Fungal and Plant Phenylalanine Ammonia-lyase

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L-Phenylalanine is one of the essential amino acids that cannot be synthesized in mammals in adequate amounts to meet the requirements for protein synthesis. Fungi and plants are able to synthesize phenylalanine via the shikimic acid pathway. 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 step in this metabolic sequence involves the action of phenylalanine ammonia-lyase (PAL). The discovery of PAL enzyme in fungi and the detection of $^{14}$CO$_2$ production from $^{14}$C-ring-labeled phenylalanine and cinnamic acid demonstrated that certain fungi can degrade phenylalanine by a pathway involving an initial deamination to cinnamic acid, as happens in plants. In this review, we provide background information on PAL and a recent update on the presence of PAL genes in fungi.

KEYWORDS: Fungi, Phenylalanine ammonia-lyase, Plant

Phenylalanine ammonia-lyase (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) [1]. The conversion of the amino acid phenylalanine to trans-cinnamic acid is the entry 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 [2]. In fungi, there is no direct evidence for the significance of this enzyme except as a catabolic function [3].

Since its discovery [1], the presence of PAL has been reported in diverse plants [4, 5] including certain algae, including Dunaliella marina [6], fungi [7-10], and a few prokaryotic organisms, including Streptomyces [11, 12]. In plants, PAL activity has been detected in many species, representing monocots, dicots, gymnosperms, ferns, and lycopsids [13]. In fungi, PAL activity has been detected only in a few basidiomycetes and deuteromycetes, and in one ascomycete, Nectria cinnabarina [7, 14]. There have been no reports of PAL in animals.

In this review, we provide background information on PAL and a recent update on the presence of PAL genes in fungi.

**Structural Properties of PAL Proteins**

PAL has been isolated and characterized from a number of plant species, some fungi and few bacterial sources. Source tissues used for PAL isolation are diverse. They include seedlings [15], shoots [1], leaf-sheath [16], cell culture [17-19], fruit [20], mycelium [21, 22], and prokaryotic cells [12]. Most known PAL sources for enzyme isolation and its properties are well tabulated and documented in several reviews [4, 23, 24]. 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

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are usually detected in analytical polyacrylamide gel electrophoresis gels under denaturing conditions. This can create confusion in the estimation of PAL subunit sizes.

Most reported PALs 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 [25], 226 kDa in the bacterium, Streptomyces [11], 250 kDa in Helianthus annuus [5], 266 kDa in Fragaria ananassa [20], 320 kDa in Ustilago maydis [26], and 560 kDa in Alternaria [21]. PAL is normally a homo-tetrameric protein consisting of four identical subunits. Hetero-tetrameric PAL, as a complex of two hetero-dimers has been reported from H. annuus (2 × 58 kDa and 2 × 68 kDa) [20] and Rhizoctonia solani (2 × 70 kDa and 2 × 90 kDa) [22]. Neumann and Schwemmle [27] 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 composed of two identical subunits with a mass of 80 kDa [28].

Isoelectric points (pIs) for PAL are usually in the acid range, from 2.5 [27] to 6.3 [26]. Isoforms with different pIs have been reported from some sources; three isoforms ranged between pI 4.8 and 5.4 in Leptosphaeria maculans fungus [29], several isoforms between pI 5.1 and 6.1 in alfalfa [17], and two isoforms between pI 4.8 and 5.4 in bean [30]. Interestingly, expression of a single cDNA of poplar PAL in a baculovirus expression system produced two isoforms with different pIs [31].

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 [32] and Rhodotorula glutinis [33]. Alfalfa PAL has been reported to be highly hydrophobic [17]. Consistent with this, the hydropathy profile of the protein sequence deduced from the cDNA sequence also predicted that alfalfa PAL would be hydrophobic [34].

The association of carbohydrate with PAL has been reported for the maize and potato enzymes [35, 36]. Through the analyses of PAL gene sequences, the presence of potential glycosylation sites has been reported from bean [37] and parsley [38], but the importance of glycosylation in PAL function has not been explored extensively. The production of active PAL in Escherichia coli cells transformed with PAL genes from the yeast Rhodosporidium [39] and from parsley [40, 41] 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 [35, 36].

