechorismic acid pathway) are common in plants and microorganisms, as shown in Fig. 1.1. The first step in the biosynthesis of phenylalanine is the condensation of erythrose 4-phosphate and phospho*enol*-pyruvate to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP). The cyclization of DAHP to 3-dehydroquinic acid requires cobalt ions and uses bound NAD^+ as cofactor. Dehydration of this 3-dehydroquinic acid yields 3-dehydroshikimic acid. 3-dehydroshikimic acid is reduced to shikimic acid in an NADP-

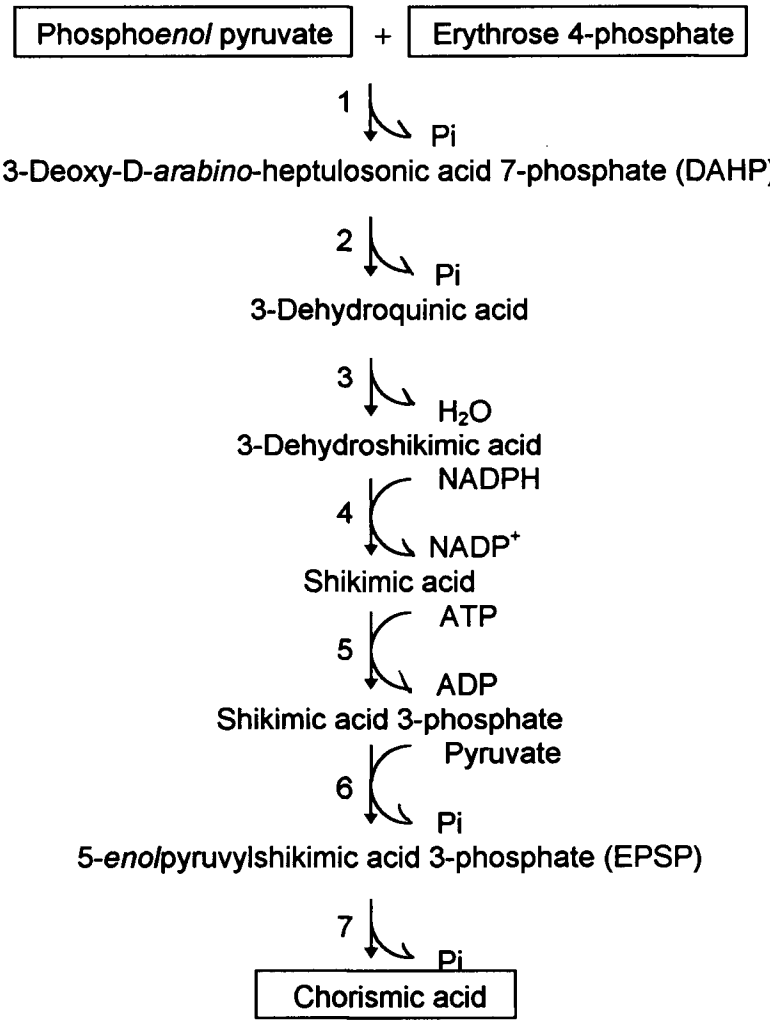


Fig. 1.1. The pathway from erythrose 4-phosphate to chorismic acid.
The enzymes are; 1: 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase, 2: 3-dehydroquinic acid synthase, 3: 3-dehydroquinic acid dehydratase, 4: shikimic acid:NADP oxidoreductase, 5: shikimic acid kinase, 6: 5-*enol*pyruvylshikimic acid 3-phosphate synthase, and 7: chorismic acid synthase.

dependent reaction catalyzed by shikimic acid dehydrogenase. After phosphorylation, shikimic acid 3-phosphate condenses with phospho*enol*-pyruvate. This condensation product, 5-*enol*pyruvylshikimic acid 3-phosphate, is converted to chorismic acid in a reaction catalyzed by chorismic acid synthase.

Beyond chorismic acid, the pathway leading to the synthesis of phenylalanine or tyrosine branched from that which produces tryptophan pathway (Fig. 1.2). In plants, the biosynthesis of phenylalanine proceeds from chorismic acid via prephenic acid and arogenic acid. In fungi, synthesis proceeds from chorismic acid via prephenic acid and either phenylpyruvic acid or arogenic acid. In bacteria, either of these pathways, or sometimes both pathways, can be operative (Bender, 1985; Jensen, 1986).

1.1.2 The metabolic fate of L-phenylalanine

1.1.2.1 In mammals

Phenylalanine can be transaminated to phenylpyruvic acid, but this is normally only a minor route of phenylalanine catabolism because the K_m of the aminotransferase is relatively high. Phenylpyruvic acid can be further catabolized via reduction to phenyllactic acid, or decarboxylation to phenylacetic acid.

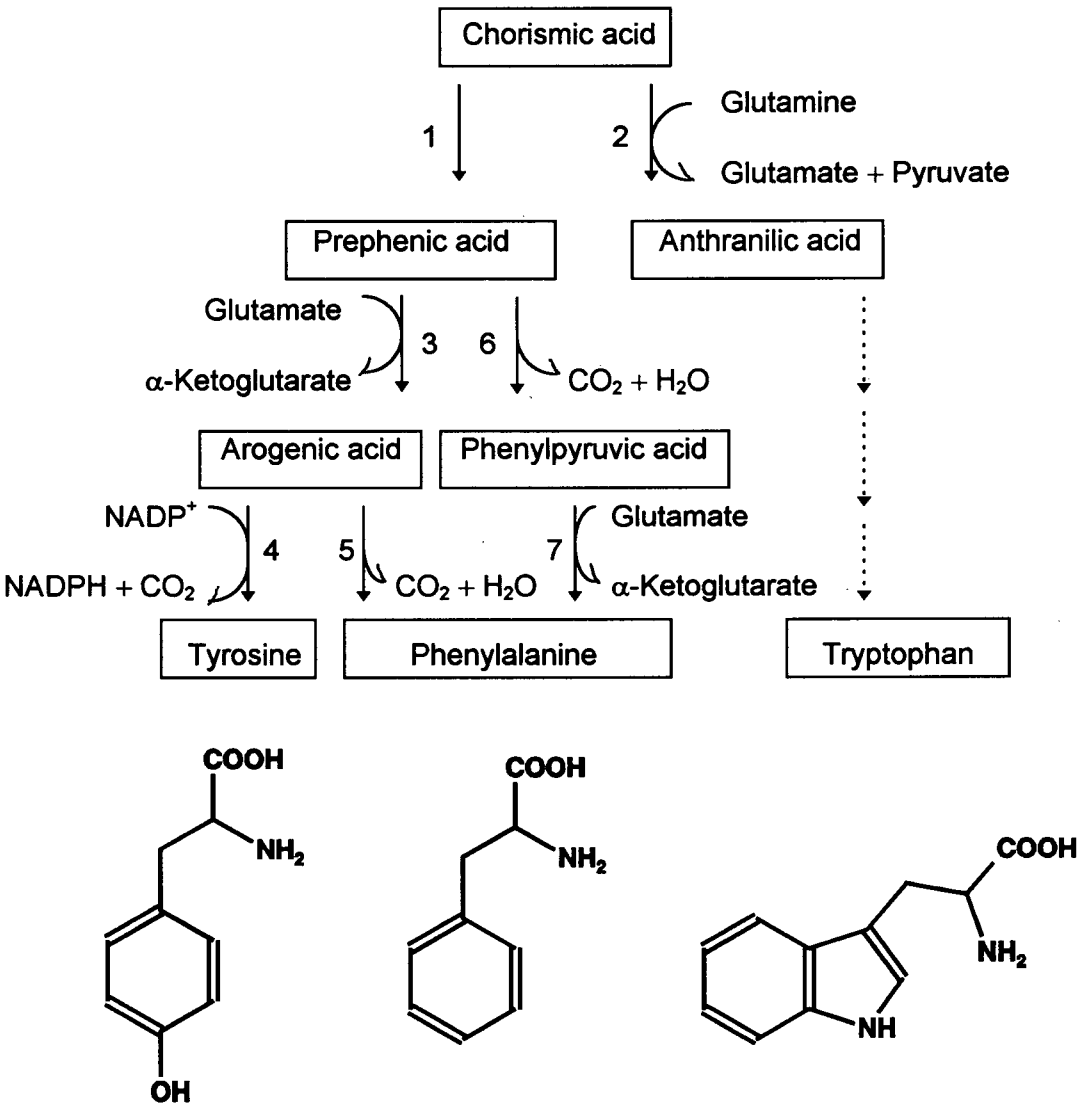


Fig. 1.2. The pathway from chorismic acid to aromatic amino acids.
The enzymes are; 1: chorismic acid mutase, 2: anthranilic acid synthase, 3: prephenic acid aminotransferase, 4: arogenic acid dehydrogenase, 5: arogenic acid dehydratase, 6: prephenic acid dehydratase, and 7: phenylpyruvate aminotransferase. Dashed arrows indicate multiple enzyme steps.

Under normal conditions, phenylalanine is metabolized by way of hydroxylation to tyrosine. Phenylalanine hydroxylase (a biopterin-dependent mixed function oxidase), dihydrobiopterin reductase, and a stimulator protein, are all involved in this hydroxylation (Fig.1.3). Failure in this hydroxylation process results in phenylketonuria (Ambrus *et al.*, 1978), an inborn metabolic disorder. In affected children, the phenylalanine concentration in blood is very high and tyrosine is low. In almost all cases of phenylketonuria, the defect is in phenylalanine hydroxylase, but a few cases, the defect is an inability to synthesize methyl-biopterin or a lack of dihydropterin reductase (Kaufman *et al.*, 1975, 1978). Under these conditions, a substantial portion of phenylalanine undergoes transamination to phenylpyruvic acid, which is further metabolized to phenylacetic acid and phenyllactic acid. These three phenylalanine metabolites are excreted in the urine.

The formation of tyrosine from phenylalanine can lead to catabolism by transamination of tyrosine to yield *p*-hydroxyphenylpyruvic acid (Fig.1.3). Normally, *p*-hydroxyphenylpyruvic acid is metabolized by way of homogentisic acid, which is cleaved eventually to fumaric acid and acetoacetic acid as shown in Fig. 1.4. *p*-Hydroxyphenylpyruvic acid can also be reduced to *p*-hydroxyphenyllactic acid, however, and this reaction becomes important when the homogentisic acid pathway from *p*-hydroxyphenylpyruvic acid to maleyl acetoacetic acid is blocked. This block results in alcaptonuria (Bender, 1985), a metabolic disorder that arises from a genetic defect in homogentisic acid oxidase. In

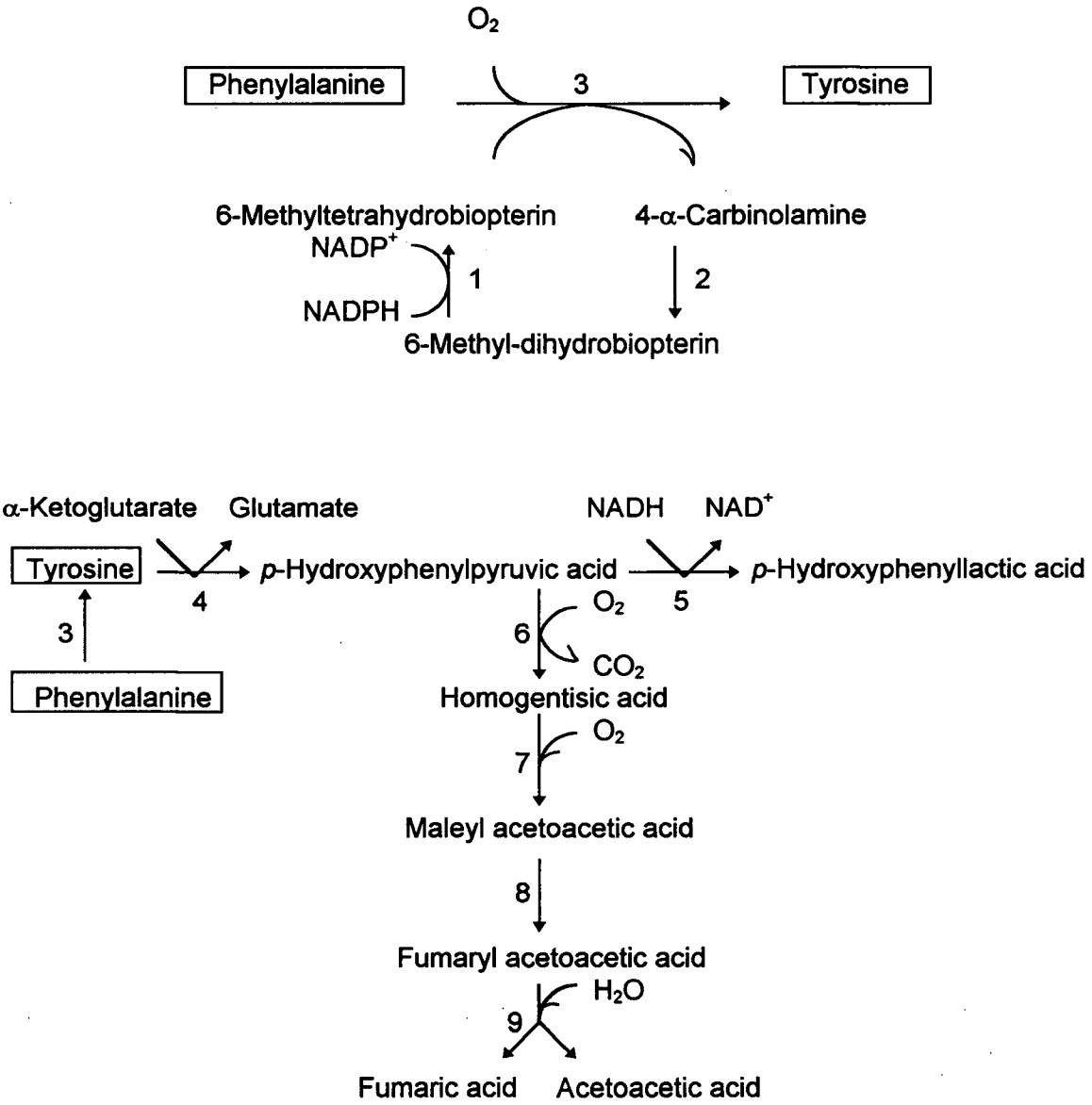


Fig. 1.3 The conversion of phenylalanine to tyrosine and other metabolites.
The enzymes; 1: dihydropterin reductase, 2: stimulator protein, 3: phenylalanine hydroxylase, 4: tyrosine aminotransferase, 5: *p*-hydroxyphenylpyruvic acid reductase, 6: *p*-hydroxyphenylpyruvic acid hydroxylase, 7: homogentisic acid oxidase, 8: maleyl acetoacetic acid isomerase, and 9: fumaryl acetoacetic acid hydrolase.

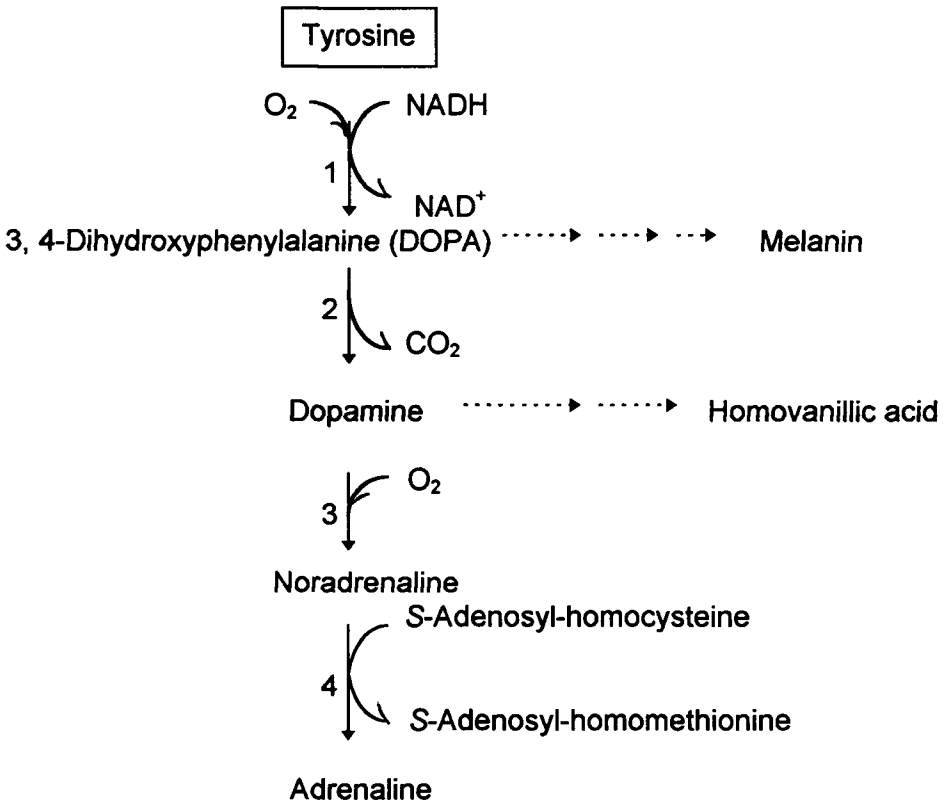


Fig. 1.4. The conversion of tyrosine to catecholamines. The enzymes; 1: tyrosine hydroxylase, 2: DOPA decarboxylase, 3: dopamine β-hydroxylase, and 4: phenylethanolamine-N-methyltransferase. Dashed arrows indicate multiple enzyme steps.

alcaptonuria patients, high amounts of *p*-hydroxyphenyllactic acid are formed and excreted in the urine.

The other important metabolic conversion of tyrosine is hydroxylation to 3,4-dihydroxyphenylalanine (DOPA), which is further metabolized to many physiologically important compounds. As shown in Fig. 1.4., DOPA serves as the precursor for the neurotransmitter, dopamine, and for the noradrenaline and adrenaline hormones (Martin, 1972). It also serves as the precursor for melanin formation, a process catalyzed by tyrosinase, a copper-dependent oxygenase, rather than by tyrosine hydroxylase (Giuseppe, 1992).

Another important product of mammalian tyrosine metabolism is the thyroid hormone thyroxine and its metabolite triiodothyronine, which stimulate energy metabolism through increasing the rate of electron transport (Metzler, 1977).

1.1.2.2 In plants

L-Phenylalanine, derived from the shikimic acid pathway, is used directly for protein synthesis in plants or metabolized through the phenylpropanoid pathway. This phenylpropanoid metabolism leads to the biosynthesis of a wide array of phenylpropanoid secondary products. The first few steps in this metabolic sequence are known as the "general phenylpropanoid pathway" (Fig. 1.5). These steps involve the actions of phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and

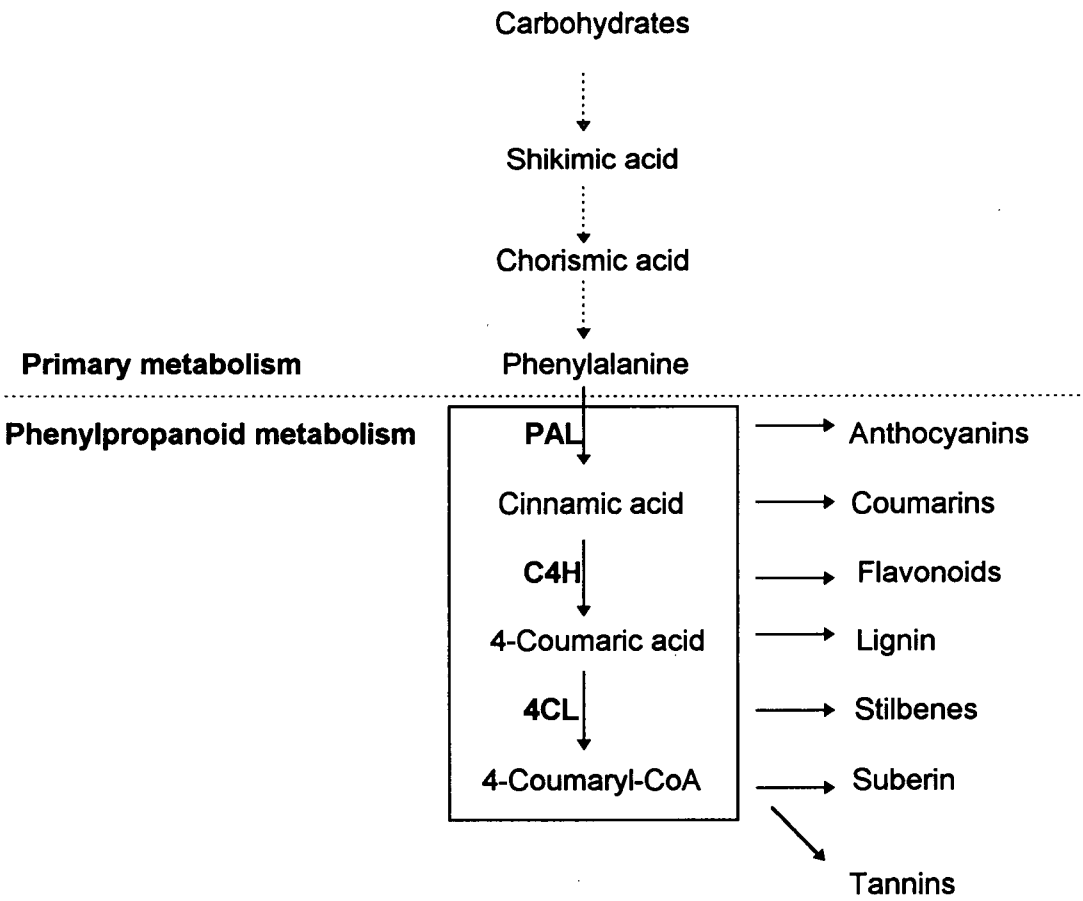


Fig. 1.5. Schematic representation of general phenylpropanoid metabolism. Dashed arrows indicate multiple enzyme steps. PAL: phenylalanine ammonia-lyase, C4H: cinnamic acid 4-hydroxylase, and 4CL: 4-coumaric acid: Coenzyme A ligase.

4-coumaric acid:CoA ligase (4CL). Following these core reactions, diverse phenylpropanoid products are derived through individual branch pathways (Hahlbrock and Scheel, 1989). These products include anthocyanins, coumarins, furanocoumarins, flavonoids, isoflavonoids, lignin, salicylic acid, stilbenes, suberin, soluble esters, tannins and other phenolics. Many of these phenylpropanoid compounds are known to have functions in allelopathy, color pigmentation, UV-protection, insect repellents, antimicrobial phytoalexins, and signaling in plant-microbe interactions. They also function as cell-wall components, waterproofing agents, and a source of structural rigidity.

Some alkaloids also arise from phenylalanine. Examples are colchicine, produced by the Liliaciae family, and ephedrine in *Ephedra* spp.. In barley, phenylalanine is converted to tyrosine and thence, via tyramine and N-methyltyramine into hordenine (Mann, 1987). Phenylalanine- or tyrosine-derived alkaloids may be grouped as four main structural types. These are simple monocyclic compounds (e.g. mescaline from 'peyote' cactus), isoquinolines (e.g. pellotine from 'peyote' cactus), benzylisoquinolines (e.g. morphine from opium poppy), and Amaryllidaceae alkaloids (e.g. lycorine from the daffodil family) (Mann, 1987).

1.1.2.3 In microorganisms

While the metabolism of phenylalanine in vascular plants and animals has been well documented, much less is known about the microbial degradation of phenylalanine. Some of the known pathways of animal and plant metabolism of phenylalanine are also

used in microorganisms. In some microorganisms, phenylalanine has often been found to be converted to homogentisic acid through the intermediary formation of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid by transamination and hydroxylation, as in the case of animals (Meister, 1965; Wat and Towers, 1979).

The discovery of PAL enzyme in fungi (Power *et al.*, 1965) and the detection of $^{14}CO_2$ production from ^{14}C-ring-labeled phenylalanine, cinnamic acid, and benzoic acid (Moore and Towers, 1967), demonstrated that certain fungi and *Streptomyces* bacteria can degrade phenylalanine by a pathway involving an initial deamination to cinnamic acid, as happens in plants. In *Streptomyces verticillatus*, *trans*-cinnamamide is formed from *trans*-cinnamic acid derived from phenylalanine (Bezanson *et al.* 1970). A metabolic pathway for the metabolism of phenylalanine via cinnamic, benzoic, *p*-hydroxybenzoic, and protocatechuic acids has been reported in several basidiomycete fungi, including *Rhodotorula* (Uchiyama *et al.*, 1969), *Ustilago hordei* (Moore *et al.*, 1967), *Schizophyllum commune* (Moore and Towers, 1967), and *Sporobolomyces roseus* (Moore *et al.*, 1968). *Schizophyllum commune* can also metabolize phenylalanine through phenylpyruvic acid, phenylacetic acid and *o*-hydroxyphenylacetic acid (Moore and Towers, 1967). Interestingly, it has been reported that another basidiomycete, *Lentinus lepideus* forms phenylpropanoid compounds (e.g. *p*-coumaric acid, caffeic acid, isoferulic acid, phloretic acid, and *p*-methoxycinnamic acid) via cinnamic acid derived from phenylalanine (Towers, 1969). In this fungus, a number of these compounds accumulate in the medium as methyl esters, but the physiological significance of these compounds is not known. The conversion of phenylalanine to benzoic acid derivatives through cinnamic acid has also been reported in Deuteromycete fungi such as *Alternaria* (Nambudiri *et al.*, 1970),

Rhizoctonia solani (Kalghatgi *et al.*, 1974), and *Penicillium brevicompactum* (Campbell *et al.*, 1987).

The fungus *Gliocladium* produces gliotoxin, an antibiotic and antiviral cyclic peptide, derived in part from phenylalanine and modified by the addition of sulfur across the peptide ring (Griffins, 1994). The conversion of phenylalanine to fungal melanin has received little attention, but was suggested to occur in *Alternaria* (Pridham and Woodhead, 1977).

1.2 PHENYLALANINE AMMONIA-LYASE (PAL)

1.2.1 Introduction

PAL (E.C. 4.3.1.5) catalyzes the nonoxidative deamination of L-phenylalanine to form *trans*-cinnamic acid and a free ammonium ion (Fig. 1.6) (Koukol and Conn, 1961). As described above, the conversion of the amino acid phenylalanine to *trans*-cinnamic acid is the entrypoint step for the channeling of carbon from primary metabolism into phenylpropanoid secondary metabolism in plants. PAL has been extensively studied because of its role in plant development and its response to a wide variety of environmental stimuli. The importance of this enzyme in plant metabolism is demonstrated by the huge diversity, and large quantities of phenylpropanoid products found in plant materials (Jones, 1984). In fungi, there is no direct evidence for the significance of this enzyme except as a catabolic function (Marusich *et al.*, 1981).

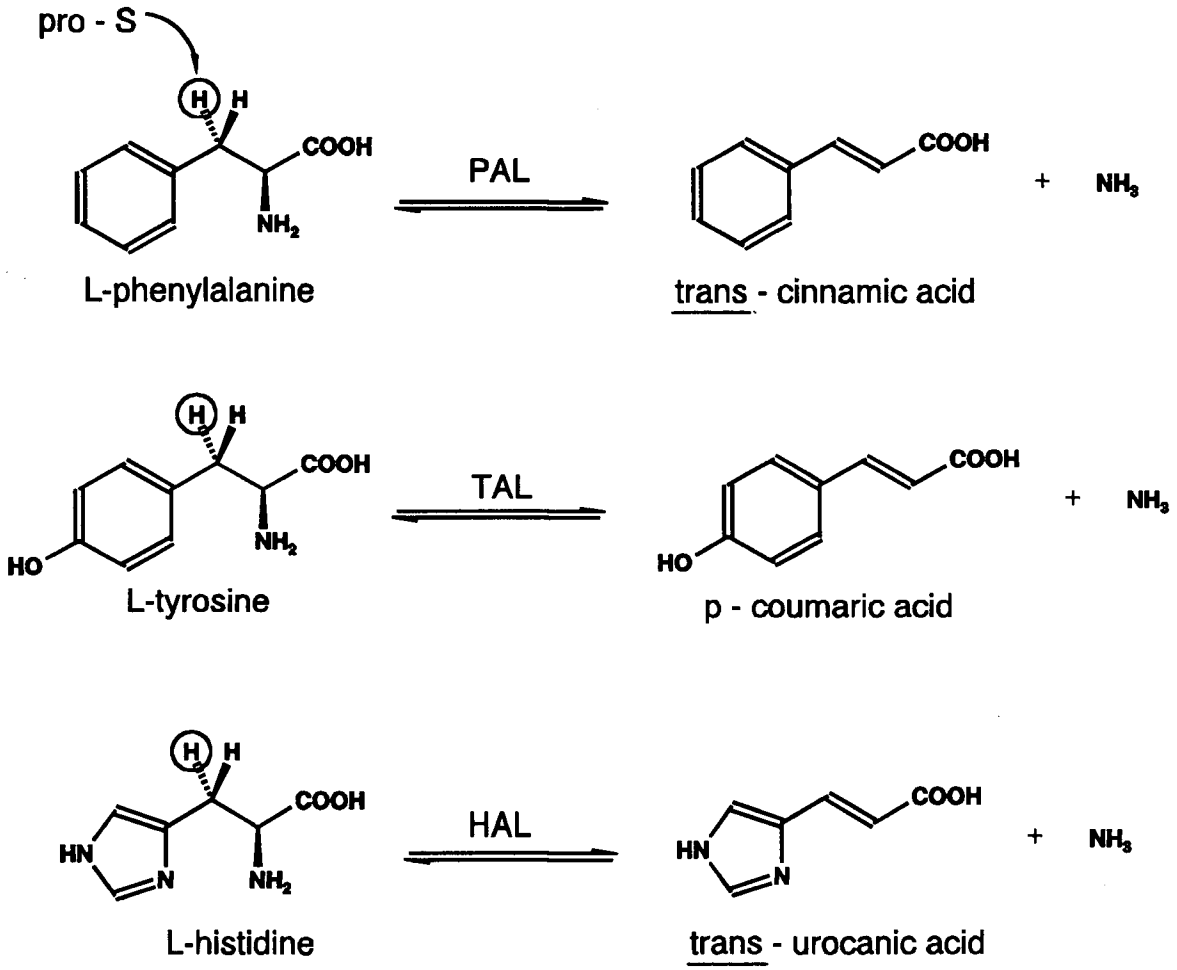


Fig. 1.6. Deamination of aromatic amino acids by ammonia-lyases.
PAL: L-phenylalanine ammonia-lyase, TAL: L-tyrosine ammonia-lyase, and HAL: L-histidine ammonia-lyase.

The presence of PAL has been reported in plants (Koukol and Conn, 1961; Camm and Towers, 1973; Jorrin *et al.*, 1988) including certain algae, e.g. *Dunaliella marina* (Czichi and Kindl, 1975), fungi (Bandoni *et al.*, 1968; Moore *et al.*, 1968; Hodgins, 1971; Sikora and Marzluf, 1982), and a prokaryotic organism, *Streptomyces verticillatus* (Emes and Vining, 1970). In plants, PAL activity has been detected in many species, representing monocots, dicots, gymnosperms, ferns, and lycopods (Young *et al.*, 1966). In fungi, PAL activity has been detected only in a few basidiomycetes and deuteromycetes, and in one ascomycete, *Nectria cinnabarina* (Bandoni *et al.*, 1968; Vance *et al.*, 1975). There have been no reports of PAL in animals.

1.2.2. Commercial and medical potential of PAL

The therapeutic potential of using PAL enzyme against neoplasms has been suggested because of its selectivity for phenylalanine (Stith *et al.*, 1973). PAL substantially inhibited neoplastic cell growth *in vitro* (Abell *et al.*, 1972), and produced cures in some mice that were inoculated with a lymphoblastic leukemia (Abell *et al.*, 1973). However, PAL is of special interest to clinicians primarily due to its potential as a treatment for the inherited metabolic disorder, phenylketonurea. A treatment involving the oral ingestion of PAL (Hoskins *et al.*, 1980) were proposed to patients to consume a normal diet, but this has not been brought to clinical trials yet.

With the increasing consumption of the aspartic acid-phenylalanine dipeptide artificial sweetener, Aspartame, the commercial demand for L-phenylalanine has led to mass

production of this amino acid (Klausner, 1985). Since the reaction is reversible, PAL can be used in a large-scale bio-conversion to produce L-phenylalanine from *trans*-cinnamic and ammonium salts acid (Hamilton *et al.*, 1985).

1.2.3 Active site and enzyme mechanism

PAL is one of the few amino acid-transforming enzymes not containing the cofactor pyridoxal 5'-phosphate. Instead, PAL contains the unusual prosthetic group dehydroalanine (Hodgins, 1971). The role of this post-translationally modified amino acid in catalysis is assumed to be the activation of the amino group of phenylalanine to form a better leaving group than NH_3^+ (Hanson and Havir, 1972). Modification of an electrophilic center at the active site of PAL by electrophilic reagents such as borohydride, cyanide, bifulfite or nitromethane, results in the complete inactivation of the enzyme. The identity of [^{3}H]-alanine and [^{14}C]-aspartic acid released following acid hydrolysis of PAL enzyme inactivated with radiolabeled reagents, NaB^3H_4 and $^{14}CN^-$, provide evidence for the presence of dehydroalanine in the active site (Hanson and Havir, 1985; Hodgins, 1971). Studies on the ability of substrates and substrate analogs of PAL to prevent inactivation by these reagents provide further evidence to support the idea that the active site contains dehydroalanine (Hanson, 1970; Hodgins, 1971). Recently, an alternative model for the role of dehydroalanin in PAL catalysis has been proposed (Schuster and Retey, 1995)

The mechanism of formation of dehydroalanine has not been determined yet. In cases of other proteins which contain dehydroalanine e.g. subtilin (Banerjee and Hansen, 1988),

thyroglobulin (Ohmiya *et al.*, 1990), and pyruvoyl enzymes (Recsei and Snell, 1984), a serine residue is considered to be the precursor of dehydroalanine. PAL amino acid sequences contain a serine residue which is completely conserved among different species (Taylor *et al.*, 1990), and is presumed to be associated with the active site of the enzyme. Recently, the precursor of the dehydroalanine residue has been identified as serine in parsley (Schuster and Retey, 1994) and poplar (McKegney *et al.*, 1996) PAL. In fungal PAL, it is likely that a similar process would account for the formation of the active site dehydroalanine from serine, but a role for serine as the precursor of dehydroalanine has not been directly demonstrated yet. Expression of PAL in *Escherichia coli* (Schulz *et al.*, 1989; Orum and Rasmussen, 1992) produced active PAL enzymes in cells in which PAL is not normally produced. The expressed PAL proteins showed similar enzyme properties compared to endogenous PAL from other sources. This suggests that the formation of dehydroalanine may be an autocatalytic process, although it cannot be ruled out that a widespread modifying enzyme is involved in the dehydroalanine formation.

1.2.4 Structural properties

1.2.4.1 PAL proteins

PAL has been isolated and characterized from a number of plant species, some fungi and one bacterial source. Source tissues used for PAL isolation are diverse. They include seedlings (Nari *et al.*, 1972), shoots (Koukol and Conn, 1961), leaf-sheath (Havir and Hanson, 1973), cell culture (Jorrin *et al.*, 1988; Bernards and Ellis, 1991; Campbell and Ellis, 1992), fruit (Given *et al.*, 1988), and mycelium (Pridham and Woodhead, 1974;

Kalghatgi and Subba Rao, 1975). Most known PAL sources for enzyme isolation and its properties are well tabulated and documented in reviews by Camm and Towers (1973), Hanson and Havir (1981), and Schomburg and Salzmann (1990). Difficulties in purification are often encountered, partly resulting from the low abundance of PAL in cells and changes in size and properties that occur during purification. Although an apparently homogeneous protein preparation can often be obtained in non-denaturing conditions, additional polypeptide bands are usually detected in analytical PAGE gel under denaturing conditions. This can create confusion in the estimation of PAL subunit sizes.

Most PALs reported range in size from 300 to 340 kDa in native molecular mass. Some examples of exceptions are reported masses of 152 kDa in *Ocimum basilicum* (Hao *et al.*, 1996), 226 kDa in a bacterium, *Streptomyces* (Emes and Vining, 1970), 250 kDa in *Helianthus annuus* (Jorrin *et al.*, 1988), 266 kDa in *Fragaria ananassa* (Given *et al.*, 1988), and 560 kDa in *Alternaria* (Pridham and Woodhead, 1974). PAL is normally a homo-tetrameric protein consisting of 4 identical subunits. Hetero-tetrameric PAL as a complex of two hetero-dimers has been reported from *Helianthus annuus* (2 × 58 kDa and 2 × 68 kDa, Given *et al.*, 1988) and *Rhizoctonia solani* (2 x 70 kDa and 2 x 90 kDa, Kalghatgi and Subba, 1975). Neumann and Schwemmle (1993) reported that *Oenothera* seedlings have two PAL isoenzymes with four identical subunits each of 75.5 kDa and 79.2 kDa. *Rhodosporidium toruloides* PAL has been reported to be a dimer being composed of two identical subunits with a mass of 80 kDa (Adachi *et al.*, 1990).

Iso-electric points (pI) for PAL are usually in the acid range from 2.5 (Neumann and Schwemmle, 1993) to 5.75 (Campbell and Ellis, 1992). Isoforms with different pIs have been reported from some sources; three isoforms ranged between pI 4.8 and 5.4 in *Leptosphaeria maculans* (Dahiya, 1993), several isoforms between pI 5.1 and 6.1 in alfalfa (Jorrin and Dixon, 1990), and two isoforms between pI 4.8 and 5.4 in bean (Bolwell and Rodger, 1991). Interestingly, expression of a single cDNA of poplar PAL in a baculovirus expression system produced two isoforms with different pIs (McKegney *et al.*, 1996).

Most PALs are considered to be hydrophobic proteins. This property has led to the use of hydrophobic affinity column chromatography for the purification of PAL from cotton (Dubery and Smit, 1994) and *Rhodotorula glutinis* (D'Cunha *et al.*, 1996). Alfalfa PAL has been reported to be highly hydrophobic (Jorrin and Dixon, 1990) and consistent with this, the hydropathy profile of the protein sequence deduced from the cDNA sequence also predicted that alfalfa PAL would be hydrophobic (Gowri *et al.*, 1991).

The association of carbohydrate with PAL has been reported for the maize and potato enzymes (Havir, 1979; Shaw *et al.*, 1990). Through the analyses of PAL gene sequences, the presence of potential glycosylation sites has been reported from bean (Cramer *et al.*, 1989) and parsley (Lois *et al.*, 1989), but the importance of glycosylation in PAL function has not been explored extensively. The production of active PAL in *E. coli* cells transformed with PAL genes from the yeast *Rhodosporidium* (Orum and Rasmussen, 1992) and from parsley (Shulz *et al.*, 1989; Appert *et al.*, 1994) suggests that PAL catalysis is not likely to be influenced by glycosylation. It has not been excluded

that glycosylation is involved in enzyme stability and in localization of the enzyme within cells (Havir, 1979; Shaw *et al.*, 1990).

1.2.4.2 PAL-encoding genes

Following the isolation of PAL cDNA from bean (Cramer *et al.*, 1989), parsley (Lois *et al.*, 1989) and sweet potato (Tanaka *et al.*,1989), PAL genes have been isolated from many sources. Currently, information on the partial or full sequences of the genes and cDNAs encoding PAL is available from several nucleic acid databases. In most plants, PAL is encoded by a small gene family of 3-5 genes. Exceptions to this are the potato PAL gene family which is made up of 40-50 genes (Joos and Hahlbrock, 1992), and the loblolly pine PAL which has been reported to be encoded by a single gene (Whetten and Sederoff, 1992). In yeasts, PAL is encoded by single gene (Anson *et al.*, 1987). In general, the sizes of PAL genes have been reported in the range from 2.1 kb to 2.4 kb. The presence of introns has been reported both in plant and yeast PAL genes. Plant PAL genes generally contain only one intron, while yeast PAL genes have five (Anson *et al.*, 1987) or six introns (Valslet *et al.*, 1988). There are two exceptions; two introns have been found in the *Arabidopsis* PAL gene (Wanner *et al.*, 1995), while no introns occur in jack pine and loblolly pine PAL genes (Campbell, 1991; S. Butland, personal communication ; Sederoff *et al.*, 1994).

1.2.4.3 PAL promoter

The organization of PAL promoters has been studied by analyses of the 5' flanking regions of plant PAL genes using DNA footprinting, S1 mapping, and primer extension methods. The presence of a TATA box in PAL gene promoters has been reported from bean (Cramer *et al.*, 1989), parsley (Lois *et al.*, 1989), and rice (Minami *et al.*, 1989). Bean and rice PAL promoters contain a CAAT box, and a GC-rich segment is present in the rice PAL promoter. As in the cases of some other phenylpropanoid enzyme-encoding genes e.g. *4CL* and *CHS* (Dron *et al.*, 1988; Zhang and Mehdy, 1994), there are H [CCTACC$(N)_7$CT] and AC Boxes upstream from the PAL transcription start site (Hatton *et al*, 1995; Loake *et al.*, 1992; Lois *et al.*, 1989). However, none of these sequences were found in the upstream region of a yeast PAL gene (Anson *et al.* ,1987).

1.2.5 Functional properties

PAL enzymes from many sources, especially from monocots and certain fungi, have activity towards L-tyrosine and can thus produce *trans-p*-coumaric acid. This has been described as tyrosine ammonia-lyase (TAL, Fig. 1.6) activity (Neish, 1960; Young *et al.*, 1966, Camm and Towers, 1973). In most PAL preparations, TAL activity is very low. The PAL/TAL ratio in PAL preparations varied from 1.35 to 5 in *Sporobolomyces pararoseus* (Parkhurst and Hodgins, 1971), from 4 to 20 in wheat (Young *et al.*, 1966), and from 0.6 to 1.3 in bean (Scott *et al.*, 1992). An even larger range in PAL/TAL ratios from several different plant species was reported by Jangaard (1974). No TAL enzyme without PAL activity has been purified. Recently, it has been demonstrated in *E. coli*-expressed maize PAL that PAL and TAL activities reside in the same polypeptide (Rösler *et al.*, 1997).

PAL preparations from a number of sources are reported to have only one Michaelis constant (K_m), but the kinetic properties of other preparations suggested that the enzyme is negatively cooperative with respect to substrate binding (Nari *et al.*, 1974). Two different K_m values for PAL have therefore been reported from many sources (Hanson and Havir, 1981). However, when individual isoforms were highly purified, kinetic analysis of each isoform revealed normal Michaelis-Menten saturation kinetics (Bolwell *et al.*, 1985; Jorrin and Dixon, 1990). The K_m values for L-phenylalanine have been reported to range from 0.011 mM (Nagai *et al.*, 1988) to 1.7 mM (Koukol and Conn, 1961). Most PAL shows no metal ion requirement although slight stimulation of PAL activity by metal ions such as Mg^{2+} and Ba^{2+} has been reported (Jorrin *et al.*, 1988). Inhibition of PAL activity can be induced by a wide range of compounds including carbonyl, sulfhydryl and thiol reagents, phenolic acids and heavy metal ions (Schomburg and Salzmann, 1990). Most PALs tested are sensitive to the synthetic PAL inhibitors such as (*S*)-2-aminooxy-3-phenylpropanoic acid (AOPP), (*R*)-(1-amino-2-phenylethyl)phosponic (APEP) acid, and 2-aminoindan-2-phosphonic acid (AIP) and thus these inhibitors have often been used to block the biosynthesis of phenylpropanoid compounds in plant cells and tissues (Hanson and Havir, 1981, Zon and Amrhein, 1992).

