# Changes in Phenylalanine Ammonia-lyase Activity and Gene Expression during Storage of Asparagus Spears

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A cDNA clone coding phenylalanine ammonia-lyase (PAL) was isolated from a cDNA library prepared from asparagus spears (*Asparagus officinalis* L. cv. Welcome) using the reverse transcription-polymerase chain reaction (RT-PCR). The partial cDNA clone encoded an mRNA of 527 bp and the derived amino acid sequence was found highly homologous to PAL from rice, maize and barley. Northern blot analysis showed an increase of pAS-PAL mRNA until 24 h at 20 °C, which coincided well with PAL activity and fiber development, suggesting that the increase is a response to the wounding associated with harvest.

*Key words:* Asparagus Spear, Gene Expression, Phenylalanine Ammonia-lyase

## Introduction

The toughening of asparagus is due to lignification of fibrovascular tissues and occurs within hours after harvest in spears stored under ambient conditions (Isherwood, 1963). Lignin, the substance that lends fibers their toughness, is polymerized from cinnamyl alcohols derived from the shikimic acid pathway (Gross, 1981). Phenylalanine ammonia-lyase (PAL), catalyzing the conversion of L-phenylalanine to *trans*-cinnamic acid, has been considered to play an important role as a key enzyme. Fluctuation in PAL activity has been shown to be a key element controlling the synthesis of lignin. An increase in the amount of PAL mRNA has been shown to underly the increase of PAL activity (Edwards *et al.*, 1985; Fritzemeier *et al.*, 1987; Lawton and Lamb, 1987; Orr *et al.*, 1993). In elicited cultured pine cells for instance, the induction of PAL activity occurs concurrently with the increase in the activities of lignin-specific enzymes and is followed by the deposition of apparently genuine gymnosperm lignin in the cell walls (Campbell and Ellis, 1992a, b). PAL transcripts increase in response to fruit ripening and wounding in the mesocarp tissue of *Cucurbita maxima* (Kato *et al.*, 2000). In freshly harvested asparagus spears PAL activity increased upon excision and incubation (Goldstein *et al.*, 1972). Increased PAL activity was also observed in intact spears given an extended period of cold treatment. However, PAL activity in asparagus spears have not yet been studied in relation to fiber development and no PAL gene expression is detected during storage of asparagus spears.

This study reports the cloning and characterization of a partial PAL cDNA from harvested asparagus spears. By RNA blot analysis and *in situ* RNA hybridization the pattern of expression of PAL gene has also been reported in relation to fiber development and PAL enzyme activity during storage.

## Materials and Methods

### *Plant material*

Green asparagus spears (*Asparagus officinalis* L. cv. Welcome) harvested from a commercially available crop in Kagawa, Japan in the year 2002 were obtained directly from farmer's field. The spears were hand harvested and trimmed to approx. 25 cm length. The spears, which were of good quality, straight with closed bracts, were put in polyethylene bags and kept at 20 °C for up to 48 h. The fiber content in both top and bottom portions of the spears was measured at harvest (0 h) and after 8, 16, 24, 36 and 48 h. For PAL enzyme analysis and RNA extraction the spears were frozen at – 80 °C.

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*Texture measurement*

The texture was measured rheologically based on the measurement of resistance to pressure or shearing. The breaking force to indicate the fiber content in spears was determined with a creep meter YAMADEN RHEONER RE-3305 (Shimizu, Tokyo, Japan) equipped with software Ver. 2.0 for automatic analysis. The thickness of the blade was 0.04 mm and it sheared at the rate of 1 mm per second with a pressure of 20 kg/cm$^2$. Spears were cut into two equal pieces and breaking force readings were made separately in the mid-point of top and bottom portions of the spears.

*Extraction and assay of PAL activity*

2 g of spear tissues were homogenized at 2 °C with a mortar and pestle in 10 ml of 0.1 M borate buffer, pH 8.8, and 1.0 g polyvinylpolypyrrolidone. A further 10 ml of the buffer were added to the homogenate which was then centrifuged at 14,000 rpm for 20 min and the supernatant was used for enzymatic assays. The protein concentration was measured after Lowry *et al.* (1951). PAL activity was determined spectrophotometrically by measuring the increase in $A_{290}$ due to the formation of *trans*-cinnamic acid. The reaction mixture consisted of 50 mM borate buffer, pH 8.8, 20 mM L-phenylalanine and the enzyme preparation in a total volume of 3 ml. A sample without L-phenylalanine was used as a blank. The activity was expressed as nmol *trans*-cinnamic acid formed per h per mg of protein.