The three-dimensional structure of the red yeast Rhodotorula PAL has been described using X-ray crystallography [42]. This homotetrameric protein contains 716 residues per subunit with a molecular mass of 76.88 kDa. A seahorse-like shape of each subunit interlocks with two other subunits, thereby maximizing adjacent subunit interactions and resulting in tetramer formation.

### PAL 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 [9]. 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$_3$ [43]. Modification of an electrophilic center at the active site of PAL by electrophilic reagents such as borohydride, cyanide, bisulfite, or nitromethane results in the complete inactivation of the enzyme. The identity of [°H]-alanine and [°C]-aspartic acid following acid hydrolysis of PAL enzyme inactivated with radiolabeled reagents, NaB$_4$H$_4$ and °CN$^-$, provides evidence for the presence of dehydroalanine in the active site [9, 44]. 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 [9, 44]. An alternative model for the role of dehydroalanin in PAL catalysis has been also proposed [45].

The mechanism of formation of dehydroalanine has not been determined yet. In cases of other proteins containing dehydroalanine, such as subtilin [46], thyroglobulin [47], and pyruvoyl enzymes [48], a serine residue is considered to be the precursor of dehydroalanine. PAL amino acid sequences contain a serine residue that is completely conserved among different species [49], 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 [45] and poplar PAL [31]. 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 E. coli produced active PAL enzymes in cells in which PAL was not normally produced [40, 47]. 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.

### Functional Properties of PAL

PAL enzymes from many sources, especially from monocots and certain fungi, have activity towards L-tyrosine and can produce trans-p-coumaric acid. This has been described as tyrosine ammonia-lyase (TAL) activity [4, 13, 50]. In most
PAL preparations, TAL activity is very low. The PAL : TAL ratio in PAL preparations varies from 1.35–5 in _Sporobolomyces pararoseus_ fungus [51], from 4–20 in wheat [13], and from 0.6–1.3 in bean [52]. An even larger range in PAL : TAL ratios from several different plant species was reported [53]. No TAL enzyme without PAL activity has been purified. It has been demonstrated in _E. coli_-expressed maize PAL that PAL and TAL activities reside in the same polypeptide [54].

PAL preparations from a number of sources are reported to have only one Michaelis constant (K_m), but the kinetic properties of other preparations suggest that the enzyme is negatively cooperative with respect to substrate binding [55]. Two different K_m values for PAL have been reported from many sources [23]. Individual isoforms were highly purified and the kinetic analysis of each isoform revealed normal Michaelis-Menten saturation kinetics [17, 56]. The K_m values for L-phenylalanine have been reported to range from 0.011 mM [57] to 1.7 mM [1]. Most PALs show no metal ion requirement, although slight stimulation of PAL activity by metal ions such as Mg^{2+} and Ba^{2+} has been reported [5]. Inhibition of PAL activity can be induced by a wide range of compounds including carbonyl, sulphydryl, and thiol reagents, phenolic acids, and heavy metal ions [24]. Most PALs tested are sensitive to synthetic PAL inhibitors such as (S)-2-aminoxy-3-phenylpropanoic acid (AOPP), (R)-(1-amino-2-phenylethyl)phosponic (APEP) acid, and 2-aminoindan-2-phosphonic acid (AIP). Thus, these inhibitors have often been used to block the biosynthesis of phenylpropanoid compounds in plant cells and tissues [23, 58].

The pH optimum for PAL is generally in the range from 8.2–9.0 [21, 23]. The temperature optimum for PAL has been reported to be 35°C in tobacco [57], 55°C in sunflower [5], and 44–46°C in _Rhizoctonia_ [22]. 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 [22]. _Rhodotorula_ PAL is apparently stable for at least 6 months when it is kept at -60°C [59].

