



Food and Health

α -Aminooxi- β -phenylpropionic acid controls phenylalanine ammonia lyase gene expression during storage of asparagus spears

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Abstract

In order to examine and regulate the phenylalanine ammonia lyase (PAL) gene expression in stored asparagus spears α -aminooxi- β -phenylpropionic acid (AOPP), a specific inhibitor of PAL was used. Freshly harvested asparagus spears were soaked in aqueous solution of AOPP for 1 h at 20°C and then stored for 48 h. AOPP at 300 μ M effectively inhibited the induction of harvest induced PAL activity in both top and bottom portion of asparagus spears. AOPP at a concentration of 100 μ M was not effective. At a much higher concentration of 600 μ M no additional effect but somewhat less inhibition of the PAL activity was observed. PAL gene expression was detected in both top and bottom portion of the spears just after harvest but AOPP decreased the level of transcripts for PAL. This was accompanied by a reduction of toughness. The data suggest that downstream products of PAL activity may be important not only for wound healing and defense reactions, but also for regulation of PAL gene expression during lignification.

Key words: *Asparagus officinalis*, α -aminooxi- β -phenylpropionic acid, phenylalanine ammonia lyase, toughness.

Introduction

The toughening of asparagus is due to lignification of fibrovascular tissues and occurs within hours after harvest in spears stored at ambient conditions¹. Lignin, the substance that lends fibers their toughness, is polymerized from cinnamyl alcohols derived from shikimic acid pathway² and PAL catalyzing the conversion of L-phenylalanine to *trans*-cinnamic acid in this pathway 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 and in many cases, an increase in the amount of PAL mRNA has been shown to underlie the increase of PAL activity³⁻⁶. Numerous reports have demonstrated a positive correlation between increased enzyme activity, PAL protein accumulation and PAL gene expression. Inhibition of PAL activity by compounds such as 2-aminoindan-2-phosphonic acid (AIP), α -aminooxyacetic acid (AOA), and α -aminooxi- β -phenylpropionic acid (AOPP) has been well characterized in several plant tissues⁷⁻¹⁰. The inhibitors are thought to directly affect PAL enzyme activity since AOPP and AIP did not affect the activity of phenylalanine-tRNA synthetase¹⁰⁻¹¹. An HPLC examination of the phenolic compounds produced by wounded and ethylene exposed lettuce revealed that the PAL inhibitors AIP and AOPP greatly reduced the accumulation of phenolic compounds¹².

We recently showed that induction of PAL activity in harvested asparagus is regulated by transcription of pAS-PAL in response to the wounding associated with harvest. In this experiment we used a specific inhibitor of PAL enzyme activity, AOPP, to determine whether reductions in enzyme activity would influence PAL gene expression and toughness of asparagus spears.

Materials and Methods

Plant material: Green asparagus spears (*Asparagus Officinalis* L. cv. Welcome) were harvested from Kagawa Agricultural Experiment Station Miki branch, Kagawa, Japan on August, 2003. Spears were hand harvested and trimmed to approximately 25 cm length. The spears, which were of good quality, straight with closed bracts, were soaked for 1 h at 20°C in aqueous solution of α -aminooxi- β -phenylpropionic acid (AOPP) at 0, 100, 300 and 600 μ M. After necessary treatments the spears were put in polyethylene bags and held at 20°C for up to 48 hours. Fiber content in both top and bottom portions of the spears was measured at harvest (0 h) and after 12, 24, and 48 h. For PAL enzyme analysis and RNA extraction the spears were frozen at -80°C.