The pH optimum for PAL is generally in the range from 8.2 to 9.0 (Pridham and Woodhead, 1974; Hanson and Havir, 1981). The temperature optimum for PAL has been reported to be 35°C in tobacco (Nagai *et al.*, 1988), 55°C in sunflower (Jorrin *et al.*, 1988), and 44-46°C in *Rhizoctonia* (Kalghatgi and Subba Rao, 1975). Plant PAL enzymes are generally sensitive to repeated freezing and thawing and lose activity as the temperature approaches 60°C. In contrast, fungal PAL is more thermally stable too

(Kalghatgi and Subba Rao, 1975). *Rhodotorula* PAL is apparently stable for at least six months when it is kept at –60°C (Fritz *et al.*, 1976).

1.2.6 Regulation

PAL enzyme activity is generally regulated at two levels - PAL synthesis, and inactivation or degradation. PAL synthesis is determined through the processes of transcription, transcript processing, and translation. PAL inactivation or degradation is mediated by several factors such as stability of enzyme, proteolytic enzyme, inhibitors, and modification of the enzyme. The rate of each process and the degree of mediation by each factor described above can affect the levels of PAL activity. These modes of PAL activity regulation are reviewed in the following sections (1.2.6.1-3).

1.2.6.1 Factors influencing PAL activity levels

PAL activity levels in plants are influenced by several environmental factors including light, pathogen attack, wounding, temperature, oxygen (Hanson and Havir, 1981; Jones, 1984). The levels also depend on the genotype, plasmotype, age, organ and tissues of the plant (Camm and Towers, 1973). In microorganisms, by contrast, there is almost no information on environmental factors involved in modulating PAL activity levels.

Light generally has a stimulatory effect on PAL activity levels although exceptions have been reported. An increased level of extractable PAL activity in response to wounding has been displayed in many plant species including bean axes, pea seedlings, potato

and sweet potato tubers, and sunflower leaves (Camm and Towers, 1973; Hanson and Havir, 1981; Shaw *et al.*, 1990). In diseased plants, increased synthesis of phenolics, lignin and isoflavonoid phytoalexins is concomitant with the increased PAL activity (Friend, 1981; Vance *et al.*, 1980; Grisebach and Ebel, 1978).

Hormonal effects on PAL activity also have been demonstrated, and these may link responses to pathogen infection with those associated with plant development. Endogenously produced ethylene appears to trigger increased PAL production in bean and tobacco infected with viruses, and in parsley, rice, sweet potato, and soybean infected with fungal pathogens (reviewed by Camm and Towers, 1973; Chappell *et al.*, 1984; Haga *et al.*, 1988). Inhibition of PAL induction by indole acetic acid (IAA) and abscisic acid has been reported in tobacco tissue cultures and soybean seedlings infected with a fungal pathogen, respectively (Innerarity *et al.*, 1972; Ward *et al.*, 1989).

1.2.6.2. Mechanisms for the regulation of PAL activity

There are many examples of PAL activity regulation at the protein level. Up-regulation could involve enhancement of *de novo* PAL synthesis and substrate pools, conversion of PAL from inactive to active forms, reduction of active PAL degradation, and/or an increase in enzyme turn-over. Among these mechanisms, the major mechanism for the increase of PAL activity levels appears to be increased *de novo* synthesis of PAL proteins. *De novo* PAL synthesis in response to environmental stimuli has been demonstrated by radio-labeling of newly synthesized PAL or by use of a protein synthesis inhibitor, cycloheximide (Lawton *et al.*, 1983; Hahlbrock and Ragg, 1975).

Down-regulation of PAL activity levels would be possible through reversal of the processes responsible for up-regulation. Namely, reduction of new PAL synthesis and depletion of substrate pools, and an increase in the inactivation and/or degradation of PAL enzyme. The direct inactivation of PAL activity by proteinaceous factors has been described in extracts from apple skin (Tan, 1980), sunflower leaves (Gupta and Creasy, 1984), and sweet potato (Tanaka *et al.*, 1977). Bolwell (1992) has indicated that cAMP-dependent phosphorylation could be involved in suppressing the induction of PAL activity. In *Phaseolus*, phosphorylation of PAL subunits was associated with a smaller subunit size (70 kDa), but with the normal size of subunit (77 kDa), suggesting that PAL degradation and inactivation may be triggered by phosphorylation of the enzyme. Bolwell and his colleagues (1986,1988) have reported that in bean suspension cultures, cinnamic acid can decrease the levels of PAL activity by increasing the degradation of PAL. In tomato, in contrast, it has been proposed that production of truncated gene products, which would presumably yield disfunctional PAL proteins, might lead to changes in PAL activity (Lee *et al.*, 1992).

1.2.6.3. Regulation of PAL gene expression

PAL activity is commonly regulated at the genetic level in response to various stimuli. PAL gene expression has been observed to be activated in plants infected with tobacco mosaic virus (Pellegrini *et al.*, 1994), bacteria (Huang and McBeath, 1994), and fungi (Bell *et al.*, 1986; Bernards and Ellis, 1991; Lamb *et al.*, 1989; Joos and Hahlbrock, 1992). Many of the responses to pathogen attacks can be mimicked by elicitors derived

from fungal cell walls or culture filtrates (Edwards *et al.*, 1985; Rohwer *et al.*, 1987; Dixon and Lamb, 1990). It has been shown that elicitor treatments can also induce PAL gene expression in bean (Shufflebottom *et al.*, 1993), parsley (Dangl *et al.*, 1987; Lois *et al.*, 1989), and in suspension cultured cells of alfalfa (Orr *et al.*, 1993; Farrendorf *et al.*, 1996), rice (Zhu *et al.*, 1995) and poplar (Moniz de Sa *et al.*, 1992).

Hormonal effects on PAL gene expression in association with pathogen infection or elicitor treatments have been reported. ABA repressed the accumulation PAL mRNA in fungal pathogen-infected soybean (Ward *et al.*, 1989), while in bean suspension cultures treated with elicitor, auxin and ethylene increased the levels of PAL mRNA (Hughes and Dickerson, 1989). Ethylene treatment increased PAL mRNA accumulation in carrot suspension cultures (Ecker *et al.*, 1987).

Parsley is one of the best studied plants with respect to the relationship between PAL gene expression and illumination. A rapid transient increase of PAL transcripts has been observed in parsley cell suspension cultures (Betz *et al.*, 1978; Shröder *et al.*, 1979) and protoplasts (Ohl *et al.*, 1990) following UV irradiation. Accumulation of PAL mRNA in tissues exposed to light has been demonstrated in dark-grown parsley seedlings which were then transferred to the light (Wu and Hahlbrock, 1992). Accumulation of PAL mRNA has also been observed in *Arabidopsis* seedlings illuminated by UVB or blue light (Kubasek *et al.*, 1992) and hybrid poplar leaves irradiated by UV (Osakabe *et al.*, 1995). Liang and his colleagues (1989) have shown that in etiolated transgenic tobacco plants, white light increases GUS activity which is expressed from a PAL promoter-GUS fusion transgene.

The response of PAL genes to wounding and heavy metals has also been studied. Increased PAL gene transcription in response to wounding has been detected in *Arabidopsis* (Ohl *et al.*, 1990), melon (Diallinas and Kanellis, 1994), parsley (Lois and Hahlbrock, 1992), and sweet potato (Tanaka *et al.*, 1989). PAL genes in *Arabidopsis* and pea respond to $HgCl_2$ and $CuCl_2$ exposure, respectively, with enhanced mRNA production (Ohl *et al.*, 1990; Preisig *et al.*,1991).

PAL gene expression is also regulated developmentally and in a tissue-specific fashions. During stem growth in *Populus kitakamiensis*, PAL genes *PAL g1* and *PAL g2a* were expressed in a tissue-specific manner. The highest levels of *PAL g1* were detected in young tissue near the shoot bud, but *PAL g2a* mRNA was mainly accumulated in mature stems (Osakabe *et al.*, 1995). *PAL* transcript changes in association with development of vascular tissue and other tissue-specific patterns of expression have been observed in other plant systems including alfalfa, *Arabidopsis* (Wanner *et al.*, 1995), bean (Leyva *et al.*, 1992), parsley (Wu and Hahlbrock, 1992), and pea (Yamada *et al.*, 1994). In melon fruit, PAL is transcriptionally induced in response to fruit ripening (Diallinas and Kanellis, 1994). The analyses of tissue-specific expression of the bean *PAL2* and *PAL3* promoters in transgenic tobacco have revealed that the AC elements play a key role in regulation of tissue-specific expression of the bean *PAL* gene family (Hatton *et al.*, 1995, 1996).

1.2.6.4 Co-ordinate regulation

It has been shown that, like other phenylpropanoid enzymes and their genes, expression of PAL enzyme is coordinately regulated with other enzymes of the phenylpropanoid pathway (reviewed by Logemann *et al.*, 1995). Cramer *et al.* (1985) have reported that PAL, chalcone synthase (CHS), and chalcone isomerase (CHI) are coordinately regulated in elicitor-treated bean cultures. Co-ordinate regulation of PAL and CHI has also been observed in illuminated mustard seedlings (Beggs *et al.*, 1987). In parsley cell suspension cultures, UV irradiation and elicitor treatment produced a coordinate increase in *PAL* and *4CL* transcript levels (Chappel and Hahlbrock, 1984; Schmelzer *et al.*, 1985). A similar coordinate expression of PAL and 4CL mRNAs in response to elicitor has been observed in alfalfa (Dalkin *et al.*, 1990) and potato (Fritzemeier *et al.*, 1987). The expression of PAL with 4CL and with other pathogenesis-related (PR) and elicitor-related (EL) genes is regulated coordinately in parsley leaves challenged with a fungal pathogen (Schmelzer *et al.*, 1989). The observation that the AC elements found in both the PAL1 and CHS promoters are involved in activation of transcription in parsley (Lois *et al.*, 1989; Schulze-Lefert *et al.*, 1989), provides a possible mechanism by which phenylpropanoid metabolism could be coordinately regulated (Cramer *et al.*,1989; Logemann *et al.*, 1995).

1.3 *USTILAGO MAYDIS*

1.3.1 Corn smut disease

Smut fungi are basidiomycetes of the order Ustilaginales. These fungal pathogens cause severe economic losses of cereal grains (such as barley, corn, millet, oats, sorghum, and wheat), other crops (such as onions, spinach), and some ornamentals (such as carnation). The most common smuts belonging to the genus *Ustilago* are smuts of barley (*U. hordei, U. nuda, U. nigra*), corn (*U. maydis*), oats (*U. avenae*), sugarcane (*U. scitaminea*), and wheat (*U. tritici*).

The infection of corn (*Zea mays*) by *U. maydis* is characterized by distinct galls (plant tumors) on stems, leaves, ears, and tassels (Christensen, 1963). The disease symptoms also include stunting, and chlorosis and anthocyanescence on stems and leaves, especially in the areas developing tumors . The fungus reproduces primarily in embryonic and protein-rich tissues of its host, and stimulates its host cells to divide and enlarge. In the end, this proliferation produces tumors containing dark, sooty, masses of black teliospores, from which the name of the disease, corn smut, is derived. *U. maydis* is distributed world-wide, but is particularly prevalent in warm and moderately dry areas, where it causes serious damage to susceptible varieties (Agrios, 1988). Losses from this disease vary from one area to another, but can reach 10% or more. Control of the disease is mainly reliant on use of resistant corn varieties, and to a lesser extent on sanitation and crop rotation measures. In addition, the maintenance of well-balanced soil fertility can reduce disease incidence (Ullstrup,1961).

1.3.2 The life cycle and mating of *U. maydis*

Several phases of the life cycle of *U. maydis* are outlined in Fig. 1.7. The fungus produces two types of cells in its life cycle. The unicellular haploid phase is saprophytic (non-pathogenic), grows by budding, and forms compact colonies with yeast-like morphology. This cell type can be easily cultured *in vitro* and produces a colony of sporidia by budding. Another type of cell is the dikaryon which is pathogenic, parasitic and dependent on growth in host tissues by infection. The dikaryon arises from mating between two compatible haploid sporidia. Through karyogamy (nuclear fusion) within the dikaryotic hyphae inside the host plant, diploid cells are produced which develop into black teliospores within galls. The teliospores are released from ruptured plant galls and dispersed by wind. After germination of teliospores, meiosis occurs and haploid sporidia are produced. Compatible mating of these sporidia leads to a new life cycle of *U. maydis* (Banuett, 1992).

The intertwined association of life cycle and pathogenicity has provided opportunities to explore the processes that govern mating, filamentous growth, and pathogenicity. It has been found that to undergo the pathogenic process involving infection and development of disease symptoms, fusion of two compatible yeast-like haploid cells is essential. Compatibility is determined by the alleles present at two different mating-type loci, the *a* locus with two alleles (*a1* and *a2*), and the *b* locus with multiple alleles (Rowell and

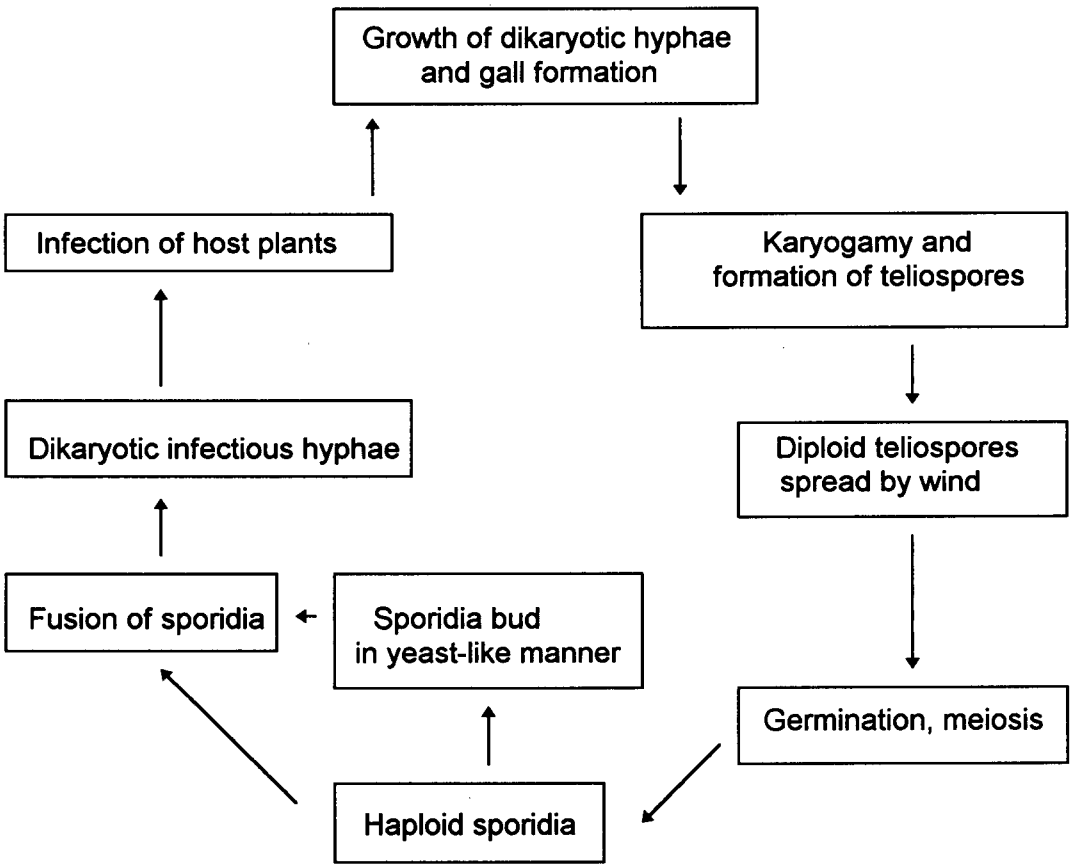


Fig. 1.7. Schematic representation of the life cycle of *Ustilago maydis*.

Devay, 1954; Puhalla, 1968, 1969; Day *et al.*, 1971). Considerable progress has been made in clarifying the mode of action of these different mating loci. The *a* mating-type locus, encoding a pheromone (*a1*) and a pheromone receptor (*a2*), has been determined to be involved in the control of haploid cell fusion (Bölker *et al.*, 1992). The *b* mating-type locus apparently has at least 25 alleles, and controls pathogenicity and dimorphism (Rowell and Devay, 1954; Day *et al.*, 1971). Two genes are found at the *b* locus, *bE* and *bW*, each encoding a protein containing a homeodomain motif (Kronstad and Leong, 1990; Schulz *et al.*, 1990). It is thought that *b* gene products have a DNA-binding function, leading to the idea that interactions between the products of the *bE* and *bW* genes from alleles of different *b* specificities are required for pathogenic development in this smut organism (Gillissen *et al.*, 1992).

1.3.3 *U. maydis* as a model fungus

The phytopathogenic fungus *U. maydis* has been used to study many aspects of eukaryotic genetics, including recombination mechanisms (Holliday, 1974), nitrogen metabolism (Lewis and Fincham, 1970), and developmental regulation (Froeliger and Kronstad, 1990). Classical genetic approaches have been productive with this fungus for several reasons. Both yeast-like haploid and diploid cell cultures can be maintained on nutrient media; teliospores germinate *in vitro*, making analysis of meiotic products reliable, and diploid strains can be constructed for the study of genetic complementation and dominance (Puhalla, 1968, 1969; Holliday, 1974). Furthermore, molecular genetic approaches are also possible in *U. maydis*, with the development of such molecular tools

as efficient transformation (Wang *et al.*, 1988), autonomously replicating vectors (Tsukuda *et al.*, 1988), genes for selection markers (Wang *et al.*, 1988; Banks and Taylor, 1988; Gold *et al.*, 1994b), and gene disruption (Kronstad *et al.*, 1989). These techniques allow genes of interest to be cloned, manipulated, silenced and/or reintroduced at a desired location in the genome. These resources have greatly facilitated analysis of the structure, expression, and function of *U. maydis* genes (Froeliger and Kronstad, 1990).

1.4 RATIONALE AND OBJECTIVES

While a huge amount of information has been accumulated on the structure, expression and function of PAL in plants, the biological role of PAL in fungi has not been established, and, in general, information on fungal PAL is very limited. Most commonly, a catabolic function for fungal PAL has been suggested, in which the enzyme is used to obtain carbon and nitrogen from external supplies of amino acids. However, fungi can also obtain carbon and nitrogen from L-phenylalanine through phenylalanine aminotransferase or amino acid oxidase, which raises the question: what selective advantage does PAL offer that has led to its retention in this group organisms? It appears that the ability to synthesize cinnamic acid is important in the life cycle of fungi.

The rationale for this work was to develop knowledge and tools that would enable me to rationalize the existence of PAL in the fungus *U. maydis*. As mentioned in the previous section, *U. maydis* is both an agriculturally important fungus and a very versatile model species in which to study the structure, expression, and function of eukaryotic genes.

Once it was established that *U. maydis* also produces PAL, I undertook detailed structural, functional and metabolic studies of this PAL. This work enabled me to clone the *U. maydis* PAL gene, which make it possible to consider gene replacement. This should reveal whether PAL is essential in *Ustilago* physiology and/or especially reveal links, if any, between PAL activity and pathogenesis. The following chapters describe the work that was carried out to these ends.

Chapter two describes the purification and characterization of the *U. maydis* PAL enzyme. *U. maydis* PAL protein data and anti-PAL antibodies were important tools that would provide a unique opportunity to pursue molecular genetic studies of PAL in a fungal species largely unrelated to the red yeasts.

Chapter three describes the isolation and sequencing analysis of a *U. maydis* genomic clone which contains a gene encoding PAL. A comparative analysis of its predicted protein sequence with some of the published PAL sequences, and with sequences for a related enzyme, histidine ammonia-lyase (HAL), provided direct evidence for the extent of evolutionary divergence in this family. Immunological evidence regarding the relatedness of the two ammonia-lyases is also presented.

Chapter four describes the *in vivo* regulation of *U. maydis* PAL by L-tryptophan, and provides an initial look at L-phenylalanine metabolism in this fungus. A biosynthetic pathway for *Ustilago* melanin formation is proposed and potential biological roles for *Ustilago* PAL are discussed in this context.

CHAPTER TWO

Purification and Characterization of Phenylalanine Ammonia-lyase from *Ustilago maydis*

2.1. INTRODUCTION

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the nonoxidative deamination of L-phenylalanine to form *trans*-cinnamic acid and a free ammonium ion. The enzyme occurs in plants (Koukol and Conn, 1961; Camm and Towers, 1973; Jorrin *et al.*, 1988), fungi (Bandoni *et al.*, 1968; Moore *et al.*, 1968; Hodgins, 1971; Sikora and Marzluf, 1982), and Actinomycetes (Emes and Vining, 1970), but not in animals. PAL has been used in experimental treatments and diagnosis of phenylketonurea, a human syndrome characterized by high levels of circulating phenylalanine (Ambrus *et al.*, 1978), and the enzyme has industrial application in the production of L-phenylalanine from *trans*-cinnamic acid, i.e. the reverse of the normal *in vivo* reaction (Yamada *et al.*, 1981).

In plants, PAL is the entrypoint enzyme into phenylpropanoid metabolism and it regulates the biosynthesis of a wide range of phenylpropanoid secondary compounds, including lignin, flavonoids, furanocoumarin and isoflavonoid phytoalexins, and wound protectant hydroxycinnamic acid esters (Jones, 1984).

Ustilago maydis is the causal agent of corn smut. The fungus reproduces primarily in embryonic and protein-rich tissues of its host (*Zea mays*), and produces galls on any above-ground part of the host plant (Agrios, 1988). The presence of PAL in the related

species, *U. hordei*, was reported earlier by Subba Rao *et al.* (1967), but nothing is known about the role of PAL in the *Ustilago* life cycle or pathogenesis.

In a yeast, *Rhodosporidium toruloides,* phenylalanine can act as the sole source of carbon, nitrogen, and energy (Marusich *et al.*, 1981). Since PAL catalyzes the initial reaction in the catabolism of the amino acid in this organism, the enzyme plays a key role in regulating phenylalanine-dependent metabolism. However, the biological function of PAL in *R. toruloides, U. maydis* and other fungi during normal (i.e. non-phenylalanine-dependent) growth and development is unclear. It is now possible to conduct gene replacement and gene disruption experiments in *U. maydis* (Kronstad *et al.*, 1989), and these techniques provide a unique opportunity to address the role of PAL. In a preliminary study, it has been established that, like *U. hordei*, cultured cells of *U. maydis* grown in synthetic medium produce substantial amounts of PAL, and the present chapter describes the purification and characterization of the *U. maydis* enzyme.

2.2 MATERIALS AND METHODS

2.2.1 Materials

DIG-Glycoprotein Detection kit and p*I* markers were purchased from Boehringer Mannheim (Laval, Canada). Molecular mass markers for native PAGE and HiTrap-NHS matrix were purchased from Pharmacia Biotechnology Products (Uppsala, Sweden). DEAE cellulose and Freund's adjuvant were purchased from Sigma (St. Louis, U.S.A.). Bio-Gel A-0.5m agarose beads, Ampholyte, alkaline phosphatase-conjugated goat anti-

rabbit antibody, SDS-PAGE molecular markers, and BCIP (5-bromo-4-chloro-3-indoyl phosphate ρ-toluidine salt)/NBT (ρ-nitro blue tetrazolium chloride) were purchased from Bio-Rad (Mississauga, Canada). Westran PVDF (polyvinylidine difluoride) membrane and Centriprep Concentrator were purchased from Schleicher & Schuell (Keene, U.S.A.) and Amicon (Beverly, U.S.A.), respectively.

2.2.2 Fungal cultures

Ustilago maydis strain 518 was maintained on potato dextrose agar medium (PDA) and grown in complete liquid medium (cm) as described by Holliday (1974). For enzyme isolation, cm (50 ml) in 250 ml Erlenmeyer flasks was inoculated with sporidia of the fungus grown on PDA and incubated at 30°C for 24 hr on a gyratory shaker (250 rpm).

2.2.3 Purification of *U. maydis* phenylalanine ammonia-lyase

All procedures were carried out at 4°C, unless otherwise mentioned. The fungal cells were harvested by centrifugation (10000 × *g*, 10 min), washed twice with distilled water, frozen in liquid nitrogen and stored at –70°C until needed. Cells (30 g) were mixed with 60 g alumina and homogenized for 30 min in a mortar and pestle. The homogenate was extracted with 120 ml 50 mM sodium phosphate buffer (pH 8.0) by stirring for 1 hr, and centrifuged (20000 × *g*, 30 min). The supernatant (115 ml) was heated at 50°C for 10 min, cooled in ice and centrifuged (20000 × *g*, 30 min). Protamine sulfate (2%, pH 7.0) was slowly added to the supernatant (final concentration 0.1%) and stirred for 30 min.

After centrifugation (20000 × *g*, 30 min), the pellet was discarded, and the supernatant was fractionated between 30% and 60% saturation with $(NH_4)_2SO_4$. The pellet was dissolved in 10 ml buffer A (1 mM sodium phosphate, pH 7.0) and dialyzed against 3 L buffer A (16 hr with 3 changes). The centrifuged dialyzate was applied to a DEAE cellulose column (2 × 28 cm), washed with buffer A, and eluted with buffer A containing NaCl in a linear 0-0.5 M gradient. The highest PAL activity fractions were pooled and brought to 70% $(NH_4)_2SO_4$ saturation. After centrifugation, the pellet was dissolved in 4 ml buffer A, dialyzed, centrifuged, and concentrated on a Amicon Centriprep concentrator. The concentrate (1 ml) was applied to a Bio-Gel A-0.5m column (1 × 110 cm) and eluted with 100 ml buffer A. The highest PAL activity fractions were combined, concentrated, and dialyzed. A cooled 6% preparative vertical polyacrylamide slab gel was used for the final purification step (see below for electrophoresis). Two 0.5 cm wide vertical strips were excised from the center of each gel. One was silver stained and the other was divided into 0.5 cm wide segments. Each segment was minced separately in 300 µl K-borate buffer (pH 8.8), shaken for 30 min, ground with a mini-pestle, centrifuged, and assayed for PAL activity and protein.

2.2.4 Enzyme assay

PAL activity was measured radiometrically as previously described (Bernard and Ellis, 1989) using L-[U-^{14}C]-phenylalanine as substrate. Protein concentration was estimated by the dye-binding method (Bradford, 1976), with bovine serum albumin as standard. All assays were done in triplicate. To test the effect of inhibitors and activators on *in vitro* activity of PAL, Bio-Gel purified PAL in PAL assay buffer was mixed with compounds to

be tested. After a 20 min incubation, the mixture was filtered through a Sephadex G25 column and assayed with the standard reaction mixture. For the detection of tyrosine ammonia-lyase (TAL) activity, crude and Bio-gel purified PAL enzyme preparations were assayed by the spectrophotometric method and by the radiometric method using tritium-labeled L-tyrosine as substrate.

2.2.5 Polyacrylamide gel electrophoresis

Vertical slab gels were used for all PAGE analyses. Polyacrylamide gels (5, 6, 7 and 8% (w/v)) were prepared for native PAGE and run with the Laemmli discontinuous buffer system (Laemmli, 1970) in which SDS was omitted from the loading and running buffer. To determine native molecular weight (M_r) of PAL protein, the mobility of PAL at each gel concentration was compared with that of thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). Subunit M_r was determined by mobility in 7.5% SDS-PAGE gels, compared to that of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa). Separated protein bands were detected by Coomassie Brilliant Blue R-250 or silver staining. To detect PAL charge isoforms, the purified PAL was run in a native IEF-PAGE system (Robertson *et al.*, 1987) using pH 3-10 Ampholyte, and silver stained. For isoelectric point (pI) determination, the mobility of the PAL band was compared with that of cytochrome c (horse heart, pI 10.6), myoglobin met (whale sperm, pI 8.3), myoglobin met (horse, pI 7.3), myoglobin met (porcine, pI 6.45), trifluoracetylated myoglobin met (porcine, pI 5.92), azurin (*P. aeruginosa*, pI 5.65,) and C-phycocyanin (*A. nidulans*, pI 4.75 and 4.85).

2.2.6 Antibody production and immuno-affinity chromatography

PAL protein-containg bands excised from preparative PAGE gels were minced in 0.5 ml Freund's adjuvant and New Zealand White rabbits were immunized with one subcutaneous and three intramuscular injections of 50 µg PAL at 10 day intervals. Specificity of the antiserum was assayed by double diffusion in agar and the titer was determined by dot immuno-blot assay. The γ-globulin fraction was isolated from *U. maydis* PAL antiserum by DEAE cellulose chromatography (Johnston and Thorpe, 1982). After incubation of a mixture of purified *U. maydis* PAL and the γ-globulin fraction at 25°C for 1hr, the solution was assayed directly for PAL activity. For affinity purification of the *U. maydis* PAL enzyme, the isolated γ-globulin was coupled to a HiTrap-NHS matrix according to the supplier's protocol. *U. maydis* PAL purified through the DEAE cellulose step was applied to the affinity column and eluted with 0.1 mM Na-citrate buffer, pH 3.0.

2.2.7 Western blot

PAL proteins separated on a 7.5% native or SDS-PAGE gel were electrophoretically transferred to Westran polyvinylidene fluoride membrane(100 V, 1 hr, 4°C). Tris-glycine buffer (25 mM Tris-HCl, 190 mM glycine, pH 8.3) was used as transfer buffer for native PAGE gels. Methanol (10%) was included in the transfer buffer for SDS-PAGE gels. After electro-blotting, the M_r marker lane was cut off the membrane and stained with Coomassie Brilliant Blue R-250. The remaining blot was washed twice with TBS (100 mM

Tris-HCl, 150 mM NaCl, pH 7.5), blocked with 3% BSA in TBS-Tween buffer (0.1% Tween-20 in TBS) for 1 hr and probed with *U. maydis* PAL polyclonal antibodies for 2 hr at room temperature. After washing three times with TBS-Tween buffer, the blot was incubated (1 hr) with an alkaline phosphatase-conjugated goat anti-rabbit antibody. The blot was washed again and the secondary antibody detected with NBT/BCIP staining.

2.3 RESULTS

2.3.1 Purification of PAL

The enzyme was most readily extracted from cultured *U. maydis* cells by grinding the cell mass with alumina powder. The degree of purification and the yield of PAL activity at each purification step are shown in Table 2.1. The elution profiles from the DEAE cellulose and Bio-Gel gel filtration columns showed one major PAL activity peak in each case (Fig. 2.1). Following chromatographic fractionation, the most active fractions were electrophoresed under non-denaturing conditions and the resulting gels were sliced and assayed. PAL was detected as a strongly trailing band of activity (Fig. 2.2), and electrophoresis in gels of different acrylamide concentrations (5-8%) showed that the mobility of the zone containing the highest PAL activity corresponded to a mass of 320±20 kDa (Fig. 2.3). Isoelectric focusing of the chromatographically purified PAL yielded one major protein band with a pI of 6.3 (Fig. 2.4A). No PAL isoforms were detected during either electrophoresis or chromatography, and the purified enzyme gave a negative reaction in the glycoprotein detection assay.

Table 2.1. Purification of PAL from *U. maydis* cells

Purification Step	Total Activity (pkat)	Protein (mg)	Specific Activity (pkat/mg protein)	Purification (-fold)	Recovery (%)
Crude extract	17670	3442	5	1	100
Protamine Sulfate	14102	2174	6.5	1.3	80
30-60% $(NH_4)_2SO_4$	5552	328	17	3.4	31
DEAE Cellulose	5132	50	103	20.6	29
Bio-Gel A-0.5m	3331	2.1	1586	318	19
Preparative PAGE	530	0.090	5889	1178	3

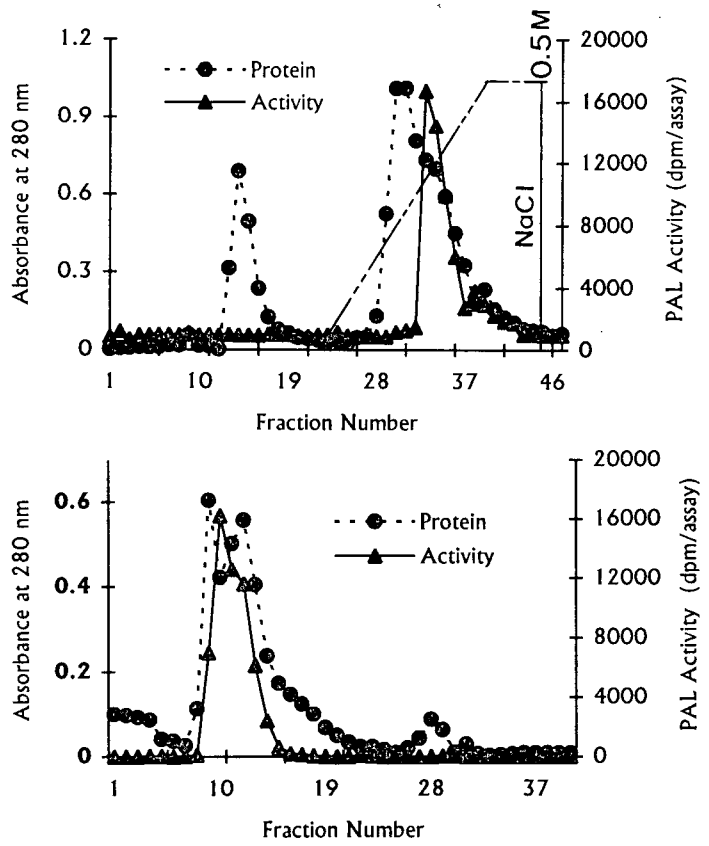


Fig. 2.1. Elution profiles of *U. maydis* PAL and protein after DEAE cellulose chromatography (upper) and Bio-Gel A-0.5m gel filtration (lower).

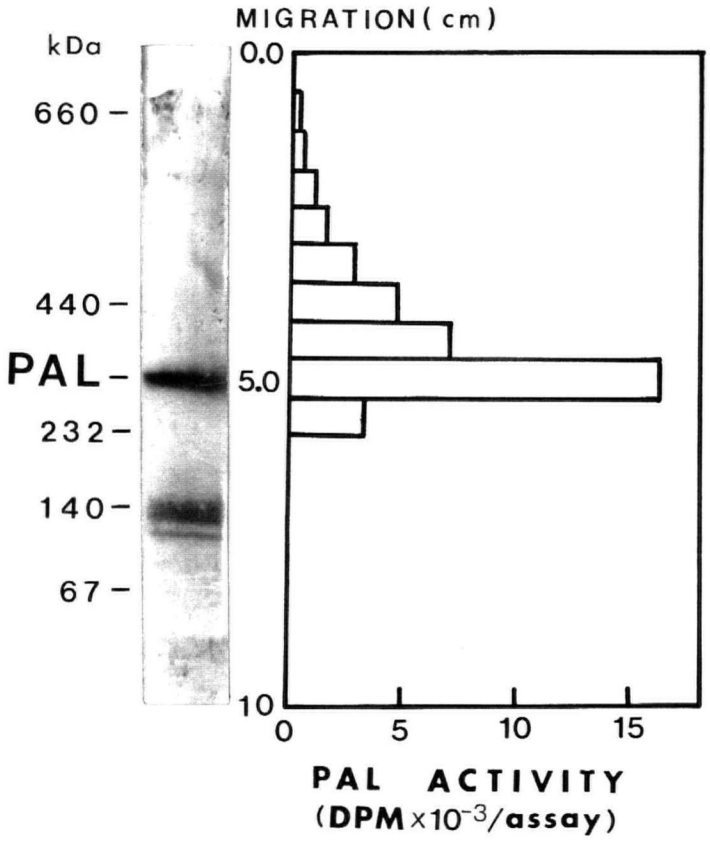


Fig. 2.2. Native PAGE analysis of purified PAL from *U. maydis*.
After preparative native PAGE (6% gel), one lane was silver stained (left) and another was sliced into 0.5 cm sections and assayed for PAL activity (histogram, right).

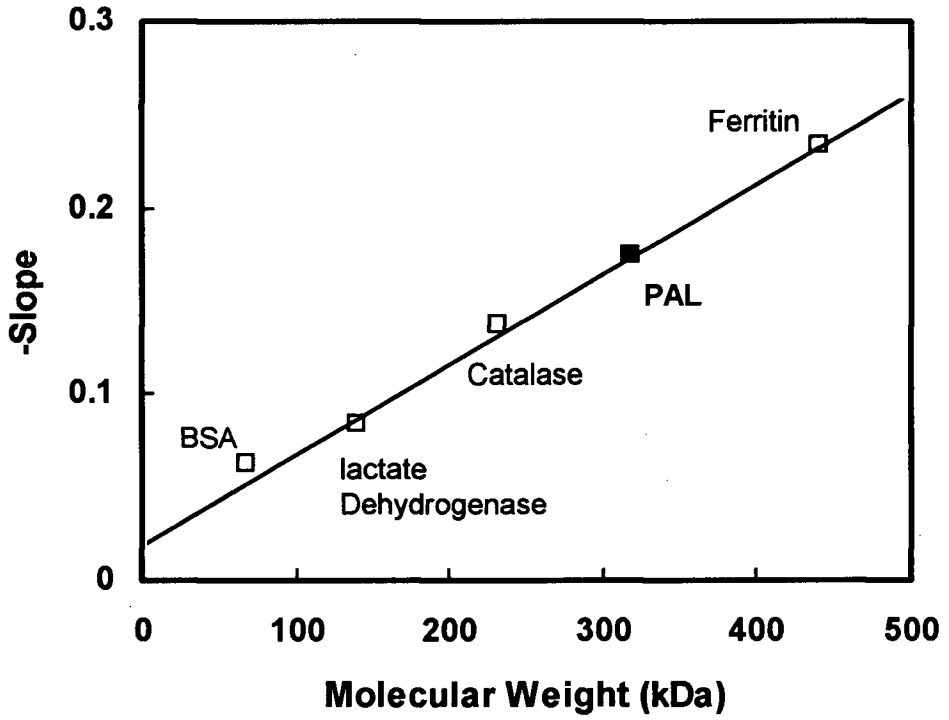


Fig. 2.3. Native molecular weight determination of *U. maydis* PAL by non-denaturing PAGE. The relative mobility of purified *U. maydis* PAL in 6, 7, 8, 9% polyacrylamide gels was compared to that of several high molecular weight standards (described in Materials and Methods). The molecular weight of PAL was determined by interpolation from the regression line.

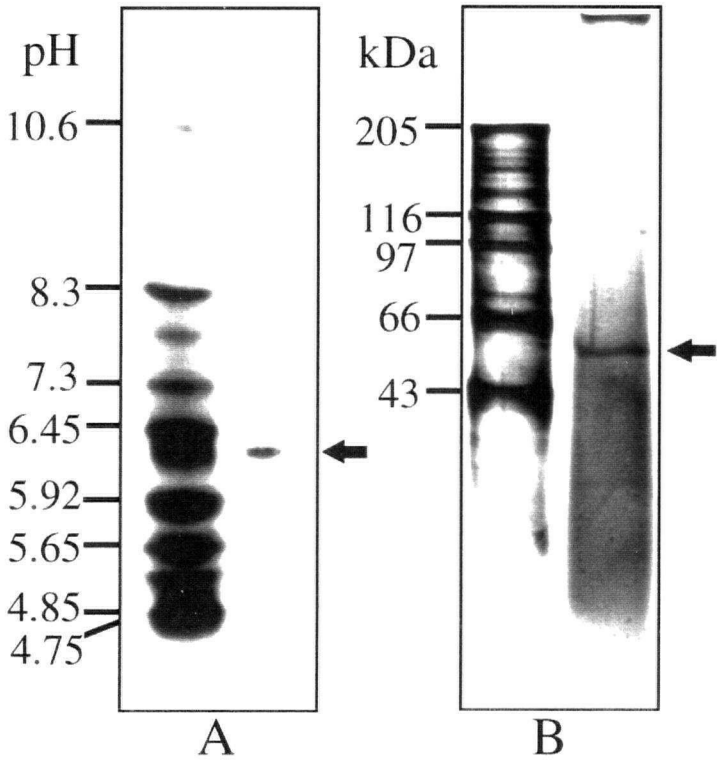


Fig. 2.4. Isoelectric focusing PAGE (A) and SDS-PAGE (B) analysis of *U. maydis* PAL enzyme. Marker proteins (left lane) and enzyme (right lane) eluted from the native PAGE gel slice revealing highest PAL activity in Fig. 2.2, were electrophoresed, side by side, and then silver stained. Arrow indicates the location of PAL enzyme.

When the native PAGE gel slice corresponding to the zone of greatest enzyme activity was electro-eluted and subjected to SDS-PAGE, a single band of protein with a mass of 55 kDa was detected by Coomassie Blue staining. Silver staining, however, revealed three additional minor bands (84, 73, and 30 kDa) (Fig. 2.4B).

2.3.2 Antibody production and characterization

Polyclonal antibodies raised against *U. maydis* PAL protein eluted from preparative non-denaturing PAGE gels produced a precipitin arc in immuno-double diffusion assays, with reaction to proteins from the crude extracts and from PAL-active fractions derived from ion-exchange and gel filtration separations (data not shown). The antibody titre was 12,000-fold as measured by dot immuno-blot assay.

To test the effect of the *U. maydis* PAL antibodies on PAL enzyme activity, the *U. maydis* PAL enzyme was co-incubated with the γ-globulin fraction of the *U. maydis* PAL antiserum. The PAL activity decreased linearly with increasing amounts of antibody, whereas the preimmune antibodies had no effect (Fig. 2.5). Immobilization of the anti-PAL γ-globulin fraction to form an immunoaffinity matrix, and chromatography of partially purified *U. maydis* PAL preparations on this matrix, allowed recovery of low levels of PAL activity. The active fractions migrated as a single protein on non-denaturing PAGE and displayed the same mobility as conventionally purified PAL (data not shown).

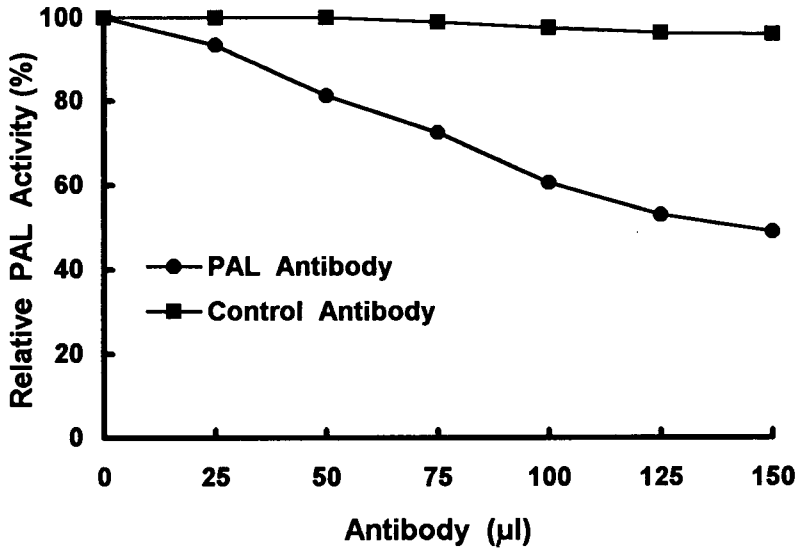


Fig. 2.5. Inhibition of *U. maydis* PAL activity by *U. maydis* PAL antibodies. The purified γ-globulin fractions from *U. maydis* PAL antiserum and from preimmune serum (control antibody) were incubated with PAL enzyme for 1 hr at 25 °C and assayed for PAL activity. Activity is expressed as a percentage of a control not treated with antibodies.