*RNA extraction and amplification of poly (A)$^+$ RNA by RT-PCR*

Total RNA was extracted according to the hot borate method of Wan and Wilkins (1994). The first strand cDNA was synthesized from 2 ng of the total RNA by reverse transcriptase with an oligo-(dT) primer according to the instruction of SUPER SCRIPT™ Preamplification System for First Strand cDNA Synthesis (GIBCOBRL, Tokyo, Japan). The polymerase chain reaction (PCR) was performed in a total volume of 25 $\mu$l containing the first strand cDNA reaction products, 10 × PCR buffer, $MgCl_2$, dNTP, First Start Taq DNA Polymerase (Roche) and primers. The primers (5′-ATYGAGGCTGCTGCYATTATG-3′ as the upstream primer and 5′-ACATCTTGGTTGTGYTGCTC-3′ as the downstream primer) were designed and synthesized on the basis of amino acid domains (IEAAAIM and AEQHNQD, respectively) conserved in various PAL genes. The *Sal*1 and *Not*1 restriction site sequences were also included at the 5′-end of the sense and antisense primer, to facilitate cloning of PCR product. The PCR procedure started with 10 min at 95 °C and was carried out for 35 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C, and 10 min at 72 °C with an ASTEC Program Temperature Control System PC-700. The PCR products were confirmed by agarose gel electrophoresis.

*Cloning and sequencing of cDNA*

The amplified cDNA was ligated to the plasmid pSPORT[1] and cloned into *Escherichia coli* (DH-5a) *Not*1- *Sal*1-cut (BRL, Tokyo, Japan). Sequencing was performed by the cycle sequencing method using a GATC$^R$-Bio Cycle sequencing Kit and a DNA sequencer GATC 1500 Long-Run system (GATC GmbH, Konstanz, Germany).

*Sequence data analysis*

Sequence analysis was performed using computer software GENETYX-MAC Ver. 7. Homology searches with the Genbank and the EMBL databases were performed using the homology program in the software. The phylogenetic tree was also constructed with the UPGMA method in the software.

*Preparation of the digoxigenin (DIG)-UTP-labelled RNA probe*

The cloned RT-PCR product including the encoded region of PAL gene was cleaved by *Not*1 and *Sal*1 from the pSPORT[1] vector that had been amplified in *Escherichia coli* (DH-5-a) and it was purified and recovered by gel electrophoresis. An antisense DIG-labelled RNA probe was prepared according to the instructions of DIG RNA Labeling Kit (Boehringer Mannheim, Germany) using SP6 RNA polymerase.

*Northern blot analysis*

10 $\mu$g of total RNA was subjected to electrophoresis on 1.0% agarose (Type II) gel containing 20 × MOPS and 37% formaldehyde. After electrophoresis for 30 min, RNA was visualized with ethidium bromide under UV light to confirm equal loading of RNA in each lane. RNA was transferred to a positively charged nylon mem-

brane Hybond$^{TM}$-N$^{+}$ (Amersham Pharmacia Biotech) by capillary action with 20 × SSC and then after drying the membrane RNA was fixed under UV light. The membrane was prehybridized at 50 °C with 5 × SSPE, 5 × Denhart's solution, formamide and 10% SDS for 3 h. Hybridization was performed at 50 °C using the gene specific antisense DIG-labelled RNA probe for 24 h and the same prehybridization buffer. After hybridization, the membrane was washed twice with 2 × SSPE containing 0.1% SDS for 10 min at room temperature, once with 1 × SSPE containing 0.05% SDS for 15 min at 65 °C and once with 0.2 × SSPE for 10 min containing 0.05% SDS at 65 °C. The membrane was further washed with buffer A containing maleic acid and Tween 20 at room temperature and was blocked with 2% blocking reagent in maleic acid buffer for 30 min. Subsequently, the membrane was incubated with Anti-Digoxygenin-AP, Fab fragments (Boehringer Mannheim) in the blocking buffer for 30 min. The signals were detected by color reaction using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride as the substrate.