Texture measurement: Texture was measured rheologically based on the measurement of resistant to pressure or shearing. Breaking force to indicate the fiber content in spears was determined with a creep meter Yamaden Rheoner RE-3305 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 kgf. 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: Two 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 PVPP. A further 10 ml of the buffer was added to the homogenate which was then centrifuged at 14,000 rpm for 20 min and the supernatant was used for enzymatic assays. Protein concentration was measured

by Lowry¹³ method. PAL activity was determined spectrophotometrically by measuring the increase in A₂₉₀ 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 Wilkins¹⁴. The first strand cDNA was synthesized from 2 g of the total RNA by reverse transcriptase with Oligo-(dT) primer according to the instruction of SUPER SCRIPT™ Pre-amplification System for First Strand cDNA Synthesis (GIBCOBRL, Tokyo, Japan). PCR was performed in a total volume of 25 µl containing the first strand cDNA reaction products, 10 x PCR Buffer, MgCl₂, 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 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 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 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 pSPORT¹ and cloned into *Escherichia coli* (DH-5α) *Not 1*-*Sal 1* - cut (BRL, Tokyo, Japan). Sequencing was performed by the cycle sequencing method using 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.

Preparation of the digoxigenin (DIG)-UTP-labeled RNA probe: The cloned RT-PCR product including the encoded region of PAL gene was cleaved by *Not 1* and *Sal 1* from the pSPORT1 vector that had been amplified in *Escherichia coli* (DH-5α) and it was purified and recovered by gel electrophoresis. Antisense DIG-labeled RNA probe was prepared according to the instructions of DIG RNA Labeling Kit (Boehringer Mannheim) using SP6 RNA polymerase.

Northern blot analysis: Ten µg of total RNA was subjected to electrophoresis on a 1.0% agarose (Type II) gel containing 20 x 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 membrane Hybond™-N⁺ (Amersham Pharmacia Biotech) by capillary action with 20

x SSC and then after drying the membrane RNA was fixed under UV light. The membrane was prehybridized at 50°C with 5 x SSPE, 5 x Denhart's solution, formamide and 10% SDS for 3 h. Hybridization was performed at 50°C using the gene specific antisense DIG-labeled RNA probe for 24 hours using the same prehybridization buffer. After hybridization, the membrane was washed twice with 2 x SSPE containing 0.1% SDS for 10 min at room temperature, once with 1 x SSPE containing 0.05% SDS for 15 min at 65°C and once with 0.2 x 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 minutes. Subsequently, the membrane was incubated with Anti-Digoxigenin-AP, Fab fragments (Boehringer Mannheim) in the blocking buffer for 30 min. Signals were detected by color reaction using 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitro blue tetrazolium chloride as the substrate.

Results

Inhibition of PAL activity: In the control samples, PAL activity started to increase after harvest and continued throughout the experimental period (Fig. 1B). Both 300 and 600 µM AOPP inhibited PAL activity maintaining the level as initial. AOPP at a concentration of 100 µM was not effective. At a much higher concentration of 600 µM no additional effect but somewhat less inhibition of the PAL activity was observed.

Expression of PAL gene: PAL gene expression was detected in both top and bottom portion of the spears beginning just after harvest and continued up to 48 h (Figs. 1B and 2B). In case of 100 µM AOPP treated spears gene expression followed almost same pattern like the control one. In contrast, for 300 and 600 µM AOPP treated spears almost no or very little expression of pAS-PAL was detected that coincided well with enzyme activity.

Fiber development: A general increase in fiber content in both the top and bottom portions of the control 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 portion. In the top portion of the spears AOPP at 300 µM reduced the breaking force by 44% and in the case of the bottom portion by 21% (Figs 1A and 2A). Spears treated with 100 µM AOPP produced the same breaking force as control. AOPP at 600 µM also inhibited PAL activity and resulted in almost similar fiber content like 300 µM AOPP treated spears.