**Structural Properties of PAL-Encoding Genes**

Following the isolation of PAL cDNA from bean [37], parsley [38] and sweet potato [60], PAL genes have been isolated from many sources. 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 [61], and the loblolly pine PAL, which has been reported to be encoded by a single gene [62]. Currently, either partial or full fungal genome data is available from more 50 species. When we searched through the DNA databases of the Broad Institute (Cambridge, MA, USA; http://www.broadinstitute.org) and National Center for Biotechnology Information (Bethesda, MD, USA) for the PAL motif [GS][STG][LIVM][STG][SAC]-S-G-[DH]-L-x-[PN]-L-[SA]-x(2,3)-[SAGVT], 45 potent PAL sequences were found in 28 fungal species (Table 1). In red yeasts such as _Rhodosporium_ spp., PAL is generally encoded by a single gene [63]. In filamentous fungi, PAL is encoded by a single, two, three, and four genes (Table 1). The _Aspergillus nidulans_ genome contains four PAL genes. The presence of introns has been reported in both plant and fungal PAL genes. Plant PAL genes generally contain only one intron, while yeast PAL genes have five [63] or six introns [64]. Two introns have been found in the _Arabidopsis_ PAL gene [65], while no introns occur in jack pine and loblolly pine and _U. maydis_ PAL genes [66-68]. The analysis of 45 PAL gene sequences from the fungi listed in Table 1 show that the number of introns varies among species. The number of introns in PAL gene also differs within a species such as _A. flavus_ and _A. oryzae_. In ascomycota, the intron number ranges from none to six introns, while in basidiomycota, intron number ranges from none to 13 introns. The highest intron number is present in the PAL genes of rust fungus _Puccinia_ (Table 1). The inferred PAL protein length varies in fun ranging from 595 to 750 amino acids, except for _Botrytis cinerea_, which has 1,131 amino acids. The position of the PAL motif on the PAL protein sequence shows little variation among species. The fungal PAL motif positions mostly between 123 and 244 amino acids, except for _Neurospora_ and _Neosartorya_ sequences, which position near the C-terminus and N-terminus, respectively. The active site serine residue that is bolded in the PAL motif in Table 1 is very well-conserved both in ascomycota and basidiomycota.

Phylogenetic analysis of the 45 PAL sequences showed that PAL could be divided into three major groups: ascomycota I, ascomycota II, and basidiomycota (Fig. 2). PAL of ascomycota I is more closely related to PAL of basidiomycota than PAL of ascomycota II. The cladogram revealed the variation in the PAL protein sequence among fungi. Comparison of the inferred protein sequences of PAL from diverse fungal species showed a 33–77% protein sequence identity among the species (Table 2). The highest identity among ascomycota PALs was 65% while the highest identify among basidiomycota PALs was 97%. The highest identity between ascomycota and basidiomycota PAL found to date is 41%. Overall, the genome information on many fungi has revealed that many species have PAL gene(s) and the structural properties of the PAL gene vary within a species and among species.

**PAL in Fungi**

While the metabolism of phenylalanine in vascular plants and animals has been well documented, much less is
known about the fungal degradation of phenylalanine. Some of the known pathways of animal and plant metabolism of phenylalanine are also used in microorganisms. In some microorganisms, phenylalanine is 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 [69]. The discovery of a PAL enzyme in fungi [70] and