2.3.3 Western blotting

On Western blots, *U. maydis* PAL polyclonal antiserum detected a 320 kDa band in crude cell extracts, and in gel filtration-purified *U. maydis* PAL preparations, as well as an equivalent band in recombinant poplar PAL preparations (Fig. 2.6). When poplar PAL polyclonal antiserum (McKegney *et al.*, 1996) was used as a probe, a weak band at 320 kDa was detected in purified *U. maydis* PAL (Fig. 2.6), but not in crude *U. maydis* cell extracts (data not shown). Activity assay of gel slices excised from the zone corresponding to the 320 kDa protein band detected on Western blots confirmed that the highest PAL activity was associated with this protein (data not shown). SDS-PAGE fractionation and Western blotting (Fig. 2.7) of *U. maydis* PAL preparations at different points in the purification revealed two strong protein bands (80 kDa and 52 kDa) and one weak band (160 kDa), with the 52 kDa protein predominating as the purification proceeded.

2.3.4 Stability

When PAL enzyme in 0.01M Na-phosphate buffer (pH 7.0) was heated for 10 min at 50 °C, cooled to room temperature and assayed at 30°C, no significant activity was lost (Fig. 2.8). Heating at 60°C or 70°C, however, resulted in activity losses of 30% and 100%, respectively (Fig. 2.8). When stored at 4°C, the enzyme retained 95% activity for a week, and 60% activity after one month.

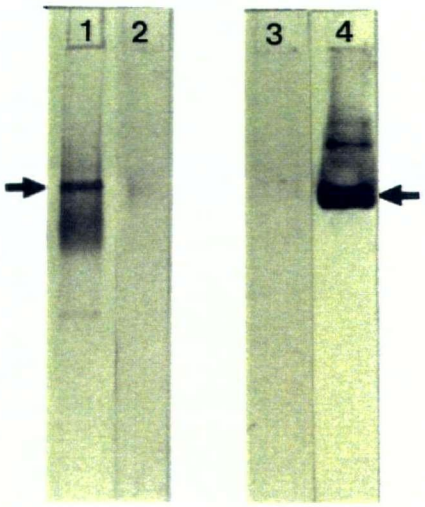


Fig. 2.6. Western blot analysis of reciprocal cross-reactivity between recombinant poplar PAL, *U. maydis* PAL and their respective antisera. *U. maydis* PAL (1 or 10 μg) from Bio-Gel filtration and purified recombinant poplar PAL (1 or 10 μg) (McKegney *et al.*, 1996) were separately electrophoresed in 7.5% native polyacrylamide gels and electro-blotted onto PVDF membrane. The blots were probed with either *U. maydis* PAL antiserum (1 μg sample of *U. maydis* PAL and 10 μg sample of poplar PAL), or poplar PAL antiserum (10 μg sample of *U. maydis* PAL and 1 μg sample of poplar PAL). After being probed with alkaline phosphatase-conjugated secondary antibodies, both the blots were developed with NBT/BCIP. Lane1: 1 μg *U. maydis* PAL/*U. maydis* PAL antiserum1:10^4. Lane 2: 10 μg poplar PAL/*U. maydis* PAL antiserum1:10^3. Lane 3: 10 μg *U. maydis* PAL/poplar PAL antiserum1:10^3. Lane 4: 1 μg poplar PAL/poplar PAL antiserum1:10^4. Arrow indicates the location of PAL enzyme.

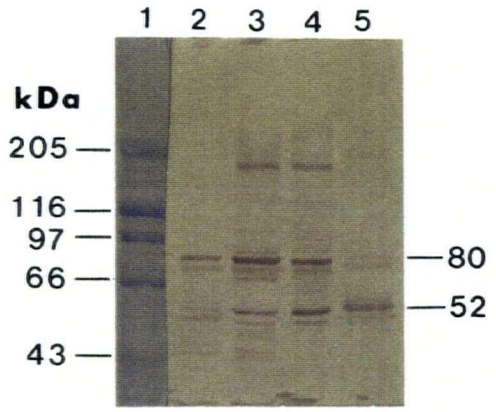


Fig. 2.7. Western blot analysis of *U. maydis* PAL subunit sizes at different stages of enzyme purification. Samples of PAL were taken from different purification stages, separated in 7.5% SDS polyacrylamide gels, and electro-blotted onto PVDF membrane. The molecular weight marker lane in the blot was stained with Coomassie Blue. Other lanes in the blot were probed with *U. maydis* PAL antibodies and alkaline phosphatase-conjugated secondary antibodies, followed by color development with NBT/BCIP. Lane 1: molecular weight markers. Lane 2: crude extract. Lane 3: 30-60% ammonium sulfate precipitate. Lane 4: Bio-Gel A-0.5m eluate. Lane 5: electro-eluate from a single band on a preparative native PAGE gel.

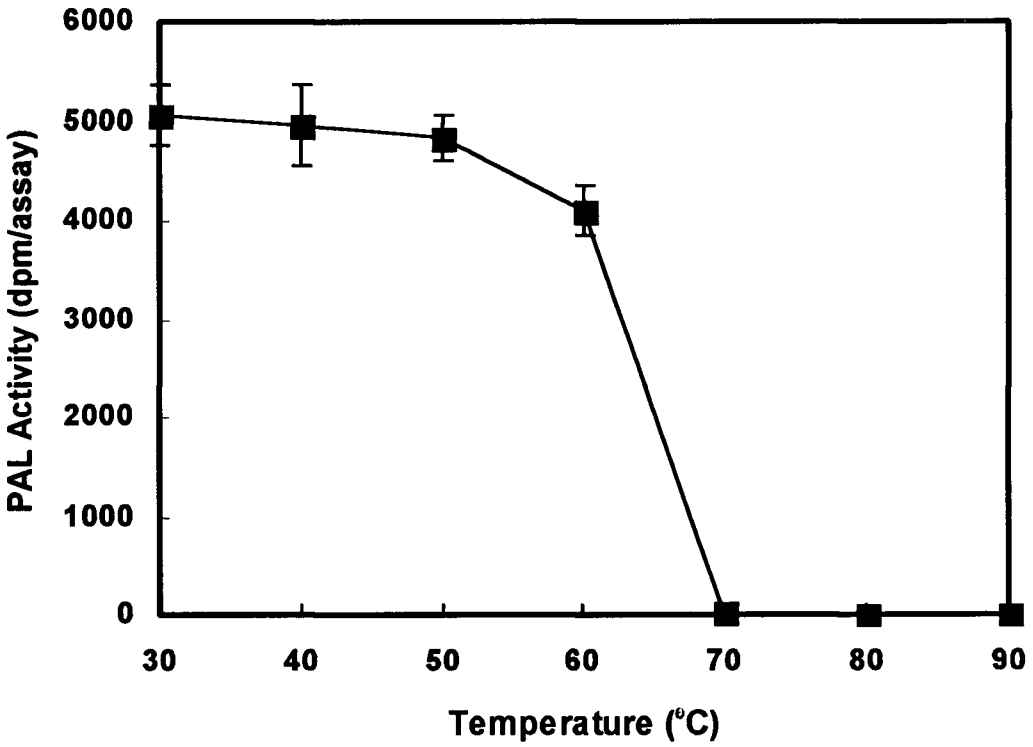


Fig. 2.8. Effect of temperature on *U. maydis* PAL activity. To measure temperature stability, the enzyme was preincubated at each different temperature for 10 min and cooled to room temperature for 30 min, and assayed at 30°C.

2.3.5 pH optimum

The optimal pH for PAL activity was pH 8.8-9.2 (Fig. 2.9). The enzyme displayed 50% of maximum activity at pH 7.2.

2.3.6 Catalytic properties

U. maydis PAL partially purified by DEAE cellulose ion-exchange chromatography displayed normal Michealis-Menten kinetics (Fig. 2.10). The apparent K_m value for L-phenylalanine was 1.05 mM, and the V_{max} was 51 pkat. mg^{-1}. The enzyme displayed no detectable activity against L-tyrosine using either the spectrophotometric or radiometric assay methods.

2.3.7 Inhibitors and activators

U. maydis PAL was readily inactivated by carbonyl reagents such as NaCN and $NaBH_4$ (Table 2.2), as well as by the sulfhydryl reagent, ρ-chloromercuribenzoate. The enzyme was moderately sensitive to its reaction product, *t*-cinnamic acid (K_i = 0.41 mM), and to the product of cinnamic acid hydroxylation, ρ-coumaric acid, but the synthetic substrate analogue, 2-aminoindan-2-phosphonic acid (AIP) (Zon amd Amrhein, 1992), was a very effective inhibitor (K_i = 0.33 μM).

None of the metal ions tested produced any enhancement of PAL activity, but heavy metal ions generally inhibited the enzyme (Table 2.3). Treatment with EDTA was not

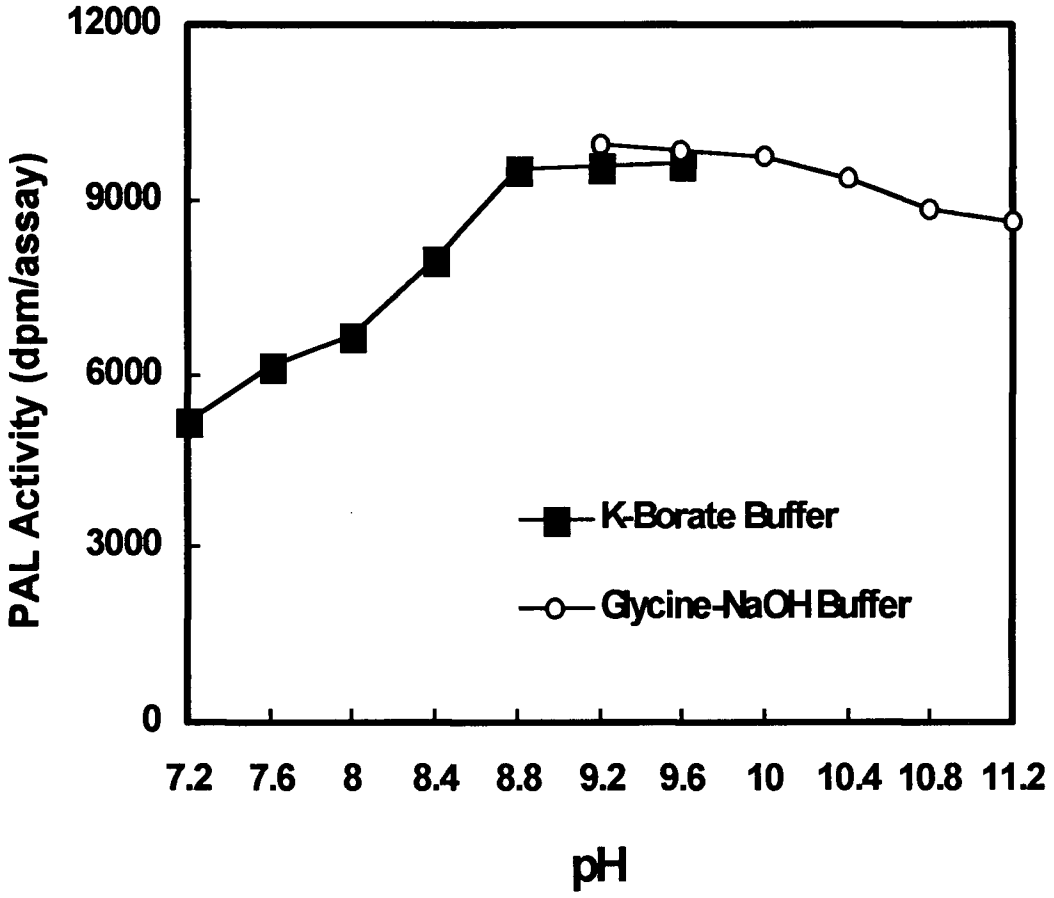


Fig. 2.9. Effect of pH on *U. maydis* PAL activity.
To measure the optimum pH, the enzyme was preincubated at each different pH for 30 min and reacted with substrate for a subsequent 30 min at 30°C.

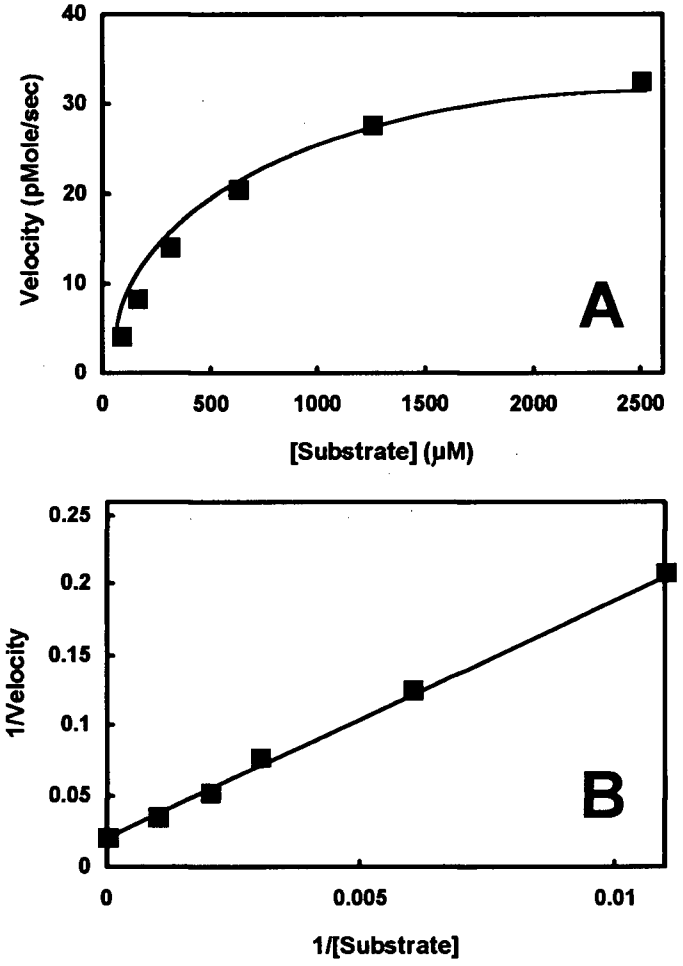


Fig. 2.10. Kinetic analysis of partially purified *U. maydis* PAL. The most active fractions from DEAE cellulose ion-exchange chromatographed PAL were analyzed. Data were plotted on Michaelis-Menten (A) and Lineweaver-Burke (B) plot.

Table 2.2. Effect of chemical modification reagents and substrate analogues on *U. maydis* PAL activity

Inhibitor	Concentration	Relative Activity (%)
None	–	100
NaCN	5 mM	0
$NaBH_4$	5 mM	13
Phenylhydrazine HCl	5 mM	91
Iodoacetate	5 mM	109
N-Ethylmaleimide	5 mM	32
p-Chloromercuribenzoate	1 mM	11
β-Mercaptoethanol	25 mM	40
AIP	0.1 μM	55
	10 μM	1
t-Cinnamic acid	1 mM	75
	10 mM	8
p-Coumaric acid	10 mM	97
	30 mM	0

Table 2.3. Effect of metal ions and chelators on *U. maydis* PAL activity

Chemicals	Concentration (mM)	Relative Activity (%)
None	–	100
$AgNO_3$	5	3
$Cd(CH_3CO_2)_2$	10	38
$CoCl_2$	10	62
CuCl	6	6
$CuCl_2$	3	1
$HgCl_2$	1	0.5
$ZnCl_2$	10	54
8-Hydroxyquinoline	12.5	15

$CaCl_2$, $FeSO_4$, $MgCl_2$, $MnCl_2$, $Na_2Cr_2O_7$, NaF, and EDTA at 10 mM concentrations did not reduce activity below 90% of control.

inhibitory, but the copper chelator, 8-hydroxyquinoline (12.5 mM), produced a moderate level of inhibition. PAL inhibition produced by Hg^{2+} could be partially reversed by treating the inhibited enzyme with β-mercaptoethanol, but 8-hydroxyquinoline was unable to reverse the inhibition produced by Cu^{+} or Cu^{2+} ions. Although PAL requires a low level of a thiol protectant for long-term stability, high concentrations (>25 mM) of β-mercaptoethanol also inhibited the enzyme.

2.4 DISCUSSION

Phenylalanine ammonia-lyase has been purified to varying degrees from numerous higher plant species but only from a limited number of fungi (Schomburg and Salzmann, 1990). It is a relatively low abundance protein and obtaining homogeneous preparations usually requires extensive fractionation of initial cell extracts. PAL is also prone to degradation during purification, and this lability can generate confusion concerning the native and subunit structure of the enzyme.

The estimated molecular mass of native *U. maydis* PAL (320 kDa) is similar to most other known PAL enzymes, which typically range in mass from 300 to 340 kDa, although exceptions have been reported for PAL from *Streptomyces* (226 kDa) (Emes and Vining, 1970), strawberry (266 kDa) (Given *et al.*, 1988), and *Alternaria* (556 kDa) (Pridham and Woodhead, 1974). There is convincing evidence from studies of the heterologous expression of both fungal and plant PAL cDNAs that PAL is normally a homotetrameric protein consisting of four copies of the same gene product (Orum and Rasmussen, 1992; Appert *et al.*, 1994). This implies that the subunit of the *U. maydis* enzyme should have a mass of 80 kDa and a protein of this size is prominent on Western blots of preparations obtained at various points during the purification procedure (Fig. 2.7). The Western blots show, however, that the original 80 kDa subunit population is accompanied by a strongly immunoreactive 52 kDa fragment throughout the normal purification process. The most highly purified active *U. maydis* PAL preparations (Fig. 2.2) consist of holoenzyme that yields almost exclusively this smaller fragment upon denaturation (Fig. 2.4.B, Fig. 2.7).

It is interesting to note that the undenatured enzyme appears to retain its native M_r even when the results of SDS-PAGE and Western blot analysis suggest that most of the subunits in the population have been cleaved to yield a discrete, substantially smaller, polypeptide fragment. This behavior, which has also been observed in other systems (Bolwell *et al.*, 1986; Dubery and Smit, 1994), implies that the cleavage fragments remain firmly bound within the tetrameric structure of PAL. Heterotetrameric quaternary structures for PAL have been reported from *Rhizoctonia* (Kalghatgi and Subba Rao, 1975) and sunflower (Jorrin *et al.*, 1988), but partial degradation of the enzyme cannot be ruled out in either case.

The *U. maydis* PAL antiserum has a high affinity for both the native fungal enzyme and its denatured subunits. The *U. maydis* PAL antiserum was also able to recognize a higher plant PAL protein, although the reaction was far weaker than with the homologous protein. The reciprocal cross-reaction between poplar PAL antiserum and *U. maydis* PAL protein (Fig. 2.6) was similarly detectable but weak. While antisera have been raised to several plant and fungal PAL proteins (e.g. alfalfa (Jorrin and Dixon, 1990), bean (Bolwell *et al.*, 1985), and *Mycosphaeria maculans* (Dahiya, 1993)), I am unaware of any other reports on the degree of cross-reactivity between plant and fungal PAL. The cross-reactivity observed here is consistent with the presence of some short stretches of highly conserved sequence within plant and fungal PAL genes (Taylor *et al.*, 1990), but the weakness of the cross-reaction also emphasizes the extent to which the structures of the *Ustilago* and higher plant PAL proteins may have diverged.

Many of the physico-chemical properties of *U. maydis* PAL are typical of this enzyme from other sources (e.g. M_r, pH optimum, sensitivity to carbonyl reagents, requirement for thiol protectants). Other properties are more unusual, including the temperature stability, a relatively high apparent K_m for phenylalanine, and a pI of 6.3. PAL proteins from other species have been reported to display apparent K_m values ranging from 11 µM to 450 µM L-phenylalanine, while the reported pI values range from 2.5 (Neumann *et al.*, 1991) to 5.75 (Campbell and Ellis, 1992).

PAL preparations from some sources, including sweet potato (Minamikawa and Uritani, 1965), tobacco (O'Neal and Keller, 1970), and yeast (Parkhurst and Hodgins, 1972), are strongly inhibited by the reaction product, *t*-cinnamic acid, but cinnamic acid has comparatively little effect on the activity of the *U. maydis* enzyme. In this, the *Ustilago* PAL resembles the enzyme from *Streptomyces* (Emes and Vining, 1970), alfalfa (Jorrin and Dixon, 1990), pine (Campbell and Ellis, 1992), and tomato (Bernard and Ellis, 1991). Like most known PALs, the *Ustilago* enzyme is sensitive to heavy metal ions and displays no metal ion requirement for catalytic activity (Table 2.3). Again, there is considerable interspecific variability in these responses, since Hg^{2+} completely inhibited the *U. maydis* PAL enzyme at 0.5 mM, whereas sunflower PAL was less strongly inhibited, retaining some activity even in 2 mM Hg^{2+} (Camm and Towers,1973). Similarly, Cu^{2+} (1mM), was a potent inactivator of the *U. maydis* PAL but had no effect on the sweet potato enzyme (Minamikawa and Uritani, 1965). The significance, if any, of these patterns will become apparent only when more is learned about the detailed structure of PAL proteins and their active site(s).

CHAPTER THREE

Cloning and Characterization of the PAL Gene from *Ustilago maydis* : Relationships with Other Phenylalanine Ammonia-lyases and with Bacterial Histidine Ammonia-lyase.

3.1 INTRODUCTION

Since its discovery by Koukol and Conn (1961), L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has been studied extensively both from the perspective of its biochemistry and its molecular biology. Plant PAL activity levels respond to an array of stresses such as wounding, chemicals, microbial infection, and UV light (Jones,1984). By catalyzing the conversion of L-phenylalanine to *t*-cinnamic acid, the enzyme regulates the biosynthesis of a huge class of phenylpropane skeleton-based natural products (Hahlbrock and Scheel, 1989). Partial or full-length genes encoding PAL have been isolated from a number of plant species, mostly from cultivated crops but including trees and *Arabidopsis*. PAL is encoded by a small gene family in most plants except in potato, where PAL is encoded by 40-50 genes (Joos and Hahlbrok, 1992), and in loblolly pine (*Pinus taeda*), where PAL is reported to be encoded by only one gene (Whetten and Sederoff, 1992). In general, PAL coding sequences range from 700 to 725 amino acids in length, with some exceptions such as 682 amino acids in poplar PAL (Osakabe *et al.*, 1995), 694 in *Arabidopsis* PAL (Wanner *et al.*, 1995), and 752 in loblolly pine PAL (Zhang and Chiang, GenBank accession no. U39792). The fungal PAL genes sequenced to date contain five or six introns (Anson *et al.*, 1987; Vaslet *et al.*, 1988), in

contrast to plant PAL sequences which generally contain only one intron. Two exceptions are the *Arabidopsis* PAL gene, which has two introns (Wanner *et al.*, 1995), and a *Pinus banksiana* PAL gene which appears to lack introns (Campbell, 1991; S. Butland, personal communication). The strict sequence conservation in the second exon of PAL genes among different plant species implies that important catalytic sites of the enzyme may be encoded in this region (Cramer *et al.*, 1989; Joos and Halbrock, 1992).

While PAL has been well-studied in plants, relatively little is known about PAL in fungi. The enzyme has been purified from a limited number of fungi (Schomburg and Salzmann, 1990), and fungal PAL genes have only been isolated from the two closely related red yeasts, *Rhodosporidium toruloides* and *Rhodotorula rubra* (Anson *et al.*, 1987; Vaslet *et al.*, 1988). The role of PAL in fungi is an area which is largely unexplored. Investigation of the structure, function, and regulation of fungal PAL will enable us to better understand the role PAL plays in fungal biology and may help us learn more about the mechanisms by which PAL is controlled in different eukaryotic systems such as plants and fungi.

The phytopathogenic fungus *Ustilago maydis* provides a good model system in which address the above question. This species has been used to study many aspects of eukaryotic genetics, including recombination mechanisms (Holliday *et al.*, 1974), nitrogen metabolism (Lewis and Fincham, 1970), and developmental regulation (Froeliger and Kronstad, 1990). Molecular genetic approaches are also possible, with the development of such molecular tools as efficient transformation (Wang *et al.*, 1988), autonomously replicating vectors (Tsukuda *et al.*, 1988), genes for selection markers

(Wang *et al.*, 1988; Banks and Taylor, 1988), and gene disruption (Kronstad *et al.*, 1989). The availability of *U. maydis* PAL protein data and anti-PAL antibodies (chapter 2) provided a unique opportunity to pursue the molecular genetic studies of PAL in a versatile fungal species largely unrelated to the red yeasts.

Histidine ammonia-lyase (HAL, EC 4.3.1.3) and PAL are the sole members of the dehydroalanine class of ammonia-lyases. HAL catalyzes the nonoxidative deamination of L-histidine to *trans*-urocanic acid and ammonia. This is the first step in the catabolism of L-histidine, which is eventually metabolized to L-glutamic acid. HAL occurs generally in bacteria and animals but may occur universally, since HAL activity has been detected in a few fungi (Hollman and Dekker, 1971; Polkinghorne and Hynes, 1975) and plants (Ruis and Kindl, 1970 and 1971; Kamel and Maksoud, 1978). In bacteria, HAL allows utilization of histidine as a carbon and/or nitrogen source. In mammals, HAL is under complex regulation that controls activity in a developmental, hormonal, and tissue-specific manner (Feigelson, 1973; Lamartiniere, 1979; Armstrong and Feigelson, 1980).

For both HAL and PAL there is strong indirect evidence that the enzyme active site contains a catalytically essential dehydroalanine (DHA) residue (Hanson and Havir, 1970; Hodgins, 1971; Consevage and Phillips, 1985). Recently, the precursor of the dehydroalanine residue in HAL and PAL has been identified as serine located at position 143 in *Pseudomonas putida* HAL (Hernandez *et al.*, 1993; Hernandez and Phillips, 1994; Langer *et al.*, 1994), 254 in rat HAL (Taylor and McInnes, 1994), and 202 in parsley (Schuster and Retey, 1994) and in poplar PAL (McKegney *et al.*, 1996). Substantial amino acid sequence conservation between HAL (from rat, *Bacillus subtilus*, and *P.*

putida) and PAL (from yeast, parsley, and kidney bean) was noted by Taylor *et al.* (1990). On the basis of functional similarity, the presence of DHA at the active site, and the observed overall sequence conservation, these researchers proposed that the present-day genes for HAL and PAL could have evolved from a common ancestral ammonia-lyase gene.

Since this proposal has been made, more HAL and PAL genes have been cloned from other sources. The relatedness of the two enzymes has been inferred from recent studies of their mechanism of action (Schuster and Retey, 1995) and the comparison of some coding sequences (Wu *et al.*, 1992), but many questions about these DHA ammonia-lyases remain unresolved, including the mechanism of active site formation and the functional significance of their multimeric structure. At this point, there have been no X-ray crystallographic analyses reported that might provide insight into these issues. However, a better understanding of the structural features common to both HAL and PAL should provide useful insights into the way an active DHA ammonia-lyase is formed.

This chapter describes the isolation and sequencing analysis of a *U. maydis* genomic clone which contains a gene encoding PAL, and a comparative analysis of its protein sequence with some of the published PAL and HAL protein sequences. Immunological evidence regarding the relatedness of the two ammonia-lyases is also presented.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms and media

Escherichia coli strains employed in this study are listed in Table 3.1. *E. coli* strains were grown in LB or TB medium with appropriate antibiotics as described (Sambrook *et al.*, 1989) and *Ustilago maydis* 518 strain was grown on potato dextrose broth or agar (PDB or PDA, Difco) and on complete medium (CM) described by Holliday (1974). *Rhodosporidium toruloides* UBC 75-0941 was grown in malt extract agar (Difco).

3.2.2 Materials

Restriction and DNA modifying enzymes were purchased from Bethesda Research Laboratories (BRL), Boehringer Mannheim and Pharmacia. The 1 Kb DNA Ladder, RNA size markers and agarose were obtained from GIBCOBRL. [α-^{32}P]dATP was from Amersham. Zeta-Probe blotting membranes for Southern and Northern blot analysis, Bio-Gel A-0.5m agarose beads, alkaline phosphatase-conjugated goat anti-rabbit antibody, SDS-PAGE molecular markers, and BCIP (5-bromo-4-chloro-3-indoyl phosphate ρ-toluidine salt)/NBT (ρ-nitro blue tetrazolium chloride) were purchased from Bio-Rad (Mississauga, Canada). Westran PVDF (polyvinylidine difluoride) membrane was purchased from Schleicher & Schuell (Keene, U.S.A.). Freund's adjuvant and other chemicals were purchased from Sigma. Degenerate oligonucleotide primers were synthesized by Nucleic Acids and Protein Services (NAPS) unit, Biotechnology Lab.,

Table 3.1. *Escherichia coli* strains used in this study

Strain	Genotype
DH5α	*endA*1, *hsdR*17(r_k^-, m_k^+), *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, *ΔlacU*169 (ϕ80d*lacZ*ΔM15)
DH10B	F' *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80d*lacZ*ΔM15), *ΔlacX*74, *deoR*, *recA*1, *araD*139, *Δ(ara, leu)*7697, *galU*, *galK*, λ^-, *rpsL*, *endA*1, *nupG*
HB101	*supE*44, *hsd*S2(r_B^-, m_B^-), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2 *rpsL*20, *xyl*-5, *mtl*-1
SOLR	e14 $^-$ (*mcrA*), Δ(*mcr*CB-*hsd*SMR-*mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn5(*kan*^{r}), *uvrC*, *lac*, *gyrA*96, *relA*1, *thi*$^-$1, *end*A1, λ^R, [F′, *proAB*, *lacI*^{q}ZΔM15], *Su*$^-$ (non-suppressing)
XL1-Blue	*endA*1, *hsdR*17(r_k^-, m_k^+), *supE*44, *thi*$^-$1, lambda$^-$, *recA*1, *gyrA*96, *relA*1, *lac*$^-$, [F′, *proAB*, *lacI*^{q}ZΔM15, Tn10(*tet*^{R})]

University of British Columbia and specific primers were synthesized using an Oligo-1000M DNA Synthesizer (Beckman).

3.2.3 Nucleic acids manipulation

Protocols used for recombinant DNA manipulations and for small scale, boiling-lysis, plasmid preparations are from Sambrook *et al.* (1989). DNA mini-preps were done by the method of Zhou *et al.* (1990). Restriction digests and ligations were carried out according to manufacturers' instructions using commercially supplied enzymes and buffers. pUC19 vector and DH5α competent *E. coli* cells were used for subcloning. Genomic DNA of *U. maydis* and *R. toruloides* was isolated as described by Wang *et al.* (1988). *U. maydis* RNA was isolated using the hot phenol method as described in Ausubel *et al.* (1995). Concentration of nucleic acids was determined by measuring the absorbance at 260 nm using a GeneQuant nucleic acids quantifier (Pharmacia).

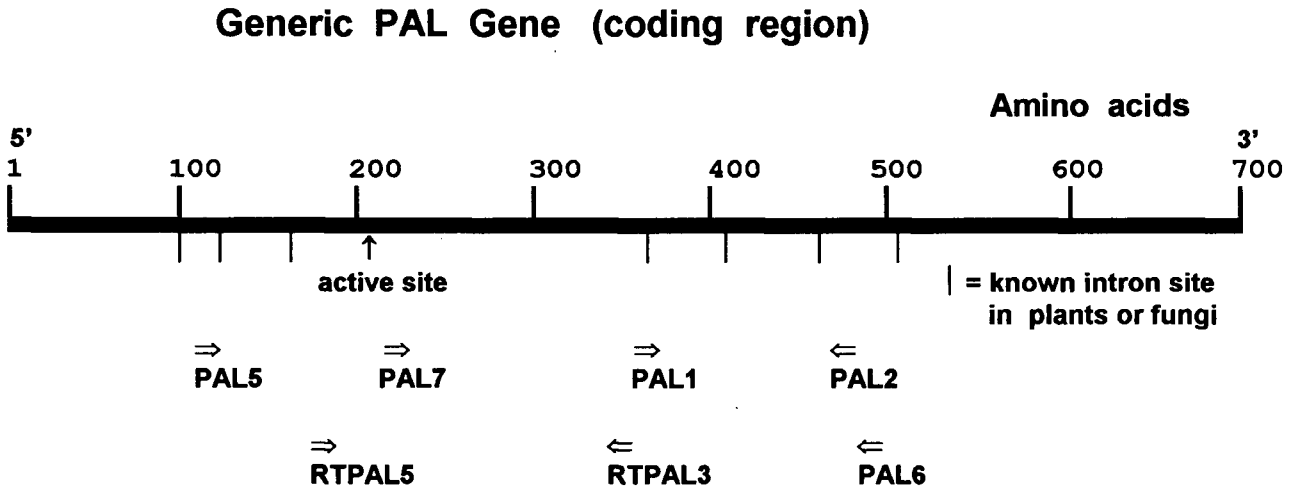
3.2.4 cDNA library screening

A λZAPII cDNA expression library (Gold *et al.*, 1994a) constructed from poly(A)$^+$ RNA from *U. maydis* cells grown on mating media was used as the source for PAL cDNA clone screening. *E. coli* XL-1 blue cells (Stratagene) were infected with the phage library, *lacZ* fusion protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), and positive clones were selected by immunoscreening with antibodies specific for *U. maydis* PAL. Immunoscreening was carried out with a

*pico*Blue immunoscreening kit (Stratagene) using alkaline phosphatase-conjugated goat anti-rabbit antibodies with BCIP and NBT as substrates for color detection. Putative positive plaques were isolated through several rounds of screening and processed to homogeneity. The plaques giving a positive signal were excised *in vivo* with the EXASSIST helper phage (Stratagene) and rescued to produce the pBluescript SK- plasmid using the *E. coli* strain, SOLR (Stratagene) which prevents coinfection of the helper phage, according to the Stratagene EXASSIST/SOLR system protocol. Transformed cells were stored as glycerol stocks at -70 °C.

3.2.5 Design of oligonucleotide primers

The DNA coding sequences and amino acid sequences for known PAL genes were compared using PC/Gene software (IntelliGenetics) and assessed to identify consensus regions which could be used as PCR primers. The sequences employed for comparison were four plant PAL sequences: *Ipomea batatas* (Genbank/EMBL accession M29232), *Oryza sativa* (accession X16090), *Phaseolus vulgaris* (accession M11937), and *Petroselinum crispum* (Lois *et al.*,1989), and two yeast PAL sequences: *Rhodotorula rubra* (accession X13094) and *Rhodosporidium toruloides* (accession M28261). Degenerate primers (PAL1, 2, 5, 6, and 7) and yeast PAL-specific primers (RTPAL3 and 5) were synthesized based on consensus region sequences (Fig. 3.1). 5' *Eco*RI or 3' *Xba*I restriction sites were incorporated in the primers to allow unidirectional cloning of amplified products.



PAL1 5'- CGGAATTC [AC]GIACI[TA][CG]ICCICA[AG]TGG[TC]T -3'
EcoRI
PAL2 5'- GCTCTAGA TG[TC]TCIGCI[GC][AT][TC]TGIAC[AG]TG -3'
XbaI
PAL5 5'- CGGAATTC TACGG[TC]GTCACIAC[TC]GGITT[TC]GG -3'
EcoRI
PAL6 5'- GCTCTAGA TTIAC[GA]TC[CT]TG[GA]TT[AG]TG[CT]TG -3'
XbaI
PAL7 5'- CGGAATTC ATC[AT]CGGCGTCGGGGGA[CT][CT]T 3'
EcoRI
RTPAL3 5'- GCTCTAGA CGCAAGGGGTAGCGGTCCT -3'
XbaI
RTPAL5 5'- CGGAATTC CACTCGGCTGTCCGCCTCGT -3'
EcoRI

Fig. 3.1. Schematic diagram indicating generic structure of a PAL gene and locations of primers (⇒) used in PCR to amplify parts of the *U. maydis* PAL gene. Degeneracy of primers is indicated by square brackets and the inosine nucleotide is indicated as I.

3.2.6 PCR amplification of a putative *U. maydis* PAL gene fragment

U. maydis genomic DNA and λZAPII-cDNA from the cDNA library were prepared using the yeast DNA isolation method and the plate lysate method, respectively, as described in Current Protocols in Molecular Biology (Ausubel *et al.*,1995). PCR reactions (50 μl) contained 200 ng of *U. maydis* genomic DNA or 20 ng of λZAPII-cDNA as template, 40 pM PAL primer pairs, 50 μM dNTPs, 1X reaction buffer (10mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl), and 1 unit *Taq* polymerase (Appligene). Each reaction was overlayed with 1 drop of mineral oil. The combinations of primers and templates for PCR reactions are listed in Table 3.2. PCR reactions were incubated in a Techne PHC2 thermal cycler. The thermal cycling conditions were as follows: initial denaturation, 94°C/4 min; followed by 35 cycles of denaturation, 94°C/1 min; annealing, 55°C/1 min; and primer extension, 72°C/2 min. The reaction product (30 μl) was analyzed by electrophoresis on a 1% agarose gel in Tris-acetate EDTA (TAE) buffer including ethidium bromide, visualized under UV light, and documented with an Image Analyzer (IS-500 Digital Imaging System, Alpha Innotech Co.). After identification of the desired size band from amplification products, this band was excised, purified using the GeneCleanII (Bio101) kit, and subcloned for further analysis.

3.2.7 Cloning of putative *U. maydis* PAL PCR product

The purified PCR amplified product was digested with *Eco*RI and *Xba*I, ligated with pUC19 vector digested with the same enzymes, and transformed into DH5α *E. coli* cells. Transformant colonies were screened using blue/white selection based on the insertional

inactivation of β-galactosidase. Twenty white colonies were picked for plasmid DNA minipreps (Zhou *et al.*, 1990), digested with *Eco*RI and *Xba*I, and subjected to electrophressis on 1% agarose gels to verify the insert presence and size. The identities of transformants with an insert size corresponding to the amplified PAL DNA fragment were confirmed by Southern blot analysis of plasmid DNA from the selected clone using the PCR-amplified PAL fragment as a probe. The amplified putative *U. maydis* PAL fragment DNAs in the confirmed clones were sequenced and their sequences were compared with other known PAL sequences. A fragment showing substantial sequence homology with yeast PALs was used as a probe to screen a *U. maydis* genomic-cosmid library.

3.2.8 DNA sequencing

Double-stranded plasmid DNA or cosmid DNA from selected clones was prepared following a mini alkaline lysis/PEG precipitation procedure (Ausubel *et al.*, 1995). Both strands of the insert were sequenced with M13 universal and/or synthetic oligonucleotide primers as needed to extend the sequence. Sequencing reactions were carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) using ABI AmpliTaq dye termination cycle sequencing chemistry according to manufacturer's instructions (Applied Biosystems) and nucleotide sequences were analyzed on Applied Biosystems' ABI 373 DNA sequencer (NAPS unit) or ABI 310 capillary DNA sequencer (at the former Pacific Agriculture Research Centre, Agriculture and Agri-Food Canada, Vancouver Station).

3.2.9 Sequence comparison and analysis

Database searches for sequence homology and comparisons of nucleotides and amino acids were performed on sequences from Genbank, EMBL, PDB, PID, PIR, and SWISS-PROT using BLAST (Altschul *et el.*, 1990). Nucleotide and amino acid sequence analysis of *U. maydis* PAL was done using PC/Gene software.

3.2.10 Genomic-cosmid library screening

A cosmid library had been constructed earlier (Barrett, 1992) in the *U. maydis* vector pJW42, using total genomic DNA isolated from *U. maydis* strain 518. The total DNA was partially digested with *Sau*3AI to produce 30 to 40 kb fragments and these fragments were ligated into the *Bam*HI site of pJW42. After being packaged *in vitro* using the Gigapack packaging extract (Stratagene), ligated DNA was transfected into *E. coli* DH5α strain. Approximately 10^6 individual transfectants were randomly pooled from transfection plates (LB/ampicillin (50 μg/ml) medium), cultured for six hours in LB/ampicillin medium, and harvested for DNA preparation (Ish-Horowicz and Burke, 1981). DNA from these nine divided pools was used as a source of genomic-cosmid library for *U. maydis* genomic PAL screening. To identify the cosmid DNA pool containing a PAL gene, PCR was employed, using nested primers (UMPAL2 and UMPAL3), which had been designed on the basis of sequence information from the PCR-amplified putative PAL DNA fragment. Cosmid DNA from the pools giving a PCR amplification product with the nested primers was transformed into *E. coli* HB101 or DH10B strain by electroporation, using a Bio-Rad Gene Pulser according to the supplier's manual, and transformants were selected on LB/ampicillin plates. The selected transformants were screened

according to Amersham's colony-lift hybridization manual using the PCR-amplified putative *U. maydis* PAL DNA fragment cloned above as a probe. After growing on antibiotic selective medium plates, bacterial DNA was transferred and fixed onto nylon membrane (Hybond N^+, Amersham). Membranes were prehybridized for 1 hour at 68°C and hybridized with the DNA probe overnight at 68°C. After washing with 2XSSC (10 min, twice) at room temperature and 0.1X SSC/0.1% SDS at 68°C (30 min, twice), the membranes were exposed to Kodak X-OMAT film. Colonies giving positive signals on autoradiograms from duplicate membranes were picked and purified to homogeneity through two more rounds of screening. Cosmid DNA from these selected clones was subjected to further analysis by PCR with UMPAL2/UMPAL3 primers to confirm the presence of target template DNA and the confirmed cosmid DNA was used for sequencing analysis.

3.2.11 Southern hybridization

U. maydis genomic DNA was digested overnight at 37°C with various restriction enzymes, fractionated in a 0.7 or 1% agarose gel, denatured in 0.25M HCl for 10 min, and transferred to a Zeta-Probe GT blotting membrane using 0.4M NaOH solution. The membrane was prehybridized at 65°C for 1 hour in 10 ml of hybridization solution (7% SDS, 0.25M Na_2HPO_4, pH 7.2) and transferred to new hybridization solution. After addition of a DNA probe which was labeled with [α-^{32}P]dATP using the Random Primers DNA Labeling System (GIBCOBRL), the membrane was incubated overnight at 65°C. The membrane then washed twice for 30 min each at 65°C in 5% SDS/20 mM Na_2HPO_4,

pH 7.2, followed by washing in the same manner in 1% SDS/20 mM Na_2HPO_4, pH 7.2, and subjected to autoradiography using Kodak X-OMAT AR film.

3.2.12 Total RNA isolation and Northern hybridization

Cells of *U. maydis* grown in complete medium were harvested 16 hr after inoculation, washed two times with sterile DEPC-treated distilled water, and frozen in liquid nitrogen. Total RNA was isolated using the hot phenol extraction method as described by Ausubel *et al.* (1995). Total RNA was fractionated on a denaturing 1% agarose gel (Sambrook *et al.*, 1989) and transferred to Zeta-Probe GT membrane using 10X SSC. The membrane was incubated in prehybridization solution (50% formamide, 0.12M Na_2HPO_4, 0.25M NaCl, and 7% SDS) for 10 min at 42°C and hybridized overnight with a random-primed radiolabeled probe in the same conditions. Following a brief rinse in 2X SSC, the membrane was washed successively in 2X SSPE/0.1% SDS, 1X SSPE/0.5% SDS, and 0.3X SSPE/1% SDS solution at 55°C for 30 min each. A 0.24- to 9.5-kb RNA ladder was used to estimate RNA size. Autoradiography was performed in the same manner as for the Southern blot.

3.2.13 N-terminal and internal peptide sequencing of *U. maydis* PAL

The purified *U. maydis* PAL (chapter 2) was run in 7.5% SDS-PAGE gels (Laemmli, 1970) and subjected to electroblotting onto Immobilon P^{sq}-PVDF membrane (Millipore) using CAPS buffer. After staining with Coomassie blue, the appropriate 80 kD and 52 kD protein bands were excised for N-terminal and internal sequencing analysis, respectively.

Protein sequencing analysis by automated Edman degradation was performed on an Applied Biosystems 470A gas phase sequencer with on-line PTH-HA analysis at the NAPS unit.