Discussion

The development of PAL activity in control spears was similar in a number of respects to other wound induced tissue systems¹⁵⁻¹⁶. There was an increase in PAL activity in both top and bottom portions of the spears which continued throughout the experimental period. Although PAL activity in both top and bottom portions followed almost the same pattern the activity was higher in bottom than top portions. High PAL activity associated with the bottom portion, or tissue near to cut surfaces,

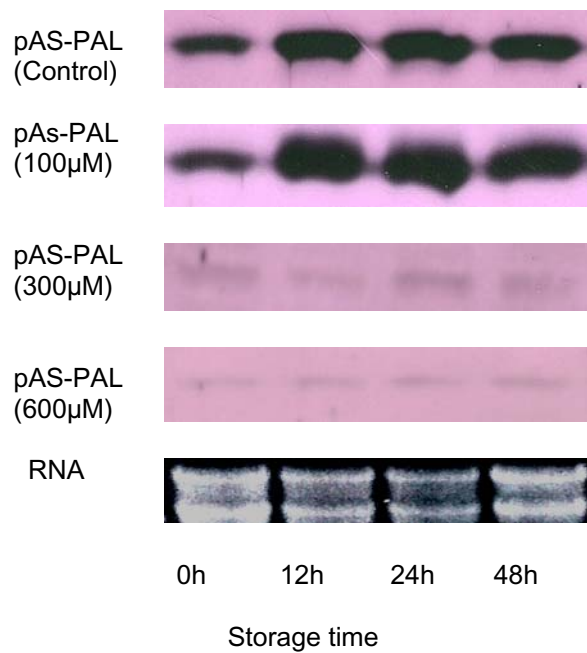
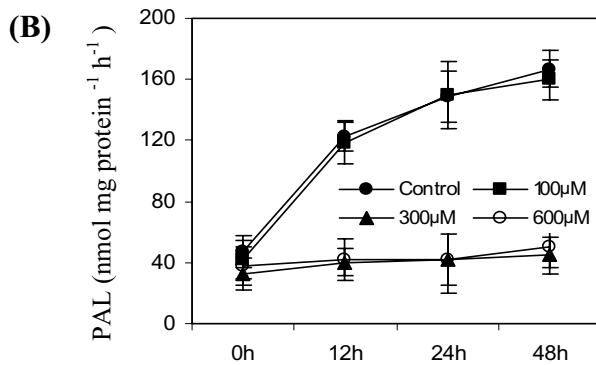
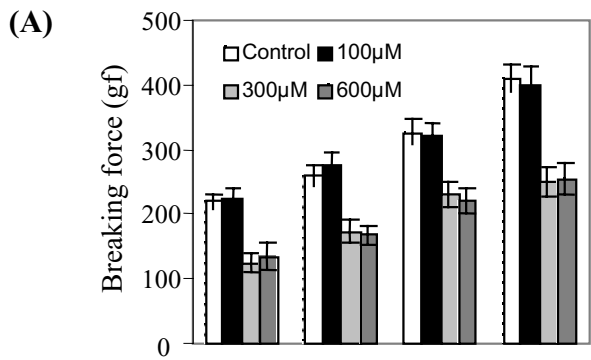


Figure 1. Effect of AOPP on PAL gene expression and fiber content in top portion of asparagus spears. (A) Changes in breaking force. Each point represents the mean of three replicates and vertical bars show SE. (B) Northern blot analysis for PAL. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

such as we observed, has been noted previously¹⁷⁻¹⁸. The basal tissue of the asparagus is highly lignified and PAL activity is known to be correlated with the degree of lignification in many tissues¹⁹⁻²⁰. To investigate the potential role of PAL in toughening of asparagus spears, AOPP a specific inhibitor of