### Table 1. Fungal species having PAL motif sequences properties

| Fungal species | Gene name | PAL motif | Position of motif | Length of protein | No. of introns |
|---------------|-----------|-----------|-------------------|-------------------|---------------|
| **Ascomycota** |           |           |                   |                   |               |
| *Aspergillus clavatus* NRRL 1 | ACLA_080920 | GSISAGDLPLSYIAA | 123-139         | 664              | 1             |
| *A. flavus* NRRL 3357 | AFL2G_05505 | GSISAGDLPLSYIAA | 189-205         | 721              | 2             |
| *A. flavus* NRRL 3357 | AFL2G_00533 | GSISAGDLTPLYAIA | 202-218         | 714              | 1             |
| *A. flavus* NRRL 3357 | AFL2G_06214 | GSISAGDLSPAYISG | 155-171         | 671              | 3             |
| *A. fumigatus* AF293 | Afl2g09110 | GSISAGDLMPYIAIA | 181-197         | 728              | 2             |
| *A. nidulans* FGSC A4 | ANID_03897 | GSISAGDLTPLYAIA | 182-198         | 687              | 1             |
| *A. nidulans* FGSC A4 | ANID_06075 | GSISAGDLMPYIAIA | 187-203         | 702              | 2             |
| *A. nigro ATCC 1015* | e_gw1_15.39 | GSISAGDLTPLYAIA | 194-210         | 719              | 1             |
| *A. nigro ATCC 1015* | e_gw1_3.237 | GSISAGDLPSYIGG | 181-197         | 720              | 2             |
| *Botrytis cinerea* B05.10 | BC1G_05296.1 | GSISAGDLPLSYIAA | 184-200         | 704              | 2             |
| *Chaetomium globosum* CBS 148.51 | CHGG_02399.1 | GSISAGDLTPLYAIA | 202-218         | 714              | 1             |
| *Fusarium graminearum* NRRL 31084 | FGSG_09311 | GSISAGDLMTMPYIA | 180-196         | 721              | 0             |
| *F. oxysporum* f. sp. lycopersici 4287 | FOXG_05297 | GSISAGDLPLSYIAA | 180-196         | 750              | 0             |
| *F. verticillioides* 7600 | FVEG_03798 | GSISAGDLPLSYIAA | 180-196         | 724              | 0             |
| *Gaetumannomyces graminis* R3-111a-1 | GGTG_00837.1 | GSISAGDLPLSYIAA | 204-220         | 743              | 2             |
| *Magnaporthe oryzae* 70-15 (MG8) | MGG_10036.7 | GSISAGDLMTPYIAA | 207-223         | 627              | 0             |
| *M. poae* ATCC 64411 | MAPP_07981.1 | GSISAGDLPLSYIAA | 198-214         | 730              | 3             |
| *Neosartorya fischeri* NRRL 181 | NFIA_08460 | GSISAGDLMTSPYIA | 48-64           | 595              | 0             |
| *Nh. nodorum* SN15 | SNOG_08528.1 | GSISAGDLPLSYIGC | 190-206         | 700              | 4             |
| *N. oryzae* RIB 40 | AAO00005000532 | GSISAGDLTPLYAIA | 189-205         | 721              | 2             |
| *N. oryzae* RIB 40 | AAO00001000788 | GSISAGDLPLSYIAA | 180-196         | 724              | 0             |
| *N. oryzae* RIB 40 | AAO00002600086 | GSISAGDLTPLAYVTG | 183-199         | 696              | 2             |
| *Neurospora crassa* OR74A (NC10) | CHGG_02399.1 | GSISAGDLMTSPYIA | 207-223         | 627              | 0             |
| *P. graminis tritici* CRL 75-36-700-3 | PTLG_12283.2 | GSISAGDLMLSYVAA | 190-206         | 691              | 1             |
| *Rhodosphoridum toruloides* | AAA33883 | GSISAGDLPLSYIAA | 207-216         | 693              | 6             |
| *R. toruloides* CBS 14 | PM544 | GSISAGDLPLSYIAA | 184-200         | 716              | 2             |
| *Rhodotorula graminis* WP1 | CAD23828 | GSISAGDLPLSYIAA | 213-229         | 713              | 1             |
| *Rhodotorula graminis* WP1 | CAD23828 | GSISAGDLPLSYIAA | 213-229         | 713              | 1             |
| *Stagonospora nodorum* SN15 | SNOG_09914.1 | GSISAGDLPLSYIAA | 179-195         | 610              | 0             |
| *S. nodorum* SN15 | SNOG_16362.1 | GSISAGDLPLSYIAA | 191-207         | 772              | 2             |
| *Uncinocarpus reesii* 1704 | VDBG_08166.1 | GSISAGDLPLSYIAA | 185-201         | 710              | 2             |
| *V. dahliae* VdLs.17 | VDAG_010581.1 | GSISAGDLPLSYIAA | 181-197         | 696              | 0             |
| *V. dahliae* VdLs.17 | VDAG_05831.1 | GSISAGDLPLSYIAA | 218-234         | 645              | 0             |
| **Basidiomycota** |           |           |                   |                   |               |
| *Coprinus cinereus* okayama7#130 | CC1G_06838.3 | GSISAGDLPLSYIAA | 212-228         | 734              | 9             |
| *C. cinerea* okayama7#130 | CC1G_14161.3 | GSISAGDLPLSYIAA | 252-268         | 770              | 6             |
| *Laccaria bicolor* S238N-H82 | LACBDRAFT_291120 | GSISAGDLPLSYIAA | 163-179         | 688              | 5             |
| *L. bicolor* S238N-H82 | LACBDRAFT_184628 | GSISAGDLPLSYIAA | 201-217         | 731              | 11            |
| *Puccinia triticum* 1-1 BBBB Race 1 | PTG_02419.1 | GSISAGDLPLSYVAA | 175-191         | 653              | 13            |
| *P. graminis tritici* CRL 75-36-700-3 | PGTG_12283.2 | GSISAGDLPLSYVAA | 190-206         | 691              | 13            |
| *Rhodosphoridum toruloides* | AAA33883 | GSISAGDLPLSYVAA | 207-213         | 693              | 6             |
| *R. toruloides* CBS 14 | PM544 | GSISAGDLPLSYIAA | 184-200         | 716              | 6             |
| *Rhodotorula graminis* WP1 | CAD23828 | GSISAGDLPLSYIAA | 213-229         | 713              | 1             |
| *R. mucilaginosa* NRRLY-1597 | CAA31486 | GSISAGDLPLSYIAA | 213-229         | 720              | 5             |
| *Ustilago maydis* 521 | UM00078 | SSISAGDLPLSYVAA | 201-217         | 724              | 0             |