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        10        20        30        40        50        60        70        80        90
ATCGAGGCTGCTGCCATTATGGAGCACATTCTGGAGGGAAGCTCGTACATGAAAATGGCAAAAAAGTTGCACGAACAAGACCCGTTACAG
I  E  A  A  A  I  M  E  H  I  L  E  G  S  S  Y  M  K  M  A  K  K  L  H  E  Q  D  P  L  Q
       →
       100       110       120       130       140       150       160       170       180
TTGCCAAAACAAGACCGTTATGCTCATCGAACCTCGCCACAGTGGCATGGTCCGCTGATTGAGGTTATCCGCTCATCGACCAAATCAATT
L  P  K  Q  D  R  Y  A  L  R  T  S  P  Q  W  H  G  P  L  I  E  V  I  R  S  S  T  K  S  I
       190       200       210       220       230       240       250       260       270
GAACGGGAGATCAATTCGGTGAACGACAACCCGTTGATCGATGTATCGAGGAACAAGGCTCTACACGGCGGGAACTTTCAAGGGACGCCG
E  R  E  I  N  S  V  N  D  N  P  L  I  D  D  S  R  N  K  A  L  H  G  G  N  F  Q  G  T  P
       280       290       300       310       320       330       340       350       360
ATCGGAGTGTCTATGGATAACACTCGGCTGGCCATCGCCGCAATCGGGAAGCTCATGTTCGCTCAGTTCTCGGAGCTCGTCAATGACTTG
I  G  V  S  M  D  N  T  R  L  A  I  A  A  I  G  K  L  M  F  A  Q  F  S  E  L  V  N  D  L
       370       380       390       400       410       420       430       440       450
TACAATAACGGGTTGCCTTCGTACCATTCGGGTGGGCGAAACCCTAGCTTGGATTACGGGTTCAAGGGTGCGGAGATCGCCATGGCATCC
Y  N  N  G  L  P  S  Y  L  S  G  G  R  N  P  S  L  D  Y  G  F  K  G  A  E  I  A  M  A  S
       460       470       480       490       500       510       520       530       540
TACTGCTCAGAACTCCAATTCCTTGCGAATCCTGTGACGAACCACGTCCAGAGCGCCGAGCAACACAACCAAGATGT
Y  C  S  E  L  Q  F  L  A  N  P  V  T  N  H  V  Q  S  A  E  Q  H  N  Q  D
                                                          ←
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the cDNA clone corresponding to pAS-PAL. The predicted amino acid sequence is given in single-letter code for each amino acid. The arrows indicate the positions of degenerated primers (→ sense, ← antisense) used for RT-PCR. The numbering refers to total nucleotide residues on each line.

Table I. Percentage of nucleotide and deduced amino acid homology between PAL from asparagus spear and other plants in the databases.

| Plants | Nucleotide (%) | Amino acid (%) |
|---|---|---|
| Rice (X87946) | 75.3 | 90.9 |
| Maize (L77912) | 75.3 | 82.3 |
| Tobacco (M84466) | 75.0 | 88.6 |
| Tomato (M90692) | 74.6 | 90.4 |
| Pea (D10001) | 72.7 | 89.1 |
| Sweet potato (M29232) | 73.8 | 87.4 |
| Barley (Z49145) | 77.0 | 96.4 |
| Sweet cherry (AF 036948) | 73.7 | 83.1 |
| Lettuce (AF411134) | 72.0 | 87.6 |
| Bean (M11939) | 71.4 | 89.2 |
| Apple (X68126) | 73.9 | 87.8 |

Asparagus PAL (AB102677) is calculated as 100%.

## Results

The cDNA pAS-PAL is a partial clone encoding a harvest-induced transcript from asparagus spears. The encoded mRNA is 527 bp long (Fig. 1) and is highly homologous to the gene of other plants. The pAS-PAL sequence is 75.3%identical to PAL from rice (X87946), 75.3% identical to PAL from maize (L77912), and 77.0% identical to the barley PAL (Z49145) all of which are wound-induced genes (Table I). Allowing for conservative amino acid substitutions, the similarities are 90.9%, 82.3%, and 96.4% for the rice, maize and barley sequences, respectively.

Fig. 2 shows the development of PAL activity in both top and bottom portions of asparagus spears. The increase in activity continued until 24 h resulting in a 30% and 55% increase over the initial level in top and bottom portions, respectively. Thereafter, the level of activity started to decline.

A general increase in fiber content in both the top and bottom portions of the spears was observed throughout the experimental period. Although the fiber development in both top and bottom portions followed almost the same pattern, the breaking force indicating the toughness of the spears was greater in the bottom than in the top part.