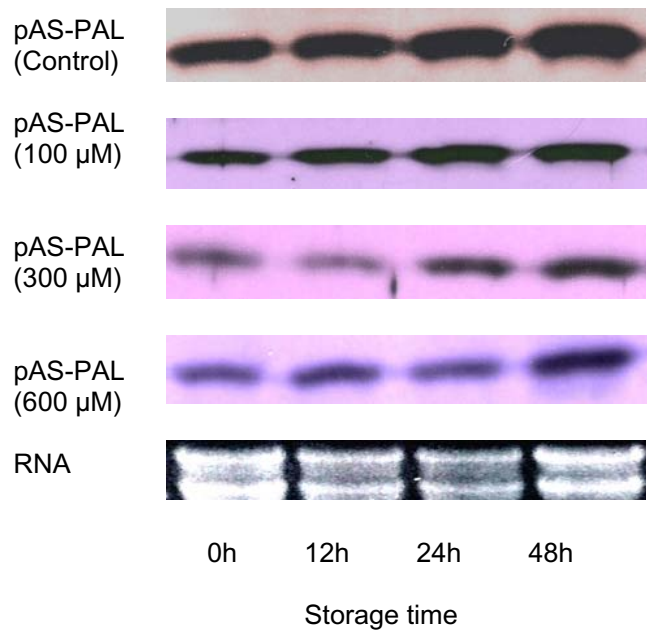
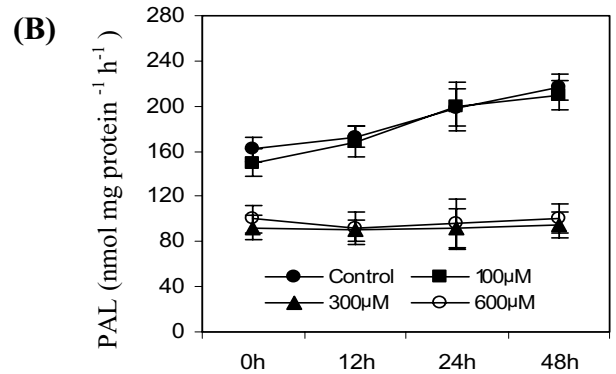
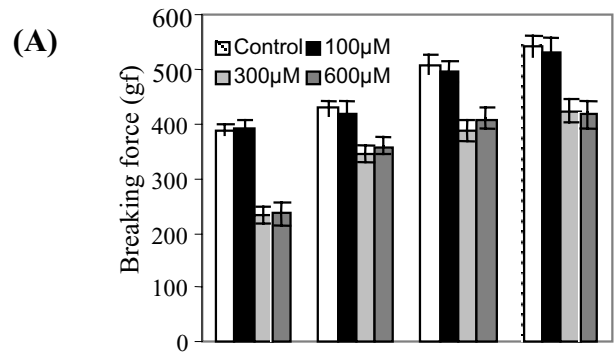


Figure 2. Effect of AOPP on PAL gene expression and fiber content in bottom portion of asparagus spears. (A) Changes in breaking force. Each point represents the mean of three replicates and vertical bars show SE. (B) Northern blot analysis for PAL. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

PAL enzyme activity was applied. AOPP has been well characterized in terms of its effect on PAL activity in other plant tissues⁸. AOPP at a concentration of 300 µM inhibited the PAL activity in both top and bottom portion of spears and this was followed by reduced fiber content. We have cloned and

sequenced a cDNA for PAL from asparagus spears. The 527-bp cDNA for PAL designated as pAS-PAL was a partial cDNA identical to the same region of the cDNA that encoded PAL from other plants and therefore, used as a probe for Northern blot analysis. RNA blot hybridization demonstrated that in both top and bottom portions PAL transcripts for 300 and 600 μ M AOPP treated spears showed almost no or very little expression during storage. In contrast, for control and 100 μ M AOPP treated spears PAL transcript accumulated just after harvest and continued up to 48 h of storage. Accumulation of PAL transcript was accompanied by increased in enzyme activity and fiber development. This suggests that PAL activity and gene expression are most influential in lignification of asparagus spears during storage. AOPP, a specific inhibitor of PAL enzyme activity, reduced PAL gene expression and decreased the fiber content of asparagus spears, suggesting that downstream products of PAL enzyme activity may have a regulatory role in the lignification process. Further research will be necessary to clarify this point.

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