3.2.14 Enzymes and antibodies

Partially purified *Pseudomonas fluorescens* HAL, *R. glutinis* PAL, and potato PAL were purchased from Sigma. *P. fluorescens* HAL was further purified from the commercial enzyme by 7.5% native PAGE gel separation and polyclonal antibodies were raised in rabbits. *U. maydis* PAL was purified by $(NH_4)_2SO_4$ precipitation, DEAE ion-exchange chromatography and Bio-Gel A-0.5m filtration (described in chapter 2). Poplar PAL was purified from baculovirus-expressed proteins (Mckegney *et al.*, 1996) by Pharmacia FPLC Mono-Q column. Polyclonal antibodies to *U. maydis* and poplar PAL were raised in New Zealand White rabbits. Anti-alfalfa PAL and anti-*P. putida* HAL polyclonal antibodies were generously provided by R.A. Dixon (The Samuel Robert Noble Foundation, Ardmore, USA) and A.T. Phillips (Pennsylvania State University, University Park, USA), respectively.

3.2.15 Western blots and enzyme Inhibition

The same amounts of protein of each PAL and HAL enzyme were electrophoresed on a 7.5% native PAGE gel, electroblotted onto PVDF membrane and probed with anti-PAL or anti-HAL antiserum. After washing, the membrane was reacted with alkaline phosphatase-conjugated secondary antibodies and immune complexes were detected with NBT/BCIP solution (Young and Davis, 1983). For enzyme inhibition by antiserum,

HAL and PAL were incubated with anti-HAL and anti-PAL antisera for 1hr at room temperature and the enzyme-antiserum reaction mixtures were assayed for HAL and PAL activity. Pre-immune sera were used as controls.

3.2.16 Assay of enzyme activity

HAL assays were done by the spectrophotometric method with L-histidine as a substrate (Rechler and Tabor, 1969) and PAL assays were done by the radiometric method with L - [*U*-^{14}C]-phenylalanine as a substrate (Bernards and Ellis, 1991). Protein concentrations were determined using the Bio-Rad dye-binding reagent with microtiter plates, based on the Bradford method (Bradford, 1976).

3.3 RESULTS

3.3.1 PCR amplification of a *U. maydis* PAL sequence from genomic DNA

Initial attempts at screening available *U. maydis* cDNA libraries with either *U. maydis* PAL polyclonal antibodies (chapter 2), or with a yeast *R. toruloides* PAL DNA fragment (PCR amplified from *R. toruloides* genomic DNA with primer RTPAL3 and RTPAL5 in this study), or with a *Pinus banksiana* PAL cDNA clone (Lam, 1995) as probe were unsuccessful. This failure may have been due to the low abundance of the PAL message in the mRNA pools from which the libraries were made or to low nucleotide sequence homology between the target *U. maydis* cDNA in the cDNA library and the

heterologous probes. This led me to choose a PCR approach, generating primers (Fig. 3.1) based on conserved amino acid sequences among several PAL proteins. Among the combinations of primers and template DNA tested (Table 3.2), only the PAL7 and RTPAL3 primer combination with genomic DNA as template amplified a fragment of the expected size (about 0.45 kb) (Fig. 3.2). This DNA fragment was isolated, subcloned into pUC19, transformed into DH5α *E. coli* cells, and sequenced. A BLAST database search showed that the 0.45 kb-sequence has homology with most known PAL genes, especially with yeast PALs (40% amino acid identity). Therefore, the PCR-amplified DNA was considered a putative *U. maydis* PAL DNA fragment and was used to screen a genomic-cosmid library.

3.3.2 Isolation and sequencing of PAL genomic-cosmid clones

Based on the sequence of the above 0.45 kb PCR product, two sequence-specific primers (UMPAL2 5'-ACGCAGCGTTCCGTTGTCTTC-3') and UMPAL3 (5'-AGCTACGTAGCCGGTGCGCTT-3') were synthesized and used for initial screening of nine genomic-cosmid pools. A 0.43 kb fragment was amplified from three pools (pool 1, 7, and 8) out of nine pools (Fig. 3.3). After transformation of DNAs of these three pools into HB101 or DH10B cells, PAL genomic clones were isolated from the transformants by colony-lift hybridization, using the 0.45 kb DNA as probe. One clone was selected for sequencing after rescreening of the above isolated PAL clones by PCR with the UMPAL2/UMPAL3 primers. DNA sequencing of both strands of this clone was performed using synthetic oligonucleotide primers designed from the DNA sequences resulting from each sequencing run (Fig. 3.4).

Table 3.2. PCR primer combinations tested for possible amplification of PAL DNA fragments from *U. maydis* and predicted size of the amplified products

Primer combination	Predicted size (bp)	
	cDNA template	Genomic DNA template
PAL1-PAL2	400	≥ 400
PAL1-PAL6	420	≥ 420
PAL5-PAL2	1100	≥1100
PAL5-PAL6	1120	≥1120
PAL5-RTPAL3	700	≥ 700
PAL7-PAL2	840	≥ 840
PAL7-PAL6	860	≥ 860
PAL7-RTPAL3	450	450
RTPAL5-PAL2	950	≥ 950
RTPAL5-PAL6	970	≥ 970
RTPAL5-RTPAL3	550	550
PAL1-M13	1050	
PAL5-M13	1740	
PAL7-M13	1480	
RTPAL5-M13	1590	

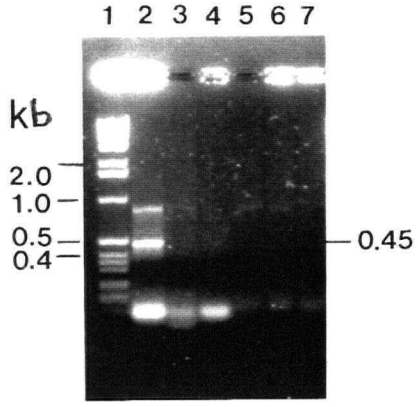


Fig. 3.2. PCR amplification of putative PAL DNA fragment from *U. maydis* genomic DNA using various PAL primer combinations. The amplified products were separated electrophoretically in a 1% agarose gel and visualized under UV following ethidium bromide staining. Lane 1: 1kb DNA ladder, Lane 2: primer PAL7/RTPAL3, Lane 3: primer PAL7/PAL2, Lane 4: primer PAL5/RTPAL3, Lane 5: primer PAL5/PAL2, Lane 6: primer PAL1/PAL2, and Lane 7: primer RTPAL5/RTPAL3.

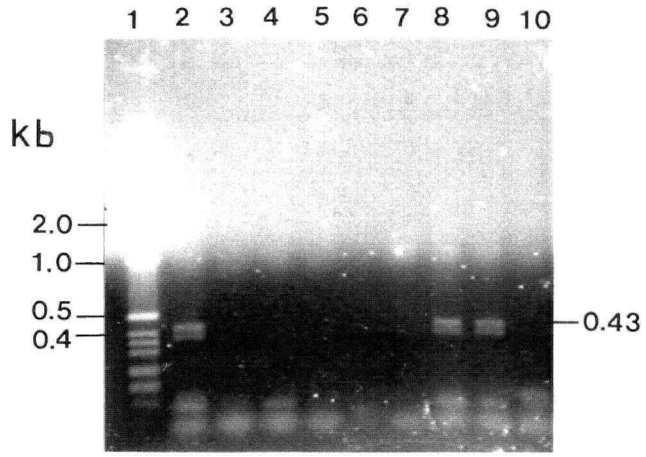


Fig. 3.3. PCR screening of *U. maydis* genomic-cosmid library pools using UMPAL3 and UMPAL2 primers. PCR products were separated electrophoretically in a 1% agarose gel and visualized under UV following ethidium bromide staining. Lane 1: 1kb DNA ladder, Lane 2: genomic-cosmid pool 1, Lane 3: pool 2, Lane 4: pool 3, Lane 5: pool 4, Lane 6: pool 5, Lane 7: pool 6, Lane 8: pool 7, Lane 9: pool 8, Lane 10: pool 9.

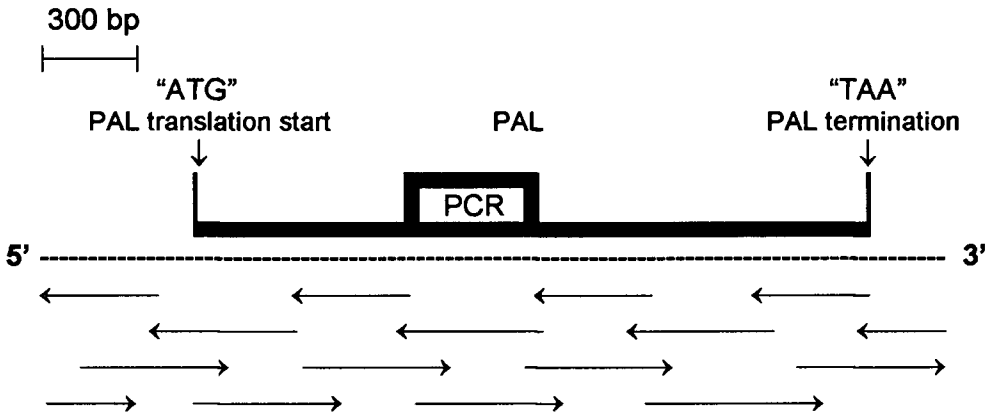


Fig. 3.4. Strategy employed in the nucleotide sequencing of the *U. maydis* PAL genomic-cosmid clone. Arrows indicate the direction and extent of DNA that was sequenced. The dotted line represents total sequenced nucleotides of the PAL genomic clone. The thick black line above the dotted line indicates the 2172 bp open reading frame encoding PAL. The sites for the presumed PAL translation initiation and termination are indicated. The box indicates the location of the PCR-amplified 0.45 kb product from Fig. 3.2.

3.3.3 Nucleotide sequence and deduced amino acid sequence of *U. maydis* PAL

A total of 3047 bp nucleotide sequence of a genomic PAL clone was determined (Fig. 3.5). This contains 495 bp of 5' untranslated sequence, a 2172 bp open reading frame encoding 724 amino acids, and 380 bp of 3' untranslated sequence. A BLAST database search for homology of the 2172 bp sequence with known genes showed that this sequence has highest homology with known PAL genes (Table 3.3). A putative 'TATA box' is present 349 bp upstream from the start codon. However, typical eukaryotic promoter sequences are not found in the usual locations upstream from the *U . maydis* PAL gene start codon. The GC content of the coding sequence is 58%. No introns are located in the *U. maydis* PAL sequence. The 3' untranslated region does not possess any of the consensus transcriptional termination signals of the higher eukaryotes (AATAAA) or of *Neurospora* (TGTCGA).

Conceptional translation of the 2172 nucleotide sequence of this *U. maydis* PAL gene is presented in Fig. 3.6. The molecular weight and isoelectric point of the translated 724 amino acid protein are estimated as 79277 and 6.3, respectively, which are in good agreement with the previously estimated subunit size and isoelectric point of purified PAL (molecular weight 80000 and pI 6.3 in chapter 2). The known enzyme active site serine residue is found at position 206 within the strictly conserved motif SGDL found in PAL genes (Schuster and Retey, 1994) (Fig. 3.6). An internal peptide fragment sequence from the purified *U. maydis* PAL matches positions 384-391 (underlined) of the deduced amino acid sequence (Fig. 3.6). Interestingly, *N*-glycosylation sites, a cAMP- or GMP-dependent protein kinase phosphorylation site, and a ATP/GTP-binding site motif

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GGTGCTCCCCAACAAATGGCGCGCTTTTTTCGGTAGCATGCAGGATAATCTGTTCATCAC      -436

TGTGAGGGTTCACGTTCGTAATTAAACAAAGCGCACATTLCCTGTTTGGATGTCATCGGA      -376

TATTCCGCGACAACTCGGTATATTATTAGTGTAGTTTGACAGAGGGAGTGGACGCGGCTG      -316

AGATGGGACCGTTCCGTGTCAGGAGAGTGGACAACGCATTGCGCGGAATGAAGTCAGAAT      -256

CGATGCATCAATGATTCACGATTGTTGCTCTGACGATCGGCTCGCCCGTTCCGTTCGCGG      -196

TGCGCATCCTGATTGCCAGATAGCCAGAGACGTGGAGCCTGAAGGTGACTATAGTATGGG      -136

ACAGCAATCCTAGCCGACTTTCTCACCTCCTATCCGCCCATATCTGCGTGCCGTGCCTCT       -76

TCGATCGTCTCTACACGACCATAACAGCTGTCCTCTCGCGTCCATACCGTTCCTCTTCCC       -16

ACCGCATCTGGCATCATGGCTCCAACCGCAGACGTGCTCCCTCCCGTCGAGGCATCCACG        45
                M  A  P  T  A  D  V  L  P  P  V  E  A  S  T         15
CGTCCAGGCTTGCTCGTCCAGCCTTCGGATACCAAACTTCGCAAAGCATCGTCCTTCCGA       105
R  P  G  L  L  V  Q  P  S  D  T  K  L  R  K  A  S  S  F  R         35
ACCGAGCAGGTCGTTATCGACGGCTACAATCTCAAGATCCAGGGTCTCGTCGCTTCCGCT       165
T  E  Q  V  V  I  D  G  Y  N  L  K  I  Q  G  L  V  A  S  A         55
CGATACGGTCACGTTACCCGTCCTCGACCCTCCGCTGAGACGCGAAAGCGTATTGATGAC       225
R  Y  G  H  V  T  R  P  R  P  S  A  E  T  R  K  R  I  D  D         75
TCGGTCCAGTCCTTAATCGCCAAGCTCGACGGTGGCGAGTCAATCTACGGCATCAACACG       285
S  V  Q  S  L  I  A  K  L  D  G  G  E  S  I  Y  G  I  N  T         95
GGGTTCGGTGGGTCCGCCGACTCGAGGACCGCCAACACACGTGCGCTTCAGCTGGCCTTG       345
G  F  G  G  S  A  D  S  R  T  A  N  T  R  A  L  Q  L  A  L        115
CTCCAGATGCAGCAGTGTGGCGTGCTCCCCGTGCCATCCACATTCCCCACGGGCGAACCC       405
L  Q  M  Q  Q  C  G  V  L  P  V  P  S  T  F  P  T  G  E  P        135
AGCTCGGCACCCTTTGCACTCCCTTTGACGGACACAGAGTCTTCACTGATCATGCCGGAG       465
S  S  A  P  F  A  L  P  L  T  D  T  E  S  S  L  I  M  P  E        155
GCATGGGTAAGGGGTGCCA4CGTGGTTAGGCTCAGCTCTCTGATGCGCGGTCATTCGGGT       525
A  W  V  R  G  A  I  V  V  R  L  S  S  L  M  R  G  H  S  G        175
GTGCGTTGGGAGGTGCTCGACAAGATGCAGAAGCTTTTCCTCCAGAACAACGTCACTCCA       585
V  R  W  E  V  L  D  K  M  Q  K  L  F  L  Q  N  N  V  T  P        195
GTCGTACCAGTCAGGTCGAGTATCTCGGCCAGTGGTGATCTTAGCCCACTTAGCTACGTA       645
V  V  P  V  R  S  S  I  S  A  S  G  D  L  S  P  L  S  Y  V        215
GCCGGTGCGCTTGCCGGTCAGCGTGGCATCTACTGCTTTGTCACCGACGGCCGTGGTCAG       705
A  G  A  L  A  G  Q  R  G  I  Y  C  F  V  T  D  G  R  G  Q        235
CGTGTCAAGGTGACTGCGGATGAGGCTTGTCGCATGCACAAGATCACCCCCGTCCAGTAT       765
R  V  K  V  T  A  D  E  A  C  R  M  H  K  I  T  P  V  Q  Y        255
GAGCCCAAGGAGGCGCTTGGTCTGCTCAACGGCACCGCTTTTTCAGCCTCTGTTGCGGGT       825
E  P  K  E  A  L  G  L  L  N  G  T  A  F  S  A  S  V  A  G        275
CTCGCTACCTACGAGGCCGAAAATCTAGCCTCTCTGACGCAGCTCACCACCGCTATGGCC       885
L  A  T  Y  E  A  E  N  L  A  S  L  T  Q  L  T  T  A  M  A        295
GTCGAAGCCCTCAAGGGTACCGATGCCAGCTTTGCTCCTTTCATTCACGAAATCGCCCGC       945
V  E  A  L  K  G  T  D  A  S  F  A  P  F  I  H  E  I  A  R        315
CCGCATCCTGGTCAGATCAAGAGCGCCAAGTTTATCCGCGCGCATCTTTCCGGCTCTAGG      1005
P  H  P  G  Q  I  K  S  A  K  F  I  R  A  H  L  S  G  S  R        335
CTAGCAGAGCATCTCGAAAACGAAAAGCACGTCCTCTTCTCCGAAGACAACGGAACGCTG      1065
L  A  E  H  L  E  N  E  K  H  V  L  F  S  E  D  N  G  T  L        355
CGTCAGGACCGTTACACGCTGCAAACCGCCTCCCAGTGGGTCGGCCCGGGTCTCGAGGAC      1125
R  Q  D  R  Y  T  L  Q  T  A  S  Q  W  V  G  P  G  L  E  D        375
ATCGAAAACGCAAAGCGATCCGTCGACTTTGAGATTAACAGCACCACAGATAACCCCATG      1185
I  E  N  A  K  R  S  V  D  F  E  I  N  S  T  T  D  N  P  M        395
ATCGACCCGTACGACGGCGACGGTCGCATCCACCACGGAGGCAACTTCCAGGCCATGGCC      1245
I  D  P  Y  D  G  D  G  R  I  H  H  G  G  N  F  Q  A  M  A        415
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ATGACGAATGCCGTCGAGAAGATCCGCCTCGCCTTGTGTGCTATGGGCAAAATGACGTTC     1305
M  T  N  A  V  E  K  I  R  L  A  L  C  A  M  G  K  M  T  F       435
CAGCAGATG1CAGAGCTCGTCAACCCGGCAATGAACCGAGGATTGCCCGCCAACTTGGCT     1365
Q  Q  M  T  E  L  V  N  P  A  M  N  R  G  L  P  A  N  L  A       455
TCCACGCCTGATCTGTCGCTCAACTTCCACGCCAAGGGAATCAATATTGCGCTTGCCAGT     1425
S  T  P  D  L  S  L  N  F  H  A  K  G  I  N  I  A  L  A  S       475
GTCACTTCGGAACTCATGTTCCTCGGCAACCCCGTTTCAACGCATGTACAAAGTGCAGAG     1485
V  T  S  E  L  M  F  L  G  N  P  V  S  T  H  V  Q  S  A  E       495
ATGGCCAACCAGGCCTTCAACTCGCTGGCGCTCATCAGC1GCCGCCAGACGCTGCAGGCG     1545
M  A  N  Q  A  F  N  S  L  A  L  I  S  G  R  Q  T  L  Q  A       515
ATCGAGTGCCTCTCGATGATTCAGGCTTGGTCGCTCTACCTCTTGTGCCAAGCACTCGAT     1605
I  E  C  L  S  M  I  Q  A  W  S  L  Y  L  L  C  Q  A  L  D       535
ATTCGCGCTTTGCAGTATAAGGTTGCTGAGCAGCTGCCCACGCTCATCTTGGCATCGCTG     1665
I  R  A  L  Q  Y  K  V  A  E  Q  L  P  T  L  I  L  A  S  L       555
CACAGTCACTTTGGCGAGTGGATGGATGAGACCAAGCAGCAAGAGATTGCAGCACAGGTG     1725
H  S  H  F  G  E  W  M  D  E  T  K  Q  Q  E  I  A  A  Q  V       575
CTCAAGAGCATGAGCAAGCGTCTCGACGAAACCTCGTCCAAGGACCTTCGCGATCGACTG     1785
L  K  S  M  S  K  R  L  D  E  T  S  S  K  D  L  R  D  R  L       595
GTCGAGACGTACCAAGACGCGTCGTCTGTGCTTGTGAGGTACTTTTCCGAGCTGCCTAGC     1845
V  E  T  Y  Q  D  A  S  S  V  L  V  R  Y  F  S  E  L  P  S       615
GGTGGTGGTGCGGATCCGCTGAGGAACATTGTCAAGTGGCGCGCCACCGGTGTAGCTGAC     1905
G  G  G  A  D  P  L  R  N  I  V  K  W  R  A  T  G  V  A  D       635
ACGGAAAAGATTTACAGGCAGGTAACGATCGAATTTCTTGACAACCCATACGCTTGCCAT     1965
T  E  K  I  Y  R  Q  V  T  I  E  F  L  D  N  P  Y  A  C  H       655
GCCAGCCACCTGTTGGGCAAGACCAAGCGCGCCTACGAGTTTGTCAGGAAGACGCTGGGT     2025
A  S  H  L  L  G  K  T  K  R  A  Y  E  F  V  R  K  T  L  G       675
GTGCCCATGCATGGTAAGGAGAACCTCAACGAATTCAAGGGCGAATTTGAGCAATGGAAC     2085
V  P  M  H  G  K  B  N  L  N  E  F  K  G  E  F  E  Q  W  N       695
ACGACGGGCGGTTACGTCTCGGTCATCTATGCTAGTATTCGAGATGGCGAGTTGTATAAC     2145
T  T  G  G  Y  V  S  V  I  Y  A  S  I  R  D  G  E  L  Y  N       715
ATGCTGAGCGAGCTCGAAAGGGATTTGTAAAGGGGTGCAAGCAGCGTATTAATAGTTAGT     2205
M  L  S  E  L  E  R  D  L                                        724
ATAAATTGGCCATCTACGGTGACAAATTGCGTGTGAGTGCCAAAAGGGCCATCGAAATGA     2265

TCATGGACAGCGACAGACTGTGTGTTGATTTGTCAAAGTGATTTGGCACTACCGAATATG     2325

ACCGTGTGTACCGGCACCAAGGCGAGGTGATGCGAATGCATGTTTTTGCGTGGCGTCAAA     2385

GGGGGATGCAGGACATGGTCGACTGCTTGTCGGAGCTGATGAGGTCGTAGCGGATTCGGA     2445

ATTTGGGTTCGAGGGCTGTGAAGGGATGTTGAGGTGTATCAAAGGGACTTGGCTTGTGCT     2505

GCGCTTGGGAGTGGGAGGGACATTTCAGGTGCATCTGCTTTCGGGAT                  2552
```

Fig. 3.5. Complete nucleotide sequence of *U. maydis* PAL gene. Deduced amino acid sequence of the PAL protein is indicated below their respective codons. Numbers in the right margin indicate position relative to the A nucleotide of the start codon, or amino acid position. The start codon (ATG) and stop codon (TAA) are underlined. The putative TATA box in the 5' untranslated leader sequence is boxed. The PCR-amplified sequence is lettered in bold.

Table 3.3. BLAST search results with the deduced *U. maydis* PAL protein sequence. The results show the 30 most similar protein sequences in the GenBank for the deduced *U. maydis* PAL protein sequence.

Rank	Accession Number	Organism	Sequence Description	Probability
1	P11544	*Rhodosporidium toruloides*	PAL	2.7e-178
2	P10248	*Rhodotorula rubra*	PAL	1.2e-172
3	P27991	*Glycine max*	PAL 1	9.9e-94
4	P27990	*Medicago sativa*	PAL	1.8e-90
5	P45726	*Solanum tuberosum*	PAL	6.4e-90
6	P35510	*Arabidopsis thaliana*	PAL 1	1.4e-89
7	P45732	*Stylosanthes humilis*	PAL	1.5e-89
8	P31426	*Camellia sinensis*	PAL 2	5.6e-89
9	P45730	*Arabidopsis thaliana*	PAL	2.6e-88
10	P45728	*Petroselinum crispum*	PAL 2	9.1e-88
11	P24481	*Petroselinum crispum*	PAL 1	1.4e-87
12	P19142	*Phaseolus vulgaris*	PAL Class II	2.5e-87
13	P25872	*Nicotiana tabacum*	PAL	2.8e-87
14	P45729	*Petroselinum crispum*	PAL 3	1.6e-86
15	Q01861	*Pisum sativum*	PAL 1	4.3e-86
16	P45734	*Trifolium subterraneum*	PAL	1.0e-85
17	P26600	*Lycopersicon esculentum*	PAL	3.5e-84
18	P14717	*Oryza sativa*	PAL	5.9e-84
19	P45733	*Nicotiana tabacum*	PAL	7.0e-84
20	P45724	*Arabidopsis thaliana*	PAL 2	1.7e-82
21	Q04593	*Pisum sativum*	PAL 2	1.4e-82
22	P31425	*Solanum tuberosum*	PAL 1	4.2e-82
23	P35511	*Lycopersicon esculentum*	PAL	1.9e-81
24	P35513	*Nicotiana tabacum*	PAL	3.9e-81
25	P45725	*Arabidopsis thaliana*	PAL 3	6.5e-79
26	P45727	*Persea americana*	PAL	5.6e-78
27	P45731	*Populus kitakamiensis*	PAL	1.4e-77
28	P19143	*Phaseolus vulgaris*	PAL Class III	4.7e-77
29	P14166	*Ipomea batatas*	PAL	1.4e-75
30	P07218	*Phaseolus vulgaris*	PAL Class I	3.4e-58