PAL, phenylalanine ammonia-lyase; NRRL, Northern Regional Research Laboratory; FGSC, Fungal Genetics Stock Center; ATCC, American Type Culture Collection; RIB, Research Institute of Brewing; NIH, National Institutes of Health; CBS, Centraalbureau voor Schimmelcultures.
the detection of $^{14}$CO$_2$ production from $^{14}$C-ring-labeled phenylalanine, cinnamic acid, and benzoic acid [71] have demonstrated that certain fungi can degrade phenylalanine by a pathway involving an initial deamination to cinnamic acid, as happens in plants. 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* [72], *Ustilago hordei* [71], *Schizophyllum commune* [71], and *Sporobolomyces roseus* [8]. *S. commune* can also metabolize phenylalanine through phenylpyruvic acid, phenylacetic acid, and $o$-hydroxyphenylacetic acid [71]. Interestingly, it has been reported that another basidiomycete, *Lentinus lepideus*, forms phenylpropanoid compounds (e.g., $p$-coumaric acid, caffeic acid, isoferrulic acid, phloretic acid, and $p$-methoxycinnamic acid) via cinnamic acid derived from phenylalanine [73]. 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* [74], *R. solani* [75], and *Penicillium brevicompactum* [76]. 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 [77].

### Commercial and Medical Potential of PAL

The therapeutic potential of using PAL enzyme against neoplasms has been suggested because of its selectivity for phenylalanine [78]. PAL substantially inhibited neoplastic cell growth in vitro [79], and produced cures in some mice that were inoculated with a lymphoblastic leukemia [80]. However, PAL is of special interest to clinicians primarily due to its potential as a treatment for the inherited metabolic disorder, phenylketonuria. A treatment involving the oral ingestion of PAL [81] were proposed to patients to consume a normal diet. Preclinical evaluation of multiple species of PEGylated recombinant phenylalanine ammonia lyase for the treatment of phenylketonuria has been performed with mouse model [82]. In 2011, BioMarin Pharmaceutical has announced PEG-PAL (PEGylated recombinant phenylalanine ammonia lyase) is currently in Phase II clinical development for the treatment of PKU.

With the increasing consumption of the aspartic acid-phenylalanine dipeptide artificial sweetener, aspartame, the commercial demand for L-phenylalanine has led to mass Fig. 2. Cladogram of the inferred amino acid sequences of L-phenylalanine ammonia-lyase (PAL) from diverse fungal species. Cladogram was constructed by the neighbor-joining method using PAUP v.4.0b10. *Arabidopsis thaliana* PAL sequence was used as an out-group.
production of this amino acid [83]. Since the reaction is reversible, PAL can be used in a large-scale bio-conversion to produce L-phenylalanine from trans-cinnamic acid and ammonium salts acid [84]. Commercial production of PAL is available from *R. glutinis* (Sigma-Aldrich, St. Louis, MO, USA).

### Conclusions

While a huge amount of information has 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. What selective advantage does PAL offer that has led to its retention in this group of organisms? It appears that the ability to synthesize cinnamic acid is important in the life cycle of fungi. Now with the full sequencing of fungal genome(s) in diverse fungal species including human and plant pathogens, saprophytes, and mushrooms, it is possible to compare and predict the potent pathways for phenylalanine degradation among different fungal species. Molecular genetic studies such as gene replacement should reveal whether PAL is essential in fungal physiology and especially reveal links, if any, between PAL activity and pathogenesis, development, and secondary metabolic activities. Further work to develop knowledge and tools that would enable us to rationalize the existence of PAL in certain fungi is needed.

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