The expression of pAS-PAL was induced by wounding due to harvest. Maximum pAS-PAL expression appeared after 24 h of storage and coincided with the peak of enzyme activity (Figs. 2, 3). A phylogenetic tree (not included) was generated from the alignment of the deduced amino acid sequences of pAS-PAL and other PAL genes in the database. The pAS-PAL (accession no. AB102677), PAL from barley (Z49145) and maize

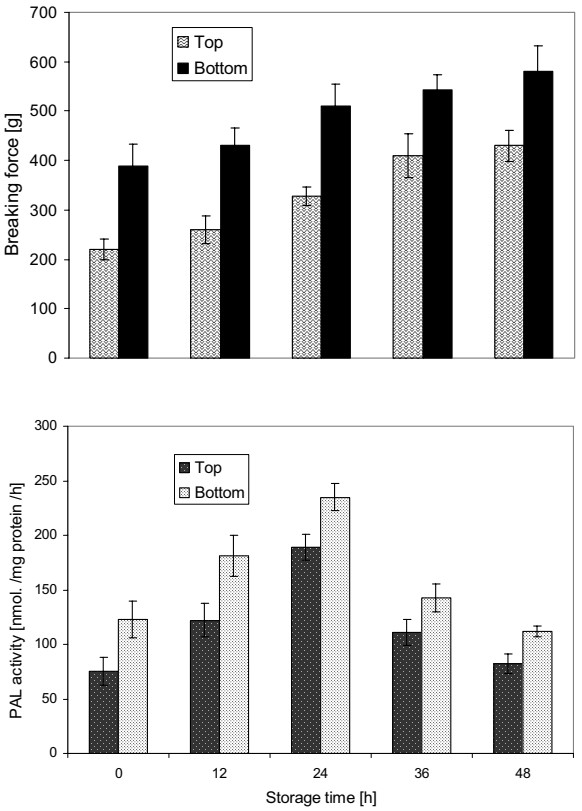


Fig. 2. Changes in fiber content and PAL activity in the top and bottom portions of asparagus spears. Each point represents the mean of three replications. Vertical bars indicate SEs.

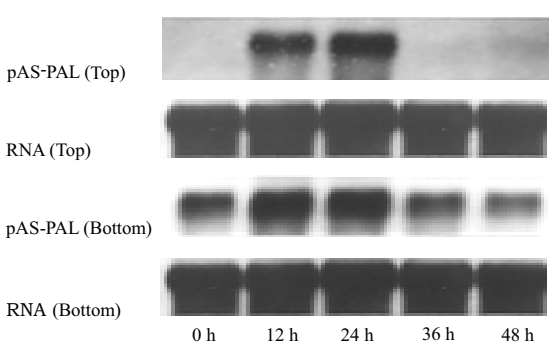


Fig. 3. Northern blot analysis of total RNA (10 $\mu$g/lane) showing the expression of pAS-PAL in the top and bottom portions of asparagus spears.

(L77912), strongly clustered together in a subgroup belonging to the monocotyledon, having closest relationship with the rice PAL gene.

## Discussion

The basal tissue of the asparagus is highly lignified and PAL activity is known to be correlated with the degree of lignification in many tissues (Higuchi, 1966; Yoshida and Shimokoriyama, 1965). Alternatively, ethylene produced by the wounded tissue may induce PAL activity as observed in some other plant tissues (Imaseki *et al.*, 1968; Kato *et al.*, 2000).

Phylogenetic analysis of PAL sequences has revealed the existence of at least two major branches that contain characteristic conserved amino acid sequences, monocotyledon and dicotyledon. The pAS-PAL belonged to the subgroup wound-induced monocotyledon as it was highly homologous to rice PAL and was closely related to PAL from maize. Our results suggest that induction of PAL activity in harvested asparagus is regulated by transcription of pAS-PAL in response to the wounding associated with harvest.

Northern blot analysis revealed that the expression of pAS-PAL increased in harvested asparagus until 24 h of storage period at 20 °C and afterwards it started to decline although the toughness determined as the value of breaking force was still increasing. The increase in fiber content after 24 h might be due to the higher activity of other lignin-specific enzymes like peroxidase and cinnamyl alcohol dehydrogenase. Further research is necessary to investigate the role of other lignin-specific enzymes controlling the toughness of asparagus.

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