```
1    MAPTADVLPP VEASTRPGLL VQPSDTKLRK ASSFRTEQVV IDGYNLKIQG LVASARYGHV
                                      ↑
61   TRPRPSAETR KRIDDSVQSL IAKLDGGESI YGINTGFGGS ADSRTANTRA LQLALLQMQQ
121  CGVLPVPSTF PTGEPSSAPF ALPLTDTESS LIMOEAWVRG AIVVRLAALM RGHSGVRWEV
                           ⇓
181  LDKMQKLFLQ NNVTPVVPVR SSISASGDLS PLSYVAGALA GQRGIYCFVT DGRGQRVKVT
                           *
241  ADEACRMHKI TPVQYEPKEA LGLLNGTAFS ASVAGLATYE AENLASLTQL TTAMAVEALK
                                                                  *
301  GTDASFAPFI HEIARPHPGQ LKSAKFIRAH LSGSRLAEHL ENEKHVLFSE DNGTLRQDRY
                                *
361  TLQTASQWVG PGLEDIENAK RSVDFEINST TDNPMIDPYD GDGRIHHGGN FQAMAMTNAV
421  EKIRLALCAM GKMTFQQMTE LVNPAMNRGL PANLASTPDL SLNFHAKGIN IALASVTSEL
481  MFLGNPVSTH VQSAEMANQA FNSLALISGR QTLQAIECLS MIQAWSLYLL CQALDIRALQ
541  YKVAEQLPTL ILASLHSHFG EWMDETKQQE IAAQVLKSMS KRLDETSSKD LRDRLVETYQ
601  DASSVLVRYF SELPSGGGAD PLRNIVKWRA TGVADTEKIY RQVTIEFLDN PYACHASHLL
                                                  *
661  GKTKRAYEFV RKTLGVPMHG KENLNEFKGE FEQWNTTGGY VSVIYASIRD GELYNMLSEL
721  ERDL
```

Fig. 3.6. Deduced amino acid sequence of *U. maydis* PAL. Amino acid sequence of an internal peptide fragment of the purified *U. maydis* PAL is underlined and the enzyme active site is indicated as ⇓. Phenylalanine and histidine ammonia-lyases signature motif is boxed. Predicted N-glycosylation sites (* above line), cAMP- or cGMP- dependent protein kinase phosphorylation site (↑), and ATP/GTP-binding site motif A (letters in bold) are shown.

are also predicted in the sequence (Fig. 3.6). The other potentially biologically significant sites are given in Table 3.4. Many putative phosphorylation sites are predicted. Codon usage of the *U. maydis* PAL gene is shown in Table 3.5. Codons that have a A in the wobble position are used infrequently, indicating that codon bias is operative in the *U. maydis* PAL gene, as in other fungal genes. Leucine, alanine, and serine appear as the three major amino acids in the deduced amino acid composition of *U. maydis* PAL (Table 3.6), while cysteine and tryptophan constitute less than 1% of the gene. The hydropathic index of the PAL amino acid sequence in Fig. 3.7 shows that most of the amino acids are located in the positive end of the scale, suggesting the *U. maydis* PAL is a hydrophobic protein.

3.3.4 Genomic organization of PAL in *U. maydis*

Southern blot hybridization using the 0.45 kb PAL fragment DNA as a probe was carried out to determine the number of PAL genes in the *U. maydis* genome. A single band was observed in each restriction digestion (Fig. 3.8). This suggests that *U. maydis* PAL is present as a single copy gene, as in the case of *Rhodosporidium* yeast PAL (Anson *et al.*, 1987).

3.3.5 Northern blot analysis of PAL transcript

Northern blot hybridization was performed by analyzing total RNA from the *U. maydis* cells harvested at the mid-log phase of growth. The ^{32}P-labeled 0.45 kb PAL DNA probe hybridized to a 2.2 kb size of RNA (Fig. 3.9). This detected size is consistent with the

Table 3.4. Detection of potentially biologically significant sites in the protein sequence (724 aa) of *U. maydis* PAL. The putative sites were identified using PROSITE program from PC/GENE software system.

Site	Location : aa residues
Tyrosine sulfation	399 : pmidp Y dgdgr
	714 : rdgel Y nmlse
Protein kinase C phosphorylation	14 : ppvea S trpgl
	69 : dpsad T rkrid
	323 : pgqik S akfir
	354 : sedng T lrqdr
	508 : slali S grqtl
	580 : vlksm S krlde
	587 : rldet S skdlr
	636 : tgvad T ekiyr
	663 : hllgk T kraye
	707 : sviya S irdge
Casein kinase II phosphorylation	32 : klrka S sseps
	145 : falpl T dtess
	240 : qrvkv T adeac
	389 : dfein S ttdnp
	456 : panla S tpdls
	587 : rldet S skdlr
	598 : drlve T yqdas
	707 : sviya S irdge
	718 : lynml S elerd
N-myristoylation	92 : gesiy G intgf
	99 : ntgfg G sadsr
	217 : lsyva G alagq
	262 : pkeal G llngt
	266 : lglln G tafsa
	301 : vealk G tdasf
	319 : arphp G qiksa
	333 : rahls G srlae
	409 : rihhg G nfqam
	449 : pamnr G lpanl
	468 : nfhak G inial
	484 : elmfl G npvst
	632 : kwrat G vadte
	698 : qwntt G gyvsv

Table 3.5. Codon usage in the *U. maydis* PAL gene

CD	AA	No.	PC	CD	AA	No.	PC	CD	AA	No.	PC	CD	AA	No.	PC
TTT	Phe	11	1.5%	TCT	Ser	6	.8%	TAT	Tyr	4	.5%	TGT	Cys	3	.4%
TTC	Phe	12	1.6%	TCC	Ser	14	1.9%	TAC	Tyr	15	2%	TGC	Cys	4	.5%
TTA	Leu	1	.1%	TCA	Ser	4	.5%	TAA	---	1	.1%	TGA	---	0	0%
TTG	Leu	12	1.6%	TCG	Ser	17	2.3%	TAG	---	0	0%	TGG	Trp	7	.9%
CTT	Leu	12	1.6%	CCT	Pro	8	1.1%	CAT	His	7	.9%	CGT	Arg	11	1.5%
CTC	Leu	33	4.5%	CCC	Pro	13	1.7%	CAC	His	10	1.3%	CGC	Arg	12	1.6%
CTA	Leu	2	.2%	CCA	Pro	7	.9%	CAA	Gln	6	.8%	CGA	Arg	8	1.1%
CTG	Leu	18	2.4%	CCG	Pro	6	.8%	CAG	Gln	29	4%	CGG	Arg	0	0%
ATT	Ile	10	1.3%	ACT	Thr	3	.4%	AAT	Asn	4	.5%	AGT	Ser	6	.8%
ATC	Ile	23	3.1%	ACC	Thr	18	2.4%	AAC	Asn	24	3.3%	AGC	Ser	13	1.7%
ATA	Ile	0	0%	ACA	Thr	5	.6%	AAA	Lys	3	.4%	AGA	Arg	0	0%
ATG	MET	21	2.9%	ACG	Thr	20	2.7%	AAG	Lys	27	3.7%	AGG	Arg	10	1.3%
GTT	Val	6	.8%	GCT	Ala	17	2.3%	GAT	Asp	13	1.7%	GGT	Gly	24	3.3%
GTC	Val	23	3.1%	GCC	Ala	29	4%	GAC	Asp	20	2.7%	GGC	Gly	21	2.9%
GTA	Val	6	.8%	GCA	Ala	14	1.9%	GAA	Glu	15	2%	GGA	Gly	4	.5%
GTG	Val	11	1.5%	GCG	Ala	11	1.5%	GAG	Glu	29	4%	GGG	Gly	2	.2%

CD: codon, AA: amino acid, No.: number of given codons in the PAL gene, PC: percentage of given codons in the PAL gene, and ---: stop codon.

Table 3.6. Inferred amino acid composition of *U. maydis* PAL

Amino Acid	No.	%
Ala	71	9.8
Arg	41	5.6
Asn	28	3.8
Asp	33	4.5
Cys	7	.9
Gln	35	4.8
Glu	44	6.0
Gly	51	7.0
His	17	2.3
Ile	33	4.5
Leu	78	10.7
Lys	30	4.1
Met	21	2.9
Phe	23	3.1
Pro	34	4.6
Ser	60	8.2
Thr	46	6.3
Trp	7	.9
Tyr	19	2.6
Val	46	6.3

No.: number of times an amino acid occurs.

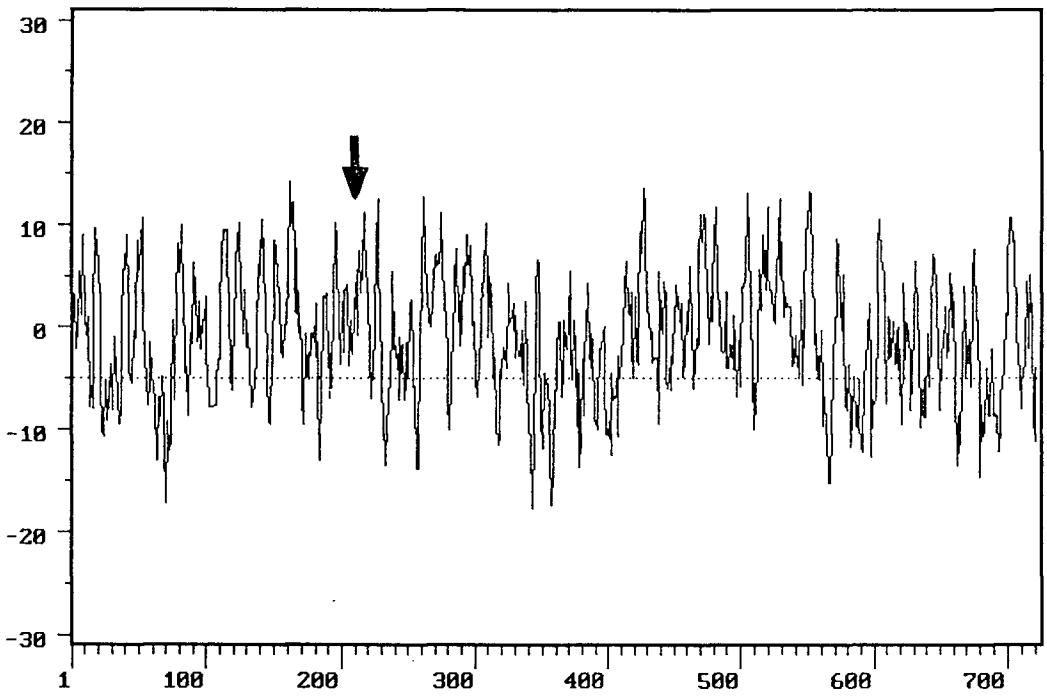

Fig. 3.7. Hydropathy profile of *U. maydis* PAL from amino acid 1 to amino acid 724. Computation of the hydropathic index was performed with an interval of 5 amino acids, using the SOPE program in PC/GENE software system. A line at the -5 value divides hydrophobic regions above it from hydrophilic regions below. Arrow indicates the position of active site.

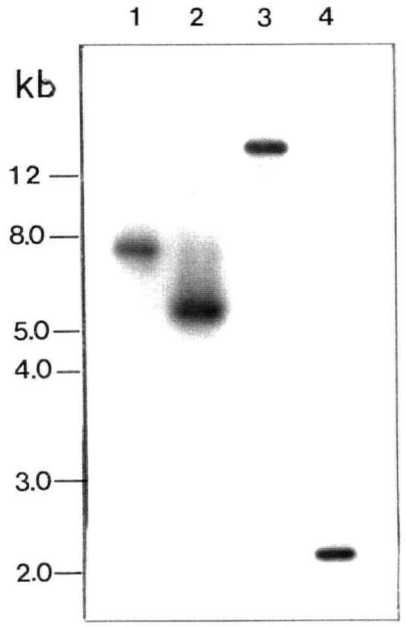

Fig. 3.8. Southern blot analysis of the *U. maydis* PAL gene. Genomic DNA from *U. maydis* strain 518 cells was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Xba*I (lane 3), and *Xho*I (lane 4), fractionated by electrophoresis in a 0.7% agarose gel, transferred to Zeta-Probe membrane, and hybridized with ^{32}P-labeled 0.45 kb PAL DNA fragment. Sizes of DNA markers are indicated in kb.

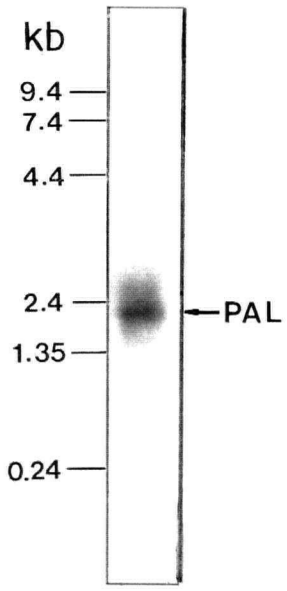

Fig. 3.9. Northern blot analysis of PAL transcript. Total RNA (20 μg) was resolved in a 1% agarose-formaldehyde gel, blotted onto Zeta-Probe membrane, and hybridized with ^{32}P-labeled 0.45 kb *U. maydis* PAL DNA fragment. Sizes of RNA markers are indicated in kb.

size of the open reading frame in the PAL DNA sequence in Fig. 3.5, and confirms that no introns are present in the *U. maydis* PAL gene.

3.3.6 Protein sequence comparison

Protein sequences were obtained from the PIR and SWISS-PROT databases and used for the alignment and sequence identity comparison, using PC/GENE software. Four HAL protein sequences used were from *Streptomyces griseus* (a bacterium, database accession P24221), *Pseudomonas putida* (a bacterium, P21310), *Rattus norvegicus* (rat, P21213), and *Homo sapiens* (human, P42357). The ten PAL protein sequences were from *Rhodosporidium toruloides* (yeast, P11544), *Rhodotorula rubra* (yeast, P10248), *Arabidopsis thaliana* (P35510), *Pinus taeda* (pine, g1143312), *Oryza sativa* (rice, P14717), *Triticum aestivum* (wheat, g1483610), *Bromheadia finlaysoniana* (orchid, g1491619), *Solanum tuberosum* (potato, P31426), *Populus trichocarpa* × *Populus deltoides* (poplar, P45730), and *Medicago sativa* (alfalfa, P27990). The percentage of protein sequence identity and similarity from a pairwise comparison of the above four HAL and eleven PAL protein sequences (including *U. maydis* PAL sequence) shows the extent of homology over various organisms (Table 3.7). As expected, sequences from closely- related organisms display the greatest protein sequence identity and similarity, e.g. between human and rat, or among potato, poplar and alfalfa. The lowest sequence conservation values (13-18%) are found between mammalian HALs and plant PALs. Overall, the *U. maydis* PAL shows low amino acid sequence identity with other PALs (23-26% with plant PALs, 39-40% with yeast PALs). It is quite noticeable that the level of homology (25%) shown between *U. maydis* PAL and bacterial HALs is similar to its

Table 3.7. Comparison of protein sequence identity and similarity of phenylalanine ammonia-lyase (PAL) and histidine ammonia-lyase (HAL) among different organisms. Numbers indicate the percentages of protein sequence identity/similarity. Sequences were analyzed based on inferred amino acid sequences from cDNAs encoding the two enzymes, using PALIGN program in the PC/GENE sequence analysis software.

	Plants							Fungi			Bacteria		Mammals	
	Alfalfa PAL	*A. t.* PAL	Potato PAL	Orchid PAL	Wheat PAL	Rice PAL	Pine PAL	*U. m.* PAL	*R. t.* PAL	*R. r.* PAL	*S. g.* HAL	*P. p.* HAL	Rat HAL	Human HAL
Poplar PAL	86/92	85/91	82/90	80/89	77/85	69/80	64/76	**25/37**	31/42	31/42	21/31	25/40	18/25	16/22
Alfalfa PAL		80/88	79/88	79/88	76/84	68/78	64/75	**24/35**	34/44	30/42	22/32	27/41	15/20	16/22
***A. t.* PAL**			78/88	81/89	76/85	68/79	63/75	**23/35**	32/43	33/43	22/32	26/40	13/18	17/25
Potato PAL				78/88	76/85	68/79	63/75	**24/35**	31/41	28/38	19/30	24/39	15/21	15/21
Orchid PAL					77/86	69/80	64/76	**26/38**	30/42	27/38	19/30	26/39	14/20	15/21
Wheat PAL						70/81	65/76	**25/36**	33/45	31/42	22/31	24/37	13/18	13/19
Rice PAL							60/70	**23/37**	33/45	31/42	23/34	27/40	14/21	15/23
Pine PAL								**25/38**	28/39	28/39	22/32	22/34	14/20	13/17
***U. m*. PAL**									**40/54**	**39/53**	**25/36**	**25/36**	**14/21**	**15/23**
***R. t.* PAL**										77/87	25/36	25/38	20/27	18/25
***R. r.* PAL**											25/36	26/39	17/26	17/26
***S. g.* HAL**												38/53	37/52	38/52
***P. p*. HAL**													41/55	42/55
Rat HAL														94/97

A.t.: Arabidopsis thaliana, U. m.: Ustilago maydis, R. t.: Rhodosporidium toruloides, R. r.: Rhodotorula rubra, S. t.: Streptomyces griseus, and, P. p.: Pseudomonas putida.

identity with plant PALs (23-26%). This suggests a possible relatedness between *U. maydis* PAL and bacterial HAL. The protein sequence alignment results are in Fig. 3.10. The major conserved motifs are YGXXXGFG (X is any amino acid), ASGDLXPLS, PKEGLXXXNG, and EXNSXXDNP. Except for the ASGDLXPLS active site motif, the functional importance of these conserved motifs remains unknown. More variation among sequences is associated with in the amino- and carboxyl-terminal regions. The overall relation between HAL and PAL sequences over diverse organisms is displayed as a dendrogram in Fig. 3.11, which illustrates the divergence of these two ammonia-lyase enzymes from the putative ancestral form.

3.3.7 Immunological relationships

To obtain further insights into the structural relationships between PAL and HAL proteins from different sources, Western blot analysis was used to determine the extent of cross-reactivity between these proteins from different species. Cross-reactivity was found among all the PAL proteins tested, using antibodies raised against both plant and fungal PALs (Fig. 3.12). Both samples of plant PAL antibodies strongly recognized plant PALs but only weakly recognized fungal PALs. Interestingly, however, antibodies raised against another fungal PAL (*Ustilago*), only weakly recognized the *Rhodotorula glutinis* (yeast) fungal PAL. Even more strikingly, although the anti-*U. maydis* PAL antibodies showed low affinity for the plant PALs, they bound strongly to *Pseudomonas* bacterial

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POPLARPAL    ME-------------------------TVTKNGYQNGSLES--LC----    18
PALY_MEDSA   ME-------------------------TISAAITKNNANES--FCLIHA    22
PALY_ARATA   ME-------------------------INGAHKSNGGGVDA-MLCGGDI    23
PAL1_SOLTU   MA-------------------------PSIAQNGHVNGEVEE-VL-----    19
ORCHIDPAL    ME-------------------------VSK----------EN-GLCL---    11
WHEATPAL     MA---------------------------------------------CAWRS     7
PALY_ORYSA   MA-------------------------GNGP--------------------     6
PINEPAL      MV-------------------------AAAEITQANEVQVKSTGLC----    21
PALY_USTMA   MA-------------------------PTADVLPPVEASTRPGLL---VQ    22
PALY_RHOTO   MA-------------------------PSLDSISHSFANGVASAKQAVNG    25
PALY_RHORB   MA-------------------------PSVDSIATSVANSLSNGLHAAAA    25
HUTH_STRGR   MDMHTVVV-------------------------------------------     8
HUTH_PSEPU   ME-------------------------------------------------     2
HUTH_RAT     MPRYTVHVRGEWLAVPCQDGKLSVGWLGREAVRRYMKNKPDNGGFTSVDE    50
HUMHISP      MPRYTVHVRGEWLAVPCQDAQLTVGWLGREAVRRYIKNKPDNGGFTSVDD    50

POPLARPAL    -------VNQRDPLSWGVAAE-AMKGSHLDEVKR----------------    44
PALY_MEDSA   KNNNNMKVNEADPLNWGVAAE-AMKGSHLDEVKR----------------    55
PALY_ARATA   KTKN-MVINAEDPLNWGAAAE-QMKGSHLDEVKR----------------    55
PAL1_SOLTU   -----WKKSIHDPLNWEMAVD-SLRGSHLDEVKK----------------    47
ORCHIDPAL    --------QGRDPLNWGAAAA-ELQGSHLDEVKK----------------    36
WHEATPAL     RSRA-------DPLNWGKAAE-ELSGSHLEAVKR----------------    33
PALY_ORYSA   -------INKEDPLNWGAAAA-EMAGSHLDEVKR----------------    32
PINEPAL      ---TDFGSSGSDPLNWVRAAK-AMEGSHFEEVKA----------------    51
PALY_USTMA   PSDTKLRK-------------------------------------------    30
PALY_RHOTO   AST-----NLAVA---GSHLP-TTQVTQVDIVEK----------------    50
PALY_RHORB   ANGGDVHKKTAGA---GSLLP-TTETTQLDIVER----------------    55
HUTH_STRGR   -------------------------------------------------     8
HUTH_PSEPU   -------------------------------------------------     2
HUTH_RAT     VRFLVRRCKGLGLLDNEDLLEVALEDNEFVEVVIEGDVMSPDFIPSQPEG   100
HUMHISP      AHFLVRRCKGLGLLDNEDRLEVALENNEFVEVVIEGDAMSPDFIPSQPEG   100

POPLARPAL    --MVADYRKPV--VKLGGETLTIAQVASIAGHDT--GDVKVELSESA-RP    87
PALY_MEDSA   --MVAEYRKPV--VRLGGETLTISQVAAIAAHDH--G-VQVDLSESA-RD    97
PALY_ARATA   --MVAEFRKPV--VNLGGETLTIGQVAAISTIGN--S-VKVELSETA-RA    97
PAL1_SOLTU   --MVDEFRKPI--VKLWGETLTVAQVASIANADNKTSGFKVELSESA-RA    92
ORCHIDPAL    --MVEEFRRPV--VKLEGVKLKISQVAAVAFGG---GASAVELAESA-RA    78
WHEATPAL     --MVEEYRKPV--VTMEGAT-TIAMVAAVAAG----SDTRVELDESA-RG    73
PALY_ORYSA   --MVAQFREPL--VKIQGATLRVGQVAAVA-QAKDAARVAVELDEEA-RP    76
PINEPAL      --MVDSYFGAKE-ISIEGKSLT-SDVAAVA----RRSQVKVKLDAAAAKS    93
PALY_USTMA   ----ASSFRTEQ-VVIDGYNLKIQGLVASARYGHVTRPR----PSAETRK    71
PALY_RHOTO   --MLAAPTDST--LELDGYSLNLGDVVSAARKGRPVRVK----DSDEIRS    92
PALY_RHORB   --ILADAGATDQ-IKLDGYTLTLGDVVGAARRGRSVKVA----DSPHIRE    98
HUTH_STRGR   ----------------GTSGTTAEDVVAVAR-----HGARVELSAAAVEA    37
HUTH_PSEPU   -------------LTLKPGTLTLAQLRAIHA-----APVRLQLDASAAPA    34
HUTH_RAT     VFLYSKYREPEKYIALDGDSLSTEDLVNLGK-----GHYKIKLTSIAEKK   145
HUMHISP      VYLYSKYREPEKYIELDGDRLTTEDLVNLGK-----GRYKIKLTPTAEKR   145
                                                  - -

POPLARPAL    GVKASSDWVMDSMDKGTDS**YGVTTGFG**ATSHRR--TKQGGALQKELIRFL   135
PALY_MEDSA   GVKASSEWVMESMNKGTDS**YGVTTGFG**ATSHSR--TKQGGALQKELIRFL   145
PALY_ARATA   GVNASSDWVMESMNKGTDS**YGVTTGFG**ATSHRR--TKNGVALQKELIRFL   145
PAL1_SOLTU   GVKASSDWVMDSMSKGTDS**YGVTTGFG**ATSHRR--TKNGGALQKELIKFL   140
ORCHIDPAL    GVKASSDWVLESVDKGTDS**YGVTTGFG**ATSHRR--TKQGGALQKELIKFL   126
WHEATPAL     RVKESSDWVMNSMMNGTDS**YGVTTGFG**ATSHRR--TKEGGALQRELIRFL   121
PALY_ORYSA   RVKASSEWILTCIAHGGDI**YGVTTGFG**GTSHRR--TKDGPALQVELLRYL   124
PINEPAL      RVEESSNWVLTQMTKGTDT**YGVTTGFG**ATSHRR--TNQGAELQKELIRFL   141
PALY_USTMA   RIDDSVQSLIAKLDGGESI**YGIN**T**GFG**GSADSR--TANTRALQLALLQMQ   119
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PALY_RHOTO    KIDKSVEFLRSQLS--MSVYGVTTGFGGSADTR--TEDAISLQKALLEHQ    138
PALY_RHORB    KIDASVEFLRTQLD--NSVYGVTTGFGGSADTR--TEDAISLQKALLEHQ    144
HUTH_STRGR    -LAAARLIVDALAAKPEPVYGVSTGFGALASRHIGTELRAQLQRNIVRSH     86
HUTH_PSEPU    -IDASVACVEQIIAEDRTAYGINTGFGLLASTRIASHDLENLQRSLVLSH     83
HUTH_RAT      -VQQSREVIDSIIKERTVVYGITTGFGKFARTVIPANKLQELQVNLVRSH    194
HUMHISP       -VQKSREVIDSIIKEKTVVYGITTGFGKFARTVIPINKLQELQVNLVRSH    194
                - - -          **---***  -          **    ---

POPLARPAL     NAGIFGNG--------------------TETCHTLPHSATRAAMLVRINT    165
PALY_MEDSA    NAGIFGNG--------------------TESNHTLPKTATRAAMLVRINT    175
PALY_ARATA    NAGIFGST--------------------KETSHTLPHSATRAAMLVRINT     75
PAL1_SOLTU    NAGVFGNG--------------------TESTHTLPHSATRAAMLVRINT    170
ORCHIDPAL     NAGIFGSG--------------------N--SNTLPSAATRAAMLVRINT    154
WHEATPAL      NAGAFGTG--------------------TDG-HVLPAAATRAAMLVRVNT    150
PALY_ORYSA    NAGIFGTG--------------------SDG-HTLPSETVRAAMLVRINT    153
PINEPAL       NAGVLGKC--------------------PE--NVLSEDTT-AAMLVRTNT    168
PALY_USTMA    QCGVLPVP-STFPTGEPSSAPFALPLTDTESSLIMPEAWVRGAIVVRLSS    168
PALY_RHOTO    LCGVLPSSFDSFRLGRGLENSLPLEV-------------VRGAMTIRVNS    175
PALY_RHORB    LCGVLPTSMDGFALGRGLENSLPLEV-------------VRGAMTIRVNS    181
HUTH_STRGR    AAGM---------------------------GPRVEREVVRALMFLRLKT    109
HUTH_PSEPU    AAGI---------------------------GAPLDDDLVRLIMVLKINS    106
HUTH_RAT      SSGV---------------------------GKPLSPERCRMLLALRINV    217
HUMHISP       SSGV---------------------------GKPLSPERCRMLLALRINV    217
                *                                          --  - -

POPLARPAL     LLQGYSGIRFEILEAITRL-LNNNITPCLPLRGTITASGDLVPLSYIAGL    214
PALY_MEDSA    LLQGYSGIDFEILEAITKP-LNKTVTPCLPLRGTITASGDLVPLSYIAGL    224
PALY_ARATA    LLQGFSGIRFEILEAITSF-LNNNITPSLPLRGTITASGDLVPLSYIAGL    224
PAL1_SOLTU    LLQGYSGIRFEILEAITKL-INSNITPCLPLRGTVTASGDLVPLSYIAGL    219
ORCHIDPAL     LLQGYSGIRFEILKAIATL-LNKNITPCLPLRGTITASGDLVPLSYLAGI    203
WHEATPAL      LLQGYSGIRFEILETIATL-LNANVTPCLPLRGTITASGDLVPLSYIAGL    199
PALY_ORYSA    LLQGYSGIRFEILEAITKL-LNTGVTPCLPLRGTITASGDLVPLSYIAGL    202
PINEPAL       LLQGYSGVRWDILETVEKL-LNAWLTPKLPLRGTITASGDLVPLSYIAGL    217
PALY_USTMA    LMRGHSGVRWEVLDKMQKLFLQNNVTPVVPVRSSISASGDLSPLSYVAGA    218
PALY_RHOTO    LTRGHSAVRLVVLEALTN-FLNHGITPIVPLRGTISASGDLSPLSYIAAA    224
PALY_RHORB    LTRGHSAVRIVVLEALTN-FLNHGITPIVPLRGTISASGDLSPLSYIAAS    230
HUTH_STRGR    VASGHTGVRPEVAQTMADVL-NAGITPVVHEYGSLGCSGDLAPLSHCALT    158
HUTH_PSEPU    LSRGFSGIRRKVIDALIALV-NAEVYPHIPLKGSVGASGDLAPLATMSLV    155
HUTH_RAT      LAKGYSGISLETLKQVIEVF-NASCLSYVPEKGTVGASGDLAPLSHLALG    266
HUMHISP       LAKGYSGISLETLKQVIEMF-NASCLPYVPEKGTVGASGDLAPLSHLALG    266
                - * ---   - - -     -    - --  ---- **** **-  -

POPLARPAL     LTGRPNSKATGPTGE----VLDAAEAFKAAGIESGFFELQPKEGLALVNG    260
PALY_MEDSA    LTGRPNSKAHGPSGE----VLNAKEAFNLAGINAEFFELQPKEGLALVNG    270
PALY_ARATA    LTGRPNSKATGPNGE----ALTAEEAFKLAGISSGFFDLQPKEGLALVNG    270
PAL1_SOLTU    LTGRPNSKAVGPSGS----KLDADEAFRVAAVSGGFFELQPKEGLALVNG    265
ORCHIDPAL     LTGRPNSKARTPNGS----TVDATTAFRLAGISSGFFDLQPKEGLALVNG    249
WHEATPAL      VTGRPNSMATAPDGS----KVNAAEAFKIAGIQHGFFELQPKEGLAMVNG    245
PALY_ORYSA    ITGRPNAQAISPDGR----KVDAAEAFKLAGIEGGFFTLNPKEGLAIVNG    248
PINEPAL       LTGRPNSRVRSRDGI----EMSGAEALKKVGLEKPF-ELQPKEGLAIVNG    262
PALY_USTMA    LAGQRGIYCFVTDGRGQRVKVTADEACRMHKITP--VQYEPKEALGLLNG    266
PALY_RHOTO    ISGHPDSKVHVVHEGKEKI-LYAREAMALFNLEP--VVLGPKEGLGLVNG    271
PALY_RHORB    ITGHPDSKVHV----DGKI-MSAQEAIALKGLQP--VVLGPKEGLGLVNG    273
HUTH_STRGR    LMG--EGEAEGPDGT----VRPAGELLAAHGIAP--VELREKEGLALLNG    200
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HUTH_PSEPU   LLG--EGKARY-KGQ----WLSATEALAVAGLEP--LTLAAKEGLALLNG   196
HUTH_RAT     LIG--EGKMWSPKSG----WADAKYVLEAHGLKP--IVLKPKEGLALING   308
HUMHISP      LVG--EGKMWSPKSG----WADAKYVLEAHGLKP--VILKPKEGLALING   308
             - *  -                 -        -          **-*---**

POPLARPAL    TAVGSGLASMVLFETNVLAVLSELLSAIFAEVMNGKPE-FTDHLTHKLKH   309
PALY_MEDSA   TAVGSGLASIVLFEANILAVLSEVLSAIFAEVMQGKPE-FTDHLTHKLKH   319
PALY_ARATA   TAVGSGMASMVLFETNVLSVLAEILSAVFAEVMSGKPE-FTDHLTHRLKH   319
PAL1_SOLTU   TAVGSGMASIVLYDSNILAVMFEVLSAIFAEVMNGKPE-FTDYLTHKLKH   314
ORCHIDPAL    TAVGSGVASIVLFETNILAVMAELLSALFCEVMQGKPE-FTDHLTHKLKH   298
WHEATPAL     TAVGSGLASMVLFEANVLSLLAEVLSGVFCEVMNGKPE-FTDHLTHKLKH   294
PALY_ORYSA   TSVGSALAATVMFDANILAVLSEVLSAVFCEVMNGKPE-YTDHLTHKLKH   297
PINEPAL      TSVGAALASIVCFDANVLALLSEVISAMFCEVMNGKPE-FTDPLTHKLKH   311
PALY_USTMA   TAFSASVAGLATYEAENLASLTQLTTAMAVEALKGTDASFAPFIHEIARP   316
PALY_RHOTO   TAVSASMATLALHDAHMLSLLSQSLTAMTVEAMVGHAGSFHPFLHDVTRP   321
PALY_RHORB   TAVSASMATLALTDAHVLSLLAQALTALTVEAMVGHAGSFHPFLHDVTRP   323
HUTH_STRGR   TDGMLGMLVMALADLRNLYTSADITAALSLEALLGTDKVLAPEL-HAIRP   249
HUTH_PSEPU   TQASTAYALRGLFYAEDLYAAAIACGGLSVEAVLGSRSPFDARI-HEARG   245
HUTH_RAT     TQMITSLGCEAVERASAIARQADIVAALTLEVLKGTTKAFDTDI-HAVRP   357
HUMHISP      TQMITSLGCEAVERASAIARQADIVAALTLEVLKGTTKAFDTDI-HALRP   357
             *    -           -       ---  *-- *        - -  -

POPLARPAL    HPGQ-IEAAAIMEHILDGSAYMKAAKKLHETDPLQKP-----KQDRYALR   353
PALY_MEDSA   HPGQ-IEAAAIMEHILDGSSYVKAAKKLHEIDPLQKP-----KQDRYALR   363
PALY_ARATA   HPGQ-IEAAAVMEHILDGSSYMKLAQKLHEMDPLQKP-----KQDRYALR   363
PAL1_SOLTU   HPGQ-IEAAAIMEHILDGSSYVKAAQKLHEMDPLQKP-----KQDRYALR   358
ORCHIDPAL    HPGQ-IEAAAVMEHILEGSSYMKMAKKLHEMDPLQKP-----KQDRYALR   342
WHEATPAL     HPGQ-IEAAAIMEHILEGSSYMMLAKKLGELDPLMKP-----KQDRYALR   338
PALY_ORYSA   HPGS-IDAAAIMEHILAGSSFMSHAKKVNEMDPLLKP-----KQDRYALR   341
PINEPAL      HPGQ-MEAAAIMEYVLDGSSYMKHAAKLHEMNPLQKP-----KQDRYGLR   355
PALY_USTMA   HPGQ-IKSAKFIRAHLSGSRLAEHLE--NEKHVLFSEDNGTLRQDRYTLQ   363
PALY_RHOTO   HPTQ-IEVAGNIRKLLEGSRFAVHHE--EEVKV--KDDEGILRQDRYPLR   366
PALY_RHORB   HPTQ-IEVARNIRTLLEGSKYAVHHE--TEVKV--KDDEGILRQDRYPLR   368
HUTH_STRGR   HPGQGVSADNMSRVLAGSG--------LTGHHQDDAPR----VQDAYSVR   287
HUTH_PSEPU   QRGQ-IDTAACFRDLLG----------DSSEVSLSHKNCDKVQDPYSLR   283
HUTH_RAT     HRGQ-IEVAFRFRSLLDSD--------HHPSEIAESHRFCDRVQDAYTLR   398
HUMHISP      HRGQ-IEVAFRFRSLLDSD--------HHPSEIAESHRFCDRVQDAYTLR   398
             ---  -  -       -              -             ** * --

POPLARPAL    TSPQWLGPQIEVIRFSTKSIEREI-NSVNDNPLIDV--SRNKAIHGGNFQ   400
PALY_MEDSA   TSPQWLGPLVEVIRFSTKSIEREI-NSVNDNPLIDV--SRNKALHGGNFQ   410
PALY_ARATA   TSPQWLGPQIEVIRYATKSIEREI-NSVNDNPLIDV--SRNKAIHGGNFQ   410
PAL1_SOLTU   TSPQWLGPQIEVIRAATKMIEREI-NSVNDNPLIDV--SRNKAIHGGNFQ   405
ORCHIDPAL    TSPQWLGPQIEVIRAATKSIEREI-NSVNDNPLIDV--SRNKALHGGNFQ   389
WHEATPAL     TSPQWLGPQIEVIRAATKSIEREI-NSVNDNPLIDV--SRGKAIHGGNFQ   385
PALY_ORYSA   TSPQWLGPQIQVIRAATKSIEREV-NSVNDNPVIDV--HRGKALHGGNFQ   388
PINEPAL      TSPQWLGPQVEIIRSATHMIEREI-NSVNDNPVIDV--ARDKALHGGNFQ   402
PALY_USTMA   TASQWVGPGLEDIENAKRSVDFEI-NSTTDNPMIDPYDGDGRIHHGGNFQ   412
PALY_RHOTO   TSPQWLGPLVSDLIHAHAVLTIEAGQSTTDNPLIDV--ENKTSHHGGNFQ   414
PALY_RHORB   CSPQWLGPLVSDMIHAHAVLSLEAGQSTTDNPLIDL--ENKMTHHGGAFM   416
HUTH_STRGR   CAPQVNGAGRDTLDHAALVAGREL-ASSVDNPVV--LPD-GRVESNGNFH   333
HUTH_PSEPU   CQPQVMGACLTQLRQAAEVLGIEA-NAVSDNPLV--FAAEGDVISGGNFH   330
HUTH_RAT     CCPQVHGVVNDTIAFVKDIITTEL-NSATDNPMV--FASRGETISGGNFH   445
HUMHISP      CCPQVHGVVNDTIAFVKNIITTEL-NSATDNPMV--FANRGETVSGGNFH   445
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POPLARPAL    GTPIGVSMDNVRLAIASIGKLLFAQFSELVNDFYNNGLPSNLTASRNPSL    450
PALY_MEDSA   GTPIGVSMDNTRLALASIGKLMFAQFSELVNDFYNNGLPSNLSASRNPSL    460
PALY_ARATA   GTPIGVSMDNTRLAIRAIGKLMFAQFSELVNDFYNNGLPSNLTASRNPSL    460
PAL1_SOLTU   GTPIGVSMDNTRLALASIGKLMFAQFSELVNDYYNNGLPSNLTAGRNPSL    455
ORCHIDPAL    GTPIGVSMDNTRLAIAAIGKLMFAQFSELVNDFYNNGLPSNLSSGRNPSL    439
WHEATPAL     GTPIGVSMDNTRLAIAAIGKLMFAQFSELVNDFYNNGLPSNLSGGRNPSL    435
PALY_ORYSA   GTPIGVSMDNARLAIANIGKLMFAQFSELVNEFYNNGLTSNLAGSRNPSL    438
PINEPAL      GTPIGVSMDNLRLSISAIGKLMFAQFSELVNDYYNGGLPSNLSGGPNPSL    452
PALY_USTMA   AMAMTNAVEKIRLALCAMGKMTFQQMTELVNPAMNRGLPANLASTPDLSL    462
PALY_RHOTO   AAAVANTMEKTRLGLAQIGKLNFTQLTEMLNAGMNRGLPSCLAAE-DPSL    463
PALY_RHORB   ASSVGNTMEKTRLAVALMGKVSFTQLTEMLNAGMNRALPSCLAAE-DPSL    465
HUTH_STRGR   GAPVAYVLDFLAIVAADLGSICERRTDRLLDKNRSHGLPPFLAD--DAGV    381
HUTH_PSEPU   AEPVAMAADNLALAIAEIGSLSERRISLMMDKHMSQ-LPPFLVE--NGGV    377
HUTH_RAT     GEYPAKALDYLAIGVHELAAISERRIERLCNPSLSE-LPAFLVA--EGGL    492
HUMHISP      GEYPAKALDYLAIGIHELAAISERRIERLCNPSLSE-LPAFLVA--EGGL    492
             -   -   -     -  -- -   - - - -   -  *-- * -  - --

POPLARPAL    DYGFKGAEIAMASYCSELQYLANPVTTHVQSAEQHNQDVNSLGLISSRKT    500
PALY_MEDSA   DYGFKGAEIAMASYCSELQYLANPVTTHVQSAEQHNQDVNSLGLISARKT    510
PALY_ARATA   DYGFKGAEIAMASYCSELQYLANPVTSHVQSAEQHNQDVNSLGLISSRKT    510
PAL1_SOLTU   DYGFKGAEIAMASYCSELQFLANPVTNHVQSAEQHNQDVNSLGLISARKT    505
ORCHIDPAL    DYGFKGAEIAMASYCSELQALANPVTNHVQSAEQHNQDVNSLGLISSRKT    489
WHEATPAL     DYGFKGAEIAMASYCSELQFLGNPVTNHVQSAEQHNQDVNSLGLISSRKT    485
PALY_ORYSA   DYGFKGTEIAMASYSSELQYLANPITNHVQSAEQHNQDVNSLGLVSARKT    488
PINEPAL      DYGLKGAEIAMASYTSELLYLANPVTSHVQSAEQHNQDVNSLGLVSARKS    502
PALY_USTMA   NFHAKGINIALASVTSELMFLGNPVSTHVQSAEMANQAFNSLALISGRQT    512
PALY_RHOTO   SYHCKGLDIAAAAYTSELGHLANPVTTHVQPAEMANQAVNSLALISARRT    513
PALY_RHORB   SYHCKGLDIAAAAYTSELGHLANPVSTHVQPAEMGNQAINSLALISARRT    515
HUTH_STRGR   DSGLMIAQYTQAALVSEMKRLAVPASADSIPSSAMQEDHVSMGWSAARKL    431
HUTH_PSEPU   NSGFMIAQVTAAALASENKALSHPHSVDSLPTSANQEDHVSMAPAAGKRL    427
HUTH_RAT     NSGFMIAHCTAAALVSESKALCHPSSVDSLSTSAATEDHVSMGGWAARKA    542
HUMHISP      NSGFMIAHCTAAALVSENKALCHPSSVDSLSTSAATEDHVSMGGWAARKA    542
             -   -  - - *-  **   *  * - -  ---   --  *--  ----

POPLARPAL    AEAVDILKLMSTTFLVALCQAIDLRHLEENLKSAVKNTVSQVSKRVLTTG    550
PALY_MEDSA   NEAIEILQLMSSTFLIALCQAIDLRHLEENLKNSVKNTVSQVAKKTLTMG    560
PALY_ARATA   SEAVDILKLMSTTFLVAICQAVDLRHLEENLRQTVKNTVSQVAKKVLTTG    560
PAL1_SOLTU   AEAVDILKLMSSTYLVALCQAIDLRHLEENLKSVVKNTVSQVAKRTLTIG    555
ORCHIDPAL    AEAVDILKLMSTTFLVGLCQAVDLRHLEENLKNAVKNTVSQVAKRVLTMG    539
WHEATPAL     AEAIDILKLMSSTFLVALCQAIDLRHLEENVKNAVKSCVKTVARKTLSTD    535
PALY_ORYSA   LEAVDILKLMTSTYIVALCQAVDLRHLEENIKSSVKNCVTQVAKKVLTMN    538
PINEPAL      AEAIDILKLMLSTYLTALCQAVDLRHLEENMLATVKQIVSQVAKKTLSTG    552
PALY_USTMA   LQAIECLSMIQAWSLYLLCQALDIRALQYKVAEQLPTLILASLHSHFGEW    562
PALY_RHOTO   TESNDVLSLLLATHLYCVLQAIDLRAIEFEFKKQFGPAIVSLIDQHFGSA    563
PALY_RHORB   AEANDVLSLLLATHLYCVLQAVDLRAMEFEHTKAFEPMVTELLKQHFGAL    565
HUTH_STRGR   RTAVDNLARIVAVELYAATRAIELRAAE-GLTPAPA---SEAVVAALRAA    477
HUTH_PSEPU   WEMAENTRGVPAIEWLGACQGLDLR--K-GLKTSAK---LEKARQALRSE    471
HUTH_RAT     LRVIEHVEQVLAIELLAACQGIEFL--R-PLKTTTP---LEKVYDLVRSV    586
HUMHISP      LRVIEHVEQVLAIELLAACQGIEFL--R-PLKTTTP---LEKVYDLVRSV    586
                 -   - -       -----

POPLARPAL    ANGELHPSRFCE---KELLKVVDREYVFAYVDDPCSATYPLMQKLRQVFV    597
PALY_MEDSA   VNGELHPSRFCE---KDLLKVVDREHVFAYIDDPCSATYPLSQKLRQVLV    607
PALY_ARATA   VNGELHPSRFCE---KDLLKVVDREQVYTYADDPCSATYPLIQKLRQVIV    607
PAL1_SOLTU   AIGELHPARFCE---KELLRVVDREYLFTYADDPCSSTYPLMQKLRQVLV    602
ORCHIDPAL    VNGELHPSRFCE---KDLIKVIDREYVFAYADDPCSSTYPLMQKLRAVIV    586
WHEATPAL     NNGHLHNARFCE---KDLLLTIDREAVFAYADDPCSANYPLMQKMRAVLV    582
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PALY_ORYSA    PTGDLSSARFSE---KNLLTAIDREAVFSYADDPCSANYPLMQKLRAVLV    585
PINEPAL       LNGELLPGRFCE---KDLLQVVDNEHVFSYIDDPCNASYPLTQKLRNILV    599
PALY_USTMA    MDETK-QQEIAAQVLKSMSKRLDETSSKDLRDRLVETYQDASSVLVRYFS    611
PALY_RHOTO    MTGSNLRDELVEKVNKTLAKRLEQTNSYDLVPRWHDAFSFAAGTVVEVLS    613
PALY_RHORB    ATA-----EVEDKVRKSIYKRLQQNNSYDLEQRWHDTFSVATGAVVEALA    610
HUTH_STRGR    GAEGPGPDRFLAPDLAAADTFVREGRLVAAVE-------PVTG-------    513
HUTH_PSEPU    -VAHYDRDRFFAPDIEKAVELLAKGSLTGL--------------------    500
HUTH_RAT      -VRPWIKDRFMAPDIEAAHRLLLDQKVWEVAA-------PYIEKYR----    624
HUMHISP       -VRPWIKDRFMAPDIEAAHRLLLEQKVWEVAA-------PYIEKYR----    624

POPLARPAL     DHALENGENEKNFSTSVFQKIEAFEEELKALLPKEVESARAAYDSGNSAI    647
PALY_MEDSA    DHALVNGESEKNFNTSIFQKIATFEEELKTLLPKEVESARTAYESGNPTI    657
PALY_ARATA    DHALVNGESEKNAVTSIFHKIGAFEEELKAVLPKEVEAARAAYDNGTSAI    657
PAL1_SOLTU    DHAMKNGESEKNINSSIFQKIGAFEDELNAVLPKEVESARALLESGNPSI    652
ORCHIDPAL     EHALNNGVKEKDSNTSIFQKISSFENELKAALPKEVEAARAEFENGSPAI    636
WHEATPAL      EHALANGE-EAHVETSVFAKLAMFEQELRAVLPKEVEAARSAVENGTAAQ    631
PALY_ORYSA    EHALTSGDR-RARGLRVLQDHQVRGGAPLCAAPGDRGRPRRRRQR-TAPV    633
PINEPAL       EHAFKNAEGEKDPNTSIFNKIPVFEAELKAQLEPQVSLARESYDKGTSPL    649
PALY_USTMA    E--LPSGGGADPLRNIVKWRATGVADTEK--IYRQVTIEFLDNPYACHAS    657
PALY_RHOTO    -------STSLSLAAVNAWKVAAAESAIS--LTRQVRETFWSAASTSSPA    654
PALY_RHORB    -------GQEVSLASLNAWKVACAEKAIA--LTRSVRDSFWAAPSSSSPA    651
HUTH_STRGR    ----------------------------------------------------    513
HUTH_PSEPU    ----------------------------------------------------    500
HUTH_RAT      ----------------------------------------------------    624
HUMHISP       ----------------------------------------------------    624

POPLARPAL     DNKIKECRSYPLYKFVREELGTVLLTGEKVQSPGEEFDKVFTAMCQGKII    697
PALY_MEDSA    PNKINGCRSYPLYKFVREELGTGLLTGENVISPGEECDKLFSAMCQGKII    707
PALY_ARATA    PNRIKECRSYPLYRFVREELGTELLTGEKVTSPGEEFDKVFTAICEGKII    707
PAL1_SOLTU    PNRITECRSYPLYRLVRQELGTELLTGEKVRSPGEEIEKVFTAMCNGQIN    702
ORCHIDPAL     ENRIKDCRSYPLYKFVKE-VGSGFLTGEKVVSPGEEFDKVFNAICEGKAI    685
WHEATPAL      QNRIAECRSYPLYRFVRKELGTEYLTGEKTRSPGEEVDKVFVAMNQGKHI    681
PALY_ORYSA    ANRIVESRSFPLYRFVREELGCVFLTGEKLKSPGEECNKVFLGISQGKLI    683
PINEPAL       PDRIQECRSYPLYEFVRNQLGTKLLSGTRTISPGEVIEVVYDAISEDKVI    699
PALY_USTMA    --HLLG-KTKRAYEFVRKTLGV------PMHGKEN--------------L    684
PALY_RHOTO    LSYLSP-RTQILYAFVREELGV------KAR-RGD--------------V    682
PALY_RHORB    LKYLSP-RTRVLYSFVREEVGV------KAR-RGD--------------V    679
HUTH_STRGR    --------P-----LA-----------------------------------    516
HUTH_PSEPU    --------------LPAGVLP-----------------------------    507
HUTH_RAT      MEHIPESRP-----LSPTAFSLESLRKNSATIPESD--------------    655
HUMHISP       MEHIPESRP-----LSPTAFSLQFLHKKSTKIPESE--------------    655

POPLARPAL     DPMLECLGEWNGS-------PLPIC-------------------------    715
PALY_MEDSA    DPLLECLGEWNGA-------PLPIC-------------------------    725
PALY_ARATA    DPMMECLNEWNGA-------PIPIC-------------------------    725
PAL1_SOLTU    DPLLECLKSWNGA-------PLPIC-------------------------    720
ORCHIDPAL     DPMLDCLKEWNGA-------PLPIC-------------------------    703
WHEATPAL      DALLECLKEWNGE-------PLPLC-------------------------    699
PALY_ORYSA    DPMLDCLKEWNGE-------PLPIN-------------------------    701
PINEPAL       VPLFKCLDGWKGTLAHSEINNLPRSPLYNDCYDLSPRMLLLMLLFSDPEF    749
PALY_USTMA    NEFKGEFEQWNTTGGY-------VSVIYA-----SIRDGELYNMLSELER    722
PALY_RHOTO    --FLGKQEV--TIGSN-------VSKIYE-----AIKSGRINNVL--LKM    714
PALY_RHORB    --YLGKQEV--TIGTN-------VSRIYE-----AIKSGCIAPVL--VKM    711
HUTH_STRGR    ---------------------------------SL----------------    516
HUTH_RAT      ---------------------------------DL----------------    509
HUMHISP       ---------------------------------DL----------------    657
```

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POPLARPAL     ---     715
PALY_MEDSA    ---     725
PALY_ARATA    ---     725
PAL1_SOLTU    ---     720
ORCHIDPAL     ---     703
WHEATPAL      ---     699
PALY_ORYSA    ---     701
PINEPAL       DWS     752
PALY_USTMA    D-L     724
PALY_RHOTO    L-A     716
PALY_RHORB    M-A     713
HUTH_STRGR    ---     516
HUTH_PSEPU    ---     509
HUTH_RAT      ---     657
HUMHISP       ---     657
```

Fig. 3.10. Comparison of the deduced amino acid sequence of *U. maydis* PAL with the deduced amino acid sequences of PAL and HAL from various organisms.
PALs; Poplar (POPLARPAL), Alfalfa (PALY_MEDSA), *A. thaliana* (PALY_ARATA), Potato (PAL1_SOLTU), Orchid (ORCHIDPAL), Wheat (WHEATPAL), Rice (PALY_ORYSA), Pine (PINEPAL), *U. maydis* (PALY_USTMA), *R. toruloides* (PALY_RHOTO), and *R. rubra* (PALY_RHORB). HALs; *S. griseus* (HUTH_STRGR), *P. putida* (HUTH_PSEPU), Rat (HUTH_RAT), and Human (HUMHISP). The amino acid sequences are in one-letter code and have been aligned using the Clustal program in PC/GENE software. The protein sequences were obtained from the SWISS-PROT database. Their database accession numbers are described in Materials and Methods. Perfectly conserved and well- conserved positions in the alignment are indicated as * and - , respectively. Bold sequences indicate highly conserved regions.

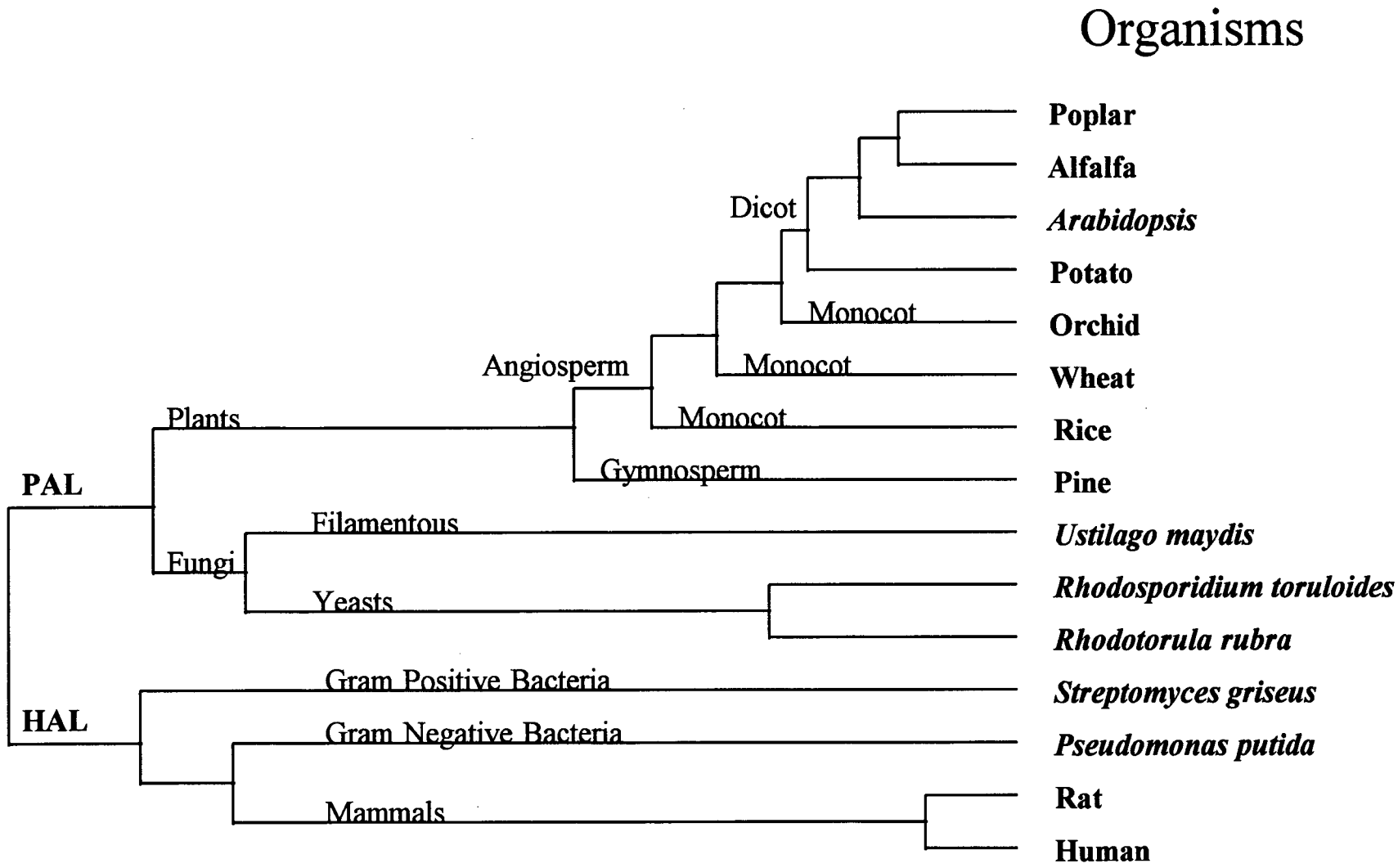
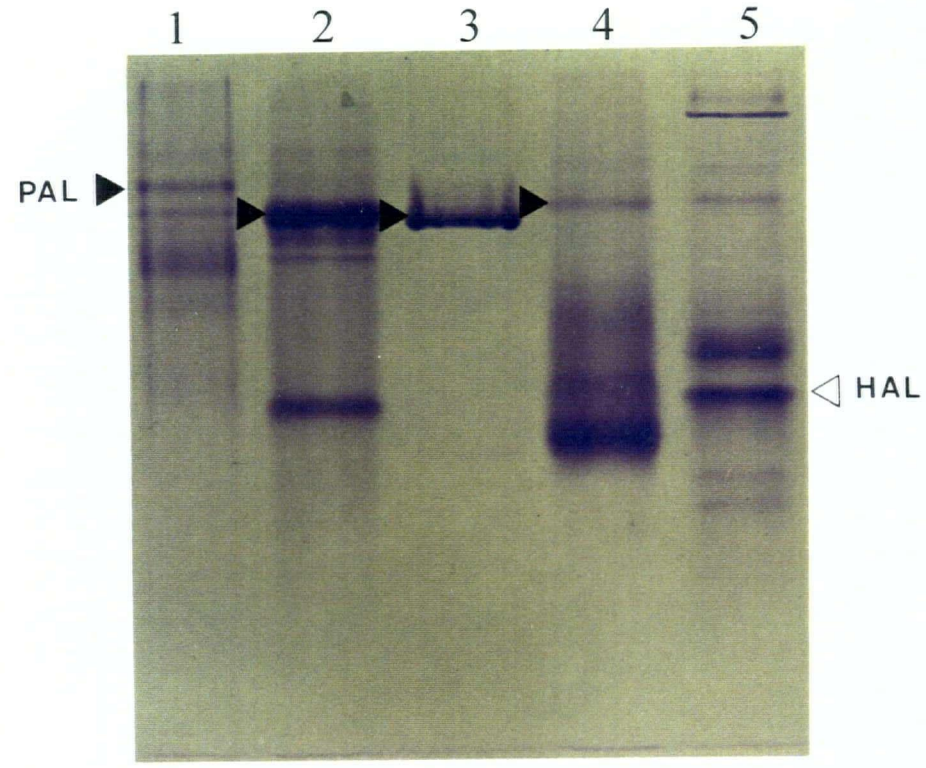
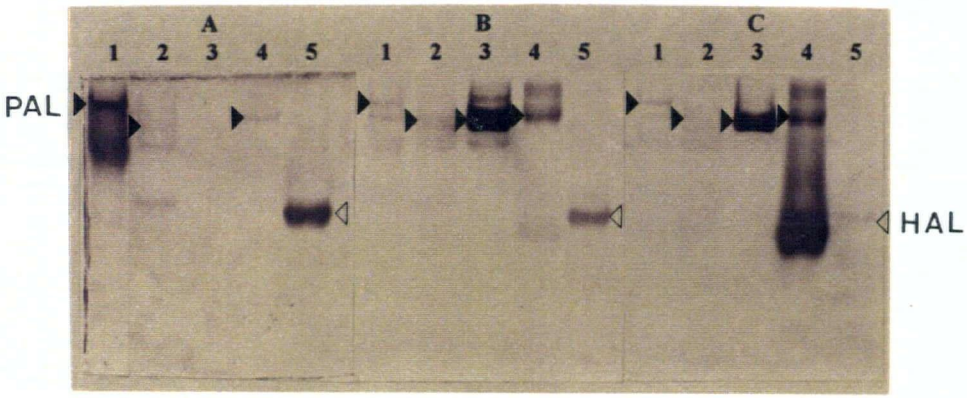
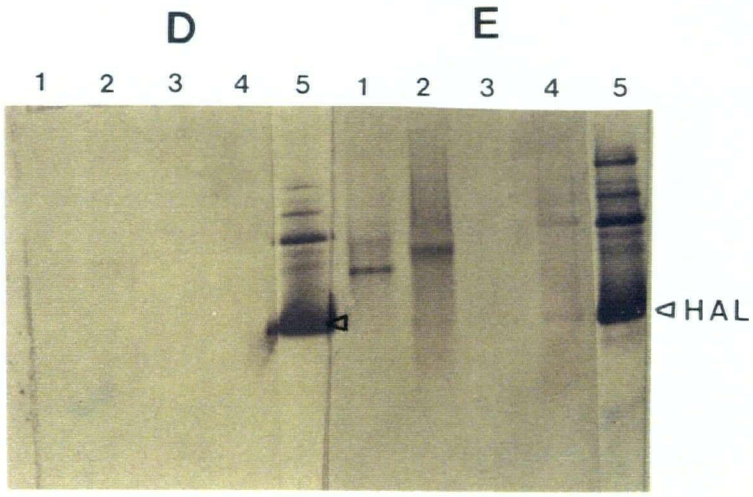


Fig. 3.11. Dendrogram of the inferred protein sequences of PALs and HALs from various organisms using PC-GENE Clustal Program. PALs; Poplar (POPLARPAL), Alfalfa (PALY_MEDSA), *Arabidopsis thaliana* (PALY_ARATA), Potato (PAL1_SOLTU), Orchid (ORCHIDPAL), Wheat (WHEATPAL), Rice (PALY_ORYSA), Pine (PINEPAL). *U. maydis* (PALY_USTMA), *R. toruloides* (PALY_RHOTO), and *R. rubra* (PALY_RHORB). HALs; *S. griseus* (HUTH_STRGR), *P. putida* (HUTH_PSEPU), Rat (HUTH_RAT), and Human (HUMHISP).

Fig. 3.12. Native PAGE (top) and Western blots (center and bottom) of fungal PAL, plant PAL and bacterial HAL using polyclonal antibodies raised against *U. maydis* PAL (A), poplar PAL (B), alfalfa PAL (C), *P. fluorescens* HAL (D), and *P. putida* HAL (E). Proteins loaded: Lane1-*U. maydis* PAL, Lane 2-*R. toruloides* PAL, Lane 3-poplar PAL, Lane 4-potato PAL, and Lane 5-*P. fluorescens* HAL. Arrow indicates the location of PAL or HAL protein.


1
2
3
4
5
PAL
HAL


A
B
C
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
PAL
HAL


D
E
1 2 3 4 5 1 2 3 4 5
HAL

HAL. Significant cross-reactivity between two plant PAL antibodies and the bacterial HAL was also observed.

Immunological enzyme inhibition tests were conducted to see whether the pattern of cross-reactivity observed on western blots carried over to solution reactions (Table 3.8). In this system, both the anti-*Ustilago* PAL and the anti-poplar PAL antibodies displayed similar enzyme inhibition patterns, including moderate inhibition of bacterial HAL activity. However, the bacterial HAL antibody inhibited only *Ustilago* PAL. The PAL and HAL antibodies tested showed no inhibition against yeast PAL.

The hydrophilicity profiles of the entire deduced amino acid sequences of poplar PAL, alfalfa PAL, *Rhodotorula* yeast PAL, *Ustilago* PAL, *Pseudomonas* bacteria HAL and rat HAL were analyzed using PC/GENE software program to compare the location of putative antigenic determinant sites (Fig. 3.13). *Ustilago* PAL contains two sites with high hydrophilicity but the other sequences contain only one site, suggesting that at least some antigenic determinant sites of *Ustilago* PAL would be different from those of the other enzymes compared. An overall pattern of similarity is present only between the two plant PALs and yeast PAL in that the most hydrophilic point is located in the middle of the sequences in each case. By contrast, the most hydrophilic sites of *U. maydis* PAL are located closer to the amino- and carboxyl-terminal ends. It is noticeable that the most hydrophilic site of bacterial HAL is located closer to the carboxyl-terminal of the sequence, as in *Ustilago* PAL.

Table 3.8. Inhibition of PAL and HAL activity by antisera raised against the two ammonia-lyases

Enzymes	*P. fluorescens* HAL antiserum	*U. maydis* PAL antiserum	Poplar PAL antiserum
P. fluorescens HAL	+ (68%)	+ (84%)	+ (80%)
U. maydis PAL	+ (90%)	+ (60%)	+ (92%)
R. toruloides PAL	–	–	–
Potato PAL	–	+ (39%)	+ (67%)
Poplar PAL	–	+ (74%)	+ (80%)

+: Inhibition, –: no inhibition, (%): inhibition of enzyme activity by antiserum expressed as a percentage of a control activity with preimmune serum.

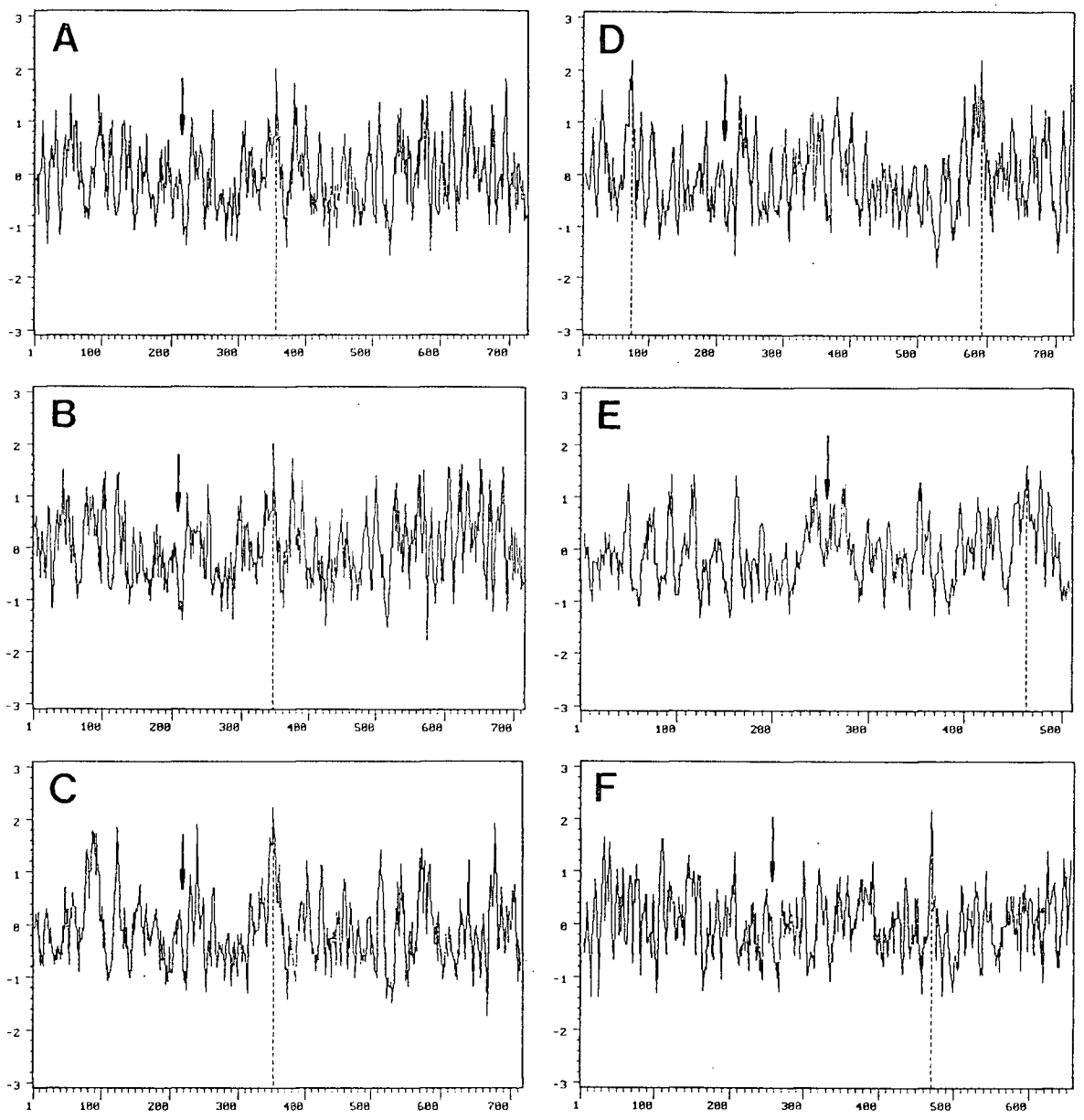


Fig. 3.13. Hydrophilicity profiles of PALs and HALs from different organisms.
A: alfalfa PAL, B: poplar PAL, C: *R. toruloides* PAL, D: *U. maydis* PAL, E: *P. putida* HAL, and rat HAL. Positive values indicate hydrophilic regions. Vertically dotted lines indicate the most hydrophilic amino acid residues. Arrows indicate the position of acive site.

3.4 DISCUSSION

PAL genes have been cloned from numerous cultivated plant species as well as two trees and a weed but only from two red yeasts. Today many potential methods are available to clone PAL genes from plants and yeast. So far, most PAL genes described in the literature have been cloned by one of three approaches: 1) using antibodies raised against the purified enzyme from the same species, 2) using heterologous DNA probes from other species, or 3) using PCR amplification with degenerate oligonucleotide primers based on amino-terminal peptide sequences of purified PAL or conserved amino acid sequences among different species. Initial trials with these approaches for the isolation of a PAL cDNA clone from *U. maydis* cDNA libraries were unsuccessful. Immunoscreening of libraries with anti-*U. maydis* PAL antibodies produced non-specific background, while no hybridization signal was detected with poplar and yeast PAL DNA probes. Purified *U. maydis* PAL enzyme was found to be blocked at the amino terminus, preventing protein sequencing. Internal peptide sequences obtained by tryptic digestion were found to have high codon degeneracy and primers based on these sequences had low calculated annealing temperatures, so cloning based on this information was also unsuccessful.

Based on the known primary structure of PAL genes from plants and yeast, several degenerate oligonucleotide primers were designed, and two of these succeeded in amplifying a *U. maydis* PAL DNA fragment (0.45 kb) by PCR from a *U. maydis* genomic DNA template. Subsequently, a PAL gene was isolated by using this 0.45 kb fragment as a probe to screen a *U. maydis* genomic library. As shown in Fig. 3.2, this PCR approach

was successful only with the combination of the enzyme active-site primer, UMPAL7, and a yeast sequence-specific primer, RTPAL3. It is worth noting that combinations of oligonucleotide primers based on other highly conserved sequences among PAL genes from various species failed to amplify *U. maydis* PAL in PCR. PAL is known to be a difficult enzyme to purify sufficiently for the production of antibodies (Jones, 1984), and even then, the antibody screening methods are not always successful (as in the case of this study). The failing of most of the degenerate primer combinations shows how challenging the cloning of highly diverged PAL genes can be.

The 2172 bp nucleotide genomic DNA sequence determined in this study is confirmed to be a PAL gene on the basis of its homology with other known PALs, conservation of enzyme active site sequences, identity with the internal peptide sequence of purified PAL protein, and similarity of physico-chemical properties (e.g. molecular weight and isoelectric point) predicted from the deduced protein sequence with those of the purified enzyme. Among PAL enzymes purified to date, the isoelectric point of 6.3 found for purified *U. maydis* PAL (chapter 2) makes this the least acidic of the known PAL proteins. Interestingly, this value corresponds to the calculated value of 6.3 from the deduced protein sequence. Protein data from many sources show that PAL is a homo-tetrameric protein consisting of four identical subunits with molecular weights in the range of 72000-83000 (Schomburg and Salzmann, 1990). In a previous study (chapter 2), it was estimated that *U. maydis* PAL protein has a 80000 subunit size. This is essentially identical to the calculated molecular weight (79277) of the deduced protein. *U. maydis* PAL, consisting of 724 deduced amino acids, is longer than most known PALs

but shorter than the 752 amino acids of pine (Zhang and Chiang, GenBank accession no. U39792), and the 725 amino acids of alfalfa PAL (Gowri *et al.*, 1991).

PAL from maize and potato are known to be glycoproteins (Havir, 1973; Shaw *et al.*, 1990). Analysis of PAL gene sequences from alfalfa (Gowri, 1991), bean (Cramer *et al.*, 1989), parsley (Lois *et al.*, 1989), and *Ustilago* (this study) reveal several potential N-glycosylation sites, but their functional significance remains to be elucidated. Protein data from SDS-PAGE (chapter 2) suggest that posttranslational modification through glycosylation is unlikely in *Ustilago* PAL, as it migrates at a molecular weight corresponding to the size of deduced protein. Heterologous expression in *E. coli* of PAL genes from yeast (Orum and Rasmussen, 1992) and parsley (Shultz *et al.*, 1989; Appert *et al.*, 1994) has demonstrated that glycosylation is not essential for the catalytic activity of the enzyme. However, it has been postulated that glycosylation may contribute to enzyme stability, to the correct localization of the enzyme within the cell, and to positioning of the active sites for optimal activity (Havir, 1973; Shaw *et al.*, 1990). Bolwell (1992) reported a role for phosphorylation in the regulation of PAL from bean. Detection of a number of phosphorylation sites in the *U. maydis* PAL sequence provides the possibility that phosphorylation may have a role in modulating the *Usilago* PAL protein activity. The observation of a synergistic effect of cAMP with tryptophan on PAL induction in *U. maydis* cell cultures (chapter 4), and the presence of a cAMP-dependent protein kinase phosphorylation site in the *U. maydis* PAL sequence, support this possibility, but the process and mechanisms of any such phosphorylation remain unknown.

In contrast to most plant PAL genes or yeast PAL genes, no A+C- or C+T- rich sequences are found in the upstream region of the *Ustilago* PAL coding sequence. As in bean (Cramer *et al.*, 1989), parsley (Lois *et al.*, 1989), and rice PALs (Minami *et al.*, 1989; Zhu *et al.*, 1995), a TATA box is present upstream from the translation start site in *Ustilago* PAL. In contrast, the *Rhodosporidium* PAL gene has no TATA box (Anson *et al.*, 1987). The higher eukaryotic consensus polyadenylation signal AATAAA in the 3' noncoding region is found in most plant PAL genes and in the *Rhodosporidium* PAL gene. The absence of this sequence in *Ustilago* PAL genes is one of the structural features which distinguishes the *Ustilago* PAL gene from other known PAL genes. Amino acid sequence identities of 39-40% with yeast fungal PALs and 23-26% with plant PALs (Table 3.7) indicate that the *Usilago* PAL structure has diverged markedly from other PALs. Furthermore, the absence of introns in the *Ustilago* PAL coding region is in striking contrast to virtually all known PAL genes. Thus far, most PAL-encoding genes in plants contain a single intron in the 5' end of the gene; the exception is an *Arabidopsis* gene, *PAL3* (Wanner *et al.*, 1995), which contains a second intron further downstream. The tendency in other fungi seems to be the retention of numerous introns, since the *R. rubra* PAL gene has five introns (Filpula *et al.*, 1988) while that of *R. toruloides* has six (Anson *et al.*, 1987). Interestingly, as in the case of *Ustilago*, there is apparently no intron in PAL genes in conifers, *Pinus banksiana* (Campell, 1991; S. Butland, personal communication) and *Pinus taeda* (Sederoff *et al.*, 1994).

PAL is encoded by a small gene family in most plants, with the exceptions of the large PAL gene family (40-50 genes) in potato (Joos and Hahlbrock, 1992) and the single PAL gene in *Pinus taeda* (Whetton and Sederoff, 1992). In fungi, the only report available is

from *R. toruloides*, which possesses a single copy of the PAL gene in its gemome (Gilbert *et al.*, 1985). Genomic Southern blot analysis (Fig. 3.8) indicates that only one PAL gene is present in the *U. maydis* genome. The substantial difference in gene sequence and organization, and the distinct kinetic (i.e. high K_m) and physico-chemical (i.e. high pI) properties (chapter 2), are all consistent with the highly diverged *Ustilago* PAL gene appearing as a distinct branch in the PAL dendrogram (Fig. 3.11). These unique features of *U. maydis* PAL raise the interesting question of how these differences might influence the function and regulation of PAL activity in *Ustilago*.

Examination of the patterns of conserved sequences between all the HALs and PALs analyzed in this study showed that sequence conservation is restricted to relatively few regions within the protein, consistent with the analysis of Taylor *et al.* (1990). Given that both proteins catalyze a similar reaction, albeit with different substrates, it seems likely that these conserved regions are important for establishing the architecture and function of a dehydroalanine-containing catalytic centre. Regions of sequence divergence, on the other hand, would be predicted to reflect the ability of the two enzymes to utilize different substrates, as well as differing patterns of regulation and cellular organization. I have confirmed (using *P. fluorescens* HAL, *U. maydis* PAL and poplar PAL) that there is no evidence of HAL activity associated with PAL, and *vice versa* (data not shown). It thus appears that modern HAL and PAL genes share a common ammonia-lyase ancestor, but have diverged extensively to serve very different functions in different organisms, or within the same organisms, as shown in the dendrogram of Fig. 3.11. Unfortunately, information on HAL in plants and fungi is very limited, as is sequence data for PAL in *Streptomyces* and the true fungi. A more detailed evolutionary study of these two

enzymes will require cDNA sequence data from more taxa, particularly in the fungi and prokaryotes.

Immunological comparison of proteins can provide useful indications of tertiary and quaternary structural relatedness between proteins from different sources. The patterns of weak recognition (Fig. 3.12) between the *U. maydis* fungal PAL antibodies and plant PALs, and between plant PAL antibodies and fungal PALs, are consistent with the divergence observed between fungal PAL and plant PAL protein sequences (Fig. 3.11). In addition, the weak detection of yeast fungal PAL by *Ustilago* PAL antibodies correlates with the extensive protein sequence divergence between these two fungi, as shown in Fig. 3.11.

U. maydis PAL and *Pseudomonas* HAL differ greatly in their properties (e.g. *Ustilago* PAL has a subunit molecular mass of 80 kDa, whereas the *Pseudomonas* HAL subunit has a mass of 55 kDa). However, the strong binding of the *Ustilago* PAL antibodies to *Pseudomonas* HAL implies that both enzymes possess common epitopes. Their sequence homology (25% identity in protein sequence), and the presence of the most hydrophilic residues closer to the carboxy terminus location in their hydrophilicity profiles (Fig. 3.12. D and E) of the deduced amino acid sequences, also suggest that there could be an unexpected level of structural relatedness between the *Ustilago* PAL and *Pseudomonas* HAL. Significant cross-reactivity between the three PAL antibodies tested and the bacterial HAL confirms that the two enzymes are immunologically related. Curiously, however, cross-reactivity was not observed between antibodies raised against bacterial HAL (from either *Pseudomonas putida* or *P. fluorescens*) and PAL proteins from

Ustilago, yeast, poplar or potato. The reason for this lack of reciprocity is unknown, and may require identification of the main epitopes on both groups of proteins. There has been little success to date in raising stable monoclonal antibodies to PAL proteins, for reasons that are not clear. The immunological enzyme inhibition tests corroborated the presence of immunological relationships between PAL and HAL enzymes detected by Western blot analysis. Again, in this test system, while the anti-bacterial HAL antiserum did not inhibit any of the plant or yeast PALs, it did react with *Ustilago* PAL, providing further evidence for surprising structural relatedness between this *Ustilago* PAL and *Pseudomonas* HAL. None of the anti-PAL or anti-HAL antibodies tested were able to inhibit yeast PAL activity, consistent with the other data showing that yeast PAL is immunologically distinct from PAL of *Ustilago* and higher plants. To extend the array of antibody probes available for these immunological comparisons, antibodies generated against the mammalian class of HAL would be very useful.

Considering the very weak cross-reactivity that the *Ustilago* PAL protein displays with plant PALs, and even with yeast PAL, these results imply that it may be quite distinct from all other known PALs. This conclusion is supported by the sequencing of the *U. maydis* PAL gene, which revealed very low sequence homology with other PAL genes. This likely accounts for the failure to clone the *U. maydis* PAL gene from a *U. maydis* cDNA library using either plant PAL cDNA or yeast PAL DNA as heterologous probes. Together, the DNA sequence data and the immuno cross-reactivity data should help define those common structural features of PAL and HAL that have been retained during their long period of divergence.

To date, PAL genes have not been cloned from plant pathogens and even information on the properties of PAL from plant pathogens is extremely limited. With the cloning of PAL gene from the corn smut pathogen, *U. maydis*, in this study, and from its host plant, corn (Rösler *et al.*, 1997), the tools are now available to explore the role and the functional divergence of both PALs in the metabolic and physiological context of this plant-pathogen interaction.

CHAPTER FOUR

Regulation of Phenylalanine Ammonia-lyase by L-Tryptophan In *Ustilago maydis*

4.1 INTRODUCTION

Flow of materials through metabolic networks is effected by controlling the presence of enzymes or by controlling the activities of these enzymes. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), the first enzyme in the major catabolic pathway of L-phenylalanine in plants and some microorganisms, catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. In plants, this reaction forms the initial step of the phenylpropanoid pathway, a major secondary metabolic pathway that provides precursors for a number of unique and important metabolites (Hanson and Havir, 1981). PAL thus controls the channeling of a substantial portion of carbon from primary into secondary metabolism in plants (Jones, 1984). Plant PAL activity is induced by pathogen attack, and by environmental stresses such as wounding and light (Hahlbrock and Scheel, 1989). The enzyme activity is modulated during plant development in different tissues (Camm and Towers, 1973; Wanner, 1995), in response to hormones (Hughes *et al.*, 1990; Reid *et al.*, 1972; Ward *et al.*, 1989) and in response to heavy metals (Ohl *et al.*, 1990; Preisig *et al.*, 1991). In gherkin and mustard, PAL induction is reported to result from the activation of a constitutive pool of inactive enzyme (Attrige *et al.*, 1974), but in other plant species, PAL induction is the result of *de novo* synthesis of the enzyme (Hahlbrock and Schröder, 1975), or of a decrease in the rate of degradation of the enzyme (Zucker, 1971). PAL

activity is regulated, at least in part, at the level of transcription (Lawton and Lamb, 1987), but can also be regulated by the protein components of a system that inactivates the enzyme in apple skin (Tan, 1980), gherkin hypocotyls (French and Smith, 1975), lettuce (Ritenour and Saltveit, 1996), potato tuber (Zuker, 1968), sunflower leaves (Gupta and Creasy, 1984), and sweet potato roots (Tanaka, 1977).

Studies on PAL induction and regulation in microorganisms have been limited to the fungi *Rhodosporidium toruloides* (formerly *Rhodotorula glutinis*), *Rhizoctonia solani* and *Neurospora crassa*, although phenylalanine catabolism is reported in many microbial species. In *R. toruloides*, the enzyme is induced by L-phenylanine or L-tyrosine, and growth with glucose or ammonium salts represses enzyme synthesis (Gilbert and Tully, 1982). This regulation of PAL activity by phenylalanine, ammonia, and glucose has been shown to occur at the level of transcription (Gilbert *et al.*, 1983). *R. toruloides* PAL is also inducible in response either to a signal of nitrogen deprivation or to a signal of carbon limitation (Marusich *et al.*, 1982). In *N. crassa*, PAL synthesis requires specific induction by L-phenylalanine and nitrogen derepression (Sikora and Marzluf, 1982). *R. solani* PAL synthesis requires induction by L-phenylalanine, L-tyrosine or L-tryptophan (Kalghatgi and Subba Rao, 1976).

The properties and genetic control of PAL in both plants and fungi are important factors in our general understanding of the metabolic processes that yield phenylpropanoid secondary metabolites and energy. The substrate inducibility of fungal PAL activity, and the very different symbiotic or parasitic microenvironments in which fungi exist, raise the

question of how the regulation of PAL differs among fungi, and between plants and fungi, and how these differences might be reflected in the properties of the PAL protein.

The PAL protein of *Ustilago maydis* has been characterized and its gene structure described in Chapters 2 and 3, respectively. In this chapter I describe the distinct pattern of regulation of *Ustilago* PAL, which is very different from the patterns known for plants and other fungi. The *Ustilago* PAL gene is expressed constitutively, but the enzyme activity can be induced by L-tryptophan. The metabolic fate of L-phenylalanine in this fungus has also been briefly examined.

4.2 MATERIALS AND METHODS

4.2.1 Fungal strains and cultures

Ustilago strains used in this study are listed in Table 4.1. Cultures were grown on potato dextrose agar (PDA, Difco) and on complete (Holliday, 1974) or basal medium (2% glucose, 20 mM NH_4NO_3, and salt solution (Holliday, 1974), pH 7.0). A sporidial suspension (1 ml) (Absorbance $\geq$2.2 at 600 nm) was inoculated into 50 ml complete or basal medium in 250 ml Erlenmeyer flasks and grown for 24-48 hr at 30°C with shaking (250 rpm). Alternatively, 100 µl sporidial suspension was inoculated into 5 ml complete or basal medium in 15 ml culture tubes and grown for 16-36 hr. Unless otherwise indicated, *U. maydis* strain 518 and basal medium were used for the experiments on PAL

Table 4.1. *Ustilago* species and *U. maydis* strains used in this study

Species/Strain	Relevant Genotype
U. maydis 518	*a2b2*
U. maydis 521	*a1b1*
U. maydis UM031	*a1b2*
U. maydis UM032	*a2b1*
U. maydis D132	a1/a2 b1/b2
U. maydis D132-9	*a1/a2 b2/b1::HygB*^{r}
U. maydis uac1	*a2b2 uac1*
U. maydis ubc1	*a2b2 ubc1*
U. maydis adr1	*a2b2 adr1*
U. hordei	*MAT1*
U. nigra	wild type field isolate
U. aegilopsidis	wild type field isolate
Sporisorium reilianum	wild type field isolate

regulation. Fungal cell growth was measured by counting the number of sporidia using a haemocytometer, with five replicates. Mesurements for cell growth are illustrated in Figures with column (average value) and vertical bar (± standard deviation).

4.2.2 Chemicals

[α-^{32}P]-dATP (3000 Ci/mmol) and L-[^{3}H]-amino acid mixtures were purchased from ICN Biomedical Pharmaceuticals, Inc.. L-[U-^{14}C]-phenylalanine (474 mCi/mmol) was purchased from Amersham International. Hyamine hydroxide was obtained from Fluka G. Laboratories. Diethyl pyrocarbonate (DEPC), cyclic adenosine 5'-triphosphate (cAMP), cycloheximide, actinomycin D, daunomycin, α-amanitin, protein A-Sepharose beads, bovine serum albumin, L-phenylalanine, L-tyrosine, L- or D- or DL-tryptophan, *trans*-cinnamic acid, *para*-coumaric acid, benzoic acid, 4-hydroxybenzoic acid, other phenolics, indole, and indolic derivatives were obtained from Sigma. Westran membrane was purchased from Schleicher & Schuell; Zeta-Probe membrane, from Bio-Rad, and microbial media, from Difco. All other chemicals (analytical reagent grade) used were obtained from Sigma or Aldrich.

4.2.3 Enzyme extraction and assay

Cultures were harvested in a microfuge tube and the fungal cells were washed two times with sterile distilled water. The cell pellet was homogenized for 20 seconds at 4°C with alumina and a mini-pestle (Mandel Scientific Co.). The homogenate was suspended

in 200 µl ice-cold 0.5 M sodium phosphate buffer, pH 8.0, vortexed for 5 min with frequent cooling in ice and then centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was desalted (Pharmacia PD-10 column), and used for PAL assays and other experimental analyses. Unless otherwise described, all experiments were repeated at least twice with three or five replicates. All values illustrated in Tables and Figures are the average ± standard deviation of all mesurements (in Figures, column indicates average and vertical bar indicates ± standard deviation). PAL activity (three replicates per protein extract) was measured by a radiometric method as described by Campbell and Ellis (1992) using L-[U-^{14}C]-phenylalanine as substrate. Protein was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

4.2.4 Immuno-blot analysis

Enzyme extracts previously described were separated on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels as described by Laemmli (1970) and electro-transferred to PVDF membrane. PAL proteins on the blot were detected using *U. maydis* PAL antiserum as described in Chapter 2.

4.2.5 Immunoprecipitation of radiolabeled PAL protein

U. maydis cells were grown in basal medium (50 ml) for 12 or 16 hr with or without L-tryptophan (0.3 mM), supplemented with 50 µCi [^{3}H]-radiolabeled amino acids mixture, and further incubated for 4 hr. Following harvest by centrifugation at 6000 × *g* for 10 min

at 4 °C, the cells were washed three times with sterile distilled water and frozen in liquid nitrogen. Protein crude extracts were prepared by grinding the cells with alumina as described in Chapter 2. Radiolabeled PAL proteins were immunoprecipitated with *U. maydis* PAL antiserum (or preimmune serum) and protein A-Sepharose 4B beads , using a protocol described in 'Antibodies: A Laboratory Manual' pp. 466-468, (Harlow and Lane,1988). After heating in SDS sample buffer at 85°C for 10 min, immunoprecipitated proteins were separated from the beads by centrifugation and the radioactivity was measured using a Beckman LS-5000TA scintillation counter.

4.2.6 Phenylalanine catabolism analysis

U. maydis cells were grown for 16 hr in complete medium (50 ml) which was then supplemented with 5 μCi L-[U-^{14}C]-phenylalanine (sp. act. 474 mCi/mmol) or 2 μCi L-[U-^{14}C]-cinnamic acid (sp. act. 474 mCi/mmol). After two hours incubation, the cultures were separated into medium and cells by centrifugation at 14, 000 × *g* for 15 min at 4°C. Cell extracts were prepared by boiling in 95% ethanol for 10 min, while the culture medium was extracted with diethyl ether after acidification with 6N HCl. Both solvent extracts were concentrated with a stream of gaseous nitrogen and chromatographed by TLC (1st dimension; n-butanol:ethanol:water = 4:1:1, 2nd dimension; toluene:acetic acid = 4:1), and autoradiographed on X-Omat AR film (Kodak). The metabolites produced from phenylalanine were identified on the basis of their R_f values and co-chromatography with authentic samples. $^{14}CO_2$ produced by *U. maydis* cultures growing in the presence of L-[U-^{14}C]-phenylalanine [0.25 μCi] was captured in Hyamine-hydroxide solution and the radioactivity was measured by liquid scintillation counting.

4.3 RESULTS

4.3.1 Induction experiments

In order to determine whether PAL activity is detectable in *U. maydis* cells grown in standard microbial growth media, several nutrient sources were tested. PAL activity was present in cells grown with all the nutrient sources tested, although the level varied (Fig. 4.1). Potato dextrose broth and casamino acids supported higher levels of PAL activity, as well as increased cell growth. Beef extract, on the other hand, supported cell growth but yielded low levels of PAL production in this study. Based on the results shown in Fig. 4.1, regulation of PAL was investigated in two media: a nutrient-rich (complete) medium and a minimal (basal) medium. Depending on inoculum density and freshness, the specific PAL activity levels measured at the early stationary phase of cell growth ranged from 4 to 16 pkatal/mg protein in basal medium, and from 14 to 22 pkatal/mg protein in complete medium.

Since aromatic amino acids have been shown to function as inducers of PAL in some fungi (Kalghatgi and Subba Rao, 1976; Gilbert and Tully, 1982; Sikora and Marzluf, 1982), L-phenylalanine, L-tyrosine, and L-tryptophan were tested for their ablity to induce PAL in *U. maydis* 518 strain. The PAL activity levels obtained from fungal cells grown in 1% glucose media (pH 7.0) with these amino acids (1 mM each) are presented in Fig. 4.2A. The highest PAL activity occurred in cells grown in L-tryptophan-supplemented medium, whereas, L-tyrosine and L-phenylalanine supplementation had very little effect.

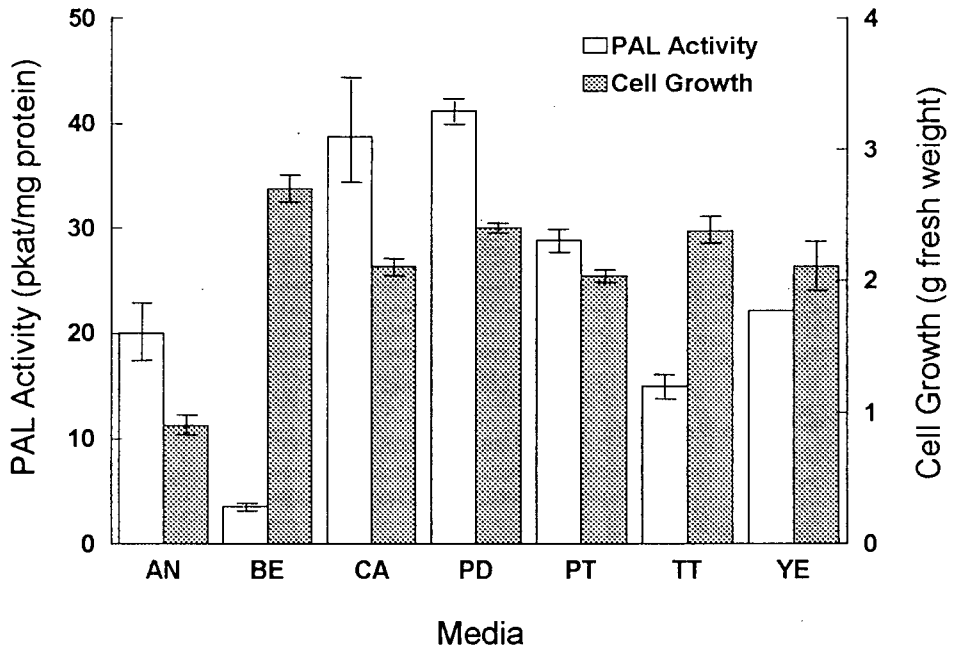


Fig. 4.1. *U. maydis* cell growth and PAL activity in different media. Cells were grown for 24 hr in each medium of 2% AN: ammonium nitrate, BE: beef extract, CA: casamino acids, PD: potato dextrose broth, PT: peptone, TT: tryptone, and YE: yeast extract.

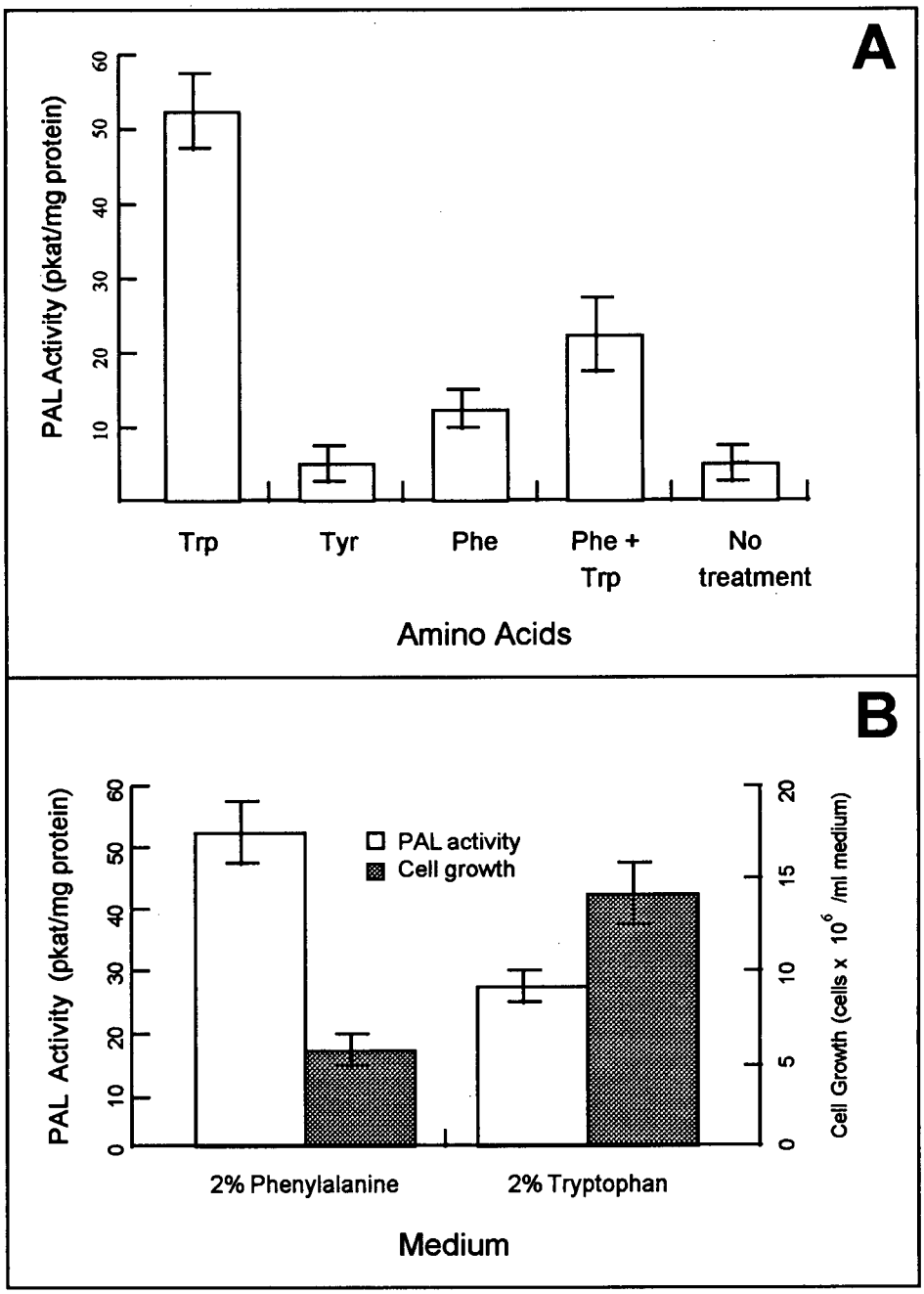


Fig. 4.2A. Effect of aromatic amino acids on PAL induction in *U. maydis*. 1 mM of each amino acid was added into 1 % glucose media and the fungal cells were grown for 36 hr, harvested, and assayed for PAL activity.
Fig. 4.2B. Cell growth and PAL activity of *U. maydis* grown on tryptophan or phenylalanine as sole carbone and nitrogen sources. The fungal cells were grown for five days in 2% tryptophan- or 2% phenylalanine-only medium containing inorganic salts (Holliday, 1974), and harvested for PAL assay and cell count.

Interestingly, PAL induction by L-tryptophan was strongly reduced by co-supplementation with L-phenylalanine.

The fungus was able to grow slowly on either tryptophan or phenylalanine (2% wt/v) as a sole carbon and nitrogen source (Fig. 4.2B). In this case, culture growth was better in tryptophan medium, but PAL activity was higher in phenylalanine medium. In absolute terms, however, these culture conditions did not result in any marked enhancement of PAL specific activity as compared to the results shown in Fig. 4.2A.

To determine whether PAL induction by tryptophan is dependent upon its concentration, different amounts (0.03, 0.1, 0.3, 1.0, and 3.0 mM) of L-tryptophan were used to supplement basal medium cultures. The increase in PAL activity responded linearly to the increase in L-tryptophan concentration up to 0.3 mM amino acid, and then declined (Fig. 4.3). The level of L-tryptophan (0.3 mM) giving the highest PAL induction in *U. maydis* is substantially lower than the level of L-phenylalanine supplemention 15 mM or 6 mM required for PAL induction in *R. toruloides* (Marusich and Zamir, 1981; Wick and Willis, 1982). Based on these results, 0.3 mM L-tryptophan was used for the following PAL regulation study.

4.3.2 PAL induction period

In order to define the time point of highest PAL inducibility during the culture cycle, *Ustilago* cultures were supplemented with 0.3 mM L-tryptophan at various time points in the growth cycle, incubated for two hours, harvested and assayed for PAL activity.

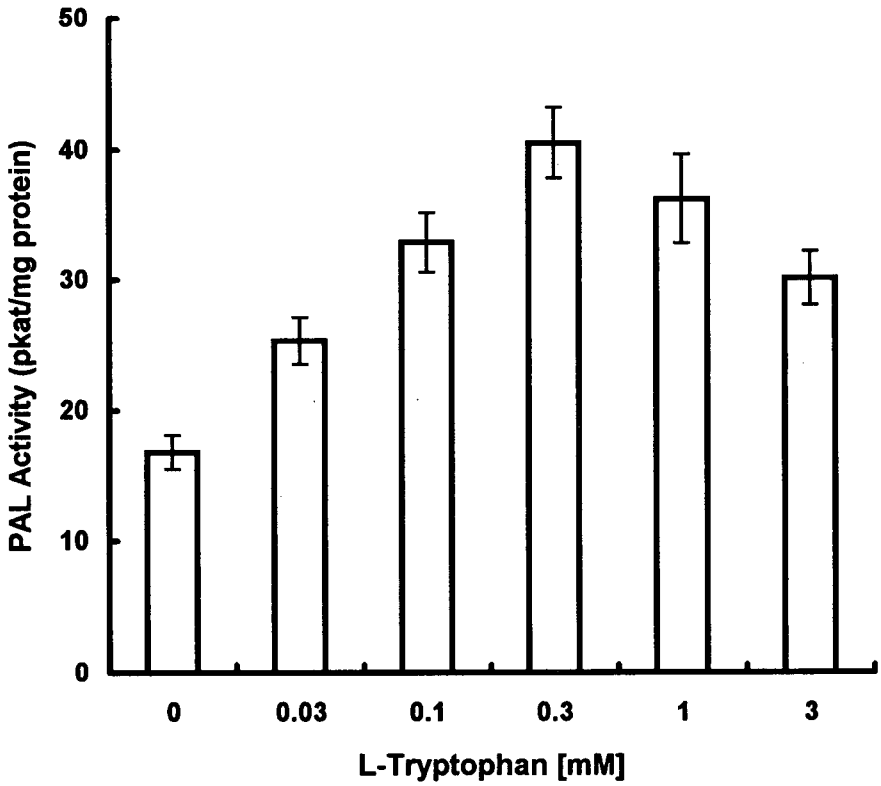


Fig. 4.3. Induction of *U. maydis* PAL at different concentrations of L-tryptophan. L-tryptophan was added 12 hr after inoculation into basal medium. Cells were incubated for 12 hr after adding tryptophan, harvested, and assayed for PAL activity.

Enhancement of PAL activity by these additions of exogenous L-tryptophan was only observed in cultures that had entered stationary phase (Fig. 4.4). This pattern was also observed when tryptophan was supplemented at the beginning of culture growth, and cultures were harvested at various time points in the growth cycle and assayed for PAL activity (data not shown). In this case, the elevated PAL levels were stable for at least twelve hours during the stationary phase of culture growth (data not shown). This pattern of highest inducibility and longer stability of *Ustilago* PAL during the stationary phase of growth differs slightly from *R. toruloides* PAL, which was highly induced during mid- to late log phase of growth and showed variation in stability during the stationary phase of growth (Wick and Willis, 1982; Gilbert *et al.*, 1983).

4.3.3 PAL inducibility by tryptophan optical isomers, precursors, and metabolites

An array of tryptophan optical isomers, precursors, and metabolites was tested for their abilty to induce PAL. All three isomeric forms of tryptophan (L, D, and DL) tested were able to induce higher levels of PAL, and to a similar degree (Fig. 4.5). On the other hand, PAL activity failed to increase in response to the various tryptophan precursors and metabolites tested, although indole-3-pyruvic acid gave a slight positive response. Therefore, PAL induction appears to be tryptophan-specific (Table. 4.2). Indole-3-acetic acid (IAA), which has been reported to occur naturally in *U. maydis* cultures (Navarre, 1990), reduced PAL activity almost 50% below control levels, while, indole (0.3 mM) itself was inhibitory to fungal cell growth, resulting in the failure of that component of the induction study.

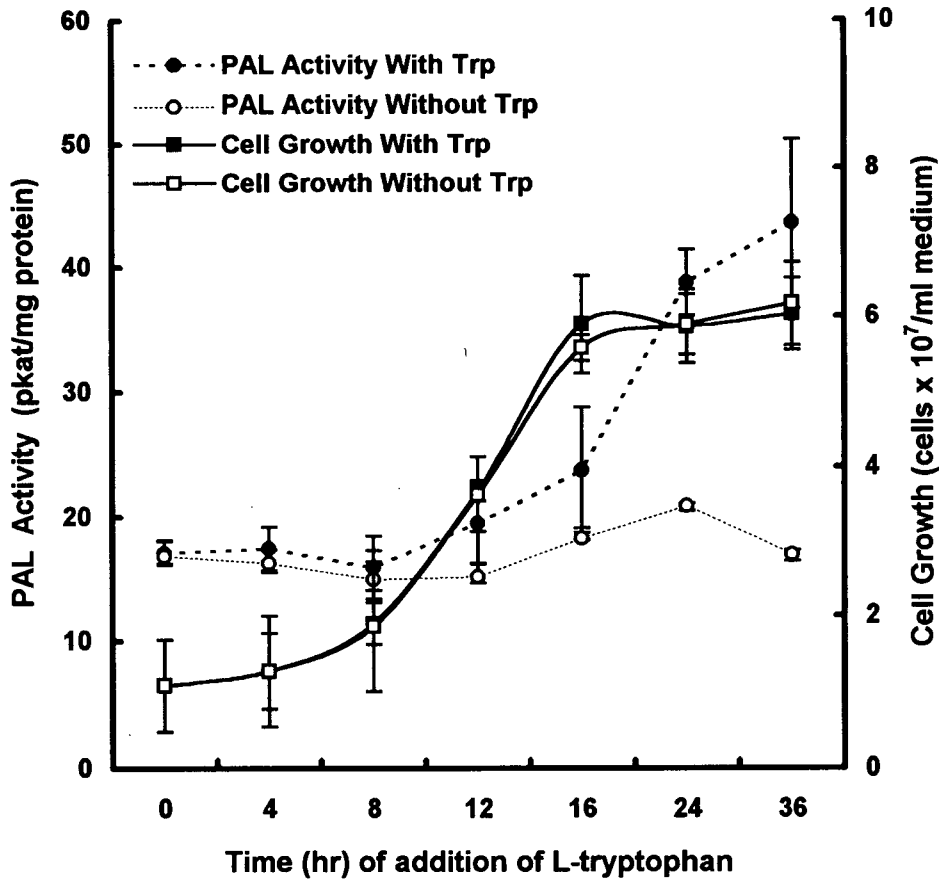


Fig. 4.4 Effect of tryptophan addition on cell growth and PAL induction of *U. maydis*. 0.3 mM L-tryptophan was added at different times in the cell cycle. Cells were harvested 2 hr later and assayed for PAL activity.

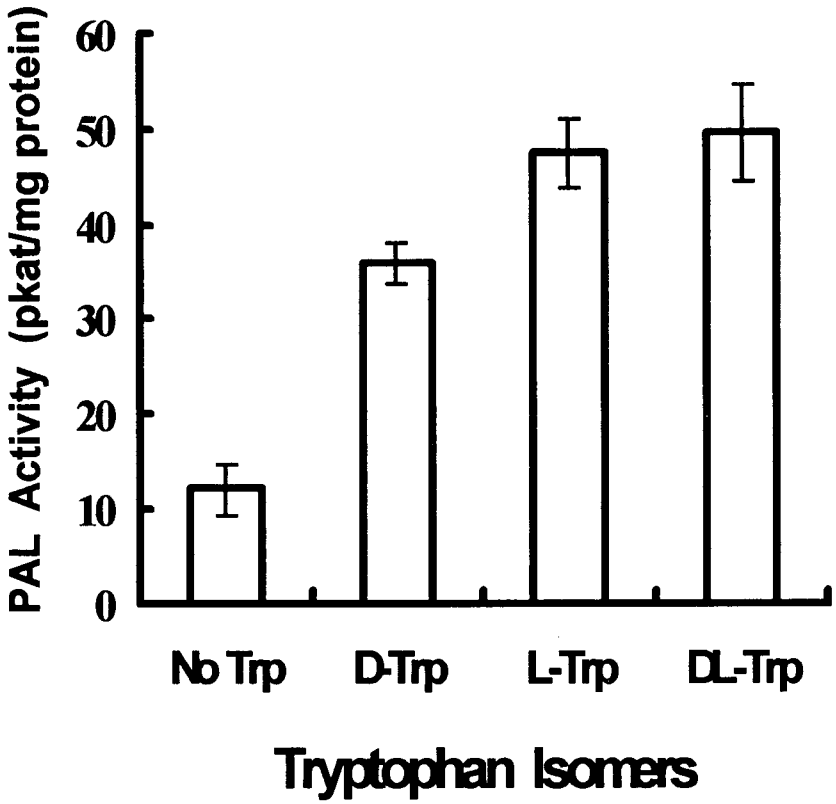


Fig. 4.5. Comparison of PAL inducibility in *U. maydis* by different optical isomers of tryptophan. 0.3 mM of each tryptophan isomer was added to 1% glucose media and the fungal cells were grown for 36 hr, harvested, and assayed for PAL activity.

4.2. Effect of tryptophan analogs and tryptophan-related metabolites on PAL induction in *U. maydis* [a]

Compounds	Relative PAL Activity (%) [b]
No supplement	100
Anthranilic acid	110 ± 10
Indole-3-acetamide	82 ± 9
Indole-3-acetic acid	55 ± 6
Indole-3-acetonitril	107 ± 11
Indole-3-butyric acid	114 ± 12
Indole-3-carbinol	116 ± 9
Indole-3-carboxylic acid	101 ± 10
DL-Indole-3-lactic acid	88 ± 7
Indole-3-pyruvic acid	135 ± 5
Kynurenic acid	76 ± 9
Tryptamine	98 ± 10
Tryptophol	86 ± 8
L-Tryptophan	310 ± 12

[a] Cells were grown in basal medium for 16 hr before addition of the exogenous compounds (0.3 mM each), harvested after 2 hr further incubation, and used for PAL assay.

[b] Relative PAL acitivity is presented as the percentage of the value of PAL activity in control which has no supplement of the compounds. Control showed 12 (pkat/mg protein) in PAL activity.

4.3.4 Tryptophan effect on PAL induction in other *U. maydis* strains and *Ustilago* species

The above observation that PAL induction was tryptophan-specific, prompted me to investigate whether PAL induction by L-tryptophan is a common phenomenon in *Ustilago* fungi. For this purpose, several *U. maydis* strains and *Ustilago* species were tested. PAL induction by L-tryptophan was observed in all the *U. maydis* strains tested (Fig. 4.6). However, among the other *Ustilago* species, only *U. nigra* revealed PAL induction by L-tryptophan (Fig. 4.7). All the *Ustilago* species tested did possess PAL, and its expression was detectable even without L-tryptophan supplementation. These results show that although PAL is widespread among *Ustilago* species, its induction by L-tryptophan is confined to certain species.

4.3.5 Effect of carbon and nitrogen sources on PAL activity induction

Various nutritional carbon and nitrogen sources were tested to examine their effect on PAL activity. Different levels of PAL activity and cell growth were obtained from the cells grown with the carbon sources tested (Fig. 4.8). Among these carbon sources, growth on lactose and galactose resulted in the highest PAL activity, even though they proved to be the poorest sources for cell growth. In general, un-induced *U. maydis* cultures displayed higher PAL activity when they were grown on carbon sources that supported poorer growth, and lower activity when grown on more readily metabolized sugars such as glucose, mannose and sucrose.

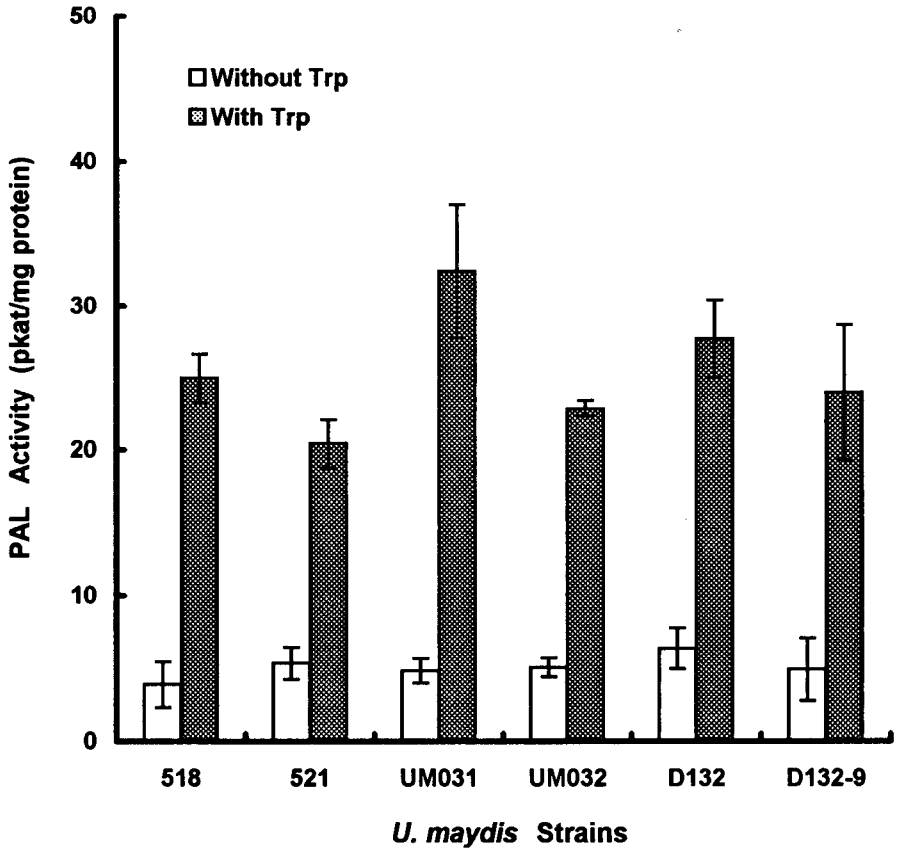


Fig. 4.6. Effect of L-tryptophan supplement on PAL induction in different *U. maydis* strains. 0.3 mM L-tryptophan was added into basal media and the fungal strains were grown for 24 hr, harvested, and assayed for PAL activity.

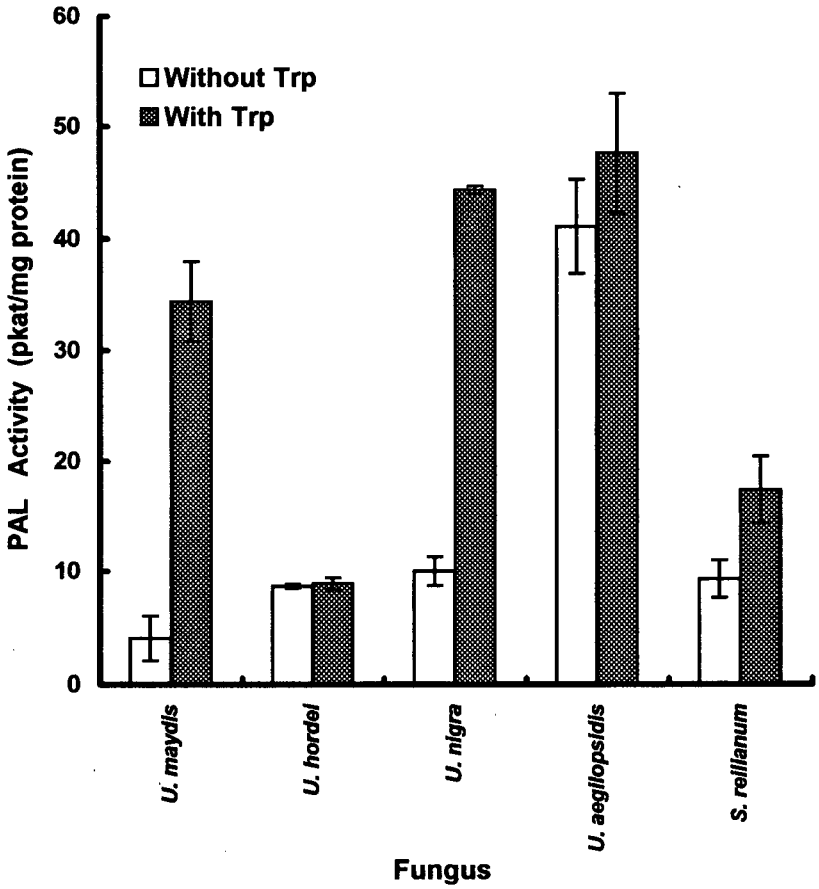


Fig. 4.7. Effect of L-tryptophan supplement on PAL induction in different *Ustilago* species. The fungal cells were grown in basal media for 36 hr with or without 0.3 mM L-tryptophan, harvested, and assayed for PAL activity.

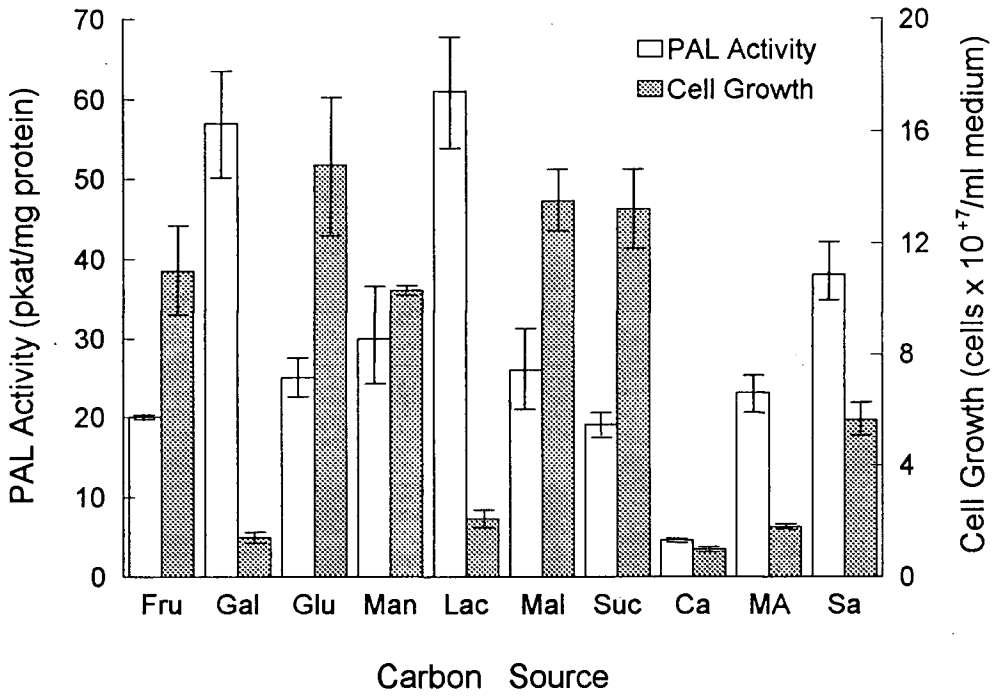


Fig. 4.8. Effect of various carbon sources on *U. maydis* cell growth and PAL activity. Fru: fructose, Gal: galactose, Glu: glucose, Man: Manose, Lac: lactose, Mal: maltose, Suc: sucrose, CA: citric acid, MA: malic acid, and SA: succinic acid. Cells were grown in 20 mM NH_4NO_3 media for 36 hr with each carbon source and harvested for PAL assay and cell count.

To understand how carbon sources influence PAL activity under tryptophan-induction conditions, glucose was selected as a representative carbon source and its effect on PAL induction in *U. maydis* was investigated. Glucose supplementation repressed PAL production both in the presence and absence of L-tryptophan, and in both the nutrient rich (complete) and minimal (glutamine only) media (Fig. 4.9A). The repressive effect of glucose was observed under tryptophan-induced conditions if the glucose was present from the beginning of the culture cycle, but not if the extra glucose was added later (16 hr) in the culture cycle (Fig. 4.9B). These results indicate that glucose repression can modulate *Ustilago* PAL expression, but that the repression effect is limited to the early growth stage.

The effects of nitrogen source on *U. maydis* cell growth and PAL activity level are shown in Fig. 4.10. Provision of nitrogen in the form of inorganic salts, or asparagine, reduced PAL activity far below the levels obtained when L-tryptophan or L-phenylalanine was provided as the sole nitrogen source for cultures. For cell growth, peptone was the best source and no significant difference was found among other nitrogen sources. Varying the ratio of ammonium nitrogen to nitrate nitrogen from 1:10 to 10:1 in the non-amino acid media did influence the level of PAL in the cultures after 24 hr growth (Fig. 4.11), but the pattern of change did not suggest a strong preference for one form of inorganic nitrogen.

4.3.6 Effect of inhibitors and cAMP on PAL activity *in vivo*

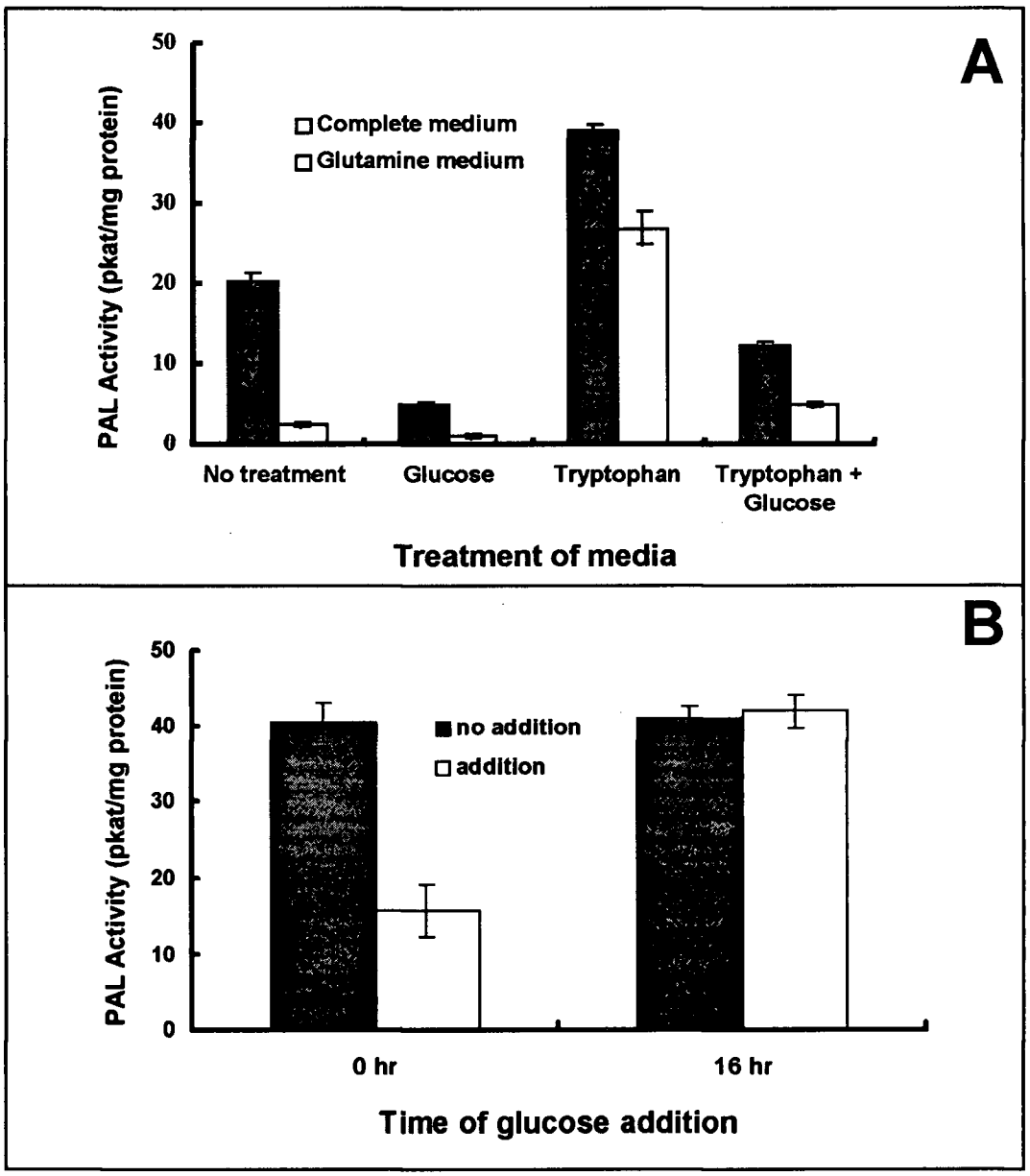


Fig. 4.9A. Effect of glucose on *U. maydis* PAL induction by L-tryptophan. Complete media and glutamine (20 mM)-only media were treated with 2% glucose or 0.3 mM tryptophan or both. The fungal cells were grown in these media for 36 hr, harvested, and assayed for PAL activity.

Fig. 4.9B. Effect of time of glucose addition on *U. maydis* PAL induction. An additional 3% glucose was added to L-tryptophan-supplemented basal media at the beginning (0 hr) or at the mid log phase (16 hr) of cell growth. The fungal cultures were incubated for a total of 36 hr, harvested, and assayed for PAL activity.

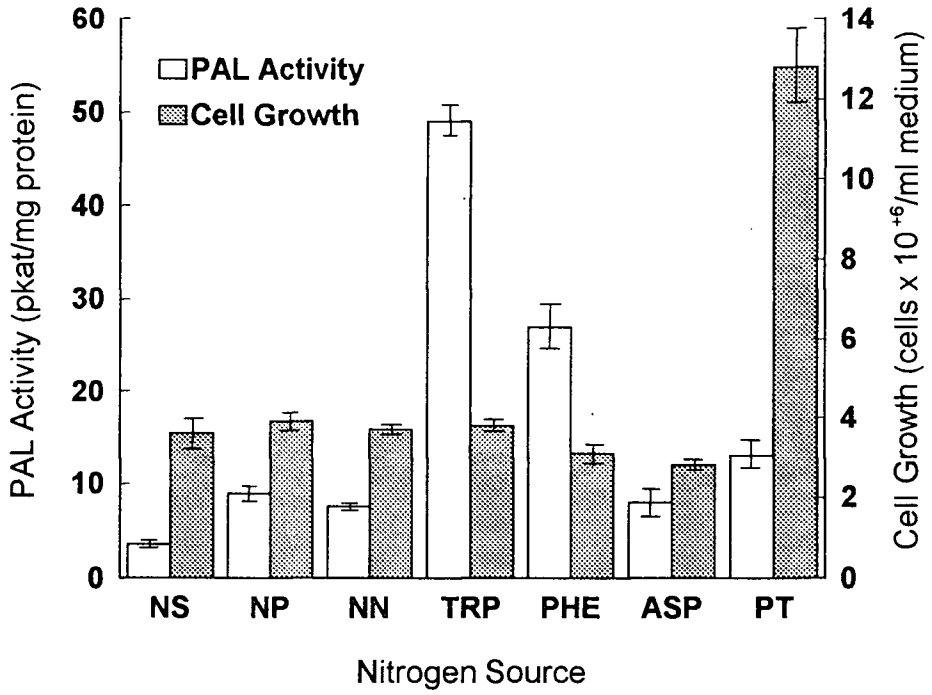


Fig. 4.10. Effect of nitrogen source on *U. maydis* cell growth and PAL activity. 0.3 mM of each nitrogen source was added to 2% glucose media and the fungal cells were grown for 36 hr and harvested for PAL assay and cell count. NS: $(NH_4)_2SO_4$, NP: $NH_4H_2PO_4$, NN: NH_4NO_3, TRP: tryptophan, PHE: phenylalanine, ASP: asparagine, and PT: peptone.

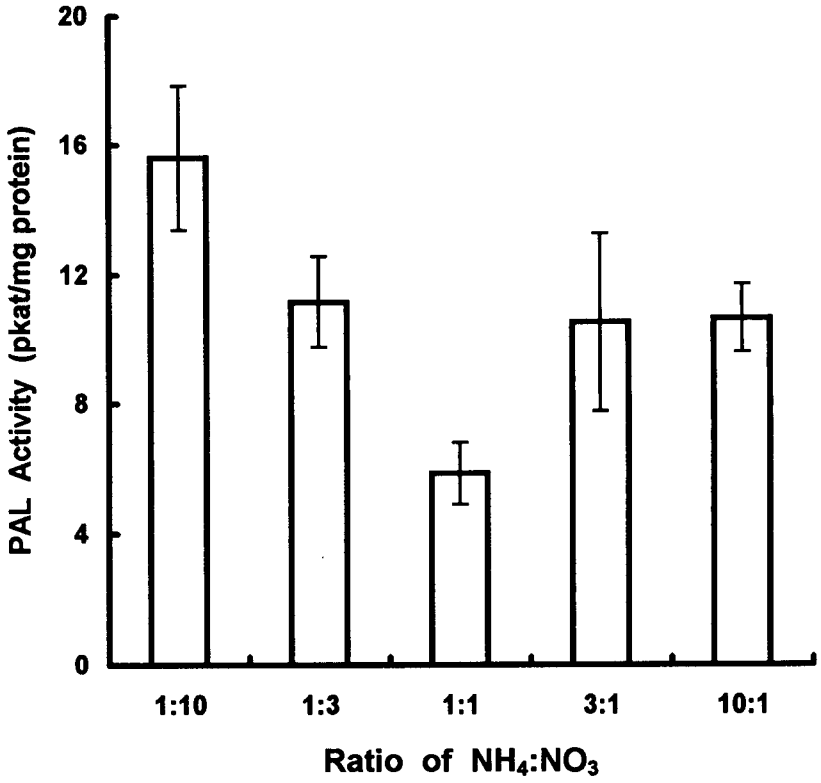


Fig. 4.11. Effect of the ratio of NH_4:NO_3 on PAL activity in *U. maydis*. The fungal cells were grown for 36 hr in 2% glucose media supplemented with various ratios of ammonium to nitrate nitrogen (total 0.3 mM), harvested and assayed for PAL activity.

It had previously been determined that purified *U. maydis* PAL could be partially inhibited by the reaction product, *t*-cinnamic acid, and strongly inhibited by the synthetic inhibitor, AIP (Chapter 2). When these compounds were added to the L-tryptophan-supplemented culture medium at appropriate concentrations, they were found to have differing effects on growth and extractable PAL activity (Fig. 4.12). Cinnamic acid substantially inhibited culture growth, but had little effect on the levels of PAL induced by 0.3 mM L-tryptophan supplementation. Addition of AIP, on the other hand, completely abolished PAL activity in the cell extracts but had no effect on culture growth over 24 hr. Interestingly, addition of cAMP (1 mM) produced a modest enhancment of PAL induction but did not affect culture growth.

With this observation, further studies were undertaken to gain insight into the possible invovement of the cAMP cascade in the regulation of PAL. For this, a *U. maydis* adenylate cyclase deficient mutant (uac1, Gold *et al.*, 1994a), and protein kinase A (PKA) deficient mutants adr1 (deficient in a catalytic subunit, Orth *et al.*, 1995) and ubc1 (deficient in the regulatory subunit, Gold *et al.*, 1994a), were tested in L-tryptophan-supplemented growth conditions for their PAL induction respose (Fig. 4.13). PAL was induced to similar levels in the adenyl cyclase mutant and in wild-type *U. maydis*, but the PAL induction was reduced by 20-30% in the PKA mutants.

4.3.7 Influence of physical environments

In order to examine the effect of environmental factors on PAL regulation, *U. maydis* cultures were grown in complete medium under various light, pH, and temperature

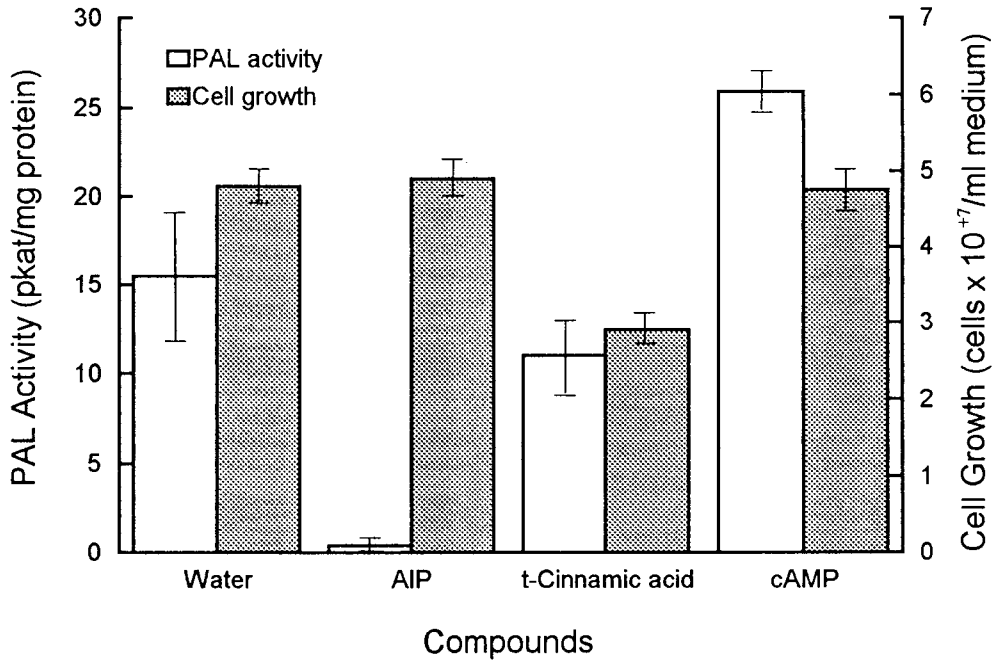


Fig. 4.12. Effect of cAMP (1 mM), AIP (100 µM), and *t*-cinnamic acid (0.3 mM) on cell growth and PAL activity of *U. maydis*. Compounds were added to basal medium, and the fungal cells were grown for 36 hr and harvested for PAL assay and cell count.

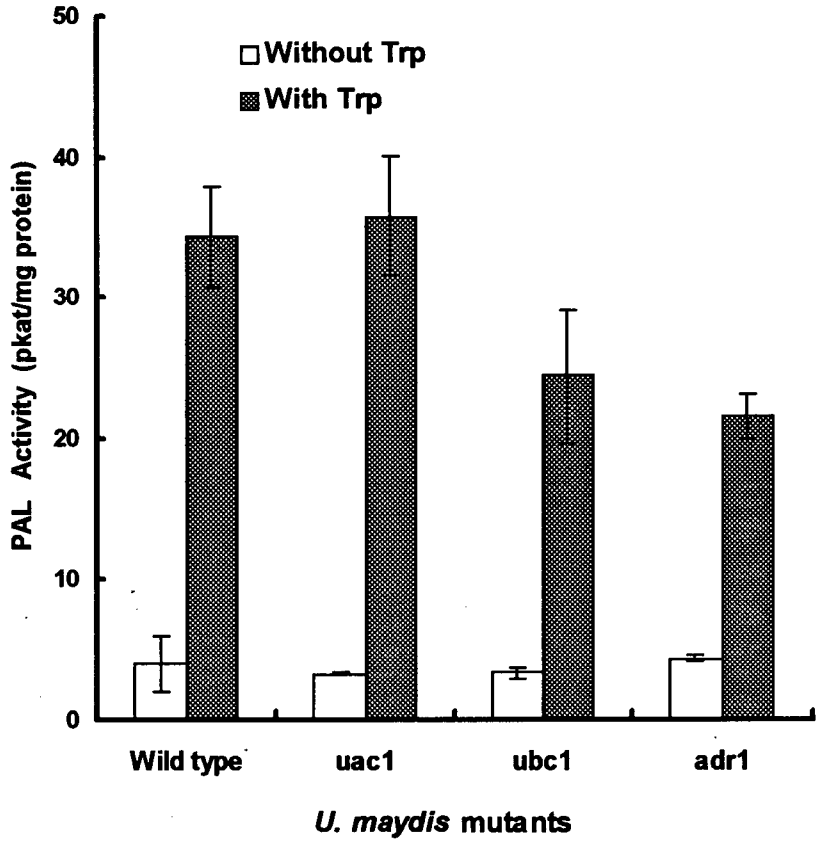


Fig. 4.13. Effect of L-tryptophan supplement on PAL induction in the *U. maydis* adenylate cyclase deficient (uac1) and protein kinase A deficient (ubc1, adr1) mutants. The mutants were grown in basal media for 36 hr with or without 0.3 mM L-tryptophan, harvested, and assayed for PAL activity.

regions. No apparent PAL induction or inhibition was observed when the cultures were incubated in light or dark, or at different medium pH values (pH 4, 5, 6, 7, 8), and/or at different temperatures (18, 24, 30°C) (data not shown). It seems that expression of *U. maydis* PAL activity is not overtly sensitive to physical environmental factors.

4.3.8 No evidence for *de novo* synthesis in PAL induction

Induction of PAL in most plants and some fungi results from *de novo* synthesis of the enzyme. The origins of induced PAL activity in *U. maydis* were investigated by the use of inhibitors of translation and transcription, and with radiotracers and immunoprecipitation. In both the L-tryptophan-induced and uninduced conditions, PAL activity was reduced when *U. maydis* cells were incubated in the presence of actinomycin D, a transcriptional inhibitor, or cycloheximide, an inhibitor of translation (Table 4.3). This implies that gene transcription and protein synthesis are necessary to maintain PAL levels in both conditions during the log phase of culture growth. However, radiolabeling of newly synthesized proteins showed that there were no differences in PAL protein labeling between the cells grown in L-tryptophan-induced and uninduced conditions (Table 4.4). This result was consistent with Western blot results which showed PAL bands of similar intensity in protein samples from L-tryptophan-induced and uninduced cultures (Fig. 4.14). Taken together, these observations suggest that the induction of increased PAL activity by L-tryptophan supplementation does not result from *de novo* synthesis of PAL, but from activation of the PAL enzyme which has been produced during the log phase of culture growth. The modest inhibition by the actinomycine and cycloheximide treatments may indicate that other proteins needed for PAL activation are turning over in the

Table. 4.3. Effect of actinomycin D and cycloheximide on *U. maydis* PAL activity

Treatment [a]	PAL Activity (pkat/mg protein)			
	Induced [b]		Uninduced	
	12hr	16hr	12 hr	16 hr
No addition	19.6 ± 1.8	32.8 ± 2.2	13.3 ± 1.6	20.5 ± 2.1
Actinomycin D	13.9 ± 1.9	23.3 ± 0.8	9.2 ± 1.2	15.1 ± 0.6
Cycloheximide	16.2 ± 1.7	26.4 ± 1.3	11.3 ± 1.0	16.8 ± 1.2

[a] Actinomycin D (100 μg/ml) and cycloheximide (10 μg/ml) were added at mid (12 hr) or late (16 hr) log phase in the cell growth cycle, and cells were harvested 4 hr later and used for PAL assay.

[b] Induced: L-tryptophan (0.3 mM) was supplemented to basal medium at the time of inoculation, uninduced: no L-tryptophan was supplemented.

Table 4.4. PAL protein synthesis in L-tryptophan-induced and -uninduced conditions[a]

Cell harvest time	Newly synthesized PAL (dpm) [b]	
	Induced	Uninduced
16 hr	382 ± 36	388 ± 47
20 hr	461 ± 56	473 ± 62

[a] Induced: L-tryptophan (0.3 mM) was supplemented to basal medium at the time of inoculation, uninduced: no L-tryptophan was supplemented.

[b] Proteins of *U. maydis* were radio-labelled *in vivo* with L-[^{3}H]-amino acid mixture. The labelled PAL proteins were immunoprecipitated with anti-PAL antibodies and counted.

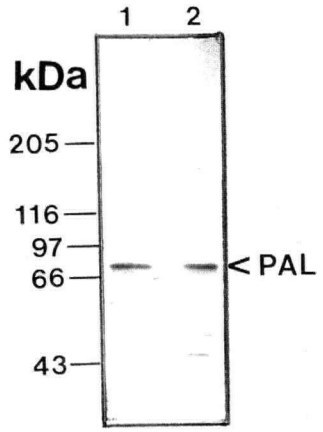


Fig. 4.14. Immunoblot detection of PAL production in *U. maydis* grown in L-tryptophan-induced and -uninduced conditions. Protein samples (20 μg) extracted from the cultures grown for 36 hr in 0.3 mM L-tryptophan-supplemented (lane 1) or unsupplemented (lane 2) basal media, were separated in a 7.5 % SDS-PAGE gel, transferred onto a PVDF membrane and immuno-detected with *U. maydis* PAL antibodies using the NBT/BCIP visualization system.

cells.The fact that the greatest enhancement of PAL activity by exogenous L-tryptophan is obtained in the stationary phase of culture growth (Fig. 4.4), when most protein synthesis has ceased, is consistent with this interpretation.

4.3.9 Metabolic fate of L-phenylalanine in *U. maydis* cultures

Chromatographic analysis of the soluble products of metabolism of L-^{14}C-phenylalanine or ^{14}C-*t*-cinnamic acid after 2 hr incubation detected radioactive label at positions on the chromatograms corresponding to *t*-cinnamic acid, benzoic acid, and 4-hydroxybenzoic acid, and possibly other hydroxybenzoic acid derivatives (Fig. 4.15). These products were detected in the culture medium as well as in the cell extracts. Incubation of the *U. maydis* cultures with L-^{14}C-phenylalanine resulted in a slow release of $^{14}CO_2$ over a 6 hr period, but this amounted to less than 0.2% of the potential label available if the phenylalanine were to be totally catabolized.

No phenylalanine transaminase activity could be detected in cell extracts, whereas 4-hydroxycinnamyl Co A-ligase activity is detectable that is active with *t*-cinnamic acid but not with 4-hydroxycinnamic acid as substrate. Taking these observations together, a plausible catabolic pathway for phenylalanine in *U. maydis* could be formulated as shown in Fig. 4.16.

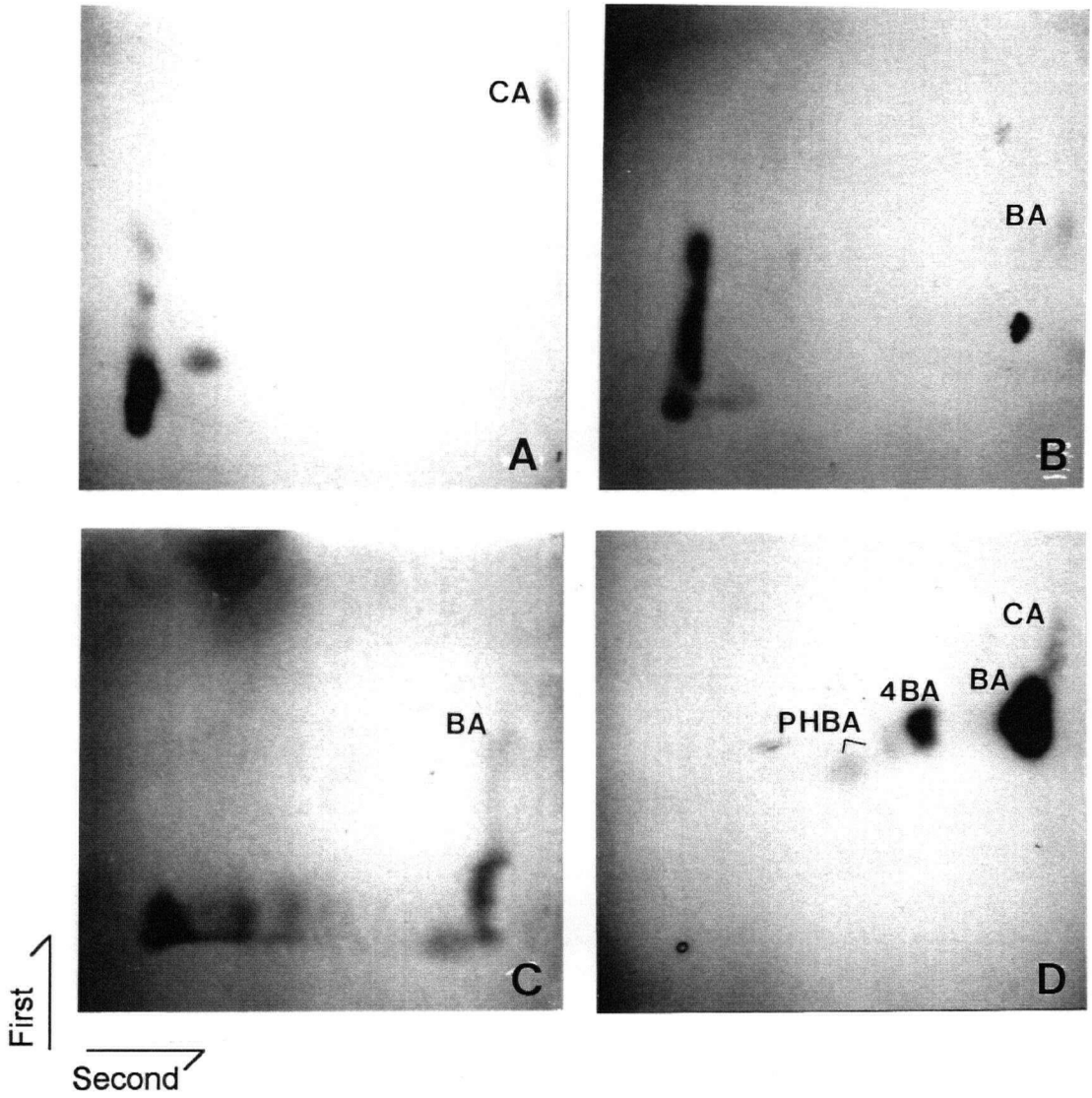


Fig. 4.15. Autoradiogram of the radioactive metabolites recovered from *U. maydis* growing in L-[U-^{14}C] phenylalanine or [U-^{14}C]*t*-cinnamic acid. A and B indicate autoradiograms of cell extract and medium extract, respectively, from the cultures grown in the presence of ^{14}C-phenylalanine. C and D indicate autoradiograms of cell extract and medium extract, respectively from the cultures grown in the presence of ^{14}C-*t*-cinnamic acid. The developed TLC plates were exposed to X-ray film for one week. CA: *t*-cinnamic acid, BA: benzoic acid, 4BA: 4-hydroxybenzoic acid, and PHBA: possible hydroxybenzoic acid derivatives.

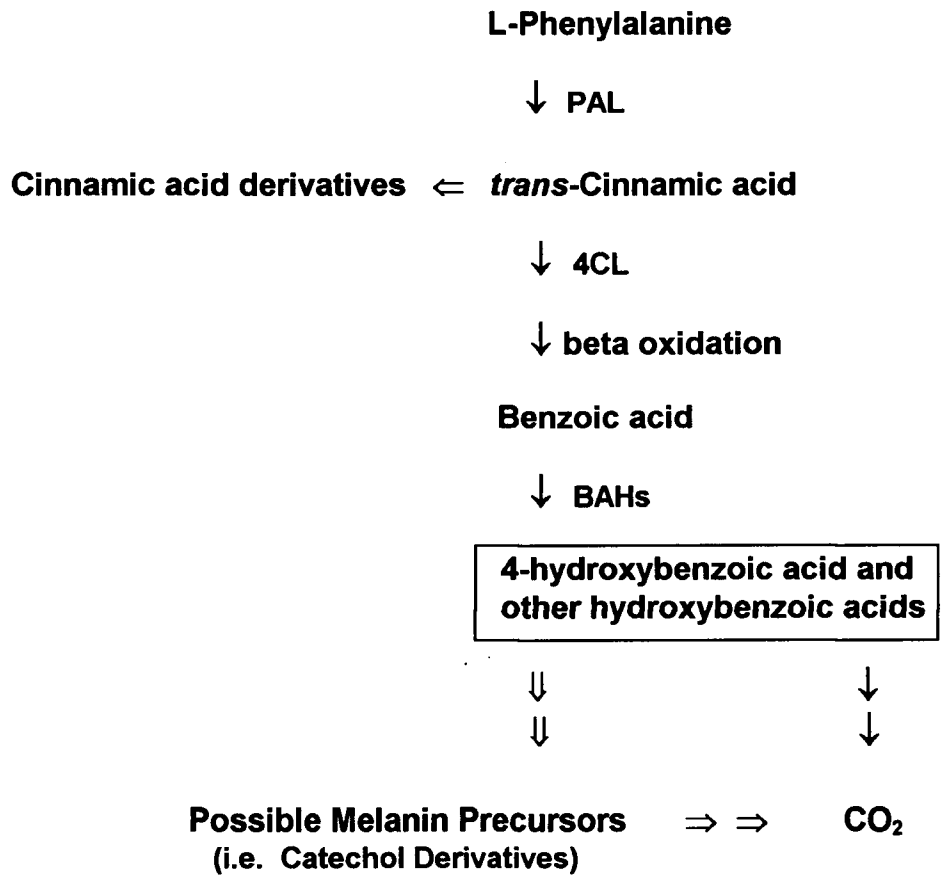


Fig. 4.16. Schematic diagram of catabolic pathway of phenylalanine in *U. maydis*. PAL:phenylalanine ammonia-lyase, 4CL:4-coumaryl Co A ligase, BAHs:benzoic aicd hydroxylases. Arrows indicate identified way (↓) and predicted way (⇓).

4.4 DISCUSSION

4.4.1 PAL induction by L-tryptophan

The influence of aromatic amino acids on fungal PAL regulation has been studied in only a few species. In the red yeast, *R. toruloides*, L-phenylalanine and L-tyrosine acted as PAL inducers (Marusich *et al.*, 1981; Gilbert *et al.*, 1983), while in *N. crassa*, L-phenylalanine (Sikora and Marzluf, 1982), and in *R. solani*, L-phenylalanine, L-tyrosine, and L-tryptophan all served as PAL inducers. In all of these cases, the best PAL inducer was L-phenylalanine. In contrast, the most effective inducer of PAL activity in *U. maydis* was L-tryptophan (Fig. 4.2), although L-phenylalanine had some effect.

However, induction of PAL in *Ustilago* cultures by L-tryptophan does not appear to be a typical microbial catabolic system directed at nutrient scavenging. First, a significant basal level of PAL is always detectable in the cultures, unlike the three fungal species mentioned above, in which PAL activity is detectable only when the fungi are grown in media supplemented with aromatic amino acids which induce PAL. Second, in contrast to other fungi like *R. toruloides* and *N. crassa*, the inducer does not appear to have a logical metabolic connection with the reaction catalyzed by the induced enzyme. L-Phenylalanine, the obvious induction candidate, is much less effective in this role than L-tryptophan. Finally, the L-tryptophan effect on PAL induction is not operative in all the *Ustilago* species tested.

Since PAL induction in *U. maydis* results only from tryptophan supplementation and not from tryptophan precursors or metabolites, it is worth asking whether the endogenous level of tryptophan in the host plants is sufficient for *U. maydis* PAL induction. There are no data for corn, but the level of free tryptophan in tobacco leaves and carrot cell cultures has been reported as 16.7 μM (Widholm, 1972) and 81 μM (Widholm, 1974), respectively. In this study, *Ustilago* PAL was measurably induced by 30 μM tryptophan supplementation (Fig. 4.3), suggesting that *Ustilago* PAL might be inducible by either the endogenous level of tryptophan or enhanced levels of tryptophan resulting from the *Ustilago* fungus infection in its host corn plants.

4.4.2 Regulatory features of PAL

The production of many inducible enzymes by microorganisms is subject to catabolite repression when the organisms are grown in the presence of a potential carbon and energy source. In addition to carbon catabolic enzymes, many proteins, including those involved in bioluminesence, photosynthesis, sporulation, antibiotic biosynthesis, pigment biosynthesis, and extracellular macromolecular degradation, have been shown to be subject to catabolite repression (Saier, 1991). Glucose repression was observed with *U. maydis* in the present study (Fig. 4.9), since both basal PAL levels and the induction of PAL by L-tryptophan were reduced by glucose addition to the medium. A similar phenomenon was reported in *R. toruloides*, where PAL induction by L-phenylalanine was repressed by glucose (Gilbert and Tully, 1982).

cAMP is widespread among eukaryotes and prokaryotes and is implicated in numerous regulatory events in fungi, including carbon catabolism, growth, and developmental phenomena (Pall, 1981; Thevelen, 1988; Uno, 1992), and in the regulation of expression of certain fungal enzymes (Herman *et al.*,1990; Terenzi *et al.*, 1992). With the observation of a stimulatory effect of exogenously supplied cAMP on PAL activity (Fig. 4.12), the possible role of cAMP in the regulation of *Ustilago* PAL by tryptophan was investigated using an adenylate cyclase deficient mutant (uac1) and cAMP-dependent protein kinase A (PKA) deficient mutants (ubc1 and adr1). Both types of mutants were able to display PAL induction in the presence of exogenous tryptophan (Fig. 4.13), implying that cAMP and PKA are not essential for this phenomenon. However, the levels of PAL in the mutants defective in *ubc1* and *adr1* were reduced compared to those in the wild type. PKA could, therefore, still be involved in the regulation of PAL induction, either by some pleiotropic effect or perhaps by mediating the phosphorylation of PAL proteins themselves. There are many predicted phosphorylation sites in the amino acid sequence of PAL deduced from the *U. maydis* PAL gene (Chapter 3), including one cAMP-dependent PKA phosphorylation site. Bolwell (1992) has suggested that the inactivation and turnover of PAL in elicitor-treated bean cells is in part regulated by phosphorylation, which is possibly cAMP-dependent. However, the evidence for occurrence of cAMP in plants is not convincing (Bolwell, 1995).

The induction of PAL in *U. maydis* by tryptophan is not likely to result from *de novo* synthesis of the protein, as shown by immunoprecipitation (Table 4.3) and Western blot assay results (Fig. 4.14). The alternative mechanisms would appear to be that some form of post-translational protein modification (such as phosphorylation of the enzyme),

or a metabolic (non-covalent) influence on the *Ustilago* PAL activity. This behavior differs from that seen in *Rhodosporidium* and *Neurospora* (Gilbert *et al.*, 1985; Sikora and Marzluf, 1982), where induction of elevated PAL activity by phenylalanine is controlled primarily at the transcriptional level.

Some aspects of the results obtained in this study are reminiscent of classic microbial secondary metabolism. For example, the tryptophan inducibility of PAL is invoked only during the stationary phase of culture growth, and the induction appears to require tryptophan itself, rather than tryptophan metabolites or precursors. It is noteworthy that IAA is most actively produced at the stationary phase in the culture cycle of *U. maydis* (Navarre, 1990). Considering that tryptophan is the major precursor for IAA production in this fungus, the induction and suppression of PAL by tryptophan and IAA, respectively, suggests that a metabolic control mechanism links phenylalanine metabolism to tryptophan metabolism during the late growing period of *U. maydis*.

It has been clearly estabilished in both prokaryotes and eukaryotes that the synthesis of these two aromatic amino acids, phenylalanine and tryptophan, is derived from chorismate through the shikimate pathway (Braus, 1991; Hrazdina and Jensen, 1992). Logically, the synthesis and catabolism of each amino acid will be regulated to reflect their demands and their levels. From this perspective, it is conceivable that *U. maydis* PAL regulation is indirectly under the influence of tryptophan levels through metabolic feedback mechanisms that control synthesis and catabolism of all the aromatic amino acids. Studies on the effect of tryptophan on the synthesis of the PAL substrate,

phenylalanine, could provide insight into potential metabolic connections between tryptophan metabolism and PAL regulation.

t-Cinnamic acid, the product of the PAL reaction, is moderately inhibitory to *U. maydis* cell growth. Toxicity of *t*-cinnamic acid was also observed in *R. toruloides* cultures (Marusich *et al.*, 1981; Kane and Fiske, 1985). In some plant systems like bean (Bowell *et al.*, 1988) and alfalfa (Orr, 1993) cell suspension cultures, addition of exogenous *t*-cinnamic acid suppressed PAL induction. This has led to the suggestion that feedback inhibition by *t*-cinnamic acid could help control diversion of L-phenylalanine from primary metabolism to secondary metabolism and thereby avoid the accumulation of *t*-cinnamic acid to levels that might become toxic to cells. In *U. maydis*, however, there was no evidence that exogenous cinnamic acid influenced PAL induction.

4.4.3 Metabolic features of phenylalanine catabolism in the context of the host-pathogen interaction

The major metabolites of *t*-cinnamic acid detectable in the *Ustilago* cultures are benzoic acid, and possibly, its derivatives, a pattern consistent with degradation of the phenylpropanoid skeleton by β-oxidation of the side-chain and oxidation of the ring. The detection of 4-coumaryl Co A-ligase (4CL) activity that was active not with *p*-coumaric acid, but with *t*-cinnamic acid, as a substrate suggests that the biosynthetic route to hydroxybenzoic acids in *U. maydis* differs somewhat from that of plants, which use *p*-coumaric acid as a preferred substrate for 4CL.

What are the roles of benzoic acid and its derivatives in *U. maydis* biology? They might serve as precursors for the synthesis of physiological effectors, analogous to signal molecules like salicylate or its derivatives in plants (Malamy *et al.*, 1990). Such roles for hydroxybenzoic acids have not been elucidated in fungi, but the recent finding in tobacco plants (Yalpani *et al.*, 1993) that salicylate is synthesized from phenylalanine via *t*-cinnamic acid and benzoic acid, provides a possible precedent for a similar process in fungi. Several fungi that contain PAL, including *Ustilago hordei*, have been reported to metabolize L-phenylalanine into benzoic acid and its derivatives (Towers *et al.*, 1969), and salicylate and its derivatives have been found in the extracts from *U. maydis* teliospores (Piattelli *et al.*, 1965).

4.4.4 Potential roles of PAL in *U. maydis*

In contrast to higher plants, the role of PAL in fungi is not obvious. Earlier information on fungal PAL regulation was restricted largely to *R. toruloides* and *N. crassa*, and no definitive biological functions were uncovered in those studies. It seems reasonable to assume, however, that PAL could provide one mechanism for catabolism of L-phenylalanine acquired during parasitic growth of plant pathogens such as *Ustilago, Rhizoctonia* or *Alternaria*, or during saprophytic growth of non-pathogenic fungi (e.g. *Rhodosporidium* and *Neurospora*). Those fungal species that possess PAL have been shown to convert exogenous phenylalanine to various phenolic metabolites.

It is also conceivable that cinnamic acid is required as a substrate for biosynthesis of some essential physiological effector or protectant for *U. maydis*, perhaps within a

specific developmental context. Derivatives of cinnamic acid which might function as germination self-inhibitors in spores, would be potential candidates, and inhibitors derived from cinnamic acid have been found in urediospores in rust fungi (Macko *et. al.*, 1970; Macko *et. al.,* 1971). Since PAL activity could also be detected in urediospores of the rust fungus (Moerschbacher *et al.*, 1988), it seems likely that PAL would be involved in the biosynthesis of these endogenous inhibitors. Self-inhibition is overcome by nutrients (including peptone or phenylalanine) in *Glomerella cingulata* (Lingappa and Lingappa, 1965), and is glucose-dependent in *Fusarium solani* (Griffin, 1970a and b), indicating that this process can be metabolically modulated in some cases. These responses at least superficially resemble the pattern of *U. maydis* PAL regulation, as reflected in the glucose repression and aromatic amino acid induction results. Since self-inhibition is also operative in teliospore germination of *Ustilago* fungi (Pritchard and Bell, 1967), *Ustilago* PAL may thus play a role in the regulation of this self-inhibition. Identification of the chemical nature of the germination self-inhibitors in *Ustilago* fungi would allow this possibility to be tested.

The function of melanin in fungi is still not clear but roles in pathogenicity and virulence (Polak, 1989), resistance against microbial stress such as hydrolytic enzymes (Kuo and Alexander, 1967) and protection from environmental stress (e.g. UV irradiation, Bell and Wheeler, 1986) have all been suggested. Formation of teliospores within the plant in the last stage of the *U. maydis* infection cycle is accompanied by melanization, which creates a UV-impermeable shield for the exposed spores. Catechol derivatives and salicylic acid have both been identified from plant melanin (Yoshida, 1969), and these phenolics were also reported as components of melanin in *U. maydis* teliospores (Piattelli

et al., 1965). Considering that catechol derivatives and salicylic acid might be synthesized via benzoic acid, and thus from phenylalanine, it is very likely that the biosynthetic route to *Ustilago* melanin will require PAL activity. Such a route has been suggested for the biosynthesis of melanin in the fungal pathogen *Alternaria* (Pridham and Woodhead, 1977).

Rhodotorula PAL has been postulated to play a role in the synthesis of another pigment which is very effective in protecting cells from UV light (Ogata *et al.*, 1967). Much more browning (melanin-like) pigmentation was observed in *U. maydis* cells grown in PAL-inducing (tryptophan-supplemented) media than in those grown in PAL-uninducing (tryptophan-unsupplemented) media. It is thus tempting to speculate that, as with plant PALs, a common role for fungal PAL might be formation of aromatic metabolites that enable the fungi to survive environmental stresses.

Clarification of the function of PAL in *U. maydis*, and of the relationship between the PAL reaction and tryptophan metabolism, will probably require a molecular genetic approach. For this, disruption of the *U. maydis* PAL gene cloned in Chapter 3 and evaluation of the resulting null phenotypes would provide valuable insights into the biological function of this well-known, but little understood, enzyme within the corn smut organism.

CHAPTER FIVE

Conclusions and Future Directions

5.1 Conclusions

For the first time, PAL has been purified to apparent homogeneity, and the corresponding gene cloned from *U. maydis*, a filamentous fungus and a plant pathogen. Analysis of the purified protein showed that *U. maydis* PAL resembles other known PALs in many respects, e.g. it is a cytosolic, homotetrameric protein with a molecular mass of 320 kDa, requires no cofactors or no thiol reducing reagents, and is stable in the absence of glycerol. In contrast to yeast PAL (Camm and Towers, 1973), *U. maydis* PAL was not able to deaminate L-tyrosine. In other respects, the *Ustilago* PAL is somewhat unusual. It has a relatively high K_m (1.05 mM) for phenylalanine, a high pI (6.3), and notable temperature stability.

Ustilago PAL appears to be composed of 724 amino acids and be encoded by a single gene. Analysis of amino acid sequence showed that *Ustilago* PAL shares low homology with other known PAL genes. This, together with the weak immunological cross-reaction between *U. maydis* PAL and plant PALs, and between *U. maydis* PAL and yeast PAL, shows that the structures of *Ustilago*, yeast, and higher plant PAL proteins have diverged substantially. It is worth noting that no introns are present in *Ustilago* PAL sequence, a pattern that is quite different from that of angiosperm plants and yeasts.

Ustilago PAL shows a modest level of amino acid sequence identity with the other ammonia-lyase, HAL. The present work also demonstrated, for the first time, the immunological relatedness between PAL and HAL. The immunological cross-reactivity between a *Pseudomonas* bacterial HAL and *Ustilago* PAL (or poplar and alfalfa PALs) is consistent with the amino acid sequence homology, and must reflect common structural features in these two ammonia-lyases that have been retained despite the long time since HAL and PAL diverged. The weakness of the cross-reactivity between *Ustilago* (or yeast) PAL and the plant PALs is consistent with the divergence observed between fungal PAL and plant PAL protein sequences. This may reflect possible divergence of PAL functions in different organisms.

The production of PAL only in phenylalanine-supplemented cultures in saprophytic fungal species suggests that their PAL expression may be dependent upon the amount of the phenylalanine available in their environmental niche. This situation differs from that of pathogenic *Ustilago* fungi which are involved in dynamic interactions with their specific hosts. The endogenous pools of amino acids in hosts are typically small, and may be insufficient to serve as an inductive signal. *Ustilago* fungi may therefore have adopted a different mode of PAL regulation. Unlike other known fungal PALs, *Ustilago* PAL can be produced constitutively at low levels by a range of *Ustilago* fungi when grown in complete media. In contrast to plant PALs, physical environmental factors such as light, pH, and temperature, do not appear to influence the induction and repression of the enzyme activity. Carbon catabolic repression of PAL activity induction is, however, operative in *U. maydis*. Analyses of growth and PAL activity induction indicate that PAL can be induced to higher level by L-tryptophan during the stationary phase of culture

growth. There was no evidence of *de novo* PAL synthesis during PAL induction time by L-tryptophan, suggesting that post-translational activation mechanism may underline this phenomenon.

The intracellular location of PAL in the biotrophic fungus *U. maydis* (this study) is in contrast to the intracellular and extracellular distribution of PAL in the saprophytic fungus *N. crassa* (Sikora and Marzluf, 1982). The secretion of PAL outside the cell can vary between different fungi and perhaps between different developmental states or environmental situation. The possible secretion of PAL by fungi *in planta* would have important implications and merits further investigation. If *U. maydis* secretes PAL during or after the penetration of its host cells, it might affect the outcome of the infection process by modifing the host response.

5.2 Future directions

The followings suggestions may be useful to guide further studies of the biological role of *Ustilago* PAL.

- Extending the present immunological analysis to include mammalian HAL and if possible plant HAL would provide further insight into the pattern of immunological relatedness between PAL and HAL proteins across different phyla.

● One of the most common mechanisms for post-translational modifications of enzyme activity is phosphorylation. Many putative phosphorylation sites can be identified in the deduced *U. maydis* PAL protein sequence. If PAL induction involves phosphorylation, or if other proteins must become phosphorylated in order to act on the PAL protein, it should be possible to interfere with these processes by use of appropriate inhibitors. It may also be possible to detect incorporation of ^{32}P into PAL in radiotracer studies.

● A tentative metabolic pathway of phenylalanine degradation has been suggested (Fig. 4.17), based on some preliminary radiotracer experiments. This pathway, combined with earlier reports on the components of melanin (Piattelli *et al.* 1965), may suggest a link between PAL and the biosynthetic pathway for melanin in *U. maydis*. Based on our understanding of melanin functions, and other physiological functions of phenolics derived from phenylalanine, PAL could be envisioned to play an important role in development and survival of *U. maydis*. Examinations of the structures and properties of compounds derived from phenylalanine through PAL in *U. maydis* would be useful to test this idea. The ultimate test of the essentiality of PAL would be elimination of the PAL function by creating a *pal*$^-$ mutant genotype through gene disruption.

In conclusion, the studies described in this thesis have provided new information concerning the properties, structure and regulation of *Ustilago* PAL, as well as the metabolic fate of L-phenylalanine in this fungus. The availability of *Ustilago* PAL-antibodies and a full-length PAL gene sequence will provide useful tools for further study of the structure and function of PAL, as a self-assembling, post-translationally modified, multimeric protein. *U. maydis* PAL is an unusual member of the PAL family,

highly diverged from plant and yeast PALs and sharing interesting similarities with HAL. Study of recombinant *U. maydis* PAL in comparison to both HAL and other PAL species, using X-ray crystallographic analysis and site-directed mutagenesis, would provide definitive insights into the structural features retained by both ammonia-lyases during their evolutionary divergence. From physiological and ecological perspectives, evaluation of the phenotype of PAL-deficient *U. maydis* mutants would help address many remaining questions regarding the role of PAL in this fungus.

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APPENDIX A

Cloning and Sequencing of a cDNA Encoding Aspartate Semialdehyde Dehydrogenase in *Ustilago maydis*

Aspartate semialdehyde dehydrogenase (ASADH) catalyzes a crucial branch point reaction in the microbial amino acid biosynthetic pathway which leads to the production of L-lysine, L-methionine, L-threonine, and L-isoleucine from L-aspartate. The essential nature of the ASADH gene has made it a target for development of new antimicrobial agents (Cirillo *et al.*, 1991). The gene encoding ASADH has therefore been cloned from several pathogenic bacterial species, but the corresponding gene from eukaryotic pathogenic organisms has yet to be cloned.

During the studies on the cloning of the gene encoding phenylalanine ammonia-lyase from *Ustilago maydis* (chapter 3), a ASADH-encoding cDNA clone was isolated while immunoscreening a λ*ZAP*II *U. maydis* cDNA library. Given the novelty of this gene, the full sequence and structural analysis of the ASADH cDNA were conducted.

The nucleotide sequence analysis of the 1182-bp *Eco*RI-*Xho*I fragment insert of the cDNA clone revealed that the cDNA contains a 42-bp 5′-untranslated region, the translation start site, a 1098-bp open reading frame encoding a protein of 366 amino acids, a TAA stop codon, and a 39-bp 3′-untranslated region (Fig. 1). The G+C content is 55%. Southern hybridization of *U. maydis* genomic DNA digested with several different restriction enzymes, using the cloned ASADH cDNA as a probe, suggests that the *U.*

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AATTCCGCACGAGCCTTGATCAACAAGAGCACGCATCATACGATGACGTCTTCTTCCTCA    60
                                                  M  T  S  S  S  S     6
CAACAGAAGCTCAAAGTCGGCCTGCTCGGCGCTACGGGCACAGTAGGTCAGCGATTCATC   120
 Q  Q  K  L  K  V  G  L  L  G  A  T  G  T  V  G  Q  R  F  I      26
CTTCAACTTGCTGATCACCCGCAATTCGAGCTCGCCGCTCTGGGTGCATCGTCTTCTTCG   180
 L  Q  L  A  D  H  P  Q  F  E  L  A  A  L  G  A  S  S  S  S     46
GCAGGTAAGCCGTACTTGGAAGCAGTACAAGGGAGGTGGAAGCAGATCCGAAGGGTTCCC   240
 A  G  K  P  Y  L  E  A  V  Q  G  R  W  K  Q  I  R  R  V  P     66
GATAATGTTGCACAGATGCCCGTATACGAATGCAAGCCGGAATACTTTGCGAGTGCAGTA   300
 D  N  V  A  Q  M  P  V  Y  E  C  K  P  E  Y  F  A  S  A  V     86
GTCTCTCCGGTCTGGATTCAGGTCCAGCGGACCGTCGAGGACGCTTTCCGTAGAGCCGAG   360
 V  S  P  V  W  I  Q  V  Q  R  T  V  E  D  A  F  R  R  A  E    106
TTGAGAGTGTTTTCCAATGCAAAGAACTACCGTACCGATCCATTGTGCCCATTGGTGGTG   420
 L  R  V  F  S  N  A  K  N  Y  R  T  D  P  L  C  P  L  V  V    126
CCACTGGTCAACCCGGAGCACATGGAGATCTTGCCATTCCAGAGACAGCAAGTGGGGACC   480
 P  L  V  N  P  E  H  M  E  I  L  P  F  Q  R  Q  Q  V  G  T    146
AAGAAGGGATTCATCGTTACCAACGCCAACTGCTCTACCACGGGCATCGTCGTACCGCTG   540
 K  K  G  F  I  V  T  N  A  N  C  S  T  T  G  I  V  V  P  L    166
AAAGCGCTCGAGGCCAAGTTTGGACCGCTGGAAAAGATTCTAGTCAACACGATGCAGGCC   600
 K  A  L  E  A  K  F  G  P  L  E  K  I  L  V  N  T  M  Q  A    186
ATTTCGGGTGCTGGTTACCCTGGAGTTTCTTCGCTCGACATCTTGGACAACGTTGTGCCA   660
 I  S  G  A  G  Y  P  G  V  S  S  L  D  I  L  D  N  V  V  P    206
TTCATCAGCGGTGAGGAGGAGAAGATCGAGTGGGAGACCGCCAAGATCTTGGGTGGCATC   720
 F  I  S  G  E  E  E  K  I  E  W  E  T  A  K  I  L  G  G  I    226
AAAACGGACAAAACCGCTTTTGACTACCATGAAGAGCACCCACTCAAGGTTTCGGCGCAC   780
 K  T  D  K  T  A  F  D  Y  H  E  E  H  P  L  K  V  S  A  H    246
TGCAACCGTGTTCCGGTCATCGATGGCCACATGGAGTGCGTTTCTGTGTCGTTCAAGAAC   840
 C  N  R  V  P  V  I  D  G  H  M  E  C  V  S  V  S  F  K  N    266
CGACCTGCACCCTCGGTTGACGAGGTCAAAAAGTGCCTGCAAGGATTTACCACGGAAGCA   900
 R  P  A  P  S  V  D  E  V  K  K  C  L  Q  G  F  T  T  E  A    286
CAGACCATCGGCGTTCACTCGGCTCCTAAGCAGGCCATTACGGTGCACGAGGAGCAGGAC   960
 Q  T  I  G  V  H  S  A  P  K  Q  A  I  T  V  H  E  E  Q  D    306
CGCCCGCAACCACGCCTCGATCGTGACTGGCAGAACGGTGCTGGTGTCAATGTCGGAAGG  1020
 R  P  Q  P  R  L  D  R  D  W  Q  N  G  A  G  V  N  V  G  R    326
GTACGCGAATGTCCCGTGTTTGACATCAAGTTTGTCGTGCTCTCGAACAATGTCATGATC  1080
 V  R  E  C  P  V  F  D  I  K  F  V  V  L  S  N  N  V  M  I    346
GGTGCTGCTACCAGCTGGTTCATGAACGCAGAGATCGCGCTCGCCAAGGGTTACCTTTCG  1140
 G  A  A  T  S  W  F  M  N  A  E  I  A  L  A  K  G  Y  L  S    366
TAATCATCATGTGTGGTCACCTGAAATCGCATACTTTTCGCC                     1182
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Fig. 1. The nucleotide sequence and deduced amino acid sequence (upper case) of the *U. maydis* ASADH cDNA (GenBank accession No. D11111). The active site cysteine residue is boxed, a potential N-glycosylation site is circled, and the stop codon is underlined. A potential mitochondrial transit peptide is predicted at amino acid position 1 to 25. The putative pyridine nucleotide binding region (amino acids 13-41) and substrate binding region (amino acids 240-260) are underlined.

maydis ASADH is likely to be encoded by one gene (Fig. 2). PCR amplification of genomic DNA using primers derived from the 5′-terminal and 3′-terminal regions of the open reading frame in the *U. maydis* ASADH cDNA, produced a band similar in length to the full length ASADH cDNA, and Southern blot analysis verified that the PCR product was derived from ASADH (Fig. 3). This implies that no substantial introns are present in the *U. maydis* ASADH gene.

The predicted isoelectric point and molecular mass of the deduced *U. maydis* ASADH protein are estimated as pH 7.6 and 40.2 kDa, respectively. The amino acid sequence identity between ASADHs in *U. maydis* and other species ranged from 53% to 21% (Fig. 4). However, conservation of ASADH amino acid identity between fungal and bacterial species is typically limited to a few regions such as the putative pyrimidine nucleotide binding domain in the N-terminal region, the substrate binding region, and the core region containing the active site (Ouyang and Viola, 1995). Interestingly, the strict conservation among ASADHs in bacteria and fungi of a Gly-XX-Gly-XX-Gly (X is any amino acid) motif in the NADP-binding region (Fig. 4) is also found in the N-terminal region of isoflavone reductase, a NADP-dependent oxidoreductase in plants (Paiva *et al.*, 1994). This pattern distinguishes it from other NADP-dependent oxidoreductases which have either a Gly-X-Gly-XX-Gly motif (homoserine dehydrogenases in microorganisms, Thomas *et al.*, 1993) or a Gly-XXX-Gly-X-Gly motif (short-chain alcohol dehydrogenases in animals, Jörnvall *et al.*, 1995). The identification of common structural domains through comparison of conserved sequences may provide the foundation necessary for subsequent structure-function analysis of the ASADH gene product domains.

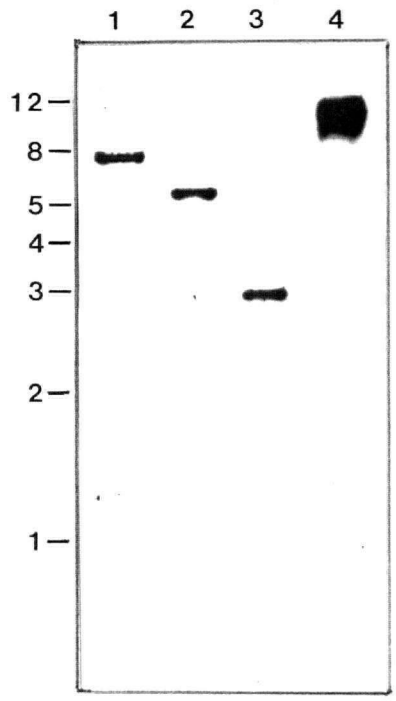

Fig. 2. Southern blot analysis of the *U. maydis* ASADH gene. Genomic DNA from *U. maydis* strain 518 cells was digested with *Eco*RI (lane1), *Pst*I (lane2), *Sma*I (lane3), and *Xba*I (lane4), fractionated by electrophoresis in a 0.7% agarose gel, transferred to Zeta-Probe membrane, and hybridized with ^{32}P-labeled 1.1kb ASADH cDNA. Sizes of DNA markers are indicated in kb.

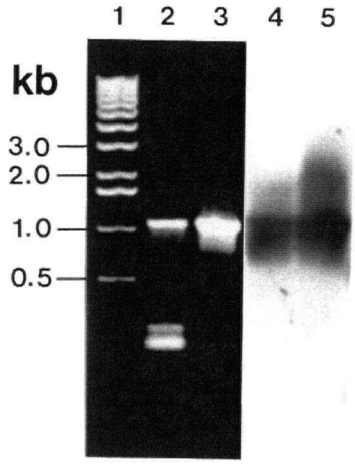

Fig. 3. PCR amplification and Southern blot analysis of ASADH gene from *U. maydis* genomic DNA and ASADH cDNA. Primers were derived from the 5' terminal (5'-ACGATGACGTCTTCTTCCTC-3') and 3' terminal (5'-CGAAAGGTAACCCTTGGCGA-3') regions of the *U. maydis* ASADH cDNA. PCR was carried out for 35 cycles of denaturation at 94 °C for 1 min, 1 min annealing at 58 °C, 1 min extension at 72 °C, followed by a final extension step for 10 min in the presence of 2.5 units of Taq-DNA polymerase. The amplified products were separated electrophoretically in a 1% agarose gel, visualized under UV following ethidium bromide staining (lane 1, 2, and 3), transferred to Zeta Probe membrane, and hybridized with ^{32}P-labeled 1.1kb ASADH cDNA (lane 4 and 5). Lane 1: 1kb DNA ladder, Lane 2: PCR product with genomic DNA as template, Lane 3: PCR product with ASADH cDNA as template, Lane 4: autoradiogram of the PCR product of lane 3, and Lane 5: autoradiogram of the PCR product of lane 4.

	NADP-binding region		Active site(C) and Core region	
Um	GLLGATGTVGQRFILQLAD	13-31	IVTNANCSTTGIVVPLKALEAKFG	151-174
Sc	GVLGATGSVGQRFILLLAN	8-26	IICISNCSTAGLVAPLKPLIEKFG	150-173
Li	AVLGATGSVGQRFIQLLDH	7-25	IITNSNCTIMGVTISLKPLLDRFG	142-165
Bs	AVVGATGAVGQQMLKTLED	8-26	IIANPNCSTIQMVAALEPIRKAYG	124-147
Sm	AIVGATGAVGTRMIQQLEQ	6-24	IIACPNCSTIQMMVALEPIRQKWG	122-145
Vc	AIFGATGAVGETMLEVLQE	8-26	IIANPNCSTIQMLVALKPIYDAVG	126-149
Cg	AVVGATGQVGQVMRTLLEE	6-23	IIANPNCTTMAAMPVLKPLHDAAG	125-148
Consensus	GATG VG L		I NC L G	

	Substrate-binding region		Total No. of aa	aa Identity
Um	LKVSAHCNRVPVIDGHMECV	241-260	366	100%
Sc	IKVSAQCNRVAVSDGHTECI	241-260	365	53%
Li	FSISAHCNRVPVFDGHTVCV	226-245	349	43%
Bs	LQVAATCVRLPIQTGHSESV	235-254	346	21%
Sm	LPVSAHCVRVPILFSHSEAV	237-256	357	23%
Vc	IMVNPTCVRVPVFYGHAEAV	230-249	337	25%
Cg	LKVSGTCVRVPVFTGHTLTI	242-261	344	21%
Consensus	C R H			

Fig. 4. Conserved regions in ASADH-encoded amino acid sequences from Um (*U. maydis*), Bs (*Bacillus subtilus*), Cg (*Corynebacterium glutamicum*), Li (*Leptospira interogans*), Sm (*Streptococcus mutans*), Sc (*Saccharomyces cerevisiae*), and Vc (*Vibrio cholerae*). The amino acid sequence identity was obtained from the PALIGN program in the PC/GENE software. The Swiss-Prot database accession number for ASADH is Q04797 for Bs, P26511 for Cg, P41394 for Li, P10539 for Sm, P13663 for Sc, and P23247 for Vc.

Disruption of the *U. maydis* ASADH gene by insertion of a phleomycin resistant gene has been undertaken. Preliminary data show that disruption of the gene appears to be lethal to *U. maydis* (Kim S.H., Durrenberger F., Kronstad J., and Ellis B., unpublished data), implying that the ASADH gene is essential in this organism.

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APPENDIX B

A Partial cDNA Sequence Encoding the *Ustilago maydis* Peroxidase-Catalase

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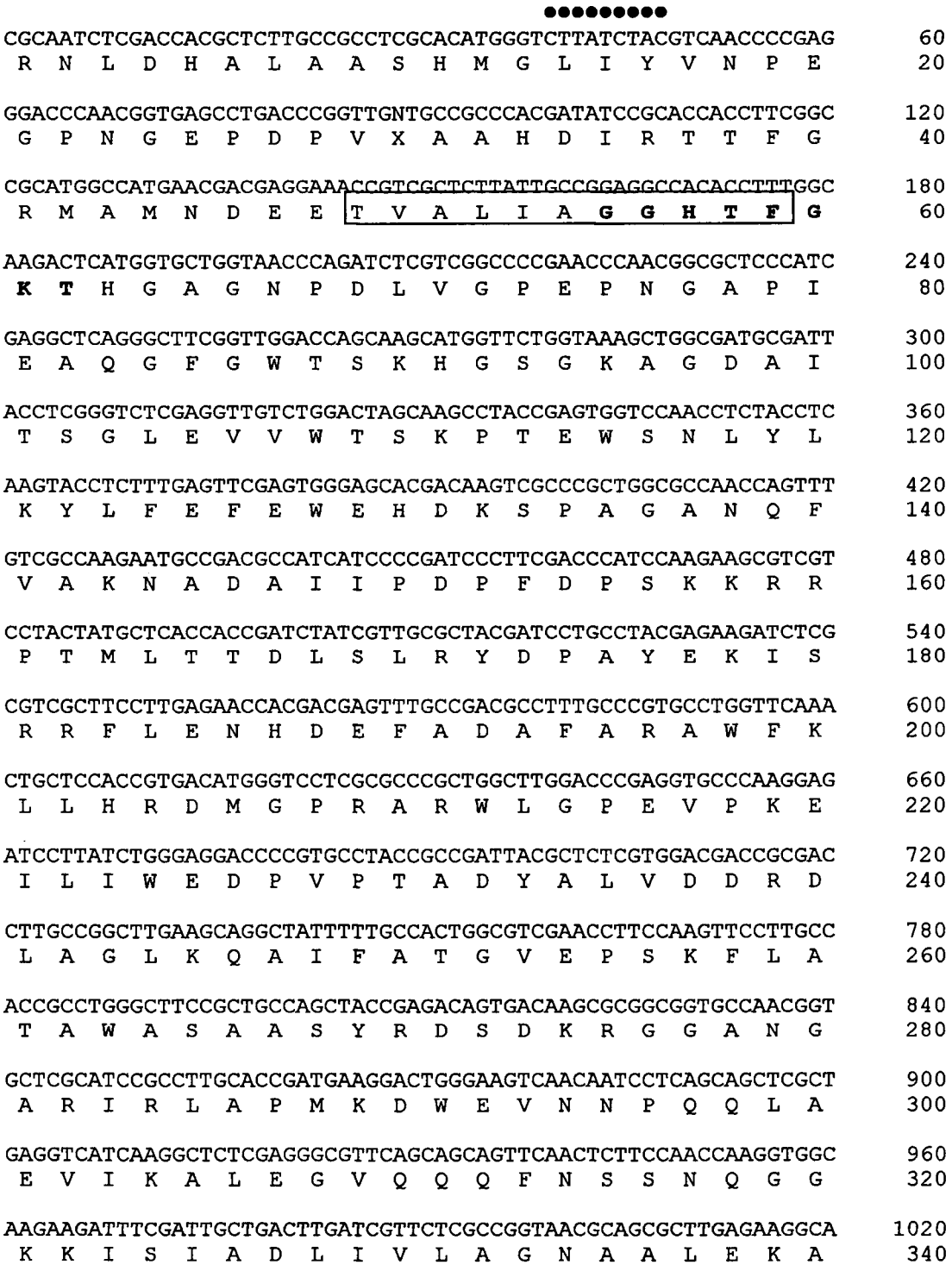
During the studies on the cloning of the gene encoding PAL from *U. maydis* (chapter 3), a cDNA clone giving a positive signal during the immunoscreening a λ*ZAP*II *U. maydis* cDNA library was isolated and its nucleotide sequence was determined.

The nucleotide sequence analysis of the 1725-bp *Eco*RI-*Xho*I fragment insert of the cDNA clone revealed that the cDNA contains a 1593-bp open reading frame encoding a protein of 531 amino acids, a TAA stop codon, and a 132-bp 3′-untranslated region (Fig. 1). BLAST search through the Swiss-Prot database shows that the deduced 551 amino acid sequence has highest homology with the amino acid sequences of catalase-peroxidases from several organisms (Table 1). Based on this homology and the presence of the peroxidase proximal heme ligand (Fig. 1), this cDNA was tentatively designated as a partial cDNA encoding *U. maydis* peroxidase/catalase.

Whether the gene product of this putative peroxidase/catalase gene is bifunctional, exhibiting a catalase and broad-spectrum peroxidase activities, remains to be determined. If it is bifunctional, this represents the first report on the partial structure of peroxidase/catalase-encoding gene from fungi. Cloning of the complete sequence of this gene would enable a more detailed study on the role of these proteins in *Ustilago* biology.


CGCAATCTCGACCACGCTCTTGCCGCCTCGCACATGGGTCTTATCTACGTCAACCCCGAG 60
R N L D H A L A A S H M G L I Y V N P E 20
GGACCCAACGGTGAGCCTGACCCGGTTGNTGCCGCCCACGATATCCGCACCACCTTCGGC 120
G P N G E P D P V X A A H D I R T T F G 40
CGCATGGCCATGAACGACGAGGAAACCGTCGCTCTTATTGCCGGAGGCCACACCTTTGGC 180
R M A M N D E E T V A L I A G G H T F G 60
AAGACTCATGGTGCTGGTAACCCAGATCTCGTCGGCCCCGAACCCAACGGCGCTCCCATC 240
K T H G A G N P D L V G P E P N G A P I 80
GAGGCTCAGGGCTTCGGTTGGACCAGCAAGCATGGTTCTGGTAAAGCTGGCGATGCGATT 300
E A Q G F G W T S K H G S G K A G D A I 100
ACCTCGGGTCTCGAGGTTGTCTGGACTAGCAAGCCTACCGAGTGGTCCAACCTCTACCTC 360
T S G L E V V W T S K P T E W S N L Y L 120
AAGTACCTCTTTGAGTTCGAGTGGGAGCACGACAAGTCGCCCGCTGGCGCCAACCAGTTT 420
K Y L F E F E W E H D K S P A G A N Q F 140
GTCGCCAAGAATGCCGACGCCATCATCCCCGATCCCTTCGACCCATCCAAGAAGCGTCGT 480
V A K N A D A I I P D P F D P S K K R R 160
CCTACTATGCTCACCACCGATCTATCGTTGCGCTACGATCCTGCCTACGAGAAGATCTCG 540
P T M L T T D L S L R Y D P A Y E K I S 180
CGTCGCTTCCTTGAGAACCACGACGAGTTTGCCGACGCCTTTGCCCGTGCCTGGTTCAAA 600
R R F L E N H D E F A D A F A R A W F K 200
CTGCTCCACCGTGACATGGGTCCTCGCGCCCGCTGGCTTGGACCCGAGGTGCCCAAGGAG 660
L L H R D M G P R A R W L G P E V P K E 220
ATCCTTATCTGGGAGGACCCCGTGCCTACCGCCGATTACGCTCTCGTGGACGACCGCGAC 720
I L I W E D P V P T A D Y A L V D D R D 240
CTTGCCGGCTTGAAGCAGGCTATTTTTGCCACTGGCGTCGAACCTTCCAAGTTCCTTGCC 780
L A G L K Q A I F A T G V E P S K F L A 260
ACCGCCTGGGCTTCCGCTGCCAGCTACCGAGACAGTGACAAGCGCGGCGGTGCCAACGGT 840
T A W A S A A S Y R D S D K R G G A N G 280
GCTCGCATCCGCCTTGCACCGATGAAGGACTGGGAAGTCAACAATCCTCAGCAGCTCGCT 900
A R I R L A P M K D W E V N N P Q Q L A 300
GAGGTCATCAAGGCTCTCGAGGGCGTTCAGCAGCAGTTCAACTCTTCCAACCAAGGTGGC 960
E V I K A L E G V Q Q Q F N S S N Q G G 320
AAGAAGATTTCGATTGCTGACTTGATCGTTCTCGCCGGTAACGCAGCGCTTGAGAAGGCA 1020
K K I S I A D L I V L A G N A A L E K A 340

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TCGGGTCTCCCCGTTCCCTTCACTCCTGGTCGTACTGATGCTACCCAGGAGCAGACCGAG    1080
 S  G  L  P  V  P  F  T  P  G  R  T  D  A  T  Q  E  Q  T  E     360

GTCGACACCTTCGAGTTCCTCAAGCCGGTCGCCGATGGCTTCCGCAATTACGGCCAGTCC    1140
 V  D  T  F  E  F  L  K  P  V  A  D  G  F  R  N  Y  G  Q  S     380

ACCGACCGTGTTTGCGCTGAACAGATCCTCATTGACCGCGCCAACCTGCTCACTCTCACC    1200
 T  D  R  V  C  A  E  Q  I  L  I  D  R  A  N  L  L  T  L  T     400

CCTCCCGAGCTCACTGTCCTCATCGGCGGTCTCCGCGCTCTTGGTCTCAACTACAACGGC    1260
 P  P  E  L  T  V  L  I  G  G  L  R  A  L  G  L  N  Y  N  G     420

TCGTCACACGGTGTCTTGACTCACCGCCGAGGCCAGCTCTCGAACGACTTCTTTGTCAAC    1320
 S  S  H  G  V  L  T  H  R  R  G  Q  L  S  N  D  F  F  V  N     440

CTCCTCGACATGAGCACCGAGTGGAAGGCTGCTGACGGTGGCAAGGGCGAAGTCTTCGAC    1380
 L  L  D  M  S  T  E  W  K  A  A  D  G  G  K  G  E  V  F  D     460

GGTGTCGACCGCAAGTCAGGCCAGAAGAAGTGGTCTGCTACCCGTGCCGATCTTGTCTTT    1440
 G  V  D  R  K  S  G  Q  K  K  W  S  A  T  R  A  D  L  V  F     480

GGCTCTCAGGCTGAGCTTCGTGCCCTCGCCGAGAACTACGCTCAGGCCGACAACGCCGAC    1500
 G  S  Q  A  E  L  R  A  L  A  E  N  Y  A  Q  A  D  N  A  D     500

AAGTTCAAGAAGGACTTTGTGACTGCCTGGAACAAGGTTATGAACCTGGATCGTTTTGAC    1560
 K  F  K  K  D  F  V  T  A  W  N  K  V  M  N  L  D  R  F  D     520

GTCAAGAAGAGCAACATTGCCCGTGCCAGGTTCTAACCATGCTCCGCTCCAATCACTGAT    1620
 V  K  K  S  N  I  A  R  A  R  F                                 531

GCTGTAGTAGTAGTAGCCATGATTTCCTTGCCCTATTCAGATTTATGGAATGTCTCTTTT    1680
ATGGAATCTTATTTTATTTTTTAACTTAAAAAAAAAAAAAAAAA                    1725
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Fig. 1. Nucleotide sequence of a 1725-bp cDNA contained in a positively selected cDNA clone during the immunoscreening of *U. maydis* PAL cDNA clone (chapter 3). Deduced amino acid sequence of this cDNA is indicated below the respective codons. Numbers on the right margin indicates position relative to the C nucleotide or R amino acid in the beginning of sequences, respectively. Stop codon TAA is underlined. Predicted peroxidases proximal heme ligand signature (boxed), ATP/GTP-binding site motif A (P-loop) (letters in bold), and 2Fe-2S ferredoxin, iron-sulfur binding region signature (marked as •) are shown.

Table 1. BLAST search results with the protein sequence deduced from the *U. maydis* 1725-bp cDNA in Fig. 1. The results show the ten most similar protein sequences from the Swiss-Prot database.

Rank	Accession Number	Organism	Sequence Description	Probability
1	P14412	*Bacillus stearothermophilus*	Peroxidase/Catalase	2.5e-218
2	Q04657	*Mycobacterium intracellulare*	Peroxidase/Catalase	6.1e-216
3	P13029	*Escherichia coli*	Catalase HPI (Hydroperoxidase I)	1.3e-210
4	P46817	*Mycobacterium vosis*	Peroxidase/Catalase	2.0e-208
5	Q08129	*Mycobacterium tuberculosis*	Peroxidase/Catalase	5.2e-208
6	P17750	*Salmonella typhimurium*	Catalase HPI (Hydroperoxidase I)	1.0e-024
7	P37743	*Rhodobacter capsultus*	Peroxidase/Catalase	9.1e-05
8	P00431	*Saccharomyces ceravisiae*	Cytochrome C Peroxidase Precursor	2.9e-05
9	Q05431	*Arabidopsis thaliana*	L-Ascorbate Peroxidase	3.9e-05
10	P48534	*Pisum sativum*	L-Ascorbate Peroxidase	